barrier function. For instance, a recombinant activated form of protein C reduces tumour necrosis factor-α-induced expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin, and inhibits the induction of apoptosis in endothelial cells.[40] Furthermore, activated protein C reduces vascular permeability through cleavage of protease-activated receptor 1 by the activated protein C/EPCR complex.[41–45] Given that SSc vasculature is characterized by proinflammatory and proapoptotic properties and increased vascular permeability, decreased EPCR expression in SSc endothelial cells may be related to the induction of the SSc vascular phenotype through the low availability of activated protein C. Further studies are required to clarify this point.

In summary, this is the first report demonstrating the potential contribution of reduced EPCR to the development of SSc. A weak inverse correlation of serum EPCR levels with MRSS and PIC suggests the role of EPCR-dependent biological events as a part of the complex pathological processes associated with SSc. The present findings further reinforce the notions that endothelial Fli1 deficiency is a key feature underlying the induction of structural and functional abnormalities of SSc vasculopathy, and that bosentan has the potential to reverse the vascular phenotype related to Fli1 deficiency. The reversal of an impaired coagulation/fibrinolysis system is a possible mechanism underlying the preventive effect of bosentan on digital ulcers in patients with SSc.

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Progranulin Overproduction Due to Fli-1 Deficiency Contributes to the Resistance of Dermal Fibroblasts to Tumor Necrosis Factor in Systemic Sclerosis

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Objective. **Progranulin is a growth factor that is active in wound repair and is an antagonist of tumor necrosis factor (TNF) receptors, regulating fibroblast activation, angiogenesis, and inflammation. Because long-standing activation of gene programs related to wound healing is a hallmark of systemic sclerosis (SSc), we sought to investigate the role of progranulin in SSc.**

Methods. **Progranulin expression levels in human and murine skin samples were determined by immunohistochemical analysis and quantitative reverse transcription–polymerase chain reaction. The role of progranulin in fibroblast activation was examined using a gene-silencing technique. Progranulin levels in serum obtained from 60 patients with SSc and 16 healthy control subjects were determined by enzyme-linked immunosorbent assay.**

Results. **Progranulin expression was increased in SSc dermal fibroblasts compared with normal dermal fibroblasts, both in vivo and in vitro. Transcription factor Fli-1, a deficiency of which is involved in the activation of SSc dermal fibroblasts, served as a potent repressor of the progranulin gene, and Fli-1$^{+/-}$ mice and bleomycin-treated wild-type mice exhibited up-regulated expression of progranulin in dermal fibroblasts. SSc dermal fibroblasts were resistant to the antifibrotic effect of TNF, but this resistance was reversed by gene silencing of progranulin. Serum progranulin levels were elevated in patients with early diffuse cutaneous SSc (dcSSc), especially in those with inflammatory skin symptoms, and were positively correlated with the C-reactive protein level.**

Conclusion. **Progranulin overproduction due to Fli-1 deficiency may contribute to the constitutive activation of SSc dermal fibroblasts by antagonizing the antifibrotic effect of TNF. Progranulin may also be involved in the inflammatory process associated with progressive skin sclerosis in early dcSSc.**

Systemic sclerosis (SSc) is a multisystem connective tissue disease characterized by immune abnormalities, vasculopathy, and fibrosis of the skin and certain internal organs (1). Although the pathogenesis of SSc currently remains elusive, dysregulated activation of fibroblasts has been thought to be the final consequence of this disease, following inflammation, autoimmune attacks, and vascular injuries. In addition to transforming growth factor β (TGFβ), which plays a central role in the fibrotic process of SSc (2), an increasing number of growth factors, cytokines, and chemokines have been shown to be involved in the complex network of signaling pathways driving the deposition of extracellular matrix proteins, especially in the early stage of this disorder (3).

Progranulin, a pleiotropic growth factor, is a ubiquitously expressed secreted glycoprotein that does not structurally belong to any of the well-established growth factor families (4). Progranulin is implicated in the regulation of cellular proliferation, cell migration, and survival and, therefore, has been well studied in the fields of wound healing (5), tumorigenesis (6,7), and neuroproliferative and degenerative diseases (8). In the

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skin, progranulin is expressed in the perivascular inflammatory infiltrate under physiologic conditions, but it is highly induced in dermal fibroblasts and endothelial cells following injury.

Exogenous progranulin promotes the infiltration of neutrophils and macrophages, fibroblast proliferation, and angiogenesis in murine cutaneous wounds and induces proliferation, migration, and tube formation in cultured dermal fibroblasts and/or endothelial cells, indicating that it is a potent wound-related growth factor (5). Progranulin also exerts an immunomodulatory effect by serving as an endogenous antagonist of tumor necrosis factor receptors (TNFRs) (9) and as a cofactor in the presentation of CpG oligonucleotides to Toll-like receptor 9 (TLR-9) (10). Importantly, *Pgrn*-deficient mice exhibit joint manifestations that are much more severe than those in wild-type (WT) mice in the collagen-induced arthritis model, and both homozygous and heterozygous loss of *Pgrn* exacerbate joint symptoms in TNF-transgenic mice, all of which are improved by administration of recombinant human progranulin (9). Thus, progranulin plays a novel role in TNF-related diseases such as rheumatoid arthritis (11).

Because various signaling pathways related to wound healing are aberrantly activated, and because the expression of TNF, a potent antifibrotic cytokine (12–15), is markedly increased in the lesional skin of patients with SSc (16,17), we hypothesized that progranulin is involved in the mechanism underlying dermal fibrosis in SSc. The aim of this study was to investigate the role of progranulin in SSc by performing experiments using both human and murine skin specimens.

PATIENTS AND METHODS

This study was approved by the ethics committee at the University of Tokyo Graduate School of Medicine. Written informed consent was obtained from all of the patients and healthy control subjects. The entire study was performed according to the Declaration of Helsinki. All animal studies and procedures were approved by the Committee on Animal Experimentation at the University of Tokyo Graduate School of Medicine.

Immunohistochemical analysis. Skin samples were obtained from the forearms of 5 patients with diffuse cutaneous SSc (dcSSc) (disease duration of <2 years) and 5 closely matched healthy control subjects. A 6-mm–diameter punch biopsy device was used to obtained sections from the back skin of WT C57BL/6 mice, WT mice treated with phosphate buffered saline (PBS) or bleomycin as described previously (18), and Fli-1$^{+/-}$ mice (a generous donation from Dr. Dennis K. Watson) (19). Immunohistochemical analysis with anti-human progranulin antibody (R&D Systems) or anti-mouse progranulin antibody (R&D Systems) was performed on formalin-fixed, paraffin-embedded tissue sections, using a Vectastain ABC kit (Vector) according to the manufacturer's instructions.

Cell cultures. Dermal fibroblasts were obtained from 8 patients with dcSSc (duration of skin thickening <2 years) and 8 healthy donors closely matched for age and sex; the fibroblasts were obtained from the same skin area in patients and controls and were maintained as described previously (20). Human dermal microvascular endothelial cells (HDMECs) were purchased from Lonza and cultured on collagen-coated tissue culture plates in EBM-2 medium supplemented with EGM-2 BulletKit medium (Lonza).

Gene silencing of Fli-1 and TNF stimulation. Cells were seeded shortly before transfection. The cells were transfected with 10 n*M* Fli-1 small interfering RNA (siRNA) or nonsilencing scrambled RNA (Santa Cruz Biotechnology) using HiPerFect transfection reagent (Qiagen) for 72 hours. Cells were serum-starved for the last 48 hours. Some cells were further stimulated with TNF (PeproTech) for the last 24 hours.

RNA isolation and quantitative reverse transcription–polymerase chain reaction (qRT-PCR). RNA was isolated from skin specimens obtained from patients and control subjects (17 patients with dcSSc, 13 with limited cutaneous SSc [lcSSc], and 8 controls), cultured fibroblasts and endothelial cells, and murine skin samples, and qRT-PCR was performed, as described previously (21). The primer sequences were as follows: for *FLI1*, forward 5′-GGATGGCAAGGAACTGTGTAA-3′ and reverse 5′-GGTTGTATAGGCCAGCAG-3′; for *GAPDH*, forward 5′-ACCCACTCCTCCACCTTTGA-3′ and reverse 5′-CATACCAGGAAATGAGCTTGACAA-3′; for *Gapdh*, forward 5′-CGTGTTCCTACCCCCAATGT-3′ and reverse 5′-TGTCATCATACTTGGCAGGTTTCT-3′; for *COL1A2*, forward 5′-GATGTTGAACTTGTTGCTGACG-3′ and reverse 5′-TCTTTCCCCATTCATTTGTCTT-3′; for *Col1a2*, forward 5′-CACCCCAGCGAAGAACTCATA-3′ and reverse 5′-GCCACCATTGATAGTCTCTCC-3′; for *PGRN*, forward 5′-CAGGGACTTCCAGTTGCTGC-3′ and reverse 5′-GCAGCAGTGATGGCCATCC-3′; for *Pgrn*, forward 5′-AGTTCGAATGTCCTGACTCCGCCA-3′ and reverse 5′-AAGCCACTGCCCTGTTGGTCCTTT-3′; for *TNFA*, forward 5′-CCCAGGGACCTCTCTCTAATCA-3′ and reverse 5′-AGCTGCCCCTCAGCTTGAG-3′; for *Tnfa*, forward 5′-CCACCACGCTCTTCTGTCTAC-3′ and reverse 3′-AGGGTCTGG-GCCATAGAACT-3′.

