sections were mounted on APES (amino-propyl-triethoxy-silane)-coated slides, deparaffinized with xylene, and rehydrated through a graded series of ethanols. Endogenous peroxidase was blocked by incubation in 3 % hydrogen peroxide for 30 min. The sections were then incubated with anti-human CXCL5 antibody (R&D Systems), anti-mouse CXCL5 antibody (PeproTech, Rocky Hill, NJ, USA), or anti-Fli1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted with blocking buffer overnight at 4 °C, followed by incubation with biotinylated secondary antibody. The immunoreactivity was visualized with diaminobenzidine and the sections were counterstained with hematoxylin or methyl green.

Cell culture

Human dermal microvascular ECs (HDMECs) were purchased from Lonza (Walkersville, MD, USA) and cultured on collagen-coated tissue culture plates in EBM-2 medium supplemented with the EGM-2 Bullet Kit (Lonza). Experiments were conducted with HDMECs in passages 3–5.

Gene silencing of Fli1

HDMECs were seeded shortly before transfection. The cells were transfected with 10 nM of Fli1 siRNA or non-silencing scrambled RNA (SCR) (Santa Cruz Biotechnology) using HiPerfect transfection reagent (Qiagen, Valencia, CA, USA) for 72 h. Cells were then serum starved for the last 24 h.

RNA isolation and quantitative real-time PCR

RNA isolation from cultured cells and quantitative real-time PCR were carried out as described previously [2]. The sequences of primers were as follows: CXCL5 forward 5′-GGTCCTTCGAGCTCCTTGT-3′ and reverse 5′-ACGC AGCTCTCTCAACACAG-3′; Fli1 forward 5′-GGATGGC AAGGAACTGTGTAA-3′ and reverse 5′-GGTTGTATAG GCCAGCAG-3′; GAPDH forward 5′-ACCCACTCCTCC ACCTTTGA-3′ and reverse 5′-CATACCAGGAAATGAG CTTGACAA-3′.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was carried out using EpiQuik ChIP kit (Epigentek, Farmingdale, NY, USA). Briefly, cells were treated with 1 % formaldehyde for 10 min. The cross-linked chromatin was then prepared and sonicated to an average size of 300–500 bp. The DNA fragments were immunoprecipitated with anti-Fli1 antibody (Santa Cruz) or normal rabbit IgG at 4 °C. After reversal of cross-linking, the immunoprecipitated chromatin was amplified by PCR amplification of specific region of the CXCL5 genomic locus. Putative Fli1 transcription factor binding site was predicted by Tfsitescan. The primers were as follows: CXCL5/F-411, 5′-TGCCATGGAGCAAGA-CAGT-3′; CXCL5/R-203, 5′-ACAGGTTTCTGGAACCA TCG-3′. The amplified DNA products were resolved by agarose gel electrophoresis.

Statistical analysis

Statistical analysis was carried out with the Mann–Whitney *U* test for two-group comparison, 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. Correlations with clinical data were assessed by Spearman's rank correlation coefficient. Statistical significance was defined as a P value of <0.05.

Results

Serum CXCL5 levels in SSc

Serum CXCL5 levels were significantly decreased in SSc patients compared with healthy controls [median (25–75 percentiles); 276.3 pg/ml (152.7–573.8) versus 548.6 pg/ml (428.1–993.6), $p = 0.0036$; Mann–Whitney *U* test). Since the expression profiles of certain growth factors and cytokines can be quite different between dcSSc and lcSSc, we also evaluated serum CXCL5 levels in these subgroups. As shown in Fig. 1a, serum CXCL5 levels were significantly lower in dcSSc patients [276.3 pg/ml (145.8–615.6)] and lcSSc patients (274.9 pg/ml (162.7–548.5)] than in healthy subjects ($p < 0.05$ for each; a Kruskal–Wallis test and a Steel–Dwass' test). Given that dcSSc is characterized by extensive fibrosis to a much greater extent than lcSSc, these results suggest that CXCL5 plays little role in fibroblast activation, but is potentially associated with other pathological events in SSc. Supporting this idea, serum CXCL5 levels did not correlate with MRSS, the percentage of predicted vital capacity and the percentage of predicted diffusion lung capacity for carbon monoxide in total SSc, dcSSc, and lcSSc (data not shown).

