Scleroderma is a fibrotic condition characterized by immunological abnormalities, vascular injury and increased accumulation of extracellular matrix proteins in the skin. Although the etiology of scleroderma has not been fully elucidated, a growing body of evidence suggests that the overproduction of extracellular matrix proteins by activated fibroblasts results from an imbalance between synthesis and degradation of connective tissues. A number of mediators, cytokines, chemokines and growth factors secreted by inflammatory cells and mesenchymal cells (fibroblasts and myofibroblasts) play an important role in the fibrotic process of scleroderma. In this article, we describe recent advances concerning immunological aspects in the pathogenesis of bleomycin-induced murine scleroderma, laying stress on the involvement of interleukin-13 (IL-13) and plasminogen activator inhibitor-1 (PAI-1).

Key words: scleroderma, cytokine, IL-13, PAI-1, bleomycin-induced murine scleroderma

1. Introduction

Systemic sclerosis (SSc) is a connective tissue disease which shows fibrosis of the skin and various internal organs. Although the pathogenesis of SSc is not yet fully elucidated, it is characterized by i) excessive accumulation of extracellular matrix (ECM) proteins in the skin and various internal organs, ii) vascular injury and iii) immunologic abnormalities. 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 for initiating and/or leading to the sequential events of fibrosis in this disease.

Animal models are useful to provide clues and therapeutic interventions for various human diseases. Although animal models which exhibit all the aspects of SSc are not currently available, several experimental animal models, such as tight skin (Tsk) mouse, Tsk2 mouse, UCD 200 chicken, bleomycin-induced murine scleroderma, sclerodermatous graft-versus-host disease (Scl-GvHD) mouse, injections of transforming growth factor-β (TGF-β)/connective tissue growth factor (CTGF)-induced murine fibrosis model, and kinase-deficient type II TGF-β receptor transgenic mouse have been mainly examined so far. In this review, we discuss the role of cytokines and proteases in the pathogenesis of bleomycin-induced murine scleroderma, and focus on the role of interleukin-13 (IL-13) and plasminogen activator inhibitor-1 (PAI-1).

2. Bleomycin-induced scleroderma model

Bleomycin is a frequently used antitumor antibiotic for various kinds of cancers, and lung fibrosis is a well-known side effect of bleomycin. Mountz et al.
reported that rats injected repeatedly with sublethal doses of bleomycin over a 58-week period developed severe dermal fibrosis similar to that found in human scleroderma, with structural abnormalities of collagen fibers. On the other hand, we established a murine scleroderma model within 4 weeks by local bleomycin treatment\textsuperscript{6-8}. Dermal sclerosis was induced by repeated subcutaneous injections of bleomycin into the back. Histopathological examination revealed definite dermal sclerosis characterized by thickened collagen bundles and deposition of homogenous material in the thickened dermis with cellular infiltrates which were mainly composed of T cells and macrophages. α-SMA-positive myofibroblasts were observed in the lesional skin, and gradually increased in tandem with the induction of dermal sclerosis\textsuperscript{6}. Lung fibrosis, with thickened alveolar walls and cellular infiltrates, was also induced at an earlier time. The induced sclerotic changes remained at least 6 weeks, when untreated. Dermal sclerosis was induced in various mice strains, although there is some variation among strains in the intensity and the period required to induce dermal sclerosis. C3H/He, DBA/2, B10.D2 and B10.A strains demonstrated intense dermal sclerosis, which are suggestive of bleomycin-“susceptibility”\textsuperscript{6}. Hydroxyproline content in the skin was significantly increased, and increased production as well as mRNA upregulation of type I collagen were demonstrated in the sclerotic skin\textsuperscript{7}. Of interest, autoantibody was detected in the serum after repeated bleomycin treatment\textsuperscript{6}. In this model, apoptosis is highly induced in keratinocytes and infiltrating cells, but not either in endothelial cells or fibroblasts.

3. Role of cytokines in bleomycin-induced murine scleroderma

3.1 Fibrogenic cytokine

\textit{TGF-β}

TGF-β plays a key role in the pathogenesis of fibrosis. TGF-β, which is found abundantly in platelets and released from activated macrophages or lymphocytes, is a strong chemottractant for fibroblasts\textsuperscript{9}. TGF-β increases the synthesis of ECM components such as collagen type I and type III or fibronectin by fibroblasts, modulates cell-matrix adhesion protein receptors, and regulates the production of proteins that can modify the ECM by proteolytic action\textsuperscript{9}. In addition, TGF-β is capable of stimulating its own synthesis by fibroblasts through autoinduction\textsuperscript{10}. Thus, maintenance of increased TGF-β production may lead to a progressive deposition of ECM, resulting in fibrosis. TGF-β mRNA is elevated in the lesional skin of SSc\textsuperscript{11,12} and also shown to co-localize with type I collagen in scleroderma skin lesions\textsuperscript{12}.

