Kazuhiko Takehara
Manabu Fujimoto
Masataka Kuwana
Editors

Systemic Sclerosis

Basic and Translational Research

Springer

Systemic Sclerosis

Kazuhiko Takehara • Manabu Fujimoto •
Masataka Kuwana
Editors

Systemic Sclerosis

Basic and Translational Research

Springer

Editors
Kazuhiko Takehara
Department of Dermatology
Kanazawa University
Kanazawa, Ishikawa
Japan

Manabu Fujimoto
Department of Dermatology
University of Tsukuba
Tsukuba, Ibaraki
Japan

Masataka Kuwana
Department of Allergy and Rheumatology
Nippon Medical School
Tokyo, Tokyo
Japan

ISBN 978-4-431-55707-4 ISBN 978-4-431-55708-1 (eBook)
DOI 10.1007/978-4-431-55708-1

Library of Congress Control Number: 2015958785

Springer Tokyo Heidelberg New York Dordrecht London

Printed on acid-free paper

Springer Japan KK is part of Springer Science+Business Media (www.springer.com)

Preface

Systemic sclerosis (scleroderma) is a most challenging disease to treat. Great progress has been made in the treatment of specific symptoms, including renal crisis with angiotensin-converting enzyme inhibitors reflux esophagitis with proton-pump inhibitors and pulmonary arterial hypertension with prostanoids, endothelin receptor antagonists, and phosphodiesterase type 5 inhibitors; however, there are still no disease-modifying drugs that are able to control the overall disease activity satisfactorily. This is partly because, whereas other connective tissue diseases appear primarily mediated by immune dysregulation, systemic sclerosis has a tremendously complex pathogenesis comprising several distinct components of microvasculopathy, excessive fibrosis, and immune activation and autoimmunity. Thus, to develop a new concept of therapy based on the pathogenesis of the disease is needed.

Recent studies nonetheless have revealed a variety of new evidence that suggests clues to the pathogenesis of the disease. This book provides a comprehensive review of the recent advances in the research of systemic sclerosis. First, Hironobu Ihn provides an overview on the pathomechanism of fibrosis, which is a hallmark of the disease, and Ayumi Yoshizaki and Shinichi Sato consider the possible relationship between systemic autoimmunity and disease manifestation. Masataka Kuwana outlines the importance of endothelial progenitor cells in a theory that defective vascular repair machinery is one of important mechanisms contributing to vasculopathy of the disease. Toshiyuki Yamamoto outlines animal models of scleroderma, including a bleomycin-induced murine scleroderma model. Genetics also appears important, as it too is associated with certain susceptibility genes and is affected by epigenetic regulation. Naoyuki Tsuchiya and Aya Kawasaki summarize the findings of genetic epidemiology. By contrast, Masatoshi Jinnin points out that inherited genetic factors cannot fully explain the pathogenesis of systemic sclerosis and that epigenetics play an assignable role by the mediating influence of the environmental factors. As in other connective tissue diseases, the critical roles of cytokines have been gathering much attention in systemic sclerosis. Yasushi Kawaguchi reviews the role of the interleukin (IL)-1 family including IL-1α, IL-1β, and IL-1 receptor antagonists as well as IL-33; and Yoshihito Shima discusses the potential involvement of IL-6 and the prospect of IL-6-targeting

therapy. Manabu Fujimoto and Kazuhiko Takehara detail the role of profibrotic cytokines, transforming growth factor β, and connective tissue growth factor. Recently, several molecules have come under the spotlight. Sei-ichiro Motegi presents the current knowledge of the endothelin/endothelin receptor system in terms of vasculopathy and fibrosis, and Yoshihide Asano discusses the importance of Fli1, a member of the ETS transcription factor family, in pathogenesis. B cells are a source of autoantibodies that are further hallmarks of the disease. Yasuhito Hamaguchi outlines the clinical importance of autoantibodies detected in the sera of patients with systemic sclerosis. Moreover, B cells may play a role independent of antibody secretion. Takashi Matsushita discusses the potential roles of pathogenic and regulatory B cells in the disease. Minoru Hasegawa provides a review of biomarkers used in the assessment of disease activity and severity as well as the monitoring of specific organ involvement.

The central objective of this book is to provide readers with the latest knowledge on the research of systemic sclerosis. We hope that this book will serve as a useful resource for all rheumatologists and dermatologists as well as basic scientists, especially immunologists, molecular biologists, and biochemists, who are interested in this disease. We sincerely hope that we will be able to develop an effective therapeutic approach by understanding the pathomechanisms and thus will eventually overcome this intractable disease.

Kanazawa, Japan — Kazuhiko Takehara
Tsukuba, Japan — Manabu Fujimoto
Tokyo, Japan — Masataka Kuwana

Contents

Fibrosis: Overview

1

Hironobu Ihn

Abstract

Fibrosis, excessive extracellular matrix deposition, in the skin, lung, and other organs is a hallmark of systemic sclerosis (SSc). Fibroblasts isolated from sclerotic lesions, such as the sclerotic skin or lung, in patients with SSc and cultured in vitro are characterized by increased synthesis of collagen and other extracellular matrix proteins, decreased synthesis and activity of matrix metalloproteinases, and increased synthesis of tissue inhibitor of metalloproteinases, consistent with the disease phenotype. The pathogenesis of SSc is still poorly understood, but increasing evidence suggests that transforming growth factor-β (TGF-β) is a key mediator of tissue fibrosis as a consequence of extracellular matrix accumulation in the pathology of SSc. TGF-β regulates diverse biological activities including cell growth, cell death or apoptosis, cell differentiation, and extracellular matrix synthesis. TGF-β is known to induce the expression of extracellular matrix proteins in mesenchymal cells and to stimulate the production of protease inhibitors that prevent enzymatic breakdown of the extracellular matrix. This chapter focuses on the mechanism of fibrosis in SSc.

Keywords

Extracellular matrix • Collagen • TGF-β • Smads • Integrins

H. Ihn (✉)
Department of Dermatology & Plastic Surgery, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Chuo-ku, Kumamoto 860-8556, Japan
e-mail: ihn-der@kumamoto-u.ac.jp

K. Takehara et al. (eds.), *Systemic Sclerosis*, DOI 10.1007/978-4-431-55708-1_1

1.1 Introduction

Scleroderma, or systemic sclerosis (SSc), is a generalized connective tissue disease that is characterized by sclerotic changes in the skin and sometimes various other organ systems (Fig. 1.1) [1]. Clinical outcomes have improved probably due to better management of the complications, but SSc is still considered to be incurable and diffuse cutaneous SSc carries high risk of fatality. Although the pathogenesis of SSc is still unknown, the basic mechanism appears to involve endothelial cell injury, aberrant immune activation, and overproduction of extracellular matrix (ECM) [2–7].

Fibrosis is the most characteristic pathological hallmark of SSc. Fibrosis is a complex biological process involving an acute inflammatory response. Transient activation of fibroblasts to proliferate and produce elevated quantities of ECM is essential to fibrosis. It is likely that such transient fibroblast activation is regulated by a variety of cytokines produced by infiltrating platelets, monocytes/macrophages, T lymphocytes, and other inflammation-associated cells [8]. Numerous in vitro and in vivo studies have suggested that cytokines such as TGF-α and TGF-β, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), IL-1-α and IL-β, tumor necrosis factor (TNF)-α and TNF-β, IL-4, IL-6, IL-13, and oncostatin M (OSM) regulate dermal fibroblast proliferation and ECM deposition [9–18].

Increasing evidence suggests that activation of lesional fibroblasts contributes to the fibrotic process [19, 20]. Numerous differences between cultured SSc and healthy skin fibroblasts that may contribute to excessive ECM deposition in vivo have been demonstrated, such as increased expression of collagen types I, III, VI, and VII, fibronectin, and glycosaminoglycans [20–28]; an elevated expression of tissue inhibitor of metalloproteinases [29]; and elevated expression of intercellular adhesion molecules [30]. SSc and healthy skin fibroblasts also differ in their response to TGF-β. SSc fibroblasts are less sensitive to the stimulatory effects of

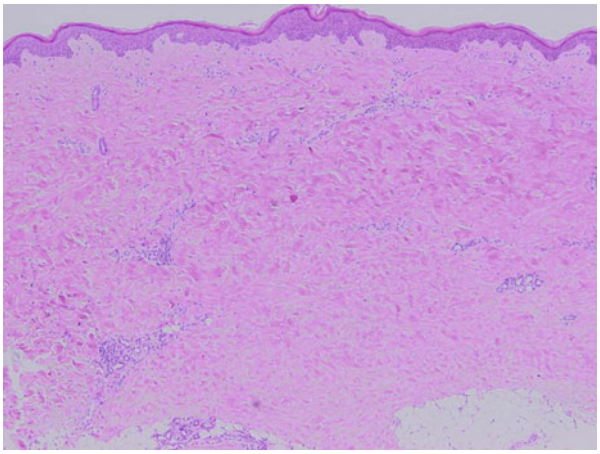

Fig. 1.1 Histopathology of the skin in a patient with SSc, showing thickened collagen bundles

cytokines that regulate collagen production, such as TGF-β, oncostatin M (OSM), interleukin (IL)-4, and IL-17 [26, 31–33].

The mechanism of fibroblast activation in SSc is presently unknown. However, many of the characteristics of SSc fibroblasts resemble those of healthy fibroblasts stimulated by TGF-β [34, 35], suggesting that TGF-β is a key mediator of tissue fibrosis in SSc.

The most potent profibrotic stimulus to fibroblasts is TGF-β. The TGF-β superfamily, which includes the prototypic factor TGF-β1, has a shared structure, similar signaling pathways, and an overlap of biological effects. TGF-β is a 25 kD homodimeric polypeptide, which participates in a broad array of biological activities such as normal development, wound repair, and pathological processes [18]. TGF-β regulates multiple cellular functions including inhibition and stimulation of cell growth, cell death or apoptosis, and cellular differentiation.

1.2 TGF-β

1.2.1 TGF-β Superfamily, Structure, and Activation

The TGF-β superfamily includes the various forms of TGF-β, bone morphogenic protein (BMP), activin, nodals, the anti-Mullerian hormone, and many other structurally related factors [36]. There are three mammalian isoforms of TGF-β, TGF-β1, TGF-β2, and TGF-β3, which are structurally almost identical. Their isoforms share a common structural knot motif consisting of six cysteine residues joined together by three intrachain disulfide bonds that stabilize β-sheet bands. One free cysteine forms an interchain disulfide bond with an identical monomeric chain to permit formation of mature TGF-β dimers. These are secreted as a large latent covalently bound complex with TGF-β-binding protein, which serves to bind TGF-β to the ECM and to enable its proteolytic activation [36]. Activation of TGF-β is a complex process involving conformational changes of latent TGF-β-binding protein, induced by either cleavage of the latency associated peptide by various proteases such as plasmin, plasma transglutaminase, thrombin, or endoglycosylases or by physical interactions of the latency associated peptide with other proteins, such as thrombospondin-1, leading to the release of bioactive and mature TGF-β. TGF-β has been shown to increase expression of collagen types I, III, VI, VII, and X, fibronectin, and proteoglycans [34]. Stimulation of ECM production by TGF-β is further enhanced by its inhibitory effect on matrix degradation, by decreasing synthesis of proteases and increasing levels of protease inhibitors [34].

Although in vitro effects of TGF-β isoforms seem to be similar, mice lacking TGF-β isoforms revealed that each TGF-β isoform plays an independent and non-redundant role in vivo. Disruption of the TGF-β1 gene results in a wasting syndrome accompanied by a multifocal, mixed inflammatory cell response and tissue necrosis, leading to organ failure and death, which indicates a critical role of TGF-β1 in immune regulation [37]. TGF-β2 null mice exhibit perinatal mortality

and a wide range of developmental defects, including cardiac, lung, craniofacial, limb, spinal column, eye, inner ear, and urogenital defects. The developmental processes most commonly involved in the affected tissues include epithelial mesenchymal interactions, cell growth extracellular matrix production, and tissue remodeling. Many affected tissues have neural crest-derived components and stimulate neural crest deficiencies [38]. Mice lacking TGF-β3 exhibit an incomplete penetrant failure of the palatal shelves to fuse leading to cleft palate. The defect appears to result from impaired adhesion of the apposing medial edge epithelia of the palatal shelves and subsequent elimination of the midline epithelial seam, suggesting that TGF-β3 is crucial for epithelial differentiation [39].

Enhanced expression of TGF-β has been well demonstrated in the tissue of SSc as well as that of a mouse model of SSc [19, 39]. However, SSc fibroblasts have been shown to produce an amount of TGF-β equivalent to that produced by normal fibroblasts in vitro [27, 40]. Furthermore, there was no significant difference between SSc fibroblasts and normal fibroblasts in the production of total TGF-β1 and active TGF-β1 [27].

1.2.2 TGF-β Receptors

TGF-β exerts its multiple biological actions by an interaction with two transmembrane serine/threonine kinase receptors, types I and II, that are coexpressed by most cells including mesenchymal and endothelial cells. From the structural point of view, type I and type II TGF-β receptors are very similar glycoproteins, characterized by a cysteine-rich extracellular domain, a single hydrophobic transmembrane domain, and a C-terminal cytoplasmic serine/threonine kinase domain. Initiation of signaling requires the binding of TGF-β to TGF-β receptor type II, a constitutively active serine/threonine kinase, which results in the recruitment and phosphorylation of the type I TGF-β receptor to produce a heteromeric complex that activates downstream signaling pathways [41]. To date, at least six distinct type I receptors of the TGF-β superfamily, named activin receptor-like kinases (ALKs), have been cloned. Type I receptors have a region between the transmembrane and the kinase domains, containing a conserved TSGSGSG motif, named the GS domain. The phosphorylation of serine and threonine residues in the GS domain of the type I receptor by type II receptor has been shown to be essential for TGF-β signaling [42]. TGF-β receptor type I is thought to determine the specificity of the cellular response to TGF-β, whereas TGF-β receptor type II is thought to determine the ligand specificity. TGF-β receptor type I alone is unable to bind TGF-β, and TGF-β receptor type II is unable to signal without TGF-β receptor type I [41]. Betaglycan, a transmembrane proteoglycan also known as TGF-β receptor type III, allows high-affinity binding of TGF-β to TGF-β receptor type II, but does not itself transduce signal.

Increased expression of TGF-β receptors has been well demonstrated in fibrosis [27, 43–45]. Increased expression of TGF-β receptors type I and type II has also been reported in SSc fibroblasts in vitro and in vivo [27, 46–49]. The

overexpression of these TGF-β receptors in SSc fibroblasts was shown to be regulated at transcriptional level [47] and to be resistant to various stimuli [48, 49]. TGF-β receptors have been shown to be upregulated by TGF-β, PDGF, and EGF [48, 50, 51]. Moreover, elevated expression of the endothelial cell-enriched TGF-β receptor endoglin was reported in SSc fibroblasts [52]. Therefore, the overexpression of these TGF-β receptors in SSc fibroblasts may be due to the exposure of SSc fibroblasts to these cytokines in vivo. The overexpression of TGF-β receptors induced collagen transcription in cultured dermal fibroblasts [43], which indicates a potential role of enhanced expression of TGF-β receptors in fibrosis. A recent report has demonstrated that an increased TGF-β receptor type I/type II ratio contributes to elevated collagen protein synthesis in SSc [53]. Furthermore, overexpression of dominant negative TGF-β receptor type II improved liver fibrosis [54], and the effects of the overexpression of dominant negative TGF-β receptor type II have also been investigated in SSc [53, 55].

1.3 SMAD

1.3.1 SMADS Proteins

Following ligand activation, signaling from TGF-β receptor type I to the nucleus occurs predominantly by phosphorylation of cytoplasmic proteins belonging to the Smad family [56]. TGF-β receptor type I specifically recognizes and phosphorylates the ligand-specific receptor activated Smad (R-Smad). R-Smads include Smad1, Smad5, and Smad8 downstream of BMP and Smad2 and Smad3 downstream of TGF-β and activin. They all consist of two Mad-homology (MH) domains and a linker region [57]. The N-terminal MH1 domain has DNA-binding activity, whereas the C-terminal MH2 domain has protein-binding properties. Phosphorylation of R-Smad by TGF-β occurs principally on serine residues within the C-terminus [56]. After phosphorylation by the TGF-β receptor type I, R-Smads form heteromeric complexes with the co-Smad, Smad4. The R-Smad/Smad4 complex is then translocated into the nucleus and functions there as transcription factors, binding DNA either directly or in association with other DNA-binding transcription factors [56]. The Smad3/Smad4 complex binds to DNA via the consensus sequence "CAGAC" [58]. However, Smad2 does not bind DNA directly, requiring other DNA-binding transcription factors and Smad4 to activate transcription in response to TGF-β. The inhibitory Smad (I-Smad), such as Smad6 or Smad7, binds to the TGF-β receptor type I and prevents R-Smad phosphorylation and subsequent nuclear translocation of the R-Smad/Smad4 heterocomplex [59]. Recent studies have focused on the role of Smad in tissue fibrosis.

Despite extensive research to elucidate the role of Smad proteins downstream of TGF-β, few direct Smad target ECM genes have been characterized. For example, the transcriptional activation of the fibronectin gene by TGF-β was shown to be regulated by a JNK-specific, Smad-independent mechanism [60]. This suggests that pathways that alternate to the Smad signaling pathway also play important roles in

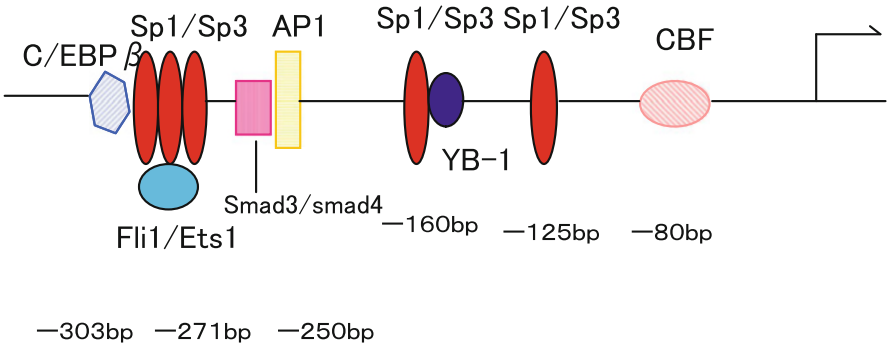

Fig. 1.2 Transcriptional regulation of the human α2(I) collagen gene. The human α2(I) collagen promoter is regulated by transcription factors Sp1/Sp3, Ets1/Fli1, C/EBP-β, AP-1, Smad3/Smad4, YB-1, and CCAAT-binding factor (CBF)

the regulation of ECM genes expression by TGF-β. Using a combined cDNA microarray/promoter transactivation approach, about 60 ECM-related genes have been identified as direct Smad target genes [61].

Smad proteins have been reported to be involved in the transcriptional regulation of the gene for type I collagen, which is excessively deposited in fibrosis. The human α2(I) collagen gene is upregulated at transcriptional level in fibrotic lesion [26] and its promoter is known to be regulated by transcription factors Sp1/Sp3 and CCAAT-binding factor (CBF) (Fig. 1.2) [62–66]. Earlier studies identified a TGF-β response element containing Sp1 binding sites in the human α2(I) collagen promoter [67], and Sp1 was shown to be required for the response of the gene to TGF-β [68]. Further studies have shown the important role of Smad3/Smad4 complex binding to the CAGACA motif near the Sp1 binding site in the human α2 (I) collagen promoter for the full TGF-β response [69–71]. In addition, recent studies have shown that synergistic cooperation between Sp1 and Smad3/Smad4 is required for response of the collagen gene to TGF-β [72–74]. Further detailed analyses showed the cooperation of p300/CBP with Smad in the TGF-β response of the collagen gene [75, 76]. Other studies indicate that the interaction of Ets transcription factors with Smad is also involved in TGF-β response of the extracellular matrix genes, such as collagen and tenascin-C [77–81]. Furthermore, MMP-1 downregulation by TGF-β is also shown to involve Smad3 [82].

Several studies showed that mice lacking Smad3 have a significantly reduced cutaneous as well as pulmonary fibrotic response [83, 84], which indicates an important role of Smad3 in fibrosis. Smad2, Smad3, and Smad4 have been reported to contribute to liver fibrosis in vitro and in vivo [85–88]. Gene transfer of Smad7 was shown to prevent renal fibrosis [89]. These not only suggest the potential role of Smad7 in fibrosis, but also may lead to the development of novel approaches for treating fibrosis.

The expression levels of Smad proteins were also investigated in SSc fibroblasts [90, 91]. Some SSc fibroblasts expressed increased levels of Smad3 and Smad7, but

others did not [90]. A recent study showed increased expression of Smad 3 and increased phosphorylation of Smad2/Smad3 in SSc fibroblasts [91]. Constitutive phosphorylation of Smad3 and the increased binding of Smad3 to the "CAGA" motif in SSc fibroblasts were also reported [109]. Another study reported the decreased expression of Smad7 in scleroderma fibroblasts [92]. In that study, SSc fibroblasts showed increased phosphorylation of Smad2 and Smad3 compared with normal fibroblasts after TGF-β treatment [92]. However, detailed analysis showed that the expression of Smad7 was increased in SSc fibroblasts in vivo and in vitro and that Smad7-Smurf-mediated negative regulation of TGF-β signaling was impaired in SSc fibroblasts [93].

1.3.2 Coactivators and Corepressors of SMAD-Dependent Gene Transcription

In the nucleus, activated Smad complexes regulate various gene expressions with the recruitment of coactivators or corepressors into transcriptional complexes. The role of CREB binding protein (CBP) and p300 as essential coactivators for Smad-driven gene expression has been well demonstrated [94]. Squelching of p300/CBP by STAT1 or c-Jun induced by IFN-γ or TNF-α was reported to inhibit Smad signaling [76, 95]. Smad proteins also recruit transcriptional corepressors, such as Ski, Ski-related novel gene N (SnoN), TG3-interacting factor (TGIF), or SNIP1 [96–99]. These proteins bind chromatin-condensating histone deacetylases, which inhibit the histone acetyltransferase activity of p300/CBP. The expression levels of these corepressors have significant roles in physiological processes. Recent studies have focused on the role of these corepressors in tissue fibrosis. Indeed, downregulation of Ski and SnoN has been reported in renal fibrosis [86]. SSc fibroblasts exhibited increased c-Ski/SnoN levels both in vitro and in vivo. Although c-Ski/SnoN constitutively formed a complex with Smads, the inhibitory effects of c-Ski/SnoN were impaired in SSc fibroblasts [100]. These impaired inhibitory effects of c-Ski/SnoN are characteristic for SSc fibroblasts and may be involved in the pathogenesis of fibrosis in SSc. However, the role of other corepressors in tissue fibrosis, especially in SSc, is still poorly understood.

1.4 The Mitogen-Activated Protein Kinase (MAPK)

Several cross-signaling mechanisms have been described that implicate Smad proteins, such as MAPK and phosphoinositide-3-kinases (PI3Ks). The MAPK is a major signaling system used by eukaryotic cells to transduce extracellular signals to intracellular responses [87]. Three major subgroups of the MAPK superfamily members have been identified to date: the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2), also known as p44/p42 MAPKs, respectively; the c-jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK); and the p38 MAPK [87].

The signal transduction cascades involved in the activation of MAPKs require a well-coordinated cascade of three protein kinase reactions that transduce signals by sequential phosphorylation and activation of the next kinase in their respective pathways. The MAPKs require dual phosphorylation at the threonine and tyrosine sites by MAPK kinases. The MAPKs are considered to play essential roles in signal transduction in many biological events, such as the regulation of cell growth, differentiation, apoptosis, and cellular responses to environmental stresses. ERK1 and ERK2 have been shown to be involved mainly in fibroblast proliferation [17], and the p38 MAPK has been reported to be involved mainly in ECM production by fibroblasts [88, 101].

TGF-β has been demonstrated in various cell types to activate each of the three major MAPK members. Recent studies have shown that p38 MAPK is involved in type I collagen synthesis by TGF-β stimulation, which suggests the possible role of p38 MAPK in fibrosis [89]. Fibronectin synthesis was shown to be upregulated via the JNK pathway [101]. On the other hand, the activation of ERK was reported to inhibit type I collagen expression [102]. Constitutive phosphorylation and activation of p38 MAPK was demonstrated in SSc fibroblasts, and the inhibition of p38 MAPK using specific p38 MAPK inhibitors or dominant negative mutant p38 MAPK abolished the upregulated expression of type I collagen or fibronectin in SSc fibroblasts. These results strongly suggest the contribution of p38 MAPK signaling to the constitutive upregulated expression of type I collagen or fibronectin in SSc fibroblasts [89].

1.5 Phosphoinositide-3-Kinase (PI3K)

Phosphoinositide-3-kinases (PI3Ks) phosphorylate inositol-containing lipids at the D-3 position of the inositol ring. They are divided into three classes in mammalian cells. Class III PI3Ks produce phosphatidylinositol (PtdIns)-3-P, which is constitutively present in all cells. Class I and class II PI3Ks can utilize PtdIns, PtdIns-4-P, and PtdIns-4,5-P_2. Class I PI3Ks are heterodimers of a 110-kDa catalytic subunit (p110α, p110β, p110δ, and p110γ) and an adaptor/regulator subunit (p85α, p85β, p55, and p101) [103]. Following PI3K activation, PIP_3 recruits the phosphoinositide-dependent kinase (PDK)-1 and Akt/PKB, bringing these proteins into proximity at the plasma membrane where Akt is phosphorylated on Thr^{308} by PDK-1 [103]. This is followed by phosphorylation at Ser^{473}. Once activated, Akt leaves the plasma membrane to phosphorylate intracellular substrates. Akt has also been shown to translocate the nucleus where it can phosphorylate transcription factors [104].

Some studies have suggested that the PI3K signaling pathway can be modulated by the TGF-β family [105, 106]. Bakin et al. have shown that the inhibition of PI3K blocks TGF-β1-induced Smad2 phosphorylation [105], suggesting that Smad proteins are potential targets of the PI3K pathway. In fact, the inhibition of PI3K was shown to result in a redistribution of Smad anchor for receptor activation (SARA), which directly interacts with Smad2/Smad3 and functions to recruit

Smad2 for phosphorylation to the activated TGF-β receptor complex [107], and the attenuation of both TGF-β-induced Smad2 phosphorylation and transcriptional activation [108]. Furthermore, the basal activity of PI3K was shown to be necessary for COL1A2 mRNA stabilization [109, 110] and to be indispensable for the establishment of the constitutive activation of TGF-β/Smad3 signaling in SSc fibroblasts [109].

1.6 Connective Tissue Growth Factor (CTGF)

Connective tissue growth factor (CTGF) is induced by TGF-β and modulates fibroblast cell growth and ECM secretion [111, 112]. CTGF has been demonstrated in experimental and human fibrosis [113, 114], in which its expression appears to correlate with the degree of fibrosis [115, 116]. These results suggest that CTGF mediates many of the profibrotic action of TGF-β.

CTGF is a 36–38 kD cysteine-rich peptide containing 349 amino acids. It belongs to the CCN (CTGF, cyr 61/cef 10, nov) family of growth factors. The gene for CTGF was originally cloned from a human umbilical endothelial cell cDNA library [111]. CTGF has been detected in endothelial cells, fibroblasts, cartilaginous cells, smooth muscle cells, and some cancer cell lines. Earlier studies revealed that TGF-β1 increases CTGF mRNA markedly in human foreskin fibroblasts [112]. PDGF, EGF, and FGF were also shown to induce CTGF expression, but their effects were only transient and weak [112].

CTGF has diverse bioactivities. Depending on cell types, CTGF has been shown to trigger mitogenesis, chemotaxis, ECM production, apoptosis, and angiogenesis. In earlier studies, CTGF was noted to have mitogenic and chemotactic effects on fibroblasts [111]. CTGF was also reported to enhance the mRNA expression of α1 (I) collagen, fibronectin, and α5 integrin in fibroblasts [117]. The finding that TGF-β increases CTGF synthesis and that TGF-β and CTGF share many functions is consistent with the hypothesis that CTGF is a downstream mediator of TGF-β.

The mechanism by which CTGF exerts its effects on cells, especially its signal transduction, is still unclear. CTGF was reported to bind to the surface of fibroblasts with high affinity, and this binding was competed with recombinant PDGF BB [111]. This suggests that either CTGF binds to certain class of PDGF receptor or there is some cross-reactivity of PDGF BB with CTGF receptors.

CTGF mRNA has been detected in the fibroblasts of sclerotic lesions of patients with SSc [115]. In patients with localized scleroderma, CTGF mRNA was detected in fibroblasts from tissue in the sclerotic stage more than the inflammatory stage, which suggests a close correlation between CTGF and fibrosis [116]. Similar results were also obtained in keloid and other fibrotic diseases [116]. Subsequently, expression of CTGF has been reported in a variety of fibrotic disorders, including hepatic fibrosis, pulmonary fibrosis, and cardiac fibrosis [118–121]. CTGF is also implicated in the dermal fibrosis of SSc [122, 123]. Furthermore, increased responsiveness of SSc fibroblasts to CTGF has been shown [124]. However, the

detailed role of CTGF in SSc is still unclear. Further studies are needed to clarify this point.

1.7 MicroRNA (miRNAs)

Recently, microRNAs (miRNAs) have attracted attention for its involvement in the pathogenesis of various human diseases such as immune diseases. miRNAs are small noncoding RNA that bind to complementary sequences in the untranslated regions (UTRs) of target mRNAs, resulting in inhibiting their translation into protein. More than 1,000 miRNAs have been identified, which corresponds to 1–5 % of all genes in the human genome, and miRNAs are thought to be the most abundant regulators. Various miRNAs have been reported to be involved in the pathogenesis of SSc [125–128]. miR-196a expression was decreased in SSc both in vitro and in vivo, and overexpression of the miR-196a resulted in the downregulation of type I collagen in SSc fibroblasts, which suggests that miR-196a is involved in the pathogenesis of SSc [125]. The constitutive upregulation of miR-92a expression was shown both in sera and in dermal fibroblasts from SSc patients which was resulted from autocrine TGF-β signaling [126]. Furthermore, forced overexpression of miR-92a in dermal fibroblasts resulted in the downregulation of matrix metalloproteinase-1 (MMP-1) expression [126]. MMP-1 is a target of miR-92a, and therefore, miR-92a overexpression plays a role in excessive collagen accumulation in SSc via downregulation of MMP-1 [126]. microRNA let-7a expression was downregulated in SSc both in vitro and in vivo, and the inhibition or overexpression of let-7a in dermal fibroblasts affected the expression of type I collagen [127]. Furthermore, the intermittent microRNA let-7a injection improved the skin fibrosis induced by bleomycin in mice [127]. miR-150 expression was decreased in SSc fibroblasts both in vitro and in vivo [128]. Transfection of miR-150 inhibition into fibroblasts induced expression of integrin b3, phosphorylated Smad3, and type I collagen, whereas forced overexpression of the miRNA resulted in their downregulation in SSc fibroblasts, which suggested that miR-150 may play an important role in the pathogenesis of fibrosis in SSc via overexpression of integrin β3 [128].

1.8 The Role of Autocrine TGF-β Signaling in SSc

Increasing evidence suggests that TGF-β is a key mediator of tissue fibrosis in SSc as a consequence of ECM accumulation. Many of the characteristics of SSc fibroblasts resemble those of normal fibroblasts stimulated by TGF-β such as elevated expression of collagen types I, III, VI, and VII, fibronectin, and glycosaminoglycans [20–28]; elevated expression of tissue inhibitor of metalloproteinases

[29]; decreased expression of collagenase (MMP-1) [129]; expression of α-smooth muscle actin [130]; and increased expression of αvβ5 integrin [131]. Dysregulation of TGF-β signaling has been reported in SSc fibroblasts.

Enhanced expression of TGF-β has been well demonstrated in the tissue of SSc as well as that of a mouse model of SSc [19, 39]. However, SSc fibroblasts have been shown to produce an amount of TGF-β equivalent to that produced by normal fibroblasts in vitro [27, 40]. Furthermore, there was no significant difference between SSc fibroblasts and normal fibroblasts in the production of total TGF-β1 and active TGF-β1 [27].

Increased expression of TGF-β receptors has been well demonstrated in fibrosis [27, 43–45]. Increased expression of TGF-β receptors type I and type II has also been reported in SSc fibroblasts in vitro and in vivo [27, 46–49]. The overexpression of these TGF-β receptors in SSc fibroblasts was shown to be regulated at transcriptional level [47] and to be resistant to various stimuli [48, 49]. TGF-β receptors have been shown to be upregulated by TGF-β, PDGF, and EGF [48, 50, 51]. Moreover, elevated expression of the endothelial cell-enriched TGF-β receptor endoglin was reported in SSc fibroblasts [52]. Therefore, the overexpression of these TGF-β receptors in SSc fibroblasts may be due to the exposure of SSc fibroblasts to these cytokines in vivo. The overexpression of TGF-β receptors induced collagen transcription in cultured dermal fibroblasts [43], which indicates a potential role of enhanced expression of TGF-β receptors in fibrosis. A recent report has demonstrated that an increased TGF-β receptor type I/type II ratio contributes to elevated collagen protein synthesis in SSc [53]. Furthermore, overexpression of dominant negative TGF-β receptor type II improved liver fibrosis [52], and the effects of the overexpression of dominant negative TGF-β receptor type II have also been investigated in SSc [53, 54]. Further studies showed that the total expression levels of TGF-β receptors were elevated in SSc fibroblasts, but that cell surface expression levels of TGF-β receptors were not elevated in SSc fibroblasts [132]. The internalization rate of TGF-β receptors was increased in SSc fibroblasts and caveolin constitutively made a complex with TGF-β receptors, and this leads to the activation state of TGF-β signaling [132].

The expression levels of Smad proteins were also investigated in SSc fibroblasts [90, 91]. Some SSc fibroblasts expressed increased levels of Smad3 and Smad7, but others did not [90]. A recent study showed increased expression of Smad 3 and increased phosphorylation of Smad2/Smad3 in SSc fibroblasts [91]. Constitutive phosphorylation of Smad3 and the increased binding of Smad3 to the "CAGA" motif in SSc fibroblasts were also reported [109]. Further study showed that constitutive increased Smad3 phosphorylation, increased interaction of Smad3 with Sp1, and p300 were detected in SSc fibroblasts [133]. The overexpression of Smad3 caused an increase of up to fivefold in COL1A2 promoter activity in normal fibroblasts, but Smad3 caused a small increase in COL1A2 promoter activity in SSc fibroblasts [133]. These results suggested that SSc fibroblasts are less sensitive to

exogenous TGF-β/Smad signaling because SSc fibroblasts are already activated by the autocrine TGF-β signaling [133]. Another study discovered the regulatory elements of the constitutive upregulated α2(I) collagen gene in SSc fibroblasts [134]. The binding sites of Sp1, Ets1, and Smads were important for the constitutive upregulated α2(I) collagen gene in SSc fibroblasts [134]. Furthermore, the binding activity of Ets1 as well as Smad3 to their binding sites was increased in SSc fibroblasts [134]. This promoter analysis emphasized that Ets1 forms a transcriptionally active complex with Smad and Sp1 by autocrine TGF-β signaling [134].

One study reported the decreased expression of Smad7 in scleroderma fibroblasts [92]. In that study, SSc fibroblasts showed increased phosphorylation of Smad2 and Smad3 compared with normal fibroblasts after TGF-β treatment [92]. However, detailed analysis showed that the expression of Smad7 was increased in SSc fibroblasts in vivo and in vitro and that Smad7-Smurf-mediated negative regulation of TGF-β signaling was impaired in SSc fibroblasts [93].

Therefore, the fibroblast activation in SSc may be a result of stimulation by autocrine TGF-β signaling. This notion is strongly supported by the recent following findings: (1) SSc fibroblasts were shown to produce an amount of TGF-β equivalent to that produced by normal fibroblasts in vitro [27, 90]; (2) SSc fibroblasts express elevated levels of TGF-β receptors, correlating with collagen expression, and the increased levels of TGF-β receptors were shown to induce the expression of ECM proteins [27, 46–49]; (3) the blockade of endogenous TGF-β signaling abolished the scleroderma phenotype [27, 131]; and (4) constitutive activation of TGF-β/ Smad signaling was shown in SSc fibroblasts [93, 109]. Thus, the development of a therapeutic approach to block TGF-β/ Smad signaling in SSc is required.

1.9 Conclusion

Great progress has been made over the past several years in the understanding of TGF-β signaling. The identification of Smad proteins and other signal pathways as downstream signal transduction mediators in TGF-β signaling has lead to the elucidation of molecular mechanisms of gene activation by TGF-β (Fig. 1.3). However, further detailed analyses are required to clarify the pathogenesis of fibrosis in SSc.

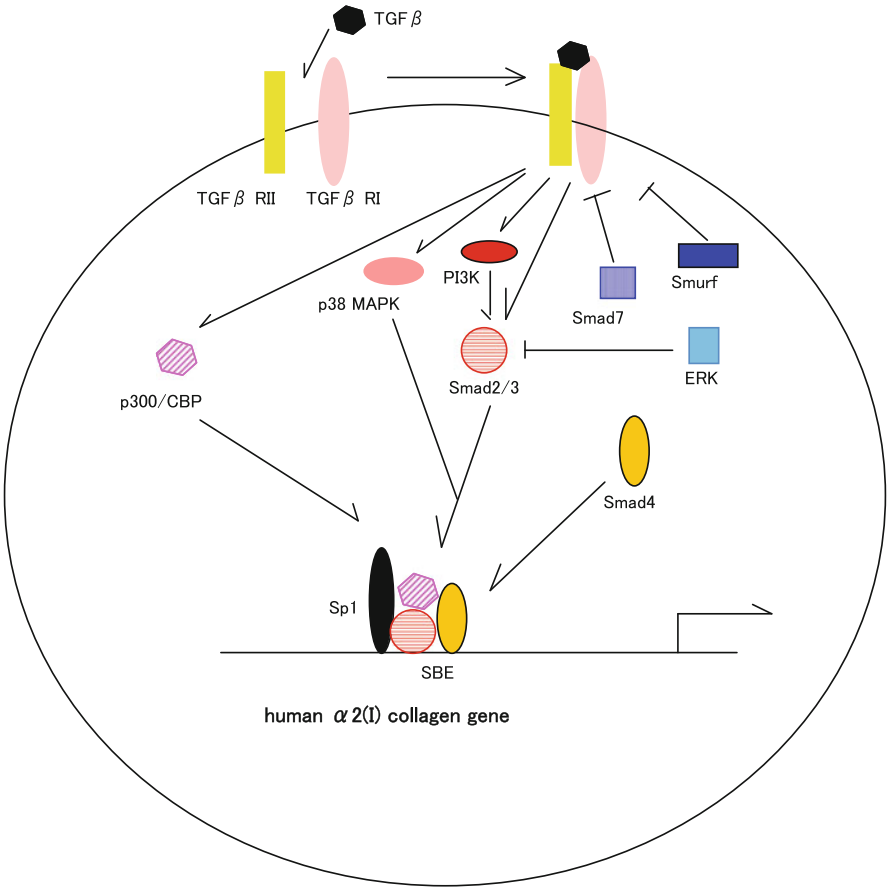

Fig. 1.3 Schematic representation of the TGF-β signaling cascade. Initiation of signaling requires the binding of TGF-β to TGF-β receptor type II (TGFβ RII), resulting in the recruitment and phosphorylation of TGF-β receptor type I (TGFβ RI) to produce a heteromeric complex that activates downstream signaling pathways. Activated TGFβ RI then recruits and phosphorylates R-Smad, such as Smad2 and Smad3, leading to the association with Smad4 and translocation of the formed heterocomplex into the nucleus. The translocated heterocomplex binds to the target gene with other transcription factors, such as Sp1, and regulates gene expression, cooperating with p300/CBP. TGF-β also activates the p38 MAPK and PI3K pathways. TGF-β upregulates Smad7 expression and Smad7 associates with ligand-activated TGFβ RI and interferes with the phosphorylation of Smad2 and Smad3 by preventing their interaction with activated TGFβ RI. Smurfs (Smurf1/Smurf2) induce polyubiquitination and degradation of TGFβ RI. Thus, Smad7-Smurf inhibits TGF-β signaling by a negative feedback system. Activation of ERK inhibits TGF-β/Smad signaling

References

1. LeRoy EC. Systemic sclerosis (scleroderma). In: Wyngaarden JB, Smith LH, Bennett JC, editors. Cecil text book of medicine. 19th ed. Philadelphia: WB Saunders; 1992. p. 1530–5.
2. Korn JH. Immunological aspects of scleroderma. Curr Opin Rheumatol. 1991;3:947–52.
3. LeRoy EC. A brief overview of the pathogenesis of scleroderma (systemic sclerosis). Ann Rheum Dis. 1992;51:286–8.
4. Ihn H. Pathogenesis of fibrosis: role of TGF-β and CTGF. Curr Opin Rheumatol. 2002;14:681–5.
5. Ihn H. The role of TGF-β signaling in the pathogenesis of fibrosis in scleroderma. Arch Immunol Ther Exp. 2002;50:325–31.
6. Ihn H. Scleroderma, fibroblasts, signaling, and excessive extracellular matrix. Curr Rheumatol Rep. 2005;7:156–62.
7. Ihn H. Autocrine TGF-β signaling in the pathogenesis of systemic sclerosis. J Invest Dermatol. 2008;49:103–13.
8. Clark RA. Cutaneous tissue repair: basic biological considerations. J Am Acad Dermatol. 1985;13:701–25.
9. Border WA, Ruoslahti E. Transforming growth factor-β: the dark side of tissue repair. J Clin Invest. 1992;90:1–7.
10. Gospodarowicz D, Moran JS. Mitogenic effect of fibroblast growth factor on early passage cultures of human murine fibroblasts. J Cell Biol. 1975;66:451–7.
11. Cohen S, Carpenter G. Human epidermal growth factor: isolation and chemical and biological properties. Proc Natl Acad Sci U S A. 1975;72:1317–21.
12. Rutherford RB, Ross R. Platelet factors stimulate fibroblasts and smooth muscle cells quiescent in plasma serum to proliferate. J Cell Biol. 1976;69:196–203.
13. Beutler B, Cerami A. Cathectin and tumor necrosis factor as two sides of the same biological coin. Nature. 1986;320:584–8.
14. Postlethwaite AE, Holness MA, Katai H, Raghow R. Human fibroblasts synthesize elevated levels of extracellular matrix protein in response to interleukin 4. J Clin Invest. 1992;90:1479–85.
15. Duncun MR, Berman B. Differential regulation of collagen, glycosaminoglycan, fibronectin and collagenase activity production in cultured human adult dermal fibroblasts by interleukin 1-α and β and tumor necrosis factor-α and β. J Invest Dermatol. 1989;92:699–706.
16. Duncun MR, Berman B. Stimulation of collagen and glycosaminoglycan production in cultured human adult dermal fibroblasts by recombinant human interleukin 6. J Invest Dermatol. 1991;97:686–92.
17. Ihn H, Tamaki K. Oncostatin M stimulates the growth of dermal fibroblasts via a mitogen-activated protein kinase-dependent pathway. J Immunol. 2000;165:2149–55.
18. Jinnin M, Ihn H, Yamane K, Tamaki K. Interleukin-13 stimulates the transcription of the human α2(I) collagen gene in human dermal fibroblasts. J Biol Chem. 2004;279:41783–91.
19. Kulozik M, Hogg A, Lankat-Buttgereit B, Kreig T. Co-localization of transforming growth factor β2 with α1(I) procollagen mRNA in tissue sections of patients with systemic sclerosis. J Clin Invest. 1990;86:917–22.
20. Jelaska A, Arakawa M, Broketa G, Korn JH. Heterogeneity of collagen synthesis in normal and systemic sclerosis skin fibroblasts: increased proportion of high collagen-producing cells in systemic sclerosis fibroblasts. Arthritis Rheum. 1996;39:1338–46.
21. LeRoy EC. Increased collagen synthesis by scleroderma skin fibroblasts in vivo. J Clin Invest. 1974;54:880–9.
22. Peltonen J, Kahari L, Uitto J, Jimenez SA. Increased expression of type VI collagen genes in systemic sclerosis. Arthritis Rheum. 1990;33:1829–35.
23. Xu W, LeRoy EC, Smith EA. Fibronectin release by systemic sclerosis and normal dermal fibroblasts in response to TGF-β. J Rheumatol. 1991;18:241–6.

24. Ludnica L, Varga J, Christiano AM, Iozzo RV, Jimenez SA, Uitto J. Elevated expression of type VII collagen in the skin of patients with systemic sclerosis. Regulation by transforming growth factor-β. J Clin Invest. 1994;93:1709–15.
25. Falanga V, Tiegs SL, Alstadt SP, Roberts AB, Sporn MB. Transforming growth factor β: selective increase in glycosaminoglycan synthesis by cultures of fibroblasts from patients with progressive systemic sclerosis. J Invest Dermatol. 1987;89:100–4.
26. Ihn H, Tamaki K. Increased phosphorylation of transcription factor Sp1 in scleroderma fibroblasts: association with increased expression of type I collagen gene. Arthritis Rheum. 2000;43:2240–7.
27. Ihn H, Yamane K, Kubo M, Tamaki K. Blockade of endogenous transforming growth factor β signaling prevents up-regulated collagen synthesis in scleroderma fibroblasts: association with increased expression of transforming growth factor β receptors. Arthritis Rheum. 2001;44:474–80.
28. Mimura Y, Ihn H, Jinnin M, Asano Y, Yamane TK. Epidermal growth factor induces fibronectin expression in human dermal fibroblasts via protein kinase C δ signaling pathway. J Invest Dermatol. 2004;122:1390–8.
29. Kirk TZ, Mark ME, Chua CC, Chau BH, Mayers MD. Myofibroblasts from scleroderma skin synthesize elevated levels of collagen and tissue inhibitor of metalloproteinase (TIMP-1) with two forms of TIMP-1. J Biol Chem. 1995;270:3423–8.
30. Ihn H, Sato S, Fujimoto M, Kikuchi K, Kadono T, Tamaki T, Tamaki K, Takehara K. Circulating intercellular adhesion molecule-1 in the sera of patients with systemic sclerosis: enhancement by inflammatory cytokines. Br J Rheumatol. 1997;36:1270–5.
31. Duncan MA, Hasan A, Berman B. Oncostatin M stimulates collagen and glycosaminoglycan production by cultured normal dermal fibroblasts: insensitivity of sclerodermal and keloidal fibroblasts. J Invest Dermatol. 1995;104:128–33.
32. Lee KS, Ro YJ, Ryoo YW, Kwon HJ, Song JY. Regulation of interleukin-4 on collagen gene expression by systemic sclerosis fibroblasts in culture. J Dermatol Sci. 1996;12:110–7.
33. Nakashima T, Jinnin M, Yamane K, Honda N, Kajihara I, Makino T, Masuguchi S, Fukushima S, Okamoto Y, Hasegawa M, Fujimoto M, Ihn H. Impaired IL-17 signaling pathway contributes to the increased collagen expression in scleroderma fibroblasts. J Immunol. 2012;188:3573–83.
34. Massague J. The transforming growth factor-β family. Annu Rev Cell Biol. 1990;6:597–641.
35. LeRoy EC, Smith EA, Kahaleh MB, Trojanowska M, Silver RM. A strategy for determining the pathogenesis of systemic sclerosis: is transforming growth factor β the answer? Arthritis Rheum. 1989;32:817–25.
36. Massague J. TGF-β signal transduction. Ann Rev Biochem. 1998;67:753–91.
37. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D, Annunziata N, Doetschman T. Targeted disruption of the mouse transforming growth factor-β1 gene results in multifocal inflammatory disease. Nature. 1992;359:693–9.
38. Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, Cardell EL, Doetschman T. TGFβ2 knockout mice have multiple developmental defects that are non-overlapping with other TGFβ knockout phenotypes. Development. 1997;124:2659–70.
39. Proetzel G, Pawlowski SA, Wiles MV, Boivin GP, Howles PN, Ding J, Ferguson MW, Doetschman T. Transforming growth factor-β3 is required for secondary palate fusion. Nat Genet. 1995;11:409–14.
40. Needleman BW, Choi J, Burrows-Menzu A, Fontana JA. Secretion and binding of transforming growth factor β by scleroderma and normal dermal fibroblasts. Arthritis Rheum. 1990;33:650–3.
41. Wrana JL, Attisano L, Weisser R, Ventura F, Massague J. Mechanism of activation of the TGF-β receptor. Nature. 1994;370:341–7.

42. Saitoh M, Nishitoh H, Amagasa T, Miyazono K, Takagi M, Ichijo H. Identification of important regions in the cytoplasmic juxtamembrane domain of type I receptor that separate signaling pathways of transforming growth factor-β. J Biol Chem. 1996;271:2769–75.
43. Kawakami T, Ihn H, Xu W, Smith E, LeRoy EC, Trojanowska M. Increased expression of TGF-β receptors by scleroderma fibroblasts: evidence for contribution of autocrine TGF-β signaling to scleroderma phenotype. J Invest Dermatol. 1998;110:47–51.
44. Kubo M, Ihn H, Yamane K, Tamaki K. Up-regulated expression of transforming growth factor β receptors in dermal fibroblasts in skin sections from patients with localized scleroderma. Arthritis Rheum. 2001;44:731–4.
45. Roulot D, Sevcsik AM, Coste T, Strosberg AD, Marullo S. Role of transforming growth factor-β type II receptor in hepatic fibrosis: studies of human chronic hepatitis C and experimental fibrosis in rats. Hepatology. 1999;29:1730–8.
46. Kubo M, Ihn H, Yamane K, Tamaki K. Upregulated expression of transforming growth factor-β receptors in dermal fibroblasts of skin sections from patients with systemic sclerosis. J Rheumatol. 2002;29:2558–64.
47. Yamane K, Ihn H, Kubo M, Tamaki K. Increased transcriptional activities of transforming growth factor β receptors in scleroderma fibroblasts. Arthritis Rheum. 2002;46:2421–8.
48. Yamane K, Ihn H, Tamaki K. Epidermal growth factor up-regulates expression of transforming growth factor TGF-β receptor type II in human dermal fibroblasts by phosphoinositide 3-kinase/Akt signaling pathway: resistance to epidermal growth factor stimulation in scleroderma fibroblasts. Arthritis Rheum. 2003;48:1652–66.
49. Yamane K, Ihn H, Asano Y, Jinnin M, Tamaki K. Antagonistic effects of TNF-α on TGF-β signaling through down-regulation of TGF-β receptor type II in human dermal fibroblasts. J Immunol. 2003;171:3855–62.
50. Bloom BB, Humphries DE, Kuang PP, Fine A, Goldstein RH. Structure and expression of the promoter for the R4/ALK5 human type I transforming growth factor-β receptor: regulation by TGF-β. Biochim Biophys Acta. 1996;1312:243–8.
51. Czuwara-Ladykowska J, Gore EA, Shegogue DA, Smith EA, Trojanowska M. Differential regulation of transforming growth factor-β receptors type I and type II by platelet-derived growth factor in human dermal fibroblasts. Br J Dermatol. 2001;145:569–75.
52. Leask A, Abraham DJ, Finlay DR, Holmes A, Pennington D, Shi-Wen X, Chen Y, Venstrom K, Dou X, Ponticos M, et al. Dysregulation of transforming growth factor-β signaling in scleroderma: overexpression of endoglin in cutaneous scleroderma fibroblasts. Arthritis Rheum. 2002;46:1857–65.
53. Pannu J, Gore-Hyer E, Yamanaka M, Smith EA, Rubinchik S, Dong JY, Jablonska M, Blaszczyk M, Trojanowska M. An increased transforming growth factor β receptor type I: type II ratio contributes to elevated collagen protein synthesis that is resistant to inhibition via a kinase-deficient transforming growth factor β receptor type II in scleroderma. Arthritis Rheum. 2004;50:1566–77.
54. Qi Z, Atsuchi N, Ooshima A, Takeshita A, Ueno H. Blockade of type β transforming growth factor signaling prevents liver fibrosis and dysfunction in the rat. Proc Natl Acad Sci U S A. 1999;96:2345–9.
55. Trojanowska M, Smith E, Dong JY, Gore E. Overexpression of a kinase-deficient TGFβ receptor II via an adenoviral vector potently inhibits extracellular matrix production in scleroderma and normal dermal fibroblasts. Arthritis Rheum. 1999;42:S203.
56. Massague J, Chen YG. Controlling TGF-β signaling. Genes Dev. 2000;14:627–44.
57. Shi Y, Hata A, Lo RS, Massague J, Pavletich NP. A structure basis for mutational inactivation of the tumor suppressor Smad4. Nature. 1997;388:87–93.
58. Zawel L, Dai JL, Buckhaults P, Zhou S, Kinzler KW, Vogelstein B, Kern SE. Human Smad3 and Smad4 are sequence-specific transcription activators. Mol Cell. 1998;1:611–7.
59. Imamura T, Takase M, Nishihara A, Oeda E, Hanai J, Kawabata M, Miyazono K. Smad6 inhibits signaling by the TGF- β superfamily. Nature. 1997;389:622–6.

60. Hocevar B, Brown TL, Howe PH. TGFβ induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. EMBO J. 1999;18:1345–56.
61. Verrecchia F, Chu ML, Mauviel A. Identification of novel TGFβ/Smad gene targets in dermal fibroblasts using a combined cDNA microarray/promoter transactivation approach. J Biol Chem. 2001;276:17058–62.
62. Ihn H, Ohnishi K, Tamaki T, LeRoy EC, Trojanowska M. Transcriptional regulation of the human α2(I) collagen gene: combined action of upstream stimulatory and inhibitory cis-acting element. J Biol Chem. 1996;271:26717–23.
63. Ihn H, LeRoy EC, Trojanowska M. Oncostatin M stimulates transcription of the human α2 (I) collagen gene via the Sp1/Sp3-binding site. J Biol Chem. 1997;272:24666–72.
64. Ihn H, Trojanowska M. Sp3 is a transcriptional activator of the human α2(I) collagen gene. Nucleic Acids Res. 1997;25:3712–7.
65. Ihn H, Tamaki K. Competition analysis of the human α2(I) collagen promoter using synthetic oligonucleotides. J Invest Dermatol. 2000;114:1011–6.
66. Ihn H, Ihn Y, Trojanowska M. Sp1 phosphorylation induced by serum stimulates the human α2(I) collagen gene. J Invest Dermatol. 2001;117:301–8.
67. Inagaki Y, Truter S, Ramirez F. Transforming growth factor-β stimulates α2(I) collagen gene expression through a cis-acting element that contains an Sp1-binding site. J Biol Chem. 1994;269:14828–34.
68. Greenwel P, Inagaki Y, Hu W, Walsh M, Ramirez F. Sp1 is required for the early response of α2(I) collagen to transforming growth factor-β1. J Biol Chem. 1997;272:19738–45.
69. Chen SJ, Yuan W, Mori Y, Levenson A, Trojanowska M, Varga J. Stimulation of type I collagen transcription in human skin fibroblasts by TGF-β: involvement of Smad3. J Invest Dermatol. 1999;112:49–57.
70. Chen SJ, Yuan W, Lo S, Trojanowska M, Varga J. Interaction of Smad3 with proximal Smad-binding element of the human α2(I) collagen gene promoter required for transcriptional activation by TGF-β. J Cell Physiol. 2000;183:381–92.
71. Mori Y, Chen SJ, Varga J. Modulation of endogenous Smad expression in normal skin fibroblasts by transforming growth factor-β. Exp Cell Res. 2000;258:374–83.
72. Zhang W, Ou J, Inagaki Y, Greenwel P, Ramirez F. Synergistic cooperation between Sp1 and Smad3/Smad4 mediates transforming growth factor-β1 stimulation of α2(I) collagen (COL1A2) transcription. J Biol Chem. 2000;275:39237–45.
73. Poncelet AC, Schnaper HW. Sp1 and Smad proteins cooperate to mediate transforming growth factor-β1-induced α2(I) collagen expression in human glomerular mesangial cells. J Biol Chem. 2001;276:6983–92.
74. Inagaki Y, Nemoto T, Nakao A, ten Dijke P, Kobayashi K, Takehara K, Greenwel P. Interaction between GC box binding factors and Smad proteins modulates cell lineage-specific α2(I) collagen gene transcription. J Biol Chem. 2001;276:16573–9.
75. Ghosh AK, Yuan W, Mori Y, Varga J. Smad-dependent stimulation of type I collagen gene expression in human skin fibroblasts by TGF-β involves functional cooperation with p300/CBP transcriptional coactivators. Oncogene. 2000;19:3546–55.
76. Ghosh AK, Yuan W, Mori Y, Chen SJ, Varga J. Antagonistic regulation of type I collagen gene expression by interferon-γ and transforming growth factor-β. Integration at the level of p300/CBP transcriptional coactivators. J Biol Chem. 2001;276:11041–8.
77. Czuwara-Ladykowska J, Sementchenko VI, Watson FK, Trojanowska M. Ets1 is an effector of the TGF-β signaling pathway and an antagonist of the profibrotic effects of TGF-β. J Biol Chem. 2002;277:20399–408.
78. Jinnin M, Ihn H, Asano Y, Yamane K, Trojanowska M, Tamaki K. Tenascin-C upregulation by transforming growth factor-β in human dermal fibroblasts involves Smad3, Sp1, and Ets1. Oncogene. 2004;23:1656–67.
79. Shirasaki F, Makhluf HA, LeRoy EC, Watson DK, Trojanowska M. Ets transcription factors cooperate with Sp1 to activate the human tenascin-C promoter. Oncogene. 1999;18:7754–64.

80. Czuwara-Ladykowska J, Shirasaki F, Jackers P, Watson DK, Trojanowska M. Fli-1 inhibits collagen type I production in human dermal fibroblasts via an Sp-dependent manner. J Biol Chem. 2001;15:20839–48.
81. Trojanowska M. Ets factors and regulation of the extracellular matrix. Oncogene. 2000;18:6464–71.
82. Yuan W, Varga J. Transforming growth factor-β repression of matrix metalloproteinase 1 in dermal fibroblasts involves Smad3. J Biol Chem. 2001;276:38502–10.
83. Flanders KC, Sullivan CD, Fujii M, Sowers A, Anzano MA, Arabshahi A, Major C, Deng C, Russo A, Mitchell JB, Roberts AB. Mice lacking Smad3 are protected against cutaneous injury induced by ionizing radiation. Am J Pathol. 2002;160:1057–68.
84. Zhao J, Shi W, Wang YL, Chen H, Bringas Jr P, Datto MB, Frederick JP, Wang XF, Warburton D. Smad3 deficiency attenuates bleomycin-induced pulmonary fibrosis in mice. Am J Physiol Lung Cell Mol Physiol. 2002;282:585–93.
85. Lee DK, Park SH, Yi Y, Choi SG, Lee C, Parks WT, Cho H, de Caestecker MP, Shual Y, Roberts AB, Kim SJ. The hepatitis B virus encoded oncoprotein pX amplifies TGF-β family signaling through direct interaction with Smad4: potential mechanism of hepatitis B virus-induced liver fibrosis. Genes Dev. 2001;15:455–66.
86. Yang J, Zhang X, Li Y, Liu Y. Downregulation of Smad transcriptional corepressors SnoN and Ski in the fibrotic kidney: an amplication mechanism for TGF-β1 signaling. J Am Soc Nephrol. 2003;14:3167–77.
87. Segar R, Krebs EG. The MAPK signaling cascade. FASEB J. 1995;9:726–98.
88. Ihn H, Yamane K, Asano Y, Kubo M, Tamaki K. IL-4 up-regulates the expression of tissue inhibitor of metalloproteinase-2 in dermal fibroblasts via the p38 mitogen-activated protein kinase-dependent pathway. J Immunol. 2002;168:1895–902.
89. Ihn H, Yamane K, Tamaki K. Increased phosphorylation and activation of mitogen-activated protein kinase p38 in scleroderma fibroblasts. J Invest Dermatol. 2005;125:247–55.
90. Holmes A, Abraham DJ, Sa S, Shiwen X, Black CM, Leask A. CTGF and SMADs, maintenance of scleroderma phenotype is independent of SMAD signaling. J Biol Chem. 2001;276:10594–601.
91. Mori Y, Chen SJ, Varga J. Expression and regulation of intracellular SMAD signaling in scleroderma skin fibroblasts. Arthritis Rheum. 2003;48:1964–78.
92. Dong C, Zhu S, Wang T, Yoon W, Alvarez RJ, ten Dijke P, White B, Wigley FM, Goldschmidt-Clemont PJ. Deficient Smad7 expression: a putative molecular defect in scleroderma. Proc Natl Acad Sci U S A. 2002;99:3908–13.
93. Asano Y, Ihn H, Yamane K, Kubo M, Tamaki K. Impaired Smad7-Smurf-mediated negative regulation of TGF-β signaling in scleroderma fibroblasts. J Clin Invest. 2004;113:253–64.
94. Janknecht R, Wells NJ, Hunter T. TGFβ stimulated cooperation of SMAD proteins with the coactivators CBP/p300. Genes Dev. 1998;12:2114–9.
95. Verrecchia F, Pessah M, Atfi A, Mauviel A. Tumor necrosis factor-α inhibits transforming growth factor-β/SMAD signaling in human dermal fibroblasts via AP-1 activation. J Biol Chem. 2000;275:30226–31.
96. Luo K, Stroschein SL, Wang W, Chen D, Martens Z, Zhou S, Zhou Q. The Ski oncoprotein interacts with the SMAD proteins to repress TGFβ signaling. Genes Dev. 1999;13:2196–206.
97. Sun Y, Liu X, Eaton EN, Lodish HF, Weinberg RA. SnoN and Ski protooncoproteins are rapidly degraded in response to transforming growth factor β signaling. Proc Natl Acad Sci U S A. 1999;96:12442–7.
98. Wotton D, Lo RS, Lee S, Massague J. A SMAD transcriptional corepressor. Cell. 1999;97:29–39.
99. Kim RH, Wang D, Tsang M, Martin J, Huff C, deCaestecker MP, Parks WT, Meng X, Lechleider RJ, Wang T, Roberts AB. A novel SMAD nuclear interacting protein, SNIP1, suppresses p300-dependent TGFβ signal transduction. Genes Dev. 2000;14:1605–16.
100. Jinnin M, Ihn H, Mimura Y, Asano Y, Tamaki K. Involvement of the constitutive complex formation of c-Ski/SnoN with Smads in the impaired negative feedback regulation of

transforming growth factor b signaling in scleroderma fibroblasts. Arthritis Rheum. 2007;56:1694–705.
101. Ihn H. p38 MAP kinase inhibitors in dermatology. Exp Rev Dermatol. 2007;2:403–7.
102. Reunanen N, Foschi M, Han J, Kahari VM. Activation of extracellular signal-regulated kinase 1/2 inhibits type I collagen expression by human skin fibroblasts. J Biol Chem. 2000;275:34634–9.
103. Cantley LC. The phosphoinositide 3-kinase pathway. Science. 2002;296:1655–7.
104. Toker A. Protein kinases as mediators of phosphoinositide 3-kinase signaling. Mol Pharmacol. 2000;57:652–8.
105. Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, Arteaga CL. Phosphatidylinositol 3-kinase function is required for transforming growth factor β-mediated epithelial to mesenchymal transition and cell migration. J Biol Chem. 2000;275:36803–10.
106. Runyan CE, Schnaper HW, Poncelet A-C. The phosphatidylinositol 3-kinase/Akt pathway enhances Smad3-stimulated mesangial cell collagen I expression in response to transforming growth factor-β1. J Biol Chem. 2004;279:2632–9.
107. Tezuka T, Chiang TA, Davison AF, Attisano L, Wrana JL. SARA, a FYVE domain protein that recruits Smad2 to the TGF-β receptor. Cell. 1998;95:779–91.
108. Itoh F, Divecha N, Brocks L, Oomen L, Janssen H, Calafat J, Itoh S, ten Dijke P. The FYVE domain in Smad anchor for receptor activation (SARA) is sufficient for localization of SARA in early endosomes and regulates TGF-β/Smad signaling. Genes Cells. 2002;7:321–31.
109. Asano Y, Ihn H, Yamane K, Jinnin M, Mimura Y, Tamaki K. Phosphatidylinositol 3-kinase is involved in α2(I) collagen gene expression in normal and scleroderma fibroblasts. J Immunol. 2004;172:7123–35.
110. Shegogou D, Trojanowska M. Mammalian target of rapamycin positively regulates collagen type I production via a phsphatidylinositol 3-kinase-independent pathway. J Biol Chem. 2004;279:23166–75.
111. Bradham DM, Igarashi A, Potter RL, Grotendorst GR. Connective tissue growth factor: a cystein-rich mitogen secreted by human vascular endothelial cells is related to the SRC induced immediate early gene product CEF-10. J Cell Biol. 1991;114:1285–94.
112. Igarashi A, Okochi H, Bradham DM, Grotendorst GR. Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. Mol Biol Cell. 1993;4:637–45.
113. Shinozaki M, Kawara S, Hayashi N, Kakinuma T, Igarashi A, Takehara K. Induction of subcutaneous tissue fibrosis in newborn mice by transforming growth factor β: simultaneous application with basic fibroblast growth factor causes persistant fibrosis. Biochem Biophys Res Commun. 1997;240:292–7.
114. Mori T, Kawara S, Shinozaki M, Hayashi N, Kakinuma T, Igarashi A, Takigawa M, Nakanishi T, Takehara K. Role and interaction of connective tissue growth factor with transforming growth factor β in persistant fibrosis: a mouse fibrosis model. J Cell Physiol. 1999;181:153–9.
115. Igarashi A, Nashiro K, Kikuchi K, Sato S, Ihn H, Grotendorst GR, Takehara K. Significant correlation between connective tissue growth factor gene expression and skin sclerosis in tissue sections from patients with systemic sclerosis. J Invest Dermatol. 1995;105:280–4.
116. Igarashi A, Nashiro K, Kikuchi K, Sato S, Ihn H, Fujimoto M, Grotendorst GR, Takehara K. Connective tissue growth factor gene expression in tissue sections from localized scleroderma, keloid, and other fibrotic skin disorders. J Invest Dermatol. 1996;106:723–33.
117. Frazer K, Williams SKD, Klapper H, Grotendorst GR. Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. J Invest Dermatol. 1996;107:404–11.
118. Paradis V, Dargere D, Vidaud M, De Gouville AC, Huet S, Martinez V, Gauthier JM, Sobesky R, Ratziu V, Bedossa P. Expression of connective tissue growth factor in experimental rat and human liver fibrosis. Hepatology. 1999;30:968–76.

119. Ziesche R, Hofbauer E, Wittmann K, Petkov V, Block LH. A preliminary study of long-term treatment with interferon γ-1β and low-dose prednisolone in patients with idiopathic pulmonary fibrosis. N Engl J Med. 1999;341:1264–9.
120. Abou-Shady M, Freiss H, Zimmermann A, di Mola FF, Guo XZ, Baer HU, Buchler MW. Connective tissue growth factor in human liver cirrhosis. Liver. 2000;20:296–304.
121. Chen MM, Lam A, Abraham JA, Schreiner GF, Joly AH. CTGF expression is induced by TGF-β in cardiac myocytes: a potential role in heart fibrosis. J Mol Cell Cardiol. 2000;32:1805–19.
122. Stratton R, Shiwen X, Martini G, Holmes A, Leask A, Haberberger T, Martin GR, Black CM, Abraham D. Iloprost suppresses connective tissue growth factor production in fibroblasts and in the skin of scleroderma patients. J Clin Invest. 2001;108:241–50.
123. Takehara K. Hypothesis: pathogenesis of systemic sclerosis. J Rheumatol. 2003;30:755–9.
124. Gore-Hyer E, Pannu J, Smith EA, Grotendorst G, Trojanowska M. Selective stimulation of collagen synthesis in the presence of costimulatory insulin signaling by connective tissue growth factor in scleroderma fibroblasts. Arthritis Rheum. 2003;48:798–806.
125. Honda N, Jinnin M, Kajihara I, Makino T, Makino K, Masuguchi S, Fukushima S, Okamoto Y, Hasegawa M, Fujimoto M, Ihn H. TGF-β-mediated down-regulation of microRNA-196a contributes to the constitutive upregulated type I collagen expression in scleroderma dermal fibroblasts. J Immunol. 2012;188:3323–31.
126. Sing T, Jinnin M, Yamane K, Honda N, Makino K, Kajihara I, Makino T, Sakai K, Masuguchi S, Fukushima S, Ihn H. microRNA-92a expression in the sera and dermal fibroblasts increases in patients with scleroderma. Rheumatology. 2012;51:1550–6.
127. Makino K, Jinnin M, Hirano A, Yamane K, Eto M, Kusano T, Honda N, Kajihara I, Makino T, Sakai K, Masuguchi S, Fukushima S, Ihn H. The down-regulation of microRNA let-7a contributes to the excessive expression of type I collagen in systemic and localized scleroderma. J Immunol. 2012;190:3905–15.
128. Honda N, Jinnin M, Kira-Etoh T, Makino K, Kajihara I, Makino T, Fukushima S, Inoue Y, Okamoto Y, Hasegawa M, Fujimoto M, Ihn H. miR-150 down-regulation contributes to the constitutive type I collagen overexpression in scleroderma dermal fibroblasts via the induction of integrin β3. Am J Pathol. 2013;182:206–16.
129. Takeda K, Hatamochi A, Ueki H, Nakata M, Oishi Y. Decreased collagenase expression in cultured systemic sclerosis fibroblasts. J Invest Dermatol. 1994;103:359–63.
130. Sappino AP, Masouye I, Saurat JH, Gabbiani G. Smooth muscle differentiation in scleroderma fibroblastic cells. Am J Pathol. 1990;137:585–91.
131. Asano Y, Ihn H, Yamane K, Kubo M, Tamaki K. Increased expression levels of integrin αvβ5 on scleroderma fibroblasts. Am J Pathol. 2004;164:1275–92.
132. Asano Y, Ihn H, Jinnin M, Tamaki K, Sato S. Altered dynamics of transforming growth factor β (TGF- β) receptors in scleroderma fibroblasts. Ann Rheum Dis. 2011;70:384–7.
133. Ihn H, Yamane K, Asano Y, Tamaki K. Constitutive phosphorylated Smad3 interacts with Sp1 and p300 in scleroderma fibroblasts. Rheumatology. 2006;45:157–65.
134. Jinnin M, Ihn H, Mimura Y, Asano Y, Tamaki K. Potential regulatory elements of the constitutive up-regulated α2(I) collagen gene in scleroderma dermal fibroblasts. Biochem Biophys Res Commun. 2006;343:904–9.

Autoimmunity in Systemic Sclerosis: Overview

2

Ayumi Yoshizaki and Shinichi Sato

Abstract

Systemic sclerosis (SSc) is characterized by autoimmunity and tissue fibrosis of several organs. Although the pathogenic relationship between systemic autoimmunity and the clinical manifestations of SSc remains unknown, SSc patients display a variety of abnormal immune activation including the production of disease-specific autoantibodies. Previous studies have demonstrated that immune cells, mainly including T and B cells, play a critical role in systemic autoimmunity and disease expression, though the role of autoimmunity in generating the clinical and pathologic phenotype in SSc remains uncertain. Activation and polarization of T cells can contribute to a profibrotic environment. Oligoclonal T cells, preferentially producing type 2 cytokines, exist in affected tissues and peripheral blood early in the disease course and seem to be participating in the establishment of fibrosis. Similarly, SSc patients have B cell abnormalities characterized by chronic hyper-reactivity of memory B cells, possibly due to CD19 overexpression. CD19 is a crucial regulator of B cell activation. Recent studies demonstrated B cells from SSc patients show an upregulated CD19 signaling pathway that induces SSc-specific autoantibody production in SSc mouse models. Although distinct subsets of autoantibodies do not have a proven pathogenic role, they are selectively associated with unique disease manifestations. Collectively, autoimmunity in SSc is most likely participating in SSc-specific tissue damage. If revealed the mechanisms of

A. Yoshizaki (✉) • S. Sato
Department of Dermatology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, 113-8655 Bunkyo-ku, Tokyo, Japan
e-mail: ayuyoshi@me.com

K. Takehara et al. (eds.), *Systemic Sclerosis*, DOI 10.1007/978-4-431-55708-1_2

autoimmunity in SSc, these knowledge could lead to new disease-modifying therapeutic strategies directed at SSc-specific immune effector pathways.

Keywords
Systemic sclerosis • Autoimmunity • B lymphocyte • T lymphocyte • Fibrosis

2.1 Introduction

Systemic sclerosis (SSc) is a connective tissue disease characterized by excessive extracellular matrix deposition in the skin and visceral organs with an autoimmune background [1]. Although the pathogenesis of SSc remains unknown, three major abnormalities (Fig. 2.1), including immune activation, collagen accumulation, and vascular injury, are considered as main features of disease [2–4]. Collagen accumulation crucially results in fibrosis of the several organs, such as the skin and lungs. Vascular injury mainly consists of Raynaud's phenomenon, digital ulcers, scleroderma renal crisis, and pulmonary hypertension. Immune activation is characterized by autoantibody production, lymphocyte activation, and release of various cytokines. The presence of autoantibodies is a central feature of immune activation associated with SSc, because antinuclear antibody (Ab) has been detected in >90 % of patients [5]. SSc patients have autoantibodies that react to various intracellular components, such as DNA topoisomerase I (topo I), centromere, RNA polymerases, U1RNP, U3RNP, Th/To, and histones, though these distinct subsets of autoantibodies do not have a proven pathogenic role [5].

The cytokines released by lymphocytes infiltrating the affected tissue may cause vascular injury and collagen production. In SSc, differentiation toward several T helper (Th) lymphocyte subsets (Fig. 2.2) has been reported [6]. The key profibrotic

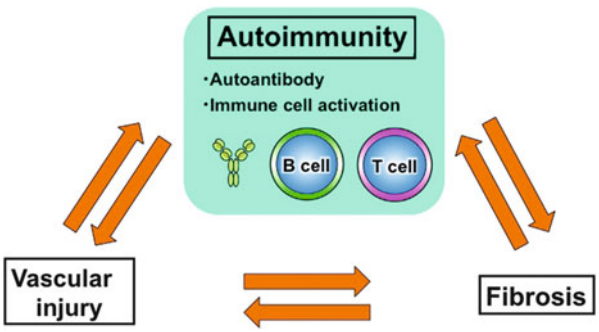

Fig. 2.1 The relationship of autoimmunity, tissue fibrosis, and vascular injury in SSc. SSc is characterized by autoimmunity, tissue fibrosis, and vascular injury. Although the pathogenic relationship between autoimmunity and clinical manifestations of SSc remains unclear, three major abnormalities contribute each other to the disease progression and development

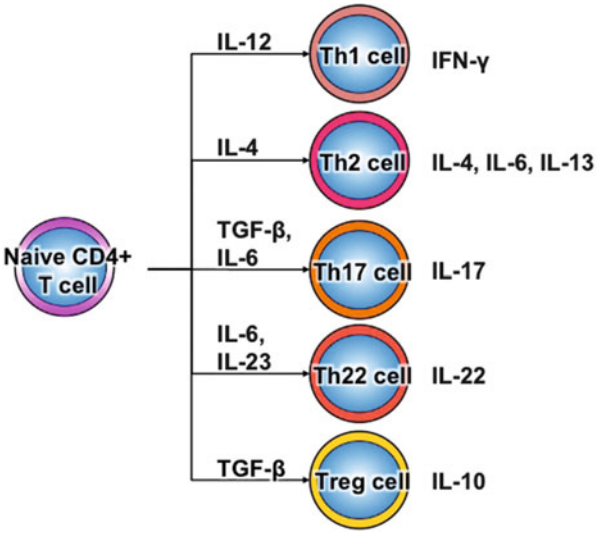

Fig. 2.2 CD4+ T cell differentiation and function. CD4+ naive T cells differentiate into several effector subsets with specific secretion of signature cytokines. The generation of these cell populations depends on particular cognate interactions and cytokines. After lineage commitment, these cells are transcriptionally regulated by many factors and secrete effector cytokines

cytokines, such as interleukin (IL)-4, IL-6, and IL-13, are secreted by Th2 cells, and their interactions with fibroblasts serve to drive the fibrotic response [7]. Substantial progress in the knowledge of T cell differentiation has been achieved in the past two decades (Fig. 2.2). IL-6 can be neutralized in vivo not only to limit IL-6-driven tissue fibrosis but simultaneously to suppress switching of regulatory T (Treg) to Th17 T cells that will provide more IL-6. Recent studies have indicated that B cells have various fundamental roles in regulating immune responses [8, 9] than had previously been appreciated (Fig. 2.3). These roles include antigen presentation, cytokine production, lymphoid organogenesis, differentiation of T cells, and influence on dendritic cell and macrophage function. Consistently, not only autoantibody production but also abnormalities of other B cell function could lead to the induction or development of autoimmune disorders. SSc patients have polyclonal B cell hyperactivity and hyper-γ-globulinemia in addition to autoantibody production [10]. Thus, immunological abnormalities are likely to be critical for the development and progression of the disease, including SSc-specific tissue fibrosis and vascular damage. However, it remains unknown how these three major abnormalities can be unified into one hypothesis. In this section, we will highlight evidence indicating that the autoimmune response may participate in initiating and/or developing fibroblast modifications that are characteristically observed in SSc.

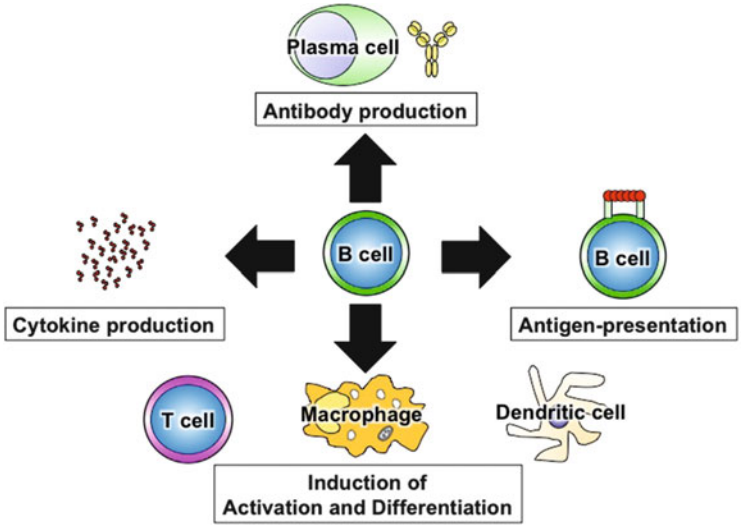

Fig. 2.3 Multiple roles of B cells in immune system and autoimmunity. B cells regulate immune responses and crucially contribute to immune system development. B cells are not only involved in natural, adaptive, and autoantibody production but also interact with T cells and other antigen-presenting cells, such as macrophages and dendritic cells, produce multiple regulatory cytokines, and are critical for lymphoid tissue development

2.2 T Cell Autoimmune Responses in SSc

2.2.1 The Th2 Polarization in SSc

CD4+ T cells consist of subgroups characterized by distinct cytokine secretion patterns. Histological examination of early SSc skin has demonstrated that an inflammatory infiltrate precedes fibrosis and development of the vasculopathy [11]. Interestingly, collagen synthesis assessed by in situ localization of procollagen appears to be higher in fibroblasts adjacent to inflammatory cells [12, 13]. These studies have suggested the hypothesis that inflammatory cells and in particular T cells provide important stimuli that drive collagen synthesis in fibroblasts. Indeed, transcriptome analysis in animal models has shown that genes involved in wound healing and fibrosis are associated to mainly Th2-polarized responses as opposed to Th1-polarized responses [14].

Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13, thereby facilitating humoral immunity. There are several reports of high levels of Th2 cytokines in SSc serum such as IL-4 and IL-13 [15–17]. IL-4 is crucial in polarizing Th2 response mediated through its receptor and intracellular signaling molecules, such as STAT6. However, IL-4 does not work alone, and IL-13 is also necessary to mount an appropriate Th2 response. Th2 cells work in a positive feedback loop

perpetuating the phenotype and secretion of Th2-type fibrogenic cytokines. IL-4, IL-6, and IL-13 stimulate the synthesis of collagen by human fibroblasts [18, 19]. Furthermore, these Th2 cytokines enhance the immunoglobulin production by B cells [20]. By contrast, Th1 cytokines, interferon (IFN)-γ and tumor necrosis factor (TNF)-α, suppress collagen production by fibroblasts in vitro [19]. Therefore, a relative shift to Th2 compared with Th1 cytokines can induce tissue fibrosis and antibody production.

2.2.2 Th1/Th2 Balance in SSc

A number of studies have been reported that Th2 cytokines increased serum IL-4, IL-6, IL-10, and IL-13 levels in SSc patients [21–23]. In addition, peripheral blood mononuclear cells from SSc patients show elevated IL-6 production in vitro [24]. Especially, CD4+ T cell clones derived from the skin of SSc patients exhibit a Th2 cytokine profile [25]. Classic Th2 cytokines include IL-4, IL-5, and IL-13. IL-4 is a multifunctional cytokine produced by activated Th2 T cells. IL-4 normally promotes humoral immunity by inducing immunoglobulin production and isotype switching of B cells and is also involved in the differentiation of naive CD4+ T cells toward a Th2 phenotype [26]. Antibody-mediated depletion of T cells in bleomycin (BLM)-induced SSc model mice, which is a well-known established mouse model of SSc, decreased fibrosis along with reduced IL-4 secretion [27]. Elevated levels of IL-4 have been demonstrated in affected tissues of SSc patients [28]. Direct stimulation of human fibroblasts with IL-4 leads to a significant increased expression of collagen and fibronectin, and SSc-derived fibroblasts are more sensitive to such induction [29]. In the tight-skin (TSK/+) mouse, which is a genetic model for human SSc, treatment with antibodies against IL-4 prevented the induction of dermal fibrosis [30]. Furthermore, in BLM-induced SSc model mice, genetic deletion of the IL-4 gene reduced fibrosis and fibroblast collagen production compared with wild-type mice [31].

IL-13 is an immunoregulatory cytokine also produced predominantly by activated Th2 cells. IL-13 and IL-4 share a common IL-4 receptor alpha chain and signal through STAT6 [32]. Therefore, IL-13 shares many functional activities with IL-4, but their function is not redundant [33]. Although IL-4 is important for polarization of naive CD4+ T cells, IL-13 is not necessary. Indeed, IL-13 was found to be directly fibrotic in a hepatic fibrosis model, and deletion of IL-13 was associated with reduced fibrosis and collagen deposition. IL-13 has also been required for the development of cutaneous fibrosis mediated by IL-33, as genetic deletion of IL-13, but not of IL-4, abrogates IL-33-mediated fibrosis and collagen deposition, and this was independent of tumor growth factor (TGF)-β expression [34].

By contrast, plasma levels of IFN-γ and IFN-γ production by peripheral mononuclear cells are markedly reduced in SSc patients compared with normal controls [23, 35]. Thus, cytokine balance is generally inclined to Th2 rather than Th1 in SSc patients. However, on the contrary, a strong shift to Th1-cytokine-producing cells

among CD8+ T cells has been shown in the peripheral blood from SSc patients [36]. Furthermore, an increased number of IFN-γ positive cells is reported in peripheral T cells from SSc patients [37]. Therefore, there is a possibility that Th1 cytokines, in addition to Th2 cytokines, also play a role in the development of SSc.

2.2.3 Other Th Subsets in SSc Pathogenesis

The presence and role of Th17 and Treg cells in SSc have attracted great interest in the last few years, and they remain an area of intense investigation. In 2005, Th17 cells, which produce IL-17A and IL-17 F, were firstly described [38]. Th17 cells have protective effect against extracellular bacteria and fungi. Moreover, Th17 cells and IL-17 cytokines are implicated in the pathogenesis of several inflammatory and autoimmune diseases, including experimental autoimmune encephalomyelitis and collagen-induced arthritis [39]. Th17 cells require retinoid-related orphan receptor-γ to differentiate in response to a combination of TGF-β and IL-6. The percentage of Th17 cells is consistently increased in SSc [40]. In fact, increased levels of IL-17A have been detected in the serum of SSc patients [41, 42], and increased IL-17A mRNA levels were identified in the involved skin and lung [43]. On the other hand, IL-17 F, which shares an amino acid sequence identity of more than 50 % with IL-17A, was not found to be increased in the involved skin and serum of SSc individuals [44].

Treg cells are reciprocally linked with Th17 cells. TGF-β appears to be involved in the generation of both subsets, whereas the presence or absence of IL-6 skews the differentiation process toward inflammatory Th17 or suppressive Treg cells, respectively. While Th17 cells have been mostly found increased in the disease, Treg cells have been reported to be reduced in number or functionally defective in several studies [45–48]. Indeed, in SSc patients, several studies showed a reduced frequency and impaired function of Treg cells [47, 49]. Furthermore, immunohistochemical approach has revealed that FoxP3+ Treg cells decreased in the skin from patients with SSc [50]. Collectively, an attractive hypothesis has been demonstrated, which Treg cells may specifically convert into Th17 cells in the context of the disease. Several studies have reported that conversion of Treg to Th17 cells is possible [51–53]. In fact, IL-6, which increased in SSc, has been shown to participate to this conversion [54]. It is also possible that Treg cell priming in SSc is skewed toward Th17 cells via increased levels of IL-6 and TGF-β production.

Th22 cells typically produce IL-22 in the absence of IL-17 and IFN-γ and are enriched in a subset of CCR4- , CCR6- , and CCR10-expressing T cells, which are skin homing T cells [55, 56]. Although the key transcription factor governing the commitment toward Th22 cells remains unknown, the aryl hydrocarbon receptor and STAT3 have been proposed [56, 57]. While IL-22 does not participate in the communication between immune cells because peripheral blood mononuclear cells do not express IL-22R, IL-22 acts mainly on epithelial cells of mucosal origin and

the skin, and it promotes antimicrobial defense, epithelial homeostasis, and regeneration [58]. Recent studies have suggested that IL-22 associates with the pathogenesis of several immune disorders, including rheumatoid arthritis, SLE, asthma, and inflammatory skin diseases, such as psoriasis and atopic dermatitis [59]. In SSc patients, Th22 increases in the peripheral blood in addition to Th2 and Th17 cells [40]. Similar to that which has been observed for Th17 cells in SSc, Th22 cell number positively correlates with skin sclerosis and lung fibrosis. In BLM-induced lung fibrosis model mice, lung inflammation was ameliorated in IL-22-deficient mice compared to wild-type mice [60]. These results suggest that Th22 cells contribute to tissue fibrosis via IL-22 production in SSc patients.

2.3 B Cell Autoimmune Responses in SSc

2.3.1 Autoantibody Production of B Cell

Autoantibodies are present in >90 % of SSc patients and selectively correlate with disease-specific clinical manifestations. However, a direct link between their presence and the pathogenesis in SSc has been suggested only recently. B cells participate in adaptive immune responses through functions beyond antibody production, such as antigen presentation, cytokine production, lymphoid organogenesis, T cell differentiation, and influence on dendritic cell and macrophage function [61]. Consistently, not only autoantibody production, but also abnormalities in other B cell functions, could lead to the induction or development of autoimmunity. SSc patients have polyclonal B cell hyperactivity in addition to undergoing autoantibody production [10]. Chronic B cell activation is likely to be critical for the development and progression of the disease. In SSc patients, circulating memory B cells were shown to exhibit a chronically activation, clonal expansion, and antibody production [62]. A defective negative selection and/or excessive positive costimulation could possibly predispose to SSc autoimmunity by prolonged survival of circulating autoreactive B cells. Indeed, elevated levels of B-cell-activating factor, which is an important mediator in B cell survival, have been detected in the plasma of SSc patients [63]. Moreover, recent studies have shown that B cell depletion therapy reduced skin sclerosis and lung fibrosis in human SSc patients [64, 65].

2.3.2 Response Regulators of B Cells

B cells respond to a lot of stimulation, which regulates the negative selection in the bone marrow, the generation of humoral immune responses in the peripheral lymphoid organs, and the establishment and maintenance of tolerance and memory. The outcome of these B cell responses is determined by signaling thresholds via B cell antigen receptor (BCR) complex signaling during B cell responses to self- and foreign antigens [66]. In addition, B cell signaling thresholds are regulated by

response regulators, which are an array of cell-surface molecules or cytoplasmic signal transduction molecules, that augment or diminish BCR signals during B cell development [66–68]. Therefore, "response regulators" could be a useful concept for understanding how autoimmunity is induced, as well as how normal humoral immune responses are regulated.

Recent studies have identified many molecules that belong to response regulators using mice lacking or overexpressing certain molecules [66–68]. CD19, CD21, and CD45 have been identified as a group of positive response regulators, which augment signals through the BCR complex, while CD22, CD72, and FcγRIIB have been grouped as negative response regulators that diminish BCR signals [66–71]. Transgenic mice that overexpress CD19 lose tolerance and generate autoantibodies spontaneously [72, 73], whereas mice lacking CD22 have chronically activated B cells with various spontaneous autoantibody production [74, 75]. These data suggest that B cell responses to foreign or self-antigens are controlled in part by interaction between positive and negative response regulators. Furthermore, abnormal regulation of the response regulator function and expression may result in autoantibody production.

2.3.3 CD19 as a Crucial Positive Response Regulator of B Cell Signaling

Among B cell response regulators, CD19, which is a critical cell-surface signal transduction molecule of B cells, is one of the most potent positive regulators. CD19 expression is restricted to B lineage cells and follicular dendritic cells that are antigen-presenting cells located in the murine spleen [76]. CD19 is a 95,000 Mr glycoprotein member of the immunoglobulin (Ig) superfamily expressed from early pre-B cells until plasma cell differentiation [62]. In the B cell activation process, CD19 expression is tightly regulated, suggesting that intrinsic CD19 expression levels may determine a predisposition to autoimmunity. CD19 has an extracellular region containing two C2-type Ig-like domains and a cytoplasmic region of ~240 amino acids with nine conserved tyrosine residues [77]. Lyn, a Src-family protein tyrosine kinase member, is the dominant kinase that phosphorylates CD19 upon various stimuli. Once tyrosyl phosphorylated, CD19 serves as a membrane-bound adaptor protein for Src homology 2-containing signaling molecules such as Lyn, Vav, and phosphatidylinositol 3-kinase, which further mediate downstream activation cascades.

In recent analysis using CD19-deficient mice and CD19-transgenic mice, in vivo CD19 function has been finely assessed [73, 78–82]. CD19-deficient B cells exhibit reduced proliferation to various transmembrane signals compared with wild-type B cells, whereas B cells from CD19-transgenic mice show augmented proliferation [78, 81]. Serum Ig levels are spontaneously increased in CD19-transgenic mice, while they are generally decreased in CD19-deficient mice. In addition, serum levels of several autoantibodies including anti-topo I, anti-DNA, and anti-histone Abs are decreased in CD19-deficient mice, whereas those in CD19-transgenic mice

are increased [73]. Moreover, analysis using CD19-transgenic mice of autoreactive B cells has revealed that CD19 overexpression disrupts peripheral tolerance in B cells and thereby induces autoantibody production and autoimmune reactions [72]. These results suggest that CD19 expression levels regulate autoantibody production through augmenting B cell signaling. Of interest, CD19 expression is not affected by B cell activation, though B cells stimulated with anti-IgM Ab or lipopolysaccharide increase the expression of I-A, a major histocompatibility complex class II molecule, which indicates B cell activation [73]. Collectively, CD19 expression is tightly regulated during the B cell activation process, suggesting that overexpression of CD19 can lead to autoimmunity.

2.3.4 CD19 Overexpression on B Cells in SSc Patients

CD19 density on surface of SSc B cells is significantly higher by 20 % than healthy individuals, revealed by flow cytometric analysis of the peripheral blood from SSc patients [83]. This CD19 overexpression is detected in both naive and memory B cells from SSc patients [84]. Although CD19 expression levels are higher on B cells from SSc patients, the increase in CD19 expression is small, and it remains unknown whether this small increase is related to autoimmunity. Therefore, the pathogenic significance of the 20 %-increased CD19 expression was assessed by generating transgenic mice, which overexpressed CD19 to a similar extent as human SSc [82, 84]. These CD19 transgenic mice, which overexpress CD19 by 29 %, have increased levels of various autoantibodies, including SSc-specific anti-topo I Ab, as well as anti-DNA Ab, anti-histone Ab, and rheumatoid factor [84, 85]. These results suggest that the small increase in CD19 expression observed in human SSc may be sufficient to induce autoantibody production. However, of interest, these transgenic mice do not develop fibrosis in the skin and visceral organs. Thus, it is still unclear whether CD19 overexpression and autoantibody production are related to disease development in SSc.