The association between serum CXCL5 levels and disease duration in dcSSc

Since serum levels of biomarkers associated with disease process are usually different in each disease stage of dcSSc [21–25, 29–32], we classified dcSSc patients into three groups, such as early stage (disease duration of <1 years), mid-stage (1–6 years), and late stage (>6 years) according to the dynamics of matrix metalloproteinase (MMP) 1 gene expression along with disease duration in this disease

subtype [17], and compared serum CXCL5 levels among these three groups and healthy controls. As shown in Fig. 1b, serum CXCL5 levels were significantly lower in early stage dcSSc than in mid-stage dcSSc and healthy controls ($p < 0.05$ for each; a Kruskal–Wallis test and a Steel–Dwass' test). Importantly, serum CXCL5 levels were uniformly and markedly decreased in early stage dcSSc, while largely distributed in mid-stage dcSSc, suggesting that serum CXCL5 levels decrease during the initiation of fibrosis and start increasing afterward along with disease duration at least up to 6 years in dcSSc. Therefore, CXCL5 may be potentially involved in disease process of dcSSc with disease duration of <6 years.

Clinical correlation of serum CXCL5 levels in dcSSc patients

Closely looking at the distribution of serum CXCL5 levels in late stage dcSSc, there were several SSc patients with quite low serum CXCL5 levels, suggesting that CXCL5 in sera keeps low levels in a certain subset of dcSSc regardless of disease duration. Since serum CXCL5 levels were uniformly decreased in early stage dcSSc, we set a cut-off value at 300.7 pg/ml (mean + 2SD of serum CXCL5 levels in early stage dcSSc, shown as a dotted line in Fig. 1b) and classified mid-stage dcSSc and late stage dcSSc into two groups; patients with serum CXCL5 levels higher than the cut-off value and those with serum CXCL5 levels lower than the cut-off value. These two groups potentially represent dcSSc patients with persistently decreased serum CXCL5 levels throughout the disease course and those with serum CXCL5 levels increasing along with disease duration, respectively. Since CXCL5 has a potent pro-angiogenic property, we focused on cutaneous and visceral vascular involvement and compared the prevalence of these clinical features between these two groups (Table 1). As for cutaneous vascular involvement, including Raynaud's phenomenon, nailfold bleeding, pitting scars, and telangiectasia, there was no significant difference in the prevalence of these clinical features between the two groups. Regarding organ involvement associated with proliferative obliterative vasculopathy, the prevalence of digital ulcers was significantly elevated in dcSSc patients with serum CXCL5 levels lower than the cut-off value compared to those with serum CXCL5 levels higher than the cut-off value (80 versus 29 %, $p = 0.036$; Fisher's exact test), while the prevalence of elevated right ventricular systolic pressure (RVSP) or scleroderma renal crisis (SRC) was not significantly different between the two groups. Taken together with the evidence that only one