Signaling by TGF-β elicits potent profibrotic responses in fibroblasts. Upon the binding of TGF-β to the type II receptor, the type I receptor becomes activated and signaling 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 human scleroderma skin and cultured scleroderma fibroblasts, basal level and TGF-β-inducible expression of Smad7 are selectively decreased, while Smad3 expression is increased relative to normal fibroblasts\textsuperscript{13}. Scleroderma fibroblasts exhibit increased Smad7 levels\textsuperscript{14}, whereas other groups report opposite results\textsuperscript{13,15}.

Blocking the bioactivity of TGF-β can suppress cutaneous fibrosis in different animal models of scleroderma\textsuperscript{16-18}. Bleomycin exposure to rat lung fibroblast cultures results in increases in TGF-β mRNA synthesis, TGF-β mRNA steady-state levels and TGF-β protein production\textsuperscript{19}. Increased TGF-β mRNA transcription is followed by TGF-β mRNA and protein accumulation, which is followed by increased procollagen gene transcription\textsuperscript{19}. 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 procollagen promoter\textsuperscript{20}. 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\textsuperscript{6}. TGF-β1 and -β2 mRNA expression was also detected in the lesional skin. Additionally, we have recently observed increased expression and synthesis of TGF-β1 in bleomycin-“susceptible” mice strains\textsuperscript{21}. Fibroblasts showed predominantly nuclear localization of Smad3 and intense staining for phospho-Smad2/3 in the bleomycin-treated skin\textsuperscript{22}. By contrast, expression of Smad7 was downregulated, which may account for sustained activation of TGF-β/Smad signaling\textsuperscript{22}.

TGF-β can contribute to the differentiation of both regulatory T cells and inflammatory Th17 cells. IL-17 is a T cell-derived cytokine, and functions to secret vari-
uous cytokines and chemokines by different cell types. Elevated levels of IL-17 have been observed in patients with SSc, especially in the early stages. IL-17 has been reported to induce fibroblast proliferation, but not collagen production in SSC fibroblasts. Further studies are necessary to clarify the role of Th17 cytokines in bleomycin-induced scleroderma.

3.2 Th2 type cytokine

**Interleukin-4 (IL-4)**

Recent hypotheses have indicated that an imbalance exists between the type 1 and type 2 cytokine response in the pathogenesis of scleroderma. The type 1 response likely promotes repair with restoration of normal tissue architecture. In contrast, type 2 response may favor exuberant fibroblast activation, proliferation, and ultimately the deposition of ECM protein and fibrogenesis. IL-4 results in an overall increase in type 2 activity and inhibition of type 1 cytokine responses. The contribution of IL-4 to scleroderma leads to the classification of this disorder as type 2 conditions. Additionally, a report shows that most CD4+ T cell clones generated from scleroderma skin biopsies exhibited type 2 cytokine profiles.

IL-4 has recently been shown to upregulate tissue inhibitor of metalloproteinase-2 (TIMP-2) in dermal fibroblasts. More collagen after IL-4 stimulation. Fibroblasts from Tsk mice exhibit increased and peaked at 4 weeks (Fig. 1A B). IL-13 is detected in the sera or by activated peripheral blood mononuclear cells of patients with SSc. IL-13 has the ability to suppress proinflammatory cytokine production in monocytes/macrophages, and is known to enhance the growth and differentiation of B cells and to promote immunoglobulin synthesis.

In addition, IL-13 has been implicated in the pathogenesis of fibrotic conditions including SSc, because in vitro studies demonstrated that IL-13 is a potent stimulator of fibroblast proliferation and collagen production. IL-13 is a potent stimulator of CCL2/monocyte chemoattractant protein-1 (MCP-1), which plays an important role in fibrosis. In human dermal fibroblasts, IL-13 induces type I collagen protein, mRNA and also promoter activity of α2(I) collagen via phosphoinositol 3-kinase (PI3K) and STAT6 signaling pathways. IL-13 induced transcriptional activity of tenascin-C, which is upregulated via the PI3K/Akt and the protein kinase C signaling pathways. Both serum levels of IL-13 and the expression levels of tenascin-C in the dermis are increased in patients with SSc. Serum levels of IL-13 are also elevated in patients with localized scleroderma, which are associated with the number of plaque lesions more than 3-cm in diameter. Granel et al. analyzed four IL-13 gene polymorphisms (rs1800925, rs20541, rs847 and rs2243204) and observed an association of IL-13 rs1800925 and rs2243204 with diffuse type SSc.