Immunoblotting. Whole cell lysates were prepared from fibroblasts treated with recombinant human progranulin (R&D Systems), progranulin siRNA, and/or TNF, as described previously (22). Samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting with antibodies against type I collagen (SouthernBiotech), progranulin (R&D Systems), or β-actin (Sigma-Aldrich). Bands were detected using enhanced chemiluminescence techniques (Thermo Scientific).

Chromatin immunoprecipitation assay. A chromatin immunoprecipitation (ChIP) assay was performed using an Epi-Quik ChIP kit (Epigentek). Briefly, cells were treated with 1% formaldehyde for 10 minutes. The cross-linked chromatin was then prepared and sonicated to an average size of 300–500 bp. The DNA fragments were immunoprecipitated with anti–Fli-1 antibody (Santa Cruz Biotechnology) or normal rabbit IgG at 4°C. After reversal of cross-linking, the immunoprecipitated chromatin was amplified by PCR amplification of a specific region of the progranulin genomic locus. The putative Fli-1 binding site was predicted by Tfsitescan. The primers were as follows:

for progranulin/F-399, 5′-CAGACACGCGCTATCATCTC-3′; for progranulin/R-597, 5′-GCCTGGAATGCTGTGTTTCT-3′. The amplified DNA products were resolved by agarose gel electrophoresis.

Oligonucleotide pull-down assay. The oligonucleotides containing biotin on the 5′ nucleotide of the sense strand were used. The sequences of these oligonucleotides were as follows: for progranulin, 5′-ATGGGGTGTGGGGCGAGAGGAAGCAGGGAGGAGAGTGATT-3′ and 5′-AATCACTCTCCTCCCTGCTTCCTCTCGCCCCACACCCCAT-3′, which correspond to basepairs −471 to −432 of the progranulin promoter containing the putative Fli-1 binding site; for mutated progranulin, 5′-ATGGGGTGTGGGGCGAGAGTCCGCAGGGAGGAGAGTGATT-3′ and 5′-AATCACTCTCCTCCCTGCGGACTCTCGCCCCACACCCCAT-3′, which have a mutation in the putative Fli-1 binding site. The putative Fli-1 binding site was predicted by Tfsitescan. These oligonucleotides were annealed to their respective complementary oligonucleotides at 95°C for 1 hour. Nuclear extracts were incubated with streptavidin-coupled agarose beads and 500 pmoles of each double-stranded oligonucleotide for 2 hours at room temperature with gentle rocking. The protein/DNA/streptavidin/agarose complex was washed 4 times with PBS containing protease inhibitors. The precipitates were subjected to immunoblotting with anti–Fli-1 antibody (Santa Cruz Biotechnology).

Quantification of early apoptotic cells. Cells were stained with fluorescein isothiocyanate–labeled annexin V (BioLegend) and propidium iodide (PI) (Molecular Probes). The number of early apoptotic cells (annexin V positive/PI negative) was quantified with a FACScan flow cytometer (BD PharMingen) (23).

Patients enrolled in the clinical study. Serum samples were obtained from 60 patients with SSc (56 women and 4 men, median age 59 years [interquartile range (IQR) 51–66.5], median disease duration 2.5 years [IQR 1.4–9.5]) and 16 healthy control subjects (15 women and 1 man, median age 55 years [IQR 51–59]). The samples were frozen at −80°C until assayed. Patients who had been treated with corticosteroids or other immunosuppressant agents prior to the first visit were excluded. Patients were grouped according to the classification system described by LeRoy and Medsger (24); 30 patients were classified as having dcSSc, and 30 patients were classified as having lcSSc. All patients fulfilled the American College of Rheumatology/European League Against Rheumatism 2013 classification criteria for SSc (25).

Measurement of serum progranulin levels. Progranulin levels in serum from patients with SSc and healthy control subjects were measured using specific enzyme-linked immunosorbent assay kits (R&D Systems) according to the instructions of the manufacturer.

Statistical analysis. Differences between 2 groups were assessed using Welch's *t*-test, and one-way analysis of variance followed by Tukey's post hoc test was used to determine differences among groups. Fisher's exact test was used to analyze frequency, and Spearman's rank correlation coefficient was used to evaluate the correlation between variables. The Shapiro-Wilk normality test was used to confirm a normal distribution. Continuous data are presented as the mean ± SD when a normal distribution was assumed and as the median (IQR) when the distribution was skewed. *P* values less than 0.05 were considered significant.

RESULTS

Role of Fli-1 deficiency in increased progranulin expression in SSc dermal fibroblasts. We initially evaluated progranulin expression by performing immunohistochemical analysis in the skin of SSc patients with a disease duration of <2 years and closely matched healthy control subjects. Progranulin was expressed abundantly in some perivascular inflammatory cells, moderately in epidermal keratinocytes, and marginally in endothelial cells, and the expression levels in those cells were comparable between patients with SSc and control subjects

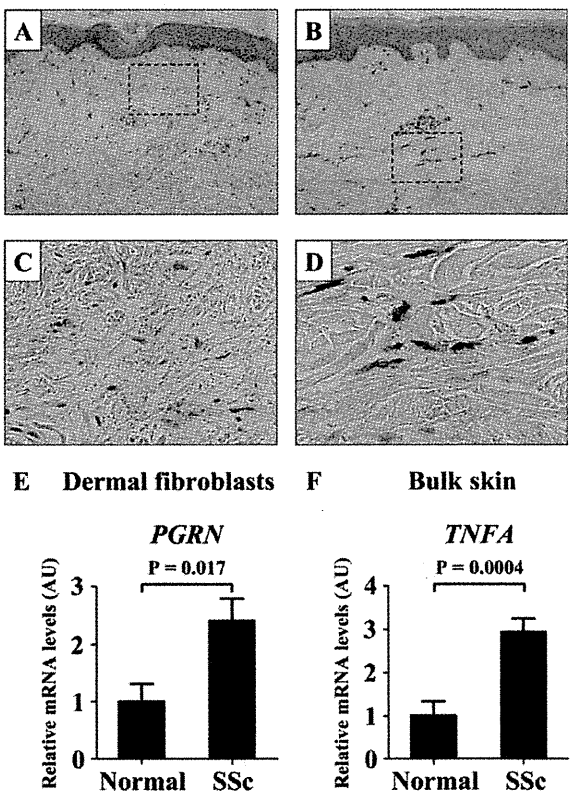

Figure 1. Expression of progranulin in the lesional skin of patients with systemic sclerosis (SSc). **A** and **B,** Progranulin expression in the epidermis and the papillary and reticular layers of the dermis from a healthy control subject (**A**) and a patient with SSc (**B**). **C** and **D,** Progranulin expression in the reticular dermis from a healthy control subject (**C**) and a patient with SSc (**D**). Boxed areas in **A** and **B** (original magnification × 100) are shown at higher magnification in **C** and **D** (original magnification × 400), respectively. Results are representative of those for 5 control subjects and 5 SSc patients. **E** and **F,** *PGRN* mRNA expression in cultivated dermal fibroblasts from control subjects (n = 3) and SSc patients (n = 3) (**E**) and *TNFA* mRNA expression in bulk skin from control subjects (n = 8) and SSc patients (n = 30) (**F**), as determined by quantitative reverse transcription–polymerase chain reaction. Expression was normalized to that of *GAPDH*. Values are the mean ± SEM.