In SSc patients, hyper-γ-globulinemia and polyclonal B cell activation are also found [86, 87]. Recent studies using DNA microarrays have revealed upregulation of gene expression related to B cell activation [10]. These observations suggest the presence of intrinsic B cell hyperactivation in SSc. To assess whether there is intrinsic B cell hyperactivation in SSc, phenotypic and functional abnormalities of peripheral blood B cell subsets were assessed [8, 88]. In SSc patients, total blood B cells are expanded, and homeostasis and subsets of B cells are disturbed. Although memory B cells and plasmablasts diminish, naive B cells expand. In addition, these memory B cells have increased expression of CD80 and CD86, which are crucial costimulatory molecules of B cells. This finding indicates that SSc memory B cells are chronically activated in vivo, possibly due to CD19 overexpression. Furthermore, CD95 (Fas) expression, which is upregulated following B cell activation, increases on SSc memory B cells. The increased Fas expression induces marked sensitivity to Fas-mediated apoptosis [89]. This enhanced sensitivity to spontaneous apoptosis of SSc memory B cells may result in a

diminished number of these cells in the blood. Taken together, it is possible that the continuous loss of memory B cells and plasmablasts increases the production of naive B cells to maintain B cell homeostasis. In addition, SSc memory B cells have enhanced ability to produce IgG, resulting in hyper-γ-globulinemia and possibly autoantibody production, though the memory B cell number is decreased in SSc patients. Thus, SSc patients have distinct abnormalities of B cell compartments characterized by expanded naive B cells and activated memory B cells, which may be related to CD19 overexpression, since memory B cells as well as naive B cells from SSc patients overexpress CD19.

2.3.5 Raynaud's Phenomenon Potentially Induces B Cell Autoimmunity in SSc

Previous studies have confirmed that human SSc patients show enhanced reactive oxygen species (ROS) production, which is probably due to ischemia and reperfusion injury following Raynaud's phenomenon, an initial clinical manifestation in human SSc [90, 91]. Enhanced ROS production, which is common features of human SSc and the BLM-induced SSc model, plays a role as an initiator for tissue injury and fibrosis [90, 92, 93]. In BLM-induced lung fibrosis model, inflammatory cells and lung epithelial cells are responsible for ROS [92]. Mice deficient for ROS production reduced BLM-induced lung fibrosis, suggesting that ROS generated by BLM induces tissue damage and fibrosis [93]. Moreover, a free radical scavenger, edaravone, inhibits skin and lung fibrosis in BLM-induced SSc model mice [94].

Furthermore, ROS are responsible for hyaluronan degradation, leading to production of hyaluronan fragments, one of the endogenous ligands of toll-like receptor 4 (TLR4), that induce inflammatory responses [95]. In fact, in BLM-induced SSc model mice, BLM induces autoantigen presentation during tissue injury and hyaluronan fragment production, which results in autoantibody production and fibrosis. Likewise, hyaluronan levels are increased in plasma or sera from human patients with SSc, especially early SSc, and correlated closely with the disease severity, progression, and degree of visceral involvement [96–98].

Collectively, we have proposed the following hypothesis previously (Fig. 2.4): enhanced ROS production, which is induced by Raynaud's phenomenon in human SSc and BLM in the BLM-induced SSc model mice, respectively, could initiate tissue damage, leading to generation of hyaluronan fragments which induce B cell activation mainly via CD19 and TLR4. Moreover, the abnormal B cell activation may be associated with autoantibody production and augmented cytokine production that may result in fibrosis. Collectively, these findings suggest that CD19 and TLR4 signals of B cells would be one of potential therapeutic targets in human SSc.

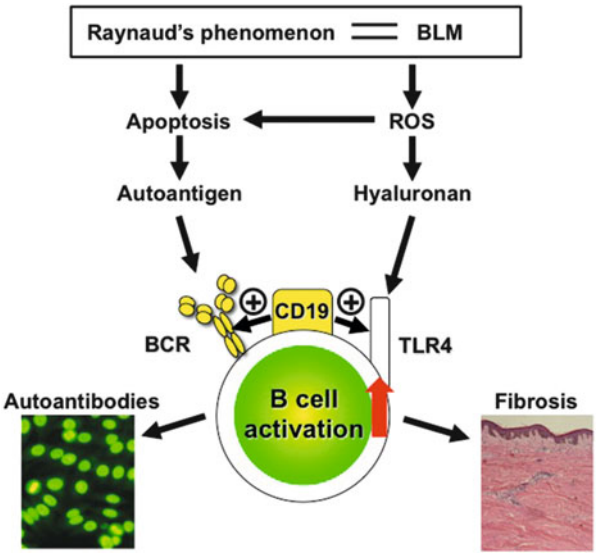

Fig. 2.4 A model linking Raynaud's phenomenon, systemic autoimmunity, and tissue fibrosis in SSc patients and BLM-induced SSc model mice. SSc patients exhibit enhanced ROS production, due to ischemia and reperfusion injury following Raynaud's phenomenon. Similarly, BLM induces the production of ROS. Consequently, ROS increase hyaluronan fragments, which induce B cell activation through the TLR4 signaling as endogenous TLR4 ligands. Simultaneously, apoptosis is detected in the skin of the early disease stage from patients with SSc and BLM-induced SSc model mice. During apoptosis process, autoantigens including topo I are concentrated on the surface apoptotic blebs, which may result in the antigen presentation of the cryptic epitopes. Remarkably, CD19 loss inhibits these B cell activation and autoantibody production. Collectively, we hypothesize that Raynaud's phenomenon and BLM induce fibrosis by enhancing hyaluronan production, which activates B cells to produce fibrogenic cytokines mainly via CD19 and TLR4 signaling and induce autoantibody production

2.4 Conclusion

Although direct roles in pathogenesis have been unclear, activation of the immune system in SSc has long been recognized. An abnormal immune activation involving both humoral and cellular autoimmunity appears to be a crucial step for disease initiation and development, such as aberrant fibrogenesis and obliterative microangiopathy. In this review, we have highlighted T-cell and B cell-mediated autoimmune responses. We have learned that Th cells have a skewed functional repertoire, which may vary along disease progression. B cells have been activated by the condition of SSc, including ischemia, which induces autoantibody production and several organ fibrosis. Although the role of autoantibodies detected in SSc is still unclear, they strongly associated with unique disease manifestations. Thus, further studies revealing the association of these immunological abnormalities and

disease progression are still required in vivo. Such advances may contribute to the finding of brand new therapeutic targets and the development of rational therapeutic strategies.

References

1. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger Jr TA, et al. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. J Rheumatol. 1988;15:202–5.
2. Furst DE, Clements PJ. Hypothesis for the pathogenesis of systemic sclerosis. J Rheumatol. 1997;24 suppl 48:53–7.
3. Yoshizaki A, Yanaba K, Iwata Y, Komura K, Ogawa A, Muroi E, et al. Elevated serum interleukin-27 levels in patients with systemic sclerosis: association with T cell, B cell and fibroblast activation. Ann Rheum Dis. 2011;70(1):194–200. doi:10.1136/ard.2009.121053.
4. Yoshizaki A, Komura K, Iwata Y, Ogawa F, Hara T, Muroi E, et al. Clinical significance of serum HMGB-1 and sRAGE levels in systemic sclerosis: association with disease severity. J Clin Immunol. 2009;29(2):180–9. doi:10.1007/s10875-008-9252-x.
5. Okano Y. Antinuclear antibody in systemic sclerosis (scleroderma). Rheum Dis Clin N Am. 1996;22(4):709–35.
6. Sakkas LI, Chikanza IC, Platsoucas CD. Mechanisms of disease: the role of immune cells in the pathogenesis of systemic sclerosis. Nat Clin Pract Rheumatol. 2006;2(12):679–85.
7. Kumar PS, Sagar VR. Drying kinetics and physico-chemical characteristics of Osmo-dehydrated Mango, Guava and Aonla under different drying conditions. J Food Sci Technol. 2014;51(8):1540–6. doi:10.1007/s13197-012-0658-3.
8. Sato S, Fujimoto M, Hasegawa M, Takehara K, Tedder TF. Altered B lymphocyte function induces systemic autoimmunity in systemic sclerosis. Mol Immunol. 2004;41(12):1123–33. doi:10.1016/j.molimm.2004.06.025.
9. Lipsky PE. Systemic lupus erythematosus: an autoimmune disease of B cell hyperactivity. Nat Immunol. 2001;2(9):764–6. doi:10.1038/ni0901-764. ni0901-764 [pii].
10. Whitfield ML, Finlay DR, Murray JI, Troyanskaya OG, Chi JT, Pergamenschikov A, et al. Systemic and cell type-specific gene expression patterns in scleroderma skin. Proc Natl Acad Sci U S A. 2003;100(21):12319–24.
11. Prescott RJ, Freemont AJ, Jones CJ, Hoyland J, Fielding P. Sequential dermal microvascular and perivascular changes in the development of scleroderma. J Pathol. 1992;166(3):255–63.
12. Kahari VM, Sandberg M, Kalimo H, Vuorio T, Vuorio E. Identification of fibroblasts responsible for increased collagen production in localized scleroderma by in situ hybridization. J Invest Dermatol. 1988;90(5):664–70.
13. Scharffetter K, Lankat-Buttgereit B, Krieg T. Localization of collagen mRNA in normal and scleroderma skin by in-situ hybridization. Eur J Clin Invest. 1988;18:9–17.
14. Hoffmann KF, McCarty TC, Segal DH, Chiaramonte M, Hesse M, Davis EM, et al. Disease fingerprinting with cDNA microarrays reveals distinct gene expression profiles in lethal type 1 and type 2 cytokine-mediated inflammatory reactions. FASEB J. 2001;15(13):2545–7. doi:10.1096/fj.01-0306fje.
15. Higashi-Kuwata N, Makino T, Inoue Y, Takeya M, Ihn H. Alternatively activated macrophages (M2 macrophages) in the skin of patient with localized scleroderma. Exp Dermatol. 2009;18(8):727–9. doi:10.1111/j.1600-0625.2008.00828.x.
16. Sato S, Hasegawa M, Takehara K. Serum levels of interleukin-6 and interleukin-10 correlate with total skin thickness score in patients with systemic sclerosis. J Dermatol Sci. 2001;27:140–6.
17. Bellisai F, Morozzi G, Scaccia F, Chellini F, Simpatico A, Pecetti G, et al. Evaluation of the effect of Bosentan treatment on proinflammatory cytokine serum levels in patients affected by Systemic Sclerosis. Int J Immunopathol Pharmacol. 2011;24(1):261–4.

18. Duncan MR, Berman B. Stimulation of collagen and glycosaminoglycan production in cultured human adult dermal fibroblasts by recombinant human interleukin 6. J Invest Dermatol. 1991;97(4):686–92.
19. Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. Nat Rev Immunol. 2004;4 (8):583–94. doi:10.1038/nri1412.
20. Hasegawa M, Fujimoto M, Takehara K, Sato S. Pathogenesis of systemic sclerosis: altered B cell function is the key linking systemic autoimmunity and tissue fibrosis. J Dermatol Sci. 2005;39(1):1–7. doi:10.1016/j.jdermsci.2005.03.013.
21. Hasegawa M, Fujimoto M, Kikuchi K, Takehara K. Elevated serum levels of interleukin 4 (IL-4), IL-10, and IL-13 in patients with systemic sclerosis. J Rheumatol. 1997;24 (2):328–32.
22. Hasegawa M, Sato S, Fujimoto M, Ihn H, Kikuchi K, Takehara K. Serum levels of interleukin 6 (IL-6), oncostatin M, soluble IL-6 receptor, and soluble gp130 in patients with systemic sclerosis. J Rheumatol. 1998;25:308–13.
23. Needleman BW, Wigley FM, Stair RW. Interleukin-1, interleukin-2, interleukin-4, interleukin-6, tumor necrosis factor-a, and interferon-g levels in sera from patients with scleroderma. Arthritis Rheum. 1992;35(1):67–72.
24. Hasegawa M, Sato S, Ihn H, Takehara K. Enhanced production of interleukin-6 (IL-6), oncostatin M and soluble IL-6 receptor by cultured peripheral blood mononuclear cells from patients with systemic sclerosis. Rheumatology. 1999;38(7):612–7.
25. Mavalia C, Scaletti C, Romagnani P, Carossino AM, Pignone A, Emmi L, et al. Type 2 helper T-cell predominance and high CD30 expression in systemic sclerosis. Am J Pathol. 1997;151 (6):1751–8.
26. Kopf M, Le Gros G, Bachmann M, Lamers MC, Bluethmann H, Kohler G. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. Nature. 1993;362(6417):245–8. doi:10.1038/362245a0.
27. Sharma SK, MacLean JA, Pinto C, Kradin RL. The effect of an anti-CD3 monoclonal antibody on bleomycin-induced lymphokine production and lung injury. Am J Respir Crit Care Med. 1996;154(1):193–200. doi:10.1164/ajrccm.154.1.8680680.
28. Salmon-Ehr V, Serpier H, Nawrocki B, Gillery P, Clavel C, Kalis B, et al. Expression of interleukin-4 in scleroderma skin specimens and scleroderma fibroblast cultures. Potential role in fibrosis. Arch Dermatol. 1996;132(7):802–6.
29. Postlethwaite AE, Holness MA, Katai H, Raghow R. Human fibroblasts synthesize elevated levels of extracellular matrix proteins in response to interleukin 4. J Clin Invest. 1992;90 (4):1479–85. doi:10.1172/JCI116015.
30. Ong C, Wong C, Roberts CR, Teh HS, Jirik FR. Anti-IL-4 treatment prevents dermal collagen deposition in the tight-skin mouse model of scleroderma. Eur J Immunol. 1998;28(9):2619–29.
31. Huaux F, Liu T, McGarry B, Ullenbruch M, Phan SH. Dual roles of IL-4 in lung injury and fibrosis. J Immunol. 2003;170(4):2083–92.
32. Aversa G, Punnonen J, Cocks BG, de Waal Malefyt R, Vega Jr F, Zurawski SM, et al. An interleukin 4 (IL-4) mutant protein inhibits both IL-4 or IL-13-induced human immunoglobulin G4 (IgG4) and IgE synthesis and B cell proliferation: support for a common component shared by IL-4 and IL-13 receptors. J Exp Med. 1993;178(6):2213–8.
33. Fallon PG, Richardson EJ, McKenzie GJ, McKenzie AN. Schistosome infection of transgenic mice defines distinct and contrasting pathogenic roles for IL-4 and IL-13: IL-13 is a profibrotic agent. J Immunol. 2000;164(5):2585–91.
34. Rankin AL, Mumm JB, Murphy E, Turner S, Yu N, McClanahan TK, et al. IL-33 induces IL-13-dependent cutaneous fibrosis. J Immunol. 2010;184(3):1526–35. doi:10.4049/jimmunol.0903306.
35. Kantor TV, Friberg D, Medsger Jr TA, Buckingham RB, Whiteside TL. Cytokine production and serum levels in systemic sclerosis. Clin Immunol Immunopathol. 1992;65(3):278–85.

36. Fujii H, Hasegawa M, Takehara K, Mukaida N, Sato S. Abnormal expression of intracellular cytokines and chemokine receptors in peripheral blood T lymphocytes from patients with systemic sclerosis. Clin Exp Immunol. 2002;130(3):548–56.
37. Valentini G, Baroni A, Esposito K, Naclerio C, Buommino E, Farzati A, et al. Peripheral blood T lymphocytes from systemic sclerosis patients show both Th1 and Th2 activation. J Clin Immunol. 2001;21(3):210–7.
38. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. Annu Rev Immunol. 2009;27:485–517. doi:10.1146/annurev.immunol.021908.132710.
39. Hemdan NY, Birkenmeier G, Wichmann G, Abu El-Saad AM, Krieger T, Conrad K, et al. Interleukin-17-producing T helper cells in autoimmunity. Autoimmun Rev. 2010;9 (11):785–92. doi:10.1016/j.autrev.2010.07.003.
40. Truchetet ME, Brembilla NC, Montanari E, Allanore Y, Chizzolini C. Increased frequency of circulating Th22 in addition to Th17 and Th2 lymphocytes in systemic sclerosis: association with interstitial lung disease. Arthritis Res Ther. 2011;13(5):R166. doi:10.1186/ar3486.
41. Kurasawa K, Hirose K, Sano H, Endo H, Shinkai H, Nawata Y, et al. Increased interleukin-17 production in patients with systemic sclerosis. Arthritis Rheum. 2000;43(11):2455–63. doi:10.1002/1529-0131(200011)43:11<2455::AID-ANR12>3.0.CO;2-K.
42. Murata M, Fujimoto M, Matsushita T, Hamaguchi Y, Hasegawa M, Takehara K, et al. Clinical association of serum interleukin-17 levels in systemic sclerosis: is systemic sclerosis a Th17 disease? J Dermatol Sci. 2008;50(3):240–2. doi:S0923-1811(08)00043-1 [pii]. 10.1016/j.jdermsci.2008.01.001.
43. Hsu E, Shi H, Jordan RM, Lyons-Weiler J, Pilewski JM, Feghali-Bostwick CA. Lung tissues in patients with systemic sclerosis have gene expression patterns unique to pulmonary fibrosis and pulmonary hypertension. Arthritis Rheum. 2011;63(3):783–94. doi:10.1002/art.30159.
44. Nakashima T, Jinnin M, Yamane K, Honda N, Kajihara I, Makino T, et al. Impaired IL-17 signaling pathway contributes to the increased collagen expression in scleroderma fibroblasts. J Immunol. 2012;188(8):3573–83. doi:10.4049/jimmunol.1100591.
45. Mathian A, Parizot C, Dorgham K, Trad S, Arnaud L, Larsen M, et al. Activated and resting regulatory T cell exhaustion concurs with high levels of interleukin-22 expression in systemic sclerosis lesions. Ann Rheum Dis. 2012;71(7):1227–34. doi:10.1136/annrheumdis-2011-200709.
46. Brembilla NC, Chizzolini C. T cell abnormalities in systemic sclerosis with a focus on Th17 cells. Eur Cytokine Netw. 2012;23(4):128–39. doi:10.1684/ecn.2013.0325.
47. Fenoglio D, Battaglia F, Parodi A, Stringara S, Negrini S, Panico N, et al. Alteration of Th17 and Treg cell subpopulations co-exist in patients affected with systemic sclerosis. Clin Immunol. 2011;139(3):249–57. doi:10.1016/j.clim.2011.01.013.
48. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity. 2009;30(6):899–911. doi:10.1016/j.immuni.2009.03.019.
49. Banica L, Besliu A, Pistol G, Stavaru C, Ionescu R, Forsea AM, et al. Quantification and molecular characterization of regulatory T cells in connective tissue diseases. Autoimmunity. 2009;42(1):41–9. doi:10.1080/08916930802282651.
50. Antiga E, Quaglino P, Bellandi S, Volpi W, Del Bianco E, Comessatti A, et al. Regulatory T cells in the skin lesions and blood of patients with systemic sclerosis and morphoea. Br J Dermatol. 2010;162(5):1056–63. doi:10.1111/j.1365-2133.2010.09633.x.
51. Koenen HJ, Smeets RL, Vink PM, van Rijssen E, Boots AM, Joosten I. Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells. Blood. 2008;112(6):2340–52. doi:10.1182/blood-2008-01-133967.
52. Osorio F, LeibundGut-Landmann S, Lochner M, Lahl K, Sparwasser T, Eberl G, et al. DC activated via dectin-1 convert Treg into IL-17 producers. Eur J Immunol. 2008;38 (12):3274–81. doi:10.1002/eji.200838950.

53. Yang XO, Nurieva R, Martinez GJ, Kang HS, Chung Y, Pappu BP, et al. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. Immunity. 2008;29 (1):44–56. doi:10.1016/j.immuni.2008.05.007.
54. Beriou G, Costantino CM, Ashley CW, Yang L, Kuchroo VK, Baecher-Allan C, et al. IL-17-producing human peripheral regulatory T cells retain suppressive function. Blood. 2009;113 (18):4240–9. doi:10.1182/blood-2008-10-183251.
55. Duhen T, Geiger R, Jarrossay D, Lanzavecchia A, Sallusto F. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. Nat Immunol. 2009;10 (8):857–63. doi:10.1038/ni.1767.
56. Trifari S, Kaplan CD, Tran EH, Crellin NK, Spits H. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. Nat Immunol. 2009;10(8):864–71. doi:10.1038/ni.1770.
57. Veldhoen M, Hirota K, Westendorf AM, Buer J, Dumoutier L, Renauld JC, et al. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. Nature. 2008;453(7191):106–9. doi:10.1038/nature06881.
58. Wolk K, Witte E, Witte K, Warszawska K, Sabat R. Biology of interleukin-22. Semin Immunopathol. 2010;32(1):17–31. doi:10.1007/s00281-009-0188-x.
59. Zhang N, Pan HF, Ye DQ. Th22 in inflammatory and autoimmune disease: prospects for therapeutic intervention. Mol Cell Biochem. 2011;353(1–2):41–6. doi:10.1007/s11010-011-0772-y.
60. Sonnenberg GF, Nair MG, Kirn TJ, Zaph C, Fouser LA, Artis D. Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A. J Exp Med. 2010;207(6):1293–305. doi:10.1084/jem.20092054.
61. Davidson A, Diamond B. Autoimmune diseases. N Engl J Med. 2001;345(5):340–50.
62. Tedder TF, Poe JC, Fujimoto M, Haas KM, Sato S. The CD19-CD21 signal transduction complex of B lymphocytes regulates the balance between health and autoimmune disease: systemic sclerosis as a model system. Curr Dir Autoimmun. 2005;8:55–90.
63. Matsushita T, Hasegawa M, Yanaba K, Kodera M, Takehara K, Sato S. Elevated serum BAFF levels in patients with systemic sclerosis: enhanced BAFF signaling in systemic sclerosis B lymphocytes. Arthritis Rheum. 2006;54(1):192–201.
64. Lafyatis R, Kissin E, York M, Farina G, Viger K, Fritzler MJ, et al. B cell depletion with rituximab in patients with diffuse cutaneous systemic sclerosis. Arthritis Rheum. 2009;60 (2):578–83. doi:10.1002/art.24249.
65. Jordan S, Distler JH, Maurer B, Huscher D, van Laar JM, Allanore Y, et al. Effects and safety of rituximab in systemic sclerosis: an analysis from the European Scleroderma Trial and Research (EUSTAR) group. Ann Rheum Dis. 2014. doi:10.1136/annrheumdis-2013-204522.
66. Goodnow CC. Balancing immunity and tolerance: deleting and tuning lymphocyte repertoires. Proc Natl Acad Sci U S A. 1996;93:2264–71.
67. Tedder TF. Introduction: response-regulators of B lymphocyte signaling thresholds provide a context for antigen receptor signal transduction. Semin Immunol. 1998;10:259–65.
68. Tedder TF, Inaoki M, Sato S. The CD19/21 complex regulates signal transduction thresholds governing humoral immunity and autoimmunity. Immunity. 1997;6:107–18.
69. Bolland S, Ravetch JV. Spontaneous autoimmune disease in Fc(gamma)RIIB-deficient mice results from strain-specific epistasis. Immunity. 2000;13(2):277–85.
70. Majeti R, Xu Z, Parslow TG, Olson JL, Daikh DI, Killeen N, et al. An inactivating point mutation in the inhibitory wedge of CD45 causes lymphoproliferation and autoimmunity. Cell. 2000;103(7):1059–70.
71. Pan C, Baumgarth N, Parnes JR. CD72-deficient mice reveal nonredundant roles of CD72 in B cell development and activation. Immunity. 1999;11(4):495–506.
72. Inaoki M, Sato S, Weintraub BC, Goodnow CC, Tedder TF. CD19-regulated signaling thresholds control peripheral tolerance and autoantibody production in B lymphocytes. J Exp Med. 1997;186:1923–31.

73. Sato S, Ono N, Steeber DA, Pisetsky DS, Tedder TF. CD19 regulates B lymphocyte signaling thresholds critical for the development of B-1 lineage cells and autoimmunity. J Immunol. 1996;157:4371–8.
74. Sato S, Miller AS, Inaoki M, Bock CB, Jansen PJ, Tang MLK, et al. CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: altered signaling in CD22-deficient mice. Immunity. 1996;5:551–62.
75. O'Keefe TL, Williams GT, Davies SL, Neuberger MS. Hyperresponsive B cells in CD22-deficient mice. Science. 1996;274:798–801.
76. Tedder TF, Zhou L-J, Engel P. The CD19/CD21 signal transduction complex of B lymphocytes. Immunol Today. 1994;15:437–42.
77. Fujimoto M, Poe JC, Hasegawa M, Tedder TF. CD19 regulates intrinsic B lymphocyte signal transduction and activation through a novel mechanism of processive amplification. Immunol Res. 2000;22(2–3):281–98.
78. Engel P, Zhou L-J, Ord DC, Sato S, Koller B, Tedder TF. Abnormal B lymphocyte development, activation and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. Immunity. 1995;3:39–50.
79. Rickert RC, Rajewsky K, Roes J. Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. Nature. 1995;376:352–5.
80. Sato S, Steeber DA, Tedder TF. The CD19 signal transduction molecule is a response regulator of B-lymphocyte differentiation. Proc Natl Acad Sci U S A. 1995;92:11558–62.
81. Sato S, Steeber DA, Jansen PJ, Tedder TF. CD19 expression levels regulate B lymphocyte development: human CD19 restores normal function in mice lacking endogenous CD19. J Immunol. 1997;158:4662–9.
82. Zhou L-J, Smith HM, Waldschmidt TJ, Schwarting R, Daley J, Tedder TF. Tissue-specific expression of the human CD19 gene in transgenic mice inhibits antigen-independent B lymphocyte development. Mol Cell Biol. 1994;14:3884–94.
83. Sato S, Hasegawa M, Fujimoto M, Tedder TF, Takehara K. Quantitative genetic variation in CD19 expression correlates with autoimmunity. J Immunol. 2000;165(11):6635–43.
84. Sato S, Fujimoto M, Hasegawa M, Takehara K. Altered blood B lymphocyte homeostasis in systemic sclerosis: expanded naive B cells and diminished but activated memory B cells. Arthritis Rheum. 2004;50(6):1918–27.
85. Asano N, Fujimoto M, Yazawa N, Shirasawa S, Hasegawa M, Okochi H, et al. B Lymphocyte signaling established by the CD19/CD22 loop regulates autoimmunity in the tight-skin mouse. Am J Pathol. 2004;165(2):641–50.
86. Famularo G, Giacomelli R, Alesse E, Cifone MG, Morrone S, Boirivant M, et al. Polyclonal B lymphocyte activation in progressive systemic sclerosis. J Clin Lab Immunol. 1989;29 (2):59–63.
87. Fleischmajer R, Perlish JS, Reeves JRT. Cellular infiltrates in scleroderma skin. Arthritis Rheum. 1977;20:975–84.
88. Fujimoto M, Sato S. B lymphocytes and systemic sclerosis. Curr Opin Rheumatol. 2005;17 (6):746–51.
89. Wang J, Watanabe T. Expression and function of Fas during differentiation and activation of B cells. Int Rev Immunol. 1999;18:367–79.
90. Ogawa F, Shimizu K, Muroi E, Hara T, Hasegawa M, Takehara K, et al. Serum levels of 8-isoprostane, a marker of oxidative stress, are elevated in patients with systemic sclerosis. Rheumatology. 2006;45(7):815–8.
91. Simonini G, Pignone A, Generini S, Falcini F, Cerinic MM. Emerging potentials for an antioxidant therapy as a new approach to the treatment of systemic sclerosis. Toxicology. 2000;155(1–3):1–15.
92. Inghilleri S, Morbini P, Oggionni T, Barni S, Fenoglio C. In situ assessment of oxidant and nitrogenic stress in bleomycin pulmonary fibrosis. Histochem Cell Biol. 2006;125(6):661–9.

93. Manoury B, Nenan S, Leclerc O, Guenon I, Boichot E, Planquois JM, et al. The absence of reactive oxygen species production protects mice against bleomycin-induced pulmonary fibrosis. Respir Res. 2005;6:11–22.
94. Yoshizaki A, Yanaba K, Ogawa A, Iwata Y, Ogawa F, Takenaka M, et al. The specific free radical scavenger edaravone suppresses fibrosis in the bleomycin-induced and tight skin mouse models of systemic sclerosis. Arthritis Rheum. 2011;63(10):3086–97. doi:10.1002/art.30470.
95. Mendoza G, Alvarez AI, Pulido MM, Molina AJ, Merino G, Real R, et al. Inhibitory effects of different antioxidants on hyaluronan depolymerization. Carbohydr Res. 2007;342(1):96–102.
96. Freitas JP, Filipe P, Emerit I, Meunier P, Manso CF, Guerra Rodrigo F. Hyaluronic acid in progressive systemic sclerosis. Dermatology. 1996;192(1):46–9.
97. Neudecker BA, Stern R, Connolly MK. Aberrant serum hyaluronan and hyaluronidase levels in scleroderma. Br J Dermatol. 2004;150(3):469–76.
98. Yoshizaki A, Iwata Y, Komura K, Hara T, Ogawa F, Muroi E, et al. Clinical significance of serum hyaluronan levels in systemic sclerosis: association with disease severity. J Rheumatol. 2008;35(9):1825–9. doi:08/13/087 [pii].

Endothelial Progenitor Cells

3

Masataka Kuwana

Abstract

Systemic sclerosis (SSc) is characterized by excessive fibrosis and microvasculopathy with deficiency in vascular formation and repair. The postnatal vascular system is constantly maintained through angiogenesis and vasculogenesis. Endothelial progenitor cells (EPCs), major players of vasculogenesis, are heterogeneous cell population containing an extremely rare number of "true EPCs" and pro-angiogenic hematopoietic cells (PHCs) that promotes vascular formation through secretion of pro-angiogenic factors and differentiation into endothelial cells and mural cells with low efficiency. We have recently proposed a theory that defective vascular repair machinery is one of the important mechanisms contributing to SSc vasculopathy, based on reduced counts and impaired function of circulating $CD34^{+}CD133^{+}CD309^{+}CD45^{dim}CD14^{-}$ EPCs, which are now regarded as immature PHCs. In contrast, monocytic PHCs increased paradoxically in SSc patients and contribute to fibrotic aspects of the disease by differentiating into fibroblast-like cells. Understanding the roles of EPCs in pathogenic process of SSc may be key to dissecting its pathogenesis and to developing novel therapeutic strategies.

Keywords

Angiogenesis • Endothelial progenitor cells • Monocytes • Statins • Vasculogenesis

M. Kuwana (✉)
Department of Allergy and Rheumatology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan
e-mail: kuwanam@nms.ac.jp

K. Takehara et al. (eds.), *Systemic Sclerosis*, DOI 10.1007/978-4-431-55708-1_3

3.1 Introduction

Maintenance of the postnatal vascular system requires constant remodeling in response to injury and senescence. This occurs by collaborative effects of two distinct processes: (1) angiogenesis, which refers to the formation of new blood vessels by sprouting from preexisting vessels through proliferation and migration of mature endothelial cells, and (2) vasculogenesis, which refers to the de novo differentiation of mature endothelial cells through the recruitment and differentiation of endothelial progenitor cells (EPCs) [1]. Nascent vessels formed by angiogenesis and vasculogenesis are subsequently maturated via the recruitment of mesenchymal cells, such as pericytes and smooth muscle cells, through a process termed arteriogenesis. Since EPCs were first defined as a circulating primitive cell population that contributes to postnatal vasculogenesis [2], numerous studies have been conducted to evaluate the contribution of EPCs to the pathogenesis of a variety of vascular diseases, including atherosclerosis and connective tissue diseases.

Systemic sclerosis (SSc) is a multisystem connective tissue disease characterized by excessive fibrosis, microvasculopathy, and autoimmunity. Vascular involvement in patients with SSc mainly affects small arteries and causes reduced blood flow and tissue ischemia, leading to clinical manifestations such as digital ulcers, pulmonary arterial hypertension, and renal crisis. Two types of typical vascular pathology include progressive intimal proliferation and fibrosis in arterioles and the loss of capillaries. The mechanism of SSc vasculopathy is not fully understood, but increasing evidence indicates that endothelial injury is a primary event that triggers subsequent complex vascular pathophysiology [3]. The persistent increase in pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), observed in SSc patients indicates a strong pro-angiogenic response to vascular damage [4]. Nailfold capillaroscopic findings reveal loss of capillaries and formation of giant capillaries in the early phase of the disease and the vascular disorganization in the late phase [5]. The capillary loss may result from vascular damage, but there is almost no evidence of vascular recovery. In addition, the formation of abnormal blood vessels like bushy and leaky capillaries indicates an inadequate vascular repair process. These findings together suggest that, in patients with SSc, the vascular repair machinery does not work properly, and the disease progresses toward irreversible structural changes, despite the strong pro-angiogenic push. Thus, defective vascular repair process mediated through angiogenesis and vasculogenesis is an intriguing hypothesis to explain the pathogenesis of SSc vasculopathy. This chapter features current understandings of roles of EPCs in the vascular and fibrotic aspects of SSc.

3.2 Current Definition of EPCs

Since the first description of EPCs as circulating primitive cells that contribute to postnatal vasculogenesis, numerous studies have been carried out to clarify the mechanisms of postnatal vascular formation and repair, as well as the contribution

Table 3.1 Current terminology of human EPCs

Terminology of human EPCs	Surface markers used for isolation or characterization	Culture methods used for isolation
Pro-angiogenic hematopoietic cells (PHCs)	$CD31^+CD45^+CD41a^+$ $CD41a^-$ $CD235a^-$	Early-outgrowth EPC
		Colony-forming unit-Hill
Immature subset	$CD34^+CD133^+CD309^+CD45^{dim}$ $CD14^-$	
Monocytic subset	$CD34^{-/dim}CD133^-CD309^{-/dim}$ $CD45^+CD14^+$	
Endothelial colony-forming cells (ECFCs) (true "EPCs")	$CD34^{bright}CD31^+CD133^-CD45^-$ $CD14^-CD41a^-CD235a^-$	Late-outgrowth EPC

of EPCs to the pathogenesis of various vascular diseases, and to develop potential therapeutic strategies that promote tissue regeneration or attenuate pathologic neovascularization. However, a great deal of controversy about EPCs and their roles in postnatal vascular formation has arisen because of discrepancies in the definition of EPCs [6]. The major problem in defining EPCs derives from the lack of specific markers. In the landmark paper by Asahara et al. [2], EPCs were characterized using endothelial cell marker-positive cells, which were selected as a cell fraction from peripheral blood mononuclear cells that was enriched in cells expressing CD34 or CD309, a VEGF receptor type 2. These cells were shown to contribute to revascularization and salvage of ischemic hind limbs in animal models.

Currently, it is widely accepted that there are at least two types of EPCs that can be discriminated based on their surface antigen expression, proliferation potential, and time of emergence in the cell culture system [7] (Table 3.1). Endothelial colony-forming cells (ECFCs) or late-outgrowth EPCs are lineage-restricted progenitor cells that only give rise to endothelium. These cells are regarded as "true EPCs," based on their potential for clonogenic expansion in vitro and their ability to form vessels in vitro and in vivo [7]. Circulating precursors of ECFCs have not been identified yet, but they express CD34 and CD31 and completely lack expression of hematopoietic markers including CD45 [8]. Whether rare ECFCs are derived from hemangioblasts in the bone marrow or from endothelial stem cells that reside in the endothelium remains still undetermined [9].

On the other hand, it has been shown that the vast majority of the cells originally identified as EPCs in various assays are in fact hematopoietic lineage cells that display pro-angiogenic properties and are now termed pro-angiogenic hematopoietic cells (PHCs) [7]. PHCs include several different circulating cell types that are identified in the literature as circulating angiogenic cells (CACs), circulating endothelial precursors, monocytic EPCs, early-outgrowth EPCs, and colony-forming unit (CFU)-ECs. In fact, they fall into at least two distinct major subsets: $CD14^+$ monocytic origin (monocytic PHCs) and $CD14^-$ cells positive for CD34, CD133, and CD309 (immature PHCs), initially termed circulating

endothelial progenitors (CEPs) [10]. Currently, it is generally accepted that PHCs do not give rise to endothelial cells efficiently but rather work as pro-angiogenic supporting cells [11]. Monocytic PHCs cannot proliferate or form tubular structures in vitro in the absence of mature endothelial cells, but several studies reported that monocytic PHCs are capable of integrating into tubular structures and differentiate into endothelial cell-like cells in vivo [12, 13]. However, it is uncertain whether these endothelial cell-like cells are able to exert the full range of endothelial functioning. On the other hand, immature PHCs derived from stem cells in the bone marrow express immature hematopoietic markers CD34 and CD133 as well as a mature hematopoietic marker CD45 with low intensity, but lack expression of a monocytic marker CD14. Immature PHCs also represent expression of endothelial markers CD309 and Tie-2. These cells have typical features of progenitors, including capacity to proliferate and to differentiate into endothelial cells, but the efficiency is much lower in comparison with ECFCs. Nevertheless, PHCs, either in monocytic or immature subset, are capable of promoting blood vessel formation through multiple mechanisms, including secretion of a series of pro-angiogenic factors, including VEGF, hepatocyte growth factor, granulocyte colony-stimulating factor (G-CSF), and stromal cell-derived factor-1 (SDF-1) [14, 15], and differentiation into other elements of the vasculature, such as pericytes and smooth muscle cells. Essentially, PHCs are involved in the very early phase of vascular repair through attaching to the injured vascular lumen immediately after injury.

There is huge difference in circulating quantities among three EPC subsets. ECFCs are extremely rare cell population and presumed to present at less than 1 out of 10^6 circulating mononuclear cells [7], which can be detectable only in long-term cultures. Immature PHCs are also rare at a frequency of 10–100 cells in 1 mL of peripheral blood in healthy individuals. In contrast, monocytic PHCs comprise approximately 0.1–2 % of peripheral blood mononuclear cells [7, 12]. Monocytic PHCs' capacity to enhance blood vessel formation is apparently inferior to immature PHCs, although they clearly predominate over immature PHCs and ECFCs in the absolute number in circulation [16]. In the neovascular microenvironment, ECFCs and monocytic and immature PHCs work in concert with platelets and residential endothelial cells to form new blood vessels [17].

Because of rarity of EPCs in circulation, cultivation of circulating mononuclear cells in medium favoring endothelial differentiation has been used to identify and expand these cells. In these cultures, it is difficult to determine which precursor cells give rise to EPCs, because the starting cell population is heterogeneous, and cellular phenotypes change over time in culture. In the original protocol by Asahara et al., peripheral blood mononuclear cells were cultured on fibronectin for 7 days [2]. These cells typically do not form colonies in culture but have endothelial features, including the ability to bind Ulex europeus agglutinin-1, to take up acetylated low-density lipoprotein, and to express CD31, CD105, CD309, and von Willebrand factor (vWF). The vast majority of the cells recovered in these cultures express both CD45 and CD14, indicating their monocytic origin. In contrast, Hill et al. developed a semisolid clonogenic assay, in which peripheral blood mononuclear cells that did not adhere to fibronectin within 48 h were

reseeded on fibronectin, and formed cell clusters [18]. These cells express endothelial cell markers as well as CD45 and CD14, but only a tiny fraction of the cells express CD34 [19]. The cells recovered from different culture protocols contain varied cell populations, but mainly consist of monocytic PHCs, since the depletion of $CD14^+$ monocytes from the mononuclear cells before seeding effectively prevents colony formation. ECFCs are apparently distinct from PHCs since they appear 10–21 days after culturing mononuclear cells in medium favoring endothelial differentiation and thus termed late-outgrowth EPCs [20, 21]. These cultured cells display cobblestone morphology and express endothelial markers but completely lack expression of hematopoietic markers. In addition, circulating precursors that give rise to ECFCs display clonal proliferative potential, self-renewal, and the ability to form vessels in vivo, compatible with features of traditional EPCs.

3.3 Circulating Counts of Immature PHCs in SSc Patients

Two approaches have been conducted to evaluate potential involvement of impaired vasculogenesis in the development of SSc vasculopathy. One utilizes EPC colonies obtained by short-term or long-term cultures of circulating mononuclear cells in medium favoring endothelial differentiation. This strategy is able to analyze function and gene expression of early-outgrowth EPCs, which correspond mainly to monocytic PHCs, and late-outgrowth EPCs or ECFCs, although direct quantification of EPCs in circulation is not feasible in these cultures. Another approach is an ex vivo analysis of circulating EPCs using peripheral blood, but this method is applicable only for immature PHCs, which can be identified by multicolor flow cytometry using freshly prepared peripheral blood. All markers currently used for identification of immature PHCs have considerable overlap with circulating endothelial cells, hematopoietic stem cells, and precursors. Nevertheless, in many studies, immature PHCs are identified as circulating cells expressing CD34, CD133, and CD309. We first reported that there is a reduced number of $CD34^+CD133^+CD309^+$ immature PHCs in SSc patients, compared with age- and sex-matched patients with rheumatoid arthritis or healthy individuals [22]. In subsequent analyses by other groups, some confirmed our finding [23–25], but others even showed an increase in immature PHCs in SSc patients [26–30] (Table 3.2). These contradictory results may result from differences in protocols used for quantifying immature PHCs. In fact, quantification of circulating immature PHCs is a complex and difficult task from technical viewpoint. Flow cytometry using freshly prepared peripheral blood is used to quantify these cells, but accurate quantification is technically difficult due to extreme rarity of this population, even a large number of events were acquired. To overcome this limitation, flow cytometry is often combined with procedures that enrich immature PHCs, such as sorting of $CD34^+$ cells and lineage (Lin)-negative cells. But, variable recovery rates among samples in the enrichment procedure significantly affect subsequent counts by flow cytometry. Furthermore, a large quantity of peripheral blood, usually at least

Table 3.2 Published reports evaluating circulating immature PHC counts in SSc patients in comparison with healthy controls

Authors	Definition of immature PHCs	Methods for quantification	Counts in SSc (range) Number of subjects	Counts in controls (range) Number of subjects	Quantity in SSc versus controls
Del Papa et al. [26]	$CD34^+CD133^+$	Direct FCM using 200 μL whole blood	122/mL blood (78–167) $n=46$	28/mL blood (0–56) $n=40$	Early SSc↑
Kuwana et al. [22]	$CD34^+CD133^+$ $CD309^+$	$CD34^+$ cell sorting from 20–50 mL whole blood followed by FCM	14/mL blood (2–26) $n=11$	54/mL blood (26–83) $n=11$	SSc↓↓
Allanore et al. [28]	$CD34^+CD133^+$	Direct FCM using 200 μL whole blood	2,300/mL blood (775–7,600) $n=32$	1,410/mL blood (500–3,000) $n=15$	SSc↑
Del Papa et al. [27]	$CD34^+CD133^+$ $CD309^+$	Direct FCM using 200 μL whole blood	2,108/mL blood (0–7,800) $n=62$	1,027/mL blood (20–5,100) $n=10$	Early SSc↑ Late SSc→
Zhu et al. [23]	$CD34^+CD133^+$ $CD309^+$	Direct FCM using 200 μL whole blood	0.006 % in PBMCs $n=44$	0.076 % in PBMCs $n=18$	SSc↓↓
Avouac et al. [29]	Lin^-CD34^+ $CD133^+CD309^+$	Lin^- cell sorting from 10 mL whole blood followed by FCM	$86/10^6$ Lin^- cells (5–282) $n=50$	$49/10^6$ Lin^- cells (5–275) $n=26$	SSc↑
Nevskaya et al. [30]	$CD34^+CD309^+$	Direct FCM using 100 μL whole blood	0.0080 % in PBMCs (0.0001–0.0092) $n=40$	0.0037 % in PBMCs (0.0001–0.0098) $n=24$	Early SSc↑ Late SSc↓
Mok et al. [24]	$CD133^+CD309^+$	Direct FCM using 100 μL whole blood	3,000/mL blood (ND) $n=52$	7,000/mL blood (ND) $n=52$	SSc↓↓
Andrigueti et al. [25]	$CD34^+CD133^+$ $CD309^+$	FCM using PBMCs from 10 mL whole blood	$155/10^6$ PBMCs $n=44$	$241/10^6$ PBMCs $n=39$	SSc↓

FCM flow cytometry, *ND* not described, *PBMCs* peripheral blood mononuclear cells

20 mL, is required as a starting material to obtain reliable and reproducible counts of immature PHCs.

In an effort to standardize the research on EPCs, the European League Against Rheumatism (EULAR) Scleroderma Trials and Research (EUSTAR) proposed recommendations on EPC researches, including both colony-forming cultures and direct detection by flow cytometry [31]. To verify these recommendations, we have directly compared several different protocols for quantifying immature PHCs [32]. In previous studies, immature PHCs were quantified using a variety of strategies and were expressed as a proportion (%) in mononuclear cells or the absolute number in peripheral blood or in 10^6 Lin^- cells, but we have found that the quantification strategy strongly impacts the consistency of the results. Especially, the inter-method concordance was very poor when $CD34^+$ or Lin^- cell counts, based on acquisitioned events by a flow cytometer, were used as a reference. This is probably because $CD34^+$ or Lin^- cells are rare cell populations that comprise less than 1 % of circulating mononuclear cells. The purity of the enriched fractions varied greatly among samples even when an intensive gating protocol was applied to eliminate dead cells or irrelevant cells. As a result, the EUSTAR recommendations are valid when combined with an accurate quantification technique, such as the use of fluorospheres as an internal calibrator, which substantially improved the reproducibility of the results. Using standardized protocols, we have shown that circulating immature PHCs are reduced in SSc patients in comparison with healthy controls, although large-scale studies that conform to EUSTAR recommendations and use an accurate calibration protocol are definitely necessary to answer the central question if immature PHCs are increased or reduced in circulation of SSc patients.

Clinical associations of immature PHC counts have been examined in SSc patients. Most studies focused on vascular manifestations, including digital ulcer, but one cross-sectional study showed that lower immature PHC counts were associated with the higher Medsger's severity score [29]. All studies evaluated found an association between the presence of digital ulcer and low immature PHC counts [24, 25, 27, 29]. A recent prospective study involving 100 patients with SSc revealed that low EPC counts in addition to high placental growth factor levels were identified as independent predictors of the occurrence of at least one new digital ulcer during follow-up [33]. A recent study by Avouac and colleagues has revealed that decreased immature PHC counts are associated with the late pattern of nailfold capillaroscopic findings [34]. On the other hand, associations of immature PHC counts with other manifestations of SSc have not been reported, but low immature PHC counts are shown to be associated with patients with pulmonary arterial hypertension [35] or those with idiopathic pulmonary fibrosis [36]. These findings together suggest that insufficient property exerted by immature PHCs leads to the formation of digital ulcers and probably other vascular manifestations of SSc. In addition, it has been reproducibly shown that disease duration of SSc is negatively correlated with circulating counts of immature PHCs [25, 27, 28, 30]. This correlation is confirmed in our series of Japanese patients with SSc as well (Fig. 3.1).

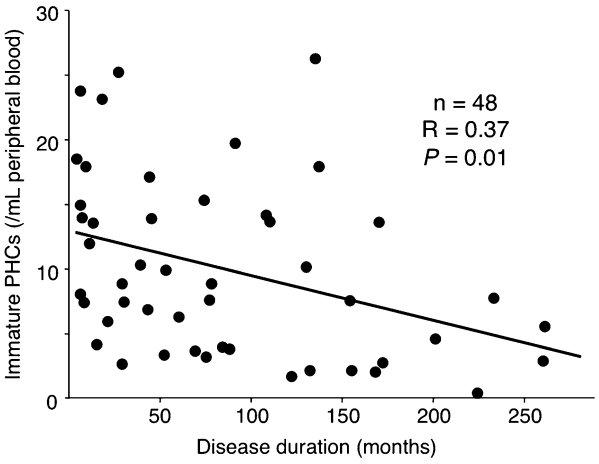

Fig. 3.1 A negative correlation between immature PHC counts and disease duration in SSc patients. These data are derived from our cohort of 48 patients with SSc

3.4 Mechanisms for Immature PHC Deficiency in SSc Patients

There are several mechanisms responsible for reduced counts of immature PHCs in SSc patients (Fig. 3.2). Tissue hypoxia caused by vascular damage and spasm induces upregulation of pro-angiogenic mediators, such as VEGF and SDF-1, which rapidly mobilize immature PHCs from the bone marrow and guide them into ischemic tissues. This response may be impaired in SSc patients potentially through mechanisms as described below. First, it is possible that persistent vascular damage eventually leads to depletion of immature PHCs. This theory is reported in patients with long-standing atherosclerosis [37]. In fact, a negative correlation between disease duration of SSc and circulating counts of immature PHCs has been reproducibly reported in several studies [25, 27, 28, 30]. This may explain reduced immature PHC counts in the later phase of the disease and may cause development of digital ulcer in clinical setting, but is irrelevant to the formation of vascular changes observed in the early SSc. Another mechanism includes impaired bone marrow microenvironment for immature PHCs despite upregulation of a series of pro-angiogenic factors. This dysregulated microenvironment within the bone marrow may suppress development and mobilization of immature PHCs. In this regard, we have recently found that immature PHC counts are inversely correlated with the level of circulating pentraxin 3, a multifunctional pattern recognition protein with capacity to inhibit angiogenesis through suppression of fibroblast growth factor-2 (FGF2) [38]. Pentraxin 3 is capable of inhibiting differentiation of bone marrow stem cells into immature PHCs in in vitro cultures with FGF2, indicating that exposure to a high concentration of pentraxin 3 suppresses the FGF2-mediated immature PHC differentiation in the bone marrow. Finally, immature PHCs in circulation may be destroyed though autoimmune mechanisms. Zhu and colleagues found that sera from SSc patients were able to induce apoptosis of

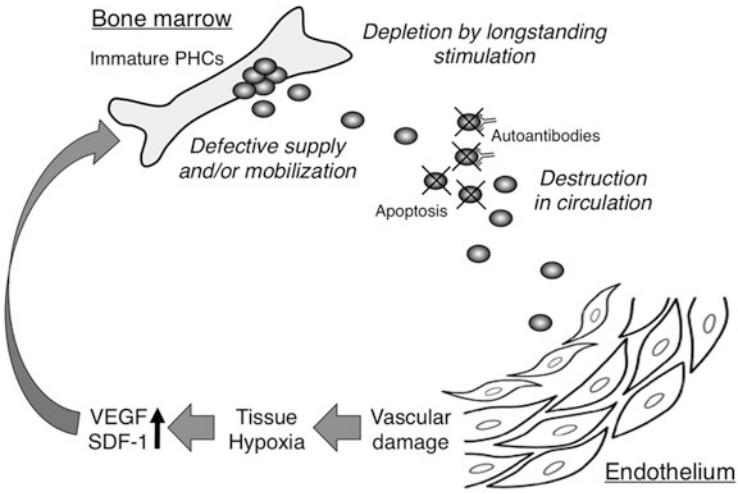

Fig. 3.2 Mechanisms responsible for reduced counts of immature PHCs in SSc patients. Tissue hypoxia caused by vascular damage induces upregulation of pro-angiogenic mediators, such as VEGF and SDF-1, which rapidly mobilize immature PHCs from the bone marrow and guide them into ischemic tissues. This response is impaired in SSc patients through mechanisms including depletion by long-standing stimulation, defective supply and/or mobilization in the bone marrow, and destruction in circulation by autoantibodies

immature PHCs, which was mediated through the Akt-FOXO3a-Bim pathway [23]. Moreover, George and co-workers recently reported the presence of IgG autoantibodies reactive with late-outgrowth EPCs in patients with a graduated atherosclerotic risk profile, using a cell-based enzyme-linked immunosorbent assay [39]. Interestingly, antigenic specificity of anti-late-outgrowth EPC antibodies was distinct from that of antibodies to mature endothelial cells (anti-endothelial cell antibodies, AECAs), whereas other studies found that AECAs induce apoptosis of bone marrow immature PHCs in SSc patients [40].

3.5 Altered Function in Immature PHCs in SSc Patients

Only few studies have evaluated the functional properties of EPCs in SSc patients, primarily because of difficulty of preparing sufficient numbers of these populations for functional assays. Nevertheless, we previously reported that the potential of SSc-derived immature PHCs to differentiate into mature endothelial cells was impaired in in vitro cultures of circulating $CD133^+$ cells containing immature PHCs [22]. Another study utilizing late-outgrowth EPCs showed impaired differentiation potential to endothelial cells in cultured cells derived from SSc patients, compared with those from healthy controls [41], although their functional properties may be altered by repeated passages and exposure to high concentrations of pro-angiogenic factors in a long-term culture. We have recently established a

system for evaluating the in vivo function of circulating immature PHCs, using a murine tumor neovascularization model, in which freshly isolated human $CD133^+$ cells are transplanted into the back of mice in conjunction with syngeneic mouse tumor cells [42]. Using this assay system, we have successfully demonstrated that neovascularization capacity of circulating immature PHCs in SSc patients is impaired, partly due to deficiency in their vasculogenesis ability. Therefore, defects in vasculogenesis observed in SSc patients are likely to be mediated through impaired immature PHC function, irrespective of its quantity.

Currently, little is known about the mechanisms behind functional aberrations in circulating immature PHCs in SSc patients. In this regard, Del Papa and colleagues reported that immature PHCs in the bone marrow from SSc patients were defective in their ability to proliferate in long-term culture with pro-angiogenic factors [27], suggesting that immature PHCs were functionally altered before their release into circulation. A recent study of the bone marrow of patients with diffuse cutaneous SSc showed markedly reduced microvascular density and increased fibrosis [43]. This dysregulated microenvironment within the bone marrow may alter the developmental process of immature PHCs.

3.6 Roles of Monocytic PHCs in SSc Pathogenesis

In contrast to immature PHCs, information on the roles of monocytic PHCs in SSc vasculopathy is limited. We recently evaluated the number of monocytic PHCs in SSc patients using a culture system developed to enrich this cell population [44]. Unexpectedly, we observed a paradoxical increase in circulating monocytic PHCs in SSc patients compared with age- and sex-matched healthy controls. Intriguingly, monocytic PHCs derived from SSc patients showed enhanced in vitro tubular structure formation compared with those from healthy controls. Furthermore, in a murine tumor neovascularization model, the transplantation of SSc-derived monocytic PHCs dramatically promoted tumor growth and tumor vessel formation in vivo, indicating that monocytic PHCs derived from SSc patients have enhanced angiogenic activity. On the other hand, vasculogenic capacity of SSc-derived monocytic PHCs was impaired, same as immature PHCs.

The increased number and enhanced pro-angiogenic potency of monocytic PHCs might be a compensatory response to impaired vasculogenesis mediated through immature PHCs. Circulating monocytic PHCs are mobilized from the bone marrow and recruited to SSc-induced lesions in response to chemokines such as MCP-1 and SDF-1, which are upregulated in the affected skin of SSc patients [45, 46]. In addition, it has been reported that hypoxic condition of the affected tissues of SSc patients stimulates the differentiation of circulating monocytic PHCs [47]. Taken together, these stimulations may promote accumulation of functionally altered monocytic PHCs into affected lesions of SSc. Since monocytic PHCs are oligopotent in terms of their capacity to differentiate into mesenchymal lineage cells [48–50], they may differentiate into fibroblast-like cells, produce collagens and other extracellular matrix proteins, and participate in the fibrotic

process of SSc. In this regard, fibrocytes derived from $CD14^+$ monocytes home to the site of tissue injury and contribute to tissue repair and fibrosis by differentiating into myofibroblasts [51]. In addition, circulating $CD14^+$ monocytes acquire the ability to produce extracellular matrix components in an MCP-1/CCR2-dependent amplification loop [52]. Our recent data on gene expression profiling of circulating $CD14^+$ monocytes identified MCP-1 and versican as genes upregulated in SSc monocytes [53]. Since versican is a chondroitin sulfate proteoglycan with capacity to bind a variety of growth factors and chemokines, including MCP-1, and works as their reservoir, increased expression of versican by SSc monocytes may amplify MCP-1-/CCR2-dependent fibrogenic process and contribute to the fibrotic aspects of SSc. The enhanced profibrotic phenotype of circulating $CD14^+$ monocytes was also reported in SSc patients with interstitial lung disease [54]. Another report described a correlation between fibrotic clinical features and the increased proportion of $CXCR4^+$ circulating cells with monocytic and endothelial markers, which correspond to monocytic PHCs, in SSc patients [55]. Therefore, monocytic PHCs accumulated at affected sites of SSc may acquire profibrotic characteristics and contribute to the promotion of fibrosis.

3.7 Development of SSc Vasculopathy Mediated Through Defective Angiogenesis and Vasculogenesis

Despite the robust pro-angiogenic responses, appropriate blood vessel formation apparently does not occur in patients with SSc. Thus, impaired angiogenesis and vasculogenesis play a critical role in the pathogenic process of SSc vasculopathy. In the early phase of SSc, a variety of processes continuously damage the endothelium, leading to subsequent expression of a series of angiogenic factors, growth factors, and chemokines, including VEGF, MCP-1, and SDF-1. Normally, the denuded vessels would be rapidly fixed by a highly regulated process of vasculogenesis through recruitment of various EPCs, but, in SSc patients, vasculogenesis mediated by immature PHCs is impaired because of their reduced counts in circulation and dysfunctional maturation potential. In compensation for defect in immature PHC-mediated vasculogenesis, monocytic PHCs are recruited into circulation and function to enhance angiogenesis. But, this mechanism eventually fails to repair vessels because of local mechanisms inhibiting angiogenesis. In this regard, in microvascular endothelial cells isolated from the skin of SSc patients, matrix metalloproteinase (MMP)-12 is overexpressed and cleaves urokinase-type plasminogen activator receptor, causing inhibition of the invasion/migration capacities of endothelial cells [56, 57]. The reduction of tissue kallikreins 9, 11, and 12, which exert a mitogenic effect on endothelial cells, and the upregulation of anti-angiogenic kallikrein 3 were reported in the SSc skin [58]. In SSc skin lesions, endothelial cells lose their expression of VE-cadherin, which is required for vascular tube formation [59]. Furthermore, selective upregulation of the anti-angiogenic VEGF-b isoform was observed in the circulation and skin of SSc patients, indicating a switch from the pro-angiogenic to the anti-angiogenic VEGF isoform

[60]. Finally, loss of epidermal growth factor-like domain 7 expression in endothelial cells and EPCs plays a role in the defective vascular repair process in patients with SSc [61]. These dysregulated endothelial features at the affected site of SSc are responsible for the disease-related defects in angiogenesis and prevent vascular repair. In addition, monocytic PHCs accumulated at affected sites acquire profibrotic characteristics and contribute to the promotion of fibrosis under strong anti-angiogenic and profibrotic environment.

3.8 Potential Therapeutic Strategies Targeting EPCs

There has been minimal success in treating the vascular manifestations of SSc with nonselective vasodilators. Given a critical role of impaired vasculogenesis in the pathogenic process of SSc vasculopathy, agents that modify counts and/or function of EPCs may be promising treatment modalities in patients with SSc. Table 3.3 lists currently available drugs that have been shown to have ability to increase EPC counts and/or improve EPC function [62–65]. One set of candidates is the statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors [66]. Besides their well-established lipid-lowering effect, statins have cholesterol-independent "pleiotropic" effects, which can restore or improve endothelial functions [67]. In a randomized, double-blind, placebo-controlled trial of atorvastatin on vascular manifestations of SSc, significant reductions in the onset and overall number of digital ulcers were observed in the atorvastatin group, compared with the placebo group [68]. In addition, in animal models of ischemia, statins were shown to induce recruitment of immature PHCs into the periphery and repair of injured endothelium in vivo [69–72]. Therefore, we conducted an open-label, prospective study of atorvastatin in patients with SSc [73]. Atorvastatin treatment resulted in an increase in immature PHCs in circulation, and the number

Table 3.3 Currently available drugs reported to increase EPC counts or improve EPC function

Drugs reported to increase EPC counts	Drugs reported to improve EPC function
Statins	Statins
Metformin	Glucagon-like peptide-1 agonists
Sulfonylurea	Dipeptidyl peptidase-4 inhibitors
Glucagon-like peptide-1 agonists	Thiazolidinediones
Dipeptidyl peptidase-4 inhibitors	Insulin
Thiazolidinediones	Angiotensin-converting enzyme inhibitors
Insulin	Angiotensin II receptor blockers
Angiotensin-converting enzyme inhibitors	Antiplatelet drugs
Angiotensin II receptor blockers	Phosphodiesterase-5 inhibitors
Phosphodiesterase-5 inhibitors	Erythropoietin
Erythropoietin	
G-CSF	

returned to baseline after discontinuation of the drug. During the treatment, Raynaud's condition score was improved, and soluble VCAM-1, an endothelial injury marker, was gradually reduced. On the other hand, simvastatin was shown to increase immature PHC counts in patients with hypercholesterolemia, but fails to increase immature PHCs in SSc patients [74], suggesting varied effects on immature PHCs among statins. We further evaluated the long-term effect of atorvastatin by an open-label study for 24 months [75]. Interestingly, immature PHC counts peaked at 1 month and gradually decreased thereafter. Nevertheless, Raynaud's condition score and soluble VCAM-1 level remained low. These long-term effects of statins on peripheral vascular disease cannot be explained by mobilization of immature PHCs alone, and it is likely that other pleiotropic effects of statins, such as improvement of endothelial function, play a role. Other small molecules and recombinant growth factors, including G-CSF and erythropoietin, may provide favorable effects on vascular manifestations in SSc patients, although there is no data supporting their efficacy to date. In addition, continuous exercise program is also shown to promote mobilization of immature PHCs [76].

We have recently found that cyclophosphamide, which is an immunosuppressant proved to be effective for treating interstitial lung disease associated with SSc, also has capacity to increase circulating immature PHCs. Cyclophosphamide is capable of mobilizing hematopoietic stem cells/progenitors from the bone marrow when a high dose was administered intravenously, but it remains unknown if low-dose regimen used for SSc patients mobilized immature PHCs as well. To evaluate the effects of intravenous cyclophosphamide (IVCY) on immature PHC counts, we performed an open-label, prospective study involving SSc patients treated with six courses of IVCY in combination with low-dose prednisolone [77]. As a result, IVCY plus corticosteroids, but not corticosteroids alone, increased the circulating counts of immature PHCs in nearly half of the patients. Immature PHC responders showed trends toward reduced levels of circulating vascular injury markers and a low probability of developing end-stage lung disease, while nonresponders did not. These findings suggest that mobilization of immature PHCs may contribute to the efficacy of IVCY for treating SSc-associated interstitial lung disease.

3.9 Conclusion

Immature and monocytic PHCs contribute to postnatal blood vessel formation and vascular repair, mainly through their immediate recruitment to the site of vascular injury, their secretion of a variety of pro-angiogenic factors, and their differentiation into endothelial cell-like cells. These cells are also oligopotent and can differentiate into fibroblast-like cells. These unique features raise the intriguing hypothesis that EPCs are involved in the pathogenesis of SSc by participating in two major pathological aspects of the disease, microvasculopathy and excessive fibrosis. Understanding the roles of EPCs in disease process of SSc may be key to dissecting its pathogenesis and to developing novel therapeutic strategies.

Acknowledgments This work was supported by a research grant on intractable diseases from the Japanese Ministry of Health, Labour and Welfare and a grant from the Japanese Ministry of Education, Science, Sports and Culture.

References

1. Fischer C, Schneider M, Carmeliet P. Principles and therapeutic implications of angiogenesis, vasculogenesis and arteriogenesis. Handb Exp Pharmacol. 2006;176:157–212.
2. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science. 1997;275:964–7.
3. Guiducci S, Giacomelli R, Matucci-Cerinic M. Vascular complications of scleroderma. Autoimmun Rev. 2007;6:520–3.
4. Liakouli V, Cipriani P, Marrelli A, Alvaro S, Ruscitti P, Giacomelli R. Angiogenic cytokines and growth factors in systemic sclerosis. Autoimmun Rev. 2011;110:590–4.
5. Herrick A, Cutolo M. Clinical implications from capillaroscopic analysis in patients with Raynaud's phenomenon and systemic sclerosis. Arthritis Rheum. 2010;62:2595–604.
6. Watt SM, Athanassopoulos A, Harris AL, Tsaknakis G. Human endothelial stem/progenitor cells, angiogenic factors and vascular repair. J R Soc Interface. 2010;7:S731–51.
7. Prater DN, Case J, Ingram DA, Yoder MC. Working hypothesis to redefine endothelial progenitor cells. Leukemia. 2007;21:1141–9.
8. Estes ML, Mund JA, Ingram DA, Case J. Identification of endothelial cells and progenitor cell subsets in human peripheral blood. Curr Protoc Cytom. 2010;52:9.33.1–11.
9. Yoder MC. Is endothelium the origin of endothelial progenitor cells? Arterioscler Thromb Vasc Biol. 2010;30:1094–103.
10. Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, et al. Expression of VEGFR-2 and AC133 by circulating human $CD34^+$ cells identifies a population of functional endothelial precursors. Blood. 2000;95:952–8.
11. Richardson MR, Yoder MC. Endothelial progenitor cells: Quo Vadis? J Mol Cell Cardiol. 2011;50:266–72.
12. Elsheikh E, Uzunel M, He Z, Holgersson J, Nowak G, Sumitran-Holgersson S. Only a specific subset of human peripheral-blood monocytes has endothelial-like functional capacity. Blood. 2005;106:2347–55.
13. Kuwana M, Okazaki Y, Kodama H, Satoh T, Kawakami Y, Ikeda Y. Endothelial differentiation potential of human monocyte-derived multipotential cells. Stem Cells. 2006;24:2733–43.
14. Rehman J, Li JL, Orschell CM, March KL. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation. 2003;107:1164–9.
15. Urbich C, Aicher A, Heeschen C, Dernbach E, Hofmann WK, Zeiher AM, et al. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. J Mol Cell Cardiol. 2005;39:733–42.
16. Yamaguchi Y, Kuwana M. Proangiogenic hematopoietic cells of monocytic origin: roles in vascular regeneration and pathogenic processes of systemic sclerosis. Histol Histopathol. 2013;28:175–83.
17. Semenza GL. Vasculogenesis, angiogenesis, and arteriogenesis: mechanisms of blood vessel formation and remodeling. J Cell Biochem. 2007;102:840–7.
18. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med. 2003;348:593–600.
19. Rohde E, Malischnik C, Thaler D, Maierhofer T, Linkesch W, Lanzer G, et al. Blood monocytes mimic endothelial progenitor cells. Stem Cells. 2006;24:357–67.

20. Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K, et al. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood. 2004;104:2752–60.
21. Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, et al. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. Blood. 2007;109:1801–9.
22. Kuwana M, Okazaki Y, Yasuoka H, Kawakami Y, Ikeda Y. Defective vasculogenesis in systemic sclerosis. Lancet. 2004;364:603–10.
23. Zhu S, Evans S, Yan B, Povsic TJ, Tapson V, Goldschmidt-Clermont PJ, et al. Transcriptional regulation of Bim by FOXO3a and Akt mediates scleroderma serum-induced apoptosis in endothelial progenitor cells. Circulation. 2008;118:2156–65.
24. Mok MY, Yiu KH, Wong CY, Qiuwaxi J, Lai WH, Wong WS, et al. Low circulating level of $CD133^{+}KDR^{+}$ cells in patients with systemic sclerosis. Clin Exp Rheumatol. 2010;28:S19–25.
25. Andrigueti FV, Arismendi MI, Ebbing PC, Kayser C. Decreased numbers of endothelial progenitor cells in patients in the early stages of systemic sclerosis. Microvasc Res. 2015;98:82–7.
26. Del Papa N, Colombo G, Fracchiolla N, Moronetti LM, Ingegnoli F, Maglione W, et al. Circulating endothelial cells as a marker of ongoing vascular disease in systemic sclerosis. Arthritis Rheum. 2004;50:1296–304.
27. Del Papa N, Quirici N, Soligo D, Scavullo C, Cortiana M, Borsotti C, et al. Bone marrow endothelial progenitors are defective in systemic sclerosis. Arthritis Rheum. 2006;54:2605–15.
28. Allanore Y, Batteux F, Avouac J, Assous N, Weill B, Kahan A. Levels of circulating endothelial progenitor cells in systemic sclerosis. Clin Exp Rheumatol. 2007;25:60–6.
29. Avouac J, Juin F, Wipff J, Couraud P, Chiocchia G, Kahan A, et al. Circulating endothelial progenitor cells in systemic sclerosis: association with disease severity. Ann Rheum Dis. 2008;67:1455–60.
30. Nevskaya T, Bykovskaia S, Lyssuk E, Shakhov I, Zaprjagaeva M, Mach E, et al. Circulating endothelial progenitor cells in systemic sclerosis: relation to impaired angiogenesis and cardiovascular manifestations. Clin Exp Rheumatol. 2008;26:421–9.
31. Distler JH, Allanore Y, Avouac J, Giacomelli R, Guiducci S, Moritz F, et al. EULAR Scleroderma Trials and Research group statement and recommendations on endothelial precursor cells. Ann Rheum Dis. 2009;68:163–8.
32. Kuwana M, Okazaki Y. Quantification of circulating endothelial progenitor cells in systemic sclerosis: a direct comparison of protocols. Ann Rheum Dis. 2012;71:617–20.
33. Avouac J, Meune C, Ruiz B, Couraud PO, Uzan G, Boileau C, et al. Angiogenic biomarkers predict the occurrence of digital ulcers in systemic sclerosis. Ann Rheum Dis. 2012;71:394–9.
34. Avouac J, Vallucci M, Smith V, Senet P, Ruiz B, Sulli A, et al. Correlations between angiogenic factors and capillaroscopic patterns in systemic sclerosis. Arthritis Res Ther. 2013;15(2):R55.
35. Chen H, Strappe P, Chen S, Wang LX. Endothelial progenitor cells and pulmonary arterial hypertension. Heart Lung Circ. 2014;23:595–601.
36. Malli F, Koutsokera A, Paraskeva E, Zakynthinos E, Papagianni M, Makris D, et al. Endothelial progenitor cells in the pathogenesis of idiopathic pulmonary fibrosis: an evolving concept. PLoS One. 2013;8:e53658.
37. Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res. 2001;89:E1–7.
38. Shirai Y, Okazaki Y, Inoue Y, Tamura Y, Yasuoka H, Takeuchi T, et al. Elevated levels of pentraxin 3 in systemic sclerosis: associations with vascular manifestations and defective vasculogenesis. Arthritis Rheumatol. 2015;67:498–507.
39. George J, Matucci-Cerinic M, Bar I, Shimoni S. Circulating autoantibodies to endothelial progenitor cells: binding characteristics and association with risk factors for atherosclerosis. PLoS One. 2014;9:e97836.

40. Del Papa N, Quirici N, Scavullo C, Gianelli U, Corti L, Vitali C, et al. Antiendothelial cell antibodies induce apoptosis of bone marrow endothelial progenitors in systemic sclerosis. J Rheumatol. 2010;37:2053–63.
41. Avouac J, Wipff J, Goldman O, Ruiz B, Couraud PO, Chiocchia G, et al. Angiogenesis in systemic sclerosis: impaired expression of vascular endothelial growth factor receptor 1 in endothelial progenitor-derived cells under hypoxic conditions. Arthritis Rheum. 2008;58:3550–61.
42. Kuwana M, Okazaki Y. Impaired *in vivo* neovascularization capacity of endothelial progenitor cells in patients with systemic sclerosis. Arthritis Rheumatol. 2014;66:1300–5.
43. Carrai V, Miniati I, Guiducci S, Capaccioli G, Alterini R, Saccardi R, et al. Evidence for reduced angiogenesis in bone marrow in SSc: immunohistochemistry and multiparametric computerized imaging analysis. Rheumatology. 2012;51:1042–8.
44. Yamaguchi Y, Okazaki Y, Seta N, Satoh T, Takahashi K, Ikezawa Z, et al. Enhanced angiogenic potency of monocytic endothelial progenitor cells in patients with systemic sclerosis. Arthritis Res Ther. 2010;12:R205.
45. Distler O, Pap T, Kowal-Bielecka O, Meyringer R, Guiducci S, Landthaler M, et al. Overexpression of monocyte chemoattractant protein 1 in systemic sclerosis: role of platelet-derived growth factor and effects on monocyte chemotaxis and collagen synthesis. Arthritis Rheum. 2001;44:2665–78.
46. Cipriani P, Franca Milia A, Liakouli V, Pacini A, Manetti M, Marrelli A, et al. Differential expression of stromal cell-derived factor 1 and its receptor CXCR4 in the skin and endothelial cells of systemic sclerosis patients: pathogenetic implications. Arthritis Rheum. 2006;54:3022–33.
47. Bellik L, Musilli C, Vinci MC, Ledda F, Parenti A. Human mature endothelial cells modulate peripheral blood mononuclear cell differentiation toward an endothelial phenotype. Exp Cell Res. 2008;314:2965–74.
48. Badorff C, Brandes RP, Popp R, Rupp S, Urbich C, Aicher A, et al. Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes. Circulation. 2003;107:1024–32.
49. Kuwana M, Okazaki Y, Kodama H, Izumi K, Yasuoka H, Ogawa Y, et al. Human circulating $CD14^+$ monocytes as a source of progenitors that exhibit mesenchymal cell differentiation. J Leukoc Biol. 2003;74:833–45.
50. Romagnani P, Annunziato F, Liotta F, Lazzeri E, Mazzinghi B, Frosali F, et al. $CD14^+CD34^{low}$ cells with stem cell phenotypic and functional features are the major source of circulating endothelial progenitors. Circ Res. 2005;97:314–22.
51. Abe R, Donnelly SC, Peng T, Bucala R, Metz CN. Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. J Immunol. 2001;166:7556–62.
52. Sakai N, Wada T, Fruichi K, Shimizu K, Kokubo S, Hara A, et al. MCP-1/CCR2-dependent loop for fibrogenesis in human peripheral CD14-positive monocytes. J Leukoc Biol. 2006;79:555–63.
53. Masuda A, Yasuoka H, Satoh T, Okazaki Y, Yamaguchi Y, Kuwana M. Versican is upregulated in circulating monocytes in patients with systemic sclerosis and amplifies a CCL2-mediated pathogenic loop. Arthritis Res Ther. 2013;15:R74.
54. Mathai SK, Gulati M, Peng X, Russell TR, Shaw AC, Rubinowitz AN, et al. Circulating monocytes from systemic sclerosis patients with interstitial lung disease show an enhanced profibrotic phenotype. Lab Invest. 2010;90:812–23.
55. Campioni D, Lo Monaco A, Lanza F, Moretti S, Ferrari L, Fotinidi M, et al. $CXCR4^+$ circulating progenitor cells coexpressing monocytic and endothelial markers correlating with fibrotic clinical features are present in the peripheral blood of patients affected by systemic sclerosis. Haematologica. 2008;93:1233–7.
56. D'Alessio S, Fibbi G, Cinelli M, Guiducci S, Del Rosso A, Margheri F, et al. Matrix metalloproteinase 12-dependent cleavage of urokinase receptor in systemic sclerosis

microvascular endothelial cells results in impaired angiogenesis. Arthritis Rheum. 2004;50:3275–85.
57. Margheri F, Manetti M, Serratì S, Nosi D, Pucci M, Matucci-Cerinic M, et al. Domain 1 of the urokinase-type plasminogen activator receptor is required for its morphologic and functional, β2 integrin-mediated connection with actin cytoskeleton in human microvascular endothelial cells: failure of association in systemic sclerosis endothelial cells. Arthritis Rheum. 2006;54:3926–38.
58. Giusti B, Serratì S, Margheri F, Papucci L, Rossi L, Poggi F, et al. The antiangiogenic tissue kallikrein pattern of endothelial cells in systemic sclerosis. Arthritis Rheum. 2005;52:3618–28.
59. Fleming JN, Nash RA, McLeod DO, Fiorentino DF, Shulman HM, Connolly MK, et al. Capillary regeneration in scleroderma: stem cell therapy reverses phenotype? PLoS One. 2008;3:e1452.
60. Manetti M, Guiducci S, Romano E, Ceccarelli C, Bellando-Randone S, Conforti ML, et al. Overexpression of VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, leads to insufficient angiogenesis in patients with systemic sclerosis. Circ Res. 2011;109:e14–26.
61. Manetti M, Guiducci S, Romano E, Avouac J, Rosa I, Ruiz B, et al. Decreased expression of the endothelial cell-derived factor EGFL7 in systemic sclerosis: potential contribution to impaired angiogenesis and vasculogenesis. Arthritis Res Ther. 2013;15:R165.
62. Roks AJ, Rodgers K, Walther T. Effects of the renin angiotensin system on vasculogenesis-related progenitor cells. Curr Opin Pharmacol. 2011;11:162–74.
63. Schwartz BG, Jackson G, Stecher VJ, Campoli-Richards DM, Kloner RA. Phosphodiesterase type 5 inhibitors improve endothelial function and may benefit cardiovascular conditions. Am J Med. 2013;126:192–9.
64. Desouza CV. Does drug therapy reverse endothelial progenitor cell dysfunction in diabetes? J Diabetes Complications. 2013;27:519–25.
65. Sanchis-Gomar F, Garcia-Gimenez JL, Pareja-Galeano H, Romagnoli M, Perez-Quilis C, Lippi G. Erythropoietin and the heart: physiological effects and the therapeutic perspective. Int J Cardiol. 2014;171:116–25.
66. Kuwana M. Potential benefit of statins for vascular disease in systemic sclerosis. Curr Opin Rheumatol. 2006;18:594–600.
67. Zhou Q, Liao JK. Statins and cardiovascular diseases: from cholesterol lowering to pleiotropy. Curr Pharm Des. 2009;15:467–78.
68. Abou-Raya A, Abou-Raya S, Helmii M. Statins: potentially useful in therapy of systemic sclerosis-related Raynaud's phenomenon and digital ulcers. J Rheumatol. 2008;35:1801–8.
69. Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, et al. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. Nat Med. 2000;6:1004–10.
70. Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, et al. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. J Clin Invest. 2001;108:391–7.
71. Llevadot J, Murasawa S, Kureishi Y, Uchida S, Masuda H, Kawamoto A, et al. HMG-CoA reductase inhibitor mobilizes bone marrow-derived endothelial progenitor cells. J Clin Invest. 2001;108:399–405.
72. Walter DH, Rittig K, Bahlmann FH, Kirchmair R, Silver M, Murayama T, et al. Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. Circulation. 2002;105:3017–24.
73. Kuwana M, Kaburaki J, Okazaki Y, Yasuoka H, Kawakami Y, Ikeda Y. Increase in circulating endothelial precursors by atorvastatin in patients with systemic sclerosis. Arthritis Rheum. 2006;54:1946–51.

74. Del Papa N, Cortiana M, Vitali C, Silvestris I, Maglione W, Comina DP, et al. Simvastatin reduces endothelial activation and damage but is partially ineffective in inducing endothelial repair in systemic sclerosis. J Rheumatol. 2008;35:1323–8.
75. Kuwana M, Okazaki Y, Kaburaki J. Long-term beneficial effects of statins on vascular manifestations in patients with systemic sclerosis. Mod Rheumatol. 2009;19:530–5.
76. Ribeiro F, Ribeiro IP, Alves AJ, do Céu Monteiro M, Oliveira NL, Oliveira J, et al. Effects of exercise training on endothelial progenitor cells in cardiovascular disease: a systematic review. Am J Phys Med Rehabil. 2013;92:1020–30.
77. Furuya Y, Okazaki Y, Kaji K, Sato S, Takehara K, Kuwana M. Mobilization of endothelial progenitor cells by intravenous cyclophosphamide in patients with systemic sclerosis: potential association with efficacy for interstitial lung disease. Rheumatology. 2010;49:2375–80.

Animal Models of Systemic Sclerosis

4

Toshiyuki Yamamoto

Abstract

Systemic sclerosis (scleroderma) is a fibrotic condition characterized by immunologic abnormalities, vascular injury, and increased accumulation of extracellular matrix proteins in the affected organs. The etiology of scleroderma has not yet been fully elucidated, and thus satisfactory therapeutic drugs are still limited. However, there are two definite and easily available mouse models so far, without genetic modification, tight skin mouse, and bleomycin-induced murine scleroderma. Making use of those mice, many research projects have been performed for a better understanding of the pathophysiology of, and exploring effective therapies for, scleroderma. Furthermore, a number of transgenic or gene-deficient mice are also produced, which mimic human scleroderma. They reproduce several, but not all, histological as well as biochemical aspects resembling human scleroderma, and we can learn lots of new findings through animal studies. This paper introduces current concepts of various animal models for scleroderma and discusses several new aspects of scleroderma pathogenesis and therapeutic approach for scleroderma.

Keywords

Scleroderma • Animal model

T. Yamamoto (✉)
Department of Dermatology, Fukushima Medical University, 1 Hikarigaoka, Fukushima 960-1295, Japan
e-mail: toyamade@fmu.ac.jp

K. Takehara et al. (eds.), *Systemic Sclerosis*, DOI 10.1007/978-4-431-55708-1_4

4.1 Introduction

Systemic sclerosis (SSc) is a connective tissue disease which shows fibrogenesis and vasculogenesis. Although the pathogenesis of SSc has not been fully elucidated yet, it is characterized by the excessive accumulation of extracellular matrix (ECM) proteins in the skin and various internal organs, vascular injury, and immunological abnormalities [1]. In early stages of SSc, activated fibroblasts in the affected areas produce high amounts of collagen. Histological analysis of the initial stage of scleroderma reveals perivascular infiltrates of mononuclear cells in the dermis, which is associated with increased collagen synthesis in the surrounding fibroblasts. A number of studies have demonstrated the crucial role of several fibrogenic cytokines released from immunocytes in initiating the sequence of events leading to fibrosis.

Animal models are useful in providing clues for the study of various human diseases and for the exploration of new treatments. Although animal models which exhibit all the aspects of SSc are not currently available, several experimental animal models, as well as several transgenic or knockout mice, have been examined so far [2]. In this chapter, recent insights into the pathogenesis and therapeutic interventions using established mice models for SSc, i.e., bleomycin-induced scleroderma and tight skin mice, are highlighted.

4.2 Bleomycin-Induced Murine Scleroderma

4.2.1 Bleomycin

Bleomycin was originally isolated from the fungus *Streptomyces verticillus* and is frequently used as an antitumor agent for the treatment of various kinds of malignancy [3]. Bleomycin hydrolase inactivates bleomycin by hydrolyzing the amide bond in the β-aminoalanineamide moiety. Due to the deficiency of the enzyme in the lungs and the skin, bleomycin-induced toxicity occurs predominantly in these organs. Pulmonary fibrosis is a well-known adverse effect of bleomycin, and bleomycin-induced lung injury in rodents is an established model for human pulmonary fibrosis. Bleomycin-induced scleroderma is also reported, although rare [4]. In 1983, Mountz et al. [5] reported that rats injected repeatedly with sublethal doses of bleomycin over a 58-week period developed severe dermal fibrosis similar to those found in human scleroderma with structural abnormalities of collagen fibers; however, histological features were not shown in their paper. In 1999, we for the first time established bleomycin-induced murine scleroderma model by repeated local injections of bleomycin into the shaved back skins [6] and published in a series of studies [7–11]. Thereafter, this model has been admitted worldwide as a representative scleroderma model, and making use of this model, a number of studies have been performed.

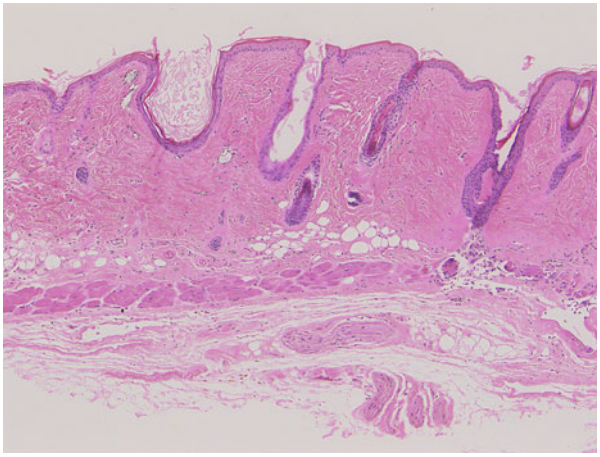

Fig. 4.1 Histological features of skin sclerosis induced by bleomycin (H&E stain) (Cited from Ref. [2])

4.2.2 Characteristics

Mice treated with local bleomycin injection develop intense dermal sclerosis, characterized by deposition of homogenous materials and thickened collagen bundles in the dermis. There is more or less difference of intensity of developing dermal sclerosis, suggestive of susceptibility for bleomycin, depending on strains. Histopathological examination revealed definite dermal sclerosis characterized by thickened collagen bundles and deposition of homogenous materials in the thickened dermis with cellular infiltrates (Fig. 4.1), which mimicked human scleroderma. Dermal thickness gradually increased with the onset of the sclerosis. Cellular infiltrates were composed of T cells, monocytes/macrophages, and mast cells, which are supposed to play an important role in the induction of dermal sclerosis. A study shows that transfer of CD4+ T cells from bleomycin-treated mice induced the same pathological changes and antibody production in untreated Balb/c nude mice [12]. However, dermal sclerosis can be compulsorily induced by bleomycin treatment, even in nude mice, severe combined immunodeficiency (SCID) mice, and mast cell-deficient mice [7, 9, 11], suggesting that T cells may not be essential for the induction of dermal sclerosis. Mast cells increased in number in tandem with the induction of dermal sclerosis. Also, a marked degranulation was found in particular in the early phase, with elevated plasma histamine levels [6]. In some strains, epidermal thickness was also induced as well [7]. Further, lung fibrosis showing thickened alveolar walls with cellular infiltrates was also induced early on. Cutaneous changes were generally localized to the area surrounding the injected site, and sclerotic changes were not induced in the remote regions, such as fingers or abdominal skin. After the stoppage of bleomycin injections, sclerotic changes remained at least 6 weeks, suggesting that the induction of dermal sclerosis is not transient but persistent. Thickness of vascular wall in the deep dermis was also observed [13]. There was some variation among strains in

the intensity of the symptoms and the period required to induce dermal sclerosis, and C3H/He, DBA/2, B10.D2, and B10.A strains demonstrated intense dermal sclerosis by bleomycin treatment, suggestive of bleomycin "susceptible" [8, 14]. Hydroxyproline contents in the bleomycin-treated skin were significantly increased in comparison with those of the PBS-treated skin. Increased production as well as upregulation of mRNA levels of type I collagen was found in the sclerotic skin [6, 8]. In the bleomycin-induced scleroderma, α-SMA-positive myofibroblasts were observed in the dermis and gradually increased in tandem with the induction of dermal sclerosis [10]. Interestingly, autoantibodies were detected in the serum after bleomycin treatment [6]. The induction of dermal sclerosis is considered to be, in part, mediated by inflammatory and fibrogenic cytokines, as well as by the direct effect of bleomycin on ECM synthesis in fibroblasts. Several methodological modifications have been tried, such as bleomycin-containing poly(L-lactic acid) microspheres [15], bleomycin-mixed methylcellulose gel [16], and also continuous infusion [17]. However, some of those techniques are difficult and can be available in limited institutes. This model has several advantages, such as an easy technique to induce dermal fibrosis, easily handling, short duration until the induction of dermal fibrosis, and high reproducibility independent of mice strains. One of the most useful advantages of this model is that this system can permit to investigate the induction of fibrosis from prior to, during, and after the development, through the fibrotic process continuously. This great advantage means that the initial event of skin fibrosis can be analyzed. By contrast, skin fibrosis is not diffusely induced, but restricted to the bleomycin-injected sites. Also, many factors, including non-specific or unessential factors in fibrosis, may be affected by bleomycin treatment.

4.2.3 Transforming Growth Factor-β

Bleomycin exerts various effects on skin-constituted cells such as fibroblasts, keratinocytes, and endothelial cells, as well as immunocytes [3]. In vitro, bleomycin upregulates collagen and transforming growth factor-β1 (TGF-β1) mRNA expression in cultured fibroblasts [18]. Also, bleomycin enhances gene expression of ECM proteins as well as fibrogenic cytokines such as TGF-β and connective tissue growth factor (CTGF) [18], which may contribute to the induction of fibrosis. It was shown that TGF-β is a mediator of the fibrotic effect of bleomycin at the transcriptional level and that the TGF-β response element is required for bleomycin stimulation of the proα1(I) collagen promoter [19]. Also, in vitro studies showed a dose-dependent stimulation of endothelial cell secretion of collagen synthesis by bleomycin, which was inhibited by the anti-TGF-β antibody [20]. TGF-β increases the synthesis of ECM by fibroblasts, modulates cell-matrix adhesion protein receptors, and regulates the production of proteins that can modify the ECM by proteolytic action, such as plasminogen activator, an inhibitor of plasminogen, or procollagenase. In addition, TGF-β is capable of stimulating its own synthesis by fibroblasts through autoinduction. Thus, maintenance of increased TGF-β production may lead to the progressive deposition of ECM, resulting in

fibrosis. In the bleomycin model, immunohistological analysis showed that TGF-β was detected on the infiltrating cells, which were predominantly composed of macrophages, as well as fibroblasts at sclerotic stages. TGF-β1 and TGF-β2 mRNA expression was also detected in the lesional skin at early phases. Additionally, expression and synthesis of TGF-β1 were increased in bleomycin-"susceptible" mice strains [14]. Thus, bleomycin-induced murine scleroderma is suggested to be a TGF-β-mediated model.

CTGF (CCN2) is selectively induced in fibroblasts after activation by active TGF-β and also regulated by TGF-β accessory receptors. CTGF may functionally coordinate the mode of action of TGF-β, such as fibroblast proliferation and ECM production in fibroblasts. High level of CTGF is shown in the lesional skin of bleomycin-induced scleroderma [21]. Plasminogen activator inhibitor-1 (PAI-1) is induced by TGF-β, and its promoter contains Smad binding elements. PAI-1 expression was upregulated in the bleomycin-treated skin; however, dermal sclerosis was similarly induced by bleomycin in even PAI-1-deficient mice [22]. PAI-1 plays an important role, but is not the prerequisite factor, in the development of bleomycin-induced scleroderma.

4.2.4 Transcriptional Factors

Signaling by TGF-β upon binding of TGF-β receptors to the nucleus occurs predominantly by phosphorylation of cytoplasmic mediators belonging to the Smad family. Three families of Smads have been identified: receptor-regulated Smad2 and Smad3 (R-Smads), common partner Smad4 (Co-Smad), and inhibitory Smad6 and Smad7 (I-Smads). Smad7 has been shown to act as an intracellular antagonist of TGF-β signaling and an inhibitor of TGF-β-induced transcriptional responses. In scleroderma skin and cultured scleroderma fibroblasts, basal level and TGF-β-inducible expression of Smad7 are selectively decreased, whereas Smad3 expression is increased. On the other hand, Smad7 expression levels in scleroderma fibroblasts are unclear and disputed. In the bleomycin-treated skin, fibroblasts showed predominantly nuclear localization of Smad3 and intense staining for phospho-Smad2/3 [23]. On the other hand, expression of Smad7 was downregulated, which may account for sustained activation of TGF-β/Smad signaling in the lesional skin. Targeted disruption of Smad3 ameliorated bleomycin-induced scleroderma, unassociated with inflammation [24]. Recently, other signaling pathways besides the Smad proteins have also been shown to mediate TGF-β signaling in scleroderma fibroblasts, such as the p38 mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and Egr-1 pathways. Other signaling pathways such as c-abl, Src kinase, and Rac1 are also involved in the induction of scleroderma by bleomycin [25, 26].

Also, recent studies suggest aberrant Wnt signaling in scleroderma, in which ß-catenin is a central mediator. Increased nuclear accumulation of ß-catenin is found in SSc fibroblasts, which was considered to result from enhanced expression of Wnt-1 and Wnt-10b in scleroderma skin [27]. Fibroblast-specific deletion of

ß-catenin reduced bleomycin-induced fibrosis [27]. In addition, inhibition of glycogen synthase kinase 3ß, which plays a pivotal role in the regulation of the canonical Wnt pathway, activates the Wnt signaling in fibroblasts, stimulates the production of collagen by fibroblasts, and deteriorates bleomycin-induced skin fibrosis in mice [28].