In the bleomycin model, mRNA expression and protein production of IL-13 in the lesional skin was increased and peaked at 4 weeks (Fig. 1A B). IL-13 was strongly detected in the infiltrating mononuclear cells, hair follicles, sweat glands and epidermis in the lesional skin (Fig. 1C). The functional IL-13 receptor complex is a heterodimer composed of the IL-4Rα-chain and a 65- to 70-kd protein termed IL-13Rα1. There is an additional IL-13-binding protein referred to as IL-13Rα2. This receptor chain binds IL-13, but not IL-4, with very high affinity but does not seem to be important in signaling. Increased expression of IL-13 receptor (IL-13R) α2 was found in the infiltrating mono-
nuclear cells and macrophages in the sclerotic skin (Fig. 2A). An increased expression of IL-13Rα2 was shown (Fig. 2B), whereas IL-13Rα1 mRNA level was not significantly enhanced. Taken together, IL-13 and its receptor may promote the progression of cutaneous fibrosis/sclerosis in the development of bleomycin-induced scleroderma. Indeed, recent studies have shown that IL-13-deficient mice failed to develop an increase in skin sclerosis after bleomycin treatment.

4. Extracellular matrix

The hallmarks of fibrosis are the accumulation of ECM proteins, including collagen, in the skin. Excessive deposition of ECM is the result of an imbalance between synthesis and degradation.
4.1. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs)

Maintenance of the normal balance of tissue turnover requires tight control of the activation of latent proenzymes and inhibition of proteolytic activity by tissue inhibitors of metalloproteinases (TIMPs). Matrix metalloproteinases (MMPs) are a family of endopeptidases involved in the remodelling of ECM. Their production is transcriptionally regulated and requires step-wise activation from inactive precursors (proMMP). MMP-1 has the specific ability (shared with MMP-8, MMP-13, and MMP-14) to cleave the triple helix of type I collagen, allowing the chains to unwind and become susceptible to further degradation.

Fibroblasts from patients with early stage SSc exhibited higher levels of gene expression of MMP-1 and MMP-3 than those from normal individuals, while mRNA levels of MMP-1 and MMP-3 were decreased in scleroderma fibroblasts from with mid-stage (2 to 4 years duration) SSc. Serum levels of MMP-1 were significantly elevated in patients with SSc.

Tumor necrosis factor-α (TNF-α) exerts its biological effects through two TNF-α receptors: TNFR1 (55 kD) and TNFR2 (75 kD). TNFRp55-deficient mice developed severe sclerotic changes of the dermis following bleomycin exposure at extremely earlier time points, as compared with wild type mice. Induction of MMP-1 expression was significantly inhibited in the bleomycin-treated skin of TNFRp55-deficient mice. The authors suggest that signaling mediated by TNFRp55 plays

Fig. 2. Expression and synthesis of IL-13 Rα2 in the lesional skin of bleomycin-induced scleroderma.

(A) Immunohistological expression of IL-13Rα2 in the skin following either PBS or bleomycin treatment. IL-13Rα2 was weakly detected in the lesional skin after PBS treatment (PBS), whereas IL-13Rα2 was detected in the epidermis, hair follicles and inflammatory cells infiltrating the dermis at 4 weeks (BLM). (Magnification, PBS: ×120, BLM: ×100) (B) Densitometric data of RT-PCR analysis of IL-13R in lesional skin. Mice were locally treated with either PBS (Cont) or bleomycin, and total RNA was isolated from skin samples. Representative data are shown from three independent experiments.
an essential role in MMP-1 expression and a key role in the collagen degradation process in the bleomycin model.

TIMPs are specific inhibitors of MMP activity and are suggested to be important for fibrogenesis. TIMP-1 expression in fibroblasts is regulated by several cytokines, among which TGF-β is the most important inducer. TIMP-1 has growth-stimulatory activity on skin fibroblasts. Increased deposition of ECM proteins together with a decrease of collagenase activity in scleroderma skin may be related to elevated levels of serum TIMP-1 in patients with SSC. Additionally, scleroderma fibroblasts produce increased amounts of TIMP-1 compared with normal fibroblasts. In the bleomycin model, TIMP-1 mRNA levels were upregulated in the lesional skin.

4.2. Plasminogen activator inhibitor-1 (PAI-1)

Plasminogen activator inhibitor-1 (PAI-1) is a 50kDa glycoprotein belonging to the serine protease superfamily. In addition to stimulating the synthesis of most ECM proteins, TGF-β also regulates the production of plasminogen activator, an inhibitor of plasminogen, or procollagenase. Plasmin can degrade fibrin, fibronectin and laminin, and activates matrix metalloproteinases and latent collagenases. PAI-1 is strongly induced by TGF-β and its promoter contains Smad binding elements. TGF-β activates transcription of the plasminogen activator type-1 gene through a major TGF-β-responsive region in the PAI-1 promoter. Recent studies show an association of PAI-1 with fibrosis. PAI-1 overexpression has been found in scleroderma fibroblasts. TGF-β signaling events, including phosphorylation of Smad-2 and -3, and transcription of the PAI-1 gene were increased in scleroderma fibroblasts, as compared with normal fibroblasts. The mean plasma PAI levels were higher in patients with SSC than control. The lung fibrosis by the intratrachial administration of bleomycin was well suppressed in PAI-1-deficient mice. It has been shown that bleomycin-induced pulmonary fibrosis is severer in transgenic mice overexpressing PAI-1. PAI-1 suppresses the dissolution of collagen and promotes its accumulation, and thus PAI-1-deficient mice are protected against bleomycin-induced pulmonary fibrosis. These observations suggest that members of the plasminogen activator system play a role in the metabolic process of ECM.