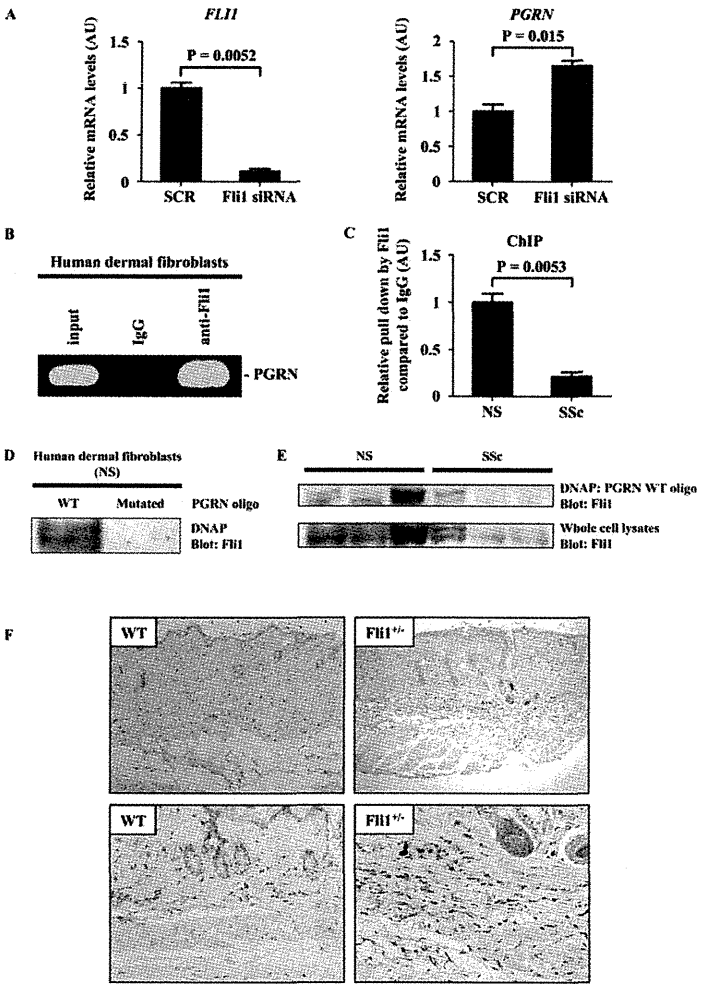

Figure 2. Contribution of Fli-1 to the regulation of progranulin expression in systemic sclerosis (SSc) dermal fibroblasts. **A,** *FLI1* and *PGRN* mRNA levels in normal dermal fibroblasts treated with nonsilencing scrambled RNA (SCR) or Fli-1 small interfering RNA (siRNA), as determined by quantitative reverse transcription–polymerase chain reaction (qRT-PCR). **B,** Immunoblot showing binding of Fli-1 to the *PGRN* promoter. **C,** Occupancy of the *PGRN* promoter by Fli-1 in dermal fibroblasts from normal subjects (NS) and SSc patients, as quantified by qRT-PCR. Results are representative of 3 independent experiments. **D** and **E,** Sequence-specific binding of Fli-1 to the *PGRN* promoter. Equal amounts (500 μg) of whole cell lysates prepared from normal dermal fibroblasts were incubated with biotin-labeled wild-type (WT) or mutated progranulin oligonucleotides (oligo) (**D**), and equal amounts (500 μg) of each whole cell lysate prepared from 3 normal and 3 SSc dermal fibroblasts were incubated with biotin-labeled WT *PGRN* oligonucleotides (**E**). Proteins bound to these nucleotides were isolated with streptavidin–agarose beads, and Fli-1 was detected by immunoblotting. To compare the levels of total Fli-1 protein in each whole cell lysate from 3 normal and 3 SSc dermal fibroblasts, immunoblotting was carried out with 50 μg of the same whole cell lysates used in the DNA oligonucleotide pull-down assay. **F,** Progranulin protein expression in skin tissue specimens obtained from WT mice and Fli-$1^{+/-}$ mice. Representative images of the epidermis and whole dermis (top) and the deep dermis (bottom) are shown. Original magnification × 100 in top row; × 400 in bottom row. Values are the mean ± SEM (n = 3 independent experiments). ChIP = chromatin immunoprecipitation; DNAP = DNA affinity precipitation.

(Figures 1A and B). In contrast, a striking difference was seen in dermal fibroblasts, with expression of progranulin being extensively high in most of the SSc dermal fibroblasts but weak or absent in normal dermal fibroblasts (Figures 1C and D). These observations were confirmed by the results of qRT-PCR in cultured dermal fibroblasts, which showed a significant increase in *PGRN* messenger RNA (mRNA) expression in patients with SSc compared with healthy controls (Figure 1E).

Because a deficiency of Fli-1 (a member of the Ets family of transcription factors regulating fibrosis and angiogenesis-related gene programs) contributes to

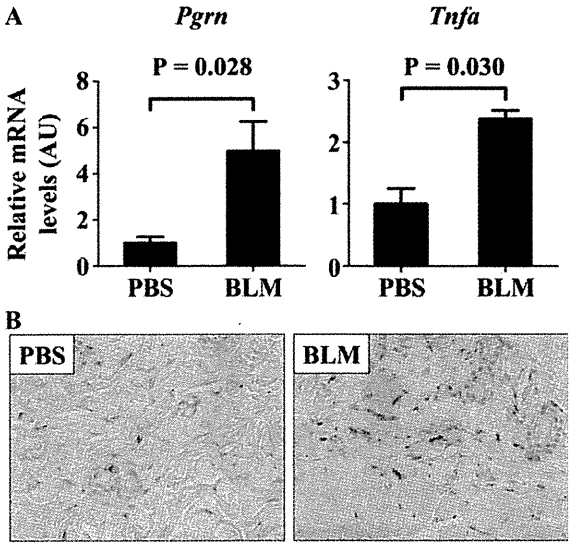

Figure 3. Up-regulated expression of *Pgrn* and *Tnfa* in the skin of bleomycin (BLM)–treated mice. **A,** *Pgrn* and *Tnfa* mRNA levels in back skin specimens obtained from phosphate buffered saline (PBS)–treated mice (n = 5) and bleomycin-treated mice (n = 5) were determined by quantitative reverse transcription–polymerase chain reaction. Values are the mean ± SEM. **B,** Progranulin protein levels in representative back skin specimens from PBS-treated mice and bleomycin-treated mice were examined by immunohistochemical analysis. Original magnification × 400.

the activation of fibroblasts and endothelial cells in SSc due to epigenetic suppression (26–30), we next evaluated the contribution of Fli-1 to the regulation of progranulin expression in those cells. As shown in Figure 2A, gene silencing of Fli-1 resulted in a significant increase in *PGRN* mRNA levels in normal dermal fibroblasts but did not affect these levels in HDMECs (data not shown). Furthermore, Fli-1 bound to the promoter of *PGRN* (Figure 2B), and Fli-1 occupancy of the *PGRN* promoter (as quantified by qRT-PCR) was decreased much more in SSc dermal fibroblasts than in normal dermal fibroblasts (Figure 2C). Moreover, the sequence-specific binding of Fli-1 to the *PGRN* promoter, as confirmed by DNA oligonucleotide pull-down assay (Figure 2D), was remarkably decreased in SSc dermal fibroblasts compared with normal dermal fibroblasts (Figure 2E).

Consistent with these findings, compared with WT mice, Fli-$1^{+/-}$ mice showed increased expression of progranulin protein in dermal fibroblasts but not in DMECs (Figure 2F). Collectively, these results indicate that progranulin is a direct target of Fli-1 in dermal fibroblasts, and that Fli-1 deficiency contributes to the induction of progranulin expression in SSc dermal fibroblasts.

Effect of autocrine progranulin on SSc dermal fibroblasts resistant to the antifibrotic effect of TNF. To further confirm the notion that altered expression of progranulin is involved in the mechanism underling dermal fibrosis in SSc, we examined the expression levels of progranulin in lesional skin from mice with bleomycin-induced SSc. As shown in Figure 3A, *Pgrn* mRNA levels were significantly higher in bleomycin-treated mice than in PBS-treated mice. Furthermore, progranulin expression was much greater in dermal fibroblasts from bleomycin-treated mice compared with that in dermal fibroblasts from PBS-treated mice (Figure 3B), while progranulin expression in dermal blood vessels was comparable in PBS-treated and bleomycin-treated mice (results not shown). These results suggest a potential contribution of progranulin to fibroblast activation in various fibrotic conditions.

TNF is a proinflammatory cytokine with a potent antifibrotic effect on dermal fibroblasts (12–15). The expression of *Tnfa* was increased in the skin of bleomycin-treated mice (Figure 3A), similar to what was observed in SSc skin (Figure 1F) (16), suggesting that some factors antagonize the effect of TNF in dermal fibroblasts. Given that progranulin functions as an endogenous antagonist of TNF, we hypothesized that progranulin inhibits the antifibrotic effect of TNF on activated dermal fibroblasts in pathologic fibrotic conditions. To address this issue, we investigated whether progranulin siRNA affects the antifibrotic effect of TNF in SSc dermal fibroblasts. We first confirmed the inhibitory effect of exogenous progranulin on the antifibrotic effect of TNF in normal dermal fibroblasts (Figure 4A) and the up-regulated expression of type I collagen and progranulin proteins in SSc dermal fibroblasts that were used in the subsequent experiments (Figure 4B).

Consistent with previous reports (12,14,15,31), TNF suppressed the expression of type I collagen in normal dermal fibroblasts at both the protein and mRNA levels, and progranulin siRNA did not affect the magnitude of this inhibitory effect. In contrast, SSc dermal fibroblasts were resistant to the inhibitory effect of TNF on the expression of type I collagen at both the protein and mRNA levels, as previously reported (32). However, progranulin siRNA reversed the sensitivity to TNF in SSc dermal fibroblasts (Figure 4C). It was also confirmed that the number of early apoptotic cells (annexin V positive/PI negative) was not altered in cells treated with progranulin siRNA and/or TNF (Figure 4C). These results indicate that overproduction of progranulin contributes to the resistance of SSc dermal fibroblasts to the antifibrotic effects of TNF.