4.2.5 Th2/Th17 Cytokines

Type 2 cytokines, i.e., IL-4 and IL-13, have also been suggested to be important in scleroderma. IL-4 is produced by activated memory T cells and mast cells and promotes fibroblast proliferation, gene expression, and synthesis of ECM proteins such as collagen and tenascin. IL-4 upregulates TGF-β production in eosinophils and T cells. Increased IL-4 production is detected in the sera or by activated peripheral blood mononuclear cells of patients with SSc. Scleroderma fibroblasts express more IL-4 receptor α and produce more collagen after IL-4 stimulation. In the bleomycin model, IL-4 levels in the serum [29] as well as in the lesional skin [30] were significantly elevated following bleomycin treatment. Transcription factor T-bet is a master regulator of type 1 immunity, and mice lacking T-bet showed increased sensitivity to bleomycin and exhibited significantly enhanced dermal sclerosis [31, 32]. IL-13 is a potent stimulator of fibroblast proliferation and collagen production. The profibrotic effect of IL-13 is postulated to involve irreversible fibroblast activation, triggered either directly or indirectly through TGF-β. IL-13 transgenic mice show increased lung fibrosis, as well as increased levels of TGF-β1. In the bleomycin model, IL-13 mRNA levels and protein synthesis in the lesional skin were increased, and IL-13 receptor (IL-13R)-α2 expression in the lesional skin was augmented mainly in the infiltrating mononuclear cells and macrophages after bleomycin exposure [30]. Another study showed that IL-13-deficient mice failed to develop an increase in skin sclerosis after bleomycin treatment [32]. IL-13 may promote the progression of cutaneous fibrosis/sclerosis in the development of bleomycin-induced scleroderma. Macrophages are polarized into two phenotypes, M1 and M2 macrophages. M2 macrophages are mostly induced by stimulation with IL-4, IL-13, and IL-10 and involved in fibrosis, which is true of bleomycin-induced scleroderma [33].

Recent studies have shown that IL-17 is involved in SSc. In vitro, IL-17 increases monocyte chemoattractant protein-1 (MCP-1)/CCL2, IL-8, and MMP-1 production in fibroblasts [34]. IL-17 exerts different effects on human SSc fibroblasts, as compared with normal fibroblasts. IL-17 has an inhibitory effect on collagen production in human fibroblasts, and SSc fibroblasts may escape the negative control due to reduced expression of IL-17A receptor [35]. By contrast, in the bleomycin-induced model, IL-17 levels are increased in the lesional skin and lung [36]. And the development of scleroderma by bleomycin was significantly attenuated in IL-17A-deficient mice, along with reduction of TGF-β and CTGF mRNA expression. Adhesion molecules such as L-selectin and ICAM-1 regulate

Th2 and Th17 cell accumulation in the skin and lung in bleomycin-induced scleroderma [37].

CCL2 is the most important chemokine for SSc [38]. In vitro studies show that CCL2 upregulates type I collagen mRNA expression in fibroblasts, which is indirectly mediated by endogenous upregulation of TGF-β gene expression [39]. CCL2 enhances expression of matrix metalloproteinase-1 (MMP-1), MMP-2, as well as TIMP-1 in cultured skin fibroblasts [40]. Scleroderma fibroblasts express increased levels of CCL2 mRNA and protein [41], and stimulation with platelet-derived growth factor (PDGF) resulted in a significant increase in CCL2 mRNA and protein [42]. Furthermore, the autoinduction of CCL2 was observed in scleroderma fibroblasts, but not in normal fibroblasts [43]. CCL2 acts indirectly via IL-1α [39]. IL-1α and IL-1 receptor levels, in turn, were shown to be significantly increased in scleroderma [44]. In addition, IL-1α and TNF-α are potent inducers of CCL2. Thus, in addition to a direct autocrine stimulatory loop, a mutual induction between IL-1α and CCL2 might lead to a self-perpetuating activation of scleroderma fibroblasts. CCL2 levels may also be increased by IL-13, because IL-13 is a potent stimulator of CCL2 [45]. In the bleomycin model, expression of CCL2 in the infiltrating mononuclear cells was enhanced following bleomycin treatment, and expression of CCL2 in fibroblasts was detected at later stages in the sclerotic skin [46]. Expression of CCR-2, a major receptor for CCL2, was also upregulated in the lesional skin at both protein and mRNA levels following bleomycin treatment. Administration of anti-CCL2 neutralizing antibody together with local bleomycin treatment reduced dermal sclerosis, along with collagen content in the skin as well as mRNA expression of type I collagen. These data suggest that CCL2 and CCR-2 signaling plays an important role in the pathogenesis of bleomycin-induced scleroderma. In the CCL2-deficient mice, skin fibrosis was diminished even by the bleomycin treatment [47]. CCL2 may contribute to the induction of dermal sclerosis directly, via its upregulation of mRNA expression of ECM on fibroblasts, as well as indirectly through the mediation of a number of cytokines released from immunocytes recruited into the lesional skin. On the other hand, oxidative stress transiently induces CCL2 mRNA and protein expression in cultured skin fibroblasts [48], suggesting that ROS may play a regulatory role in inflammation by modulating monocyte chemotactic activity.

4.2.6 Oxidative Stress

Chronic hypoxia stress drives fibrosis. Oxidative stress is an imbalance between oxidants (reactive oxygen and nitrogen species (ROS/RNS)) and antioxidants which affect lipids, DNA, carbohydrates, and proteins. ROS generated during various metabolic and biochemical reactions have multifarious effects that include oxidative damage to DNA. ROS can cause several abnormalities such as endothelial cell damage or enhanced platelet activation, leading to upregulation of the expression of adhesion molecules or secretion of inflammatory or fibrogenic cytokines including PDGF and TGF-β. In addition, free radicals stimulate fibroblast

proliferation with narrowing of the vessel walls and ischemia. Thus, excessive oxidative stress has been implicated in the pathogenesis of scleroderma [49]. Reduced levels of micronutrient antioxidants and increased susceptibility of serum lipoproteins to oxidation have been reported in patients with SSc [50]. Free radicals are produced by several mechanisms such as hypoxanthine-xanthine oxidase system and activation of polymorphonuclear leukocytes. Several markers which reflect free radical formation, i.e., 8-isoprostane and N(epsilon)-(hexanoyl) lysine, are elevated in the serum of patients with SSc [51]. Also, autoantibodies against antioxidant enzymes such as peroxiredoxin I and methionine sulfoxide reductase A (MSRA) are elevated in the serum of patients with SSc [52].

Scleroderma fibroblasts produce ROS constitutively. Other effects of oxygen radicals include the stimulation of skin fibroblast proliferation at low concentrations and the production of increased amounts of collagen, suggesting that low oxygen tension may contribute to the increased fibrogenic properties of scleroderma fibroblasts. Furthermore, several of the autoantigens targeted by scleroderma autoantibodies fragment in the presence of ROS and specific metals such as iron or copper [53]. The authors suggest that tissue ischemia generates ROS, which in turn induces the fragmentation of specific autoantigens. On the other hand, oxidative stress transiently induces CCL2 mRNA and protein expression in cultured skin fibroblasts [48], suggesting that ROS may play a regulatory role in inflammation by modulating monocyte chemotactic activity. Overexpression of PDGF has been reported in SSc. Elevated levels of PDGF A-chain are demonstrated in scleroderma skin, and TGF-β upregulates PDGF-α levels in scleroderma fibroblasts. On the other hand, increased expression of the PDGF B-chain and β-receptor in scleroderma skin has also been reported. PDGF stimulates Ras-ERK-1/2-ROS signaling pathway, which results in collagen synthesis [54]. A thiol antioxidant, N-acetylcysteine (NAC), prevented bleomycin-induced lung and dermal fibrosis [55], with diminished oxidative stress.

4.2.7 B Cells

Activated peripheral B cells are found in abnormally large numbers in patients with SSc. B cells contribute not only to antibody production but also to T cell activation and differentiation and the production of various cytokines. In CD 19-deficient mice, induction of dermal and lung fibrosis, cytokine expression, and autoantibody production were inhibited [56]. They speculate that hyaluronan activates B cells via toll-like receptor (TLR)-4 in this model. Also, expression of TLR-3 is increased in the dermis in this model, which may result in the increase of production of IL-6 and of differentiation into myofibroblasts [57]. TLR stimulation of fibroblasts may trigger cytokine cascades in fibrosis [58]. Further, recent studies have shown a TLR4-mediated fibrotic response, and TLR4 knockout as well as mutated TLR4-harboring mice attenuate bleomycin-induced scleroderma [59].

4.2.8 Epigenetics

Histone acetylation in regulating myofibroblast differentiation has been suggested. Trichostatin A, a histone deacetylase (HDAC) inhibitor, prevented the induction of dermal sclerosis showing excessive accumulation of ECM proteins [60], as well as prevention of Wnt inhibitor factor 1 (WIF-1) reduction [61]. Hypermethylation of the promoters of DKK1 and SFRP1 induced silencing and aberrant Wnt signaling in SSc, and reactivation of both transcriptions ameliorated bleomycin-induced fibrosis [62].

4.2.9 MicroRNA

MicroRNAs (miRNAs) are noncoding small RNAs, and dysregulation of miRNAs is recently shown. miRNA-29a is spontaneously downregulated in scleroderma fibroblasts, and fibrogenic cytokines such as TGF-β, PDGF-B, and IL-4 reduced the level of miRNA-29a in normal fibroblasts [63]. Also, expressions of miRNA-29a [63] and miRNA-30b [64] were downregulated in bleomycin-induced scleroderma skin, suggesting key regulators of collagen expression in SSc [63]. In addition, miRNA-196a is reduced in scleroderma fibroblasts, which was reversed by knockdown of TGF-β with small interfering RNA [65].

4.2.10 Epithelial-Mesenchymal Transition

Epithelial-mesenchymal transition (EMT) process is induced by TGF-β, epidermal growth factor (EGF), hepatocyte growth factor (HGF), and Wnt and suggested to be involved in fibrosis. ROS mediates TGF-β-induced EMT. EMT markers are localized in eccrine glands in the scleroderma skin [66]. Also, epithelial cells promote fibroblast activation via IL-1α in SSc [67], suggesting epithelial-mesenchymal interaction. Bleomycin treatment induced the epithelial cells superficial to the basement membrane zone to undergo EMT in the sclerotic skin [68], which was evaluated by E-cadherin and vimentin expression. A significant portion of myofibroblasts arise from the conversion of epithelial cells through an EMT process [69]; however, another possibility that adiponectin-positive intradermal progenitors give rise to dermal myofibroblasts is recently shown [70]. Adiponectin attenuates TGF-β-induced profibrotic response in fibroblasts. Bleomycin inhibits adipogenesis, which may contribute to promote fibrosis [71].

4.2.11 Role of Mast Cells in Fibrosis

Mast cell is increased in number, and especially MC_T (tryptase positive but chymase negative) are abundant, in scleroderma skin [72]. Cell-cell interaction between fibroblasts and mast cells exerts various effects mutually. Mast cells are a

source of TGF-β and also IL-17 [73] in SSc. SCF is important in mast cell proliferation, survival, and chemotaxis and stimulates to release mediators, i.e., histamine, and growth factors and cytokines. Dermal sclerosis is induced by bleomycin treatment in mast cell-deficient WBB6F1-W/Wv mice [7]. Recent studies using C57BL/6-Mcpt5Cre mice treated by diphtheria toxin have shown that genetic ablation of mast cells did not prevent the development of bleomycin-induced dermal sclerosis [74], suggesting that mast cells do not essentially contribute to the development of scleroderma. Also, IL-33 is highly detected in the inflammatory response. Mast cells express high levels of ST2, a receptor of IL-33. Also, ST2 is constitutively expressed on Th2 cells. IL-33 potentiates bleomycin-induced pulmonary fibrosis, and neutralizing anti-IL-33 antibody attenuated bleomycin-induced lung fibrosis [75]. Signaling pathway via IL-33/ST2 may be involved in the induction of scleroderma.

4.2.12 Matricellular Protein

Periostin is a novel matricellular protein and important in tissue remodeling, wound healing, and SSc [76]. Periostin serves as a ligand for integrins, and can bind to collagen and also other ECM proteins, and modulates TGF-β activation, EMT, and cytokine production. IL-13 is the main inducer of periostin. Periostin gene knockout mice are resistant to bleomycin and failed to induce dermal sclerosis and lung fibrosis [77]. Periostin is also involved in the myofibroblast differentiation [76]. Osteopontin is also a matricellular protein and increased in SSc. Osteopontin-deficient mice developed less dermal sclerosis by bleomycin treatment [78]. These studies show an important role of matricellular proteins in SSc, via modulating cell-matrix interactions.

4.3 Therapeutic Approach Using Bleomycin-Induced Scleroderma

Numerous therapeutic approaches have been investigated so far in bleomycin-induced scleroderma model. Systemic administration of anti-TGF-β neutralizing antibody in combination with local bleomycin treatment suppressed the development of scleroderma [29]. This effect was accompanied by the reduction of the number of myofibroblasts, mast cells, and eosinophils. Topical application of a peptide inhibitor of TGF-β has been shown to ameliorate skin fibrosis in bleomycin-induced scleroderma [79]. Latency-associated peptide (LAP) is released from latent TGF-β. Local LAP administration along with bleomycin treatment suppressed the induction of dermal sclerosis, as well as collagen and CTGF gene expression, in an ongoing experiment [80]. IFN-γ causes potent inhibition of collagen production in cultured skin fibroblasts. IFN-γ decreased TGF-β-induced α-SMA expression in palatal fibroblasts, as well as changes in morphology [81]. Moreover, IFN-γ inhibits the TGF-β-induced phosphorylation of Smad3 and the accumulation of Smad3 in

the nucleus, whereas induces the expression of Smad7 [82]. In the bleomycin model, systemic administration of IFN-γ together with bleomycin reduced dermal sclerosis, even after the onset of scleroderma [83]. Administration of inhibitor of TGF-β/Smad signaling by the activation of nuclear translocation of Y-box binding protein 1 suppressed skin fibrosis in this model [84]. A small molecule antagonist of CCL2 prevented skin sclerosis induced by bleomycin [85]. Bleomycin produces free radicals and induces apoptosis. A reduction of free radical formation may contribute to the decrease of collagen content by inhibition of proline hydroxylation, which leads to the improvement of scleroderma. Lecithinized superoxide dismutase (SOD) with high tissue accumulation and a long half-life in the blood showed an inhibitory effect on bleomycin-induced scleroderma, suggesting that antioxidant therapy may lead to an antifibrotic effect; however, post-onset administration of SOD could not attenuate the dermal sclerosis [86]. Also, a free radical scavenger, edaravone, inhibited dermal sclerosis [87]. Halofuginone has an inhibitory effect on collagen synthesis and shows antifibrotic effects in a few animal models of scleroderma. By contrast, bleomycin-induced scleroderma was not attenuated by treatment with halofuginone [88]. Halofuginone may not ameliorate dermal sclerosis along with strong inflammation caused by repeated application of bleomycin. Hepatocyte growth factor (HGF) prevented the progression of organ fibrosis. The promoter region of the HGF gene contains a TGF-β inhibitory element. HGF induces proteases which degradate ECM proteins, such as MMPs, membrane type 1-MMP (MT1-MMP), and urokinase-type plasminogen activator (uPA), and also decreases TIMPs [89]. Gene transfer of HGF not only prevented the ongoing dermal sclerosis induced by simultaneous local injections of bleomycin but also ameliorated the previously induced dermal sclerosis [90]. This effect was mediated by suppressing TGF-β levels. Adenosine is a nucleoside that is generated in response to cellular stresses such as chronic inflammation and hypoxic conditions. Adenosine A2A receptor-deficient and A2A receptor antagonist-treated mice were protected from developing bleomycin-induced dermal sclerosis [91]. Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that regulate cell proliferation, differentiation, and immune/inflammation response. PPAR-γ inhibits TGF-β signaling and collagen synthesis [92]. Insulin-sensitizing drugs, such as rosiglitazone and pioglitazone, activate PPAR-γ and inhibit TGF-β-induced fibrotic responses. Treatment with PPAR-γ ligand rosiglitazone prevented dermal sclerosis with downregulation of collagen gene expression and myofibroblast accumulation in the lesional skin [93]. Imatinib mesylate (Gleevec) targets specifically the TGF-β and PDGF signaling pathways by inhibiting the tyrosine kinase activity of c-abl and PDGF receptors. Imatinib reduced dermal thickness, the number of myofibroblasts, and synthesis of ECM proteins in the bleomycin-induced dermal sclerosis [94]. Further, imatinib induced regression of preexisting ECM accumulation in the skin and decreased dermal thickness [95]. Dasatinib and nilotinib, both are dual inhibitor of c-abl and PDGF receptor, potently reduced the dermal thickness, number of myofibroblasts, and collagen content of the skin in the bleomycin model [96]. Other biologics such as nintedanib [97] and targeting IL-6 treatment prevented bleomycin-induced

scleroderma [98]. An interaction between CD40 and its ligand CD40L has been suggested as a pathway for immune and inflammatory responses. Cultured scleroderma fibroblasts express CD40 [99]. In the lesional skin of bleomycin-induced scleroderma, dermal fibroblasts expressed CD40, and mast cells and CD4+ T cells expressed CD40L [100]. Anti-CD40L antibody inhibited the induction of skin sclerosis by suppressing fibroblast proliferation and downregulation of CCL2 expression in this model [100]. α-Melanocyte-stimulating hormone (α-MSH) has many effects such as immunomodulation, regulation of exocrine activity and apoptosis, and antifibrotic effect. α-MSH suppresses TGF-β-induced collagen synthesis in vitro. Treatment with α-MSH suppressed bleomycin-induced collagen accumulation in the skin [101]. The receptor melanocortin-1 (MC_1) is detected in dermal fibroblasts, hair follicles, and dermal papilla. In the MC_1 signaling-deficient mice, bleomycin treatment increased the development of scleroderma with collagen contents, suggesting that Mc1 has an endogenous regulatory role in collagen synthesis and fibrosis [102]. Intravenous immunoglobulin (IVIG) treatment significantly inhibited dermal sclerosis and collagen accumulation, by downregulating TGF-β and CCL2 [103]. Lysophosphatidic acid (LPA) is a lipid mediator that signals through specific G protein-coupled receptors (GPCRs), designated as LPA_1 to LPA_8. LPA has been demonstrated to be involved in several animal models of organ fibrosis. LPA_1 and LPA_3 receptor antagonist significantly reduced bleomycin-induced scleroderma [104]. Human-induced pluripotent stem cell-derived endothelial cells can recover the damaged vessels, leading to the reduction of collagen contents in the skin and mast cell numbers [105].

4.4 HOCl-Induced Murine Scleroderma

Recently, local injections of agents generating various types of ROS induced skin sclerosis in mice. Daily intradermal injections of hypochlorous acid (HOCl) induce skin and lung fibrosis, kidney damages, and serum topo-I antibody production in Balb/c mice [106]. On the other hand, local injections of agents generating peroxynitrite anions (ONOO-) induced limited skin, but not lung, fibrosis and anti-CENP-B antibody production. Aside from bleomycin, those agents are capable to produce ROS, which induces phenotypic differentiation into myofibroblasts and enhances production of type I collagen, possibly via the phosphorylation of ERK1/2 and activation of the Ras pathway. In the HOCl-induced fibrosis model, the overproduction of ROS activates ADAM 17, a protease involved in Notch cleavage, suggesting that Notch pathway is activated [107]. In this model system, skin fibrosis is ameliorated by sunitinib [108] and arsenic trioxide [109]. Some of the agents which induce DNA injury and ROS production exert various effects on endothelial cells and fibroblasts, as well as cause immune imbalance and mediator secretion, leading to dermal sclerosis.

4.5 Tight Skin (TSK) Mouse

4.5.1 TSK1 Mouse

Tsk mice are heterozygous for a mutation in the fibrillin-1 gene (Tsk 1/+). Fibrillin is a large ECM structural protein and the major component of microfibrils. Tsk1 mouse is characterized by thickened fibrotic skin that is firmly bound to the subcutaneous and deep muscular tissue, certain visceral changes in the lungs and hearts, and production of autoantibodies such as topoisomerase-1 (topo-1) [110]. mRNA levels of TGF-β and type I, III, and VI collagen in the skin were under temporal and spatial regulation during postnatal growth and development in the Tsk1/+ mice [111]. Collagen α1(I) and α1(III) gene-expressing fibroblasts were increased in Tsk1/+ fibrotic lesions. Transient transfections of a series of human α1 (I) procollagen promoter constructs into Tsk1/+ fibroblast cultures showed increased transcription rates caused by the lack of the strong inhibitory influence of the regulatory sequence contained in the promoter between −675 and −804 bp [111]. Additionally, Tsk1/+ nuclear extracts displayed decreased binding to a consensus AP-1 sequence [112]. Transcriptional activation of collagen genes was demonstrated in Tsk1/+ mice in vivo employing reporter transgenes harboring upstream fragments of the 5′ flanking region of the mouse α2(I) collagen genes. Wnt signaling stimulated matrix assembly of microfibrillar proteins including fibrillin-1 and increased expression of Wnt2 and SFRP2 in the Tsk1/+ mouse skin [113]. Mast cells are abundant with prominent degranulation also in Tsk1/+ mice. IL-4 and TGF-β possibly play important roles in the pathogenesis of fibrosis in Tsk mice. TGF-β was expressed during the rapid postnatal growth of the skin in parallel with high expression of α1(I), α1(III), and α2(VI) collagen genes [111]. Fibroblasts from Tsk1/+ mice are hyperresponsive to IL-4 and TGF-β, displaying increased synthesis of α1(I) collagen mRNA, collagen protein, and activity of a luciferase reporter construct containing α2(I) collagen promoter [114]. After IL-4 stimulation, JAK-1 and JAK-2 were phosphorylated to a greater degree in Tsk1/+ fibroblasts than in C57BL/6 fibroblasts [115]. Targeted mutations in either the signaling chain of the IL-4 receptor or STAT6 prevent cutaneous hyperplasia in Tsk mice, suggesting the importance of IL-4 [116]. Smad2 and Smad3 are considered to be the primary signaling molecules involved in the TGF-β signaling transduction pathway. Tsk fibroblasts have elevated Smad3 transcriptional activity compared with normal fibroblasts [117]. This may explain why Tsk fibroblasts are more responsive to TGF-β stimulation. Recent studies have shown that CCL7 is highly overexpressed by neonatal Tsk fibroblasts [118]. Increased CCL7 protein secretion by Tsk fibroblasts is observed, and CCL7 is abundantly expressed in the dermis of Tsk mice at 10 days and 3 weeks old.

CD4+ T cells have been shown to be required for the excessive accumulation of dermal collagen in Tsk1/+ mice [119]. By contrast, the Tsk phenotype fully develops in SCID mice. B cell functional defects caused by the loss of CD19 significantly decreased skin fibrosis in Tsk1/+ mice, suggesting that B cells play an important role [120]. In Tsk1/+ mice overexpressing CD19, anti-topo-I antibody

levels were significantly increased, although skin thickness was not enhanced [120]. B cell antigen receptor cross-linking augmented activation of extracellular signal-regulated kinase in Tsk1/+ B cells [121]. Further, B cell depletion by anti-CD20 antibody significantly suppressed skin fibrosis and autoantibody production in newborn Tsk1/+ mice [121]. Thus, disrupted B cell signaling may contribute to immunologic abnormalities in Tsk1/+ mice. Endothelial dysfunction has been shown in Tsk1/+ mice [122]. Expression of endothelial nitric oxide synthase (eNOS) protein and gene was significantly reduced in the Tsk1/+ skin, and NOS activity was also decreased [123]. There is abnormal nitric oxide metabolism in the Tsk1/+ skin, and expression and activity of protective antioxidant enzyme HO-1 were reduced.

Previous studies showed that administration of anti-IL-4 antibody to neonatal Tsk1/+ mice prevented skin fibrosis and dermal collagen content [124]. Also, shift of a Th2-based immune imbalance in Tsk1/+ mice by treatment with synthetic oligodeoxynucleotides (ODN) containing immunomodulatory CpG motifs prevented the skin fibrosis and thickening [125]. IVIG therapy decreased splenocyte secretion of IL-4 and TGF-β, resulting in abrogation of fibrogenesis [126]. A decrease in fibrosis was previously reported by inhibition of mast cell degranulation, and a recent report showed that intraperitoneal injection of mast cell chymase inhibitor significantly reduced skin fibrosis and the thickness of the subcutaneous fibrous layer [127]. Halofuginone has an inhibitory effect on collagen synthesis and shows antifibrotic effects in a few animal models of scleroderma. Halofuginone attenuates collagen synthesis, as well as collagen gene expression in avian and murine skin fibroblasts [128]. Halofuginone specifically inhibits α1 (I) collagen gene expression without affecting the synthesis of other types of collagen such as types II and III [129, 130]. Halofuginone inhibited TGF–βinduced upregulation of collagen protein and α2(I) promoter activity, as well as phosphorylation and subsequent activation of Smad3 after TGF-β stimulation [131]. Dermal application of halofuginone on Tsk1/+ mice for 60 days reduced dermal fibrosis as well as collagen α1(I) gene expression [132]. Intraperitoneally administered halofuginone also prevented the thickening of the dermis and eliminated the increase of skin collagen in Tsk1/+ model [132]. Intraperitoneal administration of anti-CD40L attenuated skin fibrosis and anti-topo-I antibody production [133]. HGF gene transfection in Tsk1/+ mice resulted in a marked reduction of hypodermal thickness [134]. Treatment with imatinib reduced dermal and hypodermal thickening in Tsk1/+ mice [97]. Other therapies targeting TGF-β/Smad signaling pathway or SOD are also effective for suppression of skin fibrosis in TSK mice [84, 87].

4.5.2 TSK 2 Mouse

The second murine tight skin mutation was a result of administration of the mutagenic agent, ethylnitrosourea (ENU) [135]. This mutation has been localized to mouse chromosome 1 and is inherited as an autosomal dominant trait, and only

heterozygous (Tsk2/+) animals survive. Tsk2/+ mice develop a tight skin phenotype that becomes apparent at 3–4 weeks of age. Histologic examination of the skin revealed marked accumulation of collagen similar to that observed in Tsk1/+ mice. By contrast, a prominent mononuclear cell infiltration is present in the dermis and adipose tissues of Tsk2/+, in contrast to Tsk1/+ mice. Lungs were normal. Biochemical analysis showed that the Tsk2/+ skin had 50 % more collagen contents than the normal mouse skin, and collagen synthesis in Tsk2/+ cultured dermal fibroblasts was 100 % higher compared with normal fibroblasts. Transient transfection experiments with α1(I) collagen promoter constructs demonstrated increased transcriptional activity of the gene, and Sp1 and NF-1 transcriptional factors were involved in the upregulated transcriptional activity of α1(I) collagen promoter in Tsk2/+ fibroblasts [136]. Sequences from −96 to +16 bp of the α1(III) collagen promoter play an important role in the upregulated expression in Tsk2/+ fibroblasts [137]. Analysis using a collagen promoter GFP reporter transgene also showed an increase in transgene activity of α1(I) collagen [138]. The presence of antinuclear antibody in the plasma was 92 % of the Tsk2/+ mice, and other autoantibodies were also developed [139], suggesting the aspects of autoimmunity in this model.

4.6 Other Scleroderma Models

In this chapter, all the animal models could not be described; however, a number of models are additionally developed, such as sclerodermatous graft-versus-host disease (Scl-GvHD) model, skin fibrosis model by exogenous injection of growth factors (TGF-β, CTGF, and bFGF), and UCD-200 chickens, and a number of transgenic (i.e., TGF-β receptor I, kinase-deficient type II TGF-β receptor, Fra-2) as well as knockout (i.e., relaxin, caveolin) mice. Fli1 (Friend leukemia integration-1)-deficient mice will be described in Chap. 12. Recently, fibrillin-1-targeted knock-in mouse models were generated, which exhibited skin fibrosis [140]. Integrin-modulating therapy by either β1 integrin or TGF-β neutralizing antibody prevented skin stiffness.

4.7 Conclusion

Complex networks involve cell-cell and cell-matrix interactions via mediators in the induction of cutaneous sclerosis. Activated fibroblasts are a part of the immune system and modulate immune cell behavior by conditioning the local cellular and cytokine microenvironment. The major characteristic of the bleomycin-induced experimental murine model of scleroderma is the histological mimicry to human scleroderma. Possible mechanisms of bleomycin-induced scleroderma are shown in Fig. 4.2. Because each model has its own advantages, and also disadvantages, researchers are recommended to choose depending on the purpose of studies, i.e., to study pathogenesis (fibrogenesis or vasculogenesis), to explore the antifibrotic effect of new drugs, and so on. Transgenic and knockout mice are elegant tools, but

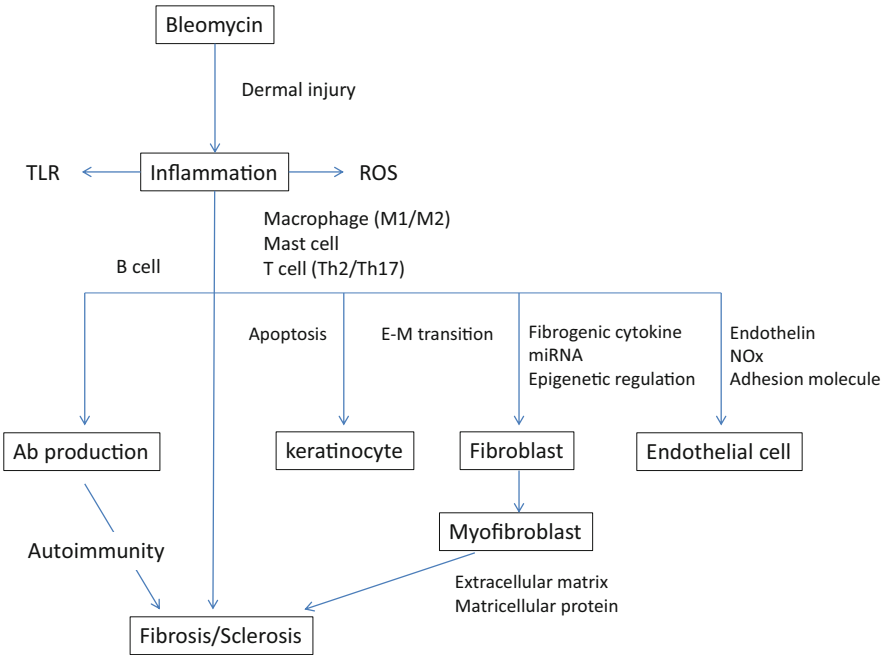

Fig. 4.2 Possible mechanisms of bleomycin-induced scleroderma

not available to anyone, and need special techniques and long periods to be made. It must be mentioned that the animal model is a simplification of the more complex human scleroderma. Nonetheless, the pathogenic mechanism discovered in the animal model may provide novel information and assist in helping us to better understand the mechanisms underlying human scleroderma. Animal models of scleroderma may also serve as promising tools for the development of new therapies specifically targeting individual cytokines, cytokine antagonists (i.e., antibodies, soluble receptors), and cytokine mutants or of drugs that specifically interfere with the signal transduction pathways involved in the fibrotic process. In these a few years, many studies related to scleroderma have been conducted using animal models, and our understanding of the pathogenesis of scleroderma has greatly progressed. Further investigation for the therapeutic drugs is needed to bring forth effective therapies for SSc in the clinical settings from bench side.

References

1. Yamamoto T. Scleroderma-pathophysiology. Eur J Dermatol. 2009;19:14–24.
2. Yamamoto T. Animal model of systemic sclerosis. J Dermatol. 2010;37:26–41.
3. Yamamoto T. Bleomycin and the skin. Br J Dermatol. 2006;155:869–75.

4. Inaoki M, Kawabata C, Nishijima C, Yoshio N, Kita T. Case of bleomycin-induced scleroderma. J Dermatol. 2012;39:482–4.
5. Mountz JD, Downs Minor MB, Turner R, Thomas MB, Richards F, Pisko E. Bleomycin-induced cutaneous toxicity in the rat: analysis of histopathology and ultrastructure compared with progressive systemic sclerosis (scleroderma). Br J Dermatol. 1983;108:679–86.
6. Yamamoto T, Takagawa S, Katayama I, et al. Animal model of sclerotic skin. I: local injections of bleomycin induce sclerotic skin mimicking scleroderma. J Invest Dermatol. 1999;112:456–62.
7. Yamamoto T, Takahashi Y, Takagawa S, Katayama I, Nishioka K. Animal model of sclerotic skin. II: bleomycin induced scleroderma in genetically mast cell deficient WBB6F1-W/Wv mice. J Rheumatol. 1999;26:2628–34.
8. Yamamoto T, Kuroda M, Takagawa S, Nishioka K. Animal model of sclerotic skin. III: histopathological comparison of bleomycin-induced scleroderma in various mice strains. Arch Dermatol Res. 2000;292:535–41.
9. Yamamoto T, Nishioka K. Animal model of sclerotic skin. IV: induction of dermal sclerosis by bleomycin is T cell independent. J Invest Dermatol. 2001;117:999–1001.
10. Yamamoto T, Nishioka K. Animal model of sclerotic skin. V: increased expression of α-smooth muscle actin in fibroblastic cells in bleomycin-induced scleroderma. Clin Immunol. 2002;102:77–83.
11. Yamamoto T, Nishioka K. Animal model of sclerotic skin. VI: evaluation of bleomycin-induced skin sclerosis in nude mice. Arch Dermatol Res. 2004;295:453–6.
12. Ishikawa H, Takeda K, Okamoto A, Matsuo SI, Isobe KI. Induction of autoimmunity in a bleomycin-induced murine model of experimental systemic sclerosis: an important role for CD4(+) T cells. J Invest Dermatol. 2009;129:1688–95.
13. Yamamoto T, Katayama I. Vascular changes in bleomycin-induced scleroderma. Int J Rheumatol. 2011;2011:270938.
14. Oi M, Yamamoto T, Nishioka K. Increased expression of TGF-β1 in the sclerotic skin in bleomycin-'susceptible' mouse strains. J Med Dent Sci. 2004;51:7–17.
15. Shibusawa Y, Negishi I, Tabata Y, Ishikawa O. Mouse model of dermal fibrosis induced by one-time injection of bleomycin-poly(L-lactic acid) microspheres. Rheumatology. 2008;47:454–7.
16. Jun J-B, Kim JK, Na Y-I, Jang SM, Paik SS, Kim Y-H. A convenient method for producing the bleomycin-induced mouse model of scleroderma by weekly injections using a methylcellulose gel. Rheumatol Int. 2012;32:1443–7.
17. Liang M, Lv J, Yang L, et al. A modified murine model of systemic sclerosis: bleomycin given by pump infusion induced skin and pulmonary inflammation and fibrosis. Lab Invest. 2015;95:342–50.
18. Yamamoto T, Eckes B, Krieg T. Bleomycin increases steady-state levels of type I collagen, fibronectin and decorin gene expression in human skin fibroblasts. Arch Dermatol Res. 2000;292:556–61.
19. King SL, Lichtler AC, Rowe DW, et al. Bleomycin stimulates proα1(I) collagen promoter through transforming growth factor-β response element by intracellular and extracellular signaling. J Biol Chem. 1994;269:13156–61.
20. Phan SH, Gharaee-Kermani M, Wolber F, Ryan US. Stimulation of rat endothelial cell transforming growth factor-β production by bleomycin. J Clin Invest. 1991;87:148–54.
21. Liu S, Taghavi R, Leask A. Connective tissue growth factor is induced in bleomycin-induced skin scleroderma. J Cell Commun Signal. 2010;4:25–30.
22. Matsushita M, Yamamoto T, Nishioka K. Plasminogen activator inhibitor-1 is elevated, but not essential, in the development of bleomycin-induced murine scleroderma. Clin Exp Immunol. 2005;139:429–38.
23. Takagawa S, Lakos G, Mori Y, Yamamoto T, Nishioka K, Varga J. Sustained activation of fibroblast transforming growth factor-β/Smad signaling in a murine model of scleroderma. J Invest Dermatol. 2003;121:41–50.

24. Lakos G, Takagawa S, Chen SJ, et al. Targeted disruption of TGF-beta/Smad3 signaling modulates skin fibrosis in a mouse model of scleroderma. Am J Pathol. 2004;165:203–17.
25. Skhirtladze C, Distler O, Dees C, et al. Src kinases in systemic sclerosis: central roles in fibroblast activation and in skin fibrosis. Arthritis Rheum. 2008;58:1475–84.
26. Liu S, Kapoor M, Shi-wen X, et al. Role of Rac1 in a bleomycin-induced scleroderma model using fibroblast-specific Rac1-knockout mice. Arthritis Rheum. 2008;58:2189–95.
27. Beyer C, Schramm A, Akhmetshina A, et al. ß-catenin is a central mediator of pro-fibrotic Wnt signaling in systemic sclerosis. Ann Rheum Dis. 2012;71:761–7.
28. Bergmann C, Akhmetshina A, Dees C, et al. Inhibition of glycogen synthase kinase 3ß induces dermal fibrosis by activation of the canonical Wnt pathway. Ann Rheum Dis. 2011;70:2191–8.
29. Yamamoto T, Takagawa S, Katayama I, Nishioka K. Anti-sclerotic effect of anti-transforming growth factor-β antibody in bleomycin-induced scleroderma. Clin Immunol. 1999;92:6–13.
30. Matsushita M, Yamamoto T, Nishioka K. Upregulation of interleukin-13 and its receptor in a murine model of bleomycin-induced scleroderma. Int Arch Allergy Immunol. 2004;135:348–56.
31. Lakos G, Melichian D, Wu M, Varga J. Increased bleomycin-induced skin fibrosis in mice lacking the Th1-specific transcription factor T-bet. Pathobiology. 2006;73:224–37.
32. Aliprantis AO, Wang J, Fathman JW, et al. Transcription factor T-bet regulates skin sclerosis through its function in innate immunity and via IL-13. Proc Natl Acad Sci U S A. 2007;104:2827–30.
33. Kato A, Yutani M, Terao M, et al. Oligosaccharide modification by N-acetylglucosaminyltransferase-V in macrophages are involved in pathogenesis of bleomycin-induced scleroderma. Exp Dermatol. 2015;24:585–90.
34. Brembilla NC, Montanari E, Truchetet M-E, et al. Th17 cells favor inflammatory responses while inhibiting type I collagen deposition by dermal fibroblasts: differential effects in healthy and systemic sclerosis fibroblasts. Arthritis Res Ther. 2013;15:R151.
35. Nakashima T, Jinnin M, Yamane K, et al. Impaired IL-17 signaling pathway contributes to the increased collagen expression in scleroderma fibroblasts. J Immunol. 2012;188:3573–83.
36. Okamoto Y, Hasegawa M, Matsushita T, et al. Potential roles of interleukin-17A in the development of skin fibrosis in mice. Arthritis Rheum. 2012;64:3726–35.
37. Yoshizaki A, Yanaba K, Iwata Y, et al. Cell adhesion molecules regulate fibrotic process via Th1/Th2/Th17 cell balance in a bleomycin-induced scleroderma model. J Immunol. 2010;185:2502–15.
38. Yamamoto T. Pathogenic role of CCL2/MCP-1 in scleroderma. Front Biosci. 2008;13:2686–95.
39. Gharaee-Kermani M, Denholm EM, Phan SH. Costimulation of fibroblast collagen and transforming growth factor β1 gene expression by monocyte chemoattractant protein-1 via specific receptors. J Biol Chem. 1996;271:17779–84.
40. Yamamoto T, Eckes B, Mauch C, Hartmann K, Krieg T. Monocyte chemoattractant protein-1 enhances gene expression and synthesis of matrix metalloproteinase-1 in human fibroblasts by an autocrine IL-1α loop. J Immunol. 2000;164:6174–9.
41. Yamamoto T, Eckes B, Hartmann K, Krieg T. Expression of monocyte chemoattractant protein-1 in the lesional skin of systemic sclerosis. J Dermatol Sci. 2001;26:133–9.
42. Distler O, Pap T, Kowal-Bielecka O, et al. Overexpression of monocyte chemoattractant protein 1 in systemic sclerosis: role of platelet-derived growth factor and effects of monocyte chemotaxis and collagen synthesis. Arthritis Rheum. 2001;44:2665–78.
43. Yamamoto T, Eckes B, Krieg T. High expression and autoinduction of monocyte chemoattractant protein-1 in scleroderma fibroblasts. Eur J Immunol. 2001;31:2936–41.
44. Kawaguchi Y, Hara M, Wright TM. Endogenous IL-1α from systemic sclerosis fibroblasts induces IL-6 and PDGF-A. J Clin Invest. 1999;103:1253–60.

45. Zhu Z, Ma B, Zheng T, et al. IL-13-induced chemokine responses in the lung: role of CCR2 in the pathogenesis of IL-13-induced inflammation and remodeling. J Immunol. 2002;168:2953–62.
46. Yamamoto T, Nishioka K. Role of monocyte chemoattractant protein-1 and its receptor, CCR-2, in the pathogenesis of bleomycin-induced scleroderma. J Invest Dermatol. 2003;121:510–6.
47. Ferreira AM, Takagawa S, Fresco R, Zhu X, Varga J, DiPietro LA. Diminished induction of skin fibrosis in mice with MCP-1 deficiency. J Invest Dermatol. 2006;126:1900–8.
48. Galindo M, Santiago B, Alcami J, Riyero M, Martin-Serrano J, Pablos JL. Hypoxia induces expression of the chemokines monocyte chemoattractant protein-1 (MCP-1) and IL-8 in human dermal fibroblasts. Clin Exp Immunol. 2001;123:36–41.
49. Tsou P-S, Talia NN, Pinney AJ, et al. Effect of oxidative stress on protein tyrosine phosphatase 1B in scleroderma dermal fibroblasts. Arthritis Rheum. 2012;64:1978–89.
50. Bruckdorfer KR, Hillary JB, Bunce T, Vancheeswaran R, Black CM. Increased susceptibility to oxidation of low-density lipoprotein isolated from patients with systemic sclerosis. Arthritis Rheum. 1995;38:1060–7.
51. Shimuzu K, Ogawa F, Akiyama Y, Muroi E, Yoshizaki A, Iwata Y, Komura K, Bae S, Sato S. Increased serum levels of N(epsilon)-(hexanoyl)lysine, a new marker of oxidative stress, in systemic sclerosis. J Rheumatol. 2008;35:2214–9.
52. Ogawa F, Shimuzu K, Hara T, Muroi E, Komura K, Takenaka M, Hasegawa M, Fujimoto M, Takehara K, Sato S. Autoantibody against one of the antioxidant repair enzymes, methionine sulfoxide reductase A, in systemic sclerosis: association with pulmonary fibrosis and vascular damage. Arch Dermatol Res. 2010;302:27–35.
53. Casciola-Rosen L, Wigley F, Rosen A. Scleroderma autoantigens are uniquely fragmented by metal-catalyzed oxidation reactions: implications for pathogenesis. J Exp Med. 1997;185:71–9.
54. Baroni SS, Santillo M, Bevilacqua F, Luchetti M, Spadoni T, Mancini M, Fraticelli P, Sambo P, Funaro A, Kazlauskas A, Avvedimento EV, Gabrielli A. Stimulatory autoantibodies to the PDGF receptor in systemic sclerosis. N Engl J Med. 2006;354:2667–76.
55. Zhou C-F, Yu JF, Zhang JX, et al. N-acetylcysteine attenuates subcutaneous administration of bleomycin-induced skin fibrosis and oxidative stress in a mouse model of scleroderma. Clin Exp Dermatol. 2013;38:403–9.
56. Yoshizaki A, Iwata Y, Mokura K, et al. CD19 regulates skin and lung fibrosis via toll-like receptor signaling in a model of bleomycin-induced scleroderma. Am J Pathol. 2008;172:1650–63.
57. Agarwal SK, Wu M, Livingston CK, et al. Toll-like receptor 3 upregulation by type I interferon in healthy and scleroderma dermal fibroblasts. Arthritis Res Ther. 2011;13:R3.
58. Bhattacharyya S, Kelley K, Melichian DS, et al. Toll-like receptor 4 signaling augments transforming growth factor-β responses: a novel mechanism for maintaining and amplifying fibrosis in scleroderma. Am J Pathol. 2013;182:192–205.
59. Takahashi T, Asano Y, Ichimura Y, et al. Amelioration of tissue fibrosis by toll-like receptor 4 knockout in murine models of systemic sclerosis. Arthritis Rheumatol. 2015;67:254–65.
60. Huber LC, Distler JHW, Moritz F, et al. Trichostatin A prevents the extracellular matrix in a mouse model of bleomycin-induced skin fibrosis. Arthritis Rheum. 2007;56:2755–64.
61. Svegliati S, Marrone G, Pezone A, et al. Oxidative DNA damage induces the ATM-mediated transcriptional suppression of the Wnt inhibitor WIF-1 in systemic sclerosis and fibrosis. Sci Signal. 2014;7:ra84.
62. Dees C, Schlottmann I, Funke R, et al. The Wnt antagonists DKK1 and SFRP1 are downregulated by promoter hypermethylation in systemic sclerosis. Ann Rheum Dis. 2014;73:1232–9.
63. Maurer B, Stanczyk J, Jüngel A, et al. MicroRNA-29, a key regulator of collagen expression in systemic sclerosis. Arthritis Rheum. 2010;62:1733–43.

64. Tanaka S, Suto A, Ikeda K, et al. Alteration of circulating miRNAs in SSc: miR-30b regulates the expression of PDGF receptor β. Rheumatology. 2013;52:1963–72.
65. Honda N, Jinnin M, Kajihara I, et al. TGF-β-mediated downregulation of microRNA-196a contributes to the constitutive upregulated type I collagen expression in scleroderma dermal fibroblasts. J Immunol. 2012;188:3323–31.
66. Nakamura M, Tokura Y. Expression of SNAI1 and TWIST1 in the eccrine glands of patients with systemic sclerosis: possible involvement of epithelial-mesenchymal transition in the pathogenesis. Br J Dermatol. 2011;164:204–5.
67. Aden N, Nuttall A, Shi-wen X, et al. Epithelial cells promote fibroblast activation via IL-1α in systemic sclerosis. J Invest Dermatol. 2010;130:2191–200.
68. Zhou C-F, Zhou D-C, Zhang J-X, et al. Bleomycin-induced epithelial-mesenchymal transition in sclerotic skin of mice: possible role of oxidative stress in the pathogenesis. Toxicol Appl Pharmacol. 2014;277:250–8.
69. Hu B, Phan SH. Myofibroblasts. Curr Opin Rheumatol. 2013;25:71–7.
70. Marangoni RG, Korman BD, Wei J, et al. Myofibroblasts in murine cutaneous fibrosis originate from adiponectin-positive intradermal progenitors. Arthritis Rheumatol. 2015;67:1062–73.
71. Ohgo S, Hasegawa S, Hasebe Y, Mizutani H, Nakata S, Akamatsu H. Bleomycin inhibits adipogenesis and accelerates fibrosis in the subcutaneous adipose layer through TGF-β1. Exp Dermatol. 2013;22:769–71.
72. Irani AM, Gruber BL, Kaufman LD, Kahaleh MB, Schwartz LB. Mast cell changes in scleroderma: presence of MCT cells in the skin and evidence of mast cell activation. Arthritis Rheum. 1992;35:933–9.
73. Truchetet M-E, Brembilla N-C, Montanari E, et al. Interleukin-17A+ cell counts are increased in systemic sclerosis skin and their number is inversely correlated with the extent of skin involvement. Arthritis Rheum. 2013;65:1347–56.
74. Willenborg S, Eckes B, Brinckmann J, et al. Genetic ablation of mast cells redefines the role of mast cells in skin wound healing and bleomycin-induced fibrosis. J Invest Dermatol. 2014;134:2005–15.
75. Li D, Guabiraba R, Besnard A-G, et al. IL-33 promotes ST2-dependent lung fibrosis by the induction of alternatively activated macrophages and innate lymphoid cells in mice. J Allergy Clin Immunol. 2014;134:1422–32.
76. Yamaguchi Y. Periostin in skin tissue and skin-related diseases. Allergol Int. 2014;63:161–70.
77. Yang L, Serada S, Fujimoto M, et al. Periostin facilitates skin sclerosis via PI3K/Akt dependent mechanism in a mouse model of scleroderma. PLoS One. 2012;7:e41994.
78. Wu M, Schneider DJ, Mayes MD, et al. Osteopontin in systemic sclerosis and its role in dermal fibrosis. J Invest Dermatol. 2012;132:1605–14.
79. Santiago B, Gutierrez-Canas I, Dotor J, et al. Topical application of a peptide inhibitor of transforming growth factor-beta1 ameliorates bleomycin-induced skin fibrosis. J Invest Dermatol. 2005;125:450–5.
80. Wakatsuki-Nakamura T, Oyama N, Yamamoto T. Local injection of latency-associated peptide (LAP), a linker peptide specific for active form of transforming growth factor-beta1 (TGF-β1), inhibits dermal sclerosis in bleomycin-induced murine scleroderma. Exp Dermatol. 2012;21:189–94.
81. Yokozeki M, Baba Y, Shimokawa H, Moriyama K, Kuroda T. Interferon-γ inhibits the myofibroblastic phenotype of rat palatal fibroblasts induced by transforming growth factor-β1 in vitro. FEBS Lett. 1999;442:61–4.
82. Ulloa L, Doody J, Massagué J. Inhibition of transforming growth factor-β/SMAD signaling by the interferon-γ/STAT pathway. Nature. 1999;397:710–3.
83. Yamamoto T, Takagawa S, Kuroda M, Nishioka K. Effect of interferon-γ on experimental scleroderma induced by bleomycin. Arch Dermatol Res. 2000;292:362–5.

84. Hasegawa M, Matsushita Y, Horikawa M, et al. A novel inhibitor of Smad-dependent transcriptional activation suppresses tissue fibrosis in mouse models of systemic sclerosis. Arthritis Rheum. 2009;60:3465–75.
85. Kimura M, Kawahito Y, Hamaguchi M, et al. SKL-2841, a dual antagonist of MCP-1 and MIP-1 beta, prevents bleomycin-induced sin sclerosis in mice. Biomed Pharmacother. 2007;61:222–8.
86. Yamamoto T, Takagawa S, Mizushima Y, Nishioka K. Effect of superoxide dismutase on bleomycin-induced dermal sclerosis: implications for the treatment of systemic sclerosis. J Invest Dermatol. 1999;113:843–7.
87. Yoshizaki A, Yanaba K, Ogawa A, et al. The specific free radical scavenger edaravone suppresses fibrosis in the bleomycin-induced and tight skin mouse models of systemic sclerosis. Arthritis Rheum. 2011;63:3086–97.
88. Yamamoto T, Nishioka K. Analysis of the effect of halofuginone on bleomycin-induced scleroderma. Rheumatology. 2002;41:594–6.
89. Matsumoto K, Nakamura T. Hepatocyte growth factor: renotropic role and potential therapeutics for renal diseases. Kidney Int. 2001;59:2023–38.
90. Wu M-H, Yokozeki H, Takagawa S, et al. Hepatocyte growth factor both prevents and ameliorates the symptoms of dermal sclerosis in a mouse model of scleroderma. Gene Ther. 2004;11:170–80.
91. Chan ES, Fernandez P, Merchant AA, et al. Adenosine A2A receptors in diffuse dermal fibrosis: pathogenic role in human dermal fibroblasts and in a murine model of scleroderma. Arthritis Rheum. 2006;54:2632–42.
92. Ghosh AK, Bhattacharyya S, Lakos G, Chen SJ, Mori Y, Varga J. Disruption of transforming growth factor beta signaling and profibrotic responses in normal skin fibroblasts by peroxisome proliferator-activated receptor gamma. Arthritis Rheum. 2004;50:1305–18.
93. Wu M, Melichian DS, Chang E, Warner-Blankenship M, Ghosh AK, Varga J. Rosiglitazone abrogates bleomycin-induced scleroderma and blocks profibrotic responses through peroxisome proliferator-activated receptor-γ. Am J Pathol. 2009;174:519–33.
94. Distler JH, Jüngel A, Huber LC, et al. Imatinib mesylate reduces production of extracellular matrix and prevents development of experimental dermal fibrosis. Arthritis Rheum. 2007;56:311–22.
95. Akhmetshina A, Venalis P, Dees C, et al. Treatment with imatinib prevents fibrosis in different preclinical models of systemic sclerosis and induces regression of established fibrosis. Arthritis Rheum. 2009;60:219–24.
96. Akhmetshina A, Dees C, Pileckyte M, et al. Dual inhibition of c-abl and PDGF receptor signaling by dasatinib and nilotinib for the treatment of dermal fibrosis. FASEB J. 2008;22:2214–22.
97. Huang J, Beyer C, Palumbo-Zerr K, et al. Nintedanib inhibits fibroblast activation and ameliorates fibrosis in preclinical models of systemic sclerosis. Ann Rheum Dis. 2015 [Epub ahead of print].
98. Desallais L, Avouac J, Fréchet M, et al. Targeting IL-6 by both passive or active immunization strategies prevents bleomycin-induced skin fibrosis. Arthritis Res Ther. 2014;16:R157.
99. Fukasawa C, Kawaguchi Y, Harigai M, et al. Increased CD40 expression in skin fibroblasts from patients with systemic sclerosis (SSc): role of CD40-CD154 in the phenotype of SSc fibroblasts. Eur J Immunol. 2003;33:2792–800.
100. Kawai M, Masuda A, Kuwana M. A CD40-CD154 interaction in tissue fibrosis. Arthritis Rheum. 2008;58:3562–73.
101. Kokot A, Sindrilaru A, Schiller M, et al. α-melanocyte-stimulating hormone suppresses bleomycin-induced collagen synthesis and reduces tissue fibrosis in a mouse model of scleroderma. Arthritis Rheum. 2009;60:592–603.
102. Böhm M, Stegemann A. Bleomycin-induced fibrosis in MC1 signalling-deficient C57BL/6J-Mc1r$^{e/e}$ mice further supports a modulating role for melanocortins in collagen synthesis of the skin. Exp Dermatol. 2014;23:431–3.

103. Kajii M, Suzuki C, Kashihara J, et al. Prevention of excessive collagen accumulation by human intravenous immunoglobulin treatment in a murine model of bleomycin-induced scleroderma. Clin Exp Immunol. 2011;163:235–41.
104. Ohashi T, Yamamoto T. Anti-fibrotic effect of lysophosphatidic acid receptors LPA1 and LPA3 antagonist on experimental murine scleroderma induced by bleomycin. Exp Dermatol. 2015;24:698–702.
105. Azhdari M, Baghaban-Eslaminejad M, Baharvand H, Aghdami N. Therapeutic potential of human-induced pluripotent stem cell-derived endothelial cells in a bleomycin-induced scleroderma mouse model. Stem Cell Res. 2013;10:288–300.
106. Servettaz A, Goulvestre C, Kavian N, et al. Selective oxidation of DNA topoisomerase 1 induces systemic sclerosis in mice. J Immunol. 2009;182:5855–64.
107. Kavian N, Servettaz A, Mongaret C, et al. Targeting ADAM-17/notch signaling abrogates the development of systemic sclerosis in a murine model. Arthritis Rheum. 2010;62:3477–87.
108. Kavian N, Servettaz A, Marut W, et al. Sunitinib inhibits the phosphorylation of platelet-derived growth factor receptor β in the skin of mice with scleroderma-like features and prevents the development of the disease. Arthritis Rheum. 2012;64:1990–2000.
109. Kavian N, Marut W, Servettaz A, et al. Reactive oxygen species-mediated killing of activated fibroblasts by arsenic trioxide ameliorates fibrosis in a murine model of SSc. Arthritis Rheum. 2012;64:3430–40.
110. Kasturi KN, Shibata S, Muryoi T, Bona C. Tight-skin mouse an experimental model for scleroderma. Int Rev Immunol. 1994;11:253–71.
111. Pablos JL, Everett ET, Harley R, LeRoy EC, Norris JS. Transforming growth factor-β1 and collagen gene expression during postnatal skin development and fibrosis in the tight-skin mouse. Lab Invest. 1995;72:180–5.
112. Philips N, Bashey RI, Jimenez SA. Increased α1(I) procollagen gene expression in tight skin (Tsk) mice myocardial fibroblasts is due to a reduced interaction of a negative regulatory sequence with AP-1 transcription factor. J Biol Chem. 1995;270:9313–21.
113. Bayle J, Fitch J, Jacobsen K, Kumar R, Lafyatis R, Lemaire R. Increased expression of Wnt2 and SFRP4 in Tsk mouse skin: role of Wnt signaling in altered dermal fibrillin deposition and systemic sclerosis. J Invest Dermatol. 2008;128:871–81.
114. MacGaha TL, Saito S, Phelps RG, et al. Lack of skin fibrosis in tight skin (TSK) mice with targeted mutation in the interleukin-4Rα and transforming growth factor β genes. J Invest Dermatol. 2001;116:136–43.
115. MacGaha TL, Le M, Kodera T, et al. Molecular mechanisms of interleukin-4-induced up-regulation of type I collagen gene expression in murine fibroblasts. Arthritis Rheum. 2003;48:2275–84.
116. Ong CJ, Ip S, The SJ, et al. A role for T helper 2 cells in mediating skin fibroblasts in tight-skin mice. Cell Immunol. 1999;196:60–8.
117. MacGaha TL, Phelps RG, Spiera H, Bona C. Halofuginone, an inhibitor of type-I-collagen synthesis and skin sclerosis, blocks transforming growth factor-beta-mediated Smad3 activation in fibroblasts. J Invest Dermatol. 2002;118:461–70.
118. Ong VH, Evans LA, Shiwen X, et al. Monocyte chemoattractant protein 3 as a mediator of fibrosis: overexpression in systemic sclerosis and the type 1 tight-skin mouse. Arthritis Rheum. 2003;48:1979–91.
119. Wallace VA, Kondo S, Kono T, et al. A role for CD4+ T cells in the pathogenesis of skin fibrosis in tight skin mice. Eur J Immunol. 1994;24:1463–6.
120. Saito E, Fujimoto M, Hasegawa M, et al. CD19-dependent B lymphocyte signaling thresholds influence skin fibrosis and autoimmunity in the tight skin mice. J Clin Invest. 2002;109:1453–62.
121. Hasegawa M, Hamaguchi Y, Yanaba K, et al. B-lymphocyte depletion reduces skin fibrosis and autoimmunity in the tight-skin mouse model for systemic sclerosis. Am J Pathol. 2006;169:954–66.

122. Marie I, Beny JL. Endothelial dysfunction in murine model of systemic sclerosis: tight-skin mouse 1. J Invest Dermatol. 2002;119:1379–87.
123. Dooley A, Low SY, Holmes A, et al. Nitric oxide synthase expression and activity in the tight-skin mouse model of fibrosis. Rheumatology. 2008;47:272–80.
124. Ong D, Wong C, Roberts CR, Teh HS, Jirik FR. Anti-IL-4 treatment prevents dermal collagen deposition in the tight-skin mouse model of scleroderma. Eur J Immunol. 1998;128:2619–29.
125. Shen Y, Ichino M, Nakazawa M, Minami M. CpG oligodeoxynucleotides prevent the development of scleroderma-like syndrome in tight-skin mice by stimulating a Th1 immune response. J Invest Dermatol. 2005;124:1141–8.
126. Blank M, Levy Y, Amital H, Shoenfeld Y, Pines M, Genina O. The role of intravenous immunoglobulin therapy in mediating skin fibrosis in tight skin mice. Arthritis Rheum. 2002;46:1689–90.
127. Shiota N, Kakizoe E, Shimoura K, Tanaka T, Okunishi H. Effect of mast cell chymase inhibitor on the development of scleroderma in tight-skin mice. Br J Pharmacol. 2005;145:424–31.
128. Granot I, Halevy O, Hurwitz S, Pines M. Halofuginone: an inhibitor of collagen type I synthesis. Biochim Biophys Acta. 1993;1156:107–12.
129. Choi ET, Callow AD, Sehgal NL, Brown DM, Ryan US. Halofuginone, a specific collagen type I inhibitor, reduces anastomotic intimal hyperplasia. Arch Surg. 1995;130:257–61.
130. Levi-Schaffer F, Nagler A, Slavin S, Knopov V, Pines M. Inhibition of collagen synthesis and changes in skin morphology in murine graft-versus-host disease and tight skin mice: effect of halofuginone. J Invest Dermatol. 1996;106:84–8.
131. McGaha TL, Phelps RG, Spiera H, Bona C. Halofuginone, an inhibitor of type-I collagen synthesis and skin sclerosis, blocks transforming-growth-factor-β-mediated Smad3 activation in fibroblasts. J Invest Dermatol. 2002;118:461–70.
132. Pines M, Domb A, Ohara M, Inbar J, Genina O, Alexiev R, Nagler A. Reduction in dermal fibrosis in the tight-skin (Tsk) mouse after local application of halofuginone. Bichem Pharmacol. 2001;62:1221–7.
133. Komura K, Fujimoto M, Yanaba K, et al. Blockade of CD40/CD40 ligand interaction attenuates skin fibrosis and autoimmunity in the tight-skin mouse. Ann Rheum Dis. 2008;67:867–72.
134. Iwasaki T, Imado T, Kitano S, Sano H. Hepatocyte growth factor ameliorates dermal sclerosis in the tight-skin mouse model of scleroderma. Arthritis Res Ther. 2006;8:R161.
135. Christner PJ, Peters J, Hawkins D, Siracusa LD, Jimenez SA. The tight skin 2 mouse: an animal model of scleroderma displaying cutaneous fibrosis and mononuclear cell infiltration. Arthritis Rheum. 1995;38:1791–8.
136. Christner PJ, Yufit Y, Peters J, MacGrath R, Jimenez SA. Transcriptional activation of the α1 (I) procollagen gene and upregulation of α1(I) and α1(III) procollagen messenger RNA in dermal fibroblasts from tight skin 2 mice. Arthritis Rheum. 1998;41:2132–42.
137. Christner PJ, Yufit Y, Peters J, MacGrath R, Conway RF, Jimenez SA. Transcriptional activation of α1(III) procollagen gene in Tsk2/+ dermal fibroblasts. Biochem Biophys Res Commun. 2003;303:406–12.
138. Barisic-Dujmovic T, Boban I, Clark SH. Regulation of collagen gene expression in the Tsk2 mouse. J Cell Physiol. 2008;215:464–71.
139. Gentiletti J, McCloskey LJ, Artlett CM, Peters J, Jimenez SA, Christner PJ. Demonstration of autoimmunity in the tight skin-2 mouse: a model for scleroderma. J Immunol. 2005;175:2418–26.
140. Gerber EE, Gallo EM, Fontana SC, et al. Integrin-modulating therapy prevents fibrosis and autoimmunity in mouse models of scleroderma. Nature. 2013;503:126–30.

Genetics of Systemic Sclerosis

5

Naoyuki Tsuchiya and Aya Kawasaki

Abstract

Genetic epidemiology suggests that the molecular background of systemic sclerosis (SSc) such as autoantibody production or gene expression profiles may be highly hereditary; however, the clinical occurrence of SSc may require non-hereditary factor(s). Genome-wide association studies (GWASs) confirmed that the *HLA* genes are the strongest genetic factors, although specific risk alleles are different among populations and among the clinical subsets such as those determined by autoantibody profiles. GWAS and candidate gene studies revealed more than a dozen of convincing candidate genes; however, thus far, all of them are shared by other diseases and do not appear to explain the unique features of SSc such as extensive fibrosis and vascular damage. Thus, something must be missing, and more studies are required to reveal the secrets of this enigmatic disease. Here, we reviewed the genetic studies of SSc, with emphasis on those performed on Asian populations.

Keywords

Systemic sclerosis • Genetics • *HLA* • Genome-wide association study • SNP

5.1 Genetic Background of Systemic Sclerosis

The pathogenesis of systemic sclerosis (SSc), characterized by fibrosis of the skin and organs, vascular damage, and autoimmunity, remains enigmatic. Because of the lack of Mendelian inheritance pattern and strong environmental factors shared by

N. Tsuchiya (✉) • A. Kawasaki
Molecular and Genetic Epidemiology Laboratory, Faculty of Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan
e-mail: tsuchiya-tky@umin.net

K. Takehara et al. (eds.), *Systemic Sclerosis*, DOI 10.1007/978-4-431-55708-1_5

the majority of patients, the role of genetic factors has been suggested. However, to what extent genetics plays a role in occurrence of SSc remains controversial.

Arnett et al. prospectively surveyed three cohorts of the SSc patients in the USA for affected first-degree relatives. As compared with prevalence of SSc in the USA (0.026 %), affected siblings were 0.4 % and affected first-degree relatives were 0.3 %. Average familial relative risk was 15 in siblings and 13 in first-degree relatives [1], which convincingly demonstrated familial clustering of SSc.

Familial clustering can occur not only from the sharing of genetic factors but also from shared environmental factors, which might partly be distinguishable by twin studies. Feghali-Bostwick et al. conducted a twin study in the USA and reported that the concordance rate for SSc was 4.2 % (1/24) in monozygotic (MZ) twins and 5.6 % (1/18) in dizygotic (DZ) twins, which does not support the strong contribution of genetic factors. On the other hand, the same study detected a clearly higher concordance for antinuclear antibody (ANA) positivity (titer of 1:40 or higher) in the MZ twins (90 %) versus the DZ twins (40 %), supporting a strong contribution of genetics for the ANA production [2].

Zhou et al. compared expression profiles between the dermal fibroblasts of 15 discordant twin pairs (ten MZ and five DZ) and five normal controls using microarray analysis. Lesional and nonlesional fibroblasts from SSc patients showed no significant differences in gene expression, but both were significantly different from those of unaffected DZ twins and normal controls. However, unaffected MZ twins were not significantly different from the affected SSc patients, thereby suggesting a stronger genetic predisposition to SSc is present at the molecular level in skin fibroblasts than is clinically detected. They also reported that normal fibroblasts incubated with serum from an SSc-affected patient or with serum from her unaffected MZ twin sister developed the increased expression of *COL1A2*, *SPARC*, and *CTGF*, typically observed in SSc fibroblasts, suggesting the role of soluble factors [3].

Taken together, these genetic epidemiological findings might suggest that the immunological and biological background is substantially influenced by genetics, but the trigger for the development of clinical SSc may not be genetically inherited. However, the number of genetic epidemiology studies of SSc is very small, and due to the low prevalence, the sample size is limited. These findings need further confirmation.

Another observation suggesting the role of genetic background is ethnic difference. In the South African populations of African ancestry, 65 % of SSc was diffuse cutaneous SSc (dcSSc), and 56 % had pulmonary fibrosis [4]. Also in Detroit, USA, 60.3 % of patients of African ancestry had dcSSc, in contrast to 26.6 % of other populations [5]. The autoantibody profiles are also remarkably different among populations. For instance, anti-topoisomerase I antibody (ATA) was detected in 76 % of the Thais and 26 % of the Australians, while 2 % of the Thais and 51 % of the Australians showed staining of the centromeres [6]. Such population difference may suggest the role of genetic factors.

5.2 Human Leukocyte Antigens (*HLAs*)

Human leukocyte antigens (*HLAs*) are the top candidate gene for any disease where immune system plays a role. Also in the case of SSc, a number of studies examined association with *HLA*. Indeed, *HLA* remains to be the strongest susceptibility gene to SSc, even in this era of genome-wide association studies, as will be discussed later.

HLA association with SSc is different among populations and with respect to disease subsets (Table 5.1) [7–22]. In this review, we will put focus on *HLA* studies in Asia and briefly mention the data from other populations.

5.2.1 *HLA* Association in Asian SSc

In a series of studies, Kuwana and colleagues examined HLA class II of Japanese patients [7, 9, 23–25] as well as other ethnicities [8]. In their analysis on 105 - Japanese patients with SSc and 104 healthy controls [7], they detected marginal increase of *DPB1*09:01* in overall SSc. In the patients positive for ATA, *DRB1*15:02-DQB1*06:01-DPB1*09:01* haplotype was significantly increased (odds ratio [OR] 6.0 vs. ATA-negative SSc, OR 7.8 vs. healthy controls), and 92 % carried either *DQB1*06:01* or *DQB1*03:01*, which uniquely share Tyr at position 26 (^{26}Y), as compared with 53 % in ATA-negative SSc and 54 % in healthy controls. In the patients with anti-U1RNP, *DQB1*03:02* was significantly increased, and at the amino acid level, *DQB1* alleles carrying ^{30}Y and ^{71}TRAELDT ^{77} sequence were increased. In the patients with anti-centromere antibody (ACA), *DRB1*01:01-DQB1*05:01-DPB1*04:02* haplotype was increased (OR 6.1), and ^{38} V was present in 91 % of the patients and 57 % in healthy controls (OR 8.0). Taken together, they concluded that HLA class II genes play a limited role in the development of SSc but play a more significant role in the production of autoantibodies [7].

Kuwana et al. also compared the HLA class II associated with ATA among 47 European-Americans, 15 African-Americans, 43 Japanese, and 12 Choctaw Native Americans. They found that the frequency of progressive pulmonary fibrosis is lower and cumulative survival rates were higher in the European-Americans when compared with African-American and Japanese patients. Interestingly, *DRB1* association with ATA was different among populations: *DRB1*15:02* in the Japanese patients, *DRB1*11:01-*11:04* in European-Americans and African-Americans, and *DRB1*16:02* in Choctaws [8].

In 2009, Zhou et al. reported the first genome-wide association study of SSc. They examined 137 Korean patients with SSc and 564 Korean healthy controls as the discovery set and 1,107 SSc and 2,747 controls in the USA as the replication set. They detected association of *HLA-DP* region, and fine mapping revealed association of *DPB1*13:01* and *DPB1*09:01* with predisposition to ATA-positive SSc. Association of *DPB1*13:01* with ATA-positive SSc was confirmed in the USA. They noted that association with autoantibody patterns was much stronger than

Table 5.1 *HLA* associations with systemic sclerosis

Population	Clinical phenotype	Associated allele or haplotype	References
Asian ancestry			
Japanese	ATA	*DRB1*15:02-DQB1*06:01-DPB1*09:01*	[7–9]
		Combination of *DQB1* ^{26}Y in *DQB1* and ^{67}FLEDR^{71} in *DRB1* or *DRB5*	
	ACA	*DRB1*01:01-DQB1*05:01-DPB1*04:02*, ^{38}V in *DQB1*	[7]
	U1RNP Ab	*DRB1*04:01* or **08:02-DQB1*03:02*	[7]
	ILD	*DRB5*01:05*	[10]
Korean	Overall SSc	*DPB1*13:01*, *DPB1*09:01*	[11]
	ATA	*DRB1*15:02*, ^{38}V-^{67}FLEDR^{71} in *DRB1* or *DRB5* including *DRB5*01:02*	[12]
Han Chinese	Overall SSc	*DRB1*11*, *DPB1*03:01*, *DPB1*13:01*	[13, 14]
	ATA	*DRB1*16:02*	[14]
	ACA	*DRB1*15:02*, *DPB1*04*	[13, 14]
	Pulmonary fibrosis	*DPB1*03:03*	[13]
European ancestry			
European-American	Overall SSc	*DRB1*11:04-DQA1*05:01-DQB1*03:01*, *DQB1* 26epi*, *DPB1*13:01*	[15]
	dcSSc	*DRB1*11:04*	[16]
	lcSSc	*DRB1*11:01*	[16]
	ATA	*DRB1*11:04-DQA1*05:01-DQB1*0301*, *DQB1* 26epi*, *DPB1*13:01*	[15]
	ACA	*DRB1*01:01*, *DQA1*01:01*, *DQB1*05:01*, *DRB1*04* (especially *DRB1*04:04*)	[15]
	Anti-RNAPIII Ab	*DRB1*04:04*, *DRB1*11* (especially *DRB1*11:04*), *DQB1*03*	[15]
North American and European	Overall SSc	*TAP2* rs17500468, *DPB1* rs9277052, *HLA-B* rs2442719, *DPB1* rs4713605, *HLA-DOA* rs44623	[17]
	ATA	*DRB1* ^{67}F, *DPB1* no (^{96}K or ^{96}R), *DRB1* ^{86}V, *DPB1* ^{76}I	[17]
	ACA	*DRB1* ^{13}F, *DQA1* ^{69}T, *TAP2* rs12528892, *HLA-DOA* rs6933319	[17]
French	dcSSc, ATA	*DRB1*11*, *DRB1*15* haplotypes	[18]
Italian	PAH	*B*35*	[19]
Italian, Spanish	Overall SSc	*DRB1*11:04-DQA1*05:01-DQB1*03:01*, *DRB1*01* protective against ATA, predispositional to ACA	[20]
Hispanic	Overall SSc	*DRB1*11:04-DQA1*05:01-DQB1*03:01*, *DQB1* 26epi*	[15]
	ATA	*DRB1*11:04*, *DQB1* 26epi*	[15]
	ACA	*DRB1*04:07*	[15]

(continued)

Table 5.1 (continued)

Population	Clinical phenotype	Associated allele or haplotype	References
African ancestry			
South African	Overall SSc	*DR2*	[21]
	ATA	*DPB1*13:01*, *DRB1*15*	[21]
	Pulmonary fibrosis	*DRB1*11:02*	[21]
African-American	SSc	*DRB1*08:04*, *DQA1*05:01*, *DQB1*03:01*	[15]
	ATA	*DRB1*11:04*, *DRB1*08:04*, *DQB1* 26epi	[15]
	Anti-RNAPIII Ab	*DRB1*08* (*DRB1*08:04*), *DQA1*05:01*, *DQB1*03:01*	[15]
Mixed			
European, Hispanic, and African-Americans	SRC	*DRB1*04:07*, **13:04*	[22]

ATA anti-topoisomerase I antibody, *ACA* anti-centromere antibody, *RNAPIII* RNA polymerase III, *ILD* interstitial lung disease, *SRC* scleroderma renal crisis, *DQB1 26epi DQB1* alleles encoding non-leucine residues at position 26. Only the predispositional associations are shown

with clinical classification of dcSSc and lcSSc [11]. Although the association with *DPB1*09:01* was also reported in Japan, that of *DPB1*13:01* was not detected [7].

Recently, several studies with larger sample size have been reported from China. Wang et al. analyzed 338 SSc and 480 control Han Chinese. *DPB1*03:01* and *DPB1*13:01* were found to be significantly increased. In addition, *DPB1*03:01* was associated with pulmonary fibrosis [13]. He et al. analyzed 585 Han Chinese SSc and 458 controls. They detected striking association of *DRB1*15:02* (OR 7.3) and *DRB1*10* (OR 5.0) with risk for ACA and *DRB1*16* for ATA (OR 6.6), pulmonary fibrosis (OR 5.2), and dcSSc (OR 5.7) [14].

Finally, Odani et al. detected upregulation of *DRB5* in the peripheral blood mononuclear cells from SSc patients with interstitial lung disease (ILD) using the transcriptome analysis. *DRB5* locus is carried only by DR51 haplotype group (*DRB1*15* and *DRB1*16*). Among the *DRB5* alleles, *DRB5*01:05* allele was increased in SSc patients with ILD than in SSc patients without ILD or in healthy controls. Although *DRB1*15:02* also showed a trend toward increase in the SSc accompanied by ILD, it did not reach statistical significance. Therefore, the authors concluded that *DRB1*01:05* allele may be responsible for the risk to ILD [10].

5.2.2 *HLA* Association with SSc in the Populations of European and African Ancestry

HLA association with SSc in the populations of European and African ancestry is also summarized in Table 5.1. Here, we will only refer to the recent large-scale analyses.

Arnett et al. [15] conducted an association study on 1,300 Caucasian, Hispanic, and African-American patients with SSc and 1,000 controls. In the patients of European ancestry, association with *DRB1*11:04-DQA1*05:01-DQB1*03:01* haplotype was detected, with *DRB1*11:04* (OR 2.48) probably playing a primary role. However, the strongest association was with *DPB1*13:01* (OR 3.18), which was not in linkage disequilibrium with *DRB1*11:04*; thus, these loci were considered to have independent contributions. They also showed that *DRB1*11:04* was associated with SSc in the Hispanic population. With respect to disease subsets, ATA-positive patients of European ancestry showed the same association with overall SSc, albeit much strongly.

Mayes used Immunochip to genotype 1,833 SSc patients and 3,466 controls and imputed the *HLA* alleles [17]. Using conditional logistic regression analysis, they claimed that six polymorphic amino acids and seven other SNPs can almost fully account for the previously known HLA class II association with SSc.

5.2.3 Summary of the *HLA* Association Studies

The implications from *HLA* association studies across various populations could be summarized as follows:

1. *HLA* is generally more strongly associated with SSc subsets defined by autoantibody profiles or clinical manifestations, rather than with the development of overall SSc.
2. Ethnic differences are present, although *DPB1*13:01* appears to be consistently associated with overall SSc or ATA in all ethnicities. In East Asian populations, ATA was associated with serological *DR2* group such as *DRB1*15:02* or *DRB1*16:02*, but in the populations of European ancestry, association with *DRB1*11* (especially *DRB1*11:04*) haplotype is consistently observed. In ACA, association with *DRB1*01:01-DQB1*05:01* haplotype was reported from both East Asian and European populations.

5.3 Non-*HLA* Genes

As in many other complex or multifactorial diseases, non-*HLA* genes have been investigated by candidate gene approach as well as genome-wide approach. Thus far, according to the GWAS Catalog [26] (http://www.ebi.ac.uk/gwas/home), five genome-wide association studies (GWASs) on SSc have been published. Four were performed in the populations of European ancestry [27–30] and one in Korea [11]. In addition, one large-scale Immunochip analysis was also reported in the populations of European ancestry [17]. These studies emphasized the strongest contribution of *MHC* region and also detected several convincing association signals in non-*HLA* genes. In addition, candidate gene analyses also detected

genes associated with susceptibility to SSc, and some of them have been replicated. Convincing candidate non-*HLA* susceptibility genes detected by these approaches are shown in Table 5.2 [17, 27–43].

Table 5.2 Non-HLA candidate genes to systemic sclerosis with $P < 5 \times 10^{-8}$ or shown to be associated in more than two studies

Gene	Potentially relevant function	Clinical phenotype	Associated conditions other than SSc[a]	References for SSc
ATG5	Autophagy	SSc	SLE, RA	[17]
CD247	T cell signaling	SSc	SLE, celiac disease, RA	[27, 28]
CSK	B cell signaling, fibrosis	SSc	SLE, blood pressure	[31]
DNASE1L3	DNA fragmentation during apoptosis	SSc, ACA	RA, eating disorder	[17]
IL12RB1	IL-12 signaling	SSc	Pulmonary function in asthma	[32]
IL12RB2	IL-12 signaling	SSc	Behcet disease, PBC, CD, IBD	[33]
IRF5	Type I interferon, proinflammatory cytokine	SSc	SLE, UC, RA, PBC	[27, 28, 34]
IRF8	Type I interferon	lcSSc	Monocyte count, MS, UC, CLL, major depressive disorder, RA, diabetic retinopathy, SLE, IBD	[29, 35]
PSORS1C1	Psoriasis candidate gene	SSc	BD, Stevens-Johnson syndrome and toxic epidermal necrolysis, multiple myeloma, CD	[28]
SCHIP1-IL12A	IL-12 signaling	lcSSc	PBC, BD, MS, celiac disease, major depressive disorder	[17]
STAT4	Type I interferon, IL-12 signaling, Th1	SSc	BD, SS, SLE, PBC, RA, celiac disease, IBD	[27, 28, 36]
TNFAIP3	Regulation of NF-κB	SSc	SLE, RA, SS, psoriasis, IBD, UC, MS	[35, 37, 38]
TNFSF4	OX40 ligand, T cell and B cell activation	ACA	SLE, celiac disease, diabetic retinopathy, CD	[39, 40]
BANK1	B cell signaling	dcSSc	SLE, blood pressure, acute urticaria and angioedema, CD, psychiatric disorders	[41, 42]
BLK	B cell signaling	SSc	SLE, RA, Kawasaki disease, periodontitis	[43, 44]

This table was created based on a review by Korman and Criswell [51]
SLE systemic lupus erythematosus, *RA* rheumatoid arthritis, *PBC* primary biliary cirrhosis, *CD* Crohn's disease, *IBD* inflammatory bowel disease, *UC* ulcerative colitis, *CLL* chronic lymphocytic leukemia, *MS* multiple sclerosis, *SS* Sjögren syndrome
[a]Based on GWAS Catalog [26] (http://www.ebi.ac.uk/gwas/home), accessed on April 29, 2015

Most of the candidate susceptibility genes in the list are those that play a role in the immune system, such as T cell and B cell activation, type I interferon and IL-12 induction and signal transduction, and NF-κB regulation (Table 5.2). Notably, all of them are associated with diseases other than SSc as well. This is partly because some of them were detected by Immunochip analysis where polymorphisms of the immune system genes including those that have already been shown to be associated with immune system disorders are targeted and others by the studies where the susceptibility genes to other diseases were examined as the candidate genes to SSc. However, in view of the unique pathophysiology and impressive clinical pictures of SSc, it is unexpected that even GWASs have not detected specific susceptibility genes to SSc, for example, genes that can explain the extensive fibrosis and vascular damage.

5.4 Genetic Studies of SSc in Japan

Genetics associations sometimes depend on the genetic background of the population. Therefore, it is important to perform association study in Japanese cases and controls. As of April 2015, GWAS of SSc has not been published in Japan, but many candidate gene studies have been published. Association study of *HLA* is already described in Sect. 5.2.1; therefore, candidate gene studies of Japanese SSc will be discussed here.

Hepatocyte growth factor (HGF) is an antifibrotic factor that inhibits fibrosis and promotes angiogenesis. Hoshino et al. examined association of single nucleotide polymorphisms (SNPs) in *HGF* with severity of ILD in Japanese patients with SSc. Although difference was not observed between SSc and healthy controls or between dcSSc and lcSSc, when the authors examined the presence or absence of end-stage lung disease (ESLD) among the patients, rs3735520T allele in the upstream flanking region was significantly increased in the patients with ESLD. Because the frequency of the risk genotype was approximately the same between SSc with and without ILD and the disease duration was also not different, this SNP was interpreted to be associated with the progression rather than the development of ILD. This association was replicated in an independent set. Furthermore, the authors demonstrated that the risk allele was associated with low expression of HGF [45]. These results will be relevant in terms of the molecular target of drug development.

Terao et al. considered nine novel susceptibility genes to RA identified by GWAS in the Japanese population as candidates for SSc susceptibility genes and conducted an association analysis in 730 patients and some 37,000 controls in Japan. They detected *PLD4* as a novel susceptibility gene to SSc and also confirmed association of *IRF8* and *TNFAIP3*, previously reported in GWAS in the European populations [35]. *PLD4* encodes phospholipase D4, and little is known about its function.

In collaboration with Takehara (Kanazawa University), Sato (The University of Tokyo), and their colleagues, our group has been pursuing the role of candidate

genes, especially those playing a role in B cell function. *CD19* rs111574654T (−499 T) allele was found to be overrepresented in SSc, especially lcSSc and ACA-positive SSc. The risk allele was associated with higher expression of *CD19* [46]. Furthermore, we detected association of a synonymous substitution of *CD22*, rs34826052 (c.2304C>A, P768P), with SSc, especially lcSSc [47]. These observations support the role of CD19/CD22 loop in SSc [48] and also suggest that the imbalance of CD19/CD22 might be in part genetically determined. Furthermore, in *IL10RB*, a nonsynonymous SNP rs2834167 (E47K) was associated with SSc, especially dcSSc and ATA-positive SSc [49]. These studies were done in a relatively small sample size and need confirmation in the future.

Furthermore, we examined the association of genes reported by GWAS of SLE in the European populations. We detected association of rs2280714, located downstream to *IRF5*, with SSc. The risk allele was associated with higher mRNA of *IRF5* [34]. We also demonstrated that the same alleles of *STAT4* [36], *BLK* [43], and *UBE2L3* [50] that are associated with SLE are also associated with SSc in the Japanese population.

5.5 Conclusion

Human genome analysis certainly opened a new field of SSc research. However, almost all non-*HLA* genes reported to be associated with SSc so far are also associated with other diseases and do not seem to explain the characteristic clinical and pathogenetic features of SSc. Something must be missing. Epigenetics may certainly play a role [51, 52], but the cause of such epigenetic changes needs to be investigated. Furthermore, the role of *HLA* and to what extent does *HLA* can explain the unique features of SSc should be evaluated in the future.

References

1. Arnett FC, Cho M, Chatterjee S, Aguilar MB, Reveille JD, Mayes MD. Familial occurrence frequencies and relative risks for systemic sclerosis (scleroderma) in three United States cohorts. Arthritis Rheum. 2001;44(6):1359–62.
2. Feghali-Bostwick C, Medsger Jr TA, Wright TM. Analysis of systemic sclerosis in twins reveals low concordance for disease and high concordance for the presence of antinuclear antibodies. Arthritis Rheum. 2003;48(7):1956–63. doi:10.1002/art.11173.
3. Zhou X, Tan FK, Xiong M, Arnett FC, Feghali-Bostwick CA. Monozygotic twins clinically discordant for scleroderma show concordance for fibroblast gene expression profiles. Arthritis Rheum. 2005;52(10):3305–14. doi:10.1002/art.21355.
4. Tager RE, Tikly M. Clinical and laboratory manifestations of systemic sclerosis (scleroderma) in Black South Africans. Rheumatology. 1999;38(5):397–400.
5. Mayes MD, Lacey Jr JV, Beebe-Dimmer J, Gillespie BW, Cooper B, Laing TJ, et al. Prevalence, incidence, survival, and disease characteristics of systemic sclerosis in a large US population. Arthritis Rheum. 2003;48(8):2246–55. doi:10.1002/art.11073.
6. McNeilage LJ, Youngchaiyud U, Whittingham S. Racial differences in antinuclear antibody patterns and clinical manifestations of scleroderma. Arthritis Rheum. 1989;32(1):54–60.

7. Kuwana M, Inoko H, Kameda H, Nojima T, Sato S, Nakamura K, et al. Association of human leukocyte antigen class II genes with autoantibody profiles, but not with disease susceptibility in Japanese patients with systemic sclerosis. Intern Med. 1999;38(4):336–44.
8. Kuwana M, Kaburaki J, Arnett FC, Howard RF, Medsger Jr TA, Wright TM. Influence of ethnic background on clinical and serologic features in patients with systemic sclerosis and anti-DNA topoisomerase I antibody. Arthritis Rheum. 1999;42(3):465–74.
9. Kuwana M, Kaburaki J, Okano Y, Inoko H, Tsuji K. The HLA-DR and DQ genes control the autoimmune response to DNA topoisomerase I in systemic sclerosis (scleroderma). J Clin Invest. 1993;92(3):1296–301. doi:10.1172/JCI116703.
10. Odani T, Yasuda S, Ota Y, Fujieda Y, Kon Y, Horita T, et al. Up-regulated expression of HLA-DRB5 transcripts and high frequency of the HLA-DRB5*01:05 allele in scleroderma patients with interstitial lung disease. Rheumatology. 2012;51(10):1765–74. doi:10.1093/rheumatology/kes149.
11. Zhou X, Lee JE, Arnett FC, Xiong M, Park MY, Yoo YK, et al. HLA-DPB1 and DPB2 are genetic loci for systemic sclerosis: a genome-wide association study in Koreans with replication in North Americans. Arthritis Rheum. 2009;60(12):3807–14. doi:10.1002/art.24982.
12. Kang SH, Park MH, Song EY, Kang SJ, Lee EB, Song YW, et al. Association of HLA class II genes with systemic sclerosis in Koreans. J Rheumatol. 2001;28(7):1577–83.
13. Wang J, Guo X, Yi L, Guo G, Tu W, Wu W, et al. Association of HLA-DPB1 with scleroderma and its clinical features in Chinese population. PLoS One. 2014;9(1):e87363. doi:10.1371/journal.pone.0087363.
14. He D, Wang J, Yi L, Guo X, Guo S, Guo G, et al. Association of the HLA-DRB1 with scleroderma in Chinese population. PLoS One. 2014;9(9):e106939. doi:10.1371/journal.pone.0106939.
15. Arnett FC, Gourh P, Shete S, Ahn CW, Honey RE, Agarwal SK, et al. Major histocompatibility complex (MHC) class II alleles, haplotypes and epitopes which confer susceptibility or protection in systemic sclerosis: analyses in 1300 Caucasian, African-American and Hispanic cases and 1000 controls. Ann Rheum Dis. 2010;69(5):822–7. doi:10.1136/ard.2009.111906.
16. Loubiere LS, Lambert NC, Madeleine MM, Porter AJ, Mullarkey ME, Pang JM, et al. HLA allelic variants encoding DR11 in diffuse and limited systemic sclerosis in Caucasian women. Rheumatology. 2005;44(3):318–22. doi:10.1093/rheumatology/keh489.
17. Mayes MD, Bossini-Castillo L, Gorlova O, Martin JE, Zhou X, Chen WV, et al. Immunochip analysis identifies multiple susceptibility loci for systemic sclerosis. Am J Hum Genet. 2014;94(1):47–61. doi:10.1016/j.ajhg.2013.12.002.
18. Azzouz DF, Rak JM, Fajardy I, Allanore Y, Tiev KP, Farge-Bancel D, et al. Comparing HLA shared epitopes in French Caucasian patients with scleroderma. PLoS One. 2012;7(5):e36870. doi:10.1371/journal.pone.0036870.
19. Grigolo B, Mazzetti I, Meliconi R, Bazzi S, Scorza R, Candela M, et al. Anti-topoisomerase II alpha autoantibodies in systemic sclerosis-association with pulmonary hypertension and HLA-B35. Clin Exp Immunol. 2000;121(3):539–43.
20. Beretta L, Rueda B, Marchini M, Santaniello A, Simeon CP, Fonollosa V, et al. Analysis of Class II human leucocyte antigens in Italian and Spanish systemic sclerosis. Rheumatology. 2012;51(1):52–9. doi:10.1093/rheumatology/ker335.
21. Tikly M, Rands A, McHugh N, Wordsworth P, Welsh K. Human leukocyte antigen class II associations with systemic sclerosis in South Africans. Tissue Antigens. 2004;63(5):487–90. doi:10.1111/j.0001-2815.2004.00199.x.
22. Nguyen B, Mayes MD, Arnett FC, del Junco D, Reveille JD, Gonzalez EB, et al. HLA-DRB1*0407 and *1304 are risk factors for scleroderma renal crisis. Arthritis Rheum. 2011;63(2):530–4. doi:10.1002/art.30111.
23. Kuwana M, Okano Y, Kaburaki J, Inoko H. HLA class II genes associated with anticentromere antibody in Japanese patients with systemic sclerosis (scleroderma). Ann Rheum Dis. 1995;54(12):983–7.