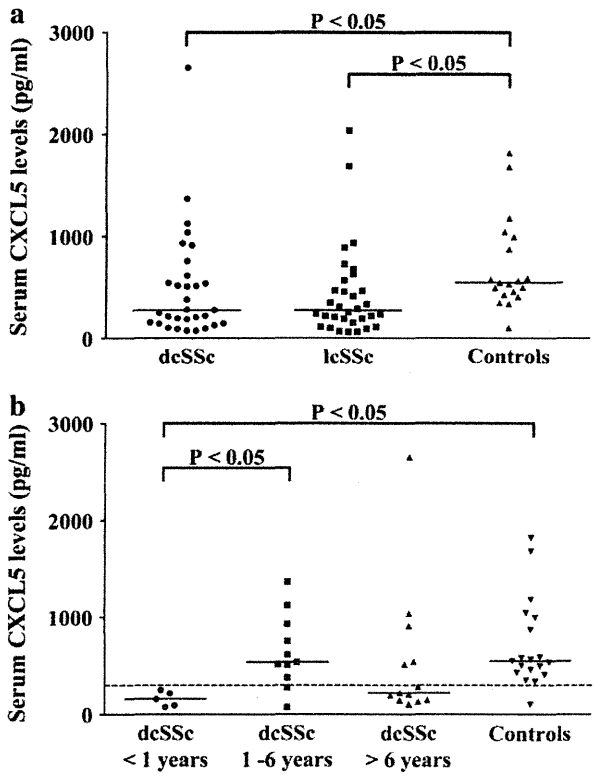

Fig. 1 Serum CXCL5 levels in SSc patients and healthy controls. **a** Serum CXCL5 levels were determined by a specific ELISA. SSc patients were classified into two subgroups, including dcSSc and lcSSc. **b** dcSSc patients were divided into three subgroups: those with disease duration of <1 years, those with disease duration of 1–6 years, and those with disease duration of >6 years. The horizontal bars indicate the median value in each group. The dotted bars indicate the cut-off value (mean + 2SD of early-stage). The statistical analysis was carried out with a Kruskal–Wallis test and a Steel–Dwass' test for multiple comparison

Table 1 Correlation of serum CXCL5 levels with clinical features in mid-stage and late stage dcSSc

	dcSSc (disease duration ≥1 year)	
	Serum CXCL5 levels lower than the cut-off value ($n = 10$)	Serum CXCL5 levels higher than the cut-off value ($n = 14$)
Nailfold bleeding	50 (5/10)	64 (9/14)
Pitting scars	70 (7/10)	36 (5/14)
Telangiectasia	56 (5/9)	58 (7/12)
Raynaud's phenomenon	90 (9/10)	86 (12/14)
Digital ulcers	80 (8/10)*	29 (4/14)
Elevated RVSP	30 (3/10)	10 (1/10)
Scleroderma renal crisis	10 (1/10)	0 (0/14)

Elevated right ventricular systolic pressure (RVSP) was defined as 35 mmHg or more 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 are percentages

* $p < 0.05$

patient had digital ulcers among five early stage dcSSc patients, these results indicate that the decrease in serum CXCL5 levels may serve as a marker for the development of digital ulcers in mid-stage and late stage dcSSc.

The expression levels of CXCL5 protein were much more decreased in dermal blood vessels of early stage dcSSc patients than in those of healthy controls

To further investigate the main source of CXCL5 explaining its decreased levels in sera, we carried out immunohistochemistry using skin sections from five dcSSc patients (<1 year) and five closely matched healthy controls. Since similar results were obtained from these skin sections, the representative results were shown in Fig. 2. The expression of CXCL5 protein was abundant in dermal blood vessels of control skin, while markedly decreased in those of SSc skin. CXCL5 expression was also observed in epidermal keratinocytes and marginally in dermal fibroblasts and perivascular inflammatory cells, but the levels were comparable between SSc and healthy controls. These results suggest that the reduction of serum CXCL5 levels in early stage dcSSc is attributable to the decreased expression of CXCL5 gene in dermal blood vessels.

Fli1 deficiency contributes to the downregulation of CXCL5 gene in SSc ECs

As described above, CXCL5 was expressed in dermal blood vessels, which consist of ECs and pericytes/vascular smooth muscle cells. Since previous reports demonstrated that the main source of CXCL5 is vascular ECs in human and mice [5, 8, 14], we next examined the mechanism by which CXCL5 expression is suppressed in dermal microvascular ECs of SSc lesional skin. To this end, we focused on the effect of Fli1 deficiency on endothelial CXCL5 expression in vitro and in vivo because Fli1 is constitutively downregulated in SSc dermal microvascular endothelial cells (the bottom panels in Fig. 2) and endothelial Fli1 deficiency reproduces the histopathological and functional abnormalities of dermal small vessels, which are