Correlation between serum progranulin levels and the clinical features of patients with SSc. To further assess the role of progranulin in SSc, we measured

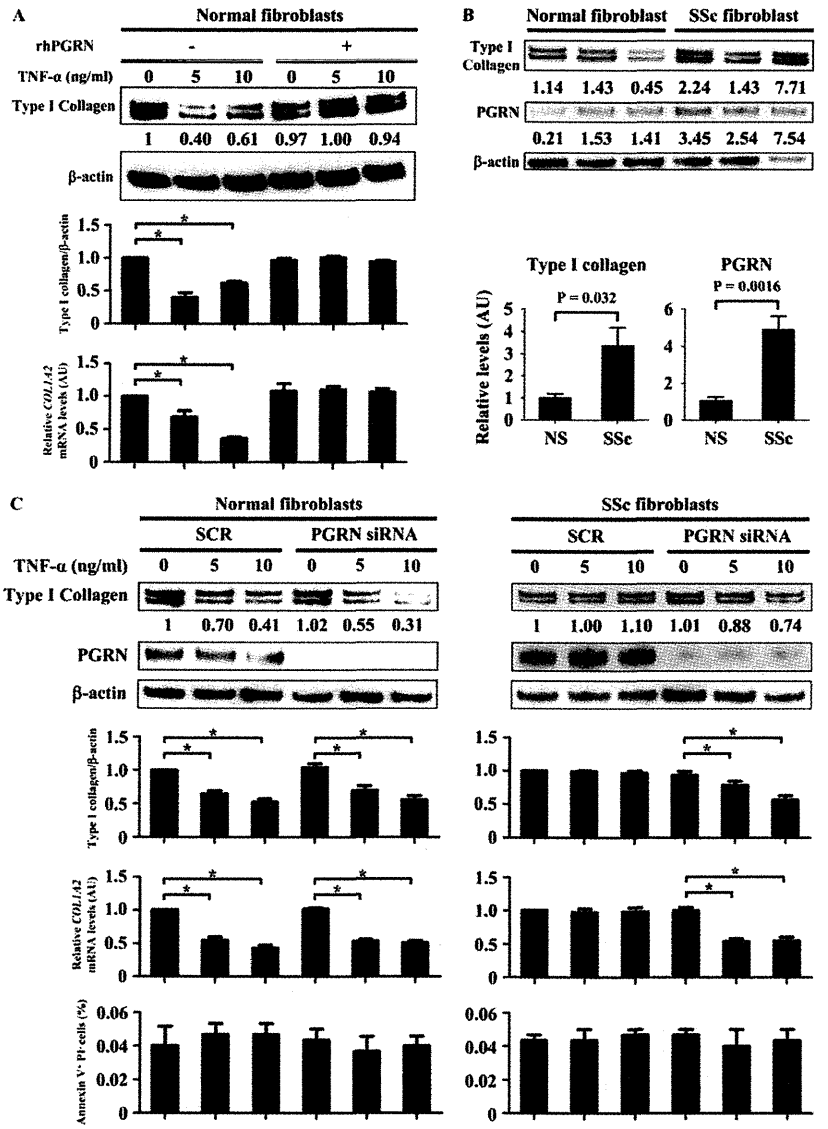

Figure 4. Autocrine progranulin renders SSc dermal fibroblasts resistant to the antifibrotic effect of tumor necrosis factor (TNF). **A**, Effect of exogenous progranulin on the antifibrotic effect of TNF in normal dermal fibroblasts. Quiescent normal dermal fibroblasts were treated with the indicated concentrations of TNF for 24 hours. Some cells were treated with 100 ng/ml of recombinant human progranulin (rhPGRN) 1 hour prior to TNF stimulation. Type I collagen protein levels and *COL1A2* mRNA levels were determined by immunoblotting and qRT-PCR, respectively (n = 3 for each group). **B**, Effect of exogenous progranulin on the expression of type I collagen and progranulin proteins in SSc dermal fibroblasts. Whole cell lysates prepared from quiescent normal and SSc dermal fibroblasts were subjected to immunoblotting with antibodies against type I collagen, progranulin, and β-actin (n = 7 for each group). **C**, Effect of progranulin siRNA on sensitivity to TNF in SSc dermal fibroblasts. Normal and SSc dermal fibroblasts (n = 7 and n = 9, respectively) were transfected with progranulin siRNA or nonsilencing scrambled RNA for 72 hours. Serum was starved for the last 48 hours. Some cells were treated with 5 or 10 ng/ml of TNF for the last 24 hours. Whole cell lysates were subjected to immunoblotting with anti–type I collagen antibody, anti-progranulin antibody, and anti–β-actin antibody. Representative immunoblots are shown. The numbers below each blot represent the relative levels of target molecules as normalized to loading controls by densitometry. In the bar graphs, values are the mean ± SEM. * = $P < 0.05$ versus controls, by one-way analysis of variance followed by Turkey's post hoc test. PI = propidium iodide (see Figure 2 for other definitions).

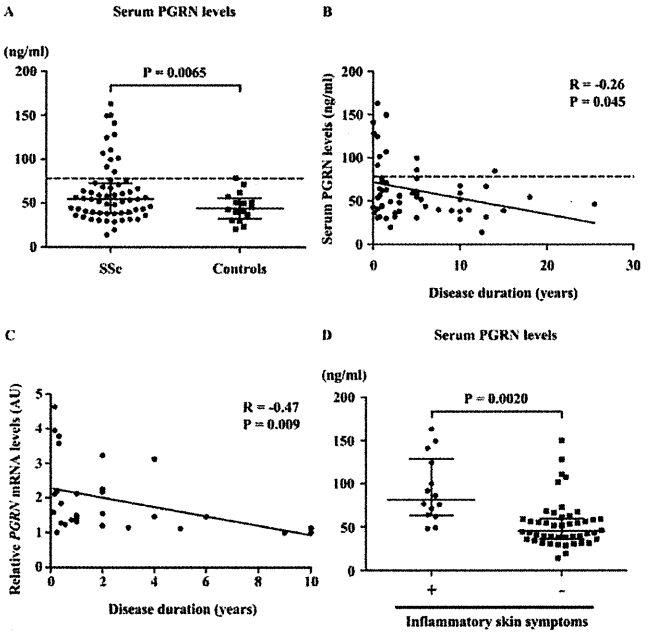

Figure 5. Correlations between serum progranulin levels and clinical features of SSc. Serum progranulin levels in 60 SSc patients and 16 healthy controls were measured with a specific enzyme-linked immunosorbent assay, and *PGRN* mRNA levels in the lesional skin of 30 SSc patients were determined by qRT-PCR. **A,** Serum progranulin levels in SSc patients and healthy control subjects. **B,** Inverse correlation between serum progranulin levels and disease duration in SSc patients. **C,** Inverse correlation between *PGRN* mRNA levels and disease duration in SSc patients. **D,** Serum progranulin levels in SSc patients with and those without inflammatory skin symptoms. Bars in **A** and **D** show the median and interquartile range. Broken lines in **A** and **B** indicate the cutoff value (mean + 2SD, as calculated from serum progranulin levels in healthy controls, which are normally distributed). See Figure 2 for definitions.

progranulin levels in serum and examined the correlation between these levels and clinical features. As shown in Figure 5A, serum progranulin levels were significantly higher in SSc patients than in healthy individuals. When we set the cutoff value at 78.1 ng/ml (mean + 2SD, as calculated from serum progranulin levels in healthy controls, which are normally distributed), serum progranulin levels were elevated in 12 of 60 patients with SSc. The clinical features of SSc patients with elevated progranulin levels compared with those of patients with normal levels are shown in Table 1.

The disease duration was significantly shorter in patients with elevated serum progranulin levels than in those with normal serum progranulin levels, while there was no significant difference between these 2 groups in terms of sex, age at disease onset, and frequency of dcSSc. Serum progranulin levels were inversely and significantly correlated with disease duration (Figure 5B), which was confirmed by the correlation between *PGRN* mRNA levels in lesional skin and disease duration in another set of 30 SSc patients who had undergone skin biopsy (Figure 5C). No significant differences between patients with an elevated serum progranulin level and those with a normal level were observed for the modified Rodnan total skin thickness score (MRSS), the frequency of vascular cutaneous symptoms and visceral involvement, and the autoantibody profile. We also examined the correlation between serum progranulin levels and parameters of dermal and pulmonary fibrosis, including the MRSS, the percentage of predicted vital capacity, and the percentage of predicted diffusing capacity for carbon monoxide, but did not detect any correlation (data not shown).

Given that progranulin has potent proinflammatory or antiinflammatory properties in a context-dependent manner (4), we assessed the association between serum progranulin levels and inflammation markers, such as the erythrocyte sedimentation rate (ESR) and the C-reactive protein (CRP) level. Notably, an elevated CRP level, but not an increased ESR, was much more frequent in patients with an elevated serum progranulin level than in those with a normal level, suggesting that progranulin serves as a proinflammatory factor in the pathologic condition of SSc. Further supporting this notion, the prevalence of inflammatory skin symptoms, such as pruritus, local heat, and erythema, was significantly higher in patients with elevated serum progranulin levels than in those with normal lev-

Table 1. Characteristics of the patients with SSc according to the progranulin level in serum*

	Progranulin level		
	Elevated (n = 12)	Normal (n = 48)	*P*
No. men/no. women	1/11	3/45	1.00
Age, median (IQR) years	56.5 (50.3–61.5)	61 (49.5–70)	0.25
Disease duration, median (IQR) years	1.5 (1–3)	6 (2–20)	0.0003
No. with dcSSc/no. with lcSSc	8/4	22/26	0.33
Clinical features			
MRSS, median (IQR)	7 (2–13.3)	6 (2.3–9)	0.36
Pitting scars	42 (5/12)	29 (14/48)	0.49
Raynaud's phenomenon	92 (11/12)	81 (39/48)	0.67
Telangiectasia	55 (6/11)	51 (23/45)	1.00
Nailfold bleeding	75 (9/12)	67 (32/48)	0.74
Organ involvement			
Esophagus	55 (6/11)	73 (35/48)	0.28
Lungs	67 (8/12)	52 (25/48)	0.52
Decreased % DLco	20 (2/10)	22 (9/44)	1.00
Decreased % VC	20 (2/10)	15 (7/46)	0.66
Elevated RVSP	27 (3/11)	39 (16/41)	0.73
Heart	8 (1/12)	23 (11/48)	0.43
Scleroderma renal crisis	8 (1/12)	4 (2/48)	0.49
Muscle	0 (0/12)	6 (3/48)	1.00
ANA specificity			
Anti–topoisomerase I antibodies	50 (6/12)	33 (16/48)	0.33
Anticentromere antibodies	17 (2/12)	35 (17/48)	0.31
Anti–U1 RNP antibodies	17 (2/12)	15 (7/48)	1.00
Laboratory findings			
Increased ESR	88 (7/8)	63 (25/40)	0.24
Elevated CRP level	45 (5/11)	15 (7/48)	0.036