24. Kuwana M, Okano Y, Kaburaki J, Tojo T, Medsger Jr TA. Racial differences in the distribution of systemic sclerosis-related serum antinuclear antibodies. Arthritis Rheum. 1994;37(6):902–6.
25. Kuwana M, Kaburaki J, Mimori T, Tojo T, Homma M. Autoantigenic epitopes on DNA topoisomerase I. Clinical and immunogenetic associations in systemic sclerosis. Arthritis Rheum. 1993;36(10):1406–13.
26. Welter D, MacArthur J, Morales J, Burdett T, Hall P, Junkins H, et al. The NHGRI GWAS Catalog, a curated resource of SNP-trait associations. Nucleic Acids Res. 2014;42(Database issue):D1001–6. doi:10.1093/nar/gkt1229.
27. Radstake TR, Gorlova O, Rueda B, Martin JE, Alizadeh BZ, Palomino-Morales R, et al. Genome-wide association study of systemic sclerosis identifies CD247 as a new susceptibility locus. Nat Genet. 2010;42(5):426–9. doi:10.1038/ng.565.
28. Allanore Y, Saad M, Dieude P, Avouac J, Distler JH, Amouyel P, et al. Genome-wide scan identifies TNIP1, PSORS1C1, and RHOB as novel risk loci for systemic sclerosis. PLoS Genet. 2011;7(7):e1002091. doi:10.1371/journal.pgen.1002091.
29. Gorlova O, Martin JE, Rueda B, Koeleman BP, Ying J, Teruel M, et al. Identification of novel genetic markers associated with clinical phenotypes of systemic sclerosis through a genome-wide association strategy. PLoS Genet. 2011;7(7):e1002178. doi:10.1371/journal.pgen.1002178.
30. Martin JE, Assassi S, Diaz-Gallo LM, Broen JC, Simeon CP, Castellvi I, et al. A systemic sclerosis and systemic lupus erythematosus pan-meta-GWAS reveals new shared susceptibility loci. Hum Mol Genet. 2013;22(19):4021–9. doi:10.1093/hmg/ddt248.
31. Martin JE, Broen JC, Carmona FD, Teruel M, Simeon CP, Vonk MC, et al. Identification of CSK as a systemic sclerosis genetic risk factor through Genome Wide Association Study follow-up. Hum Mol Genet. 2012;21(12):2825–35. doi:10.1093/hmg/dds099.
32. Lopez-Isac E, Bossini-Castillo L, Guerra SG, Denton C, Fonseca C, Assassi S, et al. Identification of IL12RB1 as a novel systemic sclerosis susceptibility locus. Arthritis Rheumatol. 2014;66(12):3521–3. doi:10.1002/art.38870.
33. Bossini-Castillo L, Martin JE, Broen J, Gorlova O, Simeon CP, Beretta L, et al. A GWAS follow-up study reveals the association of the IL12RB2 gene with systemic sclerosis in Caucasian populations. Hum Mol Genet. 2012;21(4):926–33. doi:10.1093/hmg/ddr522.
34. Ito I, Kawaguchi Y, Kawasaki A, Hasegawa M, Ohashi J, Hikami K, et al. Association of a functional polymorphism in the IRF5 region with systemic sclerosis in a Japanese population. Arthritis Rheum. 2009;60(6):1845–50. doi:10.1002/art.24600.
35. Terao C, Ohmura K, Kawaguchi Y, Nishimoto T, Kawasaki A, Takehara K, et al. PLD4 as a novel susceptibility gene for systemic sclerosis in a Japanese population. Arthritis Rheum. 2013;65(2):472–80. doi:10.1002/art.37777.
36. Tsuchiya N, Kawasaki A, Hasegawa M, Fujimoto M, Takehara K, Kawaguchi Y, et al. Association of STAT4 polymorphism with systemic sclerosis in a Japanese population. Ann Rheum Dis. 2009;68(8):1375–6. doi:10.1136/ard.2009.111310.
37. Koumakis E, Giraud M, Dieude P, Cohignac V, Cuomo G, Airo P, et al. Brief report: candidate gene study in systemic sclerosis identifies a rare and functional variant of the TNFAIP3 locus as a risk factor for polyautoimmunity. Arthritis Rheum. 2012;64(8):2746–52. doi:10.1002/art.34490.
38. Dieude P, Guedj M, Wipff J, Ruiz B, Riemekasten G, Matucci-Cerinic M, et al. Association of the TNFAIP3 rs5029939 variant with systemic sclerosis in the European Caucasian population. Ann Rheum Dis. 2010;69(11):1958–64. doi:10.1136/ard.2009.127928.
39. Coustet B, Bouaziz M, Dieude P, Guedj M, Bossini-Castillo L, Agarwal S, et al. Independent replication and meta analysis of association studies establish TNFSF4 as a susceptibility gene preferentially associated with the subset of anticentromere-positive patients with systemic sclerosis. J Rheumatol. 2012;39(5):997–1003. doi:10.3899/jrheum.111270.
40. Bossini-Castillo L, Broen JC, Simeon CP, Beretta L, Vonk MC, Ortego-Centeno N, et al. A replication study confirms the association of TNFSF4 (OX40L) polymorphisms with systemic

sclerosis in a large European cohort. Ann Rheum Dis. 2011;70(4):638–41. doi:10.1136/ard.2010.141838.
41. Rueda B, Gourh P, Broen J, Agarwal SK, Simeon C, Ortego-Centeno N, et al. BANK1 functional variants are associated with susceptibility to diffuse systemic sclerosis in Caucasians. Ann Rheum Dis. 2010;69(4):700–5. doi:10.1136/ard.2009.118174.
42. Dieude P, Wipff J, Guedj M, Ruiz B, Melchers I, Hachulla E, et al. BANK1 is a genetic risk factor for diffuse cutaneous systemic sclerosis and has additive effects with IRF5 and STAT4. Arthritis Rheum. 2009;60(11):3447–54. doi:10.1002/art.24885.
43. Ito I, Kawaguchi Y, Kawasaki A, Hasegawa M, Ohashi J, Kawamoto M, et al. Association of the FAM167A-BLK region with systemic sclerosis. Arthritis Rheum. 2010;62(3):890–5. doi:10.1002/art.27303.
44. Gourh P, Agarwal SK, Martin E, Divecha D, Rueda B, Bunting H, et al. Association of the C8orf13-BLK region with systemic sclerosis in North-American and European populations. J Autoimmun. 2010;34(2):155–62. doi:10.1016/j.jaut.2009.08.014.
45. Hoshino K, Satoh T, Kawaguchi Y, Kuwana M. Association of hepatocyte growth factor promoter polymorphism with severity of interstitial lung disease in Japanese patients with systemic sclerosis. Arthritis Rheum. 2011;63(8):2465–72. doi:10.1002/art.30415.
46. Tsuchiya N, Kuroki K, Fujimoto M, Murakami Y, Tedder TF, Tokunaga K, et al. Association of a functional CD19 polymorphism with susceptibility to systemic sclerosis. Arthritis Rheum. 2004;50(12):4002–7. doi:10.1002/art.20674.
47. Hitomi Y, Tsuchiya N, Hasegawa M, Fujimoto M, Takehara K, Tokunaga K, et al. Association of CD22 gene polymorphism with susceptibility to limited cutaneous systemic sclerosis. Tissue Antigens. 2007;69(3):242–9. doi:10.1111/j.1399-0039.2007.00801.x.
48. Fujimoto M, Sato S. B cell signaling and autoimmune diseases: CD19/CD22 loop as a B cell signaling device to regulate the balance of autoimmunity. J Dermatol Sci. 2007;46(1):1–9. doi:10.1016/j.jdermsci.2006.12.004.
49. Hikami K, Ehara Y, Hasegawa M, Fujimoto M, Matsushita M, Oka T, et al. Association of IL-10 receptor 2 (IL10RB) SNP with systemic sclerosis. Biochem Biophys Res Commun. 2008;373(3):403–7. doi:10.1016/j.bbrc.2008.06.054.
50. Hasebe N, Kawasaki A, Ito I, Kawamoto M, Hasegawa M, Fujimoto M, et al. Association of UBE2L3 polymorphisms with diffuse cutaneous systemic sclerosis in a Japanese population. Ann Rheum Dis. 2012;71(7):1259–60. doi:10.1136/annrheumdis-2011-201091.
51. Korman BD, Criswell LA. Recent advances in the genetics of systemic sclerosis: toward biological and clinical significance. Curr Rheumatol Rep. 2015;17(3):21. doi:10.1007/s11926-014-0484-x.
52. Broen JC, Radstake TR, Rossato M. The role of genetics and epigenetics in the pathogenesis of systemic sclerosis. Nat Rev Rheumatol. 2014;10(11):671–81. doi:10.1038/nrrheum.2014.128.

Role of the Interleukin-1 Family in the Fibrogenic Phenotype in Systemic Sclerosis

6

Yasushi Kawaguchi

Abstract

The IL-1 family consists of 11 cytokines. Initially, IL-1α, IL-1β, and IL-1 receptor antagonists were discovered; then, IL-1's specific receptors were identified. Subsequent studies are aimed to find molecules that bind to IL-1 receptor-like proteins (IL-1 receptor-related protein, ST2). As a result, in 2005, IL-33 was identified as a ligand of ST2. IL-33 is very similar to IL-1α in its biological properties. Our group has investigated the roles of IL-1α in the pathogenesis of tissue fibrosis in systemic sclerosis, and we are currently interested in the roles of IL-33 as well as IL-1α. Herein, I review the involvement of both of these molecules in systemic sclerosis.

Keywords

Systemic sclerosis • Interleukin-1 • Fibrosis • Fibroblasts

6.1 Introduction

Systemic sclerosis (SSc) is a connective tissue disease of unknown etiology that is characterized by the fibrosis of systemic organs. Because most patients experience skin thickening, researchers have analyzed the molecular and biological functions of fibroblasts derived from skin lesions on SSc patients [1–5]. Since 1990, many studies have been published showing that several cytokines, as well as transforming growth factor (TGF)-β, seem to be central regulators of both physiological and pathological tissue fibrosis in patients with SSc. Interleukin (IL)-1α is one of the key cytokines involved in tissue fibrosis. In 1994, we investigated the levels of IL-1α in the

Y. Kawaguchi (✉)
Institute of Rheumatology, Tokyo Women's Medical University, 10-22 Kawada-cho, Shinjuku-ku, Tokyo 162-0054, Japan
e-mail: y-kawa@twmu.ac.jp

K. Takehara et al. (eds.), *Systemic Sclerosis*, DOI 10.1007/978-4-431-55708-1_6

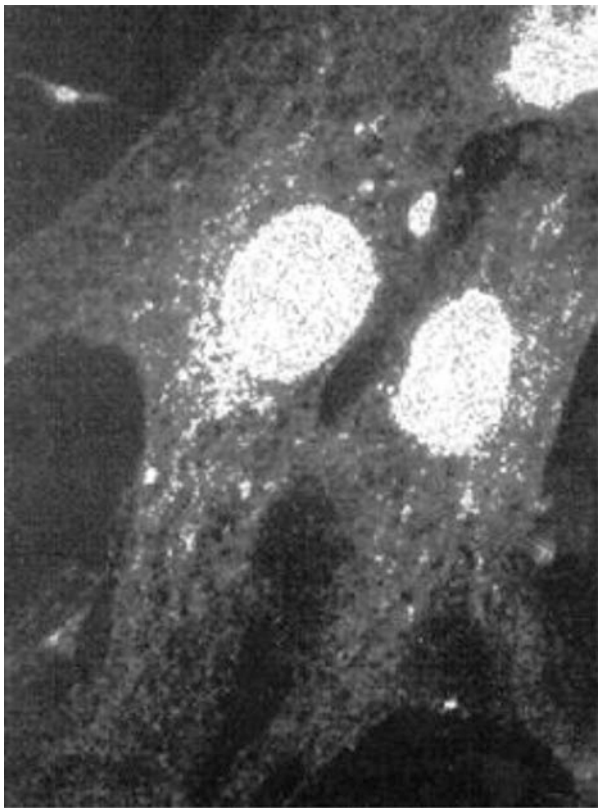

Fig. 6.1 Immunochemical staining of fibroblasts from systemic sclerosis. We used anti-IL-1α antibody and analyzed the signal using confocal microscopy. The strong signal represents IL-1α staining in the nucleus

supernatants of cultured fibroblasts and found aberrant production of pre-IL-1α in the nuclei of cultured skin fibroblasts from patients with SSc (Fig. 6.1) [6, 7]. We hypothesized that pre-IL-1α might be involved in the excessive production of collagen in skin fibroblasts, resulting in tissue fibrosis in patients with SSc. Since that time, I have investigated the precise role of IL-1α in fibrosis in patients with SSc.

6.2 IL-1 Superfamily

The history of IL-1 begins in the 1940s with studies of the pathogenesis of fever [8]. These studies investigated the central molecules of fever-producing features of proteins released from rabbit peritoneal exudate cells. Soluble factors released by these cells were discovered to augment lymphocyte proliferation in response to antigenic or mitogenic stimuli. Other researchers described macrophage products that induced the synthesis of acute phase protein [9]. At this time, the molecule was named IL-1. IL-1 is primarily a proinflammatory cytokine that induces the production of prostaglandin E2, platelet-activating factor, and nitric oxide. In addition,

Table 6.1 The IL-1 superfamily

IL-1 family	Cytokines	Receptor
IL-1F1	IL-1α	IL-1RI, IL-1RAcP
IL-1F2	IL-1β	IL-1RI, IL-1RAcP
IL-1F3	IL-1RA	IL-1RI
IL-1F4	IL-18	IL-18Rα (IL-1Rrp1)
		IL-18Rβ (IL-1RAcPL)
IL-1F5	IL-36RA	IL-36R (IL-1Rrp2)
IL-1F6	IL-36α	IL-36R (IL-1Rrp2), IL-1RAcP
IL-1F7	IL-37	IL-18Rα
IL-1F8	IL-36β	IL-36R (IL-1Rrp2), IL-1RAcP
IL-1F9	IL-36γ	IL-36R (IL-1Rrp2), IL-1RAcP
IL-1F10	IL-38	IL-36R (IL-1Rrp2)
IL-1F11	IL-33	ST2, IL-1RAcP

IL-1RA IL-1 receptor antagonist, *IL-36RA* IL-36 receptor antagonist, *IL-1RI* IL-1 receptor type I, *IL-1RAcP* IL-1 receptor accessory protein, *IL-1Rrp* IL-1 receptor-related protein, *IL-1RAcPL* IL-1 receptor accessory protein-like

IL-1 induces the production of the adhesion molecules, intracellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, on endothelial cells and mesenchymal cells [10].

Later studies reported that IL-1 comprises a superfamily [10, 11]. The four initial members of the IL-1 superfamily were IL-1α, IL-1β, IL-1 receptor antagonist (IL-1RA), and IL-18. The three-dimensional structure of IL-1α is similar to that of IL-1β and IL-18: in each, the cytokine forms an open-ended barrel composed of beta-pleated strands. In contrast, the unique structure of IL-1RA allows it to bind to the IL-1 receptor without triggering signal transduction; this is due to the lack of a second binding site on the back of the molecule. IL-1RA is able to prevent IL-1 activity by blocking the specific binding of IL-1 to IL-1 receptors [12]. Currently, the IL-1 superfamily classification includes 11 molecules, as shown in Table 6.1. Although the family name of IL-1 was established, the conventional cytokine names were used in the journal. IL-1α, IL-1β, IL-18, and IL-33 were well known as important mediators of immune responses. Recently, the new members of the IL-1 superfamily, which had conventionally been reported as IL-1F6, IL-1F7, IL-1F8, IL-1F9, and IL-1F10, were designated IL-36, IL-37, and IL-38.

6.2.1 Biological Function of IL-1α

The signal transduction properties and biological features of IL-1α have been investigated for many years [13]. IL-1α is constitutively expressed in the form of a 31-kD precursor by epithelial cells, endothelial cells, and keratinocytes. Pre-IL-1α possesses an N-terminal nuclear localization sequence that directs this cytokine to the nucleus, where it might regulate the activity of transcription factors in a

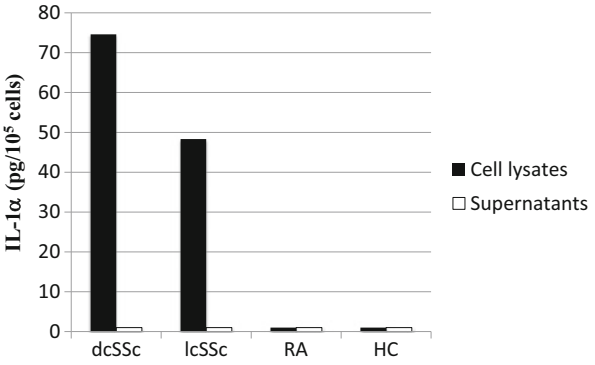

Fig. 6.2 Intracellular concentrations of IL-1α in cultured fibroblasts from systemic sclerosis (SSc). Confluent cultured fibroblasts and cell supernatants were collected. Cell lysates were prepared from cells using RIPA buffer. The abbreviations dcSSc, lcSSc, RA, and HC indicate diffuse cutaneous SSc, limited cutaneous SSc, rheumatoid arthritis, and healthy controls, respectively

receptor-independent manner. It has recently been established that the cleavage of pre-IL-1α by a number of inflammatory proteases, including calpain, elastase, granzyme B, and chymase, results in a dramatic increase in IL-1α bioactivity through the IL-1 receptor.

One of the unique features of IL-1α is that it is an autocrine factor, which accounts for the three following observations. Firstly, pre-IL-1α is translated and remains inside the cell where it localizes to the nucleus because of its nuclear localization sequence. Secondly, intracellular pre-IL-1α complexes to an intracellular pool of IL-1 receptors before exerting an effect as a ligand/receptor complex. Finally, both pre-IL-1α and mature IL-1α bound to surface IL-1 receptors are internalized and subsequently translocated into the nucleus [14]. These biological properties are specific to IL-1α and are inconsistent with the properties of IL-1β. On the other hand, IL-1α and IL-1β bind to the same receptor (IL-1 receptor type I) and exhibit a similar range of biological effects. Various biological features of these two cytokines are shown in Fig. 6.2.

6.2.2 Nuclear Localization of Pre-IL-1α in SSc

In 1994, we showed that the mRNA of the IL1A gene was constitutively expressed and that intracellular pre-IL-1α was constitutively produced by cultured skin fibroblasts from patients with SSc (Fig. 6.3) [6]. Recently, it has been reported that the levels of IL-1α in sera are significantly higher in patients with SSc than in healthy controls [15]. We have also investigated the biological effect of pre-IL-1α on the fibrogenic phenotype of skin fibroblasts [16]. To investigate the effect of constitutive expression of pre-IL-1α, we evaluated various methods for inhibiting intracellular pre-IL-1: an antibody against IL-1α, IL-1 receptor antagonist

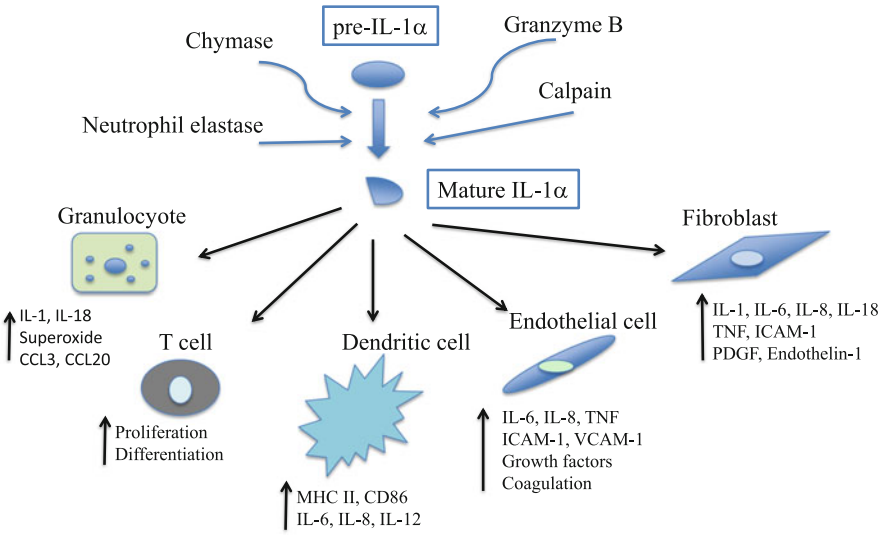

Fig. 6.3 Biological properties of IL-1α

(IL-1RA), antisense oligomers for the IL1A gene, and stable transfection of an antisense IL1A gene. Because pre-IL-1α was mainly located in the nucleus, antibodies and IL-1RA could not influence pre-IL-1α signal transduction. Eventually, we succeeded in achieving stable transfection of cultured skin fibroblasts derived from SSc [7]. In SSc fibroblasts transfected with an antisense IL1A gene, intracellular IL-1α decreased to <10 % of the levels found in SSc fibroblasts without antisense treatment. The transfected fibroblasts produced significantly lower levels of collagen type I than control fibroblasts. The results of these studies provided evidence for a direct link between the aberrant expression of pre-IL-1α and the pathogenesis of SSc. The next question raised concerned signal transduction by pre-IL-1α in the nuclei of SSc fibroblasts. In general, the IL-1 signal is able to transduce into cells by specifically binding to the IL-1 receptor on the cell surface. However, pre-IL-1α is concentrated in the nucleus, and we did not discover how signaling by pre-IL-1α was involved in the fibrogenic phenotype of SSc fibroblasts.

Several studies concerning the biological function of nuclear IL-1α have been published. A previous investigation of the nuclear target of pre-IL-1α used a yeast two-hybrid system to reveal an interaction between pre-IL-1α and necdin [17]. Necdin is a 47-kD protein that functions as a cell-growth suppressor in a manner similar to that of the retinoblastoma tumor suppressor protein, Rb. Other reports showed the biological functions of intracellular IL-1RA in SSc fibroblasts [18, 19]. These studies indicated that intracellular IL-1RA was overexpressed in SSc fibroblasts and that intracellular IL-1RA was involved in the fibrogenic phenotype of SSc fibroblasts. In 2006, we published a new concept regarding the roles of

nuclear pre-IL-1α [20]. Our study reported the formation of the pre-IL-1α complex, which consists of pre-IL-1α, IL-1 receptor type II, and HAX-1, inside fibroblasts from patients with SSc. This complex plays a crucial role in the fibrogenic phenotype of SSc fibroblasts.

6.2.3 Constitutive Expression of IL-1α at the Level of Transcription

In the previous study, aberrant expression of pre-IL-1α was observed in SSc fibroblasts. However, the mechanisms by which the production of nuclear pre-IL-1α was upregulated remained to be clarified. We explored the regulation of the transcription of the IL1A gene in fibroblasts from patients with SSc [21]. We found that the expression of the IL1A gene was upregulated at the transcriptional stage. Next, to identify the location of the cis-elements of the IL1A gene responsible for transcription in SSc fibroblasts, we performed promoter analysis and found a cis-element located around position −96 to −82, which included putative Ets binding sites.

6.2.4 IL1A Gene Polymorphisms

The precursor of IL-1α (pre-IL-1α) is initially synthesized as a 31-kDa molecule without a signal peptide from the *IL1A* gene. After the removal of the N-terminal amino acids by specific proteases (calpain and caspase-1), the resulting peptides are the mature form, IL-1α (17 kDa) [22]. Pre-IL-1α and mature IL-1α are both biologically active, and the precursor molecule is translocated into the nucleus separately from mature IL-1α because the N-terminal amino acids of pre-IL-1α contain a nuclear localization sequence [23, 24]. Single nucleotide polymorphisms (SNPs) in the gene have been reported to be associated with susceptibility to inflammatory diseases. One SNP was located at −889 (rs1800587) in the 5′-flanking region, and the other was found at +4845 (rs17561), which involved a substitution of the 114th amino acid of pre-IL-1α. Pre-IL-1α is processed into its mature form by an enzymatic cut between the 112 and 113 amino acids [25]. Those observations led us to speculate that the SNPs of the *IL1A* gene might affect the transcription and processing of IL-1α. We investigated the single nucleotide polymorphisms (SNPs) of the *IL1A* gene in patients with SSc and healthy controls in a Japanese population [26]. The minor allele frequency at rs17561 was significantly lower in SSc patients than in healthy controls ($P < 0.0001$). This SNP has alanine substituted for serine at amino acid 114, next to the enzyme-cleaving site. Alanine is nonpolar and hydrophobic, whereas serine is polar and hydrophilic. It is possible that these amino acids exhibit different chemical properties and that this amino acid substitution affects the enzymatic efficiency of the protease in cleaving pre-IL-1α. To test this hypothesis, we explored the effects of this SNP on the transcriptional activity and processing of pre-IL-1α in skin fibroblasts. We prepared promoter regions of the *IL1A* gene with either C or T at −889 inserted into a

luciferase reporter vector; these constructs were referred to as C/IL1A and T/IL1A, respectively. Skin fibroblasts were explanted from two SSc patients whose genomic DNA contained GG and TT genotypes at +4845 of the *IL1A* gene. Cell lysates were collected and reacted with various concentrations of calpain, and then the processing of pre-IL-1α was analyzed by Western blotting. A 100-fold higher concentration of calpain was required to process the pre-IL-1α containing Ala at the 114th amino acid than that to process that containing Ser. The frequency of the GG genotype was significantly higher in SSc patients than in healthy donors, whereas the frequency of the TT genotype was significantly higher in RA patients than in healthy donors. Our observation showed that the SNP at +4845 affected the enzymatic efficiency of the protease in cleaving pre-IL-1α. Pre-IL-1α with Ala, which had a high frequency in SSc patients, was more resistant to cleavage by proteases in human sera than pro-IL-1α with Ser [27].

6.3 IL-33

IL-33 is a member of the IL-1 family; it has been called IL-1F11 (Table 6.1) because it was identified as a ligand that binds a protein resembling the extracellular portion of IL-1 receptor type I [28]. This protein was named ST2. At first, ST2 was discovered as a member of the IL-1 receptor superfamily, and as a result of searching for its ligand, IL-33 was found in 2005 and seemed to be involved in type 2 immune responses. Like IL-1α, IL-33 is localized in the cell nucleus because of a nuclear localization sequence but also functions as a cell-free cytokine. Because IL-33 lacks a traditional signal sequence or a noncanonical processing and export pathway, cell death by necrosis might be the dominant mechanism by which the cytokine affects other cells, which is similar to the mechanism of IL-1α. In humans, IL-33 is constitutively expressed in epithelial cells, endothelial cells, and keratinocytes [29–31]. The biological functions of IL-33 were shown in Fig. 6.4.

6.3.1 IL-33 in SSc Patients

Recently, it was reported that serum IL-33 levels were increased in patients with SSc, and these levels were correlated with the extent of skin sclerosis in individual patients [32]. Another study by Manetti et al. found that IL-33 protein was downregulated or absent in endothelial cells and the epidermis, whereas ST2 expression was increased significantly in endothelial cells, macrophages, B cells, T cells, and activated fibroblasts in skin biopsies from patients in the early phases of SSc [33]. They speculated that IL-33 is released by the endothelial nucleus upon endothelial cell activation or damage and functions as an endogenous mediator in the early stages of SSc. They also reported increased circulating levels of IL-33 in SSc, and the levels were well correlated with early disease stage and microvascular

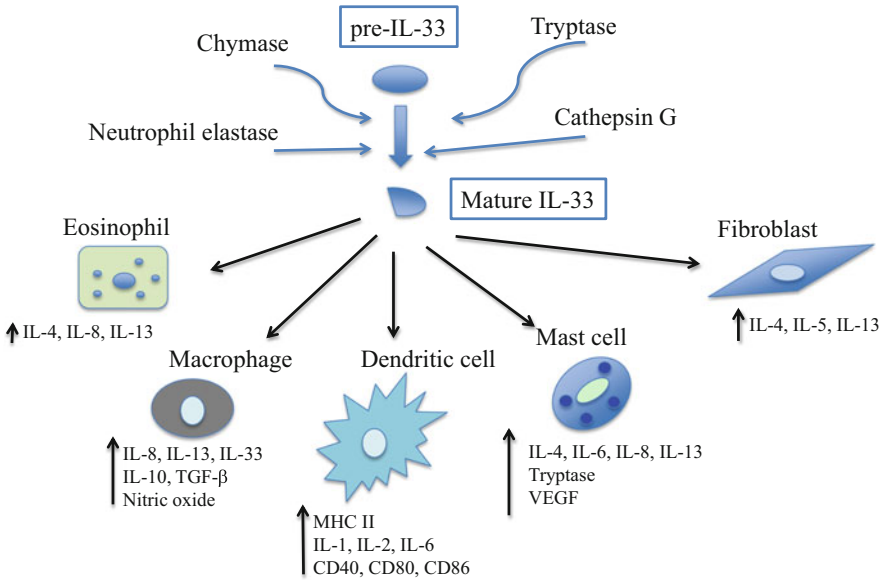

Fig. 6.4 Biological properties of IL-33

involvement [34]. Recently, Rankin et al. published an interesting report showing that IL-33 induces tissue fibrosis through the production of IL-13 [35].

Taken together, the literature suggests that IL-33 may play a crucial role in the pathogenesis or development of tissue fibrosis in SSc patients. However, IL-33 and IL-1α are potential key mediators of tissue fibrosis in SSc.

6.4 Conclusion

IL-1 family consists of IL-1, IL-18, IL-33, IL-36, IL-37, and IL-38. In these cytokines IL-1 and IL33 may be involved in the pathogenesis of SSc. In particular, IL-1α and IL-33 have a nuclear localization sequence and can be translocated into the nucleus. Although the biological functions of nuclear cytokines remain to be unclear, they may play a crucial role in excessive collagen production by fibroblasts. A novel strategy for inhibiting nuclear cytokines would provide a new therapy for tissue fibrosis in SSc.

References

1. Bhattacharyya S, Wei J, Varga J. Understanding fibrosis in systemic sclerosis: shifting paradigms, emerging opportunities. Nat Rev Rheumatol. 2012;8:42–54.
2. LeRoy EC. Increased collagen synthesis by scleroderma skin fibroblasts in vitro: a possible defect in the regulation or activation of the scleroderma fibroblast. J Clin Invest. 1974;54:880.

3. Jimenez SA, Feldman G, Bashey RI, et al. Co-ordinate increase in the expression of type I and type III collagen genes in progressive systemic sclerosis fibroblasts. Biochem J. 1986;237:837–43.
4. Ihn H. Pathogenesis of fibrosis: role of TGF-beta and CTGF. Curr Opin Rheumatol. 2002;14:681–5.
5. Kulozik M, Hogg A, Lankat-Buttgereit B, Krieg T. Co-localization of transforming growth factor β2 with α1(I) procollagen mRNA in tissue sections of patients with systemic sclerosis. J Clin Invest. 1990;86:917.
6. Kawaguchi Y. IL-1α gene expression and protein production by fibroblasts from patients with systemic sclerosis. Clin Exp Immunol. 1994;97:445.
7. Kawaguchi Y, McCarthy SA, Watkins SC, Wright TM. Autocrine activation by interleukin 1α induces the fibrogenic phenotype of systemic sclerosis fibroblasts. J Rheumatol. 2004;31:1946.
8. Dinarello CA, Goldin NP, Wolff SM. Demonstration and characterization of two distinct human leukocytic pyrogens. J Exp Med. 1974;139:1368.
9. Auron PE, Webb AC, Rosenwasser LJ, et al. Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. Proc Natl Acad Sci U S A. 1984;81:7907.
10. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. Annu Rev Immunol. 2009;27:519–50.
11. Busfield SJ, Comrack CA, Yu G, et al. Identification and gene organization of three novel members of the IL-1 family on human chromosome 2. Genomics. 2000;66:213–6.
12. Arend WP. Interleukin-1 receptor antagonist. Adv Immunol. 1993;54:167–227.
13. Dinarrello CA. Interleukin-1 and interleukin-1 antagonism. Blood. 1991;77:1627–52.
14. Mosley B, Urdal DL, Prickett KS, Larsen A, Cosman D, Conlon PJ, et al. The interleukin-1 receptor binds the human interleukin-1 alpha precursor but not the interleukin-1 beta precursor. J Biol Chem. 1987;262:2941–4.
15. Maekawa T, Jinnin M, Ohtsuki M, Ihn H. Serum interleukin-1α in patients with systemic sclerosis. J Dermatol. 2013;40:98–101.
16. Kawaguchi Y, Hara M, Wright TM. Endogenous IL-1α from systemic sclerosis fibroblasts induces IL-6 and PDGF-A. J Clin Invest. 1999;103:1253.
17. Hu B, Wang S, Zhang Y, Fegahli CA, Dingman JR, Wright TM. A nuclear target for interleukin-1 alpha: interaction with the growth suppressor necdin modulates proliferation and collagen expression. Proc Natl Acad Sci U S A. 2003;100:10008–13.
18. Higgins GC, Wu Y, Postlethwaite AE. Intracellular IL-1 receptor antagonist is elevated in human dermal fibroblasts that overexpress intracellular precursor IL-1α. J Immunol. 1999;163:3969.
19. Kanangat S, Postlethwaite AE, Higgins GC, Hasty KA. Novel functions of intracellular IL-1ra in human dermal fibroblasts: implications in the pathogenesis of fibrosis. J Invest Dermatol. 2006;126:756.
20. Kawaguchi Y, Nishimagi E, Tochimoto A, Kawamoto M, Katsumata Y, Soejima M, et al. Intracellular IL-1α-binding proteins contribute to biological functions of endogenous IL-1α in systemic sclerosis fibroblasts. Proc Natl Acad Sci U S A. 2006;103:14501.
21. Kawaguchi Y, Hara M, Kamatani N, Wright TM. Identification of IL1A gene segment that determines aberrant constitutive IL-1α expression in systemic sclerosis. Arthritis Rheum. 2003;48:193–202.
22. Kobayashi Y, Yamamoto K, Saido T, Kawasaki H, Oppenheim JJ, Matsushima K. Identification of calcium-activated neutral protease as a processing enzyme of human interleukin 1 alpha. Proc Natl Acad Sci U S A. 1990;87:5548–52.
23. Maier JA, Statuto M, Ragnotti G. Endogenous interleukin 1 alpha must be transported to the nucleus to exert its activity in human endothelial cells. Mol Cell Biol. 1994;14:1845–51.
24. Wessendorf JH, Garfinkel S, Zhan X, Brown S, Maciag T. Identification of a nuclear localization sequence within the structure of the human interleukin-1 alpha precursor. J Biol Chem. 1993;268:22100–4.
25. Dinarello CA. Biologic basis for interleukin-1 in disease. Blood. 1996;87:2095–147.

26. Kawaguchi Y, Tochimoto A, Ichikawa N, Harigai M, Hara M, Kotake S, et al. Association of IL1A gene polymorphisms with susceptibility to and severity of systemic sclerosis in Japanese population. Arthritis Rheum. 2003;48:186.
27. Kawaguchi Y, Tochimoto A, Hara M, Kawamoto M, Sugiura T, Saito S, et al. Contribution of single nucleotide polymorphisms of the IL1A gene to the cleavage of precursor IL-1α and its transcription activity. Immunogenetics. 2007;59:441.
28. Schmitz JA, Owyang E, Odkham YL, Song E, Murphy TK, McClanahan G, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity. 2005;23:479–90.
29. Cayrol C, Girard JP. The IL-1-like cytokine, IL-33 is inactivated after maturation by caspase-1. Proc Natl Acad Sci U S A. 2009;106:9021–6.
30. Garlanda C, Dinarello CA, Mantovani A. The interleukin-1 family: back to the future. Immunity. 2013;39:1003–8.
31. Luthi AU, Cullen SP, McNeela EA, Duriez PJ, Afonina IS, Sheridan C, et al. Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. Immunity. 2009;31:84–98.
32. Yanaba K, Yoshizaki A, Asano Y, Kadono T, Sato S. Serum IL-33 levels are raised in patients with systemic sclerosis: association with extent of skin sclerosis and severity of pulmonary fibrosis. Clin Rheumatol. 2011;30:825–30.
33. Manetti M, Ibba-Manneschi L, Liakouli V, Guiducci S, Milia AF, Benelli G, et al. The IL1-like cytokine IL33 and its receptor ST2 are abnormally expressed in the affected skin and visceral organs of patients with systemic sclerosis. Ann Rheum Dis. 2010;69:598–605.
34. Manetti M, Guiducci S, Ceccarelli C, Romano E, Bellando-Randome S, Conforti MJ, et al. Increased circulating levels of interleukin-33 in systemic sclerosis correlate with early disease stage and microvascular involvement. Ann Rheum Dis. 2011;70:1876–8.
35. Rnakin AL, Mumm JB, Murphy E, Turner S, Yu N, McClanahan TK, et al. IL-33 induces IL-13-dependent cutaneous fibrosis. J Immunol. 2010;184:1526–35.

Interleukin-6 in the Pathogenesis of Systemic Sclerosis

7

Yoshihito Shima

Abstract

Interleukin-6 (IL-6) is a pleiotropic cytokine that acts in the development of B-lymphocytes; the proliferation of megakaryocytes, mesangial cells, and keratinocytes; and the production of acute-phase proteins such as C-reactive protein (CRP). Many reports suggest that IL-6 plays a partial role in the pathogenesis of systemic sclerosis (SSc). Here, I describe its functions, signal transduction pathways, and involvement in SSc and then introduce an anti-IL-6 therapy for SSc using an anti-IL-6 receptor antibody, tocilizumab. To evaluate the effect of such an anti-cytokine therapy, a large-scale study is needed. And a worldwide-scale study may require a new evaluation method to suppress inter-institute variability.

Keywords

Interleukin-6 • Anti-IL-6R antibody • Tocilizumab

7.1 Biological Effects of Interleukin-6

Interleukin (IL)-6 was originally identified as a B-lymphocyte-stimulating factor by Kishimoto et al. in 1983, and its cDNA was cloned by Hirano et al. in 1986 [1, 2]. In the 1980s, several newly identified molecules were found to be involved in fever, inflammation, and plasmacytoma growth, which were referred to as interferon-β2, hepatocyte stimulatory factor, and hybridoma-plasmacytoma growth factor, respectively. Subsequently, these molecules were revealed to be the same molecule and renamed IL-6. As the variety of names indicate, IL-6 has various biological

Y. Shima (✉)
Department of Respiratory Medicine, Allergy and Rheumatic Diseases, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita 565-0871, Japan
e-mail: ryanjin@imed3.med.osaka-u.ac.jp

K. Takehara et al. (eds.), *Systemic Sclerosis*, DOI 10.1007/978-4-431-55708-1_7

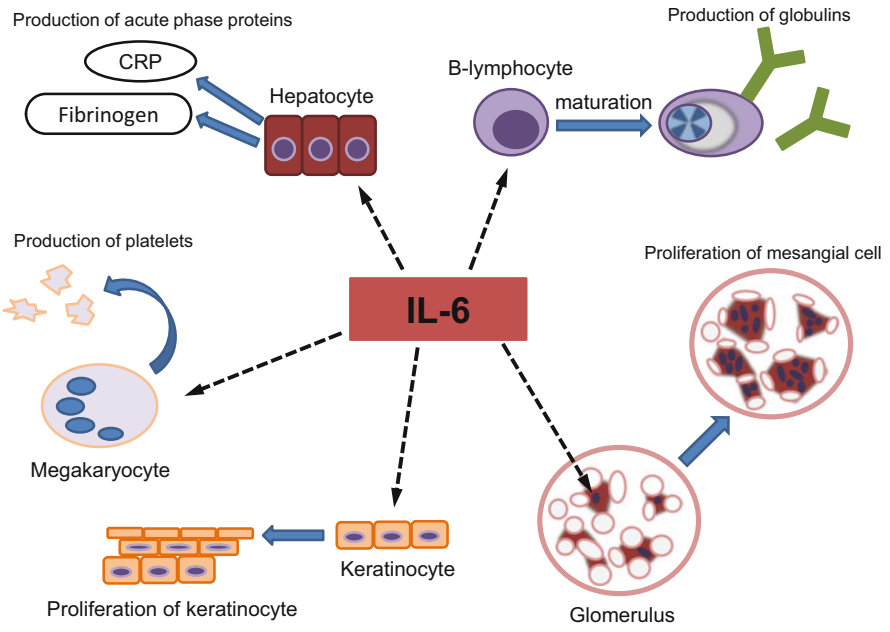

Fig. 7.1 Effects of IL-6. IL-6 affects a variety of cell types

functions (Fig. 7.1). Not only immunocompetent cells but also neural cells, keratinocytes, and mesangial cells are affected by IL-6. Simulation of hepatocytes with IL-6 results in elevation of C-reactive protein (CRP), fibrinogen, and serum amyloid A (SAA) protein levels. This finding explains why patients in an inflammatory state present with augmentation of CRP and the blood sedimentation rate. It has been reported that serum levels of IL-6 correlate with CRP and fever in patients with burn injuries [3], and administration of IL-6 to rats leads to acute-phase reactions [4]. Studies using recombinant IL-6 and an anti-IL-6 antibody show that IL-6 stimulates production of acute-phase proteins in liver cells [5]. It has been shown that IL-6 produced in an inflammatory locus is transported to the liver and binds to the IL-6 receptor (IL-6R) on hepatocytes, which stimulates production of CRP and SAA. IL-6 is well known as a stimulator of B-lymphocytes to develop into immunoglobulin-producing cells. Muraguchi et al. demonstrated that IL-6 augments immunoglobulins using pokeweed mitogen-stimulated peripheral mononuclear cells [6]. This phenomenon has been confirmed by administration of murine recombinant IL-6 to mice [7]. IL-6 is also known as one of the stimulators of megakaryocytes to regulate platelet counts. Establishment of IL-6 transgenic mice has revealed dramatic increases in the number of multinucleated megakaryocytes in their bone marrow [8]. In vitro experiments using murine megakaryocytes showed that IL-6 promotes the maturation of megakaryocytes [9], and in vivo experiments by administration of recombinant IL-6 to primates showed elevation of platelet

counts [10]. Castleman disease is a benign tumor with symptoms including chronic fever and increases in platelet counts as well as CRP, SAA, and gamma globulin levels. These symptoms are explainable in terms of IL-6 functions. Indeed, high levels of IL-6 have been reported in patients with Castleman disease, which decrease after resection of the lymph node focus. Immunohistochemical analysis has revealed positivity for IL-6 staining in the germinal center of resected lymph nodes [11]. Castleman disease can be treated with an anti-IL-6R antibody that normalizes not only the fever but also CRP, SAA, and immunoglobulins levels, and platelet counts.

Another action of IL-6 revealed from analyses of IL-6 transgenic mice includes a proliferative effect on kidney mesangial cells. Suematsu et al. established IL-6 transgenic mice and found that they had splenomegaly, peritoneal lymph node enlargement, megakaryocyte proliferation in the bone marrow, and proliferation of kidney mesangial cells [12]. It has also been reported that mesangial proliferative glomerulonephritis in IL-6 transgenic mice can be prevented by administration of an anti-IL-6R antibody [13]. In humans, a correlation has been reported between the urinary IL-6 concentration and histological severity of mesangial proliferation in IgA nephritis [14].

Kupper et al. reported that neonatal keratinocytes produce high amounts of IL-6 after stimulation with phorbol esters, IL-1, tumor necrosis factor (TNF)- α, and IL-4 [15]. Grossman et al. collected skin biopsies from patients with psoriasis, and immunohistochemistry and in situ hybridization analyses revealed production of IL-6 in their psoriatic plaques [16]. These findings suggest that IL-6 may be involved in epidermal keratinocyte proliferation.

7.2 Elevation of IL-6 in Patients with Systemic Sclerosis

Systemic sclerosis (SSc) is a connective tissue disease characterized by vascular damage and fibrosis of the skin and internal organs. Although many factors may be involved in the pathogenesis of SSc, its etiology has been unclear. IL-6 is one of the factors that might play an important role in the pathogenesis of SSc. Needleman et al. compared IL-6 levels in the sera of SSc patients with those in normal individuals and found measurable levels of IL-6 in many sera of the SSc patients [17]. Feghali et al. conducted primary culture of skin tissue isolated from SSc patients and reported high concentrations of IL-6 in the culture supernatants [18]. They analyzed the culture supernatants of skin fibroblasts isolated from an area of affected skin in a patient with SSc, which contained from 6- to 30-fold higher concentrations of IL-6 compared with unaffected skin or control skin fibroblasts. Gurram et al. reported higher production of IL-6 in peripheral mononuclear cells isolated from SSc patients than in those from normal individuals [19]. They cultured peripheral blood mononuclear cells obtained from seven SSc patients and healthy controls with heparin, concanavalin A, or type I collagen and found that the supernatants of peripheral blood mononuclear cells cultured with type I collagen contained a higher amount of IL-6 compared with the controls. Sato

et al. analyzed 32 cytokines and chemokines in sera from SSc patients and found a correlation between the skin score and IL-6 levels [20]. Hasegawa et al. analyzed serum IL-6, soluble IL-6 receptor (sIL-6R), and the soluble form of its signal transduction molecule gp130 by enzyme-linked immunosorbent assays [21]. The samples were categorized into either the early stage of SSc (within 3 years from disease onset) or the late stage (more than 3 years from onset). They found that the serum samples from the diffuse cutaneous type of SSc at the early stage contained higher levels of IL-6 than the other group. Khan et al. conducted reverse transcriptional-polymerase chain reaction analysis and found higher mRNA expression of IL-6 in cultured fibroblasts isolated from SSc patients compared with normal fibroblasts [22]. These reports suggest that IL-6 might play a role in the pathogenesis of SSc. Kawaguchi et al. reported data of in vitro experiments using an anti-IL-6 antibody. They cultured skin fibroblasts isolated from SSc patients with or without the anti-IL-6 antibody. The concentrations of type I procollagen in culture supernatants with the anti-IL-6 antibody were lower than in those without the antibody [23]. Their report supported the notion that production of sclerotic skin components can be influenced by IL-6 inhibition.

Furthermore, many reports indicate a relationship between IL-6 and the involvement of internal organs in SSc, especially lung fibrosis. Crestani et al. cultured alveolar macrophages in bronchoalveolar lavage collected from 11 lung fibrosis patients with SSc and eight normal subjects [24]. IL-6 levels in the culture supernatants were higher than normal, although there was little difference in the response to lipopolysaccharide stimulation between samples from patients with lung fibrosis and normal subjects. Hasegawa et al. also showed an inverse correlation between serum IL-6 levels and the percentage of vital capacity (%VC) in patients with early-stage diffuse cutaneous SSc [25]. Similar results were reported by Scala et al. They measured IL-6 concentrations in sera from 20 SSc patients and found that the samples from diffuse cutaneous-type SSc patients with lung involvement contained high concentrations of IL-6 [26]. These reports suggest the involvement of IL-6 in lung fibrosis, a major symptom that is frequently observed in SSc patients.

7.3 IL-6, IL-6R, and Its Signal Transducer Gp130

IL-6 might play an important role in the pathogenesis of SSc, which supports the application of therapies that inhibit IL-6. The biological functions of IL-6 are mediated through IL-6R on the cell surface. IL-6R consists of two molecules, the IL-6R α-chain, narrowly defined as IL-6R, and the signal transduction molecule gp130. These structures were determined in 1988 [27]. The extracellular region of IL-6R includes an immunoglobulin-like domain and cytokine receptor family domain, while the intracytoplasmic region of IL-6R consists of only 82 amino acids and does not have a signal transduction motif such as a tyrosine kinase domain. The signal transduction of IL-6 through IL-6R, which lacks a kinase domain, was elucidated by the discovery of a second molecule named gp130

[28]. The signal transduction molecule gp130 is 130 kD in size, and molecular cloning of its cDNA revealed a 918-amino acid sequence. Gp130 has non-receptor tyrosine kinases, Janus kinases (JAK) 1 and 2. Binding of IL-6 and IL-6R induces dimerization of gp130 and phosphorylation of JAK. After dimerization of gp130, there are two pathways for signal transduction. The main pathway operates via signal transducers and activators of transcription 3 (STAT3), and the other is a cascade through mitogen-activated protein (MAP) kinase. Interestingly, gp130 has been found to be expressed ubiquitously on all examined viable cells [29] and acts as a signal transducer not only for IL-6 but also for a variety of other cytokines such as oncostatin M, leukemia inhibitory factor, ciliary neurotrophic factor, and IL-11. Accordingly, the overlapping biological functions of these molecules are explained by their common signal transducer, gp130 [30].

IL-6R is expressed in cell types of only certain organs, including hepatocytes, skin keratinocytes, and kidney mesangial cells. However, soluble form of IL-6R (sIL-6R) exists in serum, and coupling of IL-6 and sIL-6R can induce activation of gp130 on the cell surface (Fig. 7.2). Therefore, many organs can be stimulated by IL-6. Consequently, IL-6R inhibition is a novel therapeutic strategy to inhibit IL-6 functions. Anti-gp130 therapy is the other candidate approach, but gp130 is expressed on many kinds of cells and acts as a signal transducer for other cytokines.

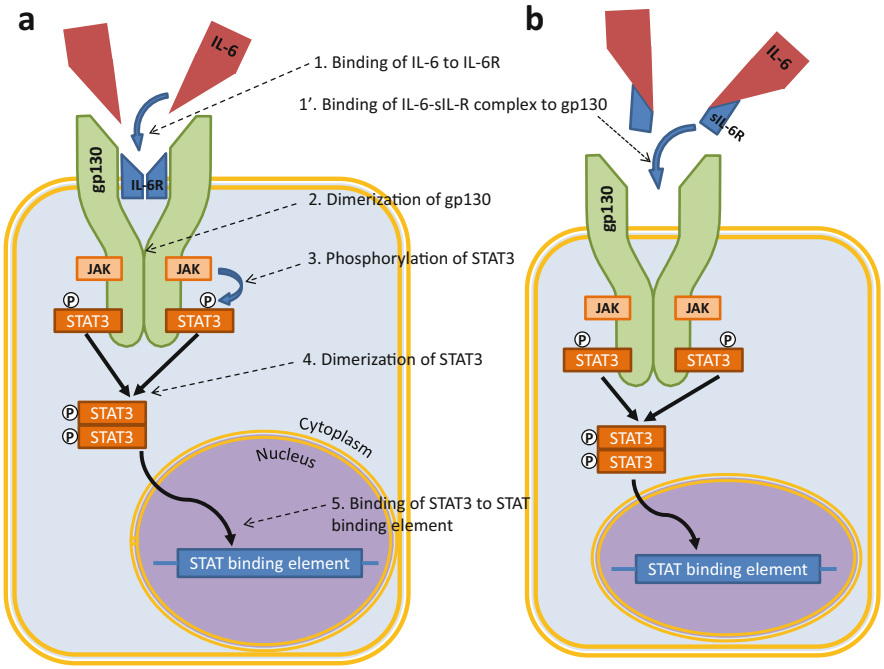

Fig. 7.2 Schemes of IL-6 signal transduction. IL-6 acts via IL-6R (IL-6R α-chain) on the cell surface (**a**). An example of this mechanism is observed in the liver. IL-6 also acts via coupling with sIL-6R in serum (**b**), resulting in stimulation of gp130 on the cell surface

Therefore, inhibition of gp130 might have various adverse effects. In fact, homozygous knockout of gp130 in mice is embryonically lethal [31].

7.4 Therapeutic Effects of Anti-IL-6R Antibodies

Kitaba et al. reported therapeutic effects of an anti-mouse IL-6R antibody in a bleomycin-induced scleroderma mouse model [32]. They established a skin-thickening model by daily subcutaneous injection of bleomycin and then administered a rat anti-mouse IL-6R monoclonal antibody or isotype-matched control antibody to assess whether the anti-IL-6R antibody suppressed the skin thickening. Skin hardness was analyzed at bleomycin-injected areas using Vesmeter which can measure material hardness objectively. Anti-mouse IL-6R antibody-injected mice showed lower hardness of the skin and histological features than control antibody-injected mice. Furthermore, they treated mice with bleomycin for 28 days and then administered the rat anti-mouse IL-6R monoclonal antibody to confirm its therapeutic effects on skin sclerosis. After 4 weeks of treatment with the anti-IL-6R antibody or isotype-matched control antibody, skin hardness at the bleomycin-injected area was low in mice treated with the anti-IL-6R antibody compared with that in mice treated with the control antibody. Histological analysis showed that the skin thickness of anti-IL-6R-treated mice was also thinner than that of control antibody-treated mice.

Therefore, anti-IL-6R antibody therapy is considered as a potential method for SSc treatment. A monoclonal antibody against human IL-6R, tocilizumab (TCZ), has already been used as a therapeutic agent for Castleman disease, idiopathic juvenile arthritis, and rheumatoid arthritis. TCZ exerts effects representative of IL-6 inhibition in these diseases. For example, Castleman disease patients treated with TCZ show decreases in CRP, SAA, and immunoglobulin levels, and patients with rheumatoid arthritis show an improvement in joint swelling, decrease in CRP levels, and correction of their augmented platelet counts. In a 2001 study, 28 patients with Castleman disease received TCZ. All patients showed decreases in inflammatory parameters such as CRP, fibrinogen, and SAA and increases in hemoglobin and serum albumin levels. CRP and fibrinogen were completely normalized in 18 (64.3 %) and 20 (71.4 %) patients, respectively, at week 16 [33]. Nishimoto et al. also reported a multicenter, randomized, and controlled trial of TCZ for the treatment of rheumatoid arthritis. Three-hundred and six patients with active rheumatoid arthritis were divided into disease-modifying antirheumatic drug (DMARD) and TCZ treatment groups. After 52 weeks of treatment, the TCZ group showed significantly less radiographic changes in the total modified Sharp score than the DMARD group [34]. These results indicate that TCZ has the capability to inhibit IL-6 functions.

TCZ is a so-called “humanized” monoclonal antibody because the whole molecule except for the complementary determining region has been replaced with human immunoglobulin to suppress immunogenic reactions. The anti-TNF-α antibody infliximab and anti-CD20 antibody rituximab are chimeric antibodies

composed of both murine and human proteins. Steroids, immunosuppressants (especially methotrexate), or antihistamines are administered when these chimeric antibodies are used to avoid xenobiotic responses. Nevertheless, infusion reactions or human anti-chimeric antibodies are occasionally observed in patients who are administered with infliximab. In contrast, the use of TCZ does not require steroids or immunosuppressants because the immunogenic potential of a humanized antibody is quite low. In fact, TCZ has a superior persistence rate than infliximab in patients with rheumatoid arthritis [35]. Hishitani et al. reported that the drug persistence rate at 5 years for infliximab and TCZ were 29.8 and 66.8 %, respectively.

7.5 Application of TCZ to Patients with SSc

Many reports show that IL-6 plays a major role in the pathogenesis of SSc, and TCZ inhibits IL-6 functions as described above. Therefore, TCZ may have the potential to treat the symptoms of SSc. We administered TCZ to patients with refractory SSc, who did not respond to conventional therapy including steroids [36]. After obtaining informed consent and permission from the ethics committee of Osaka University Hospital, three patients were intravenously administered with 8 mg/kg TCZ in 100 mL saline monthly. All patients had diffuse cutaneous SSc, two were positive for anti-RNA polymerase III antibodies, and the remaining patient was positive for anti-Scl-70 antibodies. The intervals between disease onset and the start of administration for the three patients were 3, 2, and 3 years, respectively. As reported previously, the skin scores were decreased after TCZ administration. A skin score might have some level of subjective bias because TCZ administration was not blinded. Therefore, we examined skin properties using Vesmeter device that measures skin hardness, elasticity, and viscosity. Vesmeter hardness also showed a decrease after TCZ administration. One patient had joint stiffness due to sclerosis of the skin and subcutaneous tissue at extremities but not arthritis and no inflammatory abnormalities such as CRP elevation. We measured the range of motion (ROM) of each joint every 3 months. The patient showed not only skin softening but also ROM improvements except in her ankles. This patient required a wheelchair to move before TCZ treatment, but she was able to walk within 3 years of treatment [37]. One of the patients presented with pulmonary fibrosis and a lower %VC than the normal range, but we did not find amelioration in chest computed tomography (CT) images or pulmonary functions during the treatment. The number of patients was too small to fully understand the influence of TCZ administration in patients with SSc, but some reports have presented similar results. Recently, Fernandes das Neves et al. reported three SSc patients who received 8 mg/kg TCZ every 4 weeks and observed a reduction in the modified Rodnan skin score from 17 to 10, from 41 to 25, and from 7 to 5, respectively. They also found that two patients had cessation of the progression of lung involvement as evaluated both functionally and radiologically [38]. The influences of TCZ treatment on other symptoms of SSc that include vascular damage, bowel tract damage including

esophagus dilatation, and kidney damage are still unclear. However, as mentioned above, many reports show a relationship between IL-6 and lung fibrosis. Therefore, lung involvement may be a potential target for TCZ treatment.

Patients with SSc sometimes show pulmonary hypertension. There are no data concerning TCZ treatment for pulmonary hypertension in SSc, but several reports have shown the effects of TCZ on pulmonary hypertension in Castleman disease patients. Castleman disease can lead to secondary pulmonary hypertension, and Arita et al. reported a patient with Castleman disease and pulmonary hypertension, who received TCZ [39]. The patient had received bosentan and sildenafil because of pulmonary hypertension, but their brain natriuretic peptide (BNP) level was still over 300 pg/mL. They started TCZ to suppress the inflammatory symptoms of Castleman disease, and it improved her BNP level and 6-min walking distance. Furuya et al. also presented a case report of Castleman disease, which showed amelioration of pulmonary hypertension after administration of TCZ [40].

7.6 Hypothesis for the Anti-fibrotic Effect of TCZ

Our case reports have indicated that skin hardness is ameliorated after TCZ administration. However, the mechanism has not been revealed for this change. Duncan et al. found production of collagen, glycosaminoglycans, hyaluronic acid, and chondroitin-4/6-sulfates in human dermal fibroblast cultures treated with recombinant human IL-6, but little effect on total protein production [41]. It is thought that certain cells constituting skin may be stimulated by cytokines, such as IL-6, and produce an extracellular matrix. Sappino et al. reported the existence of α-smooth muscle actin (α-SMA)-positive fibroblasts, termed myofibroblasts, in skin tissues of SSc patients. Immunohistochemistry showed that α-SMA-positive cells were observed in normally healing granulation tissue, hypertrophic scars, fibromatoses, and scleroderma lesions, but not in normal skin [42]. A correlation between the degree of α-SMA-positive myofibroblast infiltration and skin hardness has been reported by Kissin et al. [43]. They examined skin biopsies from 11 patients with diffuse SSc and 10 healthy controls and found that myofibroblasts and hyalinized collagen scores both correlated with the forearm skin score. Gallucci et al. evaluated α-SMA-positive cell infiltration in wounded IL-6 knockout mice. Because it has been reported that IL-6 knockout mice show a lack of wound healing [44], they examined α-SMA expression in wounded tissues. After wounding, α-SMA expression was increased in wild-type mice but not in IL-6 knockout mice. Exogenous addition of recombinant mouse IL-6 to primary cultures of fibroblasts from IL-6 knockout mice induces α-SMA expression [45]. Furthermore, Kitaba et al. reported therapeutic effects in a bleomycin-treated SSc model mouse by injection of an anti-mouse IL-6R antibody as described above. Bleomycin-treated model mice showed higher α-SMA-positive cell numbers, and administration of the anti-mouse IL-6R antibody decreased the number of α-SMA-positive cells [32]. Therefore, IL-6 may induce and stimulate α-SMA-positive

myofibroblasts to produce an extracellular matrix. Accordingly, IL-6 might play a pathogenetic role in sclerotic lesions.

7.7 Current State and Future Plans

In our studies, TCZ appears to improve the skin involvement. We believe that a larger-scale clinical investigation of this therapy is warranted and might provide an insight into the association between IL-6 and visceral involvement. To evaluate the therapeutic effect of TCZ on SSc, an open-label trial has been conducted in Japan (UMIN000005550), and a double-blind, placebo-controlled trial was conducted in Europe and North America (NCT01532869). Neither studies have yet been published, but their main results were presented at the 2014 Annual Meeting of American College of Rheumatology in Boston and 18th Japanese Scleroderma Study Conference in Tokyo. Interestingly, both sets of data are similar. Some patients responded well to TCZ treatment by reductions in their skin scores, but some patients did not show amelioration. Although the disease duration, age, autoantibodies, and levels of inflammatory mediators such as CRP were analyzed to reveal differences in the responses, it is currently unknown what kind of SSc patients is suitable for such treatment.

7.7.1 Study Scale of Clinical Trial for SSc

To determine whether IL-6-inhibiting therapy is effective, there are several problems to solve. The first is the scale of clinical studies. Our study was conducted at three hospitals with 13 patients. In the European study, the number of institutes committed was 50 and the number of patients registered was 87. However, patients with SSc show a variety of symptoms such as bowel tract disturbance, pulmonary hypertension, lung fibrosis, and kidney damage. The state of each patient is so different, and we do not know where the influence of TCZ treatment appears beforehand. Therefore, a larger number of patients should be needed to determine the effect of TCZ. The second problem is determining what type of SSc patients are suitable for TCZ treatment. Because the response to TCZ therapy is different among patients, we need to find predictive factors for this therapy. A global study of TCZ therapy for patients with SSc will start in 2015, which will solve these problems.

7.7.2 Evaluation Method for the Clinical Trial of SSc

The third problem is the evaluation methods to determine the effects of TCZ. In rheumatoid arthritis, numerous markers represent the disease activity, such as CRP, fibrinogen, ESR, and SAA. These values are usually combined with findings from physical examination of the joints to calculate the 28-joint disease activity score,

clinical disease activity index, and simplified disease activity index. However, there are no such biological markers for patients with SSc. Nonetheless, the disease activity of SSc can be indirectly interpreted via the nutritional condition, anemia, pulmonary function test, and CT imaging. However, in most cases, the evaluation of the disease activity depends on a modified Rodnan skin score. To examine a new therapeutic agent in SSc patients, a multicenter blinded study must be conducted. There is no doubt that the modified Rodnan skin score is clinically useful, but it may not be suitable for a multicenter trial because it contains a physician's subjective judgment. Therefore, the variability is getting bigger if the number of facilities increases, though a true result of the study is getting closer if the number of cases increases. To homogenize the object of the study and to suppress a dispersion of clinical evaluation, the assessors have to be fixed, and preliminary discussion for assessors is needed. Furthermore, as mentioned above, IL-6 is a pleiotropic cytokine, so the effect of TCZ may present in a variety of regions including the skin, lung, tendon, and bowel tissue. A multiplex scoring system and an objective "demarche" for skin tissue will be required.

7.8 Conclusion

IL-6 is a pleiotropic cytokine, and it is one of the factors that might play an important role in pathogenesis of SSc. Humanized anti-IL-6 receptor antibody, TCZ, has presented the inhibitory effect to IL-6 functions in patients with RA or Castleman disease. Therefore, TCZ is considered as a potential medicine for SSc treatment. To establish the therapeutic effect, a larger-scale study and objective evaluation methods are required.

References

1. Okada M, Sakaguchi N, Yoshimura N, Hara H, Shimizu K, Yoshida N, et al. B cell growth factors and B cell differentiation factor from human T hybridomas. Two distinct kinds of B cell growth factor and their synergism in B cell proliferation. J Exp Med. 1983;157:583–90.
2. Hirano T, Yasukawa K, Harada H, Taga T, Watanabe Y, Matsuda T, et al. Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. Nature. 1986;324:73–6.
3. Nijsten MW, de Groot ER, ten Duis HJ, Klasen HJ, Hack CE, Aarden LA. Serum levels of interleukin-6 and acute phase responses. Lancet. 1987;2:921.
4. Geiger T, Andus T, Klapproth J, Hirano T, Kishimoto T, Heinrich PC. Induction of rat acute-phase proteins by interleukin 6 in vivo. Eur J Immunol. 1988;18:717–21.
5. Gauldie J, Richards C, Harnish D, Lansdorp P, Baumann H. Interferon beta 2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. Proc Natl Acad Sci U S A. 1987;84:7251–5.
6. Muraguchi A, Hirano T, Tang B, Matsuda T, Horii Y, Nakajima K, Kishimoto T. The essential role of B cell stimulatory factor 2 (BSF-2/IL-6) for the terminal differentiation of B cells. J Exp Med. 1988;167:332–44.

7. Takatsuki F, Okano A, Suzuki C, Chieda R, Takahara Y, Hirano T, Kishimoto T, Hamuro J, Akiyama Y. Human recombinant IL-6/B cell stimulatory factor 2 augments murine antigen-specific antibody responses in vitro and in vivo. J Immunol. 1988;141:3072–7.
8. Suematsu S, Matsusaka T, Matsuda T, Ohno S, Miyazaki J, Yamamura K, Hirano T, Kishimoto T. Generation of plasmacytomas with the chromosomal translocation t(12;15) in interleukin 6 transgenic mice. Proc Natl Acad Sci U S A. 1992;89:232–5.
9. Ishibashi T, Kimura H, Uchida T, Kariyone S, Friese P, Burstein SA. Human interleukin 6 is a direct promoter of maturation of megakaryocytes in vitro. Proc Natl Acad Sci U S A. 1989;86:5953–7.
10. Asano S, Okano A, Ozawa K, Nakahata T, Ishibashi T, Koike K, Kimura H, Tanioka Y, Shibuya A, Hirano T, et al. In vivo effects of recombinant human interleukin-6 in primates: stimulated production of platelets. Blood. 1990;75:1602–5.
11. Yoshizaki K, Matsuda T, Nishimoto N, Kuritani T, Taeho L, Aozasa K, Nakahata T, Kawai H, Tagoh H, Komori T, et al. Pathogenic significance of interleukin-6 (IL-6/BSF-2) in Castleman's disease. Blood. 1989;74:1360–7.
12. Suematsu S, Matsuda T, Aozasa K, Akira S, Nakano N, Ohno S, Miyazaki J, Yamamura K, Hirano T, Kishimoto T. IgG1 plasmacytosis in interleukin 6 transgenic mice. Proc Natl Acad Sci U S A. 1989;86:7547–51.
13. Katsume A, Saito H, Yamada Y, Yorozu K, Ueda O, Akamatsu K, Nishimoto N, Kishimoto T, Yoshizaki K, Ohsugi Y. Anti-interleukin 6 (IL-6) receptor antibody suppresses Castleman's disease like symptoms emerged in IL-6 transgenic mice. Cytokine. 2002;20:304–11.
14. Horii Y, Muraguchi A, Iwano M, Matsuda T, Hirayama T, Yamada H, Fujii Y, Dohi K, Ishikawa H, Ohmoto Y, et al. Involvement of IL-6 in mesangial proliferative glomerulonephritis. J Immunol. 1989;143:3949–55.
15. Kupper TS, Min K, Sehgal P, Mizutani H, Birchall N, Ray A, May L. Production of IL-6 by keratinocytes. Implications for epidermal inflammation and immunity. Ann N Y Acad Sci. 1989;557:454–64.
16. Grossman RM, Krueger J, Yourish D, Granelli-Piperno A, Murphy DP, May LT, Kupper TS, Sehgal PB, Gottlieb AB. Interleukin 6 is expressed in high levels in psoriatic skin and stimulates proliferation of cultured human keratinocytes. Proc Natl Acad Sci U S A. 1989;86:6367–71.
17. Needleman BW, Wigley FM, Stair RW. Interleukin-1, interleukin-2, interleukin-4, interleukin-6, tumor necrosis factor alpha, and interferon-gamma levels in sera from patients with scleroderma. Arthritis Rheum. 1992;35:67–72.
18. Feghali CA, Bost KL, Boulware DW, Levy LS. Mechanisms of pathogenesis in scleroderma. I. Overproduction of interleukin 6 by fibroblasts cultured from affected skin sites of patients with scleroderma. J Rheumatol. 1992;19:1207–11.
19. Gurram M, Pahwa S, Frieri M. Augmented interleukin-6 secretion in collagen-stimulated peripheral blood mononuclear cells from patients with systemic sclerosis. Ann Allergy. 1994;73:493–6.
20. Sato S, Hasegawa M, Takehara K. Serum levels of interleukin-6 and interleukin-10 correlate with total skin thickness score in patients with systemic sclerosis. J Dermatol Sci. 2001;27:140–6.
21. Hasegawa M, Sato S, Fujimoto M, Ihn H, Kikuchi K, Takehara K. Serum levels of interleukin 6 (IL-6), oncostatin M, soluble IL-6 receptor, and soluble gp130 in patients with systemic sclerosis. J Rheumatol. 1998;25:308–13.
22. Khan K, Xu S, Nihtyanova S, Derrett-Smith E, Abraham D, Denton CP, Ong VH. Clinical and pathological significance of interleukin 6 overexpression in systemic sclerosis. Ann Rheum Dis. 2012;71:1235–42. doi:10.1136/annrheumdis-2011-200955.
23. Kawaguchi Y, Hara M, Wright TM. Endogenous IL-1alpha from systemic sclerosis fibroblasts induces IL-6 and PDGF-A. J Clin Invest. 1999;103:1253–60.

24. Crestani B, Seta N, De Bandt M, Soler P, Rolland C, Dehoux M, Boutten A, Dombret MC, Palazzo E, Kahn MF, et al. Interleukin 6 secretion by monocytes and alveolar macrophages in systemic sclerosis with lung involvement. Am J Respir Crit Care Med. 1994;149:1260–5.
25. Hasegawa M, Fujimoto M, Matsushita T, Hamaguchi Y, Takehara K, Sato S. Serum chemokine and cytokine levels as indicators of disease activity in patients with systemic sclerosis. Clin Rheumatol. 2011;30:231–7. doi:10.1007/s10067-010-1610-4.
26. Scala E, Pallotta S, Frezzolini A, Abeni D, Barbieri C, Sampogna F, De Pità O, Puddu P, Paganelli R, Russo G. Cytokine and chemokine levels in systemic sclerosis: relationship with cutaneous and internal organ involvement. Clin Exp Immunol. 2004;138:540–6.
27. Yamasaki K, Taga T, Hirata Y, Yawata H, Kawanishi Y, Seed B, Taniguchi T, Hirano T, Kishimoto T. Cloning and expression of the human interleukin-6 (BSF-2/IFN beta 2) receptor. Science. 1988;241:825–8.
28. Taga T, Hibi M, Hirata Y, Yamasaki K, Yasukawa K, Matsuda T, Hirano T, Kishimoto T. Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. Cell. 1989;58:573–81.
29. Saito M, Yoshida K, Hibi M, Taga T, Kishimoto T. Molecular cloning of a murine IL-6 receptor-associated signal transducer, gp130, and its regulated expression in vivo. J Immunol. 1992;148:4066–71.
30. Kishimoto T, Akira S, Narazaki M, Taga T. Interleukin-6 family of cytokines and gp130. Blood. 1995;86:1243–54.
31. Yoshida K, Taga T, Saito M, Suematsu S, Kumanogoh A, Tanaka T, Fujiwara H, Hirata M, Yamagami T, Nakahata T, Hirabayashi T, Yoneda Y, Tanaka K, Wang WZ, Mori C, Shiota K, Yoshida N, Kishimoto T. Targeted disruption of gp130, a common signal transducer for the interleukin 6 family of cytokines, leads to myocardial and hematological disorders. Proc Natl Acad Sci U S A. 1996;93:407–11.
32. Kitaba S, Murota H, Terao M, Azukizawa H, Terabe F, Shima Y, Fujimoto M, Tanaka T, Naka T, Kishimoto T, Katayama I. Blockade of interleukin-6 receptor alleviates disease in mouse model of scleroderma. Am J Pathol. 2012;180:165–76. doi:10.1016/j.ajpath.2011.09.013.
33. Nishimoto N, Kanakura Y, Aozasa K, Johkoh T, Nakamura M, Nakano S, Nakano N, Ikeda Y, Sasaki T, Nishioka K, Hara M, Taguchi H, Kimura Y, Kato Y, Asaoku H, Kumagai S, Kodama F, Nakahara H, Hagihara K, Yoshizaki K, Kishimoto T. Humanized anti-interleukin-6 receptor antibody treatment of multicentric Castleman disease. Blood. 2005;106:2627–32.
34. Nishimoto N, Hashimoto J, Miyasaka N, Yamamoto K, Kawai S, Takeuchi T, Murata N, van der Heijde D, Kishimoto T. Study of active controlled monotherapy used for rheumatoid arthritis, an IL-6 inhibitor (SAMURAI): evidence of clinical and radiographic benefit from an x ray reader-blinded randomised controlled trial of tocilizumab. Ann Rheum Dis. 2007;66:1162–7.
35. Hishitani Y, Ogata A, Shima Y, Hirano T, Ebina K, Kunugiza Y, Shi K, Narazaki M, Hagihara K, Tomita T, Yoshikawa H, Tanaka T, Kumanogoh A. Retention of tocilizumab and anti-tumour necrosis factor drugs in the treatment of rheumatoid arthritis. Scand J Rheumatol. 2013;42:253–9. doi:10.3109/03009742.2012.762037.
36. Shima Y, Kuwahara Y, Murota H, Kitaba S, Kawai M, Hirano T, Arimitsu J, Narazaki M, Hagihara K, Ogata A, Katayama I, Kawase I, Kishimoto T, Tanaka T. The skin of patients with systemic sclerosis softened during the treatment with anti-IL-6 receptor antibody tocilizumab. Rheumatology (Oxford). 2010;49:2408–12. doi:10.1093/rheumatology/keq275.
37. Shima Y, Hosen N, Hirano T, Arimitsu J, Nishida S, Hagihara K, Narazaki M, Ogata A, Tanaka T, Kishimoto T, Kumanogoh A. Expansion of range of joint motion following treatment of systemic sclerosis with tocilizumab. Mod Rheumatol. 2015;25:134–7. doi:10.3109/14397595.2013.874749.

38. Fernandes das Neves M, Oliveira S, Amaral MC, Delgado Alves J. Treatment of systemic sclerosis with tocilizumab. Rheumatology (Oxford). 2015;54:371–2. doi:10.1093/rheumatology/keu435.
39. Arita Y, Sakata Y, Sudo T, Maeda T, Matsuoka K, Tamai K, Higuchi K, Shioyama W, Nakaoka Y, Kanakura Y, Yamauchi-Takihara K. The efficacy of tocilizumab in a patient with pulmonary arterial hypertension associated with Castleman's disease. Heart Vessels. 2010;25:444–7. doi:10.1007/s00380-009-1215-5.
40. Furuya Y, Satoh T, Kuwana M. Interleukin-6 as a potential therapeutic target for pulmonary arterial hypertension. Int J Rheumatol. 2010. doi:10.1155/2010/720305.
41. Duncan MR, Berman B. Stimulation of collagen and glycosaminoglycan production in cultured human adult dermal fibroblasts by recombinant human interleukin 6. J Invest Dermatol. 1991;97:686–92.
42. Sappino AP, Masouyé I, Saurat JH, Gabbiani G. Smooth muscle differentiation in scleroderma fibroblastic cells. Am J Pathol. 1990;137:585–91.
43. Kissin EY, Merkel PA, Lafyatis R. Myofibroblasts and hyalinized collagen as markers of skin disease in systemic sclerosis. Arthritis Rheum. 2006;54:3655–60.
44. Gallucci RM, Simeonova PP, Matheson JM, Kommineni C, Guriel JL, Sugawara T, Luster MI. Impaired cutaneous wound healing in interleukin-6-deficient and immunosuppressed mice. FASEB J. 2000;14:2525–31.
45. Gallucci RM, Lee EG, Tomasek JJ. IL-6 modulates alpha-smooth muscle actin expression in dermal fibroblasts from IL-6-deficient mice. J Invest Dermatol. 2006;126:561–8.

Epigenetics

8

Masatoshi Jinnin

Abstract

Inherited genetic factors cannot fully explain the pathogenesis of systemic sclerosis. Environmental factors may also be involved in the pathogenesis, and recent researches have suggested that epigenetics play a role by mediating influence of the environmental factors. This chapter discusses the present-day understanding about the abnormalities in epigenetics including methylation, histone modification, and noncoding RNAs in this disease.

DNA methylation was dysregulated in fibroblasts, lymphocytes, and endothelial cells of systemic sclerosis. Histone modification in fibroblasts or lymphocytes may be correlated with skin fibrosis in systemic sclerosis. MicroRNAs have been implicated in the various symptoms of systemic sclerosis and may become potential biomarkers and therapeutic targets. The clarification of the mechanism by which epigenetic abnormalities contribute to the pathogenesis of the disease may lead to the development of new diagnostic tools and new treatments.

Keywords

Methylation • Histone modification • Noncoding RNA

8.1 Introduction

After the first description of DNA double-helix structure with Dr. James Watson in 1953, Dr. Francis Crick proposed the concept "Central dogma of molecular biology," which is that "DNA makes RNA makes proteins" and "the genetic

M. Jinnin (✉)
Department of Dermatology & Plastic Surgery, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan
e-mail: mjin@kumamoto-u.ac.jp

K. Takehara et al. (eds.), *Systemic Sclerosis*, DOI 10.1007/978-4-431-55708-1_8

information flows from DNA to protein via RNA." Genomic DNA is thought to act as a template for mRNA synthesis by a process called transcription in chromosomes, and the mRNA carries coded genetic information to ribosomes, which translates this information into protein. Accordingly, the Human Genome Project, started in 1991 and completed in 2003, was prospected to clarify the entire mechanisms of the diversity of life. However, the project revealed that DNA sequence itself cannot completely explain such diversity and indicated that genome analysis is not a goal of life science or medical science.

One of the other important research fields to explain the biological diversity is epigenetics: it studies the mechanisms by which heritable information other than DNA sequence (e.g., mutations or polymorphisms) can influence gene activity or gene expression postnatally. Epigenetics is proven to be involved in a lot of cellular processes by switching genes on or off and by deciding which proteins are transcribed. For example, all cells in the human body basically develop from a single fertilized egg and have the same DNA sequences. However, they can differentiate into various tissues and organs, because they have certain sets of genes that are turned on or off by the epigenetic changes. Epigenetics can also explain why identical twins with identical DNA sequences show different phenotypes as they grow up. Accordingly, epigenetic changes may be able to control the gene expression to the same extent as the DNA sequences themselves.

Epigenetics includes several processes such as DNA methylation or histone modification. For example, hypermethylation of promoter regions containing increased frequency of the CG sequence compared with other regions (so-called CpG islands) causes gene inactivation. Thus, the same DNA sequences with different methylated statuses may result in different promoter activities and gene expression. Acetylation, phosphorylation, or ubiquitylation of histone also altered chromatin states, which leads to the changes in promoter activities. Furthermore, RNA silencing or RNA interference (RNAi) by noncoding RNAs is recently paid attention for their regulation of gene expression in cooperation with DNA methylation or histone modifications.

Systemic sclerosis (SSc) shows similar concordance rates between monozygotic and dizygotic twin pairs, and the genetic heritability for SSc is reported to be only 0.008 [1, 2], indicating that inherited genetic factors cannot fully explain the pathogenesis of disease. Environmental factors may also be involved in the pathogenesis, and recent researches have suggested that they affect on epigenetics in autoimmune disorders or collagen diseases. This chapter discusses the present-day understanding about the epigenetic abnormalities in SSc.

8.2 DNA Methylation

DNA methylation is one of the core epigenetic mechanisms that has been widely studied. The methylation is mainly controlled by DNA methyltransferases, in cooperation with S-adenosyl-methionine as the methyl donor, which transfers a methyl group on cytosine residues of DNA to form 5-methylcytosine [2, 3]. The

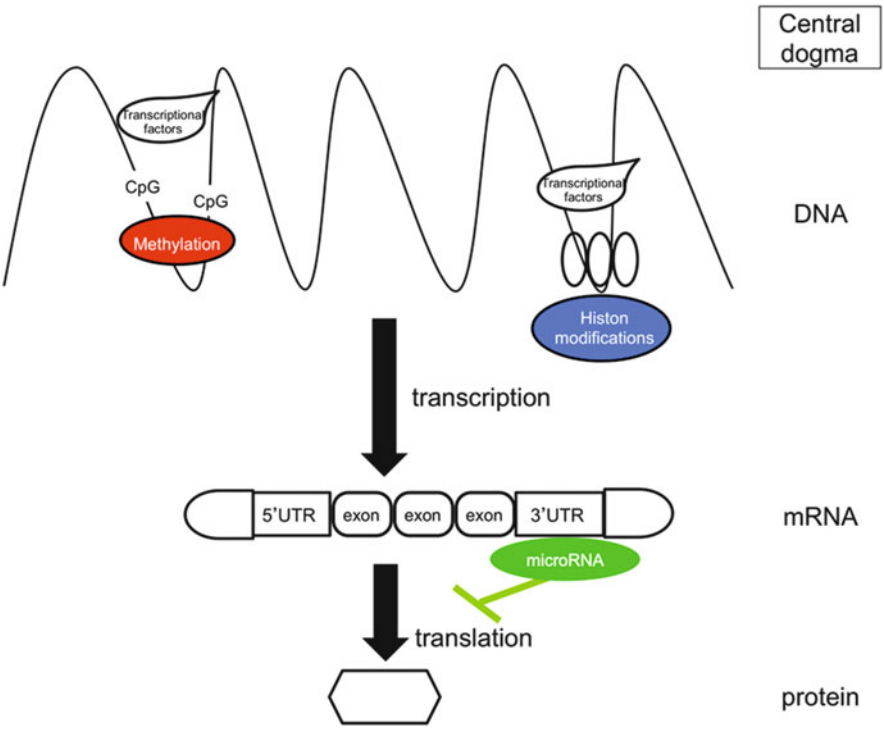

Fig. 8.1 Factors involved in epigenetics

DNA methyltransferases are also thought to be involved in the removal of methyl groups from DNA as well as the addition. They are classified into de novo DNA methyltransferases (DNMT3a, DNMT3b, and DNMT3L), which control methylation during embryonic development, and maintenance DNA methyltransferases (DNMT1, DNMT2), which maintain existing methylation pattern and regulate its inheritance during DNA replication [4]. Cytosine-phosphate-guanine (CpG) motifs are usually methylated, but CpG islands, CpG-rich regions within gene promoter regions of many genes, are basically unmethylated. If the promoter region is highly methylated, the transcription will be inhibited due to decreased binding of transcription factors and increased binding of methyl-CpG-binding domain proteins (Fig. 8.1). On the other hand, lower methylation of the promoter will result in the activated transcription. Through the posttranscriptional gene regulation, DNA methylation plays a central role in mammalian embryonic development, X-chromosome inactivation, genomic imprinting, and lineage specification [2, 5].

A lot of cell types including fibroblasts, lymphocytes, and endothelial cells are thought to be involved in the pathogenesis of SSc, and abnormality in DNA methylation has been reported in each cell type [2]. For example, Friend leukemia integration 1 (Fli1) transcription factor negatively regulates collagen transcription, and its expression is constitutively downregulated in cultured dermal fibroblasts

derived from affected SSc skin [6]. Mice with double heterozygous deficiency of Klf5 and Fli1 mimic the fibrosis as well as vasculopathy of the SSc skin and lung, B cell activation, and autoantibody production [7]. Thus, the downregulation of Fli1 may induce the pathogenic triad of SSc. The Fli1 downregulation as well as type I collagen overexpression in cultured SSc dermal fibroblasts was recovered by the treatment with the DNA methyltransferase inhibitor 2-deoxy-5-azacytidine [8]. Consistently, aberrant DNMT1 expression and DNA demethylase activity in SSc fibroblasts were also reported [9, 10]. Levels of methyl-CpG DNA-binding domain protein 1 (MBD1), MBD2, and methyl-CpG-binding protein 2 (MeCP2) were reported to be significantly increased in SSc fibroblasts compared with healthy fibroblasts [3]. These changes may result in the increased methylation status in the Fli1 promoter region and may mediate excessive collagen overexpression in SSc fibroblasts over multiple generations by cellular epigenetic inheritance [3].

DNA methylation in lymphocytes has also been well investigated in SSc patients. Different from SSc fibroblasts, the expression of several methylation-related genes including DNMT1, MBD3, and MBD4 was reported to be significantly decreased in SSc CD4+ T lymphocytes [11]. Consistently, global DNA hypomethylation was observed in the CD4+ T cells of SSc patients compared to those of normal subjects. Thus, the pattern of global methylation is different in each cell type of SSc patients, which may reflect various symptoms seen in these patients [3]. The hypomethylation in CD4+ T cells is also found in SLE patients [11]. Although the regulatory mechanism underlying in the hypomethylation is still to be clarified, several studies indicated the abnormalities in the extracellular signal-regulated kinase (ERK) signaling pathway leading to the hypomethylation in SLE [12]. There is a speculation that similar mechanisms may exist in SSc [3]. Hypomethylation of genes in CD4+ T cells may cause reactivation of LINE-1 retrotransposable elements, which may contribute to the autoimmunity [13]. Furthermore, hypomethylation of the CD70 promoter is thought to induce the overexpression of CD70 seen in SSc CD4+ T cells [14]. CD70 is one of the B cell co-stimulatory molecules expressed on activated B cells and T cells. Co-stimulation of these cells is important for the development of immune response, and its abnormality may be involved in the pathogenesis of SLE or RA [3]. Similarly, adhesion molecule integrin A_L is also overexpressed in SSc CD4+ T cells, which may be caused by the hypomethylation of its promoter region [3]. To note, the methylation may also explain the female preponderance of the disease: DNA methylation plays a main role in X-chromosome inactivation as described above, and the gene of CD40 ligand is encoded on the X chromosome. CD40 ligand is thought to contribute to the pathogenesis of SSc by its various functions including activating B cell, and its expression is increased in CD4+ T cells of female SSc patients [2]. The demethylation of CD40 ligand promoter on the inactive X chromosome was demonstrated in female SSc patients, which results in the CD40 ligand overexpression [15, 16]. The reactivation of genes by the hypomethylation of usually methylated X chromosomes of female SSc patients may cause the female gender bias in the disease. Consistent with this notion, Selmi et al. also reported reactivation of the normally methylated X chromosome in female SSc patients, by

comparing the methylation profile of all X-chromosome genes in peripheral blood mononuclear cells obtained from monozygotic twins discordant and concordant for SSc [17].

In endothelial cells of SSc patients, increased DNMT1 expression and decreased DNA demethylase activity were reported [9]. Resulting DNA hypermethylation is thought to control nitric oxide synthases (NOSs): nitric oxide (NO) can inhibit leukocyte endothelium adhesion, platelet aggregation, and vascular smooth muscle cell proliferation, via its antithrombotic and antiatherogenic effects [2]. NOSs play a central role in the synthesis of NO from L-arginine and consist of three isoforms including NOS1 (neuronal NOS), NOS2 (inducible NOS), and NOS3 (endothelial NOS, eNOS). Among them, NOS3 expression is reported to be suppressed in the vascular endothelial cells of SSc patients by the hypermethylation [3, 18]. Furthermore, the expression of bone morphogenetic protein receptor type II (BMPRII), which is involved in the pathogenesis of familial pulmonary arterial hypertension, was significantly decreased in SSc microvascular endothelial cells, due to the hypermethylation of the promoter region [19]. And the decreased level of BMPRII in SSc endothelial cells was recovered by the addition of 2-deoxy-5-azacytidine.

8.3 Histone Modifications

Chromatins consist of nucleosomes, which are comprised of 147 bp double-stranded DNA wrapped around an octamer of small basic proteins called histones. The histones are classified into linker histones (H1 and H5) and core histones (H2A, H2B, H3, and H4). These histones are highly conserved protein family that can package and organize genome. The N-terminal tails of histone proteins underwent more than 100 posttranslational modifications including acetylation, methylation, phosphorylation, deimination, ADP ribosylation, ubiquitylation, and sumoylation. These modifications affect the structure of nucleosomes and change the transcriptional activity of genes. Among them, acetylation and methylation are the two main histone changes. Histone acetylation is one of the most well-investigated modifications and usually leads to transcriptional activation. Acetylation is controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs): HATs transfer an acetyl group from acetyl coenzyme A (CoA) to the lysine side chains, which reduces the interaction between the histone and DNA, opens the chromatin structure, and promotes transcriptional activities of genes [2]. HDACs remove the acetyl group from the lysine of histone molecule and close the chromatin structure. On the other hand, histone methylation occurs on various positions of lysine. For example, methylation at histone H3 lysine 4 (H3K4) usually promotes transcriptional activities of genes, whereas trimethylation of H3K27 (H3K27me3) suppresses them [3, 20].

HDAC inhibitor, trichostatin A, reduced transforming growth factor (TGF)-β-induced mRNA expression of α1(I) collagen and fibronectin in both normal and SSc skin fibroblasts [21]. Furthermore, protein overexpression of total collagen was normalized by trichostatin A treatment in SSc skin fibroblasts, via the

inhibition of nuclear translocation and DNA binding of Smad3 or Smad4. Trichostatin A also prevented dermal accumulation of extracellular matrix in bleomycin-induced skin fibrosis mice model. Thus, histone deacetylation may contribute to the tissue fibrosis of SSc, and trichostatin A may have therapeutic potential against fibrosis. As a further detailed mechanism, among the HDAC family, HDAC-3 or HDAC-7 was up- or downregulated by trichostatin A treatment in SSc dermal fibroblasts, respectively [22]. Silencing of HDAC-7 decreased the overexpression of collagens in these cells, but not affected the expression of fibronectin. Taken together, HDAC-7 may mediate the collagen regulation by trichostatin A specifically and can represent a putative target for the anti-fibrotic therapy. To note, a report of the decreased levels of anti-HDAC-3 antibodies in the sera of SSc patients also indicate the possible involvement of HDAC in the pathogenesis of SSc [2]. Wan et al. reported that the expression of HDAC-1 and HDAC-6 is upregulated in SSc dermal fibroblasts [8]. They also showed that there was a significant reduction in the acetylated forms of histones H3 and H4 in Fli1 promoter of SSc fibroblasts. These changes may cause deacetylation of Fli1 promoter in SSc fibroblasts and resulting Fli1 downregulation. In addition, Kramer et al. found that H3K27me3 is increased in SSc fibroblasts, and the inhibition of H3K27me3 stimulates the expression of collagen [23].

In B lymphocytes of SSc patients, expression of JHDM2A was significantly upregulated, whereas HDAC-2, HDAC-7, and SUV39H2 were significantly downregulated compared to control cells [24]. On the other hand, global hyperacetylation of histone H4 was negatively correlated with the HDAC-2 expression, and global hypomethylation of histone H3K9 was positively correlated with the expression of SUV39H2 protein in SSc B cells. Furthermore, global H4 acetylation was positively correlated with disease activity, while HDAC-2 expression was negatively correlated with skin thickness. Taken together, these findings suggest that histone modifications are also dysregulated in B cells of SSc patients, which may play a part in the pathogenesis of the disease.

8.4 Noncoding RNAs

Noncoding RNAs are a lot of different classes of small or long RNAs with their own functions and properties (Fig. 8.2). Among them, long noncoding RNAs (lncRNAs) are RNA molecules more than 200-nucleotide long and can be present in both nuclear and cytosolic fractions of cells. There are thought to be more than 20,000 lncRNAs in the human genome. RNA silencing or RNA interference (RNAi) is one of the most famous functions of noncoding RNAs, but lncRNAs can also function in various biological processes other than RNA silencing, such as transcriptional activation, translational regulation, chromatin modification, nuclear-cytoplasmic trafficking, DNA methylation, cell differentiation, and cell cycle regulation [25].

Through these functions, lncRNAs are thought to play a role in the pathogenesis of several human diseases, especially malignancies [26, 27]. Although lncRNAs in collagen diseases or skin diseases have hardly been investigated so far, Cao

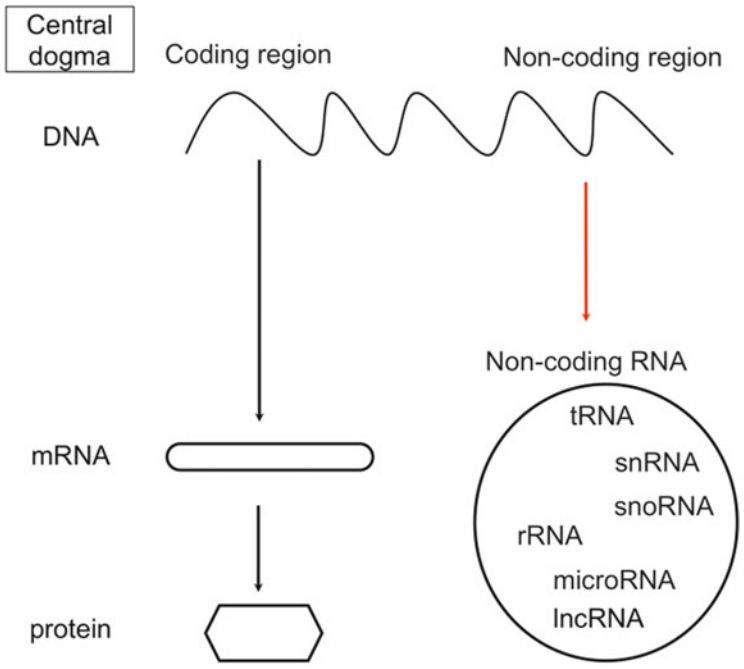

Fig. 8.2 Classification of noncoding RNAs

et al. recently analyzed the differentially expressed lncRNAs and mRNA profiles between fibrotic lungs of rat model and normal lung tissues using microarrays [28]. In the fibrotic lungs of rats, 210 and 358 lncRNAs were upregulated and downregulated, respectively. Among the upregulated lncRNAs, the authors identified two lncRNAs, namely, AJ005396 and S69206. AJ005396 and S69206 were located in the cytoplasm of the interstitial cells of the fibrotic lung tissues. Thus, lncRNAs may be involved in the etiology of lung fibrosis. We also tried to evaluate the possibility that lncRNAs play some roles in the pathogenesis of SSc, especially in the constitutive overexpression of type I collagen. Among lncRNAs, we focused on XIST, because it is X-chromosome inactivation-related gene and X-inactivation is very important in the female preponderance of this disease, as described above. However, we found TSIX, the antisense of XIST, was overexpressed in SSc dermal fibroblasts both in vivo and in vitro (Wang Z, et al. In submission). The overexpression was inhibited by the transfection of TGF-β1 siRNA. TSIX siRNA reduced the protein expression of type I collagen in SSc dermal fibroblasts, via the significant reduction of collagen mRNA stability. Accordingly, TSIX may be a new regulator of collagen in dermal fibroblasts. A better understanding of the contribution of lncRNAs to the pathogenesis of SSc will lead to new therapeutic targets.

On the other hand, small noncoding RNA includes microRNAs (miRNAs), small nuclear RNAs (snRNAs), or small nucleolar RNAs (snoRNAs). For example,

snRNAs are thought to control RNA splicing as a component of the integral cellular machinery so-called spliceosome, in the maturation of eukaryotic precursor mRNA. snoRNAs are modifier of ribosomal RNAs (rRNAs), which regulate the cleavage of long pre-rRNAs into its functional subunits such as 18S or 28S. snoRNAs can also add finishing modifications to the rRNA subunits. miRNAs are a family of small RNAs that is on average 22-nucleotide long. They bind to complementary sequences in the three-prime untranslated regions (3′ UTRs) of target mRNAs, mainly leading to the inhibition of gene expression [29, 30]. Among the miRNAs, lin-4 was first identified as a regulator of developmental timing in the nematode *Caenorhabditis elegans* (*C. elegans*) in 1993 [31]. Subsequently, lethal-7 (let-7) was discovered in the nematode in 2000 [32], and to date, there are thought to be more than 2,500 miRNAs in the human genome.