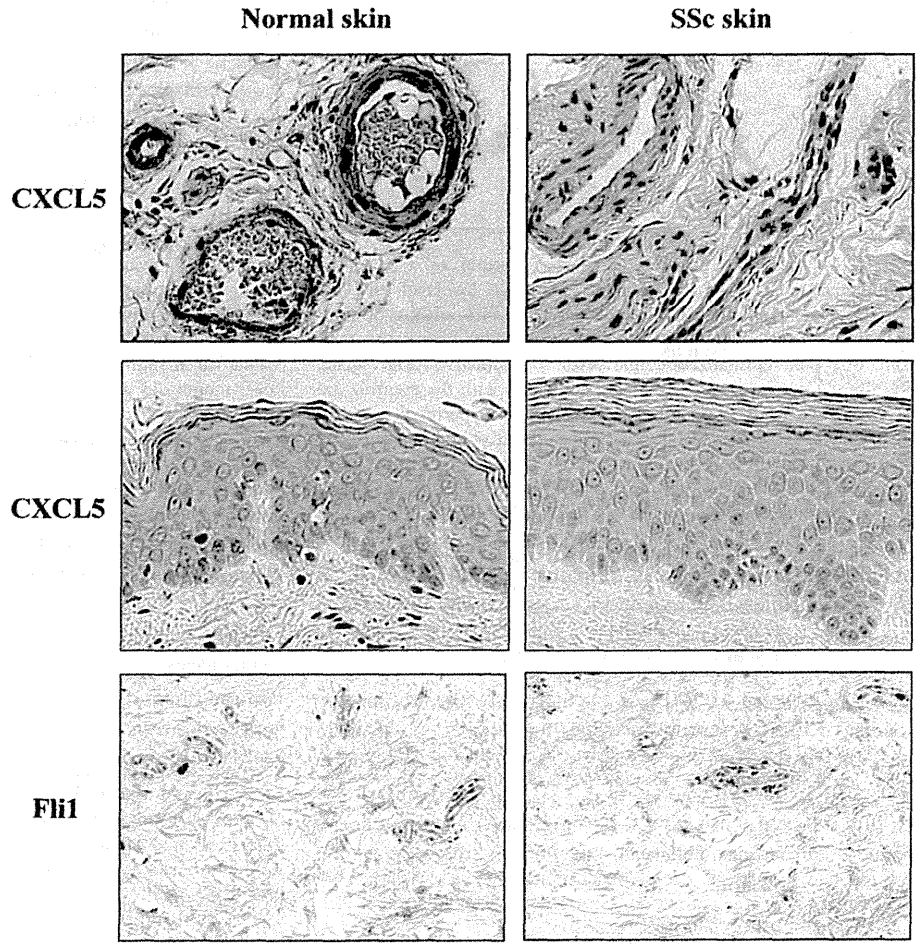

Fig. 2 CXCL5 protein levels were decreased in dermal small vessels of early dcSSc patients. CXCL5 and Fli1protein levels were determined by immunohistochemistry in the skin of five dcSSc patients with disease duration of <1 years and closely matched healthy controls. The levels of CXCL5 proteins were much more decreased in small blood vessels of SSc patients than in those of closely matched healthy controls (*top panels*), while the levels were comparable in keratinocytes (*middle panels*). As for Fli1 proteins, the levels were markedly decreased in endothelial cells and fibroblasts of SSc skin compared with those of healthy skin (*bottom panels*). A set of representative results is shown