* Disease onset was defined as the first clinical event of systemic sclerosis (SSc) other than Raynaud's phenomenon. Disease duration was defined as the interval between disease onset and the time of blood sampling. The clinical and laboratory data were obtained at the time of blood sampling. The degree of interstitial lung disease was evaluated by the percentage of predicted diffusing capacity for carbon monoxide (% DLco) and the percentage of predicted vital capacity (% VC) as determined by pulmonary function testing. Elevated right ventricular systolic pressure (RVSP) was defined as ≥35 mm Hg on echocardiography. Scleroderma renal crisis was defined as malignant hypertension and/or rapidly progressive renal failure. Except where indicated otherwise, values are the percent (no. of patients/no. of patients assessed). IQR = interquartile range; dcSSc = diffuse cutaneous SSc; lcSSc = limited cutaneous SSc; MRSS = modified Rodnan skin thickness score; ANA = antinuclear antibody; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein.

els (58% [7 of 12] versus 12% [7 of 48]; $P = 0.0037$). In addition, serum progranulin levels were significantly higher in patients with inflammatory skin symptoms than in those without such symptoms (Figure 5D). Therefore, progranulin may contribute to the inflammatory process associated with progressive skin sclerosis in early SSc.

DISCUSSION

Progranulin is a multifactorial wound-related growth factor regulating fibrosis, angiogenesis, inflammation, and innate immunity (5), all of which are critical pathologic components of SSc. A series of experiments using skin samples from patients with SSc and from murine models of SSc demonstrated that progranulin is potentially involved in the pathogenesis of SSc, especially the fibrotic and inflammatory processes of this disease.

In dermal fibroblasts, progranulin is up-regulated by stress conditions, such as hypoxia and acidosis, and protects those cells against apoptosis (33). Cell survival of myofibroblasts is tightly regulated in the process of wound healing, and prolonged survival of myofibroblasts is a hallmark of fibrotic disorders, including SSc (34–36). Therefore, dysregulated overproduction of progranulin potentially contributes to the pathologic dermal fibrosis in SSc via the protection of dermal fibroblasts against apoptosis. Another novel property of progranulin is an antagonistic effect on TNFRs, which has drawn much attention to progranulin from researchers in the field of TNF-related diseases, including autoimmune inflammatory disorders.

Among patients with SSc, TNF expression is increased in the skin and sera of those with early dcSSc with extensive and progressive skin sclerosis, as shown in the current and previous studies (16). It is generally

accepted that Th2 cell–skewed immune polarization in early dcSSc largely contributes to progressive dermal fibrosis through production of profibrotic cytokines, especially interleukin-4 (IL-4) and IL-13. However, when cocultured in vitro, Th2 cells suppress type I collagen production in normal dermal fibroblasts because of the dominant antifibrotic effect of membrane-bound TNF, which overcomes the profibrotic effect of TGFβ, IL-4, and IL-13 (37). These clinical and experimental data suggest that SSc dermal fibroblasts have some self-protective system against TNF stimulation. Indeed, SSc dermal fibroblasts have been shown to be resistant to the antifibrotic effect of TNF (32), but the molecular mechanism of this resistance has remained elusive. Importantly, the present study demonstrates that gene silencing of progranulin reverses the sensitivity to the antifibrotic effect of TNF in SSc dermal fibroblasts. Thus, progranulin overproduction may be a key pathogenic event promoting the establishment of a profibrotic phenotype in SSc dermal fibroblasts under a Th2 cell–dominant microenvironment.

A possible mechanism underlying the progranulin-dependent resistance to TNF in SSc dermal fibroblasts is the blockade of TNFRs with autocrine progranulin. This notion is based on the following findings: 1) exogenous progranulin suppressed the antifibrotic effect of TNF in normal dermal fibroblasts, 2) SSc dermal fibroblasts expressed progranulin at higher levels compared with normal dermal fibroblasts, and 3) progranulin siRNA reversed the sensitivity to the antifibrotic effect of TNF in SSc dermal fibroblasts. Taken together with the previously reported evidence that progranulin serves as an antagonist of TNFRs (9), these results suggest that the antagonistic effect of progranulin on TNFRs largely contributes to the unresponsiveness of SSc dermal fibroblasts to TNF. In the current study, however, we did not confirm that progranulin affects the impact of TNF on dermal fibroblasts through its binding to TNFRs. Therefore, we cannot rule out the possibility that autocrine progranulin competes with the antifibrotic effect of TNF through other mechanisms in SSc dermal fibroblasts. Further studies are required to clarify this point.

Fli-1 regulates the expression of various fibrosis-related genes, and gene silencing of Fli-1 strongly induces a profibrotic phenotype in normal dermal fibroblasts, including the induction of types I, III, and V collagens and the inhibition of matrix metalloproteinase 1, which largely mimics the phenotype induced by TGFβ (26,27,29,30,38,39). Importantly, SSc is a multifactorial disease involving the complex interplay between genetic factors and environmental influences, and Fli-1 is epigenetically suppressed in fibroblasts in uninvolved skin in which fibrosis develops as well as in fibroblasts in involved skin with established tissue fibrosis (26,40). Therefore, Fli-1 deficiency is a potential predisposing factor related to environmental influences in the pathogenesis of SSc. As shown in the present study, Fli-1 bound to the promoter of *PGRN*, and gene silencing of Fli-1 increased progranulin expression in normal dermal fibroblasts. Therefore, progranulin is a member of the fibrosis-related genes that is coordinately regulated by Fli-1 deficiency toward the induction of a profibrotic phenotype in dermal fibroblasts.

In the skin of Fli-$1^{+/-}$ mice, dermal fibroblasts expressed progranulin at much higher levels than expected based on the results of in vitro experiments with Fli-1 siRNA–treated dermal fibroblasts (Figures 2A and F). A possible explanation for this discrepancy is the in vivo interaction of dermal fibroblasts with other types of cells with SSc-like phenotypes. For instance, Fli-1 haploinsufficiency induces the expression of a proangiogenic gene program in endothelial cells (41–46), mild distortion of dermal arterioles (47), and endothelial cell–to–mesenchymal cell transition (48) in vivo. These vascular changes, most likely in conjunction with other inflammatory cells with SSc-like phenotypes due to Fli-1 haploinsufficiency (48), further activate Fli-$1^{+/-}$ dermal fibroblasts, leading to the remarkable expression of progranulin in vivo.

Progranulin has a dual impact on inflammation in a context-dependent manner. As shown in the present clinical analyses, serum progranulin levels positively correlated with the CRP level in patients with SSc, suggesting that progranulin has a proinflammatory role in this disease. Further supporting this idea, we observed a close association between inflammatory skin symptoms and elevated progranulin levels in serum. Regarding the association of serum progranulin levels with disease duration, a significant inverse correlation was observed. Importantly, 7 of the 8 patients with dcSSc who had an elevated serum progranulin level were evaluated within 3 years after disease onset. Among the 4 patients with lcSSc who had an elevated serum progranulin level, 3 patients were evaluated within 3 years after disease onset, suggesting that these 3 patients may have had early dcSSc with limited skin involvement at the time of blood sampling. Thus, an elevated progranulin level was predominantly observed in patients with early dcSSc.

Given that progranulin modulates the innate immune response as a cofactor in the presentation of CpG oligonucleotides to TLR-9, progranulin overproduction may accelerate the pathologic process of early SSc through activation of interferon (IFN) signaling pathways. Activation of plasmacytoid dendritic cells by self DNA derived from apoptotic cells (especially endothelial cells) via TLR-7/9 has been implicated in the

pathologic fibrosis of SSc (3). Because production of an excessive amount of IFN by plasmacytoid dendritic cells, which infiltrate around dermal small vessels in early SSc (49), potentially induces immune activation and vascular injuries, progranulin overproduction in dermal fibroblasts promotes immunologic and vascular aspects of this disease in its early stage.

In summary, this is the first report of the role of progranulin in the developmental process of SSc. Excessive production of progranulin in SSc dermal fibroblasts, which is caused at least partially by Fli-1 deficiency, may induce fibroblast activation via protecting cells against the antifibrotic effect of TNF in vivo. These observations further underscore the notion that Fli-1 is a potential predisposing factor leading to the induction of tissue fibrosis in SSc.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Asano had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Ichimura, Asano, Tada, Sugaya, Sato, Kadono.

Acquisition of data. Ichimura.

Analysis and interpretation of data. Ichimura, Asano, Akamata, Noda, Taniguchi, Takahashi, Toyama, Tada, Sugaya, Sato, Kadono.