In the maturation process of miRNA, primary miRNAs are first transcribed as longer transcripts about several hundred to a few thousand nucleotides from the noncoding regions of genome [33, 34]. Microprocessor complex consisted of Drosha and DGCR8 cleaves the primary miRNAs into precursor miRNAs, 60–70 nucleotide double-stranded stem-loop structure. Exportin 5 transports the precursor miRNAs from the nucleus to the cytoplasm, and the stem-loop of the precursor was cleaved by the endonuclease Dicer1. The cleavage leads to the production of mature double-stranded miRNA, two complementary short RNA molecules. Each strand may be degraded by Argonaute protein, the catalytically active RNAse in the RNA-induced silencing complex (RISC), or can function as a single-stranded mature miRNA by being integrated into the RISC. RISC binds to the complementary sequences in the 3′ UTRs of target mRNAs [35], leading to gene silencing by mRNA degradation or the repression of mRNA translation. The upregulation of miRNA usually results in the inhibition of target protein expression, while its downregulation leads to the induction of target proteins. Different from siRNA that knocks down transcriptional levels of the single target molecule, miRNAs have multiple targets. To predict putative target mRNAs of each miRNA, several computational programs are available on the web: microRNA.org (August 2010 Release, http://www.microrna.org/microrna/getGeneForm.do), PicTar (Last update: March 26, 2007, http://pictar.mdc-berlin.de), and TargetScan (version 6.2, http://www.targetscan.org) [36–39]. Through above functions, miRNAs are found to control cellular activities including cell proliferation, migration, or development in vitro as well as contribute to various biological events such as angiogenesis, immune response, or carcinogenesis in vivo. For example, in innate immunity, miR-155 regulates the response to inflammation in macrophages and lymphocytes [40–42]. Furthermore, miR-155 targets IL-1 signal via targeting TAK1-binding protein 2 (TAB2), a molecule involved in Toll-like receptor/IL-1 inflammatory pathway in human dendritic cells. In addition, the transcription factor PU.1 is one of the direct targets of miR-155: PU.1 regulates DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), which mediates the capture and internalization of viral, bacterial, or fungal pathogens by human dendritic cells, resulting in the modulation of pathogen-binding ability of these cells. On the other hand, in adaptive immunity, miR-181a targets multiple phosphatases, and affects

on T cell sensitivity and selection during T cell development, leading to the elevated steady-state levels of phosphorylated intermediates and the reduced T cell receptor signaling threshold [42, 43]. Furthermore, miR-155 negatively regulates the expression of suppressor of cytokine signaling (SOCS) 1, an inhibitor of IL-2 receptor signal, and maintains the homeostasis of regulatory T cells [44]. Accordingly, miR-155 is implicated in both innate immunity and adaptive immunity.

The contribution of miRNAs to the mechanisms of tissue fibrosis has also been vigorously investigated [45]. miRNAs in fibrotic tissues are first investigated in cardiac fibrosis. For example, acute myocardial infarction due to coronary artery occlusion causes pathological remodeling response including cardiac fibrosis, which impairs cardiac contractility [46]. Myocardial infarction induces the downregulation of miR-29 family in the region of the heart adjacent to the infarct. The targets of miR-29 family include several fibrosis-related molecules such as multiple collagens, fibrillins, and elastin. Accordingly, the downregulation of miR-29 family may trigger the upregulation of fibrosis-related molecules and subsequent fibrotic response.

8.5 miRNAs in the Skin of SSc

A lot of researches have shown the roles of miRNAs in the pathogenesis of SSc. In 2010, Maurer et al. described that miR-29a was strongly downregulated in cultured SSc dermal fibroblasts, involving skin of SSc and mice model of bleomycin-induced skin fibrosis as compared with the controls [47]. miR-29 forced knockdown in cultured normal dermal fibroblasts significantly increased the levels of mRNA and protein for type I and type III collagen, and forced overexpression in SSc fibroblasts decreased them. Pre-miR-29a transfection significantly downregulated the collagen luciferase activity, indicating a direct regulation of collagen by miR-29a. Fibrosis-related cytokines including TGF-β1, platelet-derived growth factor (PDGF)-B, or interleukin (IL)-4 reduced the levels of miR-29a in normal fibroblasts. This is the first evidence of contribution of miRNAs to the pathogenesis of SSc.

Next, Li et al. performed miRNA microarray chip analysis and compared the miRNA expression profiles of SSc skin and healthy controls [48]. As a result, nine miRNAs were upregulated and 15 miRNAs were downregulated in SSc skin by the array. Among them, the expression of three miRNAs (miR-206, miR-125b, and let-7g) was validated by real-time PCR. For example, miR-125b is the putative regulator of various SSc-related molecules (e.g., Smad5, IL13, CD28, IL6R, IL1F10). There is a possibility that the downregulation of miR-125b in SSc is involved in the pathogenesis of SSc via the induction of these molecules. Zhu et al. also tried to find out fibrosis-related miRNAs, and miR-21, miR-31, miR-146, miR-503, miR-145, and miR-29b were identified as the candidates [49]. They suggested that miR-21 was increased but miR-145 and miR-29b were decreased both in the skin tissues and cultured fibroblasts of SSc. TGF-β stimulation increased

the expression of miR-21 or miR-145 and decreased mRNA levels of Smad7 or Smad3, respectively. Similarly, the cytokine decreased the expression of miR-29b and increased α1(I) collagen mRNA. Accordingly, these miRNAs may contribute to the pathogenesis of SSc via the regulation of Smad7, Smad3, and α1(I) collagen.

On the other hand, we focused on miRNAs which target α1(I) collagen or α2 (I) collagen and tried to evaluate the possibility that miRNAs play a part in the mechanisms of constitutive upregulation of type I collagen in SSc fibroblasts. PCR array showed expression of several collagen-related miRNAs that were decreased in both TGF-β-stimulated normal dermal fibroblasts or cultured SSc fibroblasts compared with normal fibroblasts [50, 51]. miR-196a and let-7a, the putative regulators of both α1(I) and α2(I) collagen, were significantly decreased in SSc fibroblasts both in vivo and in vitro. The transfection of miRNA inhibitors specific for miR-196a or let-7a induced the expression of α1(I) and α2(I) collagen in normal dermal fibroblasts, while their mimics reduced them in SSc fibroblasts. Taken together, the downregulation of miR-196a or let-7a by the intrinsic activation of TGF-β in SSc dermal fibroblasts may also play a role in the constitutive upregulation of collagen.

miRNAs are likely to be involved in the regulation of other SSc-related molecules. For example, the constitutive overexpression of integrins is reported to be a key event to induce the collagen dysregulation of SSc, but the mechanism is still unknown [52]. We focused on miR-150, which is decreased in SSc dermal fibroblasts as the direct regulator of integrin β3 [53]. The forced supplementation of miR-150 downregulated the expression of integrin β3, phosphorylated Smad3, and type I collagen in SSc dermal fibroblasts, whereas the inhibition of miR-150 upregulated them in normal fibroblasts. Treatment of SSc fibroblasts with 5-aza-2-′-deoxycytidine resulted in the recovery of miR-150 expression, suggesting that its downregulation in these cells is caused by DNA methylation. On the other hand, constitutive MMP-1 downregulation is thought to accelerate the collagen accumulation in SSc fibrotic skin [52]. We found that the upregulation of miR-92a causes the downregulation of MMP-1 expression in SSc dermal fibroblasts [54]. Because MMP-1 is not predicated as the target of miR-92a by the bioinformatics programs described above, we suppose miR-92a indirectly controls MMP-1 expression via other direct targets. miR-92a expression was upregulated in SSc fibroblasts, which was normalized by the transfection with TGF-β siRNA. Thus, upregulation of miR-92a may result from the intrinsic activation of TGF-β in SSc fibroblasts, which mediate the constitutive downregulation of MMP-1.

miRNAs also mediate the effects of other cytokines or molecules in SSc fibroblasts. IL-17 signal is implicated in the pathogenesis of autoimmune diseases, and IL-17 receptor is found to be downregulated in SSc dermal fibroblasts [55]. We found that IL-17A suppressed the protein expression of α1(I) collagen. miR-129-5p, one of the miRNAs decreased in SSc fibroblasts, was upregulated by IL-17A stimulation and mediated the α1(I) collagen downregulation. Taken together, these results indicated that IL-17A signaling has an anti-fibrogenic effect by the induction of miR-129-5p and the subsequent decrease of α1(I) collagen. In SSc fibroblasts, IL-17A-miR-129-5p pathway is inhibited by the IL-17 receptor downregulation,

contributing to the excess collagen accumulation [55]. Similarly, thrombospondin family has attracted attention due to its functions in TGF-β signaling, ECM regulation, and angiogenesis. Thrombospondin-2 mRNA levels were significantly lower in cultured SSc dermal fibroblasts than in normal fibroblasts [56]. Thrombospondin-2 knockdown by the siRNA led to the downregulation of α2 (I) collagen expression via the upregulation of miR-7. Consistently, miR-7 levels were upregulated in SSc dermal fibroblasts compared to normal fibroblasts both in vivo and in vitro. Thus, thrombospondin-2 downregulation and the subsequent miR-7 upregulation in SSc fibroblasts may be due to a negative feedback mechanism against tissue fibrosis.

Taken together, these data suggest that further investigation of the regulatory mechanisms of tissue fibrosis induced by miRNAs lead to the better understanding of the pathogenesis of this disease. Furthermore, recently, single nucleotide polymorphisms associated with miRNAs, MIRSNPs, are found to alter the expression and function of the miRNAs [42, 57]. For example, miR-146a is one of the miRNAs involved in the immune system [58]. Double-stranded miR-146a is originated from mir-146a precursor (CCGAUGUGUAUCCUCAGCUUUGAGAACUGAAUUCCAUGGGUUGUGUCAGUGUCAGACCU**C/G**UGAAAUUCAGUUCUUCAGCUGGGAUAUCUCUGUCAUCGU). Mature single-stranded miR-146a (UGAGAACUGAAUUCCAUGGGUU) and its complementary strand (CCU**C/G**UGAAAUUCAGUUCUUCAG) are then separated from the double-stranded miR-146a, and MIRSNP rs2910164 is located in the complementary strand. The change from C to G allele in rs2910164 may stabilize the precursor [59], resulting in the increased expression of mature single-stranded miR-146a. By investigating the role of rs2910164 in SSc, we found that patients with CC genotype had telangiectasia at significantly higher prevalence than those with CG or GG genotype [60]. Given that miR-146a is also reportedly associated with vascular abnormalities [61], the MIRSNP may be a risk factor of vascular abnormality in SSc patients.

8.6 Extracellular miRNAs as Potential Biomarkers

In various human diseases, clinically useful biomarkers have been utilized by the recent advances of molecular biological approaches. For example, serum levels of thymus- and activation-regulated chemokine (TARC) have become available as the useful marker for determining disease activity of atopic dermatitis. Furthermore, α2 (I) collagen / PDGF-B chain fusion gene is highly specific to dermatofibrosarcoma protuberans, and detection of the fusion gene is able to differentiate dermatofibrosarcoma protuberans from dermatofibroma. On the other hand, the activation of cytokines including TGF-β and connective tissue growth factor (CTGF) in dermal fibroblasts seems to play important roles in the increased collagen production of SSc. However, serum levels of these cytokines are not correlated with the diagnosis or evaluation of disease activity. We have then focused on the possibility that miRNAs may become novel biomarkers. Although miRNAs usually exist inside

the cells, a number of miRNAs have been found in various body fluids including serum, plasma, saliva, urine, and milk [62–68]. miRNAs have been believed to be immediately degraded by RNAse in the body fluids, but a lot of papers indicated that extracellular miRNAs are protected from RNAse as well as harsh conditions including extreme temperatures, extreme pHs, or freeze-thaw cycles [69]. Currently, there seem to be at least four pathways via which miRNAs are secreted into extracellular spaces [70]: First, extracellular miRNAs are encapsulated and protected in shedding vesicles. Second, they can be enveloped in membranous micro vesicles called exosome shed from cell plasma membrane. Third, they are wrapped by apoptotic cells or broken cells, and lastly, miRNAs form complex with proteins or lipoproteins such as argonaute2, nucleophosmin 1 (NPM1), and high-density lipoprotein (HDL). Accordingly, the quantification of extracellular miRNAs can be promising biomarkers to monitor pathophysiological status. Many researchers have already revealed that extracellular miRNA levels are useful disease markers to evaluate the diagnosis, prognosis, and curative effects, especially in various cancers. Considering that RNAs or miRNAs usually respond to various stimuli more quickly than proteins, extracellular miRNA can be more sensitive markers. There have already been several papers demonstrating alteration of extracellular miRNA levels in autoimmune diseases or fibrotic diseases. For example, Michael et al. suggested the possibility of exosome-associated miRNAs in saliva as a biomarker of Sjögren's syndrome [71]. Furthermore, serum levels of miR-21, miR-20a, miR-125b-5p, miR-128, miR-30e, or miR-20b are reported as biomarkers of kidney fibrosis, liver fibrosis, or smoking-related lung fibrosis [72–74].

We have also determined levels of extracellular miRNAs in SSc patients. As described above, miR-29a is the firstly discovered miRNAs implicated in the fibrogenesis of SSc [47]. We found miR-29a was present in the serum, but there was no significant difference in the serum miR-29a levels between SSc patients and normal subjects [75]. However, patients diagnosed with scleroderma spectrum disorder (SSD), who do not fulfill the criteria of SSc but were thought to develop SSc in the future [76], had significantly lower serum miR-29a levels than SSc patients as well as healthy control subjects. Thus, serial time course measurement of serum miR-29a levels in SSD patients may lead to the early diagnosis of SSc. Downregulation of serum miR-29a levels in SSD patients indicates that the dysregulation of fibrosis-related molecules including collagen may be initiated by decreased miR-29a at the SSD stage and maintained by other factors at SSc stage. In addition, Doppler echocardiography showed significantly higher right ventricular systolic pressure in SSc patients with lower miR-29a levels than those with normal levels. Thus, miR-29a may be involved in both fibrosis and vasculopathy of SSc.

We also found the serum levels of miR-196a and let-7a were detectable and quantitative in the sera of SSc patients [50, 51]. miR-196a expression was significantly decreased in SSc dermal fibroblasts compared to normal fibroblasts as described above, but serum miR-196a levels were similar between normal subjects and SSc patients. However, modified Rodnan total skin thickness score was higher

in SSc patients with lower serum miR-196a levels than those with normal levels. On the other hand, let-7a levels were significantly downregulated in the sera of SSc patients. When we analyzed the correlation between serum let-7a levels and clinical/serological features, patients with reduced serum let-7a levels had significantly higher skin thickness score, suggesting that serum let-7a levels are inversely correlated with the extent of skin sclerosis. Furthermore, these patients showed significantly lower frequency of anti-centromere antibody than those with elevated let-7a levels. Tanaka et al. identified miR-30b as the most strongly downregulated serum miRNA of SSc patients [77]. miR-30b is the direct suppressor of PDGFR-β, and its serum levels were inversely correlated with skin thickness scores. Taken together, these results suggest that serum miRNAs are putative biomarkers of the pathogenic triad of SSc including tissue fibrosis, vasculopathy, and autoimmunity.

However, measuring only one of nearly 2,500 miRNAs may be less accurate to diagnose SSc. Our hypothesis is that expression pattern of multiple miRNAs may become more reliable and significant marker for SSc. Serum levels of 6 miRNAs (miR-7 g, miR-21, miR-29b, miR-125, miR-145, and miR-206), chosen based on previous studies [34, 47–49], were determined by real-time PCR in 15 patients with SSc and 15 normal subjects [78]. There were no significant difference in the levels of each miRNA level between SSc patients and normal subjects. However, when we ranked the six miRNA levels of each individual to determine the difference of expression patterns more clearly, there was a significant difference in the ranks among miRNAs only in patients with SSc. Furthermore, there was a strong and significant correlation between the levels of let-7g and miR-125b only in normal subjects. We also showed that the combination of miR-206 level and miR-21 level increases the area under curves (AUCs) of receiver operating characteristics curve analysis compared with either miR-206 or miR-21 alone. Accordingly, these data suggest different miRNA expression patterns in SSc patients and that the combination of miRNA levels is more reliable to diagnose SSc patients. Furthermore, investigation of the "miRNA network" in the pathogenesis may lead to the development of new diagnostic tools.

Recently we also focused on hair miRNAs, because hairs are more accessible than sera among human samples: It is sometimes difficult to take blood samples of SSc patients due to their narrowed vessels, while hair samples may be easier to obtain repeatedly. Hair shafts lose nuclear components, and DNA or RNA is believed to be degraded except for mitochondrial DNA. However, Lefkowitz et al. have proven the existence of RNAs including miRNAs in both hair roots and hair shafts [79]. If extracellular miRNAs in the hair are protected in exosome or shedding vesicles, hair miRNA levels can be stable and become novel biomarkers. We confirmed that several miRNAs in hair roots and hair shafts were detectable and quantitative using our method [80]. There was the reproducibility of hair miRNA levels, because the difference of miRNA levels in hair roots or hair shafts obtained from different places of head in each individual were less than twofold. PCR array showed different expression patterns of miRNAs in sera, hair roots, and hair shafts, indicating that they can be independent biomarkers. For example, miR-196a levels in hair shafts were significantly lower in SSc patients compared to those in normal

subjects, whereas serum and hair root miR-196a levels were not significantly changed in SSc patients. On the other hand, we also evaluated whether hair shaft miR-29a levels are able to be utilized as the disease marker [81]. As a result, hair miR-29a levels in SSc patients were significantly lower than those in control subjects or dermatomyositis patients. Furthermore, SSc patients with lower miR-29a levels tended to have contracture of the phalanges at a significantly higher prevalence than those without. Therefore, hair miRNAs are also likely to be available as the marker of the diagnosis or estimation of disease activity of SSc. As the limitation, future study of extracellular miRNAs is mandatory to clarify the difference in the expression pattern and function among miRNAs in the exosome, shedding vesicles, or apoptotic cells.

Currently, two clinical trials "Study of microRNA using stored serum of subjects in clinical trial of influenza vaccine which was added AdvaxTM" (UMIN000012979) and "Clinical Study using serum samples obtained in clinical trial of absorbed influenza vaccine" (UMIN000013074) seem to be ongoing. These studies may accelerate the clinical use of extracellular miRNAs as biomarkers.

8.7 miRNA Treatments for SSc

To date, prednisolone is sometimes used for the cutaneous fibrosis of SSc. However, it may cause significant adverse effects including renal crisis. Randomized control trials indicated that methotrexate or cyclophosphamide improves skin thickness score of SSc [82], but their effects are often limited. Accordingly, development of novel therapeutic approaches is urgently needed.

One of the first researches that examined the effect of injections of miRNA mimics or inhibitors into mice models is the report of Li et al. [83]. They showed that miR-21 targets TPM1 or PTEN and represses apoptosis of cancer cells. Repeated injection of miR-21 antisense oligonucleotide inhibited tumor formation of tongue squamous cell carcinoma cells in nude mice by reducing cell proliferation and inducing apoptosis. Furthermore, such miRNA therapy may also be effective for autoimmune diseases. Nagata et al. induced the expression of miR-15a, negative regulator of Bcl-2, in the synovium of DBA/1J mice by the double-stranded miR-15a intraarticular injection [84]. Bcl-2 protein was downregulated and cell apoptosis is induced in the synovium of these mice, suggesting the therapeutic values of miRNA injection. In addition, Nakasa et al. found that miR-146a expression is upregulated in the peripheral blood mononuclear cells of patients with rheumatoid arthritis. Forced overexpression of miR-146a leads to decreased number of TRAP-positive multinucleated cells and decreased expression of target genes including c-Jun, NFATc1, PU.1, and TRAP in vitro [85]. The authors then showed the preventive effect of systemic administration of miR-146a on the joint destruction in collagen-induced arthritis mice model.

In SSc, Peng et al. indicated the therapeutic potential of targeting miR-29 [86], and we first demonstrated that we can overexpress let-7a in the mouse skin by intermittent intraperitoneal injection of the mixtures of miRNA and atelocollagen,

which prevented the skin fibrosis induced by bleomycin [51]. Further studies need to be performed to evaluate any adverse effects.

The clinical use of miRNA treatments is under consideration. miR-122 is reported to be important for HCV RNA accumulation in cultured liver cells [87]. Lanford et al. showed that SPC3649, the oligonucleotide targeting miR-122, induces long-lasting suppression of HCV viremia in chronically infected chimpanzee model, without viral resistance or adverse effects [87]. The randomized, double-blind, placebo-controlled Phase 2a proof-of-concept study of SPC3649 (Miravirsen, Santaris Pharma) was performed to evaluate the effect, safety, and tolerability of the drug in patients with HCV. Dose-dependent, continuous, and prolonged antiviral activity was observed, and miravirsen was well tolerated in HCV patients, indicating that miravirsen can be a new antiviral therapy with a high barrier to resistance in human. To date, miravirsen seem to be the only anti-miRNA treatment under clinical trial, and its successful clinical use will further miRNA therapies in various autoimmune diseases including SSc in the future. For the treatment of each disease, miRNAs downregulated in the tissues should be supplemented using miRNA mimics, and upregulated miRNAs should be inhibited by miRNA inhibitors. Human disease is often caused by multiple factors, not by single factor. miRNAs can regulate multiple targets, and the advantages of miRNA treatments may be the normalization of such multiple factors in each disease at the same time. To avoid unexpected adverse effects, the following problems should be assessed: (1) clarification of mechanisms via which miRNAs regulate target genes, (2) identification of most essential miRNAs in the etiology of each disease, (3) development of delivery system for effective transfer of miRNAs to the target tissues, and (4) adjustment of appropriate dose of miRNAs to be administrated.

8.8 Conclusion

The abnormalities in epigenetics including methylation, histone modification, and noncoding RNAs have been reported in SSc. The clarification of the mechanism by which epigenetic abnormalities contribute to the pathogenesis of SSc may lead to the development of new diagnostic tools and new therapeutic approaches.

References

1. Feghali-Bostwick C, Medsger TA, Wright TM. Analysis of systemic sclerosis in twins reveals low concordance for disease and high concordance for the presence of antinuclear antibodies. Arthritis Rheum. 2003;48:1956–63. doi:10.1002/art.11173.
2. Luo Y, Wang Y, Wang Q, Xiao R, Lu Q. Systemic sclerosis: genetics and epigenetics. J Autoimmun. 2013;41:161–7. doi:10.1016/j.jaut.2013.01.012.
3. Altorok N, Almeshal N, Wang Y, Kahaleh B. Epigenetics, the holy grail in the pathogenesis of systemic sclerosis. Rheumatology (Oxford). 2014. doi:10.1093/rheumatology/keu155.
4. Richardson B. Primer: epigenetics of autoimmunity. Nat Clin Pract Rheumatol. 2007;3:521–7. doi:10.1038/ncprheum0573.

5. Miranda TB, Jones PA. DNA methylation: the nuts and bolts of repression. J Cell Physiol. 2007;213:384–90. doi:10.1002/jcp.21224.
6. Asano Y, Bujor AM, Trojanowska M. The impact of Fli1 deficiency on the pathogenesis of systemic sclerosis. J Dermatol Sci. 2010;59:153–62. doi:10.1016/j.jdermsci.2010.06.008.
7. Noda S, Asano Y, Nishimura S, Taniguchi T, Fujiu K, Manabe I, et al. Simultaneous downregulation of KLF5 and Fli1 is a key feature underlying systemic sclerosis. Nat Commun. 2014;5:5797. doi:10.1038/ncomms6797.
8. Wang Y, Fan PS, Kahaleh B. Association between enhanced type I collagen expression and epigenetic repression of the FLI1 gene in scleroderma fibroblasts. Arthritis Rheum. 2006;54:2271–9. doi:10.1002/art.21948.
9. Kahaleh B, Wang W. Decrease activity of DNA demethylase in SSC fibroblast and microvascular endothelial cells: a possible mechanism for persistent SSC phenotype. Rheumatology. 2012;51:5–6.
10. Gay S, Wilson AG. The emerging role of epigenetics in rheumatic diseases. Rheumatology (Oxford). 2014;53:406–14. doi:10.1093/rheumatology/ket292.
11. Lei W, Luo Y, Yan K, Zhao S, Li Y, Qiu X, et al. Abnormal DNA methylation in CD4+ T cells from patients with systemic lupus erythematosus, systemic sclerosis, and dermatomyositis. Scand J Rheumatol. 2009;38:369–74. doi:10.1080/03009740902758875.
12. Sawalha AH, Kovats S. Dehydroepiandrosterone in systemic lupus erythematosus. Curr Rheumatol Rep. 2008;10:286–91. PubMed.
13. Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor β in human disease. N Engl J Med. 2000;342:1350–8. doi:10.1056/NEJM200005043421807.
14. Jiang H, Xiao R, Lian X, Kanekura T, Luo Y, Yin Y, et al. Demethylation of TNFSF7 contributes to CD70 overexpression in CD4+ T cells from patients with systemic sclerosis. Clin Immunol. 2012;143:39–44. doi:10.1016/j.clim.2012.01.005.
15. Lian X, Xiao R, Hu X, Kanekura T, Jiang H, Li Y, et al. DNA demethylation of CD40l in CD4 + T cells from women with systemic sclerosis: a possible explanation for female susceptibility. Arthritis Rheum. 2012;64:2338–45. doi:10.1002/art.34376.
16. Valentini G, Romano MF, Naclerio C, Bisogni R, Lamberti A, Turco MC, et al. Increased expression of CD40 ligand in activated CD4+ T lymphocytes of systemic sclerosis patients. J Autoimmun. 2000;15:61–6. doi:10.1006/jaut.2000.0387.
17. Selmi C, Feghali-Bostwick CA, Lleo A, Lombardi SA, De Santis M, Cavaciocchi F, et al. X chromosome gene methylation in peripheral lymphocytes from monozygotic twins discordant for scleroderma. Clin Exp Immunol. 2012;169:253–62. doi:10.1111/j.1365-2249.2012.04621.x.
18. Romero LI, Zhang DN, Cooke JP, Ho HK, Avalos E, Herrera R, et al. Differential expression of nitric oxide by dermal microvascular endothelial cells from patients with scleroderma. Vasc Med. 2000;5:147–58. PubMed.
19. Wang Y, Kahaleh B. Epigenetic repression of bone morphogenetic protein receptor II expression in scleroderma. J Cell Mol Med. 2013;17:1291–9. doi:10.1111/jcmm.12105.
20. Kondo Y, Shen L, Cheng AS, Ahmed S, Boumber Y, Charo C, et al. Gene silencing in cancer by histone H3 lysine 27 trimethylation independent of promoter DNA methylation. Nat Genet. 2008;40:741–50. doi:10.1038/ng.159.
21. Huber LC, Distler JH, Moritz F, Hemmatazad H, Hauser T, Michel BA, et al. Trichostatin A prevents the accumulation of extracellular matrix in a mouse model of bleomycin-induced skin fibrosis. Arthritis Rheum. 2007;56:2755–64. doi:10.1002/art.22759.
22. Hemmatazad H, Rodrigues HM, Maurer B, Brentano F, Pileckyte M, Distler JH, et al. Histone deacetylase 7, a potential target for the antifibrotic treatment of systemic sclerosis. Arthritis Rheum. 2009;60:1519–29. doi:10.1002/art.24494.
23. Krämer M, Dees C, Huang J, Schlottmann I, Palumbo-Zerr K, Zerr P, et al. Inhibition of H3K27 histone trimethylation activates fibroblasts and induces fibrosis. Ann Rheum Dis. 2013;72:614–20. doi:10.1136/annrheumdis-2012-201615.

24. Wang Y, Yang Y, Luo Y, Yin Y, Wang Q, Li Y, et al. Aberrant histone modification in peripheral blood B cells from patients with systemic sclerosis. Clin Immunol. 2013;149:46–54. doi:10.1016/j.clim.2013.06.006.
25. Li G, Zhang H, Wan X, Yang X, Zhu C, Wang A, et al. Long noncoding RNA plays a key role in metastasis and prognosis of hepatocellular carcinoma. Biomed Res Int. 2014;2014:780521. doi:10.1155/2014/780521.
26. Bhan A, Mandal SS. Long noncoding RNAs: emerging stars in gene regulation, epigenetics and human disease. ChemMedChem. 2014;9:1932–56. doi:10.1002/cmdc.201300534.
27. Eades G, Zhang YS, Li QL, Xia JX, Yao Y, Zhou Q. Long non-coding RNAs in stem cells and cancer. World J Clin Oncol. 2014;5:134–41. doi:10.5306/wjco.v5.i2.134.
28. Cao G, Zhang J, Wang M, Song X, Liu W, Mao C, et al. Differential expression of long non-coding RNAs in bleomycin-induced lung fibrosis. Int J Mol Med. 2013;32:355–64. doi:10.3892/ijmm.2013.1404.
29. Bartel D. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116:281–97. doi: S0092867404000455 [pii].
30. Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ. Processing of primary microRNAs by the Microprocessor complex. Nature. 2004;432:231–5. doi:10.1038/nature03049.
31. Lee R, Feinbaum R, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993;75:843–54. doi: 0092-8674(93)90529-Y [pii].
32. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, et al. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature. 2000;403:901–6. doi:10.1038/35002607.
33. Jinnin M. Contribution of miRNAs to the pathogenesis of scleroderma and targeting them for the diagnosis and treatment. Exp Rev Dermatol. 2013;8:417–26.
34. Jinnin M. Various applications of microRNAs in skin diseases. J Dermatol Sci. 2014;74:3–8. doi:10.1016/j.jdermsci.2014.01.004.
35. Maragkakis M, Alexiou P, Papadopoulos GL, Reczko M, Dalamagas T, Giannopoulos G, et al. Accurate microRNA target prediction correlates with protein repression levels. BMC Bioinformatics. 2009;10:295. doi:10.1186/1471-2105-10-295.
36. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005;120:15–20.
37. John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS. Human microRNA targets. PLoS Biol. 2004;2, e363. doi:10.1371/journal.pbio.0020363.
38. Kiriakidou M, Nelson PT, Kouranov A, Fitziev P, Bouyioukos C, Mourelatos Z, et al. A combined computational-experimental approach predicts human microRNA targets. Genes Dev. 2004;18:1165–78. doi: 1184704 [pii].
39. Krek A, Grün D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, et al. Combinatorial microRNA target predictions. Nat Genet. 2005;37:495–500. doi: ng1536 [pii].
40. Ceppi M, Pereira PM, Dunand-Sauthier I, Barras E, Reith W, Santos MA, et al. MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. Proc Natl Acad Sci U S A. 2009;106:2735–40. doi: 0811073106 [pii].
41. Martinez-Nunez RT, Louafi F, Friedmann PS, Sanchez-Elsner T. MicroRNA-155 modulates the pathogen binding ability of dendritic cells (DCs) by down-regulation of DC-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN). J Biol Chem. 2009;284:16334–42. doi: M109.011601.
42. Furer V, Greenberg J, Attur M, Abramson S, Pillinger M. The role of microRNA in rheumatoid arthritis and other autoimmune diseases. Clin Immunol. 2010;136:1–15. doi: S1521-6616(10)00046-X [pii].
43. Li QJ, Chau J, Ebert PJ, Sylvester G, Min H, Liu G, et al. miR-181a is an intrinsic modulator of T cell sensitivity and selection. Cell. 2007;129:147–61. doi: S0092-8674(07)00319-4 [pii].

44. Lu LF, Thai TH, Calado DP, Chaudhry A, Kubo M, Tanaka K, et al. Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein. Immunity. 2009;30:80–91. doi: S1074-7613(08)00556-6 [pii].
45. Vettori S, Gay S, Distler O. Role of microRNAs in fibrosis. Open Rheumatol J. 2012;6:130–9. doi:10.2174/1874312901206010130.
46. van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, et al. Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc Natl Acad Sci U S A. 2008;105:13027–32. doi:10.1073/pnas.0805038105.
47. Maurer B, Stanczyk J, Jüngel A, Akhmetshina A, Trenkmann M, Brock M, et al. MicroRNA-29, a key regulator of collagen expression in systemic sclerosis. Arthritis Rheum. 2010;62:1733–43. doi:10.1002/art.27443.
48. Li H, Yang R, Fan X, Gu T, Zhao Z, Chang D, et al. MicroRNA array analysis of microRNAs related to systemic scleroderma. Rheumatol Int. 2010. doi:10.1007/s00296-010-1615-y.
49. Zhu H, Li Y, Qu S, Luo H, Zhou Y, Wang Y, et al. MicroRNA expression abnormalities in limited cutaneous scleroderma and diffuse cutaneous scleroderma. J Clin Immunol. 2012;32:514–22. doi:10.1007/s10875-011-9647-y.
50. Honda N, Jinnin M, Kajihara I, Makino T, Makino K, Masuguchi S, et al. TGF-β-mediated downregulation of microRNA-196a contributes to the constitutive upregulated type I collagen expression in scleroderma dermal fibroblasts. J Immunol. 2012;188:3323–31. doi: jimmunol.1100876 [pii].
51. Makino K, Jinnin M, Hirano A, Yamane K, Eto M, Kusano T, et al. The downregulation of microRNA let-7a contributes to the excessive expression of type I collagen in systemic and localized scleroderma. J Immunol. 2013;190:3905–15. doi:10.4049/jimmunol.1200822.
52. Jinnin M. Mechanisms of skin fibrosis in systemic sclerosis. J Dermatol. 2010;37:11–25.
53. Honda N, Jinnin M, Kira-Etoh T, Makino K, Kajihara I, Makino T, et al. miR-150 down-regulation contributes to the constitutive type I collagen overexpression in scleroderma dermal fibroblasts via the induction of integrin β3. Am J Pathol. 2013;182:206–16. doi:10.1016/j.ajpath.2012.09.023.
54. Sing T, Jinnin M, Yamane K, Honda N, Makino K, Kajihara I, et al. microRNA-92a expression in the sera and dermal fibroblasts increases in patients with scleroderma. Rheumatology (Oxford). 2012;51:1550–6. doi: kes120 [pii].
55. Nakashima T, Jinnin M, Yamane K, Honda N, Kajihara I, Makino T, et al. Impaired IL-17 signaling pathway contributes to the increased collagen expression in scleroderma fibroblasts. J Immunol. 2012;188:3573–83. doi: jimmunol.1100591 [pii].
56. Kajihara I, Jinnin M, Yamane K, Makino T, Honda N, Igata T, et al. Increased accumulation of extracellular thrombospondin-2 due to low degradation activity stimulates type I collagen expression in scleroderma fibroblasts. Am J Pathol. 2012;180:703–14. doi: S0002-9440(11)01022-4 [pii].
57. Bertino JR, Banerjee D, Mishra PJ. Pharmacogenomics of microRNA: a miRSNP towards individualized therapy. Pharmacogenomics. 2007;8:1625–7. doi:10.2217/14622416.8.12.1625.
58. Okada Y, Jinnin M, Makino T, Kajihara I, Makino K, Honda N, et al. MIRSNP rs2910164 of miR-146a is associated with the muscle involvement in polymyositis/dermatomyositis. Int J Dermatol. 2014;53:300–4.
59. Yamashita J, Iwakiri T, Fukushima S, Jinnin M, Miyashita A, Hamasaki T, et al. The rs2910164 G>C polymorphism in microRNA-146a is associated with the incidence of malignant melanoma. Melanoma Res. 2013;23:13–20. doi:10.1097/CMR.0b013e32835c5b30.
60. Sakoguchi A, Jinnin M, Makino T, Kajihara I, Makino K, Honda N, et al. The miR-146a rs2910164 C/G polymorphism is associated with telangiectasia in systemic sclerosis. Clin Exp Dermatol. 2012;38:99–100. doi:10.1111/j.1365-2230.2012.04453.x.
61. Nazari-Jahantigh M, Wei Y, Schober A. The role of microRNAs in arterial remodelling. Thromb Haemost. 2012;107:611–8. doi:10.1160/TH11-12-0826.

62. Valenti R, Huber V, Iero M, Filipazzi P, Parmiani G, Rivoltini L. Tumor-released microvesicles as vehicles of immunosuppression. Cancer Res. 2007;67:2912–5. doi: 67/7/2912 [pii].
63. Gilad S, Meiri E, Yogev Y, Benjamin S, Lebanony D, Yerushalmi N, et al. Serum microRNAs are promising novel biomarkers. PLoS One. 2008;3, e3148. doi:10.1371/journal.pone.0003148.
64. Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L, et al. Detection of microRNA expression in human peripheral blood microvesicles. PLoS One. 2008;3, e3694. doi:10.1371/journal.pone.0003694.
65. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res. 2008;18:997–1006. doi: cr2008282 [pii].
66. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008;105:10513–8. doi: 0804549105.
67. Hanke M, Hoefig K, Merz H, Feller AC, Kausch I, Jocham D, et al. A robust methodology to study urine microRNA as tumor marker: microRNA-126 and microRNA-182 are related to urinary bladder cancer. Urol Oncol. 2010;28:655–61. doi:10.1016/j.urolonc.2009.01.027.
68. Kosaka N, Izumi H, Sekine K, Ochiya T. microRNA as a new immune-regulatory agent in breast milk. Silence. 2010;1:7. doi:10.1186/1758-907X-1-7.
69. Chen X, Liang H, Zhang J, Zen K, Zhang CY. Horizontal transfer of microRNAs: molecular mechanisms and clinical applications. Protein Cell. 2012;3:28–37. doi:10.1007/s13238-012-2003-z.
70. van Dommelen SM, Vader P, Lakhal S, Kooijmans SA, van Solinge WW, Wood MJ, et al. Microvesicles and exosomes: opportunities for cell-derived membrane vesicles in drug delivery. J Control Release. 2012;161:635–44. doi:10.1016/j.jconrel.2011.11.021.
71. Michael A, Bajracharya SD, Yuen PS, Zhou H, Star RA, Illei GG, et al. Exosomes from human saliva as a source of microRNA biomarkers. Oral Dis. 2010;16:34–8. doi:10.1111/j.1601-0825.2009.01604.x.
72. Glowacki F, Savary G, Gnemmi V, Buob D, Van der Hauwaert C, Lo-Guidice JM, et al. Increased circulating miR-21 levels are associated with kidney fibrosis. PLoS One. 2013;8, e58014. doi:10.1371/journal.pone.0058014.
73. Shrivastava S, Petrone J, Steele R, Lauer GM, Bisceglie AM, Ray RB. Upregulation of circulating miR-20a is correlated with hepatitis C virus mediated liver disease progression. Hepatology. 2013. doi:10.1002/hep.26296.
74. Huang Y, Dai Y, Zhang J, Wang C, Li D, Cheng J, et al. Circulating microRNAs as potential biomarkers for smoking-related interstitial fibrosis. Biomarkers. 2012;17:435–40. doi:10.3109/1354750X.2012.680611.
75. Kawashita Y, Jinnin M, Makino T, Kajihara I, Makino K, Honda N, et al. Circulating miR-29a levels in patients with scleroderma spectrum disorder. J Dermatol Sci. 2011;61:67–9. doi: S0923-1811(10)00339-7 [pii].
76. Ihn H, Sato S, Tamaki T, Soma Y, Tsuchida T, Ishibashi Y, et al. Clinical evaluation of scleroderma spectrum disorders using a points system. Arch Dermatol Res. 1992;284:391–5.
77. Tanaka S, Suto A, Ikeda K, Sanayama Y, Nakagomi D, Iwamoto T, et al. Alteration of circulating miRNAs in SSc: miR-30b regulates the expression of PDGF receptor β. Rheumatology (Oxford). 2013;52:1963–72. doi:10.1093/rheumatology/ket254.
78. Koba S, Jinnin M, Inoue K, Nakayama W, Honda N, Makino K, et al. Expression analysis of multiple microRNAs in each patient with scleroderma. Exp Dermatol. 2013;22:489–91. doi:10.1111/exd.12173.
79. Lefkowitz GK, Mukhopadhyay A, Cowing-Zitron C, Yu BD. The post-apoptotic fate of RNAs identified through high-throughput sequencing of human hair. PLoS One. 2011;6, e27603. doi:10.1371/journal.pone.0027603.

80. Wang Z, Jinnin M, Kudo H, Inoue K, Nakayama W, Honda N, et al. Detection of hair-microRNAs as the novel potent biomarker: evaluation of the usefulness for the diagnosis of scleroderma. J Dermatol Sci. 2013;72:134–41. doi:10.1016/j.jdermsci.2013.06.018.
81. Takemoto R, Jinnin M, Wang Z, Kudo H, Inoue K, Nakayama W, et al. Hair miR-29a levels are decreased in patients with scleroderma. Exp Dermatol. 2013;22:832–3. doi:10.1111/exd.12245.
82. Kowal-Bielecka O, Landewé R, Avouac J, Chwiesko S, Miniati I, Czirjak L, et al. EULAR recommendations for the treatment of systemic sclerosis: a report from the EULAR Scleroderma Trials and Research group (EUSTAR). Ann Rheum Dis. 2009;68:620–8. doi:10.1136/ard.2008.096677.
83. Li J, Huang H, Sun L, Yang M, Pan C, Chen W, et al. MiR-21 indicates poor prognosis in tongue squamous cell carcinomas as an apoptosis inhibitor. Clin Cancer Res. 2009;15:3998–4008. doi:10.1158/1078-0432.CCR-08-3053.
84. Nagata Y, Nakasa T, Mochizuki Y, Ishikawa M, Miyaki S, Shibuya H, et al. Induction of apoptosis in the synovium of mice with autoantibody-mediated arthritis by the intraarticular injection of double-stranded MicroRNA-15a. Arthritis Rheum. 2009;60:2677–83. doi:10.1002/art.24762.
85. Nakasa T, Shibuya H, Nagata Y, Niimoto T, Ochi M. The inhibitory effect of microRNA-146a expression on bone destruction in collagen-induced arthritis. Arthritis Rheum. 2011;63:1582–90. doi:10.1002/art.30321.
86. Peng WJ, Tao JH, Mei B, Chen B, Li BZ, Yang GJ, et al. MicroRNA-29: a potential therapeutic target for systemic sclerosis. Expert Opin Ther Targets. 2012;16:875–9. doi:10.1517/14728222.2012.708339.
87. Lanford RE, Hildebrandt-Eriksen ES, Petri A, Persson R, Lindow M, Munk ME, et al. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science. 2010;327:198–201. doi: science.1178178 [pii].

Transforming Growth Factor-ß and Connective Tissue Growth Factor

9

Manabu Fujimoto and Kazuhiko Takehara

Abstract

Systemic sclerosis (SSc) is a disorder of connective tissue characterized by excessive fibrosis affecting different organs such as the skin, lung, and heart. Increasing evidence has demonstrated the fundamental role of cytokines in the pathogenesis. Transforming growth factor-β (TGF-β) is a very potent stimulator of collagen synthesis by fibroblasts. While TGF-β has been considered as a primary cytokine involved in the pathogenesis of SSc (LeRoy et al. Arthritis Rheum32:817–825, 1989; Takehara. J Rheumatol 30:755–759, 2003; Varga and Pasche. Nat Rev Rheumatol 5:200–206, 2009; Lafyatis. Nat Rev Rheumatol 10:706–719, 2014; Ihn. J Dermatol Sci 49:103–113, 2008), additional factors are also likely to play an important role in the initiation and maintenance. These include connective tissue growth factor (CTGF) (Takehara. J Rheumatol 30:755–759, 2003; Ihn. Curr Opin Rheumatol 14:681–685, 2002; Leask. Cell Signal 20:1409–1414, 2008; Jinnin. J Dermatol 37:11–25, 2010). This review summarizes the biology of TGF-β and CTGF and their involvement in SSc, especially in its fibrotic condition.

Keywords

Cytokine • Fibroblast • Smad • Fibrosis

M. Fujimoto (✉)
Department of Dermatology, Faculty of Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8571, Japan
e-mail: mfujimoto@md.tsukuba.ac.jp

K. Takehara
Department of Dermatology, Faculty of Medicine, Institute of Medical, Pharmaceutical and Health Sciences Kanazawa University, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8641, Japan

K. Takehara et al. (eds.), *Systemic Sclerosis*, DOI 10.1007/978-4-431-55708-1_9

9.1 TGF-β

The TGF-β superfamily consists of a variety of cytokines/hormones, including TGF-β, bone morphogenic proteins (BMPs), activins, inhibins, growth differentiation factors, and myostatin [1–6]. TGF-β is a 25 kDa homodimer. In mammals, TGF-β has three isoforms, TGF-β1, TGF-β2, and TGF-β3, encoded by different genes. TGF-β isoforms exhibit similar biological activities, if not identical, in vitro. Nonetheless, studies using mice lacking TGF-β isoforms have revealed that each TGF-β isoform plays an independent and nonredundant role in vivo [7–9]. TGF-β has pleiotropic roles in diverse biological aspects including developmental process, immune system function, vascular function, and connective tissue homeostasis. TGF-β acts as a growth inhibitor for most cell types [10], while TGF-β induces extracellular matrix (ECM) production, including type I, III, VI, VII, and X collagens, fibronectin, and proteoglycans [11–13]. The α_v integrins are the best-defined activators of TGF-β1 and TGF-β3, but do not activate TGF-β2, since TGF-β2–LAP lacks an Arg–Gly–Asp (RGD) motif that mediates integrin binding [2].

TGF-β is generally secreted from monocytes, lymphocytes, and fibroblasts as a latent precursor complex composed of the bioactive peptide of TGF-β, latency-associated peptide (LAP), and latent TGF-β-binding protein (LTBP). TGF-β and LAP form a dimeric complex noncovalently, referred to as the small latent complex (SLC). This configuration sequestrates TGF-β from the binding with its receptors. Furthermore, SLC binds with LTBP, forming the large latent complex (LLC). TGF-β activation is a complex process involving conformational changes of latent TGF-β-binding protein, induced by either cleavage of the latency-associated peptide by proteases such as plasmin, thrombin, plasma transglutaminase, and endoglycosylases or by physical interactions of the latency-associated peptide with other proteins, such as thrombospondin-1 [3]. Integrin $\alpha_v\beta_6$, expressed by epithelial cells, was the first to be demonstrated for the ability of the activation of TGF-β in the lungs [14]. Integrin $\alpha_v\beta_8$ is more widely expressed than $\alpha_v\beta_6$ and can also activate latent TGF-β [15]. Integrin $\alpha_v\beta_8$ is expressed by mesenchymal, immune, neural, and epithelial cells and is induced by IL-1β and thus provides a link between inflammation and TGF-β activation [16]. Furthermore, $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrins have been implicated in TGF-β activation by fibroblasts [2]. Additionally, thrombospondin-1 and peptides derived from it can also activate latent TGF-β in vitro and in vivo [17, 18].

9.2 TGF-β Receptor and Smad Signaling Pathway

TGF-β interacts with receptor heterodimers, TGF-β type I and type II receptors (TGFR-I and TGFR-II) [2, 3]. These receptors are expressed by most cells including mesenchymal and endothelial cells. TGFR-I and TGFR-II both possess serine/threonine kinase activity and are structurally very similar glycoproteins that have a cysteine-rich extracellular domain, a single hydrophobic transmembrane domain, and a C-terminal cytoplasmic serine/threonine kinase domain [19], although they

have distinct roles as described below. To date, at least six distinct type I receptors of the TGF-β superfamily, named activin receptor-like kinases (ALKs), have been identified. Between the transmembrane and the kinase domains, TGFR-I has a region containing a conserved TSGSGSG motif, named the GS domain. The GS domain has serine and threonine residues that undergo phosphorylation and is essential for TGF-β signaling [20]. TGFR-I alone is unable to bind TGF-β, and the binding of TGF-β to TGFR-II initiates the signaling cascade. The major known function of TGFR-II is to phosphorylate TGFR-I, as TGFR-II is unable to signal without TGFR-I [19]. Thus, TGFR-I is thought to determine the specificity of the cellular response to TGF-β whereas TGFR-II is thought to determine the ligand specificity.

Upon the activation, TGFR-I then phosphorylates cytoplasmic proteins belonging to the Smad family [21–24]. TGFR-I specifically recognizes and phosphorylates the receptor-regulated Smad (R-Smad). R-Smads include Smad1, Smad5, and Smad8 downstream of BMP and Smad2 and Smad3 downstream of TGF-β and activin. All R-Smads possess two Mad-homology (MH) domains with a linker region [25]. The N-terminal MH1 domain and the C-terminal MH2 domain have DNA-binding activity and protein-binding property, respectively. TGFR-I principally phosphorylates R-Smads on their serine residues within the C-terminal domain [37]. Upon phosphorylation, R-Smad forms heteromeric complexes with the co-Smad, Smad4. By contrast, the third group of Smad, the inhibitory Smad6 and Smad7, are known to bind to the TGFR-I to prevent phosphorylation and/or nuclear translocation of R-Smad [21–24]. Subsequently, R-Smad/ Smad4 heterocomplexes are translocated into the nucleus, where they activate target genes, binding DNA either directly or in association with other transcription factors.

A TGF-β response element has been well characterized in the mouse COL1A2 promoter. An initial study indicated that a CTF/NF-1 binding site located at –300 bp in the promoter region mediates TGF-β activation of the COL1A2 promoter [26]. In contrast, other earlier studies identified the Sp1 binding site as TGF-β response element for human ⟨2(I) collagen [27], demonstrating that Sp1 is required for the response of the gene to TGF-β [28]. Further studies have elucidated the important role of Smad3/Smad4 complex binding to the CAGACA motif near the Sp1 binding site in the human a2(I) collagen promoter for the full TGF-β response [29, 30]. Synergistic cooperation between Sp1 and Smad3/Smad4 is required for the TGF-β response of the collagen gene [31–33]. In the nucleus, activated Smad complexes regulate various gene expressions with the recruitment of coactivators or corepressors into transcriptional complexes. The role of CREB binding protein (CBP) and p300 as essential coactivators for Smad-mediated gene expression has been well demonstrated [34–36]. Another study indicated that the interaction of Ets with Smad is also involved in the TGF-β response of the collagen gene [37].

Consistent with the above findings, mice lacking Smad3 expression show a significantly reduced skin and lung fibrotic response [38, 39], indicating an important role of Smad3 in fibrosis. Smad2, Smad3, and Smad4 have been reported to contribute to liver fibrosis in vitro and in vivo [40–42]. By contrast, gene transfer of inhibitory Smad7 is shown to prevent renal fibrosis [43].

9.3 Non-Smad Pathways

While Smads play a central role in TGF-β signaling pathway, subsequent studies have revealed the presence of pathways that are not mediated by Smad proteins in the regulation of ECM genes expression by TGF-β. These alternate pathways include MAPKs and phosphoinositide 3-kinases (PI3Ks).

The MAPKs, ubiquitously expressed by eukaryotic cells, are major signaling pathways utilized to mediate a variety of intracellular signals such as cell growth, differentiation, apoptosis, and responses to stresses. Three major mitogen-activated protein kinase (MAPK) subfamilies are known: the extracellular signal-regulated kinase (ERK), p38, and Jun N-terminal kinase (JNK) subfamilies [44]. TGF-β has been demonstrated to activate each of the three major MAPK members in a variety of cell types. ERK1 and ERK2 have been shown to be involved in fibroblast proliferation [45], while ERK activation has been reported to inhibit type I collagen expression [46]. JNK activation has been shown to upregulate fibronectin synthesis [47]. p38 has been reported to be involved in type I collagen synthesis by TGF-β stimulation in fibroblasts [48].

PI3Ks are also major intracellular signaling pathways that regulate various cellular functions [49]. Following PI3K activation, PIP_3 recruits phosphoinositide-dependent kinase (PDK)-1 and Akt/PKB to the plasma membrane where PDK-1 phosphorylates Akt on Thr^{308} [49], followed by phosphorylation at Ser^{473}. Once activated, Akt leaves the plasma membrane to phosphorylate intracellular substrates or to translocate the nucleus where it phosphorylates transcription factors. PI3K signaling pathway also mediates TGF-β family signal transduction [50, 51]. The inhibition of PI3K activity attenuates TGF-β-induced Smad2 phosphorylation [50]. This may be explained by the fact that the inhibition of PI3K causes a redistribution of Smad anchor for receptor activation (SARA), which directly interacts with Smad2/Smad3 and recruits Smad2 to the activated TGF-β receptors [52]. Furthermore, the basal activity of PI3K is necessary for COL1A2 mRNA stabilization and the establishment of the constitutive activation of TGF-b/Smad3 signaling in SSc fibroblasts [53].

Abelson kinase (c-Abl) is a non-receptor tyrosine kinase that is best known for its role in chronic myelogenous leukemia [54]. c-Abl has been shown to be activated by TGF-β in fibroblasts, and to mediate some of the profibrotic effects independent of SMAD signaling [55], leading to consequent stimulation of extracellular matrix protein synthesis in vitro and in vivo [55–58]. c-Abl kinase activity is necessary for the induction of Egr-1 by TGF-β in normal fibroblasts [56].

Protein kinase C has also been reported to mediate Smad-independent pathway. PKC-δ isoform has been found to be critical in the early phase of TGF-β1 signaling related to gene transcription [59, 60]. As another study has demonstrated that c-Abl is an upstream regulator of the profibrotic PKCδ/phospho-Fli-1 pathway [61], c-Abl is likely to activate this pathway as well. Furthermore, dermal fibroblasts as well as endothelial cells from SSc patients produce increased levels of thrombospondin-1 [62], which can also activate TGF-β.

9.4 TGF-β and SSc

TGF-β has attracted much attention as a key mediator of fibrosis in SSc. Indeed, the phenotype of fibroblasts derived from SSc patients resembles that of normal fibroblasts stimulated with TGF-β in many ways. They include elevated expressions of collagen types I, III, VI, and VII, fibronectin, glycosaminoglycans, tissue inhibitor of metalloproteinases, α-smooth muscle actin, and $\alpha_v\beta_5$ integrin as well as decreased expression of MMP-1 [45, 63–72].

Dysregulated expression of TGF-β receptors has been elucidated in SSc. Overexpression of TGFR-I and THGFR-II has been reported in SSc fibroblasts than in normal fibroblasts in vitro and in vivo [3, 73–77]. The overexpression of these TGF-β receptors in SSc fibroblasts is regulated at transcriptional level and to be resistant to various stimuli [75–77]. Moreover, the expression of the endothelial cell-predominant TGF-β receptor endoglin is increased in SSc fibroblasts [78]. Since TGF-β, PDGF, and EGF can increase TGF-β receptor expression, the increased expression of these TGF-β receptors in SSc fibroblasts may be due to the exposure of SSc fibroblasts to these cytokines in vivo. There is also a report demonstrating that an increased TGFR-I:TGFR-II ratio contributes to increased collagen synthesis in SSc fibroblasts [79].

The abnormal expression and function of Smad proteins have also been reported in SSc fibroblasts [80, 81]. Increased expression of Smad3 as well as increased phosphorylation of Smad2/Smad3 has been reported in SSc fibroblasts [81]. Smad3 exhibits constitutive phosphorylation and the increased binding with the "CAGA" motif in SSc fibroblasts [53]. While Smad7 expression may be increased or decreased in SSc fibroblasts [82, 83], Smad7-Smurf-mediated negative regulation of TGF-β signaling is functionally impaired in SSc fibroblasts [82].

Collectively, SSc fibroblasts produce an amount of TGF-β equivalent to that produced by normal fibroblasts in vitro [73, 84], while SSc fibroblasts express elevated levels of TGF-β receptors, which can induce the overexpression of ECM proteins [73–76, 85]. Moreover, TGF-β/Smad signaling is constitutively activated in SSc fibroblasts [53, 82], and the blockade of endogenous TGF-β signaling abolishes the scleroderma phenotype [39, 72, 73]. Thus, exaggerated activation of SSc fibroblasts may be due to stimulation by autocrine TGF-β signaling [3].

Dysregulation in alternative pathways have also been reported. Increased phosphorylation and activation of p38 have been demonstrated in SSc fibroblasts, suggesting the possible role of p38 in fibrosis [86]. c-Abl was found to be constitutively phosphorylated in the lesional skin of patients with SSc [56, 87]. Fli-1 protein levels are markedly decreased in lesional SSc fibroblasts [88, 89]. This downregulation is due to that Fli-1 is phosphorylated at higher levels and undergoes degradation in SSc fibroblasts and supports the notion that the c-Abl/PKCδ/phospho-Fli-1 pathway is constitutively activated in these cells [61].

9.5 Therapeutic Approach

9.5.1 Anti-TGF-β Antibody

A recombinant human antibody that neutralizes TGF-β1, metelimumab (CAT-192), was evaluated in a multicenter, randomized, placebo-controlled phase I/II clinical trial to treat diffuse cutaneous SSc [90]. Forty-five early stage SS patients were enrolled and randomly assigned to the placebo group or to one of three metelimumab treatment groups (10 mg/kg, 5 mg/kg, and 0.5 mg/kg). Patients received infusions on day 0 and weeks 6, 12, and 18. There was significant morbidity and mortality, including one and three deaths in the group receiving 0.5 mg/kg and 5 mg/kg of metelimumab, respectively. More adverse events and more serious adverse events occurred in patients receiving metelimumab than in those receiving placebo, although these events were not more frequent in the high-dose treatment group. All deaths were attributed to complications of the underlying disease, and none was considered to be related to metelimumab administration. Overall improvement in skin sclerosis was observed during the study, although metelimumab showed no evidence of efficacy.

The lack of efficacy can be attributed to the fact that metelimumab only neutralizes TGF-β1, but not all TGF-β isoforms. Therefore, fresolimumab (GC-1008; Genzyme) that neutralizes all isoforms has been evaluated in a clinical trial. Also, considering that most TGF-β molecules exist in latent form, soluble TGF-β receptors that selectively block active form of TGF-β may be a reasonable approach.

9.5.2 Imatinib

Imatinib mesylate suppresses c-Abl activity. Recently, imatinib therapy in fibrosis has been assessed in a number of clinical trials. However, they have shown variable results, with some studies demonstrating improvement in skin and lung fibrosis while others failed to demonstrate the efficacy [91–98].

9.5.3 CTGF

CTGF (CCN2) belongs to the CCN family. The CCN family consists of six members: cysteine-rich 61 (Cyr-61/CCN1), connective tissue growth factor (CTGF/CCN2), nephroblastoma overexpressed gene (Nov/CCN3), CCN4 (WISP-1/Elm1), CCN5 (WISP- 2/Rcop1), and CCN6 (WISP-3) [99, 100]. CCN proteins are characterized by an extraordinarily high content of cysteine and an absolute conservation of the position of the 38 cysteine residues in the peptide sequence [101].

The CCN family proteins share significant structural homology, including an N-terminal secretory signal peptide (SP), followed by modular domains with sequence homologies to insulin-like growth factor binding protein (IGFBP, module

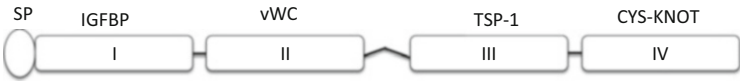

Fig. 9.1 Structure of CTGF

I), von Willebrand factor type C repeat (vWC, module II), thrombospondin type 1 repeat (TSP, module III), and the C-terminal cysteine knot-containing domain (CT, module IV) (Fig. 9.1). CTGF is a secretory protein with a molecular weight of 36–38 kDa containing 349 amino acids. Grotendorst and colleagues first identified CTGF in human umbilical endothelial cell supernatants that exhibit platelet-derived growth factor (PDGF)-like chemotactic and mitogenic activities toward mesenchymal cells [102]. CTGF c-DNA was originally cloned using anti-PDGF antibody which is thought to be contaminated with anti-CTGF antibody.

In adult mammals, constitutive expression of CTGF is rather restricted [103–106], while it can be induced in many cell types including dermal fibroblasts, myofibroblasts, and pericytes upon stimulation. TGF-β is one of the most potent inducers of CTGF expression. This is severely impaired in the fibroblasts deficient in Smad3, with a functional Smad element residing within the CTGF promoter [80]. Ets-1 is also required for the induction of CTGF by TGF-β stimulation [107, 108].

The functions of CTGF are diverse, including mitogenesis, chemotaxis, ECM production, apoptosis, and angiogenesis, depending on cell types. CTGF enhances fibroblast cell growth and ECM production as a downstream mediator of TGF-β1 [4, 109, 110]. Other factors such as endothelin-1 (ET-1) and conditions such as hypoxia also activate the CTGF proximal promoter [80, 111–114]. CTGF has been shown to enhance the mRNA expression of α1(I) collagen, fibronectin, and α_v integrin in fibroblasts [115].

Although a specific CTGF receptor has not been identified, CTGF appears to mediate many of its functions through integrins, heparin sulfate-containing proteoglycans, and the low-density lipoprotein receptor-related protein [105, 110, 116–118]. Also, CTGF has been reported to bind with the surface of fibroblasts, and this binding is competed with PDGF BB [102], suggesting that either CTGF binds with certain class of PDGF receptor or that there is some cross-reactivity of PDGF BB with CTGF receptors.

9.6 CTGF and Human SSc

Because of its ability to induce the expression of ECM molecules as one of the mediators of TGF-β, CTGF has emerged as an additional candidate responsible for excessive production of ECM proteins in SSc [1]. Igarashi et al. first reported that, when skin tissues from patients with SSc were assessed by in situ hybridization, dermal fibroblasts expressed CTGF mRNA in all 12 cases that showed histologic

sclerosis [119]. Its expression appears to correlate with the stage of the disease and the degree of fibrosis. CTGF mRNA was more abundantly expressed in the sclerotic stage than in the inflammatory stage, while no CTGF mRNA expression was observed in the atrophic stage or in the presclerotic stage. In vivo CTGF overexpression has also been observed in many fibrotic diseases, including SSc, morphea, and keloid as well as in renal, hepatic, and lung fibrosis [103, 104, 119, 120].

In accordance with the finding of increased CTGF mRNA expression in SSc lesions, cultured fibroblasts isolated from the involved skin lesions of scleroderma patients show overexpression of CTGF [80, 121]. Blocking TGF-β and Smads signaling does not affect CTGF expression, but it is dependent on BCE-1, Sp1, and endothelin-1 [80, 122–125]. By contrast, increased expression of CTGF in cell culture-activated hepatic stellate cells is dependent on TGF-β [126]. CTGF is also overexpressed by endothelial cells from SSc patients [127]. SSc MVECs recruit and activate dermal fibroblasts by induction of a CTGF/TGF-β-dependent mesenchymal-to-mesenchymal transition [128].

In SSc patients, serum concentrations of CTGF are increased, while CTGF levels are not elevated in patients with systemic lupus erythematosus and inflammatory myopathy, suggesting that elevation of CTGF levels is specific for SSc among autoimmune connective tissue diseases [129]. Furthermore, circulating CTGF levels are correlated with the extent of skin sclerosis and the severity of pulmonary fibrosis.

Moreover, a functional polymorphism has been reported in SSc patients [130, 131]. The polymorphism at the Sp1/Sp3 binding site of CTGF promoter region is reported to be responsible for the enhanced expression of CTGF [130].

In a mouse model, CTGF is upregulated in bleomycin-induced lung fibrosis [132]. Intriguingly, CTGF mRNA expression was upregulated in a bleomycin-sensitive mouse strain (C57BL/6), but not in a bleomycin-resistant mouse strain (BALB/c). Another study has shown that CTGF plays a crucial role in inducing a profibrotic environment in fibrosis-resistant BALB/c mouse lungs [133].

9.7 CTGF in Mouse Models

CTGF is also induced in bleomycin-induced skin fibrosis, in which cells that expressed CTGF are reported as α−SMA-expressing myofibroblasts. Approximately 85 % of myofibroblasts are NG2-positive, CTGF-expressing pericytes [134]. Mice with fibroblast-specific overexpression of CTGF under the control of the collagen α2(I) promoter enhancer has been generated [135]. These mice are susceptible to accelerated tissue fibrosis affecting the skin, lung, kidney, and vasculature, most notably the small arteries. A marked expansion of myofibroblast population has been observed in the dermis. Reversely, antisense oligonucleotides against CTGF alleviate fibrosis in animal models [136–138]. Also, dabigatran, a selective direct thrombin inhibitor, is shown to suppress the overexpression of CTGF by SSc fibroblasts [139].

9.8 Two-Step Fibrosis Hypothesis in SSc

Roberts et al. reported that TGF-β injection into newborn mice caused granulation tissue formation and skin fibrosis [140], although this change is only transient [141]. The injection of TGF-β1, TGF-β2, or TGF-β3 into the subcutaneous tissue of newborn mice for 7 days develops prominent granulation formation with fibrotic changes after three consecutive injections. However, the fibrotic changes cannot be observed after 7 days [141]. Thus, an injection of TGF-β alone does not develop persistent fibrosis. Likewise, bFGF injection alone causes transient formation of mildly edematous granulation tissue. Also, CTGF injection alone only causes modest edema and some cell infiltration. By contrast, the simultaneous injection of TGF-β plus CTGF or TGF-β plus bFGF or the serial injection of CTGF after TGF-β results in fibrotic tissue formation, consisting of fibroblast aggregation and ECM deposition, that persists for up to 14 days, even though the injections are discontinued on day 7 [141, 142]. In this protocol, TGF-β is injected on days 1–3, followed by CTGF or bFGF on days 4–7. Injection of CTGF or bFGF before TGF-β does not cause any significant change compared with TGF-β injection alone. Thus, single application of any cytokine is not sufficient to induce persistent dermal fibrosis, while synergic action of two cytokines appears necessary for the induction of persistent fibrosis in this model. Especially, TGF-β is likely to play an essential priming role in inducing fibrosis. Once initiated, CTGF as well as bFGF serves as an important factor for maintaining fibrosis. Based on these results, a hypothesis of two-step fibrosis mechanism in SSc has been proposed: TGF-β initiates fibrosis, and CTGF maintains it (Fig. 9.2). TGF-β induces CTGF expression, although additional factors, such as altered TGFR signaling as described above, are also likely to contribute to continuous CTGF expression. Intriguingly, selective disruption of TGFR-II in mouse skin fibroblasts increases constitutive expression of CTGF [143]. CTGF may modulate the Smad pathway indirectly through interaction with TGF-β, thereby increasing receptor binding and prolonging TGF-β signaling [109, 144].

This process may not be achieved by fibroblasts alone, but rather other cells, such as macrophages, are likely to participate. For example, TGF-β injection

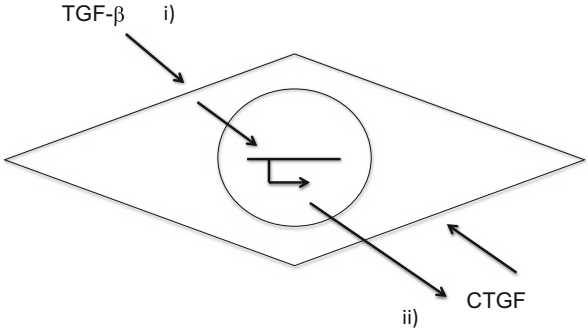

Fig. 9.2 Two-step fibrosis model in SSc

increases the expressions of MCP-1, fractalkine, CCR2, and CX3CR1 mRNA in the site [145, 146]. MCP-1-deficient mice, following bFGF and CTGF injections, exhibit decreased collagen levels in granulation tissues and decreased number of infiltrating inflammatory cells, such as mast cells, macrophages, and lymphocytes, compared with wild-type mice [147]. Furthermore, the overproduction of collagen induced by TGF-β is significantly reduced by CCR2 deficiency, while CX3CR1-deficient mice show decreased collagen production induced by TGF-β and CTGF stimulations by approximately 50 % [146]. CCR2/MCP-1 interaction is important during the TGF-β phase. In contrast, the fractalkine/CX3CR1 interaction contributes to the initiation of fibrosis by TGF-β and its maintenance by CTGF. Thus, it is suggested that two subsets of macrophages both cooperatively and independently play important roles in the development of fibrosis.

9.9 Therapeutic Approach

9.9.1 Anti-CTGF Antibody

Considering the importance of CTGF in establishing fibrosis, therapeutic approach to dampen CTGF activity by neutralizing antibody is a natural progression. A panel of anti-CTGF antibodies recognizing the native conformation of human CTGF has been generated. When newborn B6 mice received subcutaneous injections of TGF-β for 3 days with either anti-CTGF neutralizing antibodies or control purified immunoglobulin, anti-CTGF antibodies significantly reduced skin fibrosis and collagen contents compared with the control antibody group [148]. Among four discrete structural modules of CTGF, the antibody to module 2 appears the most potent. Minato et al. (2004) have demonstrated that the antibody against the C-terminal module (module 4) of human CTGF neutralizes the stimulatory effect of CTGF on chondrocytic cell proliferation efficiently but that the particular antibody bound to the von Willebrand factor type C repeat module (in our study module 2) has less inhibitory effect. Thus, the exact target structure within CTGF needs to be determined. Nonetheless, anti-CTGF antibodies are capable of exerting anti-fibrosing effects and that these anti-CTGF neutralizing antibodies may be useful as the feasible strategy to treat skin fibrotic diseases as SSc.

9.10 Conclusion

In this chapter, two fibrogenic cytokines as TGF-β and CTGF are focused as the main players of fibrotic process of SSc. Presumably TGF-β acts in the primary stage and CTGF in the maintenance process. Both anti-TGF-β and CTGF products are under process of future clinical treatment which will cure these intractable disease patients.

References

1. Takehara K. Hypothesis: pathogenesis of systemic sclerosis. J Rheumatol. 2003;30(4):755–9.
2. Lafyatis R. Transforming growth factor beta – at the centre of systemic sclerosis. Nat Rev Rheumatol. 2014;10(12):706–19.
3. Ihn H. Autocrine TGF-beta signaling in the pathogenesis of systemic sclerosis. J Dermatol Sci. 2008;49(2):103–13.
4. Jinnin M. Mechanisms of skin fibrosis in systemic sclerosis. J Dermatol. 2010;37(1):11–25.
5. Attisano L, Wrana JL. Signal transduction by members of the transforming growth factor-beta superfamily. Cytokine Growth Factor Rev. 1996;7(4):327–39.
6. Massague J. TGF-beta signal transduction. Annu Rev Biochem. 1998;67:753–91.
7. Shull MM, et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature. 1992;359(6397):693–9.
8. Sanford LP, et al. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. Development. 1997;124 (13):2659–70.
9. Proetzel G, et al. Transforming growth factor-beta 3 is required for secondary palate fusion. Nat Genet. 1995;11(4):409–14.
10. Takehara K, LeRoy EC, Grotendorst GR. TGF-beta inhibition of endothelial cell proliferation: alteration of EGF binding and EGF-induced growth-regulatory (competence) gene expression. Cell. 1987;49(3):415–22.
11. Varga J, Rosenbloom J, Jimenez SA. Transforming growth factor beta (TGF beta) causes a persistent increase in steady-state amounts of type I and type III collagen and fibronectin mRNAs in normal human dermal fibroblasts. Biochem J. 1987;247(3):597–604.
12. Roberts AB, Sporn MB. Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta). Growth Factors. 1993;8(1):1–9.
13. Massague J. The transforming growth factor-beta family. Annu Rev Cell Biol. 1990;6:597–641.
14. Munger JS, et al. The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. Cell. 1999;96(3):319–28.
15. Mu D, et al. The integrin alpha(v)beta8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-beta1. J Cell Biol. 2002;157(3):493–507.
16. Markovics JA, et al. Interleukin-1beta induces increased transcriptional activation of the transforming growth factor-beta-activating integrin subunit beta8 through altering chromatin architecture. J Biol Chem. 2011;286(42):36864–74.
17. Schultz-Cherry S, et al. Regulation of transforming growth factor-beta activation by discrete sequences of thrombospondin 1. J Biol Chem. 1995;270(13):7304–10.
18. Crawford SE, et al. Thrombospondin-1 is a major activator of TGF-beta1 in vivo. Cell. 1998;93(7):1159–70.
19. Wrana JL, et al. Mechanism of activation of the TGF-beta receptor. Nature. 1994;370 (6488):341–7.
20. Saitoh M, et al. Identification of important regions in the cytoplasmic juxtamembrane domain of type I receptor that separate signaling pathways of transforming growth factor-beta. J Biol Chem. 1996;271(5):2769–75.
21. Massague J, Chen YG. Controlling TGF-beta signaling. Genes Dev. 2000;14(6):627–44.
22. Massague J, Wotton D. Transcriptional control by the TGF-beta/Smad signaling system. EMBO J. 2000;19(8):1745–54.
23. Piek E, Heldin CH, Ten Dijke P. Specificity, diversity, and regulation in TGF-beta superfamily signaling. FASEB J. 1999;13(15):2105–24.
24. Derynck R, Zhang Y, Feng XH. Smads: transcriptional activators of TGF-beta responses. Cell. 1998;95(6):737–40.
25. Shi Y, et al. A structural basis for mutational inactivation of the tumour suppressor Smad4. Nature. 1997;388(6637):87–93.

26. Rossi P, et al. A nuclear factor 1 binding site mediates the transcriptional activation of a type I collagen promoter by transforming growth factor-beta. Cell. 1988;52(3):405–14.
27. Inagaki Y, Truter S, Ramirez F. Transforming growth factor-beta stimulates alpha 2 (I) collagen gene expression through a cis-acting element that contains an Sp1-binding site. J Biol Chem. 1994;269(20):14828–34.
28. Greenwel P, et al. Sp1 is required for the early response of alpha2(I) collagen to transforming growth factor-beta1. J Biol Chem. 1997;272(32):19738–45.
29. Chen SJ, et al. Stimulation of type I collagen transcription in human skin fibroblasts by TGF-beta: involvement of Smad 3. J Invest Dermatol. 1999;112(1):49–57.
30. Chen SJ, et al. Interaction of smad3 with a proximal smad-binding element of the human alpha2(I) procollagen gene promoter required for transcriptional activation by TGF-beta. J Cell Physiol. 2000;183(3):381–92.
31. Zhang W, et al. Synergistic cooperation between Sp1 and Smad3/Smad4 mediates transforming growth factor beta1 stimulation of alpha 2(I)-collagen (COL1A2) transcription. J Biol Chem. 2000;275(50):39237–45.
32. Poncelet AC, Schnaper HW. Sp1 and Smad proteins cooperate to mediate transforming growth factor-beta 1-induced alpha 2(I) collagen expression in human glomerular mesangial cells. J Biol Chem. 2001;276(10):6983–92.
33. Inagaki Y, et al. Interaction between GC box binding factors and Smad proteins modulates cell lineage-specific alpha 2(I) collagen gene transcription. J Biol Chem. 2001;276 (19):16573–9.
34. Ghosh AK, et al. Smad-dependent stimulation of type I collagen gene expression in human skin fibroblasts by TGF-beta involves functional cooperation with p300/CBP transcriptional coactivators. Oncogene. 2000;19(31):3546–55.
35. Ghosh AK, et al. Antagonistic regulation of type I collagen gene expression by interferon-gamma and transforming growth factor-beta. Integration at the level of p300/CBP transcriptional coactivators. J Biol Chem. 2001;276(14):11041–8.
36. Janknecht R, Wells NJ, Hunter T. TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300. Genes Dev. 1998;12(14):2114–9.
37. Czuwara-Ladykowska J, et al. Ets1 is an effector of the transforming growth factor beta (TGF-beta) signaling pathway and an antagonist of the profibrotic effects of TGF-beta. J Biol Chem. 2002;277(23):20399–408.
38. Flanders KC, et al. Mice lacking Smad3 are protected against cutaneous injury induced by ionizing radiation. Am J Pathol. 2002;160(3):1057–68.
39. Zhao J, et al. Smad3 deficiency attenuates bleomycin-induced pulmonary fibrosis in mice. Am J Physiol Lung Cell Mol Physiol. 2002;282(3):L585–93.
40. Lee DK, et al. The hepatitis B virus encoded oncoprotein pX amplifies TGF-beta family signaling through direct interaction with Smad4: potential mechanism of hepatitis B virus-induced liver fibrosis. Genes Dev. 2001;15(4):455–66.
41. Schnabl B, et al. The role of Smad3 in mediating mouse hepatic stellate cell activation. Hepatology. 2001;34(1):89–100.
42. Inagaki Y, et al. Constitutive phosphorylation and nuclear localization of Smad3 are correlated with increased collagen gene transcription in activated hepatic stellate cells. J Cell Physiol. 2001;187(1):117–23.
43. Terada Y, et al. Gene transfer of Smad7 using electroporation of adenovirus prevents renal fibrosis in post-obstructed kidney. Kidney Int. 2002;61(1 Suppl):S94–8.
44. Arthur JS, Ley SC. Mitogen-activated protein kinases in innate immunity. Nat Rev Immunol. 2013;13(9):679–92.
45. Ihn H, Tamaki K. Increased phosphorylation of transcription factor Sp1 in scleroderma fibroblasts: association with increased expression of the type I collagen gene. Arthritis Rheum. 2000;43(10):2240–7.
46. Reunanen N, et al. Activation of extracellular signal-regulated kinase 1/2 inhibits type I collagen expression by human skin fibroblasts. J Biol Chem. 2000;275(44):34634–9.

47. Hocevar BA, Brown TL, Howe PH. TGF-beta induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. EMBO J. 1999;18(5):1345–56.
48. Ihn H, et al. IL-4 up-regulates the expression of tissue inhibitor of metalloproteinase-2 in dermal fibroblasts via the p38 mitogen-activated protein kinase dependent pathway. J Immunol. 2002;168(4):1895–902.
49. Okkenhaug K. Signaling by the phosphoinositide 3-kinase family in immune cells. Annu Rev Immunol. 2013;31:675–704.
50. Bakin AV, et al. Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. J Biol Chem. 2000;275(47):36803–10.
51. Runyan CE, Schnaper HW, Poncelet AC. The phosphatidylinositol 3-kinase/Akt pathway enhances Smad3-stimulated mesangial cell collagen I expression in response to transforming growth factor-beta1. J Biol Chem. 2004;279(4):2632–9.
52. Tsukazaki T, et al. SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. Cell. 1998;95(6):779–91.
53. Asano Y, et al. Phosphatidylinositol 3-kinase is involved in alpha2(I) collagen gene expression in normal and scleroderma fibroblasts. J Immunol. 2004;172(11):7123–35.
54. Goldman JM, Melo JV. Targeting the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med. 2001;344(14):1084–6.
55. Daniels CE, et al. Imatinib mesylate inhibits the profibrogenic activity of TGF-beta and prevents bleomycin-mediated lung fibrosis. J Clin Invest. 2004;114(9):1308–16.
56. Bhattacharyya S, et al. A non-Smad mechanism of fibroblast activation by transforming growth factor-beta via c-Abl and Egr-1: selective modulation by imatinib mesylate. Oncogene. 2009;28(10):1285–97.
57. Distler JH, et al. Imatinib mesylate reduces production of extracellular matrix and prevents development of experimental dermal fibrosis. Arthritis Rheum. 2007;56(1):311–22.
58. Distler JH, Distler O. Imatinib as a novel therapeutic approach for fibrotic disorders. Rheumatology (Oxford). 2009;48(1):2–4.
59. Weiss RH, Yabes AP, Sinaee R. TGF-beta and phorbol esters inhibit mitogenesis utilizing parallel protein kinase C-dependent pathways. Kidney Int. 1995;48(3):738–44.
60. Ignotz RA, Honeyman T. TGF-beta signaling in A549 lung carcinoma cells: lipid second messengers. J Cell Biochem. 2000;78(4):588–94.
61. Bujor AM, et al. The c-Abl tyrosine kinase controls protein kinase Cdelta-induced Fli-1 phosphorylation in human dermal fibroblasts. Arthritis Rheum. 2011;63(6):1729–37.
62. Mimura Y, et al. Constitutive thrombospondin-1 overexpression contributes to autocrine transforming growth factor-beta signaling in cultured scleroderma fibroblasts. Am J Pathol. 2005;166(5):1451–63.
63. LeRoy EC. Increased collagen synthesis by scleroderma skin fibroblasts in vitro: a possible defect in the regulation or activation of the scleroderma fibroblast. J Clin Invest. 1974;54 (4):880–9.
64. Falanga V, et al. Transforming growth factor-beta: selective increase in glycosaminoglycan synthesis by cultures of fibroblasts from patients with progressive systemic sclerosis. J Invest Dermatol. 1987;89(1):100–4.
65. Peltonen J, et al. Increased expression of type VI collagen genes in systemic sclerosis. Arthritis Rheum. 1990;33(12):1829–35.
66. Xu WD, Leroy EC, Smith EA. Fibronectin release by systemic sclerosis and normal dermal fibroblasts in response to TGF-beta. J Rheumatol. 1991;18(2):241–6.
67. Rudnicka L, et al. Elevated expression of type VII collagen in the skin of patients with systemic sclerosis. Regulation by transforming growth factor-beta. J Clin Invest. 1994;93 (4):1709–15.
68. Jelaska A, et al. Heterogeneity of collagen synthesis in normal and systemic sclerosis skin fibroblasts. Increased proportion of high collagen-producing cells in systemic sclerosis fibroblasts. Arthritis Rheum. 1996;39(8):1338–46.

69. Kirk TZ, et al. Myofibroblasts from scleroderma skin synthesize elevated levels of collagen and tissue inhibitor of metalloproteinase (TIMP-1) with two forms of TIMP-1. J Biol Chem. 1995;270(7):3423–8.
70. Takeda K, et al. Decreased collagenase expression in cultured systemic sclerosis fibroblasts. J Invest Dermatol. 1994;103(3):359–63.
71. Sappino AP, et al. Smooth muscle differentiation in scleroderma fibroblastic cells. Am J Pathol. 1990;137(3):585–91.
72. Asano Y, et al. Increased expression levels of integrin alphavbeta5 on scleroderma fibroblasts. Am J Pathol. 2004;164(4):1275–92.
73. Ihn H, et al. Blockade of endogenous transforming growth factor beta signaling prevents up-regulated collagen synthesis in scleroderma fibroblasts: association with increased expression of transforming growth factor beta receptors. Arthritis Rheum. 2001;44(2):474–80.
74. Kubo M, et al. Upregulated expression of transforming growth factor-beta receptors in dermal fibroblasts of skin sections from patients with systemic sclerosis. J Rheumatol. 2002;29(12):2558–64.
75. Yamane K, et al. Increased transcriptional activities of transforming growth factor beta receptors in scleroderma fibroblasts. Arthritis Rheum. 2002;46(9):2421–8.
76. Yamane K, et al. Antagonistic effects of TNF-alpha on TGF-beta signaling through down-regulation of TGF-beta receptor type II in human dermal fibroblasts. J Immunol. 2003;171(7):3855–62.
77. Yamane K, Ihn H, Tamaki K. Epidermal growth factor up-regulates expression of transforming growth factor beta receptor type II in human dermal fibroblasts by phosphoinositide 3-kinase/Akt signaling pathway: resistance to epidermal growth factor stimulation in scleroderma fibroblasts. Arthritis Rheum. 2003;48(6):1652–66.
78. Leask A, et al. Dysregulation of transforming growth factor beta signaling in scleroderma: overexpression of endoglin in cutaneous scleroderma fibroblasts. Arthritis Rheum. 2002;46(7):1857–65.
79. Pannu J, et al. An increased transforming growth factor beta receptor type I: type II ratio contributes to elevated collagen protein synthesis that is resistant to inhibition via a kinase-deficient transforming growth factor beta receptor type II in scleroderma. Arthritis Rheum. 2004;50(5):1566–77.
80. Holmes A, et al. CTGF and SMADs, maintenance of scleroderma phenotype is independent of SMAD signaling. J Biol Chem. 2001;276(14):10594–601.
81. Mori Y, Chen SJ, Varga J. Expression and regulation of intracellular SMAD signaling in scleroderma skin fibroblasts. Arthritis Rheum. 2003;48(7):1964–78.
82. Asano Y, et al. Impaired Smad7-Smurf-mediated negative regulation of TGF-beta signaling in scleroderma fibroblasts. J Clin Invest. 2004;113(2):253–64.
83. Dong C, et al. Deficient Smad7 expression: a putative molecular defect in scleroderma. Proc Natl Acad Sci U S A. 2002;99(6):3908–13.
84. Needleman BW, et al. Secretion and binding of transforming growth factor beta by scleroderma and normal dermal fibroblasts. Arthritis Rheum. 1990;33(5):650–6.
85. Yamane K, et al. Anti-U3 snRNP antibodies in localised scleroderma. Ann Rheum Dis. 2001;60(12):1157–8.
86. Ihn H, Yamane K, Tamaki K. Increased phosphorylation and activation of mitogen-activated protein kinase p38 in scleroderma fibroblasts. J Invest Dermatol. 2005;125(2):247–55.
87. Chung L, et al. Molecular framework for response to imatinib mesylate in systemic sclerosis. Arthritis Rheum. 2009;60(2):584–91.
88. Kubo M, et al. Persistent down-regulation of Fli1, a suppressor of collagen transcription, in fibrotic scleroderma skin. Am J Pathol. 2003;163(2):571–81.
89. Asano Y, Bujor AM, Trojanowska M. The impact of Fli1 deficiency on the pathogenesis of systemic sclerosis. J Dermatol Sci. 2010;59(3):153–62.

90. Denton CP, et al. Recombinant human anti-transforming growth factor beta1 antibody therapy in systemic sclerosis: a multicenter, randomized, placebo-controlled phase I/II trial of CAT-192. Arthritis Rheum. 2007;56(1):323–33.
91. Pope J, et al. Imatinib in active diffuse cutaneous systemic sclerosis: results of a six-month, randomized, double-blind, placebo-controlled, proof-of-concept pilot study at a single center. Arthritis Rheum. 2011;63(11):3547–51.
92. Fraticelli P, et al. Low-dose oral imatinib in the treatment of systemic sclerosis interstitial lung disease unresponsive to cyclophosphamide: a phase II pilot study. Arthritis Res Ther. 2014;16(4):R144.
93. Prey S, et al. Imatinib mesylate in scleroderma-associated diffuse skin fibrosis: a phase II multicentre randomized double-blinded controlled trial. Br J Dermatol. 2012;167 (5):1138–44.
94. Bournia VK, Evangelou K, Sfikakis PP. Therapeutic inhibition of tyrosine kinases in systemic sclerosis: a review of published experience on the first 108 patients treated with imatinib. Semin Arthritis Rheum. 2013;42(4):377–90.
95. Tamaki Z, et al. Efficacy of low-dose imatinib mesylate for cutaneous involvement in systemic sclerosis: a preliminary report of three cases. Mod Rheumatol. 2012;22(1):94–9.
96. Spiera RF, et al. Imatinib mesylate (Gleevec) in the treatment of diffuse cutaneous systemic sclerosis: results of a 1-year, phase IIa, single-arm, open-label clinical trial. Ann Rheum Dis. 2011;70(6):1003–9.
97. Daniels CE, et al. Imatinib treatment for idiopathic pulmonary fibrosis: randomized placebo-controlled trial results. Am J Respir Crit Care Med. 2010;181(6):604–10.
98. Sabnani I, et al. A novel therapeutic approach to the treatment of scleroderma-associated pulmonary complications: safety and efficacy of combination therapy with imatinib and cyclophosphamide. Rheumatology (Oxford). 2009;48(1):49–52.
99. Lau LF, Lam SC. The CCN family of angiogenic regulators: the integrin connection. Exp Cell Res. 1999;248(1):44–57.
100. Perbal B. CCN proteins: multifunctional signalling regulators. Lancet. 2004;363(9402):62–4.
101. Bork P. The modular architecture of a new family of growth regulators related to connective tissue growth factor. FEBS Lett. 1993;327(2):125–30.
102. Bradham DM, et al. Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. J Cell Biol. 1991;114(6):1285–94.
103. Paradis V, et al. Expression of connective tissue growth factor in experimental rat and human liver fibrosis. Hepatology. 1999;30(4):968–76.
104. Ito Y, et al. Expression of connective tissue growth factor in human renal fibrosis. Kidney Int. 1998;53(4):853–61.
105. Chen Y, et al. Connective tissue growth factor is secreted through the Golgi and is degraded in the endosome. Exp Cell Res. 2001;271(1):109–17.
106. Chen Y, et al. CTGF expression in mesangial cells: involvement of SMADs, MAP kinase, and PKC. Kidney Int. 2002;62(4):1149–59.
107. Van Beek JP, et al. The induction of CCN2 by TGFbeta1 involves Ets-1. Arthritis Res Ther. 2006;8(2):R36.
108. Shi-wen X, et al. Endothelin is a downstream mediator of profibrotic responses to transforming growth factor beta in human lung fibroblasts. Arthritis Rheum. 2007;56 (12):4189–94.
109. Abraham D. Connective tissue growth factor: growth factor, matricellular organizer, fibrotic biomarker or molecular target for anti-fibrotic therapy in SSc? Rheumatology (Oxford). 2008;47 Suppl 5:v8–9.
110. Shi-Wen X, Leask A, Abraham D. Regulation and function of connective tissue growth factor/CCN2 in tissue repair, scarring and fibrosis. Cytokine Growth Factor Rev. 2008;19 (2):133–44.

111. Leask A, et al. The control of ccn2 (ctgf) gene expression in normal and scleroderma fibroblasts. Mol Pathol. 2001;54(3):180–3.
112. Leask A, et al. Connective tissue growth factor gene regulation. Requirements for its induction by transforming growth factor-beta 2 in fibroblasts. J Biol Chem. 2003;278 (15):13008–15.
113. Shi-Wen X, et al. Endothelin-1 promotes myofibroblast induction through the ETA receptor via a rac/phosphoinositide 3-kinase/Akt-dependent pathway and is essential for the enhanced contractile phenotype of fibrotic fibroblasts. Mol Biol Cell. 2004;15(6):2707–19.
114. Higgins DF, et al. Hypoxic induction of Ctgf is directly mediated by Hif-1. Am J Physiol Renal Physiol. 2004;287(6):F1223–32.
115. Frazier K, et al. Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. J Invest Dermatol. 1996;107(3):404–11.
116. Ball DK, et al. The heparin-binding 10 kDa fragment of connective tissue growth factor (CTGF) containing module 4 alone stimulates cell adhesion. J Endocrinol. 2003;176(2): R1–7.
117. Chen CC, Chen N, Lau LF. The angiogenic factors Cyr61 and connective tissue growth factor induce adhesive signaling in primary human skin fibroblasts. J Biol Chem. 2001;276 (13):10443–52.
118. Segarini PR, et al. The low density lipoprotein receptor-related protein/alpha2-macroglobulin receptor is a receptor for connective tissue growth factor. J Biol Chem. 2001;276 (44):40659–67.
119. Igarashi A, et al. Significant correlation between connective tissue growth factor gene expression and skin sclerosis in tissue sections from patients with systemic sclerosis. J Invest Dermatol. 1995;105(2):280–4.
120. Igarashi A, et al. Connective tissue growth factor gene expression in tissue sections from localized scleroderma, keloid, and other fibrotic skin disorders. J Invest Dermatol. 1996;106 (4):729–33.
121. Shi-wen X, et al. Autocrine overexpression of CTGF maintains fibrosis: RDA analysis of fibrosis genes in systemic sclerosis. Exp Cell Res. 2000;259(1):213–24.
122. Holmes A, et al. Constitutive connective tissue growth factor expression in scleroderma fibroblasts is dependent on Sp1. J Biol Chem. 2003;278(43):41728–33.
123. Chen Y, et al. Contribution of activin receptor-like kinase 5 (transforming growth factor beta receptor type I) signaling to the fibrotic phenotype of scleroderma fibroblasts. Arthritis Rheum. 2006;54(4):1309–16.
124. Shi-Wen X, et al. Endogenous endothelin-1 signaling contributes to type I collagen and CCN2 overexpression in fibrotic fibroblasts. Matrix Biol. 2007;26(8):625–32.
125. Leask A. Scar wars: is TGFbeta the phantom menace in scleroderma? Arthritis Res Ther. 2006;8(4):213.
126. Leask A, et al. Regulation of CCN2 mRNA expression and promoter activity in activated hepatic stellate cells. J Cell Commun Signal. 2008;2(1-2):49–56.
127. Giusti B, et al. A model of anti-angiogenesis: differential transcriptosome profiling of microvascular endothelial cells from diffuse systemic sclerosis patients. Arthritis Res Ther. 2006;8(4):R115.
128. Serrati S, et al. Systemic sclerosis endothelial cells recruit and activate dermal fibroblasts by induction of a connective tissue growth factor (CCN2)/transforming growth factor beta-dependent mesenchymal-to-mesenchymal transition. Arthritis Rheum. 2013;65(1):258–69.
129. Sato S, et al. Serum levels of connective tissue growth factor are elevated in patients with systemic sclerosis: association with extent of skin sclerosis and severity of pulmonary fibrosis. J Rheumatol. 2000;27(1):149–54.
130. Fonseca C, et al. A polymorphism in the CTGF promoter region associated with systemic sclerosis. N Engl J Med. 2007;357(12):1210–20.
131. Kawaguchi Y, et al. Association study of a polymorphism of the CTGF gene and susceptibility to systemic sclerosis in the Japanese population. Ann Rheum Dis. 2009;68(12):1921–4.