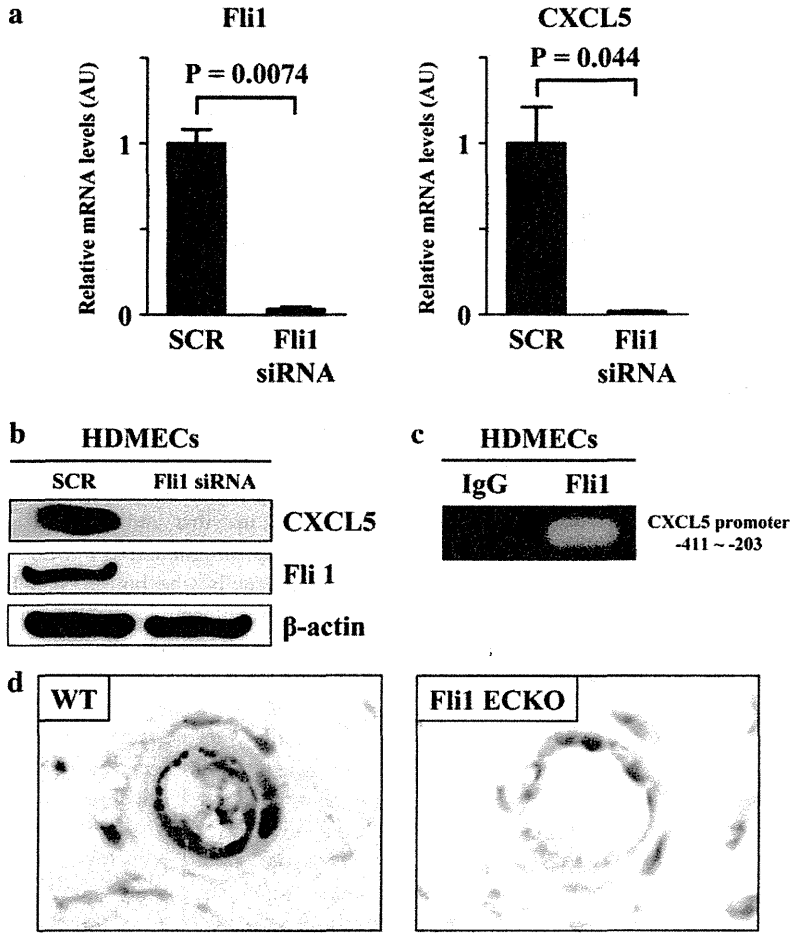

Fig. 3 Fli1 deficiency suppressed the expression of CXCL5 in endothelial cells in vivo and in vitro. **a** mRNA levels of Fli1 and CXCL5 genes in HDMECs transfected with Fli1 siRNA or non-silencing scrambled RNA (SCR) were examined by quantitative real-time PCR and normalized to the levels of human GAPDH gene. Results of controls or relative value compared with the controls are expressed as mean ± SD of three independent experiments. Statistical analysis was carried out with a two-tailed paired *t* test. **b** The levels of Fli1 and CXCL5 proteins were determined by immunoblotting in HDMECs treated with Fli1 siRNA or SCR. The levels of β-actin were examined to confirm the equal loading of samples. **c** Chromatin was isolated from HDMECs and immunoprecipitated using rabbit anti-Fli1 antibody or rabbit IgG. After isolation of bound DNA, PCR amplification was carried out using CXCL5 promoter-specific primers. One representative of three independent experiments is shown. **d** Immunostaining of CXCL5 proteins in the skin sections of 3-month-old wild type and Fli1 ECKO mice (original magnification was ×400) by Vectastain ABC kit according to the manufacturer's instruction. Representative results of arterioles in five wild type and five Fli1 ECKO mice are shown. Similar staining levels were seen in all of the other mice

characteristic of SSc vasculopathy [4]. As shown in Fig. 3a–c, gene silencing of Fli1 significantly suppressed the expression levels of CXCL5 gene at mRNA levels and at protein levels and Fli1 occupied the promoter region of CXCL5 gene in HDMECs. Furthermore, the expression levels of CXCL5 protein were markedly decreased in dermal small vessels of Fli1 ECKO mice compared with those of wild type mice (Fig. 3d). Collectively, these results indicate that CXCL5 is a member of angiogenesis-related gene program regulated by Fli1 and that endothelial CXCL5 expression is suppressed at least partially due to Fli1 deficiency in SSc skin.

Discussion

Recent studies regarding the possible contribution of CXCL5 to angiogenesis under various pathological conditions, including RA and IBD, stimulated our interest to study the role of CXCL5 in the developmental process of SSc. As an initial step to address this issue, we investigated the clinical significance of serum CXCL5 levels in patients with SSc. Serum CXCL5 levels were significantly decreased in dcSSc and lcSSc patients compared with healthy controls, but the levels were comparable between the patient subgroups, suggesting the little role of CXCL5

in the fibrotic process of SSc. When we focused on the association of serum CXCL5 levels with disease duration in dcSSc, serum CXCL5 levels were uniformly decreased in dcSSc with disease duration of <1 year and positively correlated with disease duration in dcSSc with disease duration of <6 years. Furthermore, in non-early dcSSc (≥1 year), patients with serum CXCL5 levels lower than the cut-off value had an increased risk for the development of digital ulcers than those with serum CXCL5 levels higher than the cut-off value. Collectively, these results suggest that the decrease in CXCL5 expression is associated with the initiation of disease process and the mechanism related to the development of digital ulcers in dcSSc.