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CONCISE COMMUNICATION

Serum level of circulating syndecan-1: A possible association with proliferative vasculopathy in systemic sclerosis

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ABSTRACT

Syndecan-1 is a member of the transmembrane heparan sulfate proteoglycan family, whose membrane-bound and soluble forms are involved in wound healing, inflammation and vascular biology. Because these physiological events are implicated in the pathogenesis of systemic sclerosis (SSc), we investigated the clinical association of serum syndecan-1 levels in this disease. Serum syndecan-1 levels were significantly higher in SSc patients, both in diffuse cutaneous SSc (dcSSc) and limited cutaneous SSc (lcSSc), than in healthy individuals, while comparable between dcSSc and lcSSc groups. In late stage dcSSc patients (disease duration of >6 years), but not non-late stage dcSSc patients (≤6 years), serum syndecan-1 levels were significantly higher than in normal controls. More importantly, SSc patients with elevated serum syndecan-1 levels had higher prevalence of telangiectasia, elevated right ventricular systolic pressure and decreased diffuse capacity of the lung for carbon monoxide than those with normal levels. Therefore, soluble syndecan-1 may be related to the development of proliferative vasculopathy in SSc patients.

Key words: angiogenesis, pulmonary arterial hypertension, syndecan-1, systemic sclerosis, vasculopathy.

INTRODUCTION

Systemic sclerosis (SSc) is a multisystem connective tissue disease characterized by immune abnormalities, vasculopathy and tissue fibrosis with unknown etiology.[1] Evidence has suggested that aberrant vascular activation and remodeling is a key pathological feature leading to tissue fibrosis and vasculopathy characteristic of SSc.[2]

Syndecans are a family of transmembrane heparan sulfate proteoglycans composed of four closely related proteins (syndecan 1–4). Syndecans consist of the highly conserved transmembrane and cytoplasmic domains and the variable ectodomains that can be shed from the cell surface by matrix metalloproteinases and exert paracrine and autocrine effects. Both of membrane-bound and soluble syndecans regulate a variety of cell functions and behaviors, including growth, adhesion and movement, via integrating microenvironmental signals surrounding cells. This is mainly mediated by their roles as a receptor for extracellular matrix proteins and a reservoir for growth factors through binding via heparan sulfate chains.[3] Different syndecans have distinct distributions *in vivo*. In adult tissues, syndecan-1 is stably expressed in epithelial and plasma cells, but the detection of syndecan-1 on endothelial cells and other immune cells is seemingly difficult due to its dynamic regulation. Experimental data on animal models and human samples suggest that both membrane-bound and soluble forms of syndecan-1 play roles in wound healing, inflammation and vascular biology.[4–7] Reflecting its various roles, soluble syndecan-1 levels positively correlate with disease activity of systemic lupus erythematosus and Crohn's disease.[8,9] Based on these backgrounds, to investigate the potential role of syndecan-1 in SSc we evaluated the clinical correlation of serum syndecan-1 levels in this disease.

METHODS

Patients

Serum samples were obtained from 65 SSc patients (30 diffuse cutaneous SSc [dcSSc] and 35 limited cutaneous SSc [lcSSc])[10] who fulfilled the new classification criteria[11] and 20 healthy individuals after getting informed consent and institutional approval (University of Tokyo Graduate School of Medicine). Patients who had been treated with corticosteroids or immunosuppressants were excluded. The patients' information is shown in Figure 1.

Measurement of serum syndecan-1 levels

Specific enzyme-linked immunosorbent assay kits were used to measure serum syndecan-1 levels (Abcam, Cambridge, UK). Briefly, polystyrene 96-well plates coated with antihuman syndecan-1 antibody were incubated with twofold diluted serum and biotinylated antihuman syndecan-1 antibody at room

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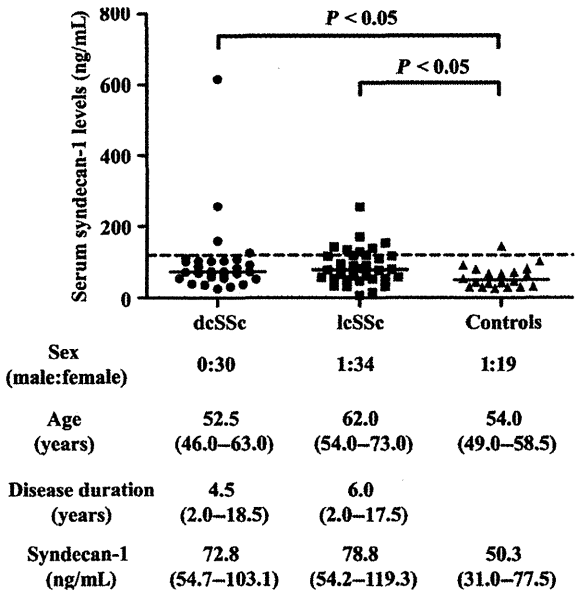

Figure 1. Serum syndecan-1 levels in systemic sclerosis (SSc) patients and healthy controls. Serum syndecan-1 levels were determined by a specific enzyme-linked immunoassay in diffuse cutaneous SSc (dcSSc), limited cutaneous SSc (lcSSc) and healthy controls. Bars indicate the median value in each group. A dotted line represents the cut-off value (119.6 ng/mL; mean + 2 standard deviations of healthy controls). Patients' information of each group is described below the graph. The values represent median with 25–75 percentiles in parenthesis. Statistical analysis was carried out with a Kruskal–Wallis test ($P = 0.027$) and a Steel–Dwass test for multiple comparison. *P*-values in the graph represent the results of a Steel–Dwass test for each pair.

temperature for 1 h. After washing the wells, horseradish peroxidase-conjugated streptavidin was added and incubated at room temperature for 30 min. Next, the wells were washed again, tetramethylbenzidine was added, and incubated at room temperature for 15 min. Finally, sulfuric acid was added to terminate the reaction and absorbance at 450 nm was measured. Serum syndecan-1 levels were calculated using a standard curve. Because epitope information is available for only one antibody recognizing the epitope close to aa107–aa110, some syndecan-1 sub-products including this epitope were measured in this assay.

Clinical assessment

Disease onset was defined as the first clinical event related to SSc other than Raynaud's phenomenon. Disease duration was defined as the interval between the onset and the time of blood sampling. The details of assessment for organ involvement are described in the Table 1 legend and a previous report.[12]

Statistical analysis

Statistical analysis was done with Mann–Whitney *U*-test for the comparison of skewed distribution, with a Kruskal–Wallis test and a Steel–Dwass test for multiple comparison, and with a Fisher's exact probability test for the analysis of frequency. Statistical significance was defined as a *P*-value of less than 0.05.

Table 1. Correlation of serum syndecan-1 levels with clinical symptoms in SSc patients

	Patients with elevated syndecan-1 levels (n = 12)	Patients with normal syndecan-1 levels (n = 53)
Sex, male:female	0:12	1:52
Age (years)	59.0 (46.8–70.3)	59.0 (49–68)
Disease duration (years)	15.5 (9.0–23.0)*	4.0 (2.0–11.3)
dcSSc : lcSSc	4:8	26:27
Cutaneous vascular symptoms		
Raynaud's phenomenon	92 (11/12)	92 (49/53)
Nail-fold bleeding	75 (9/12)	69 (36/52)
Telangiectasia	83 (10/12)*	46 (21/46)
Pitting scars	42 (5/12)	38 (20/53)
Organ involvements associated with proliferative vasculopathy		
Digital ulcers	58 (7/12)	36 (19/53)
Elevated RVSP	45 (5/11)*	10 (5/51)
Scleroderma renal crisis	8 (1/12)	2 (1/53)
Pulmonary function test		
Decreased %VC	20 (2/10)	12 (6/50)
Decreased %DLco	36 (4/11)*	10 (5/51)

Telangiectasia was examined on the hands and face. Elevated RVSP was defined as ≥35 mmHg on echocardiogram. Scleroderma renal crisis was defined as malignant hypertension and/or rapidly progressive renal failure. For age and disease duration, the values represent median with 25–75 percentiles in parenthesis. The frequencies of each symptom are shown as percentage with the exact number of evaluated patients in parenthesis. Statistical analysis was carried out with Mann–Whitney *U*-test or Fisher's exact probability test. *$P < 0.05$. dcSSc, diffuse cutaneous SSc; DLco, diffusing capacity of the lung for carbon monoxide; lcSSc, limited cutaneous SSc; RVSP, right ventricular systolic pressure; SSc, systemic sclerosis; VC, vital capacity.

RESULTS

Serum syndecan-1 levels in SSc patients

Serum syndecan-1 levels were significantly higher in SSc patients than in healthy controls (76.8 [54.2–109.8] vs 50.3 ng/mL [31.0–77.5]; $P = 0.0074$). Furthermore, serum syndecan-1 levels were significantly elevated in dcSSc and lcSSc patients (72.8 [54.7–103.1] and 78.8 ng/mL [54.2–119.3], respectively) compared with healthy controls (Fig. 1), while comparable between dcSSc and lcSSc patients. Given that dcSSc is characterized by extensive fibrosis, syndecan-1 may be associated with the pathological process of SSc other than tissue fibrosis. Supporting this idea, serum syndecan-1 levels did not correlate with modified Rodnan total skin thickness score in total SSc patients ($r = -0.01$ [$P = 0.93$]) and with scores of ground-glass opacity

and fibrosis in SSc patients with interstitial lung disease (r = 0.15 [P = 0.40] and r = −0.06 [P = 0.72], respectively).