132. Lasky JA, et al. Chrysotile asbestos induces PDGF-A chain-dependent proliferation in human and rat lung fibroblasts in vitro. Chest. 1996;109(3 Suppl):26S–8.
133. Bonniaud P, et al. Connective tissue growth factor is crucial to inducing a profibrotic environment in "fibrosis-resistant" BALB/c mouse lungs. Am J Respir Cell Mol Biol. 2004;31(5):510–6.
134. Liu S, Taghavi R, Leask A. Connective tissue growth factor is induced in bleomycin-induced skin scleroderma. J Cell Commun Signal. 2010;4(1):25–30.
135. Sonnylal S, et al. Selective expression of connective tissue growth factor in fibroblasts in vivo promotes systemic tissue fibrosis. Arthritis Rheum. 2010;62(5):1523–32.
136. Uchio K, et al. Down-regulation of connective tissue growth factor and type I collagen mRNA expression by connective tissue growth factor antisense oligonucleotide during experimental liver fibrosis. Wound Repair Regen. 2004;12(1):60–6.
137. Okada H, et al. Connective tissue growth factor expressed in tubular epithelium plays a pivotal role in renal fibrogenesis. J Am Soc Nephrol. 2005;16(1):133–43.
138. Lang C, et al. Connective tissue growth factor: a crucial cytokine-mediating cardiac fibrosis in ongoing enterovirus myocarditis. J Mol Med (Berl). 2008;86(1):49–60.
139. Bogatkevich GS, Ludwicka-Bradley A, Silver RM. Dabigatran, a direct thrombin inhibitor, demonstrates antifibrotic effects on lung fibroblasts. Arthritis Rheum. 2009;60(11):3455–64.
140. Roberts AB, et al. Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci U S A. 1986;83(12):4167–71.
141. Shinozaki M, et al. Induction of subcutaneous tissue fibrosis in newborn mice by transforming growth factor beta--simultaneous application with basic fibroblast growth factor causes persistent fibrosis. Biochem Biophys Res Commun. 1997;240(2):292–7.
142. Mori T, et al. Role and interaction of connective tissue growth factor with transforming growth factor-beta in persistent fibrosis: a mouse fibrosis model. J Cell Physiol. 1999;181 (1):153–9.
143. Denton CP, et al. Inducible lineage-specific deletion of TbetaRII in fibroblasts defines a pivotal regulatory role during adult skin wound healing. J Invest Dermatol. 2009;129 (1):194–204.
144. de Winter P, Leoni P, Abraham D. Connective tissue growth factor: structure-function relationships of a mosaic, multifunctional protein. Growth Factors. 2008;26(2):80–91.
145. Chujo S, et al. Connective tissue growth factor causes persistent proalpha2(I) collagen gene expression induced by transforming growth factor-beta in a mouse fibrosis model. J Cell Physiol. 2005;203(2):447–56.
146. Arai M, et al. Chemokine receptors CCR2 and CX3CR1 regulate skin fibrosis in the mouse model of cytokine-induced systemic sclerosis. J Dermatol Sci. 2013;69(3):250–8.
147. Chujo S, et al. Role of connective tissue growth factor and its interaction with basic fibroblast growth factor and macrophage chemoattractant protein-1 in skin fibrosis. J Cell Physiol. 2009;220(1):189–95.
148. Ikawa Y, et al. Neutralizing monoclonal antibody to human connective tissue growth factor ameliorates transforming growth factor-beta-induced mouse fibrosis. J Cell Physiol. 2008;216(3):680–7.

Endothelin

10

Sei-ichiro Motegi

Abstract

ET-1 has various biological functions, including vasodilation/vasoconstriction, extracellular matrix production, and proliferation and migration on vascular endothelial cells, vascular smooth muscle cells, fibroblasts, and macrophages/immune cells. Experimental and clinical studies have highlighted the involvement of ET-1 in the pathogenesis of pulmonary arterial hypertension, vasculopathy, and fibrosis of the skin, lungs, and other organs in systemic sclerosis as well as the potential therapeutic role for endothelin antagonists in these conditions in systemic sclerosis. This chapter reviews the current knowledge of the endothelin/endothelin receptor system and focuses on the effects of ET-1 and its antagonism on vasculopathy and fibrosis in systemic sclerosis.

Keywords

Endothelin • Endothelin receptor • Bosentan • Pulmonary arterial hypertension • Skin fibrosis

10.1 Endothelins and Endothelin Receptors

Endothelin-1 (ET-1) is a 21-amino acid peptide that was first reported to be a potent vasoconstrictor secreted from cultured porcine endothelial cells [1]. Three isotypes of ET, including ET-1, ET-2, and ET-3, constitute a gene family and share common receptors with different affinities [2]. ET-3 is abundant in the brain and may be a specific neural endothelin [3]. ET-1 is processed sequentially from the 212-amino

S.-i. Motegi (✉)
Department of Dermatology, Gunma University Graduate School of Medicine, 39-22, Showa-machi 3-chome, Maebashi, Gunma 371-8511, Japan
e-mail: smotegi@gunma-u.ac.jp

K. Takehara et al. (eds.), *Systemic Sclerosis*, DOI 10.1007/978-4-431-55708-1_10

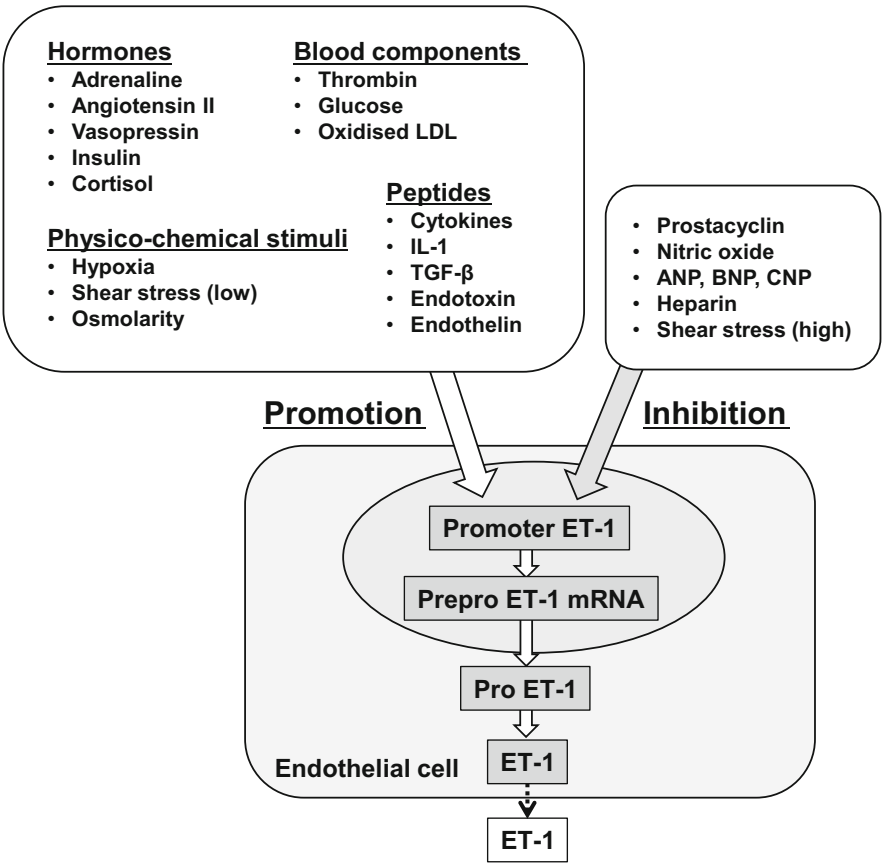

Fig. 10.1 Regulation of the synthesis of endothelin-1 (ET-1). *ANP* atrial natriuretic peptide, *BNP* brain natriuretic peptide, *CNP* C-type natriuretic peptide

acid precursor, prepro-ET-1, to the 21-amino acid biologically active ET-1 by an endopeptidase and the endothelin-converting enzyme (Fig. 10.1).

ET-1 is produced primarily by endothelial cells and other cells, such as fibroblasts, monocytes, and macrophages [1, 4]. Among the subtypes of ET, only ET-1 is secreted from vascular endothelial cells. The process is elicited by several stimulations, including those of blood flow, vessel wall tension, hypoxia, cytokines, thrombin, and growth factors [5–8]. Transforming growth factor beta (TGF-β) also stimulates the production of ET-1 in endothelial cells [9]. ET-1, however, is also produced from fibroblasts and is induced by the stimulation of angiotensin II, superoxide, and TGF-β [10–12].

ET-1 exerts biological effects through the ET receptors A (ETR-A) and B (ETR-B), which are seven-transmembrane G protein-coupled membrane proteins. ETR-A, which is mainly present in vascular smooth muscle cells (VSMC) and

fibroblasts, is responsible for α-SMA production and extracellular matrix (ECM) contraction. In contrast, the ETR-B receptor is expressed by endothelial cells where they can mediate vasodilatation by releasing nitric oxide.

10.2 Biological Functions of ET-1

Biological functions of ET-1 on vascular endothelial cells, VSMC, fibroblasts, and macrophages/immune cells are summarized in Table 10.1. ET-1 acts on endothelial cells in an autocrine manner, inducing the release of an endothelium-derived relaxing factor, nitric oxide (NO), and prostacyclin via ETR-B [13]. These effects of ET-1 contribute to the maintenance of homeostasis in the circulatory system. It has been reported that ET-1 induces the proliferation of endothelial cells, thus inhibiting migration and promoting tube formation [14–16].

ET-1 performs numerous biological functions. For example, it promotes cell growth and cell migration of VSMC and can inhibit apoptosis of VSMC [17, 18], thus being associated with the regulation of vascular contraction. In fibroblasts, ET-1 enhances cell proliferation and migration [19, 20] and also inhibits apoptosis of fibroblasts via Akt activation [21]. Furthermore, ET-1 induces the expression of

Table 10.1 Biological functions of ET-1 on vascular endothelial cell, vascular smooth muscle cell, fibroblast, and macrophage/immune cell

Target cell	Biological functions of ET-1
Endothelial cell	Proliferation ↑
	Vasodilation ↑
	(Produce NO, PGI_2 via ETR-B)
	Cell migration ↓
	Tube formation ↑
Vascular smooth muscle cell	Proliferation ↑
	Cell migration ↑
	Apoptosis ↓
	Vasoconstriction ↑
Fibroblast	Proliferation ↑
	Cell migration ↑
	Apoptosis ↓
	α-SMA expression ↑
	ECM production ↑
	Adhesion molecule expression ↑
	MMP production ↓
Macrophage/immune cell	Chemotaxis ↑
	Production of free radicals ↑
	Production of cytokine/chemokines ↑

NO nitric oxide, *PGI_2* prostaglandin I_2, *SMA* smooth muscle actin, *ECM* extracellular matrix, *MMP* matrix metalloproteinase

α-SMA in fibroblasts via ETR-A [22] and enhances the expression of intercellular adhesion molecule-1 (ICAM-1) on the surface of fibroblasts [23]. With respect to fibroblasts in vitro, ET-1 induces the production of collagen type I and III and fibronectin via ETR-A and ETR-B [24, 25]. In addition, ET-1 reduces the expression of matrix metalloproteinase-1 (MMP-1) [26]. These findings suggest that ET-1 might be involved in the pathogenesis of fibrosis in many organs.

ET-1 induces the chemotaxis of inflammatory cells, such as macrophages [27], by activating NF-κB signaling in macrophages, which is associated with the pathogenesis of atherosclerosis [28]. ET-1 also enhances the secretion of free radicals and inflammatory cytokines, such as IL-1β and TNF-α, from macrophages and/or monocytes [29, 30]. There is increasing evidence that the dysregulations of these ET-1 biological functions might be associated with the pathogenesis of vasculopathy, pulmonary arterial hypertension, heart failure, systemic hypertension, renal dysfunction, and fibrosis in systemic sclerosis (SSc).

10.3 ET-1 and Receptor Expressions in Systemic Sclerosis

There are many studies regarding the plasma ET-1 levels in patients with SSc. The findings from these studies indicate that plasma ET-1 levels were significantly higher in patients with SSc than in normal controls [31]. Yamane et al. report that plasma ET-1 levels in 31 patients with SSc (1.90 ± 0.47 pg/ml) were higher than those (1.31 ± 0.1 pg/ml) in 25 healthy individuals of corresponding age and gender [32]. Patients with diffuse cutaneous type SSc revealed higher levels of ET-1 compared with those with limited cutaneous type. In addition, plasma ET-1 levels correlated inversely with carbon monoxide diffusing capacity (DLCO).

In histological analyses, ET-1 expression was enhanced in the skin, blood vessels, lungs, and kidneys of patients with early SSc compared with those of normal individuals [33, 34]. It was also reported that ET-1 expression in dermal fibroblasts from SSc patients was elevated in comparison with that in normal control fibroblasts in vitro [22, 35, 36]. These findings suggest that ET-1 might be associated with the pathogenesis of fibrosis in SSc.

Moreover, it was found that the ETR-A expression of dermal fibroblasts in SSc patients was markedly increased. In addition, in SSc patients, ETR-B was downregulated in endothelial cells, thereby diminishing their vasodilatory role, while ETR-B was upregulated in VSMC. These conditions may result in the abnormal response to the stimulation by ET-1, including vasoconstriction, VSMC proliferation, pro-fibrotic factor production, and extracellular matrix deposition [37].

10.4 ET-1 and Pulmonary Arterial Hypertension

ET-1 synthesized by vascular endothelial cells is a highly potent vasoconstrictor and key mediator of vasculopathy. Abnormal regulation of ET-1/ETR signaling promotes arterial wall thickening, abnormal constriction, and endothelial cell

dysfunction, thus resulting in decreased levels of NO. In addition, ET-1 stimulates the secretion of vascular endothelial growth factor (VEGF), thereby causing vascular damage [38]. Thus, it can be concluded that ET-1 is associated with the pathogenesis of cardiovascular disease, including hypertension, vasospasms, and atherosclerosis.

Serum ET-1 levels are elevated in patients with pulmonary arterial hypertension (PAH); increased plasma ET-1 levels correlate with increased right atrial pressure, pulmonary vascular resistance, and mortality in patients with PAH and decreased pulmonary artery oxygen saturation [39]. ET-1 expression is increased in the vascular cells of patients with PAH [40], which can induce an inflammatory cascade effect by elevating the plasma levels of proinflammatory cytokines in patients with PAH [41].

10.5 Endothelin Receptor Antagonists and Pulmonary Arterial Hypertension

10.5.1 Endothelin Receptor Antagonists

Endothelin receptor antagonists (ERAs), such as bosentan, sitaxsentan (sitaxentan), and ambrisentan, can block the interaction of ET-1 with ETR-A and/or ETR-B (Table 10.2). As bosentan, a nonspecific ETR antagonist, can block the effect of ET, it has been found to be effective in the treatment of PAH in patients with SSc [42]. Bosentan, an orally administered ERA that has been approved for use in patients with WHO class II (mildly symptomatic) PAH, is beneficial and generally well tolerated in patients with mildly symptomatic PAH. Bosentan has been associated with dose-dependent teratogenicity in rats at plasma concentrations >1.5 times those achieved with therapeutic doses in humans. Therefore, the use of bosentan requires careful monitoring due to the dose-dependent liver toxicity and is contraindicated in pregnancy because of teratogenicity. The awareness of the adverse effects of bosentan, including teratogenicity, liver toxicity, and edema, is important for physicians. Selective ETR-A antagonists, sitaxsentan and ambrisentan, have also been proven to be effective for patients with primary PAH [43–45]. While both bosentan and ambrisentan are currently approved for use in patients with PAH in the US, sitaxsentan was withdrawn from the market in 2010 due to severe liver toxicity.

10.5.2 Bosentan and Pulmonary Arterial Hypertension

In a double-blind, placebo-controlled trial of 213 adult patients with idiopathic PAH or PAH associated with connective tissue disease, exercise capacity (6-min walk distance), the Borg dyspnea index, the WHO functional class, and the time to clinical worsening were all significantly improved with bosentan (62.5 mg of bosentan twice daily) compared with the placebo [46]. In addition, Denton

Table 10.2 Endothelin receptor antagonists

Endothelin receptor antagonist	ETR-A/ETR-B selectivity	Information
Bosentan	Nonselective	FDA approved for use in the USA in November 2001 for WHO functional class III/IV PAH then extended to include WHO class II in 2009
		Approved in the EU for WHO functional class III PAH in May 2002. EU approved and extended the indication of bosentan as a therapy to reduce the number of new DU in patients with SSc and ongoing DU disease in June 2007
Ambrisentan	ETR-A selective	FDA approved for the once-daily treatment of WHO functional class II/III PAH in June 2007. It was later approved by the European Medicines Agency for the same indication in the EU in April 2008
Sitaxsentan	ETR-A selective	Approved in the EU in August 2006, then in Canada and Australia in March 2007 for the once-daily treatment of WHO functional class III PAH. On December 10, 2010, the manufacturer voluntarily removed sitaxsentan from the market and halted clinical trials due to concerns about liver toxicity

FDA Food and Drug Administration, *EU* European Union, *PAH* pulmonary arterial hypertension, *DU* digital ulcers, *RP* Raynaud's phenomenon, and *SSc* systemic sclerosis

et al. reported the randomized control trials demonstrated that bosentan treatment was associated with the improvement or stability of clinical status of PAH patients and with the 92 % estimate for survival at 48 weeks. The result was considered to be a significant achievement among this patient population [42, 47–50].

In the pediatric patients with PAH, several phase III trials were performed. Bosentan 31.25, 62.5, or 125 mg (in pediatric patients weighing 10–20, >20–40, or >40 kg, respectively) administered twice daily for 12 weeks significantly ($p < 0.05$) improved the majority of hemodynamic measures evaluated as an exploratory measure in pediatric patients (aged 3–15 years) with a WHO functional class II or III PAH in another noncomparative, multicenter, and pharmacokinetic trial. However, there was no significant change in the peak oxygen consumption or mean walk distance in those patients who were capable of performing the 6-min walk test [51].

Furthermore, there is growing evidence that combination therapies targeting different pathophysiological steps may be necessary to effectively treat SSc-PAH. The COMPASS-1 study reported an acute hemodynamic benefit in PAH treated with sildenafil in combination with bosentan [52].

10.5.3 Endothelin-1 and Digital Ulcers

Patients with SSc usually develop Raynaud's phenomenon, persistent digital ischemia, and sometimes digital ulcers (DU). We previously reported that there were no

differences in serum lipid levels, carotid intima-media thickness (IMT), or plaque scores between SSc patients with and without DU, suggesting that atherosclerotic changes are not primarily involved in the development of DU [53]. Because ET-1 is thought to be involved in the pathogenesis of vasculopathy and PAH, ET-1 also might be involved in the pathogenesis of DU in SSc patients.

The efficacy of bosentan in patients with DU was evaluated in two randomized, double-blind, placebo-controlled, multicenter studies, RAPIDS-1 (randomized placebo-controlled study on prevention of ischemic digital ulcers in scleroderma) ($n = 122$) and RAPIDS-2 ($n = 188$) trials [38, 54]. The RAPIDS-1 trial involved 122 patients with SSc and current DU or a history of at least one in the prior 12 months at 17 centers in Europe and North America. Of the 122 patients, 79 were randomly selected to receive bosentan and 43 selected to receive a placebo during the 16-week study period. The mean number of new ulcers during the treatment period was 1.4 for patients treated with bosentan versus 2.7 for patients receiving the placebo ($P = 0.0083$), thus representing a 48 % reduction in the number of new DU. However, there were no differences between treatment groups in the healing of existing DU. The notable adverse event occurring in more patients on bosentan than in those on the placebo was elevated transaminase levels (11.4 % versus 0 %). The transaminase values returned to normal when bosentan was discontinued in all cases. The RAPIDS-2 trial included 188 SSc patients with current DU at 41 centers in Europe and North America. Of these 144 patients, 98 were randomly selected to receive bosentan and 90 were selected to receive a placebo during the 24-week study period. Over the 24 weeks, bosentan treatment was associated with a 30 % reduction in the number of new DU compared with the patients receiving the placebo. There was no difference between treatments in the healing rate of the cardinal ulcer or pain/disability. These two trials indicated that bosentan treatment reduced the occurrence of new DU in SSc patients, but had no effect on healing DU. In the RAPIDS-2 study, the most common adverse effects that occurred more frequently with bosentan than with the placebo were peripheral edema and elevated aminotransferases.

In addition to these studies, Arefiev et al. summarized previously reported case reports and studies that evaluated the efficacy of bosentan for SSc-associated RP and/or DU [45]. Ichimura et al. report that a 5-week bosentan treatment improved ulnar artery stenosis and multiple DU in SSc patients [55]. We also previously reported that six SSc patients with severe DU who were treated with a daily dose of 62.5–125 mg of oral bosentan experienced a significant reduction in pain and in the development of new DU [56].

Oral bosentan is already approved in the European Union (EU) as a therapy to reduce the number of new DU in patients with SSc and ongoing DU. However, bosentan is not yet approved for use in patients with DU in SSc patients in Japan. To assess the efficacy of bosentan for treatment of DU in SSc patients, large-scale randomized controlled trials are needed.

10.5.4 Endothelin-1 and Raynaud's Phenomenon

Sulli et al. report that the ET-1 plasma levels were significantly higher ($p = 0.001$) in patients with both primary Raynaud's phenomenon (RP) and SSc patients with secondary RP in comparison with the controls. Significant correlations were observed in patients with SSc between the ET-1 plasma levels and digital ulcers as well as in the score values of single nailfold videocapillaroscopy measures, including the number of capillaries, ramified capillaries, and enlarged capillaries ($P < 0.05$) [57].

Bosentan has been studied in the treatment of RP. Selenko-Gebauer et al. report that bosentan significantly reduced pain and reduced the number and the severity of RP attacks as well as the peripheral thermoregulation in three patients [58]. Hettema et al. analyzed 15 patients with limited type SSc and reported that bosentan significantly reduced the frequency, duration, and severity of RP attacks [59].

Only one single-centered, randomized, prospective, double-blind trial of bosentan that evaluates the effect of bosentan for the treatment of RP in patients with SSc has been published to date [60]. In 17 patients without preexisting DU, bosentan did not improve the frequency, duration, pain, or severity of RP attacks. However, in contrast to the placebo, bosentan significantly improved the functional scores assessed by the scleroderma health assessment questionnaire ($P = 0.03$ and $P = 0.01$ at weeks 12 and 20, respectively) and the UK functional score ($P = 0.038$ and $P = 0.039$ at weeks 8 and 16, respectively) compared with those treated with a placebo. Guiducci et al. reported that bosentan improved nailfold videocapillaroscopy (NVC) pattern in patients with SSc from a late NVC pattern to an active pattern [61]. No serious adverse events were noted, and only one patient withdrew due to treatment-related peripheral edema. Because this study involved a small number of patients, a larger study might show more clearly the benefits of bosentan in improving the functional impairment in SSc patients.

10.6 Endothelin-1 and Fibrosis

ET-1 has been implicated in the pathogenesis of fibrosis in many organ systems, including those of the skin, lung, and heart [24, 62]. The ET-1 transgenic mice, in which ET-1 is overexpressed in the lungs, showed progressive pulmonary fibrosis [63]. The continuous overexpression of ET-1 in SSc lung fibroblasts is driven through a TAK1/JNK-dependent and ALK5-independent mechanism which then resulted in the overexpression of collagen and CTGF [64]. Bleomycin-injected fibrotic skin of the vascular endothelial cell-specific ET-1 knockout mice showed significantly decreased skin thickness and collagen content compared to that of wild-type mice, indicating that bleomycin-induced skin fibrosis is attenuated in the vascular endothelial cell-specific ET-1 of knockout mice [65]. In addition, ET-1 induced the production of collagen types I and III and fibronectin via ET receptors

A and B on fibroblasts in vitro [24, 25] and reduced the expression of matrix metalloproteinase-1 (MMP-1) [26]. The ETR blocker, bosentan, inhibits the expressions of collagen type I and the fibronectin in fibroblasts in SSc patients [12].

Furthermore, ET-1 is essential for the regulation of alpha smooth muscle actin (α-SMA) expression in fibroblasts. Abnormal α-SMA-positive myofibroblasts in the dermis are thought to be a key feature of fibrotic disease, including SSc. ET-1 induces the expression of α-SMA and other contractility-related factors via ETR-A and a rac/phosphoinositide 3-kinase/Akt-dependent pathway [22]. In addition, the ETR blocker, bosentan, inhibited the expressions of α-SMA and the connective tissue growth factor (CTGF)/CCN2 in fibroblasts in SSc patients [12], thus suggesting that ET-1 might induce the differentiation of myofibroblasts from fibroblasts in fibrotic lesions.

Transforming growth factor-β (TGF-β) plays a major role in the pathogenesis of fibrosis in SSc, and ET-1 plays an important role in the fibrosis process as a downstream regulator or cofactor of TGF-β signaling [66]. ET-1 is induced by TGF-β through SMAD-independent but JNK- and ALK5-dependent pathways in fibroblasts, and TGF-β is induced by ET-1 in other cells, including endothelial cells [67]. Thus, ET-1 and TGF-β synergistically enhance the fibrotic process [24]. The blockade of ETRs suppresses the ability of TGF-β, inducing the expression of fibrogenic cytokines [64]. In addition, the blockade of ETRs by bosentan also suppresses TGF-β-induced skin fibrosis in vivo [68]. It has been reported that ET-1 and connective tissue growth factor (CTGF) play an essential role in this process as downstream regulators or cofactors of TGF-β signaling.

ET-1 also regulates the expression of proinflammatory cytokines, as ET-1 stimulation has been found to induce the inflammatory cytokines, such as IL-1β and TNF-α, from human monocytes [29]. In addition, IL-6 in cultured human smooth muscle cells is also induced by ET-1 [69]. A lot of in vivo and in vitro studies have shown that IL-6 plays an important role in the pathogenesis of fibrosis in SSc. For example, the serum IL-6 levels, which are significantly elevated in patients with early stage SSc [70, 71], correlate with the total skin thickness score in SSc patients [72]. These findings indicate that ET-1-induced IL-6 from fibroblasts may be involved in the pathogenesis of fibrosis in SSc.

The use of imatinib mesylate, which inhibits both c-Abl and platelet-derived growth factor receptor (PDGFR) signaling, was reported to be effective for skin fibrosis in SSc patients, suggesting that the activation of PDGFR signaling might be involved in the pathogenesis of fibrosis in SSc. Interestingly, it has been reported that ET-1 induces the transactivation and signaling of the tyrosine kinase receptors, such as the PDGFR, the vascular endothelial growth factor receptor (VEGFR), and the epidermal growth factor receptor (EGFR) [73, 74]. ET-1 receptor stimulation causes the activation of metalloproteinase, which sheds ligands, such as heparin-binding epidermal-like growth factor (HB-EGF), and leads to the activation of tyrosine kinase receptors [74]. An increase in Ca^{2+} by ET-1 also might be involved in the mechanisms of transactivation of tyrosine kinase receptors. In addition, ET-1 is recognized to be chemotactic for fibrocytes [4]. Fibrocytes belong to

mesenchymal stem cells (MSCs) derived from bone marrow and are involved in the pathogenesis of fibrosis in patients with SSc and other fibrosing diseases [75, 76].

Taken together, these findings indicate that ET-1 alone or together with TGF-β can induce the fibrosis in vivo and in vitro and may play pivotal roles in the pathogenesis of fibrotic diseases.

10.6.1 Endothelin Receptor Antagonists and Skin Fibrosis

Bosentan was reported to be effective in reducing skin fibrosis in patients with SSc [77]. Kuhn et al. conducted a prospective, open-label, noncomparative trial of ten patients with SSc who were administered bosentan for 20 weeks [77]. The total skin score and the number of total digital ulcers at 24 weeks after bosentan treatment were significantly decreased compared with baseline ($P < 0.001$). Other studies have also reported improvement in skin sclerosis among SSc patients who took bosentan treatment [78]. However, further large-scale clinical studies are warranted to identify the efficacy of bosentan with respect to skin fibrosis.

10.6.2 Endothelin Receptor Antagonist and Pulmonary Fibrosis

Various animal models have been employed to demonstrate the role of ET-1 in lung fibrosis [79], and it was found that ET-1 overexpressing transgenic mice spontaneously develop lung fibrosis in conjunction with the accumulation of perivascular inflammatory cells [63]. While ET-1 levels, as well as ETR-A and ETR-B receptor levels, are elevated in bleomycin-induced pulmonary fibrosis, bosentan was found to inhibit this fibrosis [80, 81]. These results suggest that ET-1 might be involved in the pathogenesis of pulmonary fibrosis in the rodent model and that bosentan might reduce fibrosis.

In an in vivo study, the ET-1 serum levels were significantly elevated in patients with idiopathic pulmonary fibrosis. They were also elevated in the broncho-alveolar lavage fluid and breath condensate of SSc patients [82]. In addition, SSc patients with interstitial lung disease (ILD) demonstrated expression of ET-1 on the airway bronchiolar and alveolar epithelia, the interstitium and interstitial vessels, and the alveolar macrophages [34].

In a double-blind, randomized, placebo-controlled study, 77 SSc patients with ILD were administered bosentan and 86 were randomized to administer a placebo. No significant difference between treatment groups was observed for change in the 6-min walk distance up to 12 months. There was also no effect on forced vital capacity (FVC), DLCO, or patient functional status. These results suggest that the use of bosentan as treatment for ILD in SSc patients might not be effective.

10.7 ET-1 and Calcification

Regarding the relationship between calcification and ET-1, it has been reported that the expression of mRNA and protein levels of ET-1 are increased in calcified VSMCs [83]. Furthermore, ETR-A is enhanced in the stenotic aortic valves with fibrosis and calcification [84].

ET-1 induces the intracellular calcium concentration and stimulates both the proliferation and the formation of bone nodules in osteoblastic progenitor cells [85, 86]. Furthermore, it has been reported that ET-1 increases the intracellular $Ca2^{+}$ concentration and DNA synthesis and enhances proliferation and calcium deposition in MSC via ETR-A in vitro and in vivo [83, 85]. These data indicate that ET-1/ETR signaling is involved in the regulation of fibrosis and calcification.

10.8 Endothelin-1 and Fli1

Fli1 is a member of the ETS transcriptional factor family and functions as a repressor of the *COL1A1* and *COL1A2* genes [87–89]. The expression of Fli1 genes is significantly decreased in SSc dermal fibroblasts and dermal microvascular endothelial cells in SSc skin lesions [90, 91]. Akamata et al. report that bosentan restores the pro-fibrotic phenotype of SSc dermal fibroblasts by increasing the DNA-binding ability and the expression levels of Fli1 [92]. In addition, it was recently reported that ET-1 induced the phosphorylation of Fli1 through the sequential activation of c-Abl and protein kinase C-δ, thereby leading to a decrease in Fli1 levels as well as in the Fli1 occupancy of the targeted gene promoters in human dermal microvascular endothelial cells, and bosentan treatment reversed those effects [93]. In endothelial cell-specific Fli1 knockout mice, bosentan treatment increased Fli1 expression as well as the vascular stabilization and restoration of impaired leaky vessels. These results suggest that bosentan might have a beneficial effect on vasculopathy in SSc.

10.9 Conclusion

Many experimental and clinical studies have investigated the role of ET-1 in the pathogenesis of pulmonary arterial hypertension and fibrosis of the skin, lung, or other organs in systemic sclerosis. The potential benefit of ET-1 antagonists in PAH, vasculopathy, and fibrosis in SSc has been identified in recent clinical analyses, and further basic researches and clinical studies, such as the assessment of the efficacy of the combination therapy of ET-1 and other agents, are expected.

References

1. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature. 1988;332:411–5.
2. Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K, Masaki T. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc Natl Acad Sci U S A. 1989;86:2863–7.
3. Mayes MD. Endothelin and endothelin receptor antagonists in systemic rheumatic disease. Arthritis Rheum. 2003;48:1190–9.
4. Schiffrin EL. State-of-the-Art lecture. Role of endothelin-1 in hypertension. Hypertension. 1999;34(4 Pt 2):876–81.
5. Li M, Stenmark KR, Shandas R, Tan W. Effects of pathological flow on pulmonary artery endothelial production of vasoactive mediators and growth factors. J Vasc Res. 2009;46 (6):561–71.
6. MacLean MR, McCulloch KM. Influence of applied tension and nitric oxide on responses to endothelins in rat pulmonary resistance arteries: effect of chronic hypoxia. Br J Pharmacol. 1998;123:991–9.
7. Wort SJ, Mitchell JA, Woods M, Evans TW, Warner TD. The prostacyclin-mimetic cicaprost inhibits endogenous endothelin-1 release from human pulmonary artery smooth muscle cells. J Cardiovasc Pharmacol. 2000;36(5 Suppl 1):S410–3.
8. Golden CL, Nick HS, Visner GA. Thrombin regulation of endothelin-1 gene in isolated human pulmonary endothelial cells. Am J Physiol. 1998;274(5 Pt 1):L854–63.
9. Kurihara H, Yoshizumi M, Sugiyama T, Takaku F, Yanagisawa M, Masaki T, Hamaoki M, Kato H, Yazaki Y. Transforming growth factor-beta stimulates the expression of endothelin mRNA by vascular endothelial cells. Biochem Biophys Res Commun. 1989;159:1435–40.
10. An SJ, Boyd R, Wang Y, Qiu X, Wang HD. Endothelin-1 expression in vascular adventitial fibroblasts. Am J Physiol Heart Circ Physiol. 2006;290:H700–8.
11. An SJ, Boyd R, Zhu M, Chapman A, Pimentel DR, Wang HD. NADPH oxidase mediates angiotensin II-induced endothelin-1 expression in vascular adventitial fibroblasts. Cardiovasc Res. 2007;75:702–9.
12. Shi-Wen X, Renzoni EA, Kennedy L, Howat S, Chen Y, Pearson JD, Bou-Gharios G, Dashwood MR, du Bois RM, Black CM, Denton CP, Abraham DJ, Leask A. Endogenous endothelin-1 signaling contributes to type I collagen and CCN2 overexpression in fibrotic fibroblasts. Matrix Biol. 2007;26:625–32.
13. de Nucci G, Thomas R, D'Orleans-Juste P, Antunes E, Walder C, Warner TD, Vane JR. Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. Proc Natl Acad Sci U S A. 1988;85:9797–800.
14. Kern JM, Maass V, Rupp J, Maass M. Proliferative stimulation of the vascular Endothelin-1 axis in vitro and ex vivo by infection with Chlamydia pneumoniae. Thromb Haemost. 2009;102:743–53.
15. Salani D, Taraboletti G, Rosanò L, Di Castro V, Borsotti P, Giavazzi R, Bagnato A. Endothelin-1 induces an angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. Am J Pathol. 2000;157:1703–11.
16. Daher Z, Noël J, Claing A. Endothelin-1 promotes migration of endothelial cells through the activation of ARF6 and the regulation of FAK activity. Cell Signal. 2008;20:2256–65.
17. Tchekneva E, Quertermous T, Christman BW, Lawrence ML, Meyrick B. Regional variability in Preproendothelin-1 gene expression in sheep pulmonary artery and lung during the onset of air-induced chronic pulmonary hypertension. Participation of arterial smooth muscle cells. J Clin Invest. 1998;101:1389–97.
18. Jankov RP, Kantores C, Belcastro R, Yi M, Tanswell AK. Endothelin-1 inhibits apoptosis of pulmonary arterial smooth muscle in the neonatal rat. Pediatr Res. 2006;60:245–51.

19. Gallelli L, Pelaia G, D'Agostino B, Cuda G, Vatrella A, Fratto D, Gioffrè V, Galderisi U, De Nardo M, Mastruzzo C, Salinaro ET, Maniscalco M, Sofia M, Crimi N, Rossi F, Caputi M, Costanzo FS, Maselli R, Marsico SA, Vancheri C. Endothelin-1 induces proliferation of human lung fibroblasts and IL-11 secretion through an ET(A) receptor-dependent activation of MAP kinases. J Cell Biochem. 2005;96:858–68.
20. Peacock AJ, Dawes KE, Shock A, Gray AJ, Reeves JT, Laurent GJ. Endothelin-1 and endothelin-3 induce chemotaxis and replication of pulmonary artery fibroblasts. Am J Respir Cell Mol Biol. 1992;7:492–9.
21. Kulasekaran P, Scavone CA, Rogers DS, Arenberg DA, Thannickal VJ, Horowitz JC. Endothelin-1 and transforming growth factor-beta1 independently induce fibroblast resistance to apoptosis via AKT activation. Am J Respir Cell Mol Biol. 2009;41:484–93.
22. Shi-Wen X, Chen Y, Denton CP, Eastwood M, Renzoni EA, Bou-Gharios G, Pearson JD, Dashwood M, du Bois RM, Black CM, Leask A, Abraham DJ. Endothelin-1 promotes myofibroblast induction through the ETA receptor via a rac/phosphoinositide 3-kinase/Akt-dependent pathway and is essential for the enhanced contractile phenotype of fibrotic fibroblasts. Mol Biol Cell. 2004;15:2707–19.
23. Xu SW, Denton CP, Dashwood MR, Abraham DJ, Black CM. Endothelin-1 regulation of intercellular adhesion molecule-1 expression in normal and sclerodermal fibroblasts. J Cardiovasc Pharmacol. 1998;31 Suppl 1:S545–7.
24. Horstmeyer A, Licht C, Scherr G, Eckes B, Krieg T. Signalling and regulation of collagen I synthesis by ET-1 and TGF-beta1. FEBS J. 2005;272:6297–309.
25. Xu S, Denton CP, Holmes A, Dashwood MR, Abraham DJ, Black CM. Endothelins: effect on matrix biosynthesis and proliferation in normal and scleroderma fibroblasts. J Cardiovasc Pharmacol. 1998;31 Suppl 1:S360–3.
26. Shi-Wen X, Denton CP, Dashwood MR, Holmes AM, Bou-Gharios G, Pearson JD, Black CM, Abraham DJ. Fibroblast matrix gene expression and connective tissue remodeling: role of endothelin-1. J Investig Dermatol. 2001;116:417–25.
27. Anggrahini DW, Emoto N, Nakayama K, Widyantoro B, Adiarto S, Iwasa N, Nonaka H, Rikitake Y, Kisanuki YY, Yanagisawa M, Hirata K. Vascular endothelial cell-derived endothelin-1 mediates vascular inflammation and neointima formation following blood flow cessation. Cardiovasc Res. 2009;82:143–51.
28. Wilson SH, Simari RD, Lerman A. The effect of endothelin-1 on nuclear factor kappa B in macrophages. Biochem Biophys Res Commun. 2001;286:968–72.
29. Juergens UR, Racké K, Uen S, Haag S, Lamyel F, Stöber M, Gillissen A, Novak N, Vetter H. Inflammatory responses after endothelin B (ETB) receptor activation in human monocytes: new evidence for beneficial anti-inflammatory potency of ETB-receptor antagonism. Pulm Pharmacol Ther. 2008;21:533–9.
30. Kojima T, Hattori K, Hirata Y, Aoki T, Sasai-Takedatsu M, Kino M, Kobayashi Y. Endothelin-1 has a priming effect on production of superoxide anion by alveolar macrophages: its possible correlation with bronchopulmonary dysplasia. Pediatr Res. 1996;39(1):112–6.
31. Yamane K, Kashiwagi H, Suzuki N, Miyauchi T, Yanagisawa M, Goto K, Masaki T. Elevated plasma levels of endothelin-1 in systemic sclerosis. Arthritis Rheum. 1991;34:243–4.
32. Yamane K, Miyauchi T, Suzuki N, Yuhara T, Akama T, Suzuki H, Kashiwagi H. Significance of plasma endothelin-1 levels in patients with systemic sclerosis. J Rheumatol. 1992;19:1566–71.
33. Vancheeswaran R, Azam A, Black C, Dashwood MR. Localization of endothelin-1 and its binding sites in scleroderma skin. J Rheumatol. 1994;21:1268–76.
34. Abraham DJ, Vancheeswaran R, Dashwood MR, Rajkumar VS, Pantelides P, Xu SW, du Bois RM, Black CM. Increased levels of endothelin-1 and differential endothelin type A and B receptor expression in scleroderma-associated fibrotic lung disease. Am J Pathol. 1997;151:831–41.

35. Kawaguchi Y, Suzuki K, Hara M, Hidaka T, Ishizuka T, Kawagoe M, Nakamura H. Increased endothelin-1 production in fibroblasts derived from patients with systemic sclerosis. Ann Rheum Dis. 1994;53:506–10.
36. Shi-wen X, Howat SL, Renzoni EA, Holmes A, Pearson JD, Dashwood MR, Bou-Gharios G, Denton CP, du Bois RM, Black CM, Leask A, Abraham DJ. Endothelin-1 induces expression of matrix-associated genes in lung fibroblasts through MEK/ERK. J Biol Chem. 2004;279:23098–103.
37. Shetty N, Derk CT. Endothelin receptor antagonists as disease modifiers in systemic sclerosis. Inflamm Allergy Drug Targets. 2011;10:19–26.
38. Korn JH, Mayes M, Matucci Cerinic M, Rainisio M, Pope J, Hachulla E, Rich E, Carpentier P, Molitor J, Seibold JR, Hsu V, Guillevin L, Chatterjee S, Peter HH, Coppock J, Herrick A, Merkel PA, Simms R, Denton CP, Furst D, Nguyen N, Gaitonde M, Black C. Digital ulcers in systemic sclerosis: prevention by treatment with bosentan, an oral endothelin receptor antagonist. Arthritis Rheum. 2004;50:3985–93.
39. Cacoub P, Dorent R, Nataf P, Carayon A. Endothelin-1 in pulmonary hypertension. N Engl J Med. 1993;329:1967–8.
40. Giaid A, Yanagisawa M, Langleben D, Michel RP, Levy R, Shennib H, Kimura S, Masaki T, Duguid WP, Stewart DJ. Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. N Engl J Med. 1993;328:1732–9.
41. Dorfmüller P, Perros F, Balabanian K, Humbert M. Inflammation in pulmonary arterial hypertension. Eur Respir J. 2003;22:358–63.
42. Denton CP, Pope JE, Peter HH, Gabrielli A, Boonstra A, van den Hoogen FH, Riemekasten G, De Vita S, Morganti A, Dölberg M, Berkani O, Guillevin L. Long-term effects of bosentan on quality of life, survival, safety and tolerability in pulmonary arterial hypertension related to connective tissue diseases. Ann Rheum Dis. 2008;67:1222–8.
43. Barst RJ, Langleben D, Frost A, Horn EM, Oudiz R, Shapiro S, McLaughlin V, Hill N, Tapson VF, Robbins IM, Zwicke D, Duncan B, Dixon RA, Frumkin LR, STRIDE-1 Study Group. Sitaxsentan therapy for pulmonary arterial hypertension. Am J Respir Crit Care Med. 2004;169:441–7.
44. Galié N, Badesch D, Oudiz R, Simonneau G, McGoon MD, Keogh AM, Frost AE, Zwicke D, Naeije R, Shapiro S, Olschewski H, Rubin LJ. Ambrisentan therapy for pulmonary arterial hypertension. J Am Coll Cardiol. 2005;46:529–35.
45. Arefiev K, Fiorentino DF, Chung L. Endothelin receptor antagonists for the treatment of Raynaud's phenomenon and digital ulcers in systemic sclerosis. Int J Rheumatol. 2011;2011:201787.
46. Rubin LJ, Badesch DB, Barst RJ, Galie N, Black CM, Keogh A, Pulido T, Frost A, Roux S, Leconte I, Landzberg M, Simonneau G. Bosentan therapy for pulmonary arterial hypertension. N Engl J Med. 2002;346(12):896–903. Erratum in: N Engl J Med 2002; 346: 1258.
47. Rubin LJ. Therapy of pulmonary hypertension: the evolution from vasodilators to antiproliferative agents. Am J Respir Crit Care Med. 2002;166:1308–9.
48. Humbert M, Barst RJ, Robbins IM, Channick RN, Galiè N, Boonstra A, Rubin LJ, Horn EM, Manes A, Simonneau G. Combination of bosentan with epoprostenol in pulmonary arterial hypertension: BREATHE-2. Eur Respir J. 2004;24:353–9.
49. McLaughlin VV, Sitbon O, Badesch DB, Barst RJ, Black C, Galiè N, Rainisio M, Simonneau G, Rubin LJ. Survival with first-line bosentan in patients with primary pulmonary hypertension. Eur Respir J. 2005;25:244–9.
50. Sitbon O, McLaughlin VV, Badesch DB, Barst RJ, Black C, Galiè N, Humbert M, Rainisio M, Rubin LJ, Simonneau G. Survival in patients with class III idiopathic pulmonary arterial hypertension treated with first line oral bosentan compared with an historical cohort of patients started on intravenous epoprostenol. Thorax. 2005;60:1025–30.
51. Carter NJ, Keating GM. Bosentan: in pediatric patients with pulmonary arterial hypertension. Paediatr Drugs. 2010;12:63–73.

52. Gruenig E, Michelakis E, Vachiéry JL, Vizza CD, Meyer FJ, Doelberg M, Bach D, Dingemanse J, Galiè N. Acute hemodynamic effects of single-dose sildenafil when added to established bosentan therapy in patients with pulmonary arterial hypertension: results of the COMPASS-1 study. J Clin Pharmacol. 2009;49:1343–52.
53. Motegi S, Toki S, Hattori T, Yamada K, Uchiyama A, Ishikawa O. No association of atherosclerosis with digital ulcers in Japanese patients with systemic sclerosis: evaluation of carotid intima-media thickness and plaque characteristics. J Dermatol. 2014;41:604–8.
54. Matucci-Cerinic M, Denton CP, Furst DE, Mayes MD, Hsu VM, Carpentier P, Wigley FM, Black CM, Fessler BJ, Merkel PA, Pope JE, Sweiss NJ, Doyle MK, Hellmich B, Medsger Jr TA, Morganti A, Kramer F, Korn JH, Seibold JR. Bosentan treatment of digital ulcers related to systemic sclerosis: results from the RAPIDS-2 randomised, double-blind, placebo-controlled trial. Ann Rheum Dis. 2011;70:32–8.
55. Ichimura Y, Asano Y, Hatano M, Tamaki Z, Takekoshi T, Kogure A, Tomita M, Kawashima T, Miyazaki M, Taniguchi T, Takahashi T, Mitsui H, Sugaya M, Yao A, Kinugawa K, Sato S. Significant attenuation of macrovascular involvement by bosentan in a patient with diffuse cutaneous systemic sclerosis with multiple digital ulcers and gangrene. Mod Rheumatol. 2011;21:548–52.
56. Nagai Y, Hasegawa M, Hattori T, Okada E, Tago O, Ishikawa O. Bosentan for digital ulcers in patients with systemic sclerosis. J Dermatol. 2012;39:48–51.
57. Sulli A, Soldano S, Pizzorni C, Montagna P, Secchi ME, Villaggio B, Seriolo B, Brizzolara R, Cutolo M. Raynaud's phenomenon and plasma endothelin: correlations with capillaroscopic patterns in systemic sclerosis. J Rheumatol. 2009;36:1235–9.
58. Selenko-Gebauer N, Duschek N, Minimair G, Stingl G, Karlhofer F. Successful treatment of patients with severe secondary Raynaud's phenomenon with the endothelin receptor antagonist bosentan. Rheumatology (Oxford). 2006;45 Suppl 3:iii45–8.
59. Hettema ME, Zhang D, Bootsma H, Kallenberg CG. Bosentan therapy for patients with severe Raynaud's phenomenon in systemic sclerosis. Ann Rheum Dis. 2007;66:1398–9.
60. Nguyen VA, Eisendle K, Gruber I, Hugl B, Reider D, Reider N. Effect of the dual endothelin receptor antagonist bosentan on Raynaud's phenomenon secondary to systemic sclerosis: a double-blind prospective, randomized, placebo-controlled pilot study. Rheumatology (Oxford). 2010;49:583–7.
61. Guiducci S, Bellando Randone S, Bruni C, Carnesecchi G, Maresta A, Iannone F, Lapadula G, Matucci Cerinic M. Bosentan fosters microvascular de-remodelling in systemic sclerosis. Clin Rheumatol. 2012;31:1723–5.
62. Teder P, Noble PW. A cytokine reborn? Endothelin-1 in pulmonary inflammation and fibrosis. Am J Respir Cell Mol Biol. 2000;23:7–10.
63. Hocher B, Schwarz A, Fagan KA, Thöne-Reineke C, El-Hag K, Kusserow H, Elitok S, Bauer C, Neumayer HH, Rodman DM, Theuring F. Pulmonary fibrosis and chronic lung inflammation in ET-1 transgenic mice. Am J Respir Cell Mol Biol. 2000;23:19–26.
64. Shi-Wen X, Rodríguez-Pascual F, Lamas S, Holmes A, Howat S, Pearson JD, Dashwood MR, du Bois RM, Denton CP, Black CM, Abraham DJ, Leask A. Constitutive ALK5-independent c-Jun N-terminal kinase activation contributes to endothelin-1 overexpression in pulmonary fibrosis: evidence of an autocrine endothelin loop operating through the endothelin A and B receptors. Mol Cell Biol. 2006;26:5518–27.
65. Makino K, Jinnin M, Aoi J, Kajihara I, Makino T, Fukushima S, Sakai K, Nakayama K, Emoto N, Yanagisawa M, Ihn H. Knockout of endothelial cell-derived endothelin-1 attenuates skin fibrosis but accelerates cutaneous wound healing. PLoS One. 2014;9:e97972.
66. Leask A. Signaling in fibrosis: targeting the TGF beta, endothelin-1 and CCN2 axis in scleroderma. Front Biosci (Elite Ed). 2009;1:115–22.
67. Castañares C, Redondo-Horcajo M, Magán-Marchal N, ten Dijke P, Lamas S, Rodríguez-Pascual F. Signaling by ALK5 mediates TGF-beta-induced ET-1 expression in endothelial cells: a role for migration and proliferation. J Cell Sci. 2007;120:1256–66.

68. Lagares D, García-Fernández RA, Jiménez CL, Magán-Marchal N, Busnadiego O, Lamas S, Rodríguez-Pascual F. Endothelin 1 contributes to the effect of transforming growth factor beta1 on wound repair and skin fibrosis. Arthritis Rheum. 2010;62:878–89.
69. Han JH, Lee MY, Myung SC. The effect of endothelin-1 on the production of interleukin-6 in cultured human detrusor smooth muscle cells, and the effect of interleukin-6 on the contractile response of bladder smooth muscle strips from rats. BJU Int. 2009;104:707–12.
70. Stuart RA, Littlewood AJ, Maddison PJ, Hall ND. Elevated serum interleukin-6 levels associated with active disease in systemic connective tissue disorders. Clin Exp Rheumatol. 1995;13:17–22.
71. Khan K, Xu S, Nihtyanova S, Derrett-Smith E, Abraham D, Denton CP, Ong VH. Clinical and pathological significance of interleukin 6 overexpression in systemic sclerosis. Ann Rheum Dis. 2012;71:1235–42.
72. Sato S, Hasegawa M, Takehara K. Serum levels of interleukin-6 and interleukin-10 correlate with total skin thickness score in patients with systemic sclerosis. J Dermatol Sci. 2001;27:140–6.
73. Spinella F, Caprara V, Di Castro V, Rosanò L, Cianfrocca R, Natali PG, Bagnato A. Endothelin-1 induces the transactivation of vascular endothelial growth factor receptor-3 and modulates cell migration and vasculogenic mimicry in melanoma cells. J Mol Med (Berl). 2013;91:395–405.
74. Ivey ME, Osman N, Little PJ. Endothelin-1 signalling in vascular smooth muscle: pathways controlling cellular functions associated with atherosclerosis. Atherosclerosis. 2008;199:237–47.
75. Borie R, Quesnel C, Phin S, Debray MP, Marchal-Somme J, Tiev K, Bonay M, Fabre A, Soler P, Dehoux M, Crestani B. Detection of alveolar fibrocytes in idiopathic pulmonary fibrosis and systemic sclerosis. PLoS One. 2013;8:e53736.
76. Motegi S, Okada E, Uchiyama A, Yamada K, Ogino S, Yokoyama Y, Takeuchi Y, Monma F, Suzuki T, Ishikawa O. Role of endothelin-1/endothelin receptor signaling in fibrosis and calcification in nephrogenic systemic fibrosis. Exp Dermatol. 2014;23:664–9.
77. Kuhn A, Haust M, Ruland V, Weber R, Verde P, Felder G, Ohmann C, Gensch K, Ruzicka T. Effect of bosentan on skin fibrosis in patients with systemic sclerosis: a prospective, open-label, non-comparative trial. Rheumatology (Oxford). 2010;49:1336–45.
78. Nishibu A, Sakai E, Oyama N, Yamamoto T. Endothelin receptor antagonist bosentan improves the dermal sclerosis in a patient with systemic sclerosis. Australas J Dermatol. 2012;53:e32–3.
79. Erdbruegger U, Grossheim M, Hertel B, Wyss K, Kirsch T, Woywodt A, Haller H, Haubitz M. Diagnostic role of endothelial microparticles in vasculitis. Rheumatology (Oxford). 2008;47:1820–5.
80. Mutsaers SE, Foster ML, Chambers RC, Laurent GJ, McAnulty RJ. Increased endothelin-1 and its localization during the development of bleomycin-induced pulmonary fibrosis in rats. Am J Respir Cell Mol Biol. 1998;18:611–9.
81. Park SH, Saleh D, Giaid A, Michel RP. Increased endothelin-1 in bleomycin-induced pulmonary fibrosis and the effect of an endothelin receptor antagonist. Am J Respir Crit Care Med. 1997;156(2 Pt 1):600–8.
82. Reichenberger F, Schauer J, Kellner K, Sack U, Stiehl P, Winkler J. Different expression of endothelin in the bronchoalveolar lavage in patients with pulmonary diseases. Lung. 2001;179:163–74.
83. Wu SY, Zhang BH, Pan CS, Jiang HF, Pang YZ, Tang CS, Qi YF. Endothelin-1 is a potent regulator in vivo in vascular calcification and in vitro in calcification of vascular smooth muscle cells. Peptides. 2003;24:1149–56.
84. Peltonen T, Taskinen P, Näpänkangas J, Leskinen H, Ohtonen P, Soini Y, Juvonen T, Satta J, Vuolteenaho O, Ruskoaho H. Increase in tissue endothelin-1 and ETA receptor levels in human aortic valve stenosis. Eur Heart J. 2009;30:242–9.

85. Tykocki NR, Watts SW. The interdependence of endothelin-1 and calcium: a review. Clin Sci (Lond). 2010;119:361–72.
86. von Schroeder HP, Veillette CJ, Payandeh J, Qureshi A, Heersche JN. Endothelin-1 promotes osteoprogenitor proliferation and differentiation in fetal rat calvarial cell cultures. Bone. 2003;33:673–84.
87. Asano Y, Czuwara J, Trojanowska M. Transforming growth factor-beta regulates DNA binding activity of transcription factor Fli1 by p300/CREB-binding protein-associated factor-dependent acetylation. J Biol Chem. 2007;282:34672–83.
88. Asano Y, Trojanowska M. Phosphorylation of Fli1 at threonine 312 by protein kinase C delta promotes its interaction with p300/CREB-binding protein-associated factor and subsequent acetylation in response to transforming growth factor beta. Mol Cell Biol. 2009;29:1882–94.
89. Asano Y, Trojanowska M. Fli1 represses transcription of the human α2(I) collagen gene by recruitment of the HDAC1/p300 complex. PLoS One. 2013;8:e74930.
90. Wang Y, Fan PS, Kahaleh B. Association between enhanced type I collagen expression and epigenetic repression of the FLI1 gene in scleroderma fibroblasts. Arthritis Rheum. 2006;54:2271–9.
91. Kubo M, Czuwara-Ladykowska J, Moussa O, Markiewicz M, Smith E, Silver RM, Jablonska S, Blaszczyk M, Watson DK, Trojanowska M. Persistent down-regulation of Fli1, a suppressor of collagen transcription, in fibrotic scleroderma skin. Am J Pathol. 2003;163:571–81.
92. Akamata K, Asano Y, Aozasa N, Noda S, Taniguchi T, Takahashi T, Ichimura Y, Toyama T, Sato S. Bosentan reverses the pro-fibrotic phenotype of systemic sclerosis dermal fibroblasts via increasing DNA binding ability of transcription factor Fli1. Arthritis Res Ther. 2014;16:R86.
93. Akamata K, Asano Y, Yamashita T, Noda S, Taniguchi T, Takahashi T, Ichimura Y, Toyama T, Trojanowska M, Sato S. Endothelin receptor blockade ameliorates vascular fragility in endothelial cell-specific fli-1-knockout mice by increasing fli-1 DNA binding ability. Arthritis Rheum. 2015;67:1335–44.

The Role of B Cells in Systemic Sclerosis

11

Takashi Matsushita and Kazuhiko Takehara

Abstract

Systemic sclerosis (SSc) is an autoimmune disease marked by excessive extracellular matrix deposition in the skin and internal organs. Three major abnormalities, including autoimmunity, vasculopathy, and fibrosis, are considered to play important roles in the pathophysiology of SSc. A variety of immunological abnormalities of T and B cells have been detected in SSc. Over 90 % of the patients are positive for autoantibodies, which react to various intracellular components. Hyper-γ-globulinemia and B cell hyperactivity have also been detected in SSc patients. SSc patients have been reported to show distinct abnormalities in blood B lymphocyte compartments, characterized by expanded naive B cells and activated memory B cells. In addition, B cells from SSc patients overexpress CD19, a critical cell-surface signal transduction molecule, by ~20 %. Furthermore, a B cell activating factor belonging to the tumor necrosis factor family (BAFF) has been reported to be elevated in patients with SSc and to be correlated with the severity of SSc. In the tight-skin mouse model of SSc, chronic B cell activation is critical not only for the induction of autoantibodies but also for the development of skin fibrosis. In particular, an absence of CD19 or B cell depletion therapy, with an anti-CD20 antibody or a BAFF antagonist, suppresses the development of skin fibrosis and autoimmunity in mouse models of SSc. A number of recent studies on human SSc have shown that B cell depletion therapy has beneficial effects on skin and lung fibrosis in patients with SSc. However, B cells play not only a disease-promoting role but also an inhibitory role in autoimmune diseases. Regulatory B cells negatively regulate inflammation and autoimmunity and are present in human SSc patients.

T. Matsushita (✉) • K. Takehara
Department of Dermatology, Faculty of Medicine, Institute of Medical, Pharmaceutical, and Health Sciences, Kanazawa University, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8641, Japan
e-mail: t-matsushita@med.kanazawa-u.ac.jp

K. Takehara et al. (eds.), *Systemic Sclerosis*, DOI 10.1007/978-4-431-55708-1_11

Furthermore, regulatory B cells have been shown to have an inhibitory function in the mouse model of SSc. Thus, depletion of regulatory B cells may worsen autoimmune diseases. Therefore, selective B cell depletion, which retains regulatory B cells, may be a potential therapy for autoimmune diseases, including SSc.

Keywords
Systemic sclerosis • B cell • Regulatory B cell • CD19 • Rituximab

11.1 Introduction

Systemic sclerosis (SSc) is an autoimmune disease marked by excessive extracellular matrix deposition in the skin and internal organs [1]. Three major abnormalities, as autoimmunity, vasculopathy, and fibrosis, are considered to play important roles in the pathophysiology of SSc [2] and thus, should be thoroughly investigated in order to identify new targets for effective therapies. A variety of immunological abnormalities of T and B lymphocytes have been detected in SSc [3]. Although several studies have focused on T cells as pathogenic mediators of SSc, recent studies have shed light on the critical role of B cells in the development of systemic autoimmune diseases [4–6]. Indeed, over 90 % of patients are positive for autoantibodies, which react to various intracellular components. The autoantibodies associated with SSc include anti-DNA topoisomerase I, anticentromere, and anti-RNA polymerases (Abs) [7]. Hyper-γ-globulinemia and B cell hyperactivity have been detected in SSc patients [8, 9]. SSc patients have been reported to show distinct abnormalities in blood B lymphocyte compartments, characterized by expanded naive B cells and activated memory B cells [10]. It has also been reported that a B cell activating factor belonging to the tumor necrosis factor family (BAFF) is elevated in patients with SSc and is correlated with the severity of SSc [11]. In the tight-skin mouse model of SSc, chronic B cell activation is critical not only for the induction of autoantibodies but also for the development of skin fibrosis [12]. Furthermore, B cell depletion using an antimouse CD20 antibody or a BAFF antagonist suppresses the development of skin fibrosis in the tight-skin mouse [13, 14]. In addition, several recent studies have revealed a possible beneficial effect of antihuman CD20 antibody (rituximab) therapy for human SSc patients [15–21]. Thus, B cells play an important role in the development of SSc in humans, and B cell abnormalities are observed in mouse models of SSc. However, in addition to their positive regulatory role, B cells also play a negative regulatory role. Regulatory B cells that produce IL-10 are potent negative regulators of inflammation and autoimmunity in human and mouse models of disease [22, 23]. Our recent study revealed that regulatory B cells play an inhibitory role in murine sclerodermatous chronic graft-versus-host disease (Scl-cGVHD), a mouse model of scleroderma [23]. It has been reported that pan-B cell depletion, including regulatory B cell depletion, worsens autoimmunity in humans [24, 25]. Pan-B cell depletion is not always a beneficial effect in autoimmunity.

Therefore, it is necessary to investigate the role of regulatory B cells in systemic sclerosis. In this context, we review the positive and negative regulatory roles of B cells in the pathogenesis of SSc in human and mouse models.

11.2 B Cell Function

B cell development is tightly regulated, from their early progenitors to their terminal differentiation into plasma cells [26]. The pathway of B cell development depends on signals that control both negative and positive selection of B cells in the bone marrow and in peripheral lymphoid tissues. The signaling thresholds of B cells are controlled by response regulators, such as CD19 and CD22, that augment or diminish B cell signals during responses to self- and foreign antigens [27, 28]. Among these response regulators, the cell-surface signal transduction molecule CD19 is the most potent positive regulator. In contrast, CD22 plays a negative regulatory role in B cell signaling [29]. Thus, dysregulation of these response regulators may accelerate autoimmunity. Historically, B cells have primarily been studied in their role as effector B cells, which positively regulate the immune response via antibody production. B cells can also serve as antigen-presenting cells (APCs), with the capacity to present Ag 10^3- to 10^4-fold more efficiently than other APCs [30]. Therefore, abnormalities in these B cell functions could contribute to the induction of systemic autoimmunity independent of autoantibody production. However, regulatory B cells have also been identified, and these B cells were subsequently revealed to negatively control the immune response [31, 32]. Effector B cells accelerate autoimmunity, whereas regulatory B cells inhibit autoimmunity. Thus, these two subsets of B cells play two conflicting roles in autoimmunity.

11.3 B Cell Roles in Mouse Models of SSc

SSc is an autoimmune disease marked by excessive extracellular matrix deposition in the skin and internal organs. Although the etiology of SSc remains unclear, three major abnormalities are considered to play important roles in its pathophysiology: autoimmunity, vasculopathy, and fibrosis. Mouse models are critical tools for furthering our understanding of the pathophysiology underlying this disease. The bleomycin-induced scleroderma model and TSK/+ mice are the primary SSc models. Chronic graft-versus-host disease (cGVHD) emerges from alloreactive reactions between donor-derived immune cells and host cell populations. Transplantation across minor histocompatibility loci of B10.D2 bone marrow (BM) and splenocytes into sublethally irradiated BALB/c recipients is a well-established animal model for human Scl-cGVHD and SSc, both of which show many clinical similarities to human SSc [33]. Importantly, a growing number of studies suggest a role for B cells, including regulatory B cells, in the pathogenesis of SSc. Autoantibodies against SSc-specific target autoantigens, including DNA topoisomerase-I, fibrillin 1, and RNA polymerase I, have been detected in many

scleroderma mouse models [34–37]. Constitutive CD19 tyrosine phosphorylation is augmented in TSK/+ B cells compared with wild-type B cells, and TSK/+ B cells exhibit a phenotype consistent with chronic activation [12]. In addition, intracellular Ca^{2+} responses generated by CD19 ligation are elevated in TSK/+ B cells. Thus, the CD19 signaling pathway appears to be constitutively activated in TSK/+ B cells, and this abnormal basal signaling likely results in the characteristic hyperresponsiveness of TSK/+ B cells. Serum levels of autoantibodies are significantly elevated in TSK/+ mice, whereas the lack of CD19 in TSK/+ mice completely abrogates autoantibody production [12]. Furthermore, CD19 deficiency attenuates skin fibrosis in TSK/+ mice. Similar to TSK/+ mice, the loss of CD19 attenuates the development of skin and lung fibrosis in the bleomycin-induced scleroderma model [36]. CD19 has also been reported to regulate fibrogenic cytokine production and autoantibody production by B cells via Toll-like receptor 4 signaling [36]. Thus, B cells regulate skin fibrosis in TSK/+ mice via the CD19 signaling pathway [12]. Similarly, B cell hyperresponsiveness in TSK/+ mice also results from abnormalities in the function of CD22, a critical negative regulator of BCR signaling [38]. CD19 is a major target of the CD22 inhibitory pathway [29]. CD19 may also be indirectly influenced by CD22, in that disruption of negative regulation of signaling by CD22 in TSK/+ B cells may result in abnormal activation of downstream signal transduction molecules, including CD19. Hasegawa et al. showed that B cell depletion using an antibody against mouse CD20 attenuated skin fibrosis in TSK/+ mice [14]. Thus, B cells play critical roles in the development of skin fibrosis and autoimmunity in TSK/+ mice. Collectively, these results show that B cell depletion therapy may be a promising treatment for SSc. Serum BAFF levels have been reported to be significantly elevated in TSK/+ mice, similar to what has been observed in SSc patients [11, 13]. Moreover, administration of a BAFF antagonist inhibits the development of skin fibrosis and autoantibody production in TSK/+ mice. Additionally, BAFF-stimulated TSK/+ B cells show a significantly enhanced ability to produce IL-6, a cytokine that may contribute to fibrosis and autoimmunity. An anti-BAFF antibody (belimumab) has recently been approved for the treatment of systemic lupus erythematosus (SLE), and BAFF may be a candidate for the treatment of SSc in the near future. Thus, multiple murine studies have revealed a critical role for B cells in the pathogenesis of SSc.

11.4 B Cell Roles in SSc

B cells have been reported to be chronically activated in SSc, as the frequency of B cells expressing activation markers, including HLA-DR and CD25, is increased in SSc [8, 39, 40]. Indeed, systematic analysis of the patterns of gene expression in biopsies from scleroderma patients revealed upregulation of genes related to B cells [41]. B cells have been reported to infiltrate the lesional skin in the lungs of patients with SSc [15, 41, 42]. Sato et al. showed that B cells from SSc patients overexpress CD19 by ~20 % [43]. CD19 is a critical cell-surface signal transduction molecule on B cells that regulates basal signaling thresholds and accelerates signaling

through B cell antigen receptors [44]. Furthermore, a polymorphism of the CD19 promoter region has been identified and found to be correlated with higher CD19 expression on B cells and increased susceptibility to SSc [45]. SSc patients show distinct abnormalities in blood B lymphocyte compartments, characterized by expanded naive B cells and activated memory B cells, whereas they show diminished memory B cells and plasmablasts/early plasma cells due to increased apoptosis [10]. Therefore, it is plausible that the continuous loss of memory B cells and plasmablasts/early plasma cells leads to increased production of naive B cells in the bone marrow in order to maintain B cell homeostasis. This CD22 polymorphism is also associated with susceptibility to limited cutaneous SSc [46]. This CD22 polymorphism is correlated with reduced CD22 expression on B cells in patients with SSc. Thus, expression of CD19 and CD22, positive and negative regulators of B cell signal transduction, is altered in SSc patients. Our study revealed that elevated BAFF levels are associated with the severity of skin sclerosis [11]. BAFF mRNA expression was upregulated in affected skin from patients in the early stages of SSc. BAFF-R expression on B cells was also increased in SSc patients. Furthermore, SSc B cells exhibited an enhanced ability to produce IL-6 by BAFF stimulation. Another study reported that serum levels of BAFF and CXCL13, a chemokine which is responsible for the migration of B cells to the site of inflammation, were increased in patients with SSc [47]. Thus, BAFF and its signaling in B cells contribute to B cell abnormalities and disease development in SSc (Fig. 11.1).

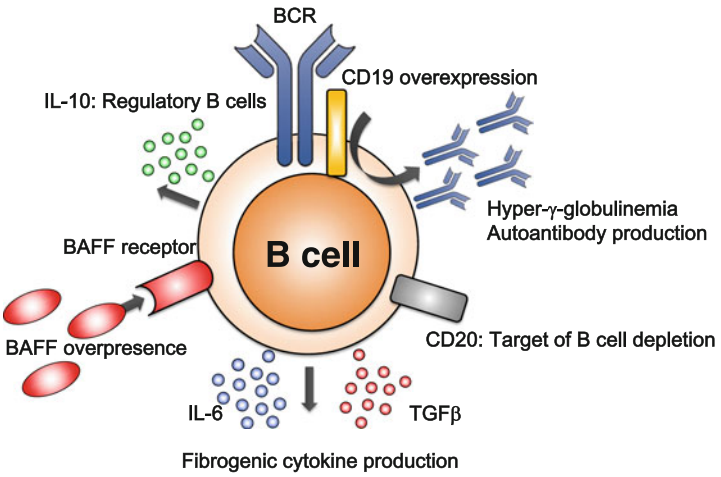

Fig. 11.1 B cell roles in SSc. Chronic B cell activation is found in SSc with CD19 overexpression. Enhanced BAFF expression in SSc patients can maintain the chronic B cell activation that leads to increased production of autoantibody and fibrogenic cytokines. Thus, B cells play critical roles in the development of SSc and could be a therapeutic target in human SSc. Several recent studies have revealed a possible beneficial effect of antihuman CD20 antibody (rituximab) therapy for SSc. On the other hand, IL-10-producing regulatory B cells negatively regulate inflammation and autoimmunity and are present in human SSc patients. Thus, depletion of regulatory B cells may worsen autoimmune diseases

11.5 B Cells and Fibrosis

Stimulated naïve T cells differentiate into memory/effector T cells that are classified into T-helper type 1 (Th1) and Th2 subsets, based on their cytokine production profiles [48]. Th1 cells mainly secrete interferon (IFN)-γ and interleukin (IL)-2, whereas Th2 cells predominantly release IL-4, IL-5, IL-6, IL-10, and IL-13 [48]. These Th2 cytokines enhance Ig production by B cells. It has been suggested that Th1 cytokines generally decrease extracellular matrix deposition, whereas Th2 cytokines increase it [49–51]. Activated B cells can produce IL-6, which induces Th2-dominant immune responses [52, 53]. IL-6 has been reported to promote tissue fibrosis [50, 54, 55]. Increased IL-6 production from activated B cells could directly promote fibrosis in patients with SSc. In a mouse model of SSc, stimulated B cells produced high levels of IL-6 [12]. Activated B cells also produce active TGF-β in vitro [56]. Recently, it was reported that B cells could directly stimulate dermal fibroblasts to produce collagen, IL-6, and TGF-β [57]. Furthermore, this induction was dependent on cell–cell contact and could be increased by the addition of BAFF to the coculture. In contrast, B cell-induced collagen production was significantly inhibited by anti-TGF-β antibodies. TGF-β from activated B cells in SSc patients induces excessive collagen synthesis in fibroblasts, which is further enhanced by BAFF stimulation [57]. Thus, B cells may have a direct effect on the production of collagen by dermal fibroblasts. Collectively, these results show that IL-6 and TGF-β from activated B cells in SSc patients accelerate collagen synthesis (Fig. 11.1).

11.6 B Cell Depletion Therapy in SSc

As previously described, B cells play critical roles in the development of SSc and could be a therapeutic target in human SSc. Rituximab, a chimeric IgG1 monoclonal antibody that specifically binds to CD20 on B cells, has been approved for the treatment of certain non-Hodgkin's lymphomas and autoimmune diseases, such as rheumatoid arthritis, granulomatosis with polyangiitis, and microscopic polyangiitis [58]. Lafyatis et al. reported that rituximab caused depletion of B cells in both peripheral blood and skin in a small open-label study of systemic sclerosis [15]. However, B cell depletion therapy did not show significant effects on skin sclerosis or autoantibody titers. Nonetheless, rituximab treatment prevented the development of progressive pulmonary disease. Bosello et al. conducted a 12-week open-label study on nine dcSSc patients treated with rituximab and methylprednisolone [16]. They reported an improvement in skin sclerosis, measured using the modified Rodnan total skin thickness score (mRSS), in patients treated with rituximab, representing a 43 % improvement. Smith et al. conducted a 24-week open-label study in eight dcSSc patients treated with rituximab and methylprednisolone [17]. In this study, B cell depletion therapy significantly improved the mRSS and histological findings, representing a 40 % improvement. The participants in this cohort study were then re-treated with rituximab at 6 months and followed for 2 years [18]. B cell depletion therapy significantly reduced the

mRSS at 24 months, representing a 45 % improvement [18]. However, all of these were open-label studies. Daoussis et al. conducted a randomized controlled small study with eight dcSSc patients [19, 20]. B cell depletion therapy significantly improved lung function in SSc patients with interstitial lung diseases. The mRSS was reduced at 2 years compared with baseline (−8.63 score). In this study, the median percentage of FVC in the rituximab group was significantly improved compared with controls (10.3 % vs. 5.0 %; $P = 0.002$). Similarly, the median percentage of DLco in the rituximab group was significantly improved compared with controls (19.5 % vs. 7.5 %; $P = 0.023$). Recently, the European Scleroderma Trials and Research (EUSTAR) group conducted a multicenter nested case–control study in which 63 SSc patients were treated with rituximab [21]. In this study, mRSS changes in patients with severe dcSSc were larger in the rituximab-treated group than in matched controls (-24.0 ± 5.2 % vs. -7.7 ± 4.3 %; $P = 0.03$). The mean mRSS in the rituximab-treated group was significantly reduced at 6 months, compared with the baseline (15 % improvement, $P = 0.008$). In addition, rituximab treatment significantly prevented the further decline of FVC in SSc patients with interstitial lung disease compared with matched controls (0.4 ± 4.4 % vs. -7.7 ± 3.6 %; $P = 0.02$). Collectively, these results show that B cell depletion therapy with rituximab could have beneficial effects on skin and lung fibrosis in patients with SSc (Fig. 11.1 and Table 11.1). Phase III randomized controlled studies will be needed to confirm the efficacy and safety of rituximab for the treatment of SSc.

11.7 Regulatory B Cells

B cells positively regulate immune responses through activation of T cells and production of antibodies. Notably, specific minor subsets of B cells can negatively regulate inflammation and autoimmunity [59–61]. Tedder et al. identified a rare and phenotypically unique $CD1d^{hi}CD5^{+}CD19^{hi}$ subset of regulatory B cells in the murine spleen of both normal and autoimmune mice [59]. These regulatory B cells that produce IL-10 are now recognized as negative regulators of the immune system, inflammation and autoimmunity in mouse models of diseases such as rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis [60, 62, 63]. Furthermore, regulatory B cells that produce IL-10 have also been identified in humans [22]. Regulatory B cells in humans normally represent <1 % of peripheral blood B cells [22]. The phenotype of human regulatory B cells is reported to be the $CD24^{hi}CD27^{+}$ B cell subset [22] or $CD19^{+}CD24^{hi}CD38^{hi}$ B cells [64]. Thus, it is necessary to investigate the role of regulatory B cells in SSc and its murine models. We reported that donor-derived regulatory B cells are important for the suppression of SSc in the Scl-cGVHD model [23]. The absence of regulatory B cells induced severe Scl-cGVHD, whereas replacement of regulatory B cells in the early phase restored the exacerbated Scl-cGVHD. However, replacement of regulatory B cells in the late phase had no effect on Scl-cGVHD. This suggests that regulatory B cells may play an important role in the onset of Scl-cGVHD rather

Table 11.1 Studies on the efficacy of rituximab in SSc

First author, year (ref)	Type of study	Number of patients	Rituximab treatment	Skin result	Lung result
Lafyatis et al. 2009 [15]	Open label	15	1 g/2 weeks apart	No change in skin score	Increased in FVC, 3.5 % improvement (at 6 months) no significant change in DLco
Bosello et al. 2010 [16]	Open label	9	1 g/2 weeks apart	Decreased in skin score, 43.3 % improvement (at 6 months)	No significant change in FVC and DLco
Smith et al. 2010, 2013 [17, 18]	Open label	8	1 g/2 weeks apart	Decreased in skin score, 45 % improvement (at 2 years)	No significant change in FVC and DLco
Daoussis et al. 2010, 2012 [19, 20]	Open label, randomized controlled study	8	4×375 mg/m^2, at baseline and 24 weeks	Decreased in skin score, 64 % improvement (at 2 years)	Increased in FVC, 10.3 % improvement (at 2 years) increased in DLco, 19.5 % improvement (at 2 years)
Jordan et al. 2014 [21]	Multicenter nested case–control study	25	1 g/2 weeks apart or single shot of 1 g	Decreased in skin score, 15 % improvement compared with baseline (at 7 months) decreased in skin score, 7 % improvement compared with matched controls (at 7 months)	Rituximab prevented further decline of FVC compared with matched controls (0.4 ± 4.4 % vs -7.7 ± 3.6 %, at 7 months)

than disease progression. Thus, these results suggested that regulatory B cells may also have an inhibitory function in SSc pathogenesis. Recently, we investigated regulatory B cells in patients with SSc [65]. The frequency of blood regulatory B cells was significantly lower in patients with SSc than in healthy controls (5.43 % vs 11.60 %, $P < 0.0001$). Furthermore, regulatory B cell levels were negatively

correlated with the titer of anti-topoisomerase I Ab and anticentromere Ab in SSc patients. These results suggest that decreased levels of regulatory B cell levels may contribute to the development of SSc (Fig. 11.1). Two large randomized controlled trials of rituximab, which depletes human pan-B cells, were conducted in SLE patients, with the expectation that it would be effective. However, it failed to achieve the primary endpoints [66, 67]. This ineffectiveness may have been due to the presence of regulatory B cells. B cell depletion will worsen the condition of the patient, if regulatory B cells are dominant in a patient with SLE. Thus, the outcome of pan-B cell depletion depends on the balance of effector B cells versus regulatory B cells in the patient. A protocol that selectively depletes effector B cells while sparing regulatory B cells may offer a particularly potent therapy for autoimmune diseases, including SSc.

11.8 Conclusion

A number of recent studies in human and mouse models of SSc have revealed critical roles for B cells in the development of SSc. B cells contribute to the pathogenesis of SSc, via production not only of autoantibodies but also of cytokines, such as IL-6 and TGF-β. B cell depletion therapy with rituximab could have beneficial effects on skin and lung fibrosis in patients with SSc. However, B cells play not only a disease-promoting role but also an inhibitory role in autoimmune diseases. Regulatory B cells negatively regulate inflammation and autoimmunity, and are known to be present in human SSc patients. Furthermore, regulatory B cells have an inhibitory function in the mouse model of SSc. Thus, depletion of regulatory B cells may worsen autoimmune diseases. Therefore, selective B cell depletion, which retains regulatory B cells, may offer a particularly potent therapy for autoimmune diseases, including SSc.

References

1. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger Jr TA, et al. Scleroderma (systemic sclerosis): classification, subsets, and pathogenesis. J Rheumatol. 1988;15:202–5.
2. Furst DE, Clements PJ. Hypothesis for the pathogenesis of systemic sclerosis. J Rheumatol. 1997;24 suppl 48:53–7.
3. White B. Immunopathogenesis of systemic sclerosis. Rheum Dis Clin North Am. 1996;22 (4):695–708.
4. Martin F, Chan AC. Pathogenic roles of B cells in human autoimmunity; insights from the clinic. Immunity. 2004;20(5):517–27.
5. Hasegawa M. B lymphocytes: shedding new light on the pathogenesis of systemic sclerosis. J Dermatol. 2010;37(1):3–10. doi:10.1111/j.1346-8138.2009.00763.x.
6. Sato S, Fujimoto M, Hasegawa M, Takehara K, Tedder TF. Altered B lymphocyte function induces systemic autoimmunity in systemic sclerosis. Mol Immunol. 2004;41(12):1123–33.
7. Okano Y. Antinuclear antibody in systemic sclerosis (scleroderma). Rheum Dis Clin North Am. 1996;22:709–35.

8. Famularo G, Giacomelli R, Alesse E, Cifone MG, Morrone S, Boirivant M, et al. Polyclonal B lymphocyte activation in progressive systemic sclerosis. J Clin Lab Immunol. 1989;29 (2):59–63.
9. Fleischmajer R, Perlish JS, Reeves JRT. Cellular infiltrates in scleroderma skin. Arthritis Rheum. 1977;20:975–84.
10. Sato S, Fujimoto M, Hasegawa M, Takehara K. Altered blood B lymphocyte homeostasis in systemic sclerosis: expanded naive B cells and diminished but activated memory B cells. Arthritis Rheum. 2004;50(6):1918–27.
11. Matsushita T, Hasegawa M, Yanaba K, Kodera M, Takehara K, Sato S. Elevated serum BAFF levels in patients with systemic sclerosis: enhanced BAFF signaling in systemic sclerosis B lymphocytes. Arthritis Rheum. 2006;54(1):192–201.
12. Saito E, Fujimoto M, Hasegawa M, Komura K, Hamaguchi Y, Kaburagi Y, et al. CD19-dependent B lymphocyte signaling thresholds influence skin fibrosis and autoimmunity in the tight-skin mouse. J Clin Invest. 2002;109(11):1453–62. doi:10.1172/JCI15078.
13. Matsushita T, Fujimoto M, Hasegawa M, Matsushita Y, Komura K, Ogawa F, et al. BAFF antagonist attenuates the development of skin fibrosis in tight-skin mice. J Invest Dermatol. 2007;127(12):2772–80. doi:10.1038/sj.jid.5700919.
14. Hasegawa M, Hamaguchi Y, Yanaba K, Bouaziz JD, Uchida J, Fujimoto M, et al. B-lymphocyte depletion reduces skin fibrosis and autoimmunity in the tight-skin mouse model for systemic sclerosis. Am J Pathol. 2006;169(3):954–66.
15. Lafyatis R, Kissin E, York M, Farina G, Viger K, Fritzler MJ, et al. B cell depletion with rituximab in patients with diffuse cutaneous systemic sclerosis. Arthritis Rheum. 2009;60 (2):578–83. doi:10.1002/art.24249.
16. Bosello S, De Santis M, Lama G, Spano C, Angelucci C, Tolusso B, et al. B cell depletion in diffuse progressive systemic sclerosis: safety, skin score modification and IL-6 modulation in an up to thirty-six months follow-up open-label trial. Arthritis Res Ther. 2010;12(2):R54. doi:10.1186/ar2965.
17. Smith V, Van Praet JT, Vandooren B, Van der Cruyssen B, Naeyaert JM, Decuman S, et al. Rituximab in diffuse cutaneous systemic sclerosis: an open-label clinical and histopathological study. Ann Rheum Dis. 2010;69(1):193–7. doi:10.1136/ard.2008.095463.
18. Smith V, Piette Y, van Praet JT, Decuman S, Deschepper E, Elewaut D, et al. Two-year results of an open pilot study of a 2-treatment course with rituximab in patients with early systemic sclerosis with diffuse skin involvement. J Rheumatol. 2013;40(1):52–7. doi:10.3899/jrheum.120778.
19. Daoussis D, Liossis SNC, Tsamandas AC, Kalogeropoulou C, Kazantzi A, Sirinian C, et al. Experience with rituximab in scleroderma: results from a 1-year, proof-of-principle study. Rheumatology (Oxford). 2010;49(2):271–80. doi:10.1093/rheumatology/kep093.
20. Daoussis D, Liossis SN, Tsamandas AC, Kalogeropoulou C, Paliogianni F, Sirinian C, et al. Effect of long-term treatment with rituximab on pulmonary function and skin fibrosis in patients with diffuse systemic sclerosis. Clin Exp Rheumatol. 2012;30(2 Suppl 71):S17–22.
21. Jordan S, Distler JH, Maurer B, Huscher D, van Laar JM, Allanore Y, et al. Effects and safety of rituximab in systemic sclerosis: an analysis from the European Scleroderma Trial and Research (EUSTAR) group. Ann Rheum Dis. 2014. doi:10.1136/annrheumdis-2013-204522.
22. Iwata Y, Matsushita T, Horikawa M, Dilillo DJ, Yanaba K, Venturi GM, et al. Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. Blood. 2011;117(2):530–41. doi:10.1182/blood-2010-07-294249.
23. Le Huu D, Matsushita T, Jin G, Hamaguchi Y, Hasegawa M, Takehara K, et al. Donor-derived regulatory B cells are important for suppression of murine sclerodermatous chronic graft-versus-host disease. Blood. 2013;121(16):3274–83. doi:10.1182/blood-2012-11-465658.
24. Olivieri I, D'Angelo S, Leccese P, Vertone D, Olivieri A. Worsening of psoriasis with rituximab therapy. Clin Exp Rheumatol. 2010;28(6):926.
25. Dass S, Vital EM, Emery P. Development of psoriasis after B cell depletion with rituximab. Arthritis Rheum. 2007;56(8):2715–8. doi:10.1002/art.22811.

26. St Clair EW, Tedder TF. New prospects for autoimmune disease therapy: B cells on death-watch. Arthritis Rheum. 2006;54(1):1–9. doi:10.1002/art.21525.
27. Tedder TF, Inaoki M, Sato S. The CD19-CD21 complex regulates signal transduction thresholds governing humoral immunity and autoimmunity. Immunity. 1997;6(2):107–18.
28. Fearon DT, Carter RH. The CD19/CR2/TAPA-1 complex of B lymphocytes: linking natural to acquired immunity. Annu Rev Immunol. 1995;13:127–49. doi:10.1146/annurev.iy.13.040195.001015.
29. Fujimoto M, Sato S. B cell signaling and autoimmune diseases: CD19/CD22 loop as a B cell signaling device to regulate the balance of autoimmunity. J Dermatol Sci. 2007;46(1):1–9. doi:10.1016/j.jdermsci.2006.12.004.
30. Liu Y, Wu Y, Ramarathinam L, Guo Y, Huszar D, Trounstine M, et al. Gene-targeted B-deficient mice reveal a critical role for B cells in the CD4 T cell response. Int Immunol. 1995;7:1353–62.
31. Bouaziz JD, Yanaba K, Tedder TF. Regulatory B cells as inhibitors of immune responses and inflammation. Immunol Rev. 2008;224:201–14. doi:10.1111/j.1600-065X.2008.00661.x.
32. Fujimoto M. Regulatory B, cells in skin and connective tissue diseases. J Dermatol Sci. 2010;60(1):1–7. doi:10.1016/j.jdermsci.2010.08.010.
33. Yamamoto T. Characteristics of animal models for scleroderma. Curr Rheumatol Rev. 2005;1(1):101–9. doi:10.2174/1573397052954109.
34. Menton DN, Hess RA, Lichtenstein JR, Eisen A. The structure and tensile properties of the skin of tight-skin (Tsk) mutant mice. J Invest Dermatol. 1978;70(1):4–10.
35. Ishikawa H, Takeda K, Okamoto A, Matsuo S, Isobe K. Induction of autoimmunity in a bleomycin-induced murine model of experimental systemic sclerosis: an important role for CD4+ T cells. J Invest Dermatol. 2009;129(7):1688–95. doi:10.1038/jid.2008.431.
36. Yoshizaki A, Iwata Y, Komura K, Ogawa F, Hara T, Muroi E, et al. CD19 regulates skin and lung fibrosis via toll-like receptor signaling in a model of bleomycin-induced scleroderma. Am J Pathol. 2008;172(6):1650–63. doi:10.2353/ajpath.2008.071049.
37. Casciola-Rosen L, Wigley F, Rosen A. Scleroderma autoantigens are uniquely fragmented by metal-catalyzed oxidation reactions: implications for pathogenesis. J Exp Med. 1997;185:71–9.
38. Asano N, Fujimoto M, Yazawa N, Shirasawa S, Hasegawa M, Okochi H, et al. B lymphocyte signaling established by the CD19/CD22 loop regulates autoimmunity in the tight-skin mouse. Am J Pathol. 2004;165(2):641–50. doi:10.1016/s0002-9440(10)63328-7.
39. Frieri M, Angadi C, Paolano A, Oster N, Blau SP, Yang S, et al. Altered T cell subpopulations and lymphocytes expressing natural killer cell phenotypes in patients with progressive systemic sclerosis. J Allergy Clin Immunol. 1991;87(4):773–9.
40. Freundlich B, Jimenez SA. Phenotype of peripheral blood lymphocytes in patients with progressive systemic sclerosis: activated T lymphocytes and the effect of D-penicillamine therapy. Clin Exp Immunol. 1987;69(2):375–84.
41. Whitfield ML, Finlay DR, Murray JI, Troyanskaya OG, Chi JT, Pergamenschikov A, et al. Systemic and cell type-specific gene expression patterns in scleroderma skin. Proc Natl Acad Sci U S A. 2003;100(21):12319–24. doi:10.1073/pnas.1635114100.
42. Lafyatis R, O'Hara C, Feghali-Bostwick CA, Matteson E. B cell infiltration in systemic sclerosis-associated interstitial lung disease. Arthritis Rheum. 2007;56(9):3167–8. doi:10.1002/art.22847.
43. Sato S, Hasegawa M, Fujimoto M, Tedder TF, Takehara K. Quantitative genetic variation in CD19 expression correlates with autoimmunity. J Immunol. 2000;165(11):6635–43.
44. Fujimoto M, Fujimoto Y, Poe JC, Jansen PJ, Lowell CA, DeFranco AL, et al. CD19 regulates Src family protein tyrosine kinase activation in B lymphocytes through processive amplification. Immunity. 2000;13(1):47–57.
45. Tsuchiya N, Kuroki K, Fujimoto M, Murakami Y, Tedder TF, Tokunaga K, et al. Association of a functional CD19 polymorphism with susceptibility to systemic sclerosis. Arthritis Rheum. 2004;50(12):4002–7. doi:10.1002/art.20674.

46. Hitomi Y, Tsuchiya N, Hasegawa M, Fujimoto M, Takehara K, Tokunaga K, et al. Association of CD22 gene polymorphism with susceptibility to limited cutaneous systemic sclerosis. Tissue Antigens. 2007;69(3):242–9. doi:10.1111/j.1399-0039.2007.00801.
47. Wutte N, Kovacs G, Berghold A, Reiter H, Aberer W, Aberer E. CXCL13 and B-cell activating factor as putative biomarkers in systemic sclerosis. Br J Dermatol. 2013;169 (3):723–5. doi:10.1111/bjd.12411.
48. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature. 1996;383(6603):787–93.
49. Chizzolini C. T lymphocyte and fibroblast interactions: the case of skin involvement in systemic sclerosis and other examples. Springer Semin Immunopathol. 1999;21:431–50.
50. Duncan MR, Berman B. Stimulation of collagen and glycosaminoglycan production in cultured human adult dermal fibroblasts by recombinant human interleukin 6. J Invest Dermatol. 1991;97(4):686–92.
51. Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. Nat Rev Immunol. 2004;4 (8):583–94. doi:10.1038/nri1412.
52. Rincon M, Anguita J, Nakamura T, Fikrig E, Flavell RA. Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4(+) T cells. J Exp Med. 1997;185(3):461–9. doi:10.1084/jem.185.3.461.
53. Hsieh CS, Heimberger AB, Gold JS, O'Garra A, Murphy KM. Differential regulation of T helper phenotype development by interleukins 4 and 10 in an alpha beta T-cell-receptor transgenic system. Proc Natl Acad Sci U S A. 1992;89(13):6065–9.
54. Demols A, Van Laethem JL, Quertinmont E, Degraef C, Delhaye M, Geerts A, et al. Endogenous interleukin-10 modulates fibrosis and regeneration in experimental chronic pancreatitis. Am J Physiol-Gastrointest Liver Physiol. 2002;282(6):G1105–12. doi:10.1152/ajpgi.00431.2001.
55. Lee CG, Homer RJ, Cohn L, Link H, Jung S, Craft JE, et al. Transgenic overexpression of interleukin (IL)-10 in the lung causes mucus metaplasia, tissue inflammation, and airway remodeling via IL-13-dependent and -independent pathways. J Biol Chem. 2002;277 (38):35466–74. doi:10.1074/jbc.M206395200.
56. Snapper CM, Waegell W, Beernink H, Dasch JR. Transforming growth factor-beta 1 is required for secretion of IgG of all subclasses by LPS-activated murine B cells in vitro. J Immunol. 1993;151(9):4625–36.
57. Francois A, Chatelus E, Wachsmann D, Sibilia J, Bahram S, Alsaleh G, et al. B lymphocytes and B-cell activating factor promote collagen and profibrotic markers expression by dermal fibroblasts in systemic sclerosis. Arthritis Res Ther. 2013;15(5):R168. doi:10.1186/ar4352.
58. Edwards JCW, Cambridge G. B-cell targeting in rheumatoid arthritis and other autoimmune diseases. Nat Rev Immunol. 2006;6(5):394–403. doi:10.1038/nri1838.
59. Yanaba K, Bouaziz JD, Haas KM, Poe JC, Fujimoto M, Tedder TF. A regulatory B cell subset with a unique CD1d(hi)CD5(+) phenotype controls T cell-dependent inflammatory responses. Immunity. 2008;28(5):639–50. doi:10.1016/j.immuni.2008.03.017.
60. Matsushita T, Yanaba K, Bouaziz JD, Fujimoto M, Tedder TF. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. J Clin Invest. 2008;118 (10):3420–30. doi:10.1172/jci36030.
61. Fillatreau S, Gray D, Anderton SM. Not always the bad guys: B cells as regulators of autoimmune pathology. Nat Rev Immunol. 2008;8(5):391–7. doi:10.1038/nri2315.
62. Evans JG, Chavez-Rueda KA, Eddaoudi A, Meyer-Bahlburg A, Rawlings DJ, Ehrenstein MR, et al. Novel suppressive function of transitional 2 B cells in experimental arthritis. J Immunol. 2007;178(12):7868–78.
63. Watanabe R, Ishiura N, Nakashima H, Kuwano Y, Okochi H, Tamaki K, et al. Regulatory B cells (B10 cells) have a suppressive role in murine lupus: CD19 and B10 cell deficiency exacerbates systemic autoimmunity. J Immunol. 2010;184(9):4801–9. doi:10.4049/jimmunol.0902385.

64. Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, et al. CD19 (+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. Immunity. 2010;32 (1):129–40. doi:10.1016/j.immuni.2009.11.009.
65. Matsushita T, Hamaguchi Y, Hasegawa M, Takehara K, Fujimoto M. Decreased levels of regulatory B cells in patients with systemic sclerosis: association with autoantibody production and disease activity. Rheumatology (Oxford). 2015. doi:10.1093/rheumatology/kev331.
66. Merrill JT, Neuwelt CM, Wallace DJ, Shanahan JC, Latinis KM, Oates JC, et al. Efficacy and safety of rituximab in moderately-to-severely active systemic lupus erythematosus: the randomized, double-blind, phase II/III systemic lupus erythematosus evaluation of rituximab trial. Arthritis Rheum. 2010;62(1):222–33. doi:10.1002/art.27233.
67. Rovin BH, Furie R, Latinis K, Looney RJ, Fervenza FC, Sanchez-Guerrero J, et al. Efficacy and safety of rituximab in patients with active proliferative lupus nephritis: the Lupus Nephritis Assessment with Rituximab study. Arthritis Rheum. 2012;64(4):1215–26. doi:10.1002/art.34359.

Fli1

12

Yoshihide Asano

Abstract

Systemic sclerosis (SSc) is a multifactorial connective tissue disease caused by the complex interplay between genetic factors and environmental influences. Fli1, a member of Ets transcription factor family, is constitutively suppressed in various cell types in the lesional and non-lesional skin of SSc patients at least partially by an epigenetic mechanism. Therefore, Fli1 deficiency is a potential predisposing factor of SSc reflecting environmental influences. Consistent with this notion, *Fli1*$^{+/-}$ mice reproduce SSc-like phenotypes in dermal fibroblasts and dermal microvascular endothelial cells at molecular levels. Importantly, bleomycin, a potential environmental factor of SSc, induces more prominent SSc-like phenotypes in various cell types of *Fli1*$^{+/-}$ mice than in those of wild-type mice. Furthermore, endothelial cell-specific Fli1 knockout mice reproduce the functional and structural vascular abnormalities characteristic of SSc vasculopathy. Moreover, double heterozygous mice for *Fli1* and *Klf5*, another transcription factor epigenetically suppressed in SSc dermal fibroblasts, spontaneously develop three cardinal features of SSc including immune abnormalities, vasculopathy, and tissue fibrosis of the skin and lung. Thus, Fli1 deficiency is a key feature in the pathogenesis of SSc. On the other hand, accumulating evidence has suggested that Fli1 is a potential therapeutic target of SSc because Fli1 expression can be reversed by bosentan, a dual endothelin receptor antagonist with a preventive effect on digital ulcers relevant to SSc. Therefore, the studies on the role of Fli1 in SSc provide us new clues to further understand the pathogenesis of SSc and to develop a new therapeutic strategy for this devastating and incurable disease.

Y. Asano (✉)
Department of Dermatology, University of Tokyo Graduate School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
e-mail: yasano-tky@umin.ac.jp

K. Takehara et al. (eds.), *Systemic Sclerosis*, DOI 10.1007/978-4-431-55708-1_12

Keywords
Fli1 • Fibroblasts • Endothelial cells • KLF5 • Bosentan

12.1 Introduction

Systemic sclerosis (SSc) is a multisystem chronic connective tissue disease characterized by initial vascular injury and resultant fibrosis of the skin and various internal organs with variable degrees of autoimmunity and inflammation. Although the pathophysiology of SSc still remains largely elusive, it has been believed that SSc is caused by a complex interaction between genetic factors and environmental influences. The critical role of genetic factors is primarily proved by the evidence that the highest risk factor for the development of SSc is a family history [1]. However, according to a twin study by Feghali-Bostwick et al. [2], concordance for SSc is low in the twins (4.7 %) and comparable between monozygotic and dizygotic twins (4.2 % versus 5.6 %), while concordance for the presence of antinuclear antibodies is much higher in the healthy monozygotic twin sibling than in the healthy dizygotic twin sibling of an SSc patient (95 % versus 60 %, $P < 0.05$), suggesting that genetic factors are related to autoimmunity increasing the susceptibility to SSc, but not enough for the development of clinically definite SSc. In line with this notion, most of susceptibility genes for SSc are HLA haplotypes and non-HLA immune and inflammatory genes, including interferon regulatory factor 5 (IRF5) and signal transducer and activator of transcription 4 (STAT4), which are also identified as disease susceptibility genes in other collagen diseases such as rheumatoid arthritis and systemic lupus erythematosus [3]. Therefore, it is speculated that a certain set of environmental factors triggers the development of SSc in genetically predisposed individuals.

Environmental factors, including silica dust, solvents, chemicals, drugs, and infectious agents, potentially modulate the behavior of various cell types through the alteration of gene expression profiles by epigenetic mechanisms, such as DNA methylation, histone modifications (acetylation and methylation), and microRNA regulation. As for DNA methylation, DNA hypermethylation of the *FLI1* and *KLF5* genes in dermal fibroblasts [4, 5] and the *BMPRII* gene in microvascular endothelial cells [6, 7] and DNA hypomethylation of the *ACTA* gene in lung fibroblasts [8] and the *CD11a*, *CD40L*, and *CD70* genes in $CD4^+$ T cells [9–11] have been reported in SSc [3]. Furthermore, a recent study by Altorok et al. has disclosed that 2,710 and 1,021 CpG sites are differentially methylated in diffuse cutaneous SSc and limited cutaneous SSc, respectively, by genome-wide DNA methylation analysis with dermal fibroblasts [12]. With respect to acetylation, hypoacetylation of histone H3 and H4 is shown in the *FLI1* promoter using SSc lesional skin samples and cultivated SSc dermal fibroblasts and in the *KLF5* promoter using cultivated SSc dermal fibroblasts [4, 5]. These genes with epigenetic modifications may be predisposing factors of SSc reflecting environmental influences. Among them, the potential contribution to the development of SSc has been well studied on the *FLI1* gene.

12.2 The Nature of Transcription Factor Fli1

FLI1 gene encodes Friend leukemia virus integration 1 (Fli1), a member of the Ets (E26 transformation-specific) transcription factor family characterized by a winged helix-turn-helix domain that is responsible for nuclear targeting and specific binding to a DNA element containing a purine-rich GGAA/T core sequence. *Fli1* gene was initially identified as a proto-oncogene in Friend virus-induced erythroleukemia in mice. Also, in humans, Fli1 is involved in the development of tumors, that is, *FLI1* gene is rearranged in 90 % of Ewing sarcoma family of tumors including morphological variants of Ewing sarcoma and peripheral primitive neuroectodermal tumor [13]. On the other hand, hemizygous loss of *FLI1* gene is pathogenic for Jacobsen/Paris-Trousseau syndrome, a rare congenital disorder caused by partial deletion of the long arm of chromosome 11 and featured by variable degrees of growth and mental retardation, cardiac defects, dysmorphogenesis of the digits and face, pancytopenia, and thrombocytopenia [14, 15]. Among various genes on chromosome 11q, *FLI1* appears to be closely associated with bone deformity and platelet dysfunction of this syndrome because $Fli1^{+/-}$ mice exhibit a characteristic cranial deformity and mild thrombocytopenia with abnormal megakaryocytes characterized by a disorganized demarcation membrane system, vacuolization, and fused α-granules [16]. Thus, genetic modification of *FLI1* gene is involved in the progression of at least two distinct tumors and the development of Jacobsen/Paris-Trousseau syndrome.

Under physiological conditions, Fli1 is expressed at high levels in both endothelial and hematopoietic cells as revealed by comparative gene expression profile analysis of various human cell lines [17]. Fli1 is also expressed in dermal fibroblasts even though the levels are relatively lower compared with endothelial cells and hematopoietic cells [18, 19]. Consistent with these data, in addition to megakaryocytic differentiation, Fli1 plays a pivotal role in myelomonocytic, erythroid, and NK cell development [20], T cell activation [21, 22], B cell proliferation and apoptosis [23–27], vascular development and angiogenesis [14, 28, 29], and extracellular matrix (ECM) remodeling [18, 30].

Reflecting the important role of Fli1 in immune cells, H-2Kk-Fli1 transgenic mice, which overexpress Fli1 in various tissues especially in the thymus and spleen, develop a progressive immunological renal disease reminiscent of systemic lupus erythematosus [24]. Importantly, Fli1 expression levels in lymphoid tissues of these mice positively correlate with the prevalence of renal involvements. The hypergammaglobulinemia, splenomegaly, B cell hyperplasia, accumulation of abnormal $CD3^+$ $B220^+$ T lymphoid cells and $CD5^+B220^+$ B cells in peripheral lymphoid tissues, and various autoantibodies in *Fli1* transgenic mice suggest the involvement of an immune dysfunction in the pathogenesis of the renal disease in these mice. Furthermore, splenic B cells from *Fli1* transgenic mice exhibit increased proliferation and prolonged survival in vitro in response to mitogens. Thus, Fli1 regulates normal lymphoid cell function and cell survival, and its dysregulation may result in the development of autoimmune diseases, such as systemic lupus erythematosus.

In addition to the impact on immune system and megakaryocytes/platelets, Fli1 regulates endothelial cell behavior and ECM metabolism. The roles of Fli1 in dermal fibroblasts and endothelial cells have been well investigated in the context of SSc and its animal models.

12.3 The Expression Levels of Fli1 in the Skin of SSc Patients

The first study reporting the expression levels of Fli1 in the lesional skin of SSc patients came out in 2003, demonstrating the following findings: (1) the expression levels of Fli1 protein are decreased in dermal fibroblasts and dermal microvascular endothelial cells in the lesional and non-lesional skin of SSc patients compared with those cells in healthy control skin, (2) the decrease in Fli1 expression is much greater in the lesional skin than in the non-lesional skin of SSc patients, (3) there is an inverse correlation between mRNA levels of the *COL1A2* gene and those of the *FLI1* gene in dermal fibroblasts of healthy control skin in vivo, (4) stable transfection of Fli1 into normal dermal fibroblasts markedly decreases the production of type I and type III collagen proteins, and (5) Fli1 expression negatively correlates with type I collagen expression among $Fli1^{+/+}$, $Fli1^{+/-}$, and $Fli1^{-/-}$ murine embryonic fibroblasts [19]. Prior to this report, the same group demonstrated that Fli1 serves as a potent repressor of the *COL1A2* gene in human dermal fibroblasts [18]. Therefore, Fli1 downregulation seems to be involved in the activation of SSc dermal fibroblasts. Importantly, Fli1 expression is moderately decreased even in the non-lesional skin of SSc patients compared with healthy control skin [19], suggesting that Fli1 downregulation may function as a predisposing factor for the activation of SSc dermal fibroblasts. Following these reports, Wang et al. [4] revealed the epigenetic downregulation of *FLI1* gene in SSc lesional skin and cultivated SSc dermal fibroblasts. Taken together, it is speculated that Fli1 is a potential predisposing factor of SSc reflecting the influence of environmental factors.

12.4 The Regulation of Fli1 Transcriptional Activity by Posttranslational Modifications

The molecular mechanism by which Fli1 regulates the transcription of target genes has been well studied in the context of *COL1A2* promoter. Fli1 suppresses the transcriptional activity of the *COL1A2* promoter by binding to GGAT motif at the −285 to −282 bp region together with p300 and histone deacetylase 1 (HDAC1), leading to the chromatin condensation due to histone deacetylation [18, 31]. The posttranslational modifications, such as phosphorylation and acetylation, regulate the DNA-binding ability and the protein stability of Fli1 and the remodeling of Fli1/p300/HDAC1 transcription repressor complex [31–33].

Transcriptional activity of Fli1 is tightly regulated by "phosphorylation-acetylation cascade." This cascade has been well investigated in human dermal fibroblasts stimulated with transforming growth factor (TGF)-β. Upon TGF-β stimulation, c-Abl and protein kinase C (PKC)-δ are sequentially activated, leading to the nuclear translocation of PKC-δ. In nucleus, activated PKC-δ directly interacts with Fli1 on the *COL1A2* promoter and phosphorylates it at threonine 312. Phosphorylation of Fli1 at threonine 312 increases its affinity for p300/CREB-binding protein-associated factor (PCAF) [33], which has histone acetyltransferase activity, subsequently resulting in acetylation of Fli1 at lysine 380. Upon acetylation Fli1 is released from the *COL1A2* promoter possibly due to a conformational change [34]. Once dissociated from the *COL1A2* promoter, Fli1 is rapidly degraded [32]. As a result, Fli1 no longer holds its inhibitory effect on the *COL1A2* promoter. Thus, TGF-β regulates transcriptional activity of Fli1 through "c-Abl-PKC-δ pathway" and "phosphorylation-acetylation cascade" (Fig. 12.1).

Phosphorylation of Fli1 is a key event regulating the remodeling of transcription repressor complex on the Ets binding site of the *COL1A2* promoter. In the absence of Fli1 phosphorylation at threonine 312, Fli1 makes complex with p300 and HDAC1 on the *COL1A2* promoter. Under this situation, acetylation levels of histones are decreased around the Ets binding site of the *COL1A2* promoter probably because histone deacetylase activity of HDAC1 competes with histone acetyltransferase activity of p300. Therefore, the *COL1A2* promoter activity is suppressed through chromatin condensation by histone deacetylation. When Fli1 is phosphorylated at threonine 312 by PKC-δ, the dissociation of the complex occurs. Then, p300 makes transcription factor complex with Ets1 on the *COL1A2* promoter, which results in the increased acetylation of histones around the Ets binding site of the *COL1A2* promoter, leading to the increased promoter activity through the chromatin decondensation [31] (Fig. 12.2).

A similar "phosphorylation-acetylation cascade" regulates the transcriptional activity of Smad2/Smad3, the principal intracellular second messengers of the TGF-β signaling pathway. Upon TGF-β stimulation, Smad2 and Smad3 are phosphorylated by serine/threonine kinase of TGF-β type I receptor and then translocate into the nucleus, where p300/CREB-binding protein or PCAF acetylates Smad2/Smad3 and increases its DNA-binding affinity [35–37]. Thus, "phosphorylation-acetylation cascade" is the critical posttranslational modification by which TGF-β coordinately regulates the DNA-binding activity of transcriptional activators such as Smad3 and repressors such as Fli1, resulting in increased ECM production.

In addition to the regulation of *COL1A2* gene expression, Fli1 also functions as a transcriptional repressor of *COL1A1* gene. Rottlerin, a specific inhibitor of PKC-δ, strongly suppresses the expression of *COL1A1* gene at the transcriptional level, and the corresponding responsive element is located at the 130 bp segment, encompassing nucleotides −804 to −675 of the *COL1A1* promoter [38]. This region contains a putative Ets transcription factor binding site at nucleotides −712 to −709, and Fli1 occupies this site in quiescent dermal fibroblasts. Furthermore, inhibition of endogenous PKC-δ enhances Fli1 binding to the *COL1A1*

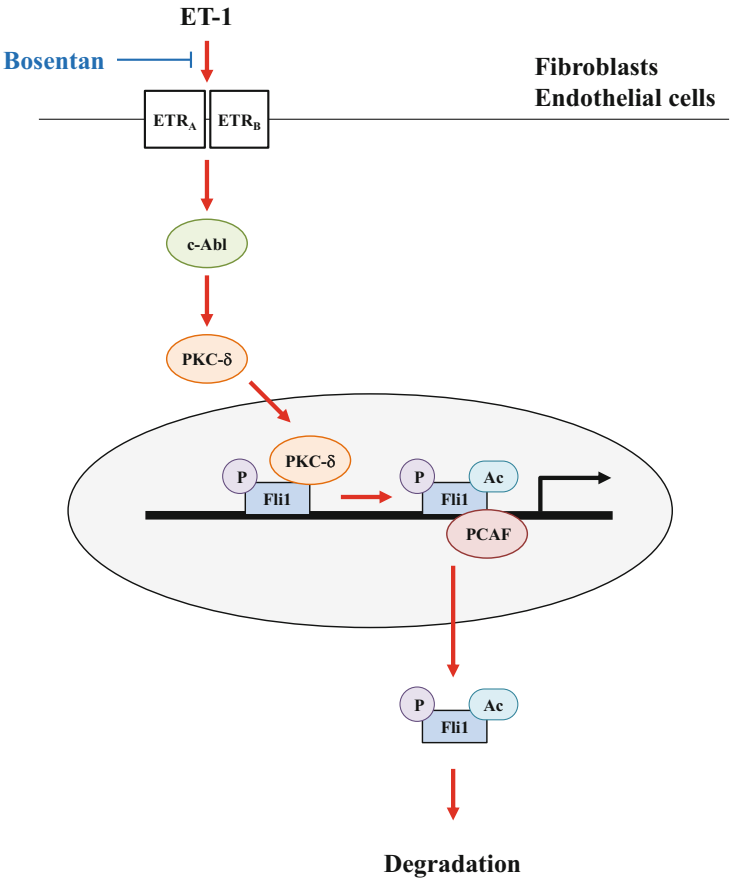

Fig. 12.1 "c-Abl-PKC-δ pathway" and "phosphorylation-acetylation cascade" regulate the transcriptional activity of Fli1. In dermal fibroblasts and endothelial cells, ET-1 sequentially activates c-Abl and PKC-δ. Activated PKC-δ translocates into the nucleus and phosphorylates Fli1 at threonine 312. Then, phosphorylated Fli1 interacts with PCAF, resulting in Fli1 acetylation at lysine 380. Acetylated Fli1 loses its DNA-binding ability and is rapidly degraded. Bosentan, a dual endothelin receptor antagonist, increases the DNA-binding ability of Fli1, leading to the increased expression of Fli1 protein by preventing its degradation

promoter [33]. These data indicate that Fli1 coordinately regulates *COL1A1* and *COL1A2* expression in dermal fibroblasts.

In dermal fibroblasts, TGF-β stimulation rapidly induces Smad2/Smad3 phosphorylation, while it takes about 2 h to induce Fli1 phosphorylation [33]. This is because the "c-Abl-PKC-δ pathway" is activated by autocrine endothelin-1 (ET-1) which is induced by TGF-β stimulation [39]. In endothelial cells, in which autocrine ET-1 maintains homeostasis, ET-1 also regulates the transcriptional activity of Fli1 through the "c-Abl-PKC-δ pathway" [40]. Given that ET-1 decreases Fli1 expression by promoting its degradation, the blockade of ET-1 increases Fli1 protein

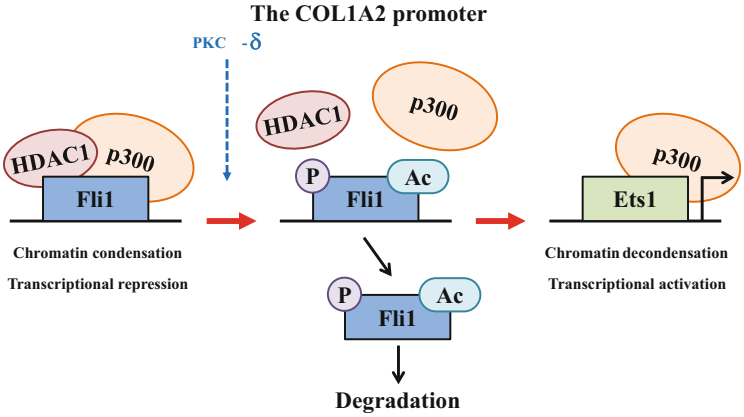

Fig. 12.2 Phosphorylation of Fli1 at threonine 312 regulates the remodeling of Fli1/p300/HDAC1 transcription repressor complex. In the absence of Fli1 phosphorylation, Fli1 stays on the *COL1A2* promoter with p300 and HDAC1. Since HDAC1 inhibits p300-dependent acetylation of histones, the transcription of *COL1A2* gene is suppressed by chromatin condensation. When Fli1 is phosphorylated at threonine 312 by PKC-δ, Fli1 dissociates from p300 and HDAC1. Then, the complex of Est1 and p300 stays on the *COL1A2* promoter and induces histone acetylation, resulting in transcriptional activation by chromatin decondensation

levels without affecting its mRNA levels [40, 41]. This property of Fli1 may explain the mechanism by which the blockade of endothelin receptors exerts a potential disease-modifying effect on SSc vasculopathy (Figs. 12.1 and 12.3) (see Sect. 12.11).

12.5 Fli1 Haploinsufficiency Partially Mimics the Property of SSc Dermal Fibroblasts

The impact of Fli1 on biological processes in vivo has been investigated in *Fli1*$^{+/-}$ mice or other *Fli1*-mutated mice because mice with homozygous deletion of *Fli1* gene are embryonic lethal. In the skin, *Fli1*$^{+/-}$ mice express *Col1a1* and *Col1a2* mRNA and type I collagen protein at higher levels than wild-type mice [5, 42]. In line with this, the number of α-SMA-positive dermal fibroblasts is increased in *Fli1*$^{+/-}$ mice compared with wild-type mice [42]. Therefore, *Fli1* haploinsufficiency is enough to activate dermal fibroblasts in vivo. However, the thickness of dermis is comparable between *Fli1*$^{+/-}$ mice and wild-type mice even though densely packed collagen bundles are characteristically seen in *Fli1*$^{+/-}$ mice [42], suggesting that *Fli1* haploinsufficiency is not enough to highly mimic the pro-fibrotic phenotype of SSc dermal fibroblasts. Consistent with this notion, *Fli1*$^{+/-}$ mice express connective tissue growth factor (CTGF) at similar levels to those of wild-type mice. Given that CTGF upregulation is a characteristic of SSc dermal fibroblasts [5], *Fli1* haploinsufficiency partially mimics the property of SSc dermal fibroblasts,

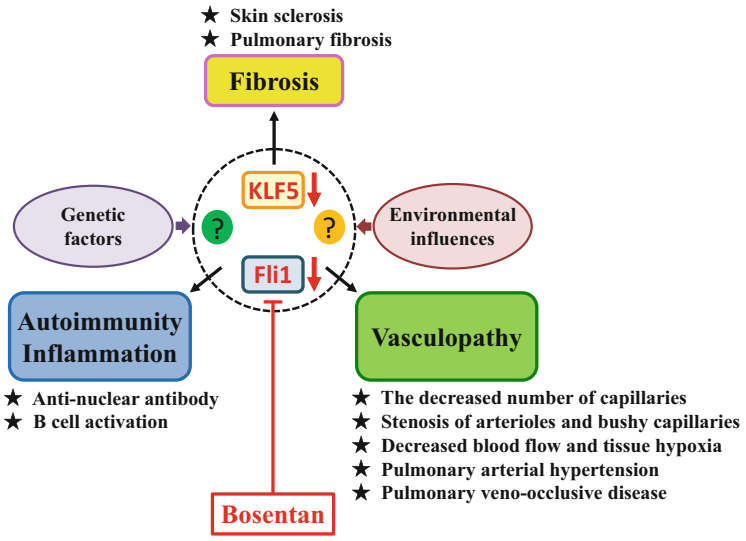

Fig. 12.3 The role of Fli1 and KLF5 in the pathogenesis of SSc. SSc is a multifactorial disease caused by the complex interplay between genetic factors and environmental influences. Fli1 and KLF5 are predisposing factors potentially reflecting environmental influences, both of which are suppressed in SSc dermal fibroblasts by an epigenetic mechanism. Double heterozygous mice for *Klf5* and *Fli1* recapitulate three cardinal features of SSc, suggesting that simultaneous downregulation of KLF5 and Fli1 is involved in the pathogenesis of SSc, at least in a certain subset of this disease

consistent with the notion that Fli1 deficiency is a predisposing factor of SSc, but not enough for the development of clinically definite SSc.

12.6 Mice with Homozygous Targeted Deletion of Fli1 Carboxyl Terminal Activation (CTA) Domain Partially Recapitulate Dermal Fibrosis of SSc

The embryonic lethality of *Fli1* null mice limits functional studies in vivo, and $Fli1^{+/-}$ mice just show mild cranial deformity, mild thrombocytopenia, mild vascular changes (see Sect. 12.7) and moderately increased dermal collagen deposits with normal dermal thickness [5, 16, 42]. Under this situation, to further elucidate the contribution of Fli1 deficiency to the development of SSc, another genetic murine model with homozygous targeted deletion of Fli1 carboxyl terminal activation (CTA) domain ($Fli1^{\Delta CTA/\Delta CTA}$) was generated [34]. Depending on the promoter and cellular context, Fli1 functions as a transcription activator or a repressor and the CTA domain mediates either the activator or repressor function of this transcription factor. Fortunately, $Fli1^{\Delta CTA/\Delta CTA}$ mice develop normally and viable, allowing us to investigate the impact of the absence of functional Fli1 in vivo.

Fli1$^{\Delta CTA/\Delta CTA}$ mice are characterized by a significant upregulation of fibrillar collagen genes, including *Col1a1*, *Col1a2*, *Col3a1*, and *Col5a1* genes, at mRNA levels as well as an increased collagen content measured by acetic acid extraction and hydroxyproline assays in the skin. In addition, collagen fibrils exhibit ultrastructural abnormalities including immature thin fibrils and very thick irregularly shaped fibrils, which are quite similar to the ultrastructural features of collagen fibers in the skin of SSc patients [43–46]. However, dermal thickness is comparable between *Fli1*$^{\Delta CTA/\Delta CTA}$ mice and wild-type counterparts, as is the case with *Fli1*$^{+/-}$ mice [5, 34, 42]. Thus, homozygous targeted deletion of the CTA domain of *Fli1* gene induces the ultrastructural abnormalities of collagen fibrils in the dermis to a much greater extent than heterozygous deletion of *Fli1* gene, but the increased dermal thickness, a hallmark of skin sclerosis in SSc, is absent in both *Fli1*-mutated mice, further supporting the idea that Fli1 deficiency is just a part of predisposing factors of SSc.

12.7 Endothelial Fli1 Deficiency Contributes to the Development of SSc Vasculopathy

The involvement of Fli1 in vascular biology was initially demonstrated in the studies on *Fli1* null mice [14, 28]. The most striking observation was that Fli1 null mice die at E11.5 during embryogenesis due to the cranial and spinal hemorrhages. Since E11.5 is a time point when the development of vasculature shifts from "embryonic vasculogenesis," a de novo synthesis of vascular network from hemangioblasts, to "angiogenesis," the progression of new vessels from preexisting ones, the lethality at E11.5 due to bleeding suggests the critical role of Fli1 in "angiogenesis," but not in "embryonic vasculogenesis." Consistent with this notion, studies on zebra fish and *Xenopus* embryos have demonstrated that Fli1 functions as a master regulator of the transcriptional network driving blood and endothelial cell lineages [29]. In humans, Fli1 is expressed in the healthy skin microvasculature [17] while greatly reduced in endothelial and peri-endothelial cells of SSc lesional and non-lesional skin [19]. Therefore, SSc vasculopathy may be caused by aberrant angiogenesis due to Fli1 deficiency.

The direct impact of Fli1 deficiency on postnatal vascular homeostasis has been suggested in *Fli1*-mutated mice. *Fli1*$^{+/-}$ mice exhibit a mild distortion of arterioles and increased vascular permeability of small vessels in the skin [5]. In contrast to a mild morphological similarity to SSc vasculature, *Fli1*$^{+/-}$ mice highly reproduce an SSc vascular phenotype at molecular levels [47–53]. Furthermore, the SSc-like vascular phenotype is much more greatly induced in *Fli1*$^{+/-}$ mice treated with bleomycin [42]. Moreover, endothelial cell-specific *Fli1* knockout (*Fli1* ECKO) mice (*Fli1*$^{flox/flox}$;*Tie2*-Cre), in which endothelial Fli1 expression is much more suppressed than in *Fli1*$^{+/-}$ mice (50–80 % versus 50 %), display stenosis of arterioles, dilation of capillaries, and remarkably increased vascular permeability of small vessels [47], which are structurally and functionally similar to SSc

vasculopathy. Therefore, Fli1 deficiency seems to be directly related to the development of SSc vasculopathy.

The detailed molecular mechanism by which Fli1 deficiency affects vascular structure and function has been investigated in *Fli1* ECKO mice [47]. Consistent with the vascular changes such as stenosis of arterioles, the number of endothelial cells in arterioles is significantly increased in *Fli1* ECKO mice compared with wild-type counterparts, suggesting that Fli1 deficiency promotes the proliferation of endothelial cells. In addition to structural abnormalities, vascular permeability is dramatically increased and vessels are quite fragile in *Fli1* ECKO mice. These structural and functional abnormalities of vasculature in *Fli1* ECKO mice appear to be caused by the constitutive activation of pro-angiogenic gene program, that is, the decreased expression of *Cdh5*, *Pecam1*, *Pdgfb*, and $S1p_1$ genes and the increased expression of *Mmp9* gene. The decrease in VE-cadherin and PECAM-1 expression may result in a weak endothelial cell-endothelial cell interaction, leading to dilation of capillaries, vascular fragility, and increased vascular permeability. Increased expression of matrix metalloproteinase (MMP)-9 may lead to alterations in vascular basement remodeling, such as loss of type IV collagen and compensatory increase of proteoglycans. The pro-angiogenic status of vasculature is also confirmed by the decreased expression of α-SMA, a marker of PCs with angiostatic phenotype, in dermal small vessels in *Fli1* ECKO mice, which is related to the downregulation of VE-cadherin, PDGFB, and $S1P_1$ in endothelial cells. Also, the proliferation of endothelial cells represents the activation of pro-angiogenic gene program. Therefore, Fli1 deficiency induces the constitutive activation of pro-angiogenic gene program in dermal microvascular endothelial cells in vivo.

Vascular phenotype of SSc patients is quite similar to that of *Fli1* ECKO mice [47]. VE-cadherin and PECAM-1 are markedly decreased in endothelial cells of dermal small vessels of SSc patients compared with those of closely matched healthy controls. The levels of type IV collagen are also decreased in the vascular basement membranes of dermal small vessels of SSc patients. This may be due to the increased production of MMP-9 by SSc endothelial cells because Fli1 does not influence type IV collagen expression in dermal microvascular endothelial cells. Consistent with this notion, serum levels of MMP-9 are increased in SSc patients compared with healthy controls [54]. α-SMA expression of dermal small vessels is uniformly decreased in SSc lesional skin, especially in capillaries, compared with healthy control skin. Thus, *Fli1* ECKO mice recapitulate the vascular phenotype of SSc patients, supporting the involvement of endothelial Fli1 deficiency in the development of SSc vasculopathy.

12.8 Fli1 Haploinsufficiency Induces Fibrosis, Vascular Activation, and Immune Abnormalities Resembling SSc in Bleomycin-Treated Mice

As described so far, Fli1 deficiency induces SSc-like phenotypes at least in dermal fibroblasts and dermal microvascular endothelial cells at molecular levels, but $Fli1^{+/-}$ mice do not show any clinical features of SSc, just mimicking a part of

morphological features of dermis and skin vasculature. These findings support the idea that Fli1 deficiency is a predisposing factor of SSc and some additional factors are required for the development of definite SSc. In line with this idea, *Fli1* haploinsufficiency exacerbates three cardinal features of SSc in mice treated with bleomycin (BLM), a potential environmental factor inducing human SSc.

Four-week BLM injection increases dermal thickness, collagen content, and mRNA levels of the *Col1a1*, *Col1a2*, and *Ctgf* genes in $Fli1^{+/-}$ mice to a greater extent than in wild-type mice. Furthermore, α-SMA-positive myofibroblasts are more frequently seen in BLM-treated $Fli1^{+/-}$ mice than in BLM-treated wild-type mice [42]. In human SSc, TGF-β expression is detectable primarily around dermal small vessels in its early stage while under detectable limit through the whole dermis in the fibrotic stage [55]. Given that the expression and phosphorylation levels of Smad3 are elevated in dermal fibroblasts of SSc fibrotic skin [56], it is speculated that SSc dermal fibroblasts effectively activate latent TGF-β in vivo. The upregulated expression of integrins αVβ3 and αVβ5, receptors for latency-associated peptide of latent form TGF-β, in SSc dermal fibroblasts is involved in this process. Importantly, αVβ3 and αVβ5 integrins are upregulated in dermal fibroblasts of BLM-treated $Fli1^{+/-}$ mice compared with those of BLM-treated wild-type mice. Furthermore, dermal fibroblasts transfected with Fli1 siRNA oligonucleotide activate latent TGF-β on cell surface by these integrins, resulting in the upregulated expression of CTGF through autocrine TGF-β stimulation [42]. Thus, Fli1 haploinsufficiency promotes the induction of an SSc-like phenotype in dermal fibroblasts in response to BLM.

Aberrant vascular activation plays a pivotal role in tissue fibrosis by inducing inflammation and endothelial-to-mesenchymal transition (EndoMT) in SSc [57]. The correlation of immune polarization of $CD4^+$ T cells with tissue fibrosis has been well studied in diffuse cutaneous SSc. In the early and sclerotic phases of diffuse cutaneous SSc, Th2/Th17 immune response is predominant, while immune polarization shifts from Th2/Th17 to Th1 in parallel with the resolution of skin sclerosis in diffuse cutaneous SSc [58]. This is plausible because Th2 cytokines such as IL-4 and IL-13 exert a pro-fibrotic effect on dermal fibroblasts, while IFN-γ, a Th1 cytokine, reduces type I collagen expression. Although IL-17A suppresses type I collagen production in normal dermal fibroblasts, SSc fibroblasts are unresponsive to the anti-fibrotic effect of this cytokine [59]. In BLM-treated mice, the infiltration of $CD4^+$ T cells in the lesional skin is tightly regulated by cell adhesion molecules. For instance, ICAM-1 and GlyCAM-1 promote the infiltration of Th2/Th17 cells, while P-selectin and E-selectin induce the infiltration of Th1 cells [60]. Of note, the relative fold induction of ICAM-1 and GlyCAM-1 to E-selectin and P-selectin in response to BLM is elevated in $Fli1^{+/-}$ mice compared with wild-type mice. Furthermore, gene silencing of Fli1 results in the induction of ICAM-1 and GlyCAM-1 and the suppression of E-selectin and P-selectin in human dermal microvascular endothelial cells [42]. Therefore, Fli1 deficiency is attributable to the increased ratio of ICAM-1 and GlyCAM-1 to E-selectin and P-selectin in BLM-treated $Fli1^{+/-}$ mice, leading to the promotion of Th2/Th17 cell infiltration in the skin. As for EndoMT, the number of double-positive cells for fibroblast-specific

protein-1 and VE-cadherin is increased in PBS- and BLM-treated *Fli1*$^{+/-}$ mice compared with wild-type counterparts. Furthermore, Fli1 binds to the promoter of *SNAIL1* gene, a master regulator of EndoMT, and gene silencing of Fli1 enhances the expression of *SNAIL1* mRNA in human dermal microvascular endothelial cells, indicating that Fli1 deficiency directly induces EndoMT [42]. Thus, Fli1 haploinsufficiency potentially promotes the induction of a pro-fibrotic phenotype in dermal microvascular endothelial cells especially in the presence of certain environmental influences.

Consistent with the notion that M2 differentiation of macrophages promotes tissue fibrosis in various pathological conditions including SSc [61], mRNA levels of M2 macrophage markers, *Arg1*, *Fizz1*, and *Ym1*, and the number of arginase 1-positive macrophages in the lesional skin are significantly elevated in BLM-treated *Fli1*$^{+/-}$ mice compared with BLM-treated wild-type mice while comparable at baseline. Of note, IL-4 and IL-13 induce polarization of macrophages toward the M2 phenotype to a greater extent in *Fli1*$^{+/-}$ peritoneal macrophages than in wild-type peritoneal macrophages, indicating that Fli1 haploinsufficiency directly contributes to the differentiation of M2 macrophages [42]. Given that M2 macrophages represent the predominant macrophage subset in the lesional skin of early diffuse cutaneous SSc [61], Fli1 haploinsufficiency may also serve as a predisposing factor to induce an SSc phenotype in response to environmental influences in macrophages.

These data on BLM-treated *Fli1*$^{+/-}$ mice indicate that bleomycin-induced factors augment Fli1 deficiency-dependent phenotypical alterations of fibroblasts, endothelial cells, and immune cells.

12.9 Fli1 Deficiency Regulates the Expression of Various Molecules Related to Fibrosis and Vasculopathy of SSc

A series of studies with human clinical samples and *Fli1*-mutated mice have demonstrated the pivotal role of Fli1 in the induction of SSc-like phenotypes in dermal fibroblasts and endothelial cells at molecular levels. These molecules include cathepsin B (CTSB) [48], cathepsin V (CTSV) [49], CXCL5 [50], CCN1 [51], chemerin [52], and lipocalin-2 [53].

12.9.1 CTSB and CTSV

Cathepsins, a family of proteolytic enzymes mainly composed of papain-like cysteine proteases, comprise 11 family members in human, cathepsins B, C, F, H, K, L, O, S, V, W, and X. These enzymes are mainly localized in endosomes and lysosomes but also function extracellularly as regulators of various biological processes, including ECM degradation and angiogenesis. As well as MMPs, cathepsins have been shown to be involved in tissue fibrosis and vasculopathy associated with SSc. For instance, upregulation of CTSB and downregulation of

CTSV in endothelial cells may contribute to the development of proliferative obliterative vasculopathy, such as pulmonary arterial hypertension, digital ulcers, and scleroderma renal crisis, while reduced expression of CTSB and CTSV in lesional dermal fibroblasts is likely to be associated with skin sclerosis in early diffuse cutaneous SSc. Importantly, the altered expression of CTSB and CTSV in dermal fibroblasts and dermal microvascular endothelial cells of SSc is recapitulated in the skin of *Fli1*$^{+/-}$ mice, and Fli1 occupies the promoters of *CTSB* and *CTSV* genes, suggesting the contribution of Fli1 deficiency to the altered expression of these molecules in SSc [48, 49].

12.9.2 CXCL5

CXCL5 is a member of CXC chemokines with neutrophilic chemoattractant and pro-angiogenic properties. CXCL5 has been implicated in the pathological angiogenesis of rheumatoid arthritis and inflammatory bowel diseases, which is characterized by the pro-angiogenic state of vasculature with neutrophil infiltration in the lesional tissues. SSc is featured by the constitutive pro-angiogenic status, but generally lacks the infiltration of neutrophils. Relevant to this, CXCL5 expression is decreased in dermal small vessels of SSc patients. Notably, CXCL5 expression is also decreased in dermal small vessels of *Fli1*$^{+/-}$ mice. Furthermore, Fli1 occupies the *CXCL5* promoter and gene silencing of Fli1 suppresses CXCL5 expression in human dermal microvascular endothelial cells [50]. These findings suggest that Fli1 deficiency selectively suppresses the expression of pro-angiogenic molecules also serving as a chemoattractant for neutrophils, such as CXCL5, while increasing most of other pro-angiogenic molecules in endothelial cells, further supporting the idea that Fli1 is a critical factor determining the vascular phenotype of SSc.

12.9.3 CCN1

CCN1 is a pleiotropic molecule involved in angiogenesis and postnatal vasculogenesis [62–66], both of which are impaired in SSc. CCN1 expression is markedly decreased in dermal small blood vessels of SSc patients compared with those of healthy controls. Fli1 occupies the *CCN1* promoter, and gene silencing of Fli1 results in the reduction of CCN1 expression in human dermal microvascular endothelial cells. In concordance with these data, CCN1 expression is remarkably suppressed in dermal blood vessels of *Fli1*$^{+/-}$ mice. Furthermore, serum CCN1 levels are significantly decreased in SSc patients with the past and current history of digital ulcers as compared to those without [51]. Therefore, endothelial CCN1 downregulation at least partially due to Fli1 deficiency may contribute to the development of digital ulcers in SSc by interfering with angiogenesis and postnatal vasculogenesis.

12.9.4 Chemerin

Chemerin is a member of adipocytokines with a chemoattractant effect on plasmacytoid dendritic cells and macrophages and a pro-angiogenic property. In SSc lesional skin, chemerin is upregulated in small blood vessels while downregulated in fibroblasts surrounded with thickened collagen bundles. The decreased expression of chemerin is significantly reversed by TGF-β1 antisense oligonucleotide in cultured SSc dermal fibroblasts. In human dermal microvascular endothelial cells, Fli1 binds to the promoter of *RARRES2* gene encoding chemerin, and gene silencing of Fli1 induces chemerin expression. Furthermore, $Fli1^{+/-}$ mice exhibit elevated chemerin expression in dermal blood vessels. Of note, SSc patients with digital ulcers have higher serum chemerin levels than those without [52]. Thus, chemerin is downregulated in SSc dermal fibroblasts by autocrine TGF-β while upregulated in SSc dermal blood vessels through endothelial Fli1 deficiency. Since Fli1 deficiency induces autocrine TGF-β signaling in dermal fibroblasts [42], Fli1 deficiency is involved in the regulation of chemerin expression in dermal fibroblasts and dermal microvascular endothelial cells of SSc patients. Since plasmacytoid dendritic cells are located around perivascular area in the lesional skin of SSc patients [67] and may contribute to vasculopathy through interferon-α production [68–70], Fli1 deficiency potentially induces vascular injury by recruiting plasmacytoid dendritic cells to perivascular region.

12.9.5 Lipocalin-2

Lipocalin-2 is an adipocytokine implicated in apoptosis, innate immunity, angiogenesis, and the development of chronic kidney disease. Reflecting its involvement in chronic kidney disease, the prevalence of scleroderma renal crisis is significantly higher in SSc patients with elevated serum lipocalin-2 levels than in those with normal levels and serum lipocalin-2 levels inversely correlate with estimated glomerular filtration rate in SSc patients with renal dysfunction. Among SSc patients with normal renal function, serum lipocalin-2 levels inversely correlate with estimated right ventricular systolic pressure in total SSc patients. In SSc lesional skin, lipocalin-2 expression is increased in endothelial cells. In human dermal microvascular endothelial cells, Fli1 binds to the promoter of *LCN2* gene encoding lipocalin-2, and gene silencing of Fli1 induces lipocalin-2 expression [53]. Given that lipocalin-2 stabilizes MMP-9, which is also upregulated by gene silencing of Fli1 in endothelial cells [47], by forming a complex and thereby prevents its autodegradation, dysregulated MMP-9-/lipocalin-2-dependent angiogenesis due to Fli1 deficiency may contribute to the development of pulmonary arterial hypertension associated with SSc.

12.10 Simultaneous Downregulation of Fli1 and KLF5 Is a Key Feature Underlying SSc

If Fli1 serves as a predisposing factor of SSc, the addition of other predisposing factors theoretically induces SSc-like phenotypes to a greater extent in *Fli1*$^{+/-}$ mice. This hypothesis has been proved by the identification of Krüppel-like factor 5 (KLF5) as another predisposing factor involved in the development of SSc and the generation of double heterozygous mice for *Klf5* and *Fli1* genes (*Klf5*$^{+/-}$;*Fli1*$^{+/-}$ mice).

KLF5 is a member of Sp1/KLF transcription factor family, which is involved in the mechanism of tissue fibrosis of heart and kidney [71, 72]. In human SSc, KLF5 expression is decreased in dermal fibroblasts in vivo and in vitro as shown by DNA microarray analysis, immunohistochemistry, and immunoblotting [5, 73]. Importantly, epigenetic inhibitors reverse KLF5 levels in SSc dermal fibroblasts. Furthermore, the decreased acetylation of histone H3 and H4 and the increased methylation of CpG islands are seen in the promoter region of *KLF5* gene in SSc dermal fibroblasts [5]. Therefore, KLF5 deficiency is likely to be a predisposing factor of SSc as well as Fli1 deficiency.

Through a series of in vivo studies with *Klf5*$^{+/-}$ mice and in vitro studies with dermal fibroblasts treated with KLF5 siRNA, it is proved that KLF5 serves as a potent repressor of *CTGF* gene in dermal fibroblasts. KLF5 binding site is located at −124 bp ~ −117 bp of the *CTGF* promoter which is close to Fli1 binding site. Of note, KLF5 and Fli1 physically interact and synergistically suppress the activity of *CTGF* promoter in dermal fibroblasts [5].

TGF-β and connective tissue growth factor (CTGF) are the key soluble factors regulating the development of tissue fibrosis. The subcutaneous injection of TGF-β followed by CTGF is enough to induce the persistent dermal fibrosis in mice [74]. In SSc dermal fibroblasts, the constitutive activation of TGF-β signaling and the increased expression of CTGF are the hallmark underlying the induction and maintenance of their pro-fibrotic phenotype [73, 75]. Given that Fli1 deficiency partially mimics TGF-β stimulation and that simultaneous deficiency of KLF5 and Fli1 remarkably induces CTGF expression, simultaneous downregulation of KLF5 and Fli1 is likely to be capable of resembling the stimulation of TGF-β and CTGF in dermal fibroblasts, which may be closely linked to the pro-fibrotic phenotype of SSc dermal fibroblasts.

Supporting this notion, *Klf5*$^{+/-}$;*Fli1*$^{+/-}$ mice spontaneously develop remarkable dermal fibrosis at the age of 3 months. Dermal thickness, the amount of collagen content, and mRNA levels of the *Col1a1*, *Col1a2*, and *Ctgf* genes are markedly increased in *Klf5*$^{+/-}$;*Fli1*$^{+/-}$ mice compared with wild-type littermates. Furthermore, the ultrastructure of dermal collagen fibrils is irregular and thick in longitudinal sections and highly variable in diameter in transverse sections, which are similar to collagen structure in SSc lesional skin [76]. Importantly, the skin of *Klf5*$^{+/-}$;*Fli1*$^{+/-}$ mice and SSc patients shares similar fibrillogenesis-associated gene expression profiles, including decreased decorin expression, increased levels of lumican and ADAMTS-2, and comparable levels of fibromodulin, biglycan, bone morphogenetic protein-1, and lysyl oxidase. Since the decreased expression of

decorin and the increased expression of lumican and ADAMTS-2 depend on the deficiency of Fli1 and KLF5, respectively, simultaneous downregulation of these genes is required to reproduce an SSc-like ultrastructure of dermis. In addition, *Klf5*$^{+/-}$;*Fli1*$^{+/-}$ mice exhibit nonspecific interstitial pneumonia, the main histological pattern of interstitial lung disease associated with SSc characterized by diffuse uniform expansion of alveolar septa with patchy septal lymphocytic infiltration, which becomes evident at the age of 8 months. Collectively, simultaneous downregulation of *Klf5* and *Fli1* genes is enough to induce SSc-like dermal and pulmonary fibrosis in mice [5].

In addition to fibrotic changes, *Klf5*$^{+/-}$;*Fli1*$^{+/-}$ mice also exhibit vascular changes simulating those of SSc, that is, stenosis of arterioles and bushy capillaries occur as early as the age of 1 month, and a progressive decrease in the number of subcutaneous vascular density starts from 2 months of age. Reduced arteriolar blood flow velocity and interstitial hypoxia are also evident in the skin of *Klf5*$^{+/-}$;*Fli1*$^{+/-}$ mice before the age of 4 months. Furthermore, pulmonary arterioles develop markedly thickened vascular walls with expansion of α-SMA-positive cells at 8 months of age, a typical histologic feature of pulmonary arterial hypertension, and interseptal venules show intimal fibrosis, focal luminal narrowing, and perivascular lymphocytic infiltrates, all suggestive of pulmonary veno-occlusive disease. Thus, the haploinsufficiency of *Klf5* and *Fli1* genes is enough to reproduce vascular alterations characteristic of SSc, such as destructive vasculopathy (loss of capillaries) and proliferative obliterative vasculopathy (stenosis of arterioles with fibro-proliferative change) [5].

As well as fibrotic and vascular changes, *Klf5*$^{+/-}$;*Fli1*$^{+/-}$ mice mimic B cell activation of SSc, which is a part of immune abnormality characteristically seen in this disease [77]. In concordance with the evidence that interstitial lung disease associated with SSc frequently accompanies diffuse or aggregated B cell infiltrates [78], *Klf5*$^{+/-}$;*Fli1*$^{+/-}$ lungs reveal perivascular B cell accumulation starting from the age of 3 months, which progresses to prominent B cell lymphoid aggregates and diffuse interstitial infiltrates at the age of 8 months. As for the activation status of B cells, in response to lipopolysaccharide and/or anti-CD40 antibody *Klf5*$^{+/-}$;*Fli1*$^{+/-}$ B cells produce much greater amount of IL-6 which is overexpressed in the skin, serum, and peripheral blood mononuclear cells of SSc patients [79, 80]. Furthermore, *Klf5*$^{+/-}$;*Fli1*$^{+/-}$ mice show circulating antinuclear antibody. Relevant to the production of IL-6 and autoantibodies, *Klf5*$^{+/-}$;*Fli1*$^{+/-}$ B cells express CD19 at higher levels than wild-type B cells, which is critical in the activation of SSc B cells [77]. Notably, both KLF5 and Fli1 interact with the *Cd19* promoter in murine B cells, suggesting that transcription factor complex of KLF5 and Fli1 intrinsically regulates CD19 expression in B cells [5].

In the pathogenesis of SSc, environmental influences have been believed to play a central role in the induction of SSc properties in various cell types, while genetic factors determine the susceptibility to and the severity of SSc [3]. This canonical idea is strongly supported by the evidence that the three cardinal features of SSc are recapitulated in mice by simultaneous downregulation of *Klf5* and *Fli1* genes, both of which are epigenetically suppressed in SSc dermal fibroblasts (Fig. 12.3). Most

importantly, *Klf5*$^{+/-}$;*Fli1*$^{+/-}$ mice develop immune activation, vasculopathy, and tissue fibrosis sequentially in this order as seen in typical SSc [5]. Therefore, further studies on *Klf5*$^{+/-}$;*Fli1*$^{+/-}$ mice and the role of these transcription factors in each cell type may provide us with a useful clue to further understand the pathological process of SSc.

12.11 Bosentan Ameliorates Vascular Fragility in *Fli1* ECKO Mice by Reversing the Expression of Fli1

The reversal of gene expression profiles by epigenetic inhibitors is a potential therapeutic strategy recently attracting much attention especially in the field of malignancy and epigenetically induced diseases. Since *FLI1* gene is epigenetically suppressed in SSc bulk skin and cultivated SSc dermal fibroblasts [4], Fli1 is a potential target for the treatment of SSc. The systemic administration of epigenetic inhibitors raises serious concerns associated with the nonspecific activation of unrelated genes; therefore, another strategy to restore Fli1 expression is required. A series of clinical and experimental data suggest that bosentan, a dual endothelin receptor antagonist, reverses the expression of Fli1 by increasing its protein stability without affecting its mRNA expression [40, 41]. In dermal fibroblasts and dermal microvascular endothelial cells, ET-1 activates c-Abl and PKC-δ sequentially, leading to Fli1 phosphorylation at threonine 312 by PKC-δ [33, 40, 41, 81]. Phosphorylated Fli1 is acetylated by PCAF at lysine 380, loses DNA-binding ability, and is rapidly degraded (Fig. 12.1) [32]. When these cells are treated with bosentan, Fli1 phosphorylation levels are markedly decreased, leading to the increase in its DNA-binding ability and protein stability [40, 41]. Importantly, the reversal of Fli1 expression is observed in endothelial cells of *Fli1* ECKO mice treated with bosentan for 4 weeks. Among the structural and functional abnormalities of vasculature in *Fli1* ECKO mice, such as stenosis of arterioles, dilation of capillaries, and increased vascular permeability of small vessels, vascular permeability is clearly normalized by bosentan in parallel with the increased expression of Fli1 in endothelial cells [40]. Of note, in human SSc, a similar disease-modifying effect of bosentan is reported. For example, 1-year administration of bosentan increases the number of nailfold capillaries with early and active patterns and decreases the number of nailfold capillaries with late pattern [82]. In addition, the 3-year combination therapy of bosentan and iloprost increases the number of nailfold capillaries, while iloprost alone results in the significant decrease in the number of nailfold capillaries [83]. Thus, the reversal of Fli1 expression may partially explain the molecular mechanism underlying the disease-modifying effect of bosentan on SSc vasculopathy (Fig. 12.3). Indeed, SSc patients treated with bosentan display the elevated expression of Fli1 in dermal microvascular endothelial cells compared with SSc patients untreated with bosentan (R. Saigusa and Y. Asano, unpublished data). However, the beneficial effect of bosentan on SSc vasculopathy is only modest and limited, which seems to be plausible because Fli1 deficiency is a part of predisposing factors of SSc.

12.12 Future Directions

So far, the contribution of Fli1 deficiency to the induction of SSc-like phenotypes has been well investigated in dermal fibroblasts, endothelial cells, and macrophages. At the time of writing, the role of Fli1 deficiency in other types of cells still remains unknown, but Th2-/Th17- skewed immune polarization in BLM-treated *Fli1*$^{+/-}$ mice and B cell activation in *Klf5*$^{+/-}$;*Fli1*$^{+/-}$ mice suggest the potential role of Fli1 deficiency in the induction of SSc-like phenotypes in T cells and B cells. Furthermore, Fli1 deficiency is also seen in SSc epidermal keratinocytes which also have been shown to possess an SSc-specific property [84]. Therefore, further studies on Fli1 deficiency in various cell types may provide us a new clue to understand the developmental process of SSc.

12.13 Conclusion

A series of data have suggested the involvement of Fli1 deficiency in the pathogenesis of SSc. However, SSc is a heterogeneous disease and possibly caused by a variety of combination of genetic factors and environmental influences. Indeed, SSc dermal fibroblasts show variable degrees of Fli1 downregulation [19]. Therefore, it is important to be aware that the insights obtained from *Fli1*-mutated mice and *Klf5*$^{+/-}$; *Fli1*$^{+/-}$ mice are applicable to a certain subset of SSc, but not to all. Given that the reversal of Fli1 expression is a potential mechanism explaining the efficacy of bosentan for SSc-related digital ulcers, variable degrees of Fli1 deficiency may explain a variety of therapeutic effects of bosentan on SSc vasculopathy. Therefore, further studies on the association between the degrees of Fli1 deficiency and the clinical features, including the severity of disease and the responsiveness to therapies, would provide us helpful information to translate basic and clinical research into clinical practice.

References

1. Arnett FC, Cho M, Chatterjee S, Aguilar MB, Reveille JD, Mayes MD. Familial occurrence frequencies and relative risks for systemic sclerosis (scleroderma) in three United States cohorts. Arthritis Rheum. 2001;44(6):1359–62.
2. Feghali-Bostwick C, Medsger TA, Wright TM. Analysis of systemic sclerosis in twins reveals low concordance for disease and high concordance for the presence of antinuclear antibodies. Arthritis Rheum. 2003;48(7):1956–63.
3. Broen JC, Radstake TR, Rossato M. The role of genetics and epigenetics in the pathogenesis of systemic sclerosis. Nat Rev Rheumatol. 2014;10(11):671–81.
4. Wang Y, Fan PS, Kahaleh B. Association between enhanced type I collagen expression and epigenetic repression of the FLI1 gene in scleroderma fibroblasts. Arthritis Rheum. 2006;54 (7):2271–9.
5. Noda S, Asano Y, Nishimura S, Taniguchi T, Fujiu K, Manabe I, et al. Simultaneous downregulation of KLF5 and Fli1 is a key feature underlying systemic sclerosis. Nat Commun. 2014;5:5797.

6. Wang Y, Kahaleh B. Epigenetic repression of bone morphogenetic protein receptor II expression in scleroderma. J Cell Mol Med. 2013;17(10):1291–9.
7. Matouk CC, Marsden PA. Epigenetic regulation of vascular endothelial gene expression. Circ Res. 2008;102(8):873–87.
8. Hu B, Gharaee-Kermani M, Wu Z, Phan SH. Essential role of MeCP2 in the regulation of myofibroblast differentiation during pulmonary fibrosis. Am J Pathol. 2011;178(4):1500–8.
9. Lian X, Xiao R, Hu X, Kanekura T, Jiang H, Li Y, et al. DNA demethylation of CD40l in CD4+ T cells from women with systemic sclerosis: a possible explanation for female susceptibility. Arthritis Rheum. 2012;64(7):2338–45.
10. Jiang H, Xiao R, Lian X, Kanekura T, Luo Y, Yin Y, et al. Demethylation of TNFSF7 contributes to CD70 overexpression in CD4+ T cells from patients with systemic sclerosis. Clin Immunol. 2012;143(1):39–44.
11. Wang Y, Shu Y, Xiao Y, Wang Q, Kanekura T, Li Y, et al. Hypomethylation and overexpression of ITGAL (CD11a) in CD4(+) T cells in systemic sclerosis. Clin Epigenetics. 2014;6(1):25.
12. Altorok N, Tsou PS, Coit P, Khanna D, Sawalha AH. Genome-wide DNA methylation analysis in dermal fibroblasts from patients with diffuse and limited systemic sclerosis reveals common and subset-specific DNA methylation aberrancies. Ann Rheum Dis. 2015;74(8):1612–20.
13. Delattre O, Zucman J, Plougastel B, Desmaze C, Melot T, Peter M, et al. Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. Nature. 1992;359(6391):162–5.
14. Hart A, Melet F, Grossfeld P, Chien K, Jones C, Tunnacliffe A, et al. Fli-1 is required for murine vascular and megakaryocytic development and is hemizygously deleted in patients with thrombocytopenia. Immunity. 2000;13(2):167–77.
15. Raslova H, Komura E, Le Couédic JP, Larbret F, Debili N, Feunteun J, et al. FLI1 monoallelic expression combined with its hemizygous loss underlies Paris-Trousseau/Jacobsen thrombopenia. J Clin Invest. 2004;114(1):77–84.
16. Carpinelli MR, Kruse EA, Arhatari BD, Debrincat MA, Ogier JM, Bories J, et al. Haploinsufficiency for *Ets1* and *Fli1* disrupts auditory and nasal development in mice and models aspects of Jacobsen syndrome. Am J Pathol. 2015;185(7):1867–76.
17. Hollenhorst PC, Jones DA, Graves BJ. Expression profiles frame the promoter specificity dilemma of the ETS family of transcription factors. Nucleic Acids Res. 2004;32(18):5693–702.
18. Czuwara-Ladykowska J, Shirasaki F, Jackers P, Watson DK, Trojanowska M. Fli-1 inhibits collagen type I production in dermal fibroblasts via an Sp1-dependent pathway. J Biol Chem. 2001;276(24):20839–48.
19. Kubo M, Czuwara-Ladykowska J, Moussa O, Markiewicz M, Smith E, Silver RM, et al. Persistent down-regulation of Fli1, a suppressor of collagen transcription, in fibrotic scleroderma skin. Am J Pathol. 2003;163(2):571–81.
20. Masuya M, Moussa O, Abe T, Deguchi T, Higuchi T, Ebihara Y, et al. Dysregulation of granulocyte, erythrocyte, and NK cell lineages in Fli-1 gene-targeted mice. Blood. 2005;105(1):95–102.
21. Mao X, Miesfeldt S, Yang H, Leiden JM, Thompson CB. The FLI-1 and chimeric EWS-FLI-1 oncoproteins display similar DNA binding specificities. J Biol Chem. 1994;269(27):18216–22.
22. Zhang XK, Watson DK. The FLI-1 transcription factor is a short-lived phosphoprotein in T cells. J Biochem. 2005;137(3):297–302.
23. Bradshaw S, Zheng WJ, Tsoi LC, Gilkeson G, Zhang XK. A role for Fli-1 in B cell proliferation: implications for SLE pathogenesis. Clin Immunol. 2008;129(1):19–30.
24. Zhang L, Eddy A, Teng YT, Fritzler M, Kluppel M, Melet F, et al. An immunological renal disease in transgenic mice that overexpress Fli-1, a member of the ets family of transcription factor genes. Mol Cell Biol. 1995;15(12):6961–70.

25. Zhang XK, Gallant S, Molano I, Moussa OM, Ruiz P, Spyropoulos DD, et al. Decreased expression of the Ets family transcription factor Fli-1 markedly prolongs survival and significantly reduces renal disease in MRL/lpr mice. J Immunol. 2004;173(10):6481–9.
26. Zhang XK, Moussa O, LaRue A, Bradshaw S, Molano I, Spyropoulos DD, et al. The transcription factor Fli-1 modulates marginal zone and follicular B cell development in mice. J Immunol. 2008;181(3):1644–54.
27. Sarrazin S, Bonod-Bidaud C, Remy P, Mehlen P, Morle F. Caspase cleavage of the transcription factor FLI-1 during preB leukemic cell death. Biochim Biophys Acta. 2002;1592 (2):123–7.
28. Spyropoulos DD, Pharr PN, Lavenburg KR, Jackers P, Papas TS, Ogawa M, et al. Hemorrhage, impaired hematopoiesis, and lethality in mouse embryos carrying a targeted disruption of the Fli1 transcription factor. Mol Cell Biol. 2000;20(15):5643–52.
29. Liu F, Walmsley M, Rodaway A, Patient R. Fli1 acts at the top of the transcriptional network driving blood and endothelial development. Curr Biol. 2008;18(16):1234–40.
30. Shirasaki F, Makhluf HA, LeRoy C, Watson DK, Trojanowska M. Ets transcription factors cooperate with Sp1 to activate the human tenascin-C promoter. Oncogene. 1999;18 (54):7755–64.
31. Asano Y, Trojanowska M. Fli1 represses transcription of the human α2(I) collagen gene by recruitment of the HDAC1/p300 complex. PLoS ONE. 2013;8(9):e74930.
32. Asano Y, Czuwara J, Trojanowska M. Transforming growth factor-beta regulates DNA binding activity of transcription factor Fli1 by p300/CREB-binding protein-associated factor-dependent acetylation. J Biol Chem. 2007;282(48):34672–83.
33. Asano Y, Trojanowska M. Phosphorylation of Fli1 at threonine 312 by protein kinase C delta promotes its interaction with p300/CREB-binding protein-associated factor and subsequent acetylation in response to transforming growth factor beta. Mol Cell Biol. 2009;29 (7):1882–94.
34. Asano Y, Markiewicz M, Kubo M, Szalai G, Watson DK, Trojanowska M. Transcription factor Fli1 regulates collagen fibrillogenesis in mouse skin. Mol Cell Biol. 2009;29(2):425–34.
35. Simonsson M, Kanduri M, Grönroos E, Heldin CH, Ericsson J. The DNA binding activities of Smad2 and Smad3 are regulated by coactivator-mediated acetylation. J Biol Chem. 2006;281 (52):39870–80.
36. Inoue Y, Itoh Y, Abe K, Okamoto T, Daitoku H, Fukamizu A, et al. Smad3 is acetylated by p300/CBP to regulate its transactivation activity. Oncogene. 2007;26(4):500–8.
37. Itoh S, Ericsson J, Nishikawa J, Heldin CH, ten Dijke P. The transcriptional co-activator P/CAF potentiates TGF-beta/Smad signaling. Nucleic Acids Res. 2000;28(21):4291–8.
38. Jimenez SA, Gaidarova S, Saitta B, Sandorfi N, Herrich DJ, Rosenbloom JC, et al. Role of protein kinase C-delta in the regulation of collagen gene expression in scleroderma fibroblasts. J Clin Invest. 2001;108(9):1395–403.
39. Lagares D, García-Fernández RA, Jiménez CL, Magán-Marchal N, Busnadiego O, Lamas S, et al. Endothelin 1 contributes to the effect of transforming growth factor beta1 on wound repair and skin fibrosis. Arthritis Rheum. 2010;62(3):878–89.
40. Akamata K, Asano Y, Yamashita T, Noda S, Taniguchi T, Takahashi T, et al. Endothelin receptor blockade ameliorates vascular fragility in endothelial cell-specific fli-1-knockout mice by increasing fli-1 DNA binding ability. Arthritis Rheumatol. 2015;67(5):1335–44.
41. Akamata K, Asano Y, Aozasa N, Noda S, Taniguchi T, Takahashi T, et al. Bosentan reverses the pro-fibrotic phenotype of systemic sclerosis dermal fibroblasts via increasing DNA binding ability of transcription factor Fli1. Arthritis Res Ther. 2014;16(2):R86.
42. Taniguchi T, Asano Y, Akamata K, Noda S, Takahashi T, Ichimura Y, et al. Fibrosis, vascular activation, and immune abnormalities resembling systemic sclerosis in bleomycin-treated fli-1-haploinsufficient mice. Arthritis Rheumatol. 2015;67(2):517–26.
43. Braun-Falco O, Rupec M. Collagen fibrils of the scleroderma in ultra-thin skin sections. Nature. 1964;202:708–9.

44. Fleischmajer R, Damiano V, Nedwich A. Alteration of subcutaneous tissue in systemic scleroderma. Arch Dermatol. 1972;105(1):59–66.
45. Fleischmajer R, Gay S, Meigel WN, Perlish JS. Collagen in the cellular and fibrotic stages of scleroderma. Arthritis Rheum. 1978;21(4):418–28.
46. Sakakibara N, Sugano S, Morita A. Ultrastructural changes induced in cutaneous collagen by ultraviolet-A1 and psoralen plus ultraviolet A therapy in systemic sclerosis. J Dermatol. 2008;35(2):63–9.
47. Asano Y, Stawski L, Hant F, Highland K, Silver R, Szalai G, et al. Endothelial Fli1 deficiency impairs vascular homeostasis: a role in scleroderma vasculopathy. Am J Pathol. 2010;176 (4):1983–98.
48. Noda S, Asano Y, Akamata K, Aozasa N, Taniguchi T, Takahashi T, et al. A possible contribution of altered cathepsin B expression to the development of skin sclerosis and vasculopathy in systemic sclerosis. PLoS ONE. 2012;7(2):e32272.
49. Noda S, Asano Y, Takahashi T, Akamata K, Aozasa N, Taniguchi T, et al. Decreased cathepsin V expression due to Fli1 deficiency contributes to the development of dermal fibrosis and proliferative vasculopathy in systemic sclerosis. Rheumatology (Oxford). 2013;52(5):790–9.
50. Ichimura Y, Asano Y, Akamata K, Takahashi T, Noda S, Taniguchi T, et al. Fli1 deficiency contributes to the suppression of endothelial CXCL5 expression in systemic sclerosis. Arch Dermatol Res. 2014;306(4):331–8.
51. Saigusa R, Asano Y, Taniguchi T, Yamashita T, Takahashi T, Ichimura Y, et al. A possible contribution of endothelial CCN1 downregulation due to Fli1 deficiency to the development of digital ulcers in systemic sclerosis. Exp Dermatol. 2015;24(2):127–32.
52. Akamata K, Asano Y, Taniguchi T, Yamashita T, Saigusa R, Nakamura K, et al. Increased expression of chemerin in endothelial cells due to Fli1 deficiency may contribute to the development of digital ulcers in systemic sclerosis. Rheumatology (Oxford). 2015;54 (7):1308–16.
53. Takahashi T, Asano Y, Noda S, Aozasa N, Akamata K, Taniguchi T, et al. A possible contribution of lipocalin-2 to the development of dermal fibrosis, pulmonary vascular involvement, and renal dysfunction in systemic sclerosis. Br J Dermatol. 2015;173(3):681–9.
54. Montagnana M, Volpe A, Lippi G, Caramaschi P, Salvagno GL, Biasi D, et al. Relationship between matrix metalloproteinases/tissue inhibitors of matrix metalloproteinases systems and autoantibody patterns in systemic sclerosis. Clin Biochem. 2007;40(12):837–42.
55. Querfeld C, Eckes B, Huerkamp C, Krieg T, Sollberg S. Expression of TGF-beta 1, -beta 2 and -beta 3 in localized and systemic scleroderma. J Dermatol Sci. 1999;21(1):13–22.
56. Dong C, Zhu S, Wang T, Yoon W, Li Z, Alvarez RJ, et al. Deficient Smad7 expression: a putative molecular defect in scleroderma. Proc Natl Acad Sci U S A. 2002;99(6):3908–13.
57. Jimenez SA. Role of endothelial to mesenchymal transition in the pathogenesis of the vascular alterations in systemic sclerosis. ISRN Rheumatol. 2013;2013:835948.
58. Matsushita T, Hasegawa M, Hamaguchi Y, Takehara K, Sato S. Longitudinal analysis of serum cytokine concentrations in systemic sclerosis: association of interleukin 12 elevation with spontaneous regression of skin sclerosis. J Rheumatol. 2006;33(2):275–84.
59. Nakashima T, Jinnin M, Yamane K, Honda N, Kajihara I, Makino T, et al. Impaired IL-17 signaling pathway contributes to the increased collagen expression in scleroderma fibroblasts. J Immunol. 2012;188(8):3573–83.
60. Yoshizaki A, Yanaba K, Iwata Y, Komura K, Ogawa A, Akiyama Y, et al. Cell adhesion molecules regulate fibrotic process via Th1/Th2/Th17 cell balance in a bleomycin-induced scleroderma model. J Immunol. 2010;185(4):2502–15.
61. Higashi-Kuwata N, Jinnin M, Makino T, Fukushima S, Inoue Y, Muchemwa FC, et al. Characterization of monocyte/macrophage subsets in the skin and peripheral blood derived from patients with systemic sclerosis. Arthritis Res Ther. 2010;12(4):R128.
62. Mo FE, Muntean AG, Chen CC, Stolz DB, Watkins SC, Lau LF. CYR61 (CCN1) is essential for placental development and vascular integrity. Mol Cell Biol. 2002;22(24):8709–20.

63. Chen N, Leu SJ, Todorovic V, Lam SC, Lau LF. Identification of a novel integrin alphavbeta3 binding site in CCN1 (CYR61) critical for pro-angiogenic activities in vascular endothelial cells. J Biol Chem. 2004;279(42):44166–76.
64. Chen CC, Mo FE, Lau LF. The angiogenic factor Cyr61 activates a genetic program for wound healing in human skin fibroblasts. J Biol Chem. 2001;276(50):47329–37.
65. Leu SJ, Lam SC, Lau LF. Pro-angiogenic activities of CYR61 (CCN1) mediated through integrins alphavbeta3 and alpha6beta1 in human umbilical vein endothelial cells. J Biol Chem. 2002;277(48):46248–55.
66. Grote K, Salguero G, Ballmaier M, Dangers M, Drexler H, Schieffer B. The angiogenic factor CCN1 promotes adhesion and migration of circulating CD34+ progenitor cells: potential role in angiogenesis and endothelial regeneration. Blood. 2007;110(3):877–85.
67. Duan H, Fleming J, Pritchard DK, Amon LM, Xue J, Arnett HA, et al. Combined analysis of monocyte and lymphocyte messenger RNA expression with serum protein profiles in patients with scleroderma. Arthritis Rheum. 2008;58(5):1465–74.
68. Sidky YA, Borden EC. Inhibition of angiogenesis by interferons: effects on tumor- and lymphocyte-induced vascular responses. Cancer Res. 1987;47(19):5155–61.
69. Indraccolo S, Pfeffer U, Minuzzo S, Esposito G, Roni V, Mandruzzato S, et al. Identification of genes selectively regulated by IFNs in endothelial cells. J Immunol. 2007;178(2):1122–35.
70. Pammer J, Reinisch C, Birner P, Pogoda K, Sturzl M, Tschachler E. Interferon-alpha prevents apoptosis of endothelial cells after short-term exposure but induces replicative senescence after continuous stimulation. Lab Invest. 2006;86(10):997–1007.
71. Takeda N, Manabe I, Uchino Y, Eguchi K, Matsumoto S, Nishimura S, et al. Cardiac fibroblasts are essential for the adaptive response of the murine heart to pressure overload. J Clin Invest. 2010;120(1):254–65.
72. Fujiu K, Manabe I, Nagai R. Renal collecting duct epithelial cells regulate inflammation in tubulointerstitial damage in mice. J Clin Invest. 2011;121(9):3425–41.
73. Whitfield ML, Finlay DR, Murray JI, Troyanskaya OG, Chi JT, Pergamenschikov A, et al. Systemic and cell type-specific gene expression patterns in scleroderma skin. Proc Natl Acad Sci U S A. 2003;100(21):12319–24.
74. Chujo S, Shirasaki F, Kawara S, Inagaki Y, Kinbara T, Inaoki M, et al. Connective tissue growth factor causes persistent proalpha2(I) collagen gene expression induced by transforming growth factor-beta in a mouse fibrosis model. J Cell Physiol. 2005;203(2):447–56.
75. Leask A. Signaling in fibrosis: targeting the TGF beta, endothelin-1 and CCN2 axis in scleroderma. Front Biosci (Elite Ed). 2009;1:115–22.
76. Perlish JS, Lemlich G, Fleischmajer R. Identification of collagen fibrils in scleroderma skin. J Invest Dermatol. 1988;90(1):48–54.
77. Fujimoto M, Sato S. B lymphocytes and systemic sclerosis. Curr Opin Rheumatol. 2005;17 (6):746–51.
78. Lafyatis R, O'Hara C, Feghali-Bostwick CA, Matteson E. B cell infiltration in systemic sclerosis-associated interstitial lung disease. Arthritis Rheum. 2007;56(9):3167–8.
79. Khan K, Xu S, Nihtyanova S, Derrett-Smith E, Abraham D, Denton CP, et al. Clinical and pathological significance of interleukin 6 overexpression in systemic sclerosis. Ann Rheum Dis. 2012;71(7):1235–42.
80. Giacomelli R, Cipriani P, Danese C, Pizzuto F, Lattanzio R, Parzanese I, et al. Peripheral blood mononuclear cells of patients with systemic sclerosis produce increased amounts of interleukin 6, but not transforming growth factor beta 1. J Rheumatol. 1996;23(2):291–6.
81. Bujor AM, Asano Y, Haines P, Lafyatis R, Trojanowska M. The c-Abl tyrosine kinase controls protein kinase Cδ-induced Fli-1 phosphorylation in human dermal fibroblasts. Arthritis Rheum. 2011;63(6):1729–37.
82. Guiducci S, Bellando Randone S, Bruni C, Carnesecchi G, Maresta A, Iannone F, et al. Bosentan fosters microvascular de-remodelling in systemic sclerosis. Clin Rheumatol. 2012;31(12):1723–5.

83. Cutolo M, Zampogna G, Vremis L, Smith V, Pizzorni C, Sulli A. Longterm effects of endothelin receptor antagonism on microvascular damage evaluated by nailfold capillaroscopic analysis in systemic sclerosis. J Rheumatol. 2013;40(1):40–5.
84. Aden N, Nuttall A, Shiwen X, de Winter P, Leask A, Black CM, et al. Epithelial cells promote fibroblast activation via IL-1alpha in systemic sclerosis. J Invest Dermatol. 2010;130 (9):2191–200.

Biomarker

13

Minoru Hasegawa

Abstract

Clinical manifestation, disease progress, and prognosis are heterogeneous in each patient with systemic sclerosis (SSc). Therefore, biomarkers that can estimate these matters are essential for clinical practice. Although SSc-specific autoantibodies are very useful markers, other biomarkers have not been established. Regarding potential biomarkers of fibrosis, some cytokines, chemokines, adhesion molecules including connective tissue growth factor, interleukin-6, CCL2, CXCL4, and circulating intercellular adhesion molecule-1 have been reported. The glycoprotein Krebs von den Lungen-6 and surfactant protein-D are currently the most reliable serum biomarkers of interstitial lung diseases of SSc. Serum or plasma levels of brain natriuretic peptide and N-terminal pro-brain natriuretic peptide have been used as useful biomarkers for SSc-related pulmonary arterial hypertension, although these are not specific for pulmonary arterial hypertension. It has been reported that interferon-inducible chemokine score correlated with the Medsger Severity Index, particularly with the severity of the skin, muscle, and lung involvement. Further large multicenter prospective studies will be needed to identify critical biomarkers of SSc.

Keywords

Systemic sclerosis • Biomarker • Fibrosis • Vascular injury

M. Hasegawa (✉)
Department of Dermatology, School of Medicine, Faculty of Medical Sciences, University of Fukui, 23-3, Matsuokashimoaizuki, Eiheiji-cho, Yoshida-gun, Fukui, Japan
e-mail: minoruha@u-fukui.ac.jp

K. Takehara et al. (eds.), *Systemic Sclerosis*, DOI 10.1007/978-4-431-55708-1_13

13.1 Necessity of Biomarker

Systemic sclerosis (SSc) is a connective tissue disease characterized by tissue fibrosis and vascular injury in the skin and internal organs. Interstitial lung diseases (ILD) and pulmonary arterial hypertension (PAH) are major causes of SSc-related death. In addition, joint contracture due to extensive skin sclerosis, intractable digital ulcer, and other severe internal organ involvement results in impaired physical function.

Severity, organ involvement, disease progress, and prognosis are heterogeneous in each patient. Therefore, biomarkers that can estimate these matters are very important and necessary for the medical examination. For example, clinicians must predict who may develop severe skin and/or ILD and who is associated with a risk of future development of PAH. Also, biomarkers can be beneficial to evaluate the response to therapy. Furthermore, assessing and identifying biomarkers can result in the understanding of SSc pathogenesis. Nonetheless, there are no other definitive markers except for SSc-specific autoantibodies [1].

13.2 Definitions of Biomarker

In 2001, Biomarkers Definitions Working Group of the National Institutes of Health proposed the definitions and characteristics of biomarkers as follows [2]. Biological marker (biomarker) is "a characteristic marker that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention." Biomarkers are useful in early efficacy and safety evaluations such as in vitro studies in tissue samples, in vivo studies in animal models, and early-phase clinical trials to establish "proof of concept." Biomarkers have many other valuable applications in disease detection and monitoring of health status. For example, biomarkers can be used for diagnosis, classification, indicator of disease activity, prognosis, prediction of organ involvement, and monitoring of clinical response to an intervention. Ideal biomarkers should be easily measureable at reasonable cost. In addition, they should be sensitive and specific to the disease. There are several excellent reviews regarding biomarkers of systemic sclerosis [3–7].

13.3 Biomarkers of Fibrosis (Table 13.1)

13.3.1 Circulating Collagen Fragments

The most characteristic pathological state of SSc is tissue fibrosis due to deposition of extracellular matrix protein (ECM). Type I collagen is the most abundant ECM protein deposited in fibrotic skin of SSc. Excessive synthesis and deposition of collagens were rationale for these turnover measurements to activity and severity in patients with SSc. The modified Rodnan total skin thickness score (MRSS) has

Table 13.1 Potential biomarkers of fibrosis

Biomarker	Clinical association	Sample
Type I procollagen peptides (C-terminal)	MRSS, CRP	Serum/plasma
Type III procollagen peptides (N-terminal)	Disease activity, HRCT score, poor prognosis	Serum/plasma
MMP-9	MRSS	Serum/plasma
MMP-12	Skin involvement, digital ulcer, severity of lung construction, nail fold bleeding	Serum/plasma
TGF-β-responsive gene signature	dcSSc>lcSSc	DNA microarray of the skin
TGF-β1, TGF-β2	dcSSc	mRNA of the skin
CTGF (N-terminal)	MRSS, ILD	Serum/plasma
IL-6	MRSS, poor prognosis, DLCO decline in SSc-ILD	Serum/plasma
CCL2	ILD	Serum/plasma, BAL fluids
CXCL4	MRSS, lung fibrosis, PAH, disease progression	Serum/plasma
VEGF	Systemic organ involvement, PAH	Serum/plasma

MRSS modified Rodnan total skin thickness score, *CRP* C-reactive protein, *HRCT* high-resolution computed tomography, *MMP* matrix metalloproteinase, *TGF* transforming growth factor, *dcSSc* diffuse cutaneous systemic sclerosis, *lcSSc* limited cutaneous systemic sclerosis, *CTGF* connective tissue growth factor, *ILD* interstitial lung disease, *IL-6* interleukin 6, *DLCO* diffusing capacity for carbon monoxide, *PAH* pulmonary arterial hypertension, *BAL* bronchoalveolar lavage, *VEGF* vascular endothelial growth factor

widely been used for evaluation of skin fibrosis in clinical practice and as a surrogate end point in clinical trials. Skin thickness at 17 distinct areas of the body is graded from 0 (normal) to 3 (prominent skin thickening) by pinching the skin [8]. The MRSS is a semi-objective parameter with very good intraobserver but imperfect interobserver reproducibility [9]. Among various studies investigating circulating and urinary levels of collagen degradations as biomarkers, one study reported that serum concentrations of C-terminal telopeptide of type I collagen, a marker of type I collagen degradation, are closely associated with the extent of skin fibrosis in patients with SSc [10]. Another study showed that serum concentrations of C-terminal telopeptide of type I collagen were significantly correlated with MRSS and acute phase reactants in patients with SSc [11]. A systematic review of 12 studies examining serum collagen type I metabolites did not find sufficient evidence as useful biomarkers of activity and severity of SSc, although this may be due to the limitations of the previous studies [12].

There are also many reports regarding collagen type III metabolites. Serum levels of the N-terminal type III procollagen peptide were associated with the disease activity of SSc [13]. Another study reported that N-terminal type III procollagen peptide correlated with high-resolution computed tomography score

in patients with SSc [14]. In a prospective follow-up study, the increased levels of N-terminal propeptide of type III procollagen were independent unfavorable prognostic signs [15]. Further longitudinal serial analysis in individual patient may be necessary to determine the utility of collagen type I or III metabolites in SSc.

13.3.2 Matrix Metalloproteinases (MMPs)

MMPs were first described as proteases that degrade ECM. However, MMPs have multiple roles such as release and activation of cytokines and growth factors [16]. MMP-9, whose substrates include type IV collagen in basement membrane, has been implicated in the pathogenesis of cancer, autoimmune disease, and various pathologic conditions characterized by excessive fibrosis. In a previous study, SSc patients had higher concentrations of MMP-9, and the concentrations were significantly higher in diffuse cutaneous SSc (dcSSc) compared with limited cutaneous SSc (lcSSc) [17]. Serum concentrations of MMP-9 correlated well with the degree of skin involvement, as determined by MRSS [17]. Moreover, dermal fibroblasts from patients with SSc produced more MMP-9 than those from healthy controls when they were stimulated with interleukin (IL)-1β, tumor necrosis factor (TNF)-α, or transforming growth factor (TGF)-β [17].

It has been known that MMP-12 degrades multiple ECM components including type IV collagen, elastin, and fibronectin [18]. Skin fibroblasts and endothelial cells from SSc patients expressed augmented MMP-12 [19]. In a study of 72 SSc patients, serum MMP-12 levels were significantly increased in dcSSc patients, and the levels were correlated with skin involvement, presence of digital ulcers, and severity of lung restriction and nail fold bleeding [20].

13.3.3 Transforming Growth Factor (TGF)-β

It has been widely accepted that TGF-β is the central player in the pathogenic mechanism of SSc, especially in the development of tissue fibrosis [21]. The main producer of TGF-β is likely macrophages, but many kinds of cells including platelets, leukocytes, and fibroblasts can produce TGF-β. Thrombospondins, plasmin, and various cell surface integrins mediate latent TGF-β activation. Although the Smad pathway has crucial roles in signaling from the TGF-β receptor, non-Smad pathways are also important for TGF-β-dependent fibrogenesis [22].

DNA microarray analysis demonstrated that a group of TGF-β-dependent genes are overexpressed in skin lesions of SSc patients [23]. In another study, DNA microarray analysis of skin biopsies has demonstrated that the TGF-β-responsive gene signature is expressed highly in dcSSc subset but not found in patients with lcSSc, morphea, or in healthy controls [24]. Serial assessment of skin biopsy samples represents the most direct way of assessing changes in scleroderma patients at a biochemical level. Regarding the biomarkers in skin biopsy specimens, CAT-192 study reported that tissue levels of mRNA for procollagens I and III

and for TGF-β1 and TGF-β2 were elevated in patients with dcSSc. Clear differences were identified between lesional and nonlesional sites as well as between SSc and healthy control samples [25].

However, circulating TGF-β levels are not consistently dependent on each study; this may be due to difficulties in accurate measurements of active TGF-β and its complex regulation and short half-life in biological fluids [26].

13.3.4 Connective Tissue Growth Factor (CTGF)

CTGF/CCN2 is another critical factor for the development of tissue fibrosis [27]. CTGF is expressed in fibroblasts induced by TGF-β1 and enhances fibroblast proliferation and ECM production as a downstream mediator of TGF-β1. A series of studies of mouse model suggest a two-step hypothesis for developing fibrosis in SSc: TGF-β1 induces fibrosis in the early stage, and afterwards CTGF contributes in maintaining the fibrotic state [28]. Serum CTGF levels were significantly elevated in patients with SSc and correlated with the severity of skin sclerosis and ILD [29]. N-terminal cleavage products of CTGF but not whole and C-terminal CTGF were increased in the plasma and dermal interstitial fluid of SSc patients and correlated positively with the severity of skin sclerosis and negatively with disease duration [30].

13.3.5 Interleukin (IL)-6

Among various cytokines, IL-6 has been considered as a potential biomarker of disease monitoring in SSc. IL-6 is a multifunctional cytokine that regulates immune responses and induces acute phase responses. Despite the critical physiological activities of IL-6, excessive production of IL-6 is pathologically involved in various immune-mediated inflammatory diseases, including rheumatoid arthritis. Augmented IL-6 expression was observed in dermal fibroblasts, mononuclear cells, and endothelial cells in patients with early dcSSc [31]. Previous studies have reported that serum IL-6 levels were significantly associated with MRSS [31, 32]. Elevated IL-6 expression was likely associated with poor prognosis in patients with early dcSSc [31]. In a recent study, serum levels of eight cytokines (IL-6, IL-8, IL-10, CCL2, CXCL10, CX3CL1, fibroblast growth factor 2, and vascular endothelial growth factor) were assessed by Luminex bead technology in exploratory cohorts of 74 patients with SSc and 58 patients with idiopathic pulmonary fibrosis [33]. In the exploratory analysis, only serum IL-6 was an independent predictor of the diffusing capacity of the lungs for carbon monoxide (DLCO) decline in both SSc-ILD and idiopathic pulmonary fibrosis.

13.3.6 Chemokine

A variety of chemokines have been reported as possible biomarkers of SSc. CCL2 (monocyte chemoattractant protein-1, MCP-1) may be one of the promising candidates. CCL2 is produced by macrophages, fibroblasts, endothelial cells, and other cells and predominant chemoattractant and activator of monocytes and T cells. Also, this chemokine induces Th2 cell polarization [34] and stimulates collagen production by fibroblasts via specific receptors and endogenous upregulation of TGF-β expression. The latter results in autocrine and/or juxtacrine stimulation of collagen gene expression [35]. Serum CCL2 levels are increased in SSc patients and have been found to correlate with the presence of ILD [36–38]. Interestingly, expression of CCL2 mRNA was most augmented among 4,507 genes when bronchoalveolar lavage (BAL) cells from SSc lung are compared with controls [39]. Consistent with this, protein levels of CCL2 are increased in BAL fluids from SSc patients with lung inflammation [39]. CCL2 concentrations in BAL fluids were associated with the presence of ILD and correlated with lung function parameters and computed tomography scores [40].

A recent proteome-wide analysis and validation has identified that CXCL4 is the predominant protein produced by plasmacytoid dendritic cells in SSc [41]. Plasma levels of CXCL4 were markedly elevated and strongly correlated with skin and lung fibrosis and pulmonary arterial hypertension in patients with SSc. In addition, plasma CXCL4 levels are likely useful to predict the disease progression in SSc [41]. In addition to its antiangiogenic activity, CXCL4 inhibits the expression of the antifibrotic cytokine interferon (IFN)-γ and upregulates profibrotic cytokines such as IL-4 and IL-13. CXCL4 exhibited direct effects for inducing SSc phenotype both in vitro and in vivo [41], suggesting its central roles in the pathogenesis of SSc.

13.4 Biomarkers of Endothelial Cell Dysfunction (Table 13.2)

Vascular involvement has been considered to be one of the earliest pathogenic features of SSc. Endothelial damage leads to vascular fibroproliferative lesions in multiple organs and can result in critical organ injury such as PAH and renal crisis. Numerous molecules have been suggested as potential biomarkers for endothelial cell injury in SSc. These include von Willebrand factor (vWF), endothelin-1, thrombomodulin, thrombospondin, brain natriuretic peptide (BNP), N-terminal propeptide of proBNP (NT-proBNP), vascular endothelial growth factor (VEGF), endostatin, plasminogen activator, prostacyclin, thromboxane, and nitrous oxide circulating metabolites.

13.4.1 von Willebrand Factor

In the pioneering study, von Willebrand factor (vWF) was markedly increased in the plasma of patients with SSc and patients with Raynaud's phenomenon

Table 13.2 Potential biomarkers of endothelial cell dysfunction

Biomarker	Clinical association	Sample
von Willebrand factor	Raynaud's phenomenon, disease severity, ILD	Serum/plasma
VEGF	Shorter disease duration, MRSS, capillary density of nail fold, PAH	Serum/plasma
HGF	Right ventricular systolic pressure	Serum/plasma
Endostatin	Right ventricular systolic pressure	Serum/plasma
Endoglin	lcSSc, telangiectasia, pulmonary artery pressure	Serum/plasma
ICAM-1	Rapidly progressive disease, digital ulcers, dcSSc, respiratory function	Serum/plasma

ILD interstitial lung disease, *VEGF* vascular endothelial growth factor, *MRSS* modified Rodnan total skin thickness score, *PAH* pulmonary arterial hypertension, *HGF* hepatocyte growth factor, *lcSSc* limited cutaneous systemic sclerosis, *ICAM* intercellular adhesion molecule, *dcSSc* diffuse cutaneous systemic sclerosis

[42]. Circulating vWF was associated with disease severity [43], pulmonary involvement [44], and the extent of radiologically demonstrated ILD [45]. However, association with clinical feature of vascular disease is not clear [46, 47]. von Willebrand factor-cleaving protease ADAMTS-13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) was significantly decreased in patients with SSc [48].

13.4.2 Growth Factors

Multiple growth factors have been considered as regulating angiogenesis. Abnormal plasma levels of proangiostatic factors including fibroblast growth factor, placental growth factor, platelet-derived growth factor, and VEGF have been reported [49]. Levels of circulating VEGF were elevated and correlated with shorter disease duration [50], skin sclerosis, and the capillary density of nail fold [51]. On the other hand, other studies did not find any association between VEGF levels and disease activity [52]. Plasma levels of the proangiogenic and antifibrotic factor, hepatocyte growth factor, were reduced in patients with SSc, but had a remarkable positive correlation with right ventricular systolic pressure as measured by echocardiogram [49].

Higher serum concentrations of endothelin-1, E-selectin, VEGF, and vascular cell adhesion molecule (VCAM)-1 were detected in SSc patients compared with healthy controls. Elevated concentrations of endothelin-1, E-selectin, VCAM-1, and VEGF dominated in the serum of SSc patients with organ systemic involvement compared to those without systemic manifestation [53]. Serum VEGF levels were correlated with systolic pulmonary artery pressure in patients with SSc [54]. Thus, the associations with clinical features are different depending on each study, although circulating VEGF levels are generally elevated in SSc patients [55]. It has been proposed that chronic and uncontrolled VEGF upregulation that is

mediated by an orchestrated expression of cytokines is the cause of the disturbed vessel morphology in the skin of SSc [55]. Furthermore, impaired VEGF receptor signaling may be contributing to vascular disturbances in SSc [56].