Increasing evidence suggests that SSc vasculopathy is caused by aberrant angiogenic process [20]. For example, pericytes/vascular smooth muscle cells are constitutively activated in SSc skin, which is characterized by high expression of regulator of G-protein signaling 5 [11] and low expression of α-smooth muscle actin [4] in those cells, and EC–EC interaction is attenuated by the down-regulation of vascular endothelial-cadherin and platelet/endothelial cell adhesion molecule 1 [4]. Furthermore, endothelial MMP9 expression, which is induced during the initiation of angiogenesis and degrades vascular basement membrane, is potentially increased in SSc ECs [4, 15]. Thus, vascular changes associated with SSc vasculopathy is characterized by immature vessels possibly caused by aberrant induction of angiogenic gene program. Fli1 is a member of Ets transcription factor family, whose deficiency in ECs has been implicated in the pathogenesis of SSc vasculopathy [4, 24, 25]. Our previous reports demonstrated that Fli1 ECKO mice reproduce the histological and functional abnormalities of SSc vasculopathy, including stenosis of arterioles, dilation of capillaries, and increased vascular permeability [4]. Importantly, in HDMECs gene silencing of Fli1 modulates the expression of various genes, including VE-Cadherin, PECAM1, MMP9, cathepsin B, and cathepsin V, towards an SSc EC phenotype [4, 24, 25]. The present observation that Fli1 bound to the CXCL5 promoter and gene silencing of Fli1 significantly suppressed the CXCL5 mRNA levels in HDMECs indicates that CXCL5 is a member of angiogenesis-related gene regulated by Fli1 and that the decreased endothelial CXCL5 expression inversely reflects the aberrant angiogenic status caused by Fli1 deficiency in SSc patients. Consistently, serum CXCL5 levels were decreased in early SSc characterized by abnormal vascular activation and decreased serum CXCL5 levels during the progression of the disease magnified the risk for the development of digital ulcers. Thus, serum CXCL5 levels may serve as an inverse marker for the severity of SSc vasculopathy.

The decrease of CXCL5 expression in SSc ECs is plausible when considering the unique property of CXCL5. Unlike other pro-angiogenic factors, CXCL5 serves as a chemoattractant for neutrophils. Therefore, CXCL5 may be involved in the pathological angiogenesis accompanied with neutrophil infiltration, which are generally seen in RA and IBD. Given that anti-CXCL5 antibody attenuates experimental arthritis and bowel inflammation, CXCL5 plays a pivotal role in the pathological angiogenesis of these autoimmune inflammatory diseases. In contrast, SSc vasculopathy is histologically characterized by perivascular infiltration of mononuclear cells, but not of neutrophils, suggesting that the expression of chemokines with neutrophil chemoattractant property is not induced during the fibrotic process of SSc. Consistently, serum and endothelial CXCL5 levels were decreased in SSc patients, especially in early dcSSc. These results indicate that the molecular mechanism of aberrant angiogenesis is quite different from the beginning of disease onset between SSc and RA even though a certain subset of RA and SSc initially shares the clinical symptoms, such as Raynaud's phenomenon and puffy fingers. The evidence that Fli1 activates a set of gene programs promoting angiogenesis while suppressing CXCL5 gene expression further supports the idea that Fli1 deficiency orchestrates the expression of angiogenesis-related genes eventually leading to the development of SSc vasculopathy.

In summary, this is the first report regarding the role of CXCL5 in the developmental process of SSc. The present data suggest the little role of CXCL5 in the pathogenesis of SSc, but provide us a new clue to further understand the difference in the pathogenesis of vascular involvement between SSc and other autoimmune diseases, including RA.

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Conflict of interest The authors declare that they have no conflict of interest.