Correlation of serum syndecan-1 levels with disease duration in dcSSc

We next assessed the link between serum syndecan-1 levels and disease duration in dcSSc. Because skin sclerosis generally progresses during the first 5–6 years and starts to regress afterwards in dcSSc,[13] we classified dcSSc patients into two groups: those with disease duration of 6 years or less (non-late stage) and those with disease duration of more than 6 years (late stage). As shown in Figure 2, serum syndecan-1 levels were higher in late stage dcSSc patients (82.9 ng/mL [46.3–141.8]), while not in non-late stage dcSSc patients (69.5 ng/mL [56.2–97.0]), than in healthy controls. Therefore, syndecan-1 seems to be poorly associated with tissue fibrosis, but contributes to certain pathological events in late stage dcSSc.

Correlation of serum syndecan-1 levels with clinical features related to vascular involvement in SSc

In contrast to dermal fibrosis, vasculopathy is gradual, but persistently progressive along with disease duration. Therefore, we further evaluate the association of serum syndecan-1 levels with SSc vasculopathy. To this end, we classified SSc patients into two groups based on the cut-off value (119.6 ng/mL [mean + 2 standard deviation of normal controls]): those with elevated syndecan-1 levels and those with normal levels. As shown in Table 1, disease duration was significantly longer in patients with elevated syndecan-1 levels than in those with normal levels, supporting the potential contribution of syndecan-1 to SSc vasculopathy. Regarding cutaneous vascular manifestations, there was no significant difference in the frequencies of Raynaud's phenomenon, nail-fold bleeding and pitting scars between these two groups, while telangiectasia was more frequently seen in patients with elevated syndecan-1 levels. As for vascular organ involvement, the frequency of elevated right ventricular systolic pressure (RSVP) was significantly greater in patients with elevated syndecan-1 levels than in the others, while the frequencies of digital ulcers and scleroderma renal crisis were comparable between the two groups. Furthermore, elevated syndecan-1 levels were linked to the higher prevalence of decreased percentage of diffusing capacity of the lung for carbon monoxide (%DLco) in SSc patients. Given that an isolated decrease in %DLco is related to pulmonary arterial hypertension (PAH),[14] these results suggest that elevation of circulating syndecan-1 levels is associated with SSc vasculopathy, especially telangiectasia and PAH.

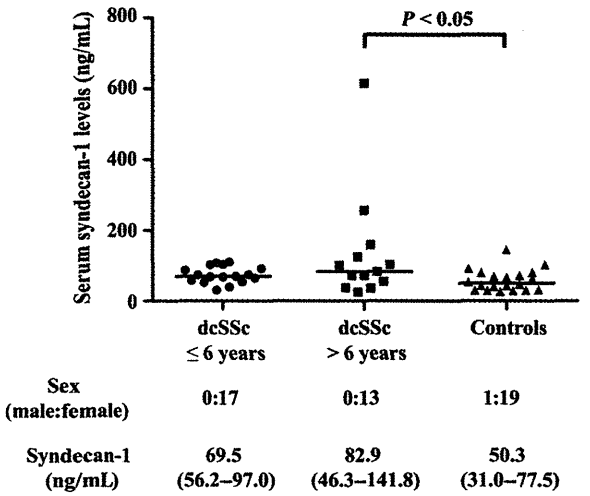

Figure 2. Serum vaspin levels in late stage and non-late stage diffuse cutaneous systemic sclerosis (dcSSc) patients. Serum vaspin levels were determined by a specific enzyme-linked immunoassay in late stage (disease duration of >6 years) and non-late stage dcSSc patients (disease duration of ≤6 years) and healthy controls. Bars indicate the median value in each group. Patients' information of each group is described below the graph. The values represent median with 25–75 percentiles in parenthesis. Statistical analysis was carried out with a Kruskal–Wallis test (P = 0.046) and a Steel–Dwass test for multiple comparison. P-values in the graph represent the results of a Steel–Dwass test for each pair.

DISCUSSION

We currently demonstrate the possible association of soluble syndecan-1 levels with the higher prevalence of telangiectasia and PAH in SSc patients, suggesting the involvement of soluble syndecan-1 in SSc vasculopathy.

The role of syndecan-1 in the physiological and pathological neovascularization has been well studied. In tumor angiogenesis, it is arguable that syndecan-1 can bind to pro-angiogenic factors such as vascular endothelial growth factor and subsequently present them to their respective receptors on endothelial cells.[4] As for wound healing, syndecan-1 is highly expressed in the vasculature of newly formed connective tissue of rat periodontal wound healing and primarily on endothelium of human neonatal skin after incisional injury.[5,6] Thus, syndecan-1 is inducible in the activated endothelial cells during wound healing, while its expression is marginal in endothelial cells under homeostatic condition. Given that SSc endothelial cells persistently show molecular changes characteristic of pro-angiogenic property,[15] SSc endothelial cells may be a potential source of syndecan-1.

Systemic sclerosis vasculopathy is believed to be caused by impaired vascular remodeling due to abnormally activated angiogenesis and defective vasculogenesis.[2] SSc vasculopathy is classified into two categories, including destructive vasculopathy and proliferative vasculopathy. Proliferative vasculopathy is characterized by proliferation of vascular cells, such as endothelial cells and pericytes/vascular smooth muscle cells, leading to the dilation of capillaries and stenosis of arterioles. These structural vascular changes are clinically seen as telangiectasia and proliferative obliterative vasculopathy (i.e. PAH, digital ulcers and scleroderma renal crisis), respectively. In this study, elevated serum syndecan-1 levels were associated with telangiectasia, elevated RVSP and decreased %DLco. Given that an isolated decrease in %DLco is an early marker of PAH,[14] an increase in serum syndecan-1 levels may be associ-

ated with proliferative vasculopathy through the activation of angiogenesis.

The limitation of this study is that the information about syndecan-1 sub-products measured in sera is incomplete. Strictly speaking, the present data revealed the potential contribution of some syndecan-1 sub-products including the epitope near aa107–aa110 to the development of proliferative vasculopathy in SSc.

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CONFLICT OF INTEREST: The authors have declared no conflicts of interest.

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CONCISE COMMUNICATION

Serum heparanase levels: A protective marker against digital ulcers in patients with systemic sclerosis

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ABSTRACT

Heparanase is an endo-β-D-glucuronidase cleaving heparan sulfate side-chains of heparin sulfate proteoglycans, which is involved in wound healing, inflammation, neovascularization and tumor progression through the degradation and remodeling of the extracellular matrix and the release of sequestered pro-angiogenic factors. Because heparanase-mediated biological processes seem to be involved in the development of systemic sclerosis (SSc), we investigated the clinical correlation of serum heparanase levels in patients with this disease. Serum heparanase levels were significantly higher in SSc patients than in healthy individuals, while comparable between diffuse cutaneous SSc and limited cutaneous SSc subgroups. On the other hand, SSc patients with digital ulcers had serum heparanase levels significantly lower than those without. These results suggest that serum heparanase levels may be elevated in SSc patients reflecting the contribution of heparanase-dependent biological processes to the development of SSc. SSc patients with high serum heparanase levels may be protected from the development of digital ulcers due to the increased release of sequestered pro-angiogenic factors such as vascular endothelial growth factor. Therefore, serum heparanase levels may serve as a protective marker against digital ulcers in SSc patients.

Key words: angiogenesis, digital ulcers, heparanase, systemic sclerosis, vascular endothelial growth factor.

INTRODUCTION

Systemic sclerosis (SSc) is a multisystem autoimmune disease characterized by vasculopathy and fibrosis of the skin and certain internal organs with unknown etiology. The injury and activation of small vasculature is proposed to be a seminal event leading to tissue fibrosis and vascular changes characteristic of SSc.[1]

Heparanase is an endo-β-D-glucuronidase capable of cleaving heparin sulfate (HS) side-chains of HS proteoglycans on cell surface and the extracellular matrix (ECM). Because HS contributes to the self-assembly and integrity of the ECM by binding structural proteins including collagens and the regulation of angiogenesis by sequestering angiogenic factors, heparanase promotes the degradation and remodeling of the ECM and the angiogenic responses by cleaving HS molecules. Under physiological condition, heparanase expression is restricted primarily to a couple of cell types including endothelial cells, keratinocytes and placental trophoblasts. During inflammation and immune responses, heparanase is induced in platelets, neutrophils, macrophages and activated lymphocytes, contributing to their extravasation.[2] In human tumor cells, heparanase expression is associated with local invasion and metastasis through promoting neovascularization, leading to poor prognosis.[3] Importantly, ectopic expression of heparanase accelerates tumor growth and heparanase gene silencing suppresses tumor progression in animal models.[4] Thus, heparanase is involved in a wide range of physiological and pathological processes related to the degradation and remodeling of the ECM and the angiogenic responses, such as wound healing, inflammation, neovascularization and tumor progression.

Because heparanase-related biological events seem to be involved in the development of SSc, we investigated the potential role of heparanase in this disease by evaluating the clinical correlation of serum heparanase levels.