13.4.3 Angiostatic Molecules

Elevated levels of circulating angiostatic molecules have been reported. These angiostatic factors include angiostatin, endoglin, endostatin, thrombospondin, and VEGF receptor. Serum levels of endostatin were not significantly elevated but associated with the presence of giant capillaries in nail fold capillaroscopy [50]. Plasma levels of endostatin were markedly elevated in patients with SSc and correlated positively with right ventricular systolic pressure [49]. In multivariate analysis of a large SSc cohort, soluble endoglin levels were significantly increased in SSc patients with cutaneous ulcerations, positive for anticentromere Ab, and with abnormal diffusing capacity for carbon monoxide divided by alveolar volume [56]. Serum soluble endoglin levels were significantly elevated in patients with lcSSc compared with dcSSc and systemic lupus erythematosus patients as well as normal controls [57]. In that study, patients with elevated soluble endoglin levels had telangiectasia more frequently than those with normal soluble endoglin levels. Furthermore, pulmonary artery pressure was positively correlated with soluble endoglin levels in patients with lcSSc. These findings are interesting, since endoglin gene encodes a transmembrane glycoprotein which acts as an accessory receptor for the TGF-β superfamily and is crucial for maintaining vascular integrity. The endoglin gene mutations are responsible for one of the two types of hereditary hemorrhagic telangiectasia (Osler–Weber–Rendu syndrome), a Mendelian autosomal vascular disorder [58], and the polymorphism is associated with SSc-related PAH [59].

13.4.4 Adhesion Molecules

SSc patients exhibit increased numbers and activation of monocytes/macrophages and T cells in the circulation and tissues [60, 61]. Leukocyte recruitment into inflammatory sites is generally achieved using multiple cell adhesion molecules [62]. Endothelial cell injury induces adhesion molecules, and this may result in further endothelial damage via recruiting inflammatory cells.

Several previous studies have demonstrated that SSc fibroblasts exhibit increased surface intercellular adhesion molecule (ICAM)-1 expression, suggesting an augmented potential for binding to T cells [63]. There are several reports that have demonstrated the increase of circulating ICAM in patients with SSc [64–66]. In one of those studies, circulating ICAM-1 levels were especially elevated in patients with diffuse rapidly progressive disease or digital ulcers [64]. Another study demonstrated that circulating ICAM-1 levels was significantly higher in dcSSc patients and was correlated with the presence of contracture of phalanges,

pulmonary fibrosis, joint involvement, and increased erythrocyte sedimentation rate [66]. In one report, serum levels of ICAM-1, P-selectin, VCAM-1, and, to a lesser degree, E-selectin correlate well with their in situ expression and with clinical disease activity [65]. Japanese multicenter, prospective, observational study demonstrated that serum ICAM-1 levels were elevated and inversely associated with the current and subsequent respiratory functions in patients with early SSc [67]. In a small study, serum levels of ICAM-1, platelet endothelial cell adhesion molecule-1, P-selectin, and VCAM-1 were higher in SSc patients compared with healthy controls at baseline and fell to normal levels after 12 months of bosentan therapy [47]. In scleroderma renal crisis, mean levels of circulating E-selectin, ICAM-1, and VCAM-1 were elevated. On the other hand, serum levels of E-selectin, ICAM-1, and VCAM-1 were not increased in lcSSc patients with PAH [68]. Serum levels of E-selectin, ICAM-1, and VCAM-1 were initially elevated and significantly reduced after infusions of iloprost (prostacyclin analogue) on Raynaud's phenomenon [69].

13.5 Biomarkers of Interstitial Lung Diseases (Table 13.3)

13.5.1 Proteins Secreted by Alveolar Epithelial Cells

Recently, ILD associated with SSc has become the main cause of SSc-related death in SSc. Serum biomarkers of ILD are critical for monitoring patients with SSc, since the evaluation using computed tomography (CT) scan cannot be frequently performed. Serum biomarkers of ILD have been focused on soluble proteins secreted by alveolar epithelial cells (the main targeted cells of ILD) and various inflammatory cytokines, chemokines, and other proteins. The glycoprotein Krebs von den Lungen-6 (KL-6) and surfactant protein-D (SP-D) may be currently the most reliable serum markers for ILD. KL-6 antigen is expressed mainly by alveolar type II pneumocytes and respiratory bronchiolar epithelial cells [70], whereas SP-D is produced and secreted by alveolar type II pneumocytes and Clara cells [71]. Several studies revealed that serum levels of KL-6 and SP-D are elevated in serum from patients with ILD, including SSc-related ILD [70, 72, 73]. These studies suggested that serum levels of SP-D and KL-6 are serologic markers of the severity and

Table 13.3 Potential biomarkers of interstitial lung diseases

Biomarker	Clinical association	Sample
KL-6	Severity and activity of ILD, maximum fibrosis scores on HRCT	Serum/plasma
SP-D	Severity and activity of ILD, maximum fibrosis scores on HRCT	Serum/plasma
CCL18	Activity of ILD, predictive worsening of ILD	Serum/plasma

KL-6 Krebs von den Lungen-6, *ILD* interstitial lung disease, *HRCT* high-resolution computed tomography, *SP-D* surfactant protein-D

activity of ILD in SSc [73–75]. In a comparative study, SP-D was more sensitive but less specific for ILD than KL-6 in SSc patients [75]. A cohort study of Scleroderma Lung Study Research Group demonstrated that both KL-6 and SP-D are highly sensitive and specific for the determination of "alveolitis." In that study, KL-6 and SP-D were significantly correlated with maximum fibrosis scores, but not with maximum ground-glass opacities, on high-resolution CT [74]. Therefore, combined use of these two markers would be more effective for diagnosis and monitoring of ILD activity in SSc patients than single use of each marker. However, some SSc patients with active ILD showed discrepancies in the serum levels of these markers. Furthermore, KL-6 or SP-D does not necessarily reflect the activity of ILD in a part of patients. KL-6 and SP-D are currently utilized in clinical practice in Japan.

13.5.2 CCL18

Another promising biomarker of SSc-ILD is CCL18, which is also known as pulmonary and activation-regulated chemokine (PARC), is constitutively expressed at high levels in the lungs, and is selectively chemotactic for T cells [76]. CCL18 in high concentrations directly stimulates intracellular signaling and collagen production in primary pulmonary fibroblasts [77]. Serum CCL18 levels were markedly elevated in association with the development of ILD, as well as with reductions in vital capacity (VC) and diffusing capacity for carbon monoxide (DLCO), and correlated closely with the activity of ILD [78]. Furthermore, serum CCL18 levels have been demonstrated as predictive biomarker for the identification of patients with a higher risk of subsequent lung disease worsening in SSc [79]. A recent study reported that SP-D correlated with concomitantly obtained forced vital capacity, while CCL18 was a predictor of short-term decline in forced VC [80]. However, neither SP-D nor CCL18 was a long-term predictor of forced VC course in patients with early SSc in that study. Furthermore, CCL18 production by BAL cells and serum CCL18 concentrations reflected pulmonary fibrotic activity in SSc patients with ILD [81].

13.6 Biomarkers of Pulmonary Arterial Hypertension (Table 13.4)

13.6.1 Brain Natriuretic Peptide (BNP)

Pulmonary arterial hypertension has become one of the most important factors that affect morbidity and mortality. However, there are currently no validated laboratory examination or serologic markers that are useful for specific diagnosis of PAH. Therefore, biomarkers are necessary to find out early asymptomatic PAH. The DLCO decreases years prior to the diagnosis of PAH in patients with lcSSc [82]. Serum BNP and serum NT-proBNP have been reported as useful biomarkers

Table 13.4 Potential biomarkers of pulmonary arterial hypertension

Biomarker	Clinical association or usefulness	Sample
BNP/NT-proBNP	Severity, stability, and prognosis of PAH, predictive of survival	Serum/plasma
Endothelin-1	Pulmonary arterial pressure, advanced microangiopathy defined by capillaroscopy	Serum/plasma
MRC1	lcSSc with PAH, pulmonary arterial pressure, mortality	mRNA of PBMC
IL-13	lcSSc with PAH	Serum/plasma

BNP brain natriuretic peptide, *NT* N-terminal, *PAH* pulmonary arterial hypertension

for pulmonary hypertension since they tend to increase in SSc patients with early PAH and correlate with hemodynamic measures [83, 84]. BNP and NT-proBNP are secreted by ventricular myocytes reflecting myocardial responses to stretch and hypoxia and by certain neurohormonal stimuli. A previous study demonstrated that SSc patients with an NT-proBNP in excess of 395 pg/mL have a very high probability of having pulmonary hypertension, and baseline and serial changes of NT-proBNP levels are highly predictive of survival [85]. A prospective cohort study demonstrated that a decreased DLCO/alveolar volume ratio and an increased NT-proBNP are predictors of PAH in SSc [86]. Furthermore, only plasma levels of BNP and NT-proBNP have been included as important parameters for assessing disease severity, stability, and prognosis of PAH in the treatment guidelines of the Task Force for the Diagnosis and Treatment of PAH of the European Society of Cardiology and European Respiratory Society [87]. However, the serum or plasma levels of BNP and NT-proBNP are not specific for PAH.

13.6.2 Endothelin-1

Endothelin-1 is a 21-amino acid polypeptide produced by various cells including endothelial cells. It is a potent vasoconstrictor and can stimulate proliferation of smooth muscle cells. It is well known that endothelin-1 has critical roles in the proliferative vasculopathy of SSc, including PAH [88]. Also, endothelin-1 receptor blockers are highly effective for the treatment of PAH. Plasma levels of endothelin-1 have been reported as elevated in patients with SSc [89, 90]. In one study, plasma levels of endothelin-1 were elevated in SSc patients with PAH and SSc patients with anticentromere antibodies. There was a positive linear correlation between endothelin-1 levels and systolic pulmonary arterial pressure [91]. High endothelin-1 plasma levels were especially detected in SSc patients with advanced microangiopathy defined by capillaroscopy [92, 93]. Therefore, circulating endothelin-1 level may be a possible biomarker for evaluating SSc-related PAH.

13.6.3 Cytokines

Increased expression of nine genes (ICAM1, IFNGR1, IL1B, IL13Ra1, JAK2, AIF1, CCR1, ALAS2, TIMP2) was found in lcSSc patients with PAH by genome-wide gene expression using peripheral blood mononuclear cell (PBMC) samples [94]. Increased circulating cytokine levels of inflammatory mediators, such as TNF-α, IL-1β, ICAM-1, and IL-6, and markers of vascular injury such as VCAM-1, VEGF, and vWF were detected in lcSSc patients with PAH by multi-analyte profiling immunoassays [94].

In another study of the same group, the mRNA expression of CCR1 and JAK2 was elevated in lcSSc patients with PAH compared with controls on PBMC but mainly on $CD14^+$ cells [95]. Expression of MRC1, a marker of alternative activation of monocyte/macrophages, was also increased in lcSSc patients with PAH and correlated with pulmonary artery pressure and higher mortality. MRC1 expression was elevated in $CD14^+$ cells and was increased by IL-13 stimulation. Plasma levels of IL-13 were markedly elevated in lcSSc patients with PAH. These findings indicate that IL-13-activated monocyte/macrophages may have a critical role in the development of PAH in lcSSc, with MRC1 as an important biomarker.

13.7 Biomarkers of Disability and Disease Activity

The Disability Index of the Health Assessment Questionnaire (HAQ-DI) was originally created to examine and quantify functional capacity in patients with arthritis [96]. The HAQ-DI is determined by a self-administered questionnaire consisting of eight categories (score 0–3). Steen and Medsger have demonstrated that changes in the HAQ-DI correlate with objective physical and laboratory variations in SSc over time [97]. Other studies have also shown that high HAQ-DI is associated with increased morbidity and mortality in patients with SSc [98, 99]. The scleroderma HAQ (SHAQ) consists of the eight domains utilized in the HAQ-DI, plus the following visual analog scales: pain, patient global assessment, vascular, digital ulcers, lung involvement, and gastrointestinal involvement [97]. The Medsger Severity Index, developed through consensus methodology, defines severity in nine organ systems (the general, vascular, skin, joint/tendon, musculoskeletal, gastrointestinal, lung, heart, and kidney) [100].

In a series of Japanese multicenter prospective studies of early dcSSc, initial serum levels of CXCL8 and P-selectin were significantly associated with the HAQ-DI at the fourth year [67, 101] (Table 13.5). Recently, IFN-inducible chemokine score has been proposed as a promising biomarker of SSc [102]. The composite chemokine score of plasma levels of IFN-γ-inducible protein 10 (IP-10/CXCL10) and IFN-inducible T cell α chemoattractant (I-TAC/CXCL11) was elevated in SSc patients and showed a correlation with the IFN gene expression

Table 13.5 Potential biomarkers of disability and disease activity

Biomarker	Clinical association	Sample
CXCL8	Subsequent HAQ-DI	Serum/plasma
P-selectin	Subsequent HAQ-DI	Serum/plasma
IFN-inducible chemokine score	Medsger Severity Index (particularly with the lung, skin, muscle involvement)	Serum/plasma

signature in 266 patients with SSc. This IFN-inducible chemokine score correlated with the Medsger Severity Index, particularly with the severity of the lung, skin, and muscle involvement (Table 13.5).

13.8 Conclusion

Clinical and serological heterogeneity of SSc and effects of medications are making identification and validation of biomarkers challenging. Large, multicenter, prospective studies of well-defined clinical cohorts must be performed to identify and validate biomarkers useful for clinical practice of SSc.

References

1. Khanna D, Clements PJ, Furst DE, Chon Y, Elashoff R, Roth MD, et al. Correlation of the degree of dyspnea with health-related quality of life, functional abilities, and diffusing capacity for carbon monoxide in patients with systemic sclerosis and active alveolitis: results from the Scleroderma Lung Study. Arthritis Rheum. 2005;52(2):592–600.
2. Biomarkers Definitions Working G. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther. 2001;69(3):89–95.
3. Castro SV, Jimenez SA. Biomarkers in systemic sclerosis. Biomark Med. 2010;4(1):133–47.
4. Hummers LK. The current state of biomarkers in systemic sclerosis. Curr Rheumatol Rep. 2010;12(1):34–9.
5. Moinzadeh P, Denton CP, Abraham D, Ong V, Hunzelmann N, Eckes B, et al. Biomarkers for skin involvement and fibrotic activity in scleroderma. J Eur Acad Dermatol Venereol: JEADV. 2012;26(3):267–76.
6. Castelino FV, Varga J. Current status of systemic sclerosis biomarkers: applications for diagnosis, management and drug development. Expert Rev Clin Immunol. 2013;9 (11):1077–90.
7. Balanescu P, Ladaru A, Balanescu E, Baicus C, Dan GA. Systemic sclerosis biomarkers discovered using mass-spectrometry-based proteomics: a systematic review. Biomarkers. 2014;19(5):345–55.
8. Clements P, Lachenbrush P, Seibold J, White B, Weiner S, Martin R, et al. Inter and intraobserver variability of total skin thickness score (modified Rodnan TSS) in systemic sclerosis. J Rheumatol. 1995;22:1281–5.
9. Czirjak L, Nagy Z, Aringer M, Riemekasten G, Matucci-Cerinic M, Furst DE, et al. The EUSTAR model for teaching and implementing the modified Rodnan skin score in systemic sclerosis. Ann Rheum Dis. 2007;66(7):966–9.

10. Hunzelmann N, Risteli J, Risteli L, Sacher C, Vancheeswaran R, Black C, et al. Circulating type I collagen degradation products: a new serum marker for clinical severity in patients with scleroderma? Br J Dermatol. 1998;139(6):1020–5.
11. Allanore Y, Borderie D, Lemarechal H, Cherruau B, Ekindjian OG, Kahan A. Correlation of serum collagen I carboxyterminal telopeptide concentrations with cutaneous and pulmonary involvement in systemic sclerosis. J Rheumatol. 2003;30(1):68–73.
12. Dziadzio M, Smith RE, Abraham DJ, Stratton RJ, Gabrielli A, Black CM, et al. Serological assessment of type I collagen burden in scleroderma spectrum disorders: a systematic review. Clin Exp Rheumatol. 2004;22(3):356–67.
13. Black CM, McWhirter A, Harrison NK, Kirk JM, Laurent GJ. Serum type III procollagen peptide concentrations in systemic sclerosis and Raynaud's phenomenon: relationship to disease activity and duration. Br J Rheumatol. 1989;28(2):98–103.
14. Diot E, Diot P, Valat C, Boissinot E, Asquier E, Lemarie E, et al. Predictive value of serum III procollagen for diagnosis of pulmonary involvement in patients with scleroderma. Eur Respir J. 1995;8(9):1559–65.
15. Nagy Z, Czirjak L. Increased levels of amino terminal propeptide of type III procollagen are an unfavourable predictor of survival in systemic sclerosis. Clin Exp Rheumatol. 2005;23 (2):165–72.
16. Ra HJ, Parks WC. Control of matrix metalloproteinase catalytic activity. Matrix Biol: J Int Soc Matrix Biol. 2007;26(8):587–96.
17. Kim WU, Min SY, Cho ML, Hong KH, Shin YJ, Park SH, et al. Elevated matrix metalloproteinase-9 in patients with systemic sclerosis. Arthritis Res Ther. 2005;7(1):R71–9.
18. Pardo A, Selman M. Matrix metalloproteases in aberrant fibrotic tissue remodeling. Proc Am Thorac Soc. 2006;3(4):383–8.
19. Serrati S, Cinelli M, Margheri F, Guiducci S, Del Rosso A, Pucci M, et al. Systemic sclerosis fibroblasts inhibit in vitro angiogenesis by MMP-12-dependent cleavage of the endothelial cell urokinase receptor. J Pathol. 2006;210(2):240–8.
20. Manetti M, Guiducci S, Romano E, Bellando-Randone S, Conforti ML, Ibba-Manneschi L, et al. Increased serum levels and tissue expression of matrix metalloproteinase-12 in patients with systemic sclerosis: correlation with severity of skin and pulmonary fibrosis and vascular damage. Ann Rheum Dis. 2012;71(6):1064–72.
21. Varga J, Abraham D. Systemic sclerosis: a prototypic multisystem fibrotic disorder. J Clin Invest. 2007;117(3):557–67.
22. Moustakas A, Heldin CH. Non-Smad TGF-beta signals. J Cell Sci. 2005;118(Pt 16):3573–84.
23. Whitfield ML, Finlay DR, Murray JI, Troyanskaya OG, Chi JT, Pergamenschikov A, et al. Systemic and cell type-specific gene expression patterns in scleroderma skin. Proc Natl Acad Sci U S A. 2003;100(21):12319–24.
24. Sargent JL, Milano A, Bhattacharyya S, Varga J, Connolly MK, Chang HY, et al. A TGFbeta-responsive gene signature is associated with a subset of diffuse scleroderma with increased disease severity. J Invest Dermatol. 2010;130(3):694–705.
25. Denton CP, Merkel PA, Furst DE, Khanna D, Emery P, Hsu VM, et al. Recombinant human anti-transforming growth factor beta1 antibody therapy in systemic sclerosis: a multicenter, randomized, placebo-controlled phase I/II trial of CAT-192. Arthritis Rheum. 2007;56 (1):323–33.
26. Dziadzio M, Smith RE, Abraham DJ, Black CM, Denton CP. Circulating levels of active transforming growth factor beta1 are reduced in diffuse cutaneous systemic sclerosis and correlate inversely with the modified Rodnan skin score. Rheumatology (Oxford). 2005;44 (12):1518–24.
27. Krieg T, Takehara K. Skin disease: a cardinal feature of systemic sclerosis. Rheumatology (Oxford). 2009;48 Suppl 3:iii14–8.
28. Takehara K. Hypothesis: pathogenesis of systemic sclerosis. J Rheumatol. 2003;30(4):755–9.
29. Sato S, Nagaoka T, Hasegawa M, Tamatani T, Nakanishi T, Takigawa M, et al. Serum levels of connective tissue growth factor are elevated in patients with systemic sclerosis: association

with extent of skin sclerosis and severity of pulmonary fibrosis. J Rheumatol. 2000;27 (1):149–54.
30. Dziadzio M, Usinger W, Leask A, Abraham D, Black CM, Denton C, et al. N-terminal connective tissue growth factor is a marker of the fibrotic phenotype in scleroderma. QJM. 2005;98(7):485–92.
31. Khan K, Xu S, Nihtyanova S, Derrett-Smith E, Abraham D, Denton CP, et al. Clinical and pathological significance of interleukin 6 overexpression in systemic sclerosis. Ann Rheum Dis. 2012;71(7):1235–42.
32. Sato S, Hasegawa M, Takehara K. Serum levels of interleukin-6 and interleukin-10 correlate with total skin thickness score in patients with systemic sclerosis. J Dermatol Sci. 2001;27 (2):140–6.
33. De Lauretis A, Sestini P, Pantelidis P, Hoyles R, Hansell DM, Goh NS, et al. Serum interleukin 6 is predictive of early functional decline and mortality in interstitial lung disease associated with systemic sclerosis. J Rheumatol. 2013;40(4):435–46.
34. Gu L, Tseng S, Horner RM, Tam C, Loda M, Rollins BJ. Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. Nature. 2000;404(6776):407–11.
35. Gharaee-Kermani M, Denholm EM, Phan SH. Costimulation of fibroblast collagen and transforming growth factor beta1 gene expression by monocyte chemoattractant protein-1 via specific receptors. J Biol Chem. 1996;271(30):17779–84.
36. Carulli MT, Ong VH, Ponticos M, Shiwen X, Abraham DJ, Black CM, et al. Chemokine receptor CCR2 expression by systemic sclerosis fibroblasts: evidence for autocrine regulation of myofibroblast differentiation. Arthritis Rheum. 2005;52(12):3772–82.
37. Hasegawa M, Sato S, Takehara K. Augmented production of chemokines (MCP-1, MIP-1α, and MIP-1β) in patients with systemic sclerosis: MCP-1 and MIP-1α may be involved in the development of pulmonary fibrosis. Clin Exp Immunol. 1999;117:159–65.
38. Antonelli A, Ferri C, Fallahi P, Ferrari SM, Giuggioli D, Colaci M, et al. CXCL10 (alpha) and CCL2 (beta) chemokines in systemic sclerosis – a longitudinal study. Rheumatology (Oxford). 2008;47(1):45–9.
39. Luzina IG, Atamas SP, Wise R, Wigley FM, Xiao HQ, White B. Gene expression in bronchoalveolar lavage cells from scleroderma patients. Am J Respir Cell Mol Biol. 2002;26(5):549–57.
40. Schmidt K, Martinez-Gamboa L, Meier S, Witt C, Meisel C, Hanitsch LG, et al. Bronchoalveoloar lavage fluid cytokines and chemokines as markers and predictors for the outcome of interstitial lung disease in systemic sclerosis patients. Arthritis Res Ther. 2009;11(4):R111.
41. van Bon L, Affandi AJ, Broen J, Christmann RB, Marijnissen RJ, Stawski L, et al. Proteome-wide analysis and CXCL4 as a biomarker in systemic sclerosis. N Engl J Med. 2014;370 (5):433–43.
42. Kahaleh MB, Osborn I, LeRoy EC. Increased factor VIII/von Willebrand factor antigen and von Willebrand factor activity in scleroderma and in Raynaud's phenomenon. Ann Intern Med. 1981;94(4 pt 1):482–4.
43. Herrick AL, Illingworth K, Blann A, Hay CR, Hollis S, Jayson MI. Von Willebrand factor, thrombomodulin, thromboxane, beta-thromboglobulin and markers of fibrinolysis in primary Raynaud's phenomenon and systemic sclerosis. Ann Rheum Dis. 1996;55(2):122–7.
44. Scheja A, Akesson A, Geborek P, Wildt M, Wollheim CB, Wollheim FA, et al. Von Willebrand factor propeptide as a marker of disease activity in systemic sclerosis (scleroderma). Arthritis Res. 2001;3(3):178–82.
45. Kumanovics G, Minier T, Radics J, Palinkas L, Berki T, Czirjak L. Comprehensive investigation of novel serum markers of pulmonary fibrosis associated with systemic sclerosis and dermato/polymyositis. Clin Exp Rheumatol. 2008;26(3):414–20.
46. Hesselstrand R, Ekman R, Eskilsson J, Isaksson A, Scheja A, Ohlin AK, et al. Screening for pulmonary hypertension in systemic sclerosis: the longitudinal development of tricuspid

gradient in 227 consecutive patients, 1992–2001. Rheumatology (Oxford). 2005;44 (3):366–71.
47. Iannone F, Riccardi MT, Guiducci S, Bizzoca R, Cinelli M, Matucci-Cerinic M, et al. Bosentan regulates the expression of adhesion molecules on circulating T cells and serum soluble adhesion molecules in systemic sclerosis-associated pulmonary arterial hypertension. Ann Rheum Dis. 2008;67(8):1121–6.
48. Mannucci PM, Vanoli M, Forza I, Canciani MT, Scorza R. Von Willebrand factor cleaving protease (ADAMTS-13) in 123 patients with connective tissue diseases (systemic lupus erythematosus and systemic sclerosis). Haematologica. 2003;88(8):914–8.
49. Hummers LK, Hall A, Wigley FM, Simons M. Abnormalities in the regulators of angiogenesis in patients with scleroderma. J Rheumatol. 2009;36(3):576–82.
50. Distler O, Del Rosso A, Giacomelli R, Cipriani P, Conforti ML, Guiducci S, et al. Angiogenic and angiostatic factors in systemic sclerosis: increased levels of vascular endothelial growth factor are a feature of the earliest disease stages and are associated with the absence of fingertip ulcers. Arthritis Res. 2002;4(6):R11.
51. Choi JJ, Min DJ, Cho ML, Min SY, Kim SJ, Lee SS, et al. Elevated vascular endothelial growth factor in systemic sclerosis. J Rheumatol. 2003;30(7):1529–33.
52. Viac J, Schmitt D, Claudy A. Plasma vascular endothelial growth factor levels in scleroderma are not correlated with disease activity. Acta Derm Venereol. 2000;80(5):383.
53. Kuryliszyn-Moskal A, Klimiuk PA, Sierakowski S. Soluble adhesion molecules (sVCAM-1, sE-selectin), vascular endothelial growth factor (VEGF) and endothelin-1 in patients with systemic sclerosis: relationship to organ systemic involvement. Clin Rheumatol. 2005;24 (2):111–6.
54. Papaioannou AI, Zakynthinos E, Kostikas K, Kiropoulos T, Koutsokera A, Ziogas A, et al. Serum VEGF levels are related to the presence of pulmonary arterial hypertension in systemic sclerosis. BMC Pulm Med. 2009;9:18.
55. Distler O, Distler JH, Scheid A, Acker T, Hirth A, Rethage J, et al. Uncontrolled expression of vascular endothelial growth factor and its receptors leads to insufficient skin angiogenesis in patients with systemic sclerosis. Circ Res. 2004;95(1):109–16.
56. Wipff J, Avouac J, Borderie D, Zerkak D, Lemarechal H, Kahan A, et al. Disturbed angiogenesis in systemic sclerosis: high levels of soluble endoglin. Rheumatology (Oxford). 2008;47(7):972–5.
57. Fujimoto M, Hasegawa M, Hamaguchi Y, Komura K, Matsushita T, Yanaba K, et al. A clue for telangiectasis in systemic sclerosis: elevated serum soluble endoglin levels in patients with the limited cutaneous form of the disease. Dermatology. 2006;213(2):88–92.
58. Shovlin CL, Guttmacher AE, Buscarini E, Faughnan ME, Hyland RH, Westermann CJ, et al. Diagnostic criteria for hereditary hemorrhagic telangiectasia (Rendu-Osler-Weber syndrome). Am J Med Genet. 2000;91(1):66–7.
59. Wipff J, Kahan A, Hachulla E, Sibilia J, Cabane J, Meyer O, et al. Association between an endoglin gene polymorphism and systemic sclerosis-related pulmonary arterial hypertension. Rheumatology (Oxford). 2007;46(4):622–5.
60. Roumm AD, Whiteside TL, Medsger Jr TA, Rodnan GP. Lymphocytes in the skin of patients with progressive systemic sclerosis. Quantification, subtyping, and clinical correlations. Arthritis Rheum. 1984;27(6):645–53.
61. Gruschwitz M, Sepp N, Kofler H, Wick G. Expression of class II-MHC antigens in the dermis of patients with progressive systemic sclerosis. Immunobiology. 1991;182(3–4):234–55.
62. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell. 1994;76(2):301–14.
63. Abraham D, Lupoli S, McWhirter A, Plater-Zyberk C, Piela TH, Korn JH, et al. Expression and function of surface antigens on scleroderma fibroblasts. Arthritis Rheum. 1991;34 (9):1164–72.

64. Sfikakis PP, Tesar J, Baraf H, Lipnick R, Klipple G, Tsokos GC. Circulating intercellular adhesion molecule-1 in patients with systemic sclerosis. Clin Immunol Immunopathol. 1993;68(1):88–92.
65. Gruschwitz MS, Hornstein OP, von den Driesch P. Correlation of soluble adhesion molecules in the peripheral blood of scleroderma patients with their in situ expression and with disease activity. Arthritis Rheum. 1995;38(2):184–9.
66. Ihn H, Sato S, Fujimoto M, Kikuchi K, Kadono T, Tamaki K, et al. Circulating intercellular adhesion molecule-1 in the sera of patients with systemic sclerosis: enhancement by inflammatory cytokines. Br J Rheumatol. 1997;36(12):1270–5.
67. Hasegawa M, Asano Y, Endo H, Fujimoto M, Goto D, Ihn H, et al. Serum adhesion molecule levels as prognostic markers in patients with early systemic sclerosis: a multicentre, prospective, observational study. PLoS One. 2014;9(2):e88150.
68. Stratton RJ, Coghlan JG, Pearson JD, Burns A, Sweny P, Abraham DJ, et al. Different patterns of endothelial cell activation in renal and pulmonary vascular disease in scleroderma. Q J Med. 1998;91:561–6.
69. Mittag M, Beckheinrich P, Haustein UF. Systemic sclerosis-related Raynaud's phenomenon: effects of iloprost infusion therapy on serum cytokine, growth factor and soluble adhesion molecule levels. Acta Derm Venereol. 2001;81(4):294–7.
70. Kohno N, Kyoizumi S, Awaya Y, Fukuhara H, Yamakido M, Akiyama M. New serum indicator of interstitial pneumonitis activity: sialylated carbohydrate antigen KL-6. Chest. 1989;96(1):68–73.
71. Hermans C, Bernard A. Lung epithelium-specific proteins: characteristics and potential applications as markers. Am J Respir Crit Care Med. 1999;159(2):646–78.
72. Kobayashi J, Kitamura S. KL-6: a serum marker for interstitial pneumonia. Chest. 1995;108 (2):311–5.
73. Asano Y, Ihn H, Yamane K, Yazawa N, Kubo M, Fujimoto M, et al. Clinical significance of surfactant protein D as a serum marker for evaluating pulmonary fibrosis in patients with systemic sclerosis. Arthritis Rheum. 2001;44(6):1363–9.
74. Hant FN, Ludwicka-Bradley A, Wang HJ, Li N, Elashoff R, Tashkin DP, et al. Surfactant protein D and KL-6 as serum biomarkers of interstitial lung disease in patients with scleroderma. J Rheumatol. 2009;36(4):773–80.
75. Yanaba K, Hasegawa M, Takehara K, Sato S. Comparative study of serum surfactant protein-D and KL-6 concentrations in patients with systemic sclerosis as markers for monitoring the activity of pulmonary fibrosis. J Rheumatol. 2004;31(6):1112–20.
76. Hieshima K, Imai T, Baba M, Shoudai K, Ishizuka K, Nakagawa T, et al. A novel human CC chemokine PARC that is most homologous to macrophage-inflammatory protein-1 alpha/LD78 alpha and chemotactic for T lymphocytes, but not for monocytes. J Immunol. 1997;159 (3):1140–9.
77. Atamas SP, Luzina IG, Choi J, Tsymbalyuk N, Carbonetti NH, Singh IS, et al. Pulmonary and activation-regulated chemokine stimulates collagen production in lung fibroblasts. Am J Respir Cell Mol Biol. 2003;29(6):743–9.
78. Kodera M, Hasegawa M, Komura K, Yanaba K, Takehara K, Sato S. Serum pulmonary and activation-regulated chemokine/CCL18 levels in patients with systemic sclerosis: a sensitive indicator of active pulmonary fibrosis. Arthritis Rheum. 2005;52(9):2889–96.
79. Tiev KP, Hua-Huy T, Kettaneh A, Gain M, Duong-Quy S, Toledano C, et al. Serum CC chemokine ligand-18 predicts lung disease worsening in systemic sclerosis. Eur Respir J. 2011;38(6):1355–60.
80. Elhaj M, Charles J, Pedroza C, Liu X, Zhou X, Estrada YMRM, et al. Can serum surfactant protein D or CC-chemokine ligand 18 predict outcome of interstitial lung disease in patients with early systemic sclerosis? J Rheumatol. 2013;40(7):1114–20.
81. Prasse A, Pechkovsky DV, Toews GB, Schafer M, Eggeling S, Ludwig C, et al. CCL18 as an indicator of pulmonary fibrotic activity in idiopathic interstitial pneumonias and systemic sclerosis. Arthritis Rheum. 2007;56(5):1685–93.

82. Steen V, Medsger Jr TA. Predictors of isolated pulmonary hypertension in patients with systemic sclerosis and limited cutaneous involvement. Arthritis Rheum. 2003;48(2):516–22.
83. Cavagna L, Caporali R, Klersy C, Ghio S, Albertini R, Scelsi L, et al. Comparison of brain natriuretic peptide (BNP) and NT-proBNP in screening for pulmonary arterial hypertension in patients with systemic sclerosis. J Rheumatol. 2010;37(10):2064–70.
84. Allanore Y, Borderie D, Meune C, Cabanes L, Weber S, Ekindjian OG, et al. N-terminal pro-brain natriuretic peptide as a diagnostic marker of early pulmonary artery hypertension in patients with systemic sclerosis and effects of calcium-channel blockers. Arthritis Rheum. 2003;48(12):3503–8.
85. Williams MH, Handler CE, Akram R, Smith CJ, Das C, Smee J, et al. Role of N-terminal brain natriuretic peptide (N-TproBNP) in scleroderma-associated pulmonary arterial hypertension. Eur Heart J. 2006;27(12):1485–94.
86. Allanore Y, Borderie D, Avouac J, Zerkak D, Meune C, Hachulla E, et al. High N-terminal pro-brain natriuretic peptide levels and low diffusing capacity for carbon monoxide as independent predictors of the occurrence of precapillary pulmonary arterial hypertension in patients with systemic sclerosis. Arthritis Rheum. 2008;58(1):284–91.
87. Galie N, Hoeper MM, Humbert M, Torbicki A, Vachiery JL, Barbera JA, et al. Guidelines for the diagnosis and treatment of pulmonary hypertension: the Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS), endorsed by the International Society of Heart and Lung Transplantation (ISHLT). Eur Heart J. 2009;30(20):2493–537.
88. Braun-Moscovici Y, Nahir AM, Balbir-Gurman A. Endothelin and pulmonary arterial hypertension. Semin Arthritis Rheum. 2004;34(1):442–53.
89. Yamane K, Kashiwagi H, Suzuki N, Miyauchi T, Yanagisawa M, Goto K, et al. Elevated plasma levels of endothelin-1 in systemic sclerosis. Arthritis Rheum. 1991;34(2):243–4.
90. Morelli S, Ferri C, Di Francesco L, Baldoncini R, Carlesimo M, Bottoni U, et al. Plasma endothelin-1 levels in patients with systemic sclerosis: influence of pulmonary or systemic arterial hypertension. Ann Rheum Dis. 1995;54(9):730–4.
91. Schmidt J, Launay D, Soudan B, Hachulla E, de Groote P, Lambert M, et al. Assessment of plasma endothelin level measurement in systemic sclerosis. Rev Med Interne. 2007;28 (6):371–6.
92. Sulli A, Soldano S, Pizzorni C, Montagna P, Secchi ME, Villaggio B, et al. Raynaud's phenomenon and plasma endothelin: correlations with capillaroscopic patterns in systemic sclerosis. J Rheumatol. 2009;36(6):1235–9.
93. Kim HS, Park MK, Kim HY, Park SH. Capillary dimension measured by computer-based digitalized image correlated with plasma endothelin-1 levels in patients with systemic sclerosis. Clin Rheumatol. 2010;29(3):247–54.
94. Pendergrass SA, Hayes E, Farina G, Lemaire R, Farber HW, Whitfield ML, et al. Limited systemic sclerosis patients with pulmonary arterial hypertension show biomarkers of inflammation and vascular injury. PLoS One. 2010;5(8):e12106.
95. Christmann RB, Hayes E, Pendergrass S, Padilla C, Farina G, Affandi AJ, et al. Interferon and alternative activation of monocyte/macrophages in systemic sclerosis-associated pulmonary arterial hypertension. Arthritis Rheum. 2011;63(6):1718–28.
96. Fries JF, Spitz P, Kraines RG, Holman HR. Measurement of patient outcome in arthritis. Arthritis Rheum. 1980;23(2):137–45.
97. Steen VD, Medsger Jr TA. The value of the Health Assessment Questionnaire and special patient-generated scales to demonstrate change in systemic sclerosis patients over time. Arthritis Rheum. 1997;40(11):1984–91.
98. Clements PJ, Roth MD, Elashoff R, Tashkin DP, Goldin J, Silver RM, et al. Scleroderma lung study (SLS): differences in the presentation and course of patients with limited versus diffuse systemic sclerosis. Ann Rheum Dis. 2007;66(12):1641–7.
99. Valentini G, Matucci Cerinic M. Disease-specific quality indicators, guidelines and outcome measures in scleroderma. Clin Exp Rheumatol. 2007;25(6 Suppl 47):159–62.

100. Medsger Jr TA, Bombardieri S, Czirjak L, Scorza R, Della Rossa A, Bencivelli W. Assessment of disease severity and prognosis. Clin Exp Rheumatol. 2003;21(3 Suppl 29):S42–6.
101. Hasegawa M, Asano Y, Endo H, Fujimoto M, Goto D, Ihn H, et al. Serum chemokine levels as prognostic markers in patients with early systemic sclerosis: a multicenter, prospective, observational study. Mod Rheumatol. 2013;23(6):1076–84.
102. Liu X, Mayes MD, Tan FK, Wu M, Reveille JD, Harper BE, et al. Correlation of interferon-inducible chemokine plasma levels with disease severity in systemic sclerosis. Arthritis Rheum. 2013;65(1):226–35.

Autoantibodies in Systemic Sclerosis

14

Yasuhito Hamaguchi

Abstract

Systemic sclerosis (SSc) is a multisystem connective tissue disorder characterized by microvascular damage and excessive fibrosis of the skin and internal organs. SSc is believed to be an autoimmune disease because of the presence of antinuclear antibodies (ANAs). ANAs are detected in more than 90 % of patients with SSc. Although the role of ANAs in the pathogenesis in SSc has not been clarified, each ANA is closely associated with specific clinical features. Therefore, it is clinically useful to classify the patients based on the type of ANAs for diagnosis and management of such patients. Anti-centromere antibodies (Abs), anti-DNA topoisomerase I Abs, and anti-RNA polymerase III Abs are representative ANAs found in patients with SSc. Other serum ANAs detected in SSc include those targeted against Th/To, U3 RNP, human upstream-binding protein, U11/U12 RNP, U1 RNP, Ku, PM-Scl, and RuvBL1/2. The prevalence of each of the ANAs and the clinical characteristics are reportedly dependent on ethnicity. Identifying several ANAs requires immunoprecipitation assays. Establishment of an easy and simple screening system for ANA specificities such as an enzyme-linked immunosorbent assay is expected.

Keywords

Systemic sclerosis • Antinuclear antibodies • Autoantibodies • Clinical feature

Y. Hamaguchi (✉)
Department of Dermatology, Faculty of Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8641, Japan
e-mail: yasuhito@med.kanazawa-u.ac.jp

K. Takehara et al. (eds.), *Systemic Sclerosis*, DOI 10.1007/978-4-431-55708-1_14

14.1 Introduction

Systemic sclerosis (SSc) is a multisystem connective tissue disorder characterized by microvascular damage and excessive fibrosis of the skin and various internal organs. It is well recognized that the clinical features of SSc are largely heterogeneous. Skin involvement is classified into two subgroups dependent on the extent of the area involved. Limited cutaneous SSc (lcSSc) includes those patients in whom skin thickening is relatively restricted to the fingers and hands with less serious internal organ involvement. In diffuse cutaneous SSc (dcSSc), skin lesions are extensive and often rapidly developing with earlier and more serious complications that include interstitial lung disease (ILD) and scleroderma renal crisis (SRC) [1].

The presence of autoantibodies (auto-Abs) is one of the hallmarks of autoimmune connective tissue diseases. SSc is considered to be an autoimmune disease because antinuclear Abs (ANAs) are detected in more than 90 % of SSc patients. These ANAs react against a variety of intracellular components [2]. There are several features regarding SSc-related ANAs. First, production of ANA is specific for each patient and multiple Abs generally do not coexist [3]. Second, once an ANA with a particular specificity develops, the type of ANA and its titer usually does not change throughout the course of disease, and other SSc-related ANAs do not arise [4]. Although the role of ANAs in the pathogenesis of SSc remains unclear, the particular type of ANA is often indicative of clinical features, severity, and prognosis. Therefore, the identification of SSc-related ANAs in each patient is clinically useful to diagnose and evaluate organ involvements and prognosis.

Anti-centromere Abs (ACA), anti-topoisomerase I (anti-topo I) Abs, formerly termed anti-Scl-70 Abs, and anti-RNA polymerase III (anti-RNAP III) Abs are representative ANAs found in SSc. In addition to these ANAs, anti-nucleolar antibodies are also found in SSc patients, though less frequently. These include anti-Th/To Abs, anti-U3 RNP Abs, and antihuman upstream-binding factor (anti-hUBF, formerly anti-NOR 90) Abs. They produce nucleolar staining patterns by indirect immunofluorescence staining (IIF) on human epithelioma type 2 (HEp-2) cells. Anti-U1 RNP Abs, anti-Ku Abs, and anti-PM-Scl Abs are detected in a clinically distinctive group of patients with SSc-myositis overlap syndrome. Recently, another SSc-myositis overlap syndrome-related Ab, anti-RuvBL1/2, was reported [5].

Previous reports have revealed that the patient's genetic background can affect the prevalence and clinical phenotype of SSc, even when the same ANA specificities are present. For example, anti-PM-Scl Abs are found in approximately 4 % of Caucasian/African American patients, but not in Japanese SSc patients [6–8]. Pulmonary arterial hypertension (PAH) is often seen and is a cause of death in ACA-positive Caucasian/African American SSc patients, but this complication is relatively rare in Japanese SSc patients [6–8]. Therefore, physicians should take genetic differences into account when considering the association of ANAs with clinical features and prognosis in patients with SSc. Below, we will focus on

SSc-related Abs and their relevance to clinical presentation, disease course, and prognosis.

14.2 Detection of Antibody

Screening for ANAs is usually conducted by IIF using HEp-2 cells. This technique should be the first ANA screening assay [9]. Representative staining patterns are shown in Fig. 14.1. Except for ACA, it is difficult to identify the ANA specificities by IIF because epitopes cannot be well defined using this technique. Therefore, additional techniques including enzyme-linked immunosorbent assay (ELISA),

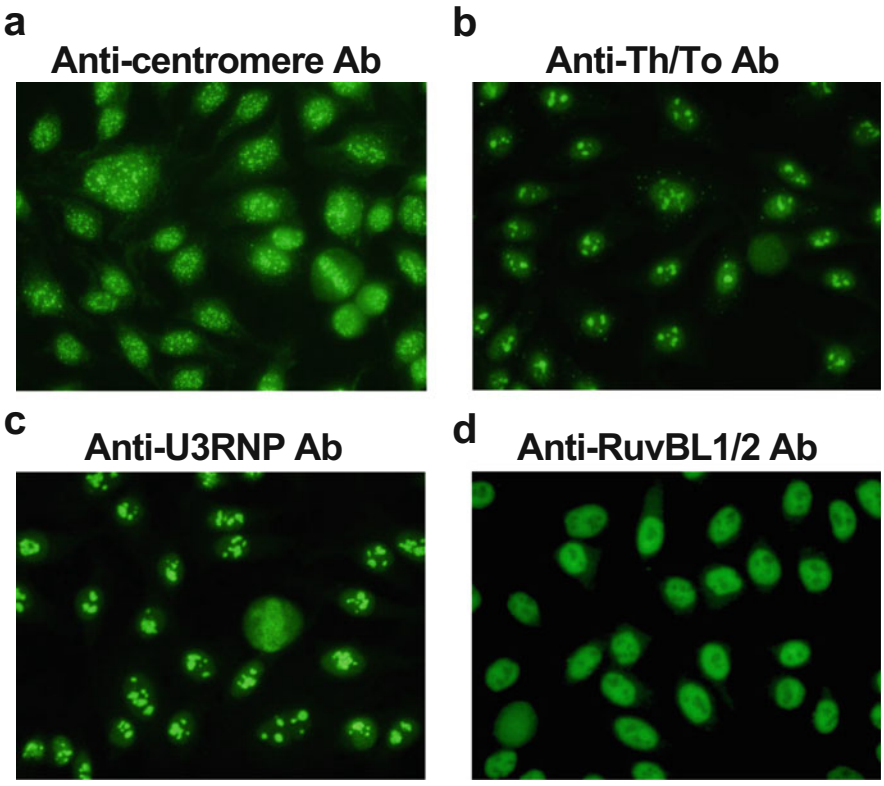

Fig. 14.1 Indirect immunofluorescence patterns observed with HEp-2 cells stained with anti-centromere antibody (*Ab*) (**a**), anti-Th/To Ab (**b**), anti-U3 RNP Ab (**c**), and anti-RuvBL1/2 Ab (**d**) sera (original magnification ×400). Anti-centromere Abs produce a homogeneously distributed and speckled pattern in the nucleus. Anti-Th/To Abs demonstrate a dotty nucleolar staining of interphase cells and mitotic chromosomes are not stained. Anti-U3 RNP Abs show clumpy nucleolar staining of interphase cells and the mitotic chromosomes are stained. Anti-RuvBL1/2 Abs show a speckled nuclear pattern of interphase cells and the mitotic chromosomes are stained

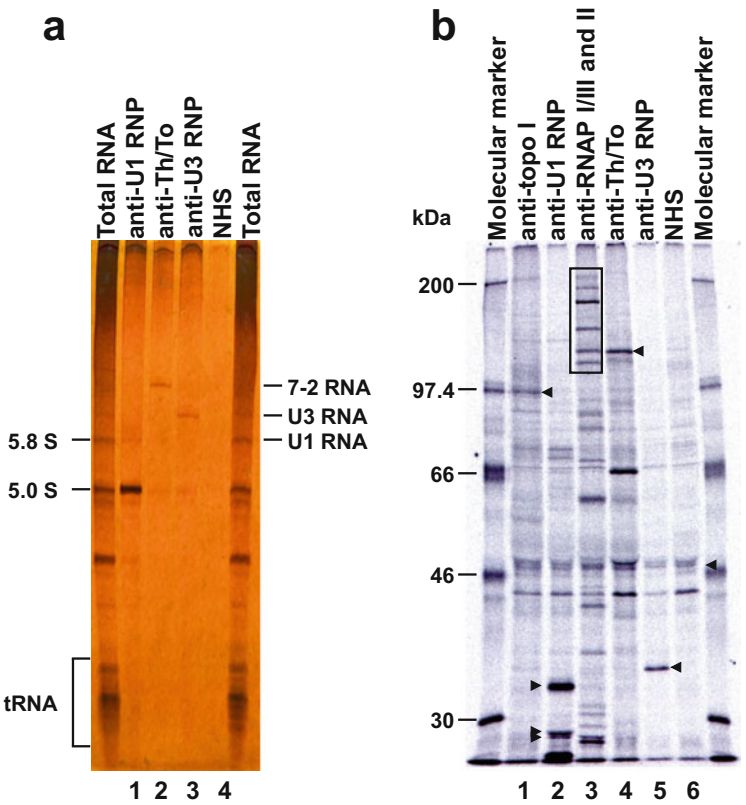

Fig. 14.2 Representative immunoprecipitation assay of autoantibodies related to systemic sclerosis (*SSc*). (**a**) Immunoprecipitation of U1RNP, Th/To, and U3RNP by sera. K562 cell extracts were immunoprecipitated with sera, and RNA was extracted, electrophoresed on 8 % urea-polyacrylamide gel, and visualized by silver staining. Total RNA, with the 5.8 and 5.0 S small ribosomal RNAs and the tRNA region indicated; Lanes 1–3, SSc patients' sera indicated, with antibodies to U1 RNP, Th/To, and U3 RNP; Lane 4, normal human serum (*NHS*) indicated. (**b**) Immunoprecipitation of 35S-methionine-labeled K562 cell extracts was performed on SSc patients' sera and NHS, separated on 10 % SDS-PAGE and analyzed by autoradiography. Topoisomerase I (topo I, lane 1), U1 RNP (lane 2), RNA polymerase I/III and II (RNAP I/III and II, lane 3), Th/To (lane 4), and U3 RNP (lane 5) proteins are shown by *arrowheads* or indicated region. Molecular weight markers include protein bands corresponding to 200, 97.4, 66, 46, and 30 kDa (Reused with permission from Ref. [10])

Western blotting, immunodiffusion, and immunoprecipitation (Fig. 14.2) [10] are required to confirm ANA specificities in patients' sera (Table 14.1). There remains a need for standardization of ANA determination, although evidence-based guidelines for the use of specific immunoassays in SSc have been proposed [11].

Table 14.1 Autoantibodies in systemic sclerosis

Antibody	Major autoantigen	IIF	Assay
Anti-centromere	CENP-A, B, and C	DC	IIF, ELISA, WB
Anti-topoisomerase I	DNA topoisomerase I	Ho and N	DID, ELISA, IP, WB
Anti-RNA polymerase	RNA polymerase I, II, and III	Sp and/or N	ELISA, IP
Anti-Th/To	H1/8-2 and Th/7-2 RNA	N	IP
Anti-U3 RNP	Fibrillarin and other U3RNP components	N	IP
Anti-hUBF (NOR 90)	Human upstream-binding factor	N	IP
Anti-U11/U12 RNP	U11/12 RNP complex	Sp	IP, WB
Anti-U1 RNP	70 kDa, A and C polypeptides of U1 snRNP	Sp	DID, ELISA, IP, WB
Anti-PM-Scl	PM-Scl-75 and 100 proteins of the human exosome	N	DID, IP
Anti-Ku	80 and 70 kDa DNA-binding dimeric protein	Sp	DID, IP
Anti-RuvBL1/2	a complex of RuvBL1 and RuvBL2 proteins	Sp	IP, WB

IIF indirect immunofluorescence staining pattern on HEp-2 cells, *DC* discrete speckled, *ELISA* enzyme-linked immunosorbent assay, *WB* Western blotting, *Ho* homogenous, *N* nucleolar, *DID* double immunodiffusion, *IP* immunoprecipitation, *Sp* speckled

14.3 Antinuclear Antibodies in SSc

14.3.1 Anti-Centromere Antibodies (ACA)

ACA were first reported by Moroi et al. in 1980 [12]. ACA can be identified by IIF, since they uniquely produce punctate spots dispersed in the interphase nucleus, localized to the constriction on metaphase chromosomes (Fig. 14.1a). At least six centromeric polypeptides, CENP-A to F, have been defined. CENP-B, an 80 kDa haploid DNA-binding protein, is the major autoantigen that reacts to virtually all ACA-positive sera [13, 14] (Table 14.1). An ELISA system using a cloned fusion protein of the CENP-B antigen is available with adequate sensitivity and specificity [15, 16]. ACA are rarely found in healthy individuals or in patients other than those with autoimmune connective tissue diseases. ACA are also detected in patients with primary biliary cirrhosis [17, 18] or those with systemic lupus erythematosus (SLE) [19].

The overall frequency of ACA in patients with SSc has been reported to be 20–30 %, but it varies among different ethnicities. Approximately 30 % of Caucasian SSc patients are positive for ACA, whereas the frequency is lower in African American and Thai patients with SSc [20, 21]. Regarding the genetic factors related to ANA specificities, ACA are associated with HLA molecules DR1, DR4, DR8,

Table 14.2 Predominant clinical features associated with major systemic sclerosis-related antinuclear antibodies

Antibody	Disease subset	Organ involvement
Anti-centromere	Limited	Digital ulcers and gangrene
		Isolated pulmonary arterial hypertension
Anti-topoisomerase I	Diffuse	Digital ulcers and gangrene
		Pulmonary fibrosis
		Severe heart disease
		Renal crisis
Anti-RNA polymerase	Diffuse	Renal crisis
Anti-Th/To	Limited	Isolated pulmonary arterial hypertension
		Pulmonary fibrosis
Anti-U3 RNP	Diffuse	Isolated pulmonary arterial hypertension
		Pulmonary fibrosis
		Severe heart disease
		Myositis
Anti-hUBF (NOR 90)	Limited	Skin ulcer
Anti-U11/U12 RNP	Limited	Pulmonary fibrosis
		Gastrointestinal involvement
Anti-U1 RNP	Limited	Isolated pulmonary arterial hypertension
		Arthritis/arthralgia
		SSc-myositis overlap
Anti-PM-Scl	Limited	SSc-myositis overlap
Anti-Ku	Limited	SSc-myositis overlap
Anti-RuvBL1/2	Diffuse	SSc-myositis overlap

DR11, and DQ7 (DQB1*0301) [22, 23]. When ACA are found in patients with Raynaud's phenomenon without skin thickening, this is predictive for the future development of lcSSc [24].

ACA in SSc patients is associated with limited skin involvement, peripheral vascular damage, and calcinosis [25] (Table 14.2). Although almost all ACA-positive patients suffer from Raynaud's phenomenon, the degree of ischemia varies among different ethnicities. While frequencies of pitting scars or ulcers were 42–61 % in Caucasian and/or African American patients [6, 26, 27], these complications occurred in only 11–17 % of Japanese patients with ACA [7, 8]. Digital gangrene is also observed more frequently in Caucasian and/or African American patients. Although esophageal dysfunction is commonly seen in ACA-positive patients, serious internal organ involvement, such as ILD, is generally rare. However, the presence of ACA can be predictive for development of PAH at a late stage [9, 28]. ACA titers measured by ELISA are stable over time in individual patients, and titers are not associated with disease activity [29, 30]. ACA-positive SSc patients had a more favorable prognosis than patients with other SSc-related ANAs [28]. Two large cohort studies have also confirmed that SSc patients with ACA had a lower mortality than those with anti-topo I or anti-nucleolar Abs [27, 31].

14.3.2 Anti-topoisomerase I Antibodies (Anti-topo I, Formerly Anti-Scl-70)

In 1979, auto-Abs against a 70–100 kDa chromatin-associated protein were first described in patients with SSc [32]. This protein was identified as DNA topoisomerase I [33] (Table 14.1). Overall, anti-topo I Abs were found in about 40 % of patients with SSc, but ethnic differences considerably affect the prevalence, which ranges from 28 to 70 % [7, 11]. For instance, the prevalence of anti-topo I Abs is low in Caucasians but higher in Japanese and Thai patients. Anti-topo I Abs are associated with HLA-DRB1, DQB1, and DPB1 [21, 23, 34, 35]. Anti-topo I Abs are rarely found in healthy individuals or in patients with other autoimmune connective tissue diseases [11]. Coexistence with ACA only occurs in about 0.5 % of SSc patients [36]. Anti-topo I Abs are detected in about 40 % of patients with dcSSc, but less than 10 % of patients with lcSSc [11, 37]. However, another study reported that one-third of anti-topo I Ab-positive patients had lcSSc [38].

It is well recognized that anti-topo I Abs are strongly associated with a higher risk for severe ILD, resulting in increasing mortality in this subgroup (Table 14.2). More than a few studies reported that granulocytosis on bronchoalveolar lavage (BAL) was associated with greater risk of deterioration and the prognostic value in SSc patients with ILD [39–41]. However, a subsequent study found that neutrophilia on BAL was linked to early mortality, but BAL findings were not linked to long-term survival or rapid progression of ILD [42]. Routine serial monitoring and rapid decline of the vital capacity by pulmonary function test provided a unique opportunity to evaluate longer-term outcome. Wells et al. have reported that the percent predicted diffusing capacity of the lungs for carbon monoxide reflected the extent of ILD and, therefore, routine measurement was recommended [43]. Anti-topo I Abs are now considered to be a marker for poor prognosis, since effective therapy for the treatment of ILD has not been sufficiently established. Other than ILD, anti-topo I Abs may be a predictor for the development of SRC. In 46 Italian SSc patients with SRC, anti-topo I Abs were detected in 30 patients (65 %), whereas anti-RNAP III Abs were positive in seven patients (15 %) [44]. Survival rates of SSc patients with SRC were significantly lower in the anti-topo I Ab-positive group than in the anti-RNAP III Ab-positive group. Regarding the association of anti-topo I Abs with malignancy, some reports described a higher risk for malignancy in patients with anti-topo I Abs [45, 46], yet this finding has not always been confirmed [47, 48].

Serial measurements of auto-Ab titers are not generally considered to be useful for monitoring disease severity. However, several studies have found that anti-topo I Ab levels determined by ELISA were related to disease severity and that seronegative conversion resulted in disease remission [49–51]. Therefore, routine measurement of anti-topo I Ab titers by ELISA may be useful for monitoring disease activity.

Although the pathophysiological role of ANAs in the development of SSc remains unclear, in vitro studies showed direct pathogenic effects of anti-topo I Abs in SSc. Henault et al. have reported that the autoantigen topo I was bound

specifically to fibroblasts, where it was recognized by anti-topo I Abs from SSc patients [52]. These topo I/anti-topo I complexes induced the adhesion and activation of monocytes that resulted in the initiation and maintenance of an inflammatory cascade, stimulating the fibrosis seen in SSc patients [53, 54].

14.3.3 Anti-RNA Polymerase I, II, and III (RNAPs) Abs

RNAPs I, II, and III are major targets of auto-Ab responses in SSc patients [4, 55, 56]. Most SSc sera that react with RNAPs recognize more than one class of RNAP, with a pattern of RNAP I/III or RNAP I/II/III. Abs to RNAP I/III routinely coexist and this pattern is highly specific for SSc [4, 55, 56] (Table 14.1). Some anti-RNAP I/III Ab-positive patients also have anti-RNAP II Abs, i.e., anti-RNAP I/II/III Abs. Anti-RNAP II Abs alone are detected at a low frequency in sera from SSc patients, often in a combination with anti-topo I Abs, and also in sera from some patients with SLE or an overlap syndrome [57, 58]. Immunoprecipitation assays were essential in the identification of anti-RNAP III Abs, but an ELISA system using recombinant RNAP III as the antigen is currently available and widely used in the clinical field [59, 60]. The HLA associations of anti-RNAP III in SSc patients has not been established [61].

The prevalence of anti-RNAP III Abs is influenced by patient ethnicity [62]. In a US cohort, 61 of 247 (25 %) patients with SSc were positive for anti-RNAP III Abs, whereas only 5 of 127 (4 %) were positive in a French cohort [63]. An Italian cohort confirmed this low prevalence of anti-RNAP III Abs: only 16 of 466 (3 %) had anti-RNAP III Abs [64]. In contrast, 69 of 451 (15.3 %) SSc patients had anti-RNAP III Abs in Australian patients with SSc [65]. In a Japanese population, anti-RNAP III Abs were found in 6 % (37 of 583 SSc patients) to 10.7 % (38 of 354 SSc patients) [60, 66].

Anti-RNAP III Abs are strongly associated with diffuse cutaneous involvement and SRC independent of ethnicity [6, 11, 55, 66] (Table 14.2). SRC occurred in 25 % of patients with anti-RNAP Abs in contrast to 12 % in other patients with dcSSc [6]. On the other hand, ILD that requires aggressive therapy rarely occurred in this subgroup. A recent study elucidated clinical and immunological predictors of SRC. Thus, anti-RNAP I/II/III Ab positivity and an ELISA index for anti-RNAP III Abs greater than 157 were independent factors associated with the development of SRC [66]. Several studies have revealed a strong association of anti-RNAP III Abs with malignancy. Shah et al. reported a close temporal relationship between the onset of cancer and SSc in patients with anti-RNAP I/III Abs [67]. They showed that the median duration of SSc at cancer diagnosis differed significantly between SSc-related Ab-based subgroups (−1.2 years in the anti-RNAP I/III Ab group, +13.4 years in the anti-topo I Ab group, +11.1 years in the ACA group, and +2.3 years in the group that was negative for all antigens tested). In addition, RNAP III Abs demonstrated a robust nucleolar staining pattern in 4 of 5 available tumors from patients with anti-RNAP I/III Abs, whereas no tumors from patients without anti-RNAP I/III displayed nucleolar RNAP III Ab staining [68]. Joseph

et al. reported that foreign antigens were encoded by somatically mutated genes in SSc patients with cancer [69]. They reported that genetic alterations of the *POLR3A* locus that encodes RPC1 were found in anti-RNAP III Ab-positive SSc patients with Abs to RPC1 but not in those without Abs to RPC1, and *POLR3A* mutations triggered cellular immunity and cross-reactive humoral immune responses in anti-RNAP III Ab-positive patients with cancers.

Despite a higher frequency of SRC, the survival rate in patients with anti-RNAP III Abs is better than in those with anti-topo I or anti-U3RNP Abs [6]. Previously, it had been reported that patients with anti-RNAP III Abs had the worst survival rate among all SSc subgroups [6]. However, mortality rates in patients with anti-RNAP III Abs have dramatically improved. This increased survival rate is explained by the fact that patients with anti-RNAP III Abs have a low risk of suffering ILD and SRC is now more readily treated with angiotensin-converting enzyme (ACE) inhibitors [70].

14.3.4 Anti-Th/To Abs (Known as Anti-7-2RNA Abs)

Okano et al. first reported Abs to Th/To (anti-Th/To) in 1990 [71]. Anti-Th/To Abs were originally specific for SSc or Raynaud's disease, but were subsequently detected in patients with localized scleroderma [72]. Anti-Th/To produce a nucleolar, dotty staining pattern in IIF (Fig. 14.1b) and their autoantigens are ribonucleoproteins (RNPs) associated with H1/8-2 and Th/7-2 RNAs (Table 14.1). H1/8-2 is a component of RNase P and TH/7-2 is that of RNase MRP and both are RNA-processing enzymes [73, 74]. Anti-Th/To Abs are associated with HLA DRB1*11, *1502, or *0802 [75, 76].

Anti-Th/To Abs are found in 2–5 % of patients with SSc. Anti-Th/To Abs are associated with lcSSc, but overall prognosis is worse since patients with anti-Th/To Abs have a higher risk for severe organ involvement, including ILD and PAH [77, 78] (Table 14.2). ACA and anti-Th/To Abs are representative auto-Abs that are associated with lcSSc, but the major difference between these two SSc-related ANAs is that anti-Th/To Ab-positive patients can suffer from both ILD and PAH [6]. In a comparison study between ACA and anti-Th/To Abs by Mitri et al., patients with anti-Th/To Abs were younger and had a shorter disease duration at their first evaluation than those with ACA. Although both subgroups had a higher frequency of PAH (28 % of anti-Th/To Abs and 19 % of ACA), patients with anti-Th/To Abs more often had ILD and had poorer survival rates than those with ACA [26]. However, severe internal organ involvement is not commonly seen in Japanese patients with anti-Th/To Abs [7, 8].

14.3.5 Anti-U3 RNP Abs

Anti-U3 RNP Abs were initially found in sera from SSc patients in 1985 [79]. The major autoantigen of anti-U3 RNP Abs is fibrillarin, which is a 34 kDa basic protein

and a component of the nucleolar U3-ribonucleoprotein complex (Table 14.1). Anti-U3 RNP Abs show a nucleolar, clumpy staining pattern in IIF (Fig. 14.1c) [80]. Anti-U3 RNP Abs were initially associated with HLA DQB1*0604 [81] or DRB1*0804 [82], but this finding was not confirmed in another study [61].

Anti-U3 RNP Abs are found in 4–10 % of all patients with SSc [80, 83, 84], but the frequency is 18.5 % in African American SSc patients [82]. Clinical characteristics are potentially influenced by ethnicity (Table 14.2). Caucasian, African American, and Japanese patients with anti-U3 RNP Abs have some common clinical phenotypes of a diffuse cutaneous form and peripheral vasculopathy, such as pitting ulcers and digital gangrene. On the other hand, internal organ involvement that includes ILD, PAH, and SRC is more frequent in Caucasians/African Americans, and the mortality of these patients is higher than in other SSc-related ANA-based subgroups [6–8, 85]. Physicians should be aware that severe gastrointestinal tract involvement can occur in patients with anti-U3 RNP Abs. It has been reported that 5 patients were positive for anti-U3 RNP Abs among 14 Japanese patients with severe gastrointestinal tract involvement that included malabsorption syndrome and/or pseudo-obstruction within 2 years of SSc disease onset [86].

14.3.6 Antihuman Upstream-Binding Factor (Anti-hUBF, Formerly Anti-NOR 90)

Auto-Abs reactive with nucleolus-organizing region (NOR) 90 (anti-NOR 90 Abs) were first described in 1987 [87]. Subsequent analysis found that the autoantigen corresponding to anti-NOR 90 specificity is the human upstream-binding factor (hUBF) [88] (Table 14.1). Anti-hUBF Abs are not specific to SSc and are found in other autoimmune connective tissue diseases such as Raynaud's disease, rheumatoid arthritis, SLE, and also in some malignancies [89–91]. Anti-hUBF Abs are linked to the HLA-DR1 allele [92]. The clinical characteristics of anti-hUBF Abs have not been clarified, since large cohort studies are lacking. Accumulated case reports suggest that anti-hUBF Abs are probably related to limited cutaneous form, mild organ involvements, and a favorable prognosis [90] (Table 14.2).

14.3.7 Anti-U11/U12 RNP Abs

U11/U12 RNP are low-abundance, small nuclear ribonucleoproteins (snRNPs) that form a two-snRNP complex [93] (Table 14.1). Fertig et al. reported clinical characteristics of 33 SSc patients with anti-U11/U12 RNP Abs [94]. Anti-U11/U12 RNP Abs are present in the sera of approximately 3 % of patients with SSc. Almost half of the patients (17 of 33, 52 %) had the limited form (Table 14.2). All patients had Raynaud's phenomenon, and gastrointestinal tract involvement was significantly increased in the anti-U11/U12 RNP-positive group. Although none had intrinsic PAH, 79 % of patients had ILD. The degree of ILD occurring in

patients with anti-U11/U12 RNP Abs is severe. Anti-U11/U12 RNP Ab-positive SSc patients with ILD had a 2.25-fold greater risk of death than anti-U11/U12 RNP Ab-negative patients with ILD.

14.4 Antinuclear Abs in SSc-Myositis Overlap

14.4.1 Anti-U1 RNP Abs

Anti-U1 RNP Abs bind to RNP, a ribonuclease-sensitive antigen involved in splicing heterogeneous nuclear RNA into mRNA (Table 14.1). Anti-U1 RNP Abs are widely detected in patients with various autoimmune connective tissue diseases, particularly those with mixed connective tissue disease (MCTD) [95]. Other than MCTD, anti-U1 RNP Abs are found in SSc patients with a prevalence of approximately 6 % (range 2–14 %) [6, 7, 11, 83]. Limited cutaneous form, puffy hands, Raynaud's phenomenon, arthritis, and esophageal dysfunction are characteristic clinical features in this subgroup. SRC rarely develops, but PAH can occur and cause an increased mortality (Table 14.2) [6]. Anti-U1 RNP Abs are generally a predictive factor for a good response to corticosteroids, resulting in a better prognosis if the patients do not develop PAH [6, 96].

14.4.2 Anti-RM-Scl Abs

Anti-PM-Scl Abs were initially found in 1977 in patients with polymyositis (PM)/SSc overlap syndrome [97]. The PM-Scl antigen consists of 11–16 polypeptides, and 75 and 100 kDa polypeptides have been identified as the major antigen [98] (Table 14.1). Anti-PM-Scl Abs are strongly associated with HLA DQA1*0501 and HLA DRB1*0301 [23]. Anti-PM-Scl Abs are found in 4–11 % of SSc patients overall, but the frequency is greatly affected by ethnicity. Large studies analyzing Japanese SSc patients determined that none of the SSc patients was positive for anti-PM-Scl Abs [7, 8]. Anti-PM-Scl Abs are detected in approximately 25 % of SSc patients with myositis overlap but in only 2 % of patients with SSc alone [11, 77, 99] (Table 14.2). Anti-PM-Scl Abs predict for limited cutaneous involvement with less serious internal organ involvements and a favorable response to low or moderate doses of corticosteroids, resulting in a favorable prognosis [100].

14.4.3 Anti-Ku Abs

Mimori et al. first reported a case of PM/SSc overlap syndrome with anti-Ku Abs in 1994 [101]. Anti-Ku Abs were originally thought to be specific to SSc, but accumulating reports revealed that anti-Ku are also found in patients with other autoimmune connective tissue diseases, including SLE and overlap syndrome [102, 103]. Franceschini et al. reported that in 14 anti-Ku Ab-positive patients,

one-half had an overlap syndrome (5 PM/SSc, 1 PM/SLE/SSc, and 1 PM/SLE). Of those patients with overlap syndrome, 85 % had clinical characteristics seen in SSc [103] (Table 14.2). Furthermore, another large cohort study confirmed that anti-Ku Abs were related to SSc with myositis [104].

14.4.4 Anti-RuvBL1/2 Abs

RuvBL1 and RuvBL2 are highly conserved eukaryotic proteins that form a double hexamer in the nucleus (Table 14.1). The RuvBL1/2 complex is involved in many cellular processes, such as transcription, DNA repair, and chromatin remodeling [105]. The clinical characteristics of 37 patients with anti-RuvBL1/2 Abs were reported in 2014 [5]. Anti-RuvBL1/2 Abs produce a speckled nuclear staining pattern by IIF (Fig. 14.1d). Anti-RuvBL1/2 Abs were present in 10 of 588 (1.7 %) SSc patients in a Japanese cohort and 27 of 585 (4.6 %) SSc patients in a Pittsburg cohort. Anti-RuvBL1/2 Abs were exclusively detected in SSc patients and highly associated with SSc in overlap with myositis in both the Japanese and Pittsburgh cohorts. Patients with anti-RuvBL1/2 Abs had higher frequencies of dcSSc. Compared with other Abs related to SSc-myositis overlap (anti-PM-Scl Abs and anti-Ku Abs), the presence of anti-RuvBL1/2 Abs was distinctive in terms of its associations with older age at SSc onset, male gender, and a high frequency of diffuse cutaneous form (Table 14.2).

14.5 Conclusion

Since clinical characteristics and prognoses in SSc are largely heterogeneous, it is clinically helpful for physicians to group SSc patients based on their auto-Ab profiles. Several SSc-related ANAs are identified only by immunoprecipitation assays, but those assays are complex and, therefore, are conducted in few facilities. Establishment of an easy and simple screening system for ANA specificities such as ELISA is needed.

Acknowledgments We thank Ms. Masako Matsubara, Yuko Yamada, and Tomoko Hayashi for technical assistance.

References

1. LeRoy EC, Black C, Fleischmajer R, et al. Scleroderma (systemic sclerosis): classification, subsets, and pathogenesis. J Rheumatol. 1988;15:202–5.
2. Tan EM. Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. Adv Immunol. 1989;44:93–151.
3. Fanning GC, Welsh KI, Bunn C, et al. HLA associations in three mutually exclusive autoantibody subgroups in UK systemic sclerosis patients. Br J Rheumatol. 1998;37:201–7.

4. Okano Y. Antinuclear antibody in systemic sclerosis (scleroderma). Rheum Dis Clin North Am. 1996;22:709–35.
5. Kaji K, Fertig N, Medsger Jr TA, et al. Autoantibodies to RuvBL1 and RuvBL2: a novel systemic sclerosis-related antibody associated with diffuse cutaneous and skeletal muscle involvement. Arthritis Care Res. 2014;66:575–84.
6. Steen VD. Autoantibodies in systemic sclerosis. Semin Arthritis Rheum. 2005;35:35–42.
7. Kuwana M, Kaburaki J, Okano Y, et al. Clinical and prognostic associations based on serum antinuclear antibodies in Japanese patients with systemic sclerosis. Arthritis Rheum. 1994;37:75–83.
8. Hamaguchi Y, Hasegawa M, Fujimoto M, et al. The clinical relevance of serum antinuclear antibodies in Japanese patients with systemic sclerosis. Br J Dermatol. 2008;158:487–95.
9. Ho KT, Reveille JD. The clinical relevance of autoantibodies in scleroderma. Arthritis Res Ther. 2003;5:80–93.
10. Hamaguchi Y. Autoantibody profiles in systemic sclerosis: predictive value for clinical evaluation and prognosis. J Dermatol. 2010;37:42–53.
11. Reveille JD, Solomon DH. Evidence-based guidelines for the use of immunologic tests: anticentromere, Scl-70, and nucleolar antibodies. Arthritis Rheum. 2003;49:399–412.
12. Moroi Y, Peebles C, Fritzler MJ, et al. Autoantibody to centromere (kinetochore) in scleroderma sera. Proc Natl Acad Sci U S A. 1980;77:1627–31.
13. Earnshaw WC, Machlin PS, Bordwell BJ, et al. Analysis of anticentromere autoantibodies using cloned autoantigen CENP-B. Proc Natl Acad Sci U S A. 1987;84:4979–83.
14. Masumoto H, Masukata H, Muro Y, et al. A human centromere antigen (CENP-B) interacts with a short specific sequence in alphoid DNA, a human centromeric satellite. J Cell Biol. 1989;109:1963–73.
15. Rothfield N, Whitaker D, Bordwell B, et al. Detection of anticentromere antibodies using cloned autoantigen CENP-B. Arthritis Rheum. 1987;30:1416–9.
16. Tan EM, Smolen JS, McDougal JS, et al. A critical evaluation of enzyme immunoassays for detection of antinuclear autoantibodies of defined specificities. I. Precision, sensitivity, and specificity. Arthritis Rheum. 1999;42:455–64.
17. Makinen D, Fritzler M, Davis P, et al. Anticentromere antibodies in primary biliary cirrhosis. Arthritis Rheum. 1983;26:914–7.
18. Lee SL, Tsay GJ, Tsai RT. Anticentromere antibodies in subjects with no apparent connective tissue disease. Ann Rheum Dis. 1993;52:586–9.
19. Caramaschi P, Biasi D, Manzo T, et al. Anticentromere antibody – clinical associations. A study of 44 patients. Rheumatol Int. 1995;14:253–5.
20. McNeilage LJ, Youngchaiyud U, Whittingham S. Racial differences in antinuclear antibody patterns and clinical manifestations of scleroderma. Arthritis Rheum. 1989;32:54–60.
21. Reveille JD, Fischbach M, McNearney T, et al. Systemic sclerosis in 3 US ethnic groups: a comparison of clinical, sociodemographic, serologic, and immunogenetic determinants. Semin Arthritis Rheum. 2001;30:332–46.
22. McHugh NJ, Whyte J, Artlett C, et al. Anti-centromere antibodies (ACA) in systemic sclerosis patients and their relatives: a serological and HLA study. Clin Exp Immunol. 1994;96:267–74.
23. Genth E, Mierau R, Genetzky P, et al. Immunogenetic associations of scleroderma-related antinuclear antibodies. Arthritis Rheum. 1990;33:657–65.
24. Kallenberg CG, Wouda AA, Hoet MH, et al. Development of connective tissue disease in patients presenting with Raynaud's phenomenon: a six year follow up with emphasis on the predictive value of antinuclear antibodies as detected by immunoblotting. Ann Rheum Dis. 1988;47:634–41.
25. Tan EM, Rodnan GP, Garcia I, et al. Diversity of antinuclear antibodies in progressive systemic sclerosis. Anti-centromere antibody and its relationship to CREST syndrome. Arthritis Rheum. 1980;23:617–25.

26. Mitri GM, Lucas M, Fertig N, et al. A comparison between anti-Th/To- and anticentromere antibody-positive systemic sclerosis patients with limited cutaneous involvement. Arthritis Rheum. 2003;48:203–9.
27. Ferri C, Valentini G, Cozzi F, et al. Systemic sclerosis: demographic, clinical, and serologic features and survival in 1,012 Italian patients. Medicine (Baltimore). 2002;81:139–53.
28. Hesselstrand R, Scheja A, Shen GQ, et al. The association of antinuclear antibodies with organ involvement and survival in systemic sclerosis. Rheumatology (Oxford). 2003;42:534–40.
29. Tramposch HD, Smith CD, Senecal JL, et al. A long-term longitudinal study of anticentromere antibodies. Arthritis Rheum. 1984;27:121–4.
30. Vazquez-Abad D, Russell CA, Cusick SM, et al. Longitudinal study of anticentromere and antitopoisomerase-I isotypes. Clin Immunol Immunopathol. 1995;74:257–70.
31. Scussel-Lonzetti L, Joyal F, Raynauld JP, et al. Predicting mortality in systemic sclerosis: analysis of a cohort of 309 French Canadian patients with emphasis on features at diagnosis as predictive factors for survival. Medicine (Baltimore). 2002;81:154–67.
32. Douvas AS, Achten M, Tan EM. Identification of a nuclear protein (Scl-70) as a unique target of human antinuclear antibodies in scleroderma. J Biol Chem. 1979;254:10514–22.
33. Shero JH, Bordwell B, Rothfield NF, et al. High titers of autoantibodies to topoisomerase I (Scl-70) in sera from scleroderma patients. Science. 1986;231:737–40.
34. Reveille JD, Durban E, MacLeod-St Clair MJ, et al. Association of amino acid sequences in the HLA-DQB1 first domain with antitopoisomerase I autoantibody response in scleroderma (progressive systemic sclerosis). J Clin Invest. 1992;90:973–80.
35. Kuwana M, Kaburaki J, Okano Y, et al. The HLA-DR and DQ genes control the autoimmune response to DNA topoisomerase I in systemic sclerosis (scleroderma). J Clin Invest. 1993;92:1296–301.
36. Dick T, Mierau R, Bartz-Bazzanella P, et al. Coexistence of antitopoisomerase I and anticentromere antibodies in patients with systemic sclerosis. Ann Rheum Dis. 2002;61:121–7.
37. Spencer-Green G, Alter D, Welch HG. Test performance in systemic sclerosis: anti-centromere and anti-Scl-70 antibodies. Am J Med. 1997;103:242–8.
38. Walker UA, Tyndall A, Czirjak L, et al. Clinical risk assessment of organ manifestations in systemic sclerosis: a report from the EULAR scleroderma trials and research group database. Ann Rheum Dis. 2007;66:754–63.
39. Silver RM, Miller KS, Kinsella MB, et al. Evaluation and management of scleroderma lung disease using bronchoalveolar lavage. Am J Med. 1990;88:470–6.
40. Behr J, Vogelmeier C, Beinert T, et al. Bronchoalveolar lavage for evaluation and management of scleroderma disease of the lung. Am J Respir Crit Care Med. 1996;154:400–6.
41. Witt C, Borges AC, John M, et al. Pulmonary involvement in diffuse cutaneous systemic sclerosis: broncheoalveolar fluid granulocytosis predicts progression of fibrosing alveolitis. Ann Rheum Dis. 1999;58:635–40.
42. Goh NS, Veeraraghavan S, Desai SR, et al. Bronchoalveolar lavage cellular profiles in patients with systemic sclerosis-associated interstitial lung disease are not predictive of disease progression. Arthritis Rheum. 2007;56:2005–12.
43. Wells AU, Hansell DM, Rubens MB, et al. Fibrosing alveolitis in systemic sclerosis: indices of lung function in relation to extent of disease on computed tomography. Arthritis Rheum. 1997;40:1229–36.
44. Codullo V, Cavazzana I, Bonino C, et al. Serologic profile and mortality rates of scleroderma renal crisis in Italy. J Rheumatol. 2009;36:1464–9.
45. Weiner ES, Earnshaw WC, Senecal JL, et al. Clinical associations of anticentromere antibodies and antibodies to topoisomerase I. A study of 355 patients. Arthritis Rheum. 1988;31:378–85.
46. Rothfield N, Kurtzman S, Vazques-Abad D, et al. Association of anti-topoisomerase I with cancer. Arthritis Rheum. 1992;35:724.

47. Derk CT, Sakkas LI, Rasheed M, et al. Autoantibodies in patients with systemic sclerosis and cancer: a case-control study. J Rheumatol. 2003;30:1994–6.
48. Higuchi M, Horiuchi T, Ishibashi N, et al. Anticentromere antibody as a risk factor for cancer in patients with systemic sclerosis. Clin Rheumatol. 2000;19:123–6.
49. Henry PA, Atamas SP, Yurovsky VV, et al. Diversity and plasticity of the anti-DNA topoisomerase I autoantibody response in scleroderma. Arthritis Rheum. 2000;43:2733–42.
50. Kuwana M, Kaburaki J, Mimori T, et al. Longitudinal analysis of autoantibody response to topoisomerase I in systemic sclerosis. Arthritis Rheum. 2000;43:1074–84.
51. Sato S, Hamaguchi Y, Hasegawa M, et al. Clinical significance of anti-topoisomerase I antibody levels determined by ELISA in systemic sclerosis. Rheumatology (Oxford). 2001;40:1135–40.
52. Henault J, Robitaille G, Senecal JL, et al. DNA topoisomerase I binding to fibroblasts induces monocyte adhesion and activation in the presence of anti-topoisomerase I autoantibodies from systemic sclerosis patients. Arthritis Rheum. 2006;54:963–73.
53. Arcand J, Robitaille G, Koenig M, et al. The autoantigen DNA topoisomerase I interacts with chemokine receptor 7 and exerts cytokine-like effects on dermal fibroblasts. Arthritis Rheum. 2012;64:826–34.
54. Arcand J, Robitaille G, Koenig M, et al. Heparin inhibits the interaction of DNA topoisomerase I/anti-topoisomerase I immune complexes with heparan sulfate on dermal fibroblasts. Arthritis Rheum. 2012;64:1632–41.
55. Kuwana M, Kaburaki J, Mimori T, et al. Autoantibody reactive with three classes of RNA polymerases in sera from patients with systemic sclerosis. J Clin Invest. 1993;91:1399–404.
56. Okano Y, Steen VD, Medsger Jr TA. Autoantibody reactive with RNA polymerase III in systemic sclerosis. Ann Intern Med. 1993;119:1005–13.
57. Satoh M, Ajmani AK, Ogasawara T, et al. Autoantibodies to RNA polymerase II are common in systemic lupus erythematosus and overlap syndrome. Specific recognition of the phosphorylated (IIO) form by a subset of human sera. J Clin Invest. 1994;94:1981–9.
58. Harvey GR, Rands AL, McHugh NJ. Anti-RNA polymerase antibodies in systemic sclerosis (SSc): association with anti-topoisomerase I antibodies and identification of autoreactive subunits of RNA polymerase II. Clin Exp Immunol. 1996;105:468–74.
59. Kuwana M, Okano Y, Pandey JP, et al. Enzyme-linked immunosorbent assay for detection of anti-RNA polymerase III antibody: analytical accuracy and clinical associations in systemic sclerosis. Arthritis Rheum. 2005;52:2425–32.
60. Satoh T, Ishikawa O, Ihn H, et al. Clinical usefulness of anti-RNA polymerase III antibody measurement by enzyme-linked immunosorbent assay. Rheumatology (Oxford). 2009;48:1570–4.
61. Falkner D, Wilson J, Fertig N, et al. Studies of HLA-DR and DQ alleles in systemic sclerosis patients with autoantibodies to RNA polymerases and U3-RNP (fibrillarin). J Rheumatol. 2000;27:1196–202.
62. Sobanski V, Dauchet L, Lefevre G, et al. Prevalence of anti-RNA polymerase III antibodies in systemic sclerosis: New data from a French cohort and a systematic review and meta-analysis. Arthritis Rheumatol. 2014;66:407–17.
63. Meyer OC, Fertig N, Lucas M, et al. Disease subsets, antinuclear antibody profile, and clinical features in 127 French and 247 US adult patients with systemic sclerosis. J Rheumatol. 2007;34:104–9.
64. Airo P, Ceribelli A, Cavazzana I, et al. Malignancies in Italian patients with systemic sclerosis positive for anti-RNA polymerase III antibodies. J Rheumatol. 2011;38:1329–34.
65. Nikpour M, Hissaria P, Byron J, et al. Prevalence, correlates and clinical usefulness of antibodies to RNA polymerase III in systemic sclerosis: a cross-sectional analysis of data from an Australian cohort. Arthritis Res Ther. 2011;13:R211.
66. Hamaguchi Y, Kodera M, Matsushita T, et al. Clinical and immunologic predictors of scleroderma renal crisis in Japanese systemic sclerosis patients with anti-RNA polymerase III autoantibodies. Arthritis Rheumatol. 2015;67:1045–52.

67. Shah AA, Rosen A, Hummers L, et al. Close temporal relationship between onset of cancer and scleroderma in patients with RNA polymerase I/III antibodies. Arthritis Rheum. 2010;62:2787–95.
68. Shah AA, Hummers LK, Casciola-Rosen L, et al. Examination of autoantibody status and clinical features associated with cancer risk and cancer-associated scleroderma. Arthritis Rheumatol. 2015;67:1053–61.
69. Joseph CG, Darrah E, Shah AA, et al. Association of the autoimmune disease scleroderma with an immunologic response to cancer. Science. 2014;343:152–7.
70. Steen VD, Medsger Jr TA. Long-term outcomes of scleroderma renal crisis. Ann Intern Med. 2000;133:600–3.
71. Okano Y, Medsger Jr TA. Autoantibody to Th ribonucleoprotein (nucleolar 7-2 RNA protein particle) in patients with systemic sclerosis. Arthritis Rheum. 1990;33:1822–8.
72. Yamane K, Ihn H, Kubo M, et al. Antibodies to Th/To ribonucleoprotein in patients with localized scleroderma. Rheumatology (Oxford). 2001;40:683–6.
73. Hashimoto C, Steitz JA. Sequential association of nucleolar 7-2 RNA with two different autoantigens. J Biol Chem. 1983;258:1379–82.
74. Reddy R, Tan EM, Henning D, et al. Detection of a nucleolar 7-2 ribonucleoprotein and a cytoplasmic 8-2 ribonucleoprotein with autoantibodies from patients with scleroderma. J Biol Chem. 1983;258:1383–6.
75. Falkner D, Wilson J, Medsger Jr TA, et al. HLA and clinical associations in systemic sclerosis patients with anti-Th/To antibodies. Arthritis Rheum. 1998;41:74–80.
76. Kuwana M, Kimura K, Hirakata M, et al. Differences in autoantibody response to Th/To between systemic sclerosis and other autoimmune diseases. Ann Rheum Dis. 2002;61:842–6.
77. Cepeda EJ, Reveille JD. Autoantibodies in systemic sclerosis and fibrosing syndromes: clinical indications and relevance. Curr Opin Rheumatol. 2004;16:723–32.
78. Van Eenennaam H, Vogelzangs JH, Bisschops L, et al. Autoantibodies against small nucleolar ribonucleoprotein complexes and their clinical associations. Clin Exp Immunol. 2002;130:532–40.
79. Lischwe MA, Ochs RL, Reddy R, et al. Purification and partial characterization of a nucleolar scleroderma antigen (Mr = 34,000; pI, 8.5) rich in NG, NG-dimethylarginine. J Biol Chem. 1985;260:14304–10.
80. Tormey VJ, Bunn CC, Denton CP, et al. Anti-fibrillarin antibodies in systemic sclerosis. Rheumatology (Oxford). 2001;40:1157–62.
81. Arnett FC, Reveille JD, Goldstein R, et al. Autoantibodies to fibrillarin in systemic sclerosis (scleroderma). An immunogenetic, serologic, and clinical analysis. Arthritis Rheum. 1996;39:1151–60.
82. Sharif R, Fritzler MJ, Mayes MD, et al. Anti-fibrillarin antibody in African American patients with systemic sclerosis: immunogenetics, clinical features, and survival analysis. J Rheumatol. 2011;38:1622–30.
83. Jacobsen S, Halberg P, Ullman S, et al. Clinical features and serum antinuclear antibodies in 230 Danish patients with systemic sclerosis. Br J Rheumatol. 1998;37:39–45.
84. Aggarwal R, Lucas M, Fertig N, et al. Anti-U3 RNP autoantibodies in systemic sclerosis. Arthritis Rheum. 2009;60:1112–8.
85. Gunduz OH, Fertig N, Lucas M, et al. Systemic sclerosis with renal crisis and pulmonary hypertension: a report of eleven cases. Arthritis Rheum. 2001;44:1663–6.
86. Nishimagi E, Tochimoto A, Kawaguchi Y, et al. Characteristics of patients with early systemic sclerosis and severe gastrointestinal tract involvement. J Rheumatol. 2007;34:2050–5.
87. Rodriguez-Sanchez JL, Gelpi C, Juarez C, et al. Anti-NOR 90. A new autoantibody in scleroderma that recognizes a 90-kDa component of the nucleolus-organizing region of chromatin. J Immunol. 1987;139:2579–84.

88. Chan EK, Imai H, Hamel JC, et al. Human autoantibody to RNA polymerase I transcription factor hUBF. Molecular identity of nucleolus organizer region autoantigen NOR-90 and ribosomal RNA transcription upstream binding factor. J Exp Med. 1991;174:1239–44.
89. Imai H, Ochs RL, Kiyosawa K, et al. Nucleolar antigens and autoantibodies in hepatocellular carcinoma and other malignancies. Am J Pathol. 1992;140:859–70.
90. Dagher JH, Scheer U, Voit R, et al. Autoantibodies to NOR 90/hUBF: longterm clinical and serological followup in a patient with limited systemic sclerosis suggests an antigen driven immune response. J Rheumatol. 2002;29:1543–7.
91. Fujii T, Mimori T, Akizuki M. Detection of autoantibodies to nucleolar transcription factor NOR 90/hUBF in sera of patients with rheumatic diseases, by recombinant autoantigen-based assays. Arthritis Rheum. 1996;39:1313–8.
92. Dick T, Mierau R, Sternfeld R, et al. Clinical relevance and HLA association of autoantibodies against the nucleolus organizer region (NOR-90). J Rheumatol. 1995;22:67–72.
93. Wassarman KM, Steitz JA. The low-abundance U11 and U12 small nuclear ribonucleoproteins (snRNPs) interact to form a two-snRNP complex. Mol Cell Biol. 1992;12:1276–85.
94. Fertig N, Domsic RT, Rodriguez-Reyna T, et al. Anti-U11/U12 RNP antibodies in systemic sclerosis: a new serologic marker associated with pulmonary fibrosis. Arthritis Rheum. 2009;61:958–65.
95. Burdt MA, Hoffman RW, Deutscher SL, et al. Long-term outcome in mixed connective tissue disease: longitudinal clinical and serologic findings. Arthritis Rheum. 1999;42:899–909.
96. Lundberg I, Hedfors E. Clinical course of patients with anti-RNP antibodies. A prospective study of 32 patients. J Rheumatol. 1991;18:1511–9.
97. Ricken D. Progressive scleroderma and dermatomyositis. Verh Dtsch Ges Inn Med. 1977;83:751–6.
98. Brouwer R, Vree Egberts WT, Hengstman GJ, et al. Autoantibodies directed to novel components of the PM/Scl complex, the human exosome. Arthritis Res. 2002;4:134–8.
99. Reichlin M, Maddison PJ, Targoff I, et al. Antibodies to a nuclear/nucleolar antigen in patients with polymyositis overlap syndromes. J Clin Immunol. 1984;4:40–4.
100. Marguerie C, Bunn CC, Copier J, et al. The clinical and immunogenetic features of patients with autoantibodies to the nucleolar antigen PM-Scl. Medicine (Baltimore). 1992;71:327–36.
101. Mimori T, Akizuki M, Yamagata H, et al. Characterization of a high molecular weight acidic nuclear protein recognized by autoantibodies in sera from patients with polymyositis-scleroderma overlap. J Clin Invest. 1981;68:611–20.
102. Takeda Y, Dynan WS. Autoantibodies against DNA double-strand break repair proteins. Front Biosci. 2001;6:D1412–22.
103. Franceschini F, Cavazzana I, Generali D, et al. Anti-Ku antibodies in connective tissue diseases: clinical and serological evaluation of 14 patients. J Rheumatol. 2002;29:1393–7.
104. Rozman B, Cucnik S, Sodin-Semrl S, et al. Prevalence and clinical associations of anti-Ku antibodies in patients with systemic sclerosis: a European EUSTAR-initiated multi-centre case-control study. Ann Rheum Dis. 2008;67:1282–6.
105. Jha S, Dutta A. RVB1/RVB2: running rings around molecular biology. Mol Cell. 2009;34:521–33.