In the bleomycin model, mRNA level of PAI-1 in the lesional skin was increased (Fig. 3A). PAI-1 was detected on fibroblastic and inflammatory cells (Fig. 3B), and functionally active PAI-1 levels were increased after bleomycin injection (Fig. 3C). After confirming enhanced PAI-1 levels in the bleomycin-induced scleroderma, we next examined whether PAI-1 deficiency prevents dermal sclerosis. Contrary to our expectation, histological examination revealed that sclerotic skin was definitely induced by bleomycin treatment even in PAI-1-deficient mice (Fig. 4A). Collagen content in the skin was significantly increased following bleomycin treatment in both wild type (WT) and PAI-1-deficient mice. TGF-β1-positive cells were increased in the bleomycin-treated skin after both WT and PAI-1-deficient mice, which were mainly infiltrating mononuclear cells and fibroblasts. TGF-β1 and type I collagen mRNA levels showed more than 2-fold increases in the lesional skin of both PAI-1-deficient and WT mice (Fig. 4B). PAI-2 mRNA expression was also elevated following bleomycin treatment in WT, but not in PAI-1-deficient mice. PAI-1 may play an important role, possibly via TGF-β pathway activation, but the fact that PAI-1 deficiency did not ameliorate skin sclerosis suggests that PAI-1 is not the essential factor in the development of bleomycin-induced scleroderma. There was no difference in plasmin activity in liver and renal fibrosis in PAI-1-deficient mice. More complex biochemical effects in the PA/plasmin system (plasminogen, alpha2-antiplasmin) and other than the PA/plasmin system are greatly suspected.

5. Perspective

Recent advances suggest there are complex networks that involve cell-cell and cell-matrix interactions via mediators in the pathogenesis of cutaneous sclerosis. Fibroblasts were previously considered to be important connective tissue cells necessary for the structure of microenvironments, whereas recent findings suggest that fibroblasts are a part of the immune system and modulate immune cell behavior by conditioning local cellular and cytokine microenvironments. Activated sclerotic fibroblasts are a major source of a number of cytokines and chemokines. Also, fibroblasts display receptors and surface markers that are involved in cell-cell and cell-matrix interactions. TGF-β plays a key role in the fibrotic process, but many other cytokines (e.g., type 2 cytokines) and chemokines also play important roles at different stages of disease progression. Further, the imbalance between production and degradation of ECM proteins shifts toward fibrosis. Possible mechanisms of bleomycin-induced murine...
Fig. 3. Expression and synthesis of PAI-1 in the lesional skin of bleomycin-induced scleroderma.

(A) Densitometric data of RT-PCR analysis of whole skin samples showing gene expression of PAI-1. Total RNA was isolated from skin samples treated with either PBS for 4 weeks (Cont) or bleomycin for 1-4 weeks. Representative data are shown from three independent experiments.

(B) Histological examination. (left) Immunohistochemical localization of PAI-1 was faintly detected in the PBS-treated skin. (middle) In contrast, PAI-1 was detected on the macrophage-like cells in the bleomycin-treated skin at 2 weeks. (right) At 4 weeks, immunoreactive cells for PAI-1 are mainly infiltrating macrophage-like cells and fibroblastic cells in the bleomycin-treated skin. (Magnification: ×60) (C) Immunoreactive PAI-1 protein levels assessed by ELISA. The skin samples (n=5) were homogenized and the supernatants were measured after centrifugation (*P< 0.05). Results were expressed as mean±SD. Significance testing was assessed by Mann Whitney U-test. A P value < 0.05 was considered as significant.
scleroderma are schematically proposed in Fig. 5. To explore the cellular and molecular mechanisms in animal models may enhance our understanding of the pathogenesis of human sclerosis. Animal models of scleroderma may also present promising tools for the evaluation of new therapeutic interventions that aim at specific targeting of individual cytokines, including cytokine antagonists (i.e., antibodies, soluble receptors), cytokine mutants, and also drugs that specifically interfere with the signal transduction pathways involved in the fibrotic process.

Acknowledgements

We would like to thank Mrs. M. Sekiya for her technical assistance.

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Fig. 5. Schematic design of the pathogenesis of bleomycin-induced scleroderma.