MATERIAL AND METHODS

Patients

Serum samples were obtained from 65 SSc patients (64 women, one man; age, median [25–75 percentiles]: 62 years [49–69]; disease duration 6 years [2–15]) and 20 healthy individuals (19 women, one man; age 54 years [49–58.5]) after obtaining informed consent and institutional approval (University of Tokyo Graduate School of Medicine). Patients having been treated with corticosteroids or immunosuppressants prior to blood sampling were excluded. Patients were grouped by

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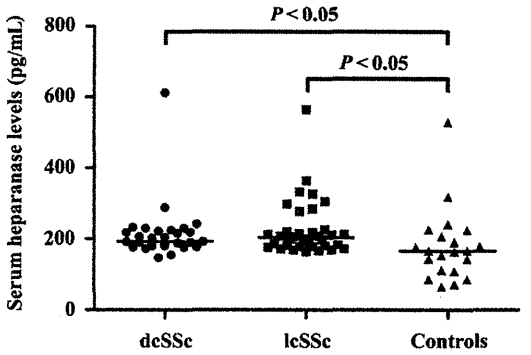
LeRoy's classification system:[5] 29 with diffuse cutaneous SSc (dcSSc) and 36 with limited cutaneous SSc (lcSSc). All patients fulfilled the new classification criteria of SSc.[6] Patients' information of disease subgroups is shown in Figure 1.

Measurement of serum human heparanase levels

Specific enzyme-linked immunosorbent assay kits were used to measure serum human heparanase levels (BlueGene Biotech, Shanghai, China). Briefly, polystyrene 96-well plates coated with anti-human heparanase antibody were incubated with two-fold diluted serum and biotinylated anti-human heparanase antibody at room temperature for 1 h. After washing the wells, horseradish peroxidase-conjugated streptavidin was added and incubated at room temperature for 30 min. Next, the wells were washed again, tetramethyl-benzidine added, and incubated at room temperature for 15 min. Finally, sulfuric acid was added to terminate the reaction and absorbance at 450 nm was measured. Serum heparanase levels were calculated using a standard curve.



	dcSSc	lcSSc	Controls
Sex (male:female)	0:29	1:35	1:19
Age (years)	57.0 (46.5–63.0)	65.5 (55.0–73.8)	54.0 (49.0–58.5)
Disease duration (years)	4.0 (2.0–12.0)	7.5 (2.0–20.0)	
Heparanase (pg/mL)	193.0 (180.5–223.5)	203.5 (179.5–224.5)	166.5 (110.0–215.5)

Figure 1. Serum heparanase levels in patients with diffuse cutaneous systemic sclerosis (dcSSc), those with limited cutaneous SSc (lcSSc) and healthy individuals (controls). Serum heparanase levels were determined using a specific enzyme-linked immunosorbent assay (ELISA). Horizontal lines indicate the median value in each group. Patients' information of each group is described below the graph. The values represent median with 25–75 percentiles in parentheses. Statistical analysis was carried out with a Kruskal–Wallis test ($P = 0.0074$) and a Steel–Dwass test for multiple comparison. *P*-values in the graph represent the results of a Steel–Dwass test for each pair.

Clinical assessment

Disease onset was defined as the first clinical event that was a clear manifestation of SSc other than Raynaud's phenomenon. The duration of disease was defined as the interval between the onset and the time of blood sampling. The details of assessment for organ involvement are described in Table 1 legends and a previous report.[7]

Statistical analysis

Statistical analysis was carried out with a Mann–Whitney *U*-test for the comparison of skewed distribution, with a Kruskal–Wallis test and a Steel–Dwass test for multiple comparison, and with Spearman's rank correlation coefficient for correlations with clinical data. Statistical significance was defined as $P < 0.05$.

RESULTS

Serum heparanase levels in SSc patients

Serum heparanase levels were significantly higher in SSc patients than in healthy individuals (202.5 pg/mL [181.1–225.3] vs 166.5 pg/mL [110.0–215.5], $P < 0.05$). When we classified patients into dcSSc and lcSSc subgroups, serum heparanase levels were also significantly higher in dcSSc (193.0 pg/mL [180.5–223.5]) and lcSSc (203.5 pg/mL [179.5–224.5]) patients than in healthy individuals ($P < 0.05$, multiple comparison), whereas there was no significant difference between dcSSc and lcSSc groups. Given that dcSSc is characterized by extensive tissue fibrosis, serum heparanase levels seem not to reflect the severity of tissue fibrosis. Consistently, serum heparanase levels did not correlate with modified Rodnan total skin thickness score ($r = -0.008$, $P = 0.95$), percentage vital capacity ($r = 0.02$, $P = 0.89$) and percentage of diffusing capacity of the lung for carbon monoxide ($r = -0.07$, $P = 0.59$). Therefore, heparanase may be associated with certain pathological processes other than fibrosis in SSc.

Clinical correlation of serum heparanase levels in SSc patients

We further examined the clinical correlation of serum heparanase levels especially by focusing on SSc vasculopathy, such as Raynaud's phenomenon, nailfold bleeding, telangiectasia, digital ulcers (DU), pulmonary arterial hypertension and scleroderma renal crisis (SRC). To this end, we compared serum heparanase levels between SSc patients with each symptom and those without. The presence of cutaneous vascular symptoms, including Raynaud's phenomenon, nailfold bleeding and telangiectasia, did not affect serum heparanase levels. Regarding organ involvement associated with proliferative obliterative vasculopathy, SSc patients with current and past history of DU had serum heparanase levels that were significantly lower than those without, while the presence of elevated right ventricular systolic pressure or SRC did not alter serum heparanase levels. Finally, we assessed the correlation of serum heparanase levels with systemic inflammatory markers, such as C-reactive protein and erythrocyte sedimentation rate, but there were no significant correlations ($r = 0.13$ [$P = 0.32$] and $r = 0.06$

Table 1. Correlation of serum heparanase levels with clinical features in systemic sclerosis patients

	Serum heparanase levels in patients with symptoms	Serum heparanase levels in patients without symptoms
Raynaud's phenomenon	201.0 [180.0–220.0] (n = 59)	208.5 [180.8–257.8] (n = 6)
Nailfold bleeding	201.0 [182.0–220.0] (n = 47)	200.0 [174.0–254.0] (n = 17)
Telangiectasia	203.5 [183.5–223.0] (n = 34)	198.5 [176.8–227.0] (n = 26)
Digital ulcers	189.0 [176.5–216.0] * (n = 25)	208.0 [182.8–231.3] (n = 40)
Elevated RVSP	201.0 [179.0–285.0] (n = 11)	201.5 [180.8–222.8] (n = 54)
Scleroderma renal crisis	191.0 [186.0–208.0] (n = 3)	202.0 [179.8–225.3] (n = 62)

Elevated right ventricular systolic pressure (RVSP) was defined as ≥35 mmHg on echocardiogram. Scleroderma renal crisis was defined as malignant hypertension and/or rapidly progressive renal failure. Statistical analysis was carried out with a Fisher's exact probability test for the analysis of frequency. Values represent median with 25–75 percentiles in square brackets and the number of patients in parentheses. *P < 0.05.

[P = 0.62], respectively). Collectively, these results suggest that the decrease in serum heparanase levels is linked to the development of DU in SSc patients.

DISCUSSION

The initial finding of this study was the elevation of serum heparanase levels in dcSSc and lcSSc patients compared with healthy controls, suggesting the contribution of heparanase to the development of SSc. Given the comparable serum heparanase levels between dcSSc and lcSSc groups and no correlation of serum heparanase levels with systemic inflammatory markers, serum heparanase levels appear to reflect neither the severity nor the activity of tissue fibrosis and inflammation characteristic of SSc. As for vasculopathy, SSc patients with the current and past history of DU showed significantly lower serum heparanase levels than those without, suggesting that SSc patients with high serum heparanase levels are protected from DU. Although these results are counterintuitive, a similar result has been reported on vascular endothelial growth factor (VEGF). Serum VEGF levels are elevated in SSc patients compared with healthy controls, suggesting the role of VEGF in the development of SSc. However, SSc patients with high serum VEGF levels are protected from DU.[8] Given that heparanase promotes the release of VEGF sequestered in the ECM, it is plausible that serum levels of VEGF and heparanase exhibit similar clinical correlation with DU in SSc patients.

Systemic sclerosis vasculopathy is thought to be caused by impaired vascular remodeling due to defective vasculogenesis and aberrantly activated angiogenesis.[9,10] Constitutive activation of angiogenesis is attributable to imbalance between pro-angiogenic and antiangiogenic factors, in which pro-angiogenic factors are persistently predominant.[11,12] VEGF is a member of pro-angiogenic factors upregulated in the lesional skin of SSc patients and has been implicated in the development of vasculopathy and fibrosis of this disease.[13] Heparanase has been shown to be involved in the regulation of angiogenesis under the physiological and pathological conditions primarily by release of ECM-resident angiogenic factors such as basic fibroblast growth factor and VEGF.[4,14,15] Furthermore, endogenous heparanase regulates the expression of VEGF through the activation of Src family members in various cell lines.[4] Therefore, heparanase expression may correlate with the availability of functionally active VEGF *in vivo*. Based on this idea, SSc patients with high serum heparanase levels are likely to have biologically active VEGF at high levels, which may result in the low frequency of DU due to the predominance of pro-angiogenic factors relative to antiangiogenic factors as previously speculated.[8]

In summary, this article is the first report of the potential contribution of heparanase to the development of SSc. A series of clinical analyses suggests that serum heparanase levels may serve as a protective marker against DU in SSc patients. Further experimental studies are required to clarify the role of heparanase in SSc.

CONFLICT OF INTEREST: None.

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