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CORRESPONDENCE

Serum levels of mannose-binding lectin in systemic sclerosis: a possible contribution to the initiation of skin sclerosis in the diffuse cutaneous subtype

Systemic sclerosis (SSc) is a multisystem connective tissue disease characterized by immune abnormalities, vasculopathy and fibrosis of the skin and certain internal organs. Recent evidence suggests the potential contribution of a dysregulated innate immune system to its pathogenesis [1]. Mannose-binding lectin (MBL), a member of the collectin family produced mainly by liver hepatocytes and, to a smaller extent, by gastrointestinal and reproductive tracts, binds to various pathogens as a pattern-recognition molecule and plays a role in innate immune defense by activating the complement system or by acting directly as an opsonin. MBL expression is decreased in certain people by mutations and promoter polymorphisms in the *MBL2* gene and genetic MBL deficiency (5-10% of the general population) and is associated with increased risk, severity and frequency of infections and autoimmunity. Low circulating MBL is associated with a poorer outcome in systemic lupus erythematosus and rheumatoid arthritis. Whether genetic MBL deficiency increases susceptibility to these diseases and is influenced by ethnicity is still controversial [2].

To evaluate the role of MBL in SSc, we measured serum MBL levels by a specific ELISA (R&D Systems, Minneapolis, MN, USA) in 63 SSc (33 diffuse cutaneous SSc (dcSSc) and 30 limited cutaneous SSc (lcSSc)) and 10 healthy subjects. All patients but 3 lcSSc fulfilled the criteria of the American College of Rheumatology. Those 3 had sclerodactyly and at least two other features; calcinosis, Raynaud's phenomenon, esophageal dysmotility and telangiectasia. No patients treated with corticosteroids and/or immunosuppressants were enrolled. The study was performed according to the Declaration of Helsinki and approved by the ethical committee of the University of Tokyo, Graduate School of Medicine. Written informed consent was obtained from all participants.

Since genetic information of participants was unavailable, we determined the frequency of MBL insufficiency among total SSc, dcSSc, lcSSc and healthy subjects, based on the cut-off level of 600 ng/mL [3], but failed to detect any differences among them (22%, 18%, 27% and 27%, respectively *(figure 1A)*, which are similar to rates in the general population [4]), suggesting that MBL insufficiency is rarely associated with the development of SSc. Serum MBL levels positively correlated with the modified Rodnan total skin thickness score [5] in dcSSc ($r = 0.51$, $p = 0.0044$; *figure 1B*), but not in lcSSc ($r = -0.15$, $p = 0.44$). Furthermore, the correlation was also seen in dcSSc with disease duration of ≤ 6 years ($r = 0.50$, $p = 0.015$), but not in dcSSc with disease duration of >6 years ($r = -0.26$, $p = 0.66$), and serum MBL levels positively correlated with disease duration in dcSSc ($r = 0.42$, $p = 0.014$; *figure 1C*). Given that skin sclerosis generally progresses during the first 5-6 years but spontaneously regresses afterwards in dcSSc [6], serum MBL levels appear to decrease during the initiation of skin fibrosis in dcSSc. However, serum MBL levels did not correlate with the values of pulmonary function tests, right ventricular systolic pressure, immunoglobulin G and inflammatory markers, and were not affected by the presence of vascular clinical symptoms (Raynaud's phenomenon, nailfold bleeding, pitting scars, digital ulcers, elevated right ventricular systolic pressure (>35 mmHg) and scleroderma renal crisis) in SSc (data not shown). Collectively, these results suggest that MBL potentially contributes to the mechanism associated with the initiation of skin fibrosis in dcSSc.

Evidence has demonstrated the pivotal role of toll-like receptors (TLRs), especially TLR4, in the activation of fibroblasts [7], macrophages [8], and B cells [9] in SSc. Importantly, MBL directly interacts with TLR4 and attenuates LPS-induced inflammatory cytokine secretion and nuclear factor-κB activity in THP-1 cells [10]. Therefore, low circulating MBL may potentially amplify activation of the TLR4-mediated pro-fibrotic system in the early stages of dcSSc. Further studies regarding the expression of MBL in the skin of SSc and healthy subjects are required to assess this hypothesis because MBL is modestly expressed in gastrointestinal and reproductive tracts, which serve as a first-line of defense as well as the skin.

Although the current interpretation is limited, because MBL expression is regulated genetically as well as pathologically to a different extent in each patient, and the number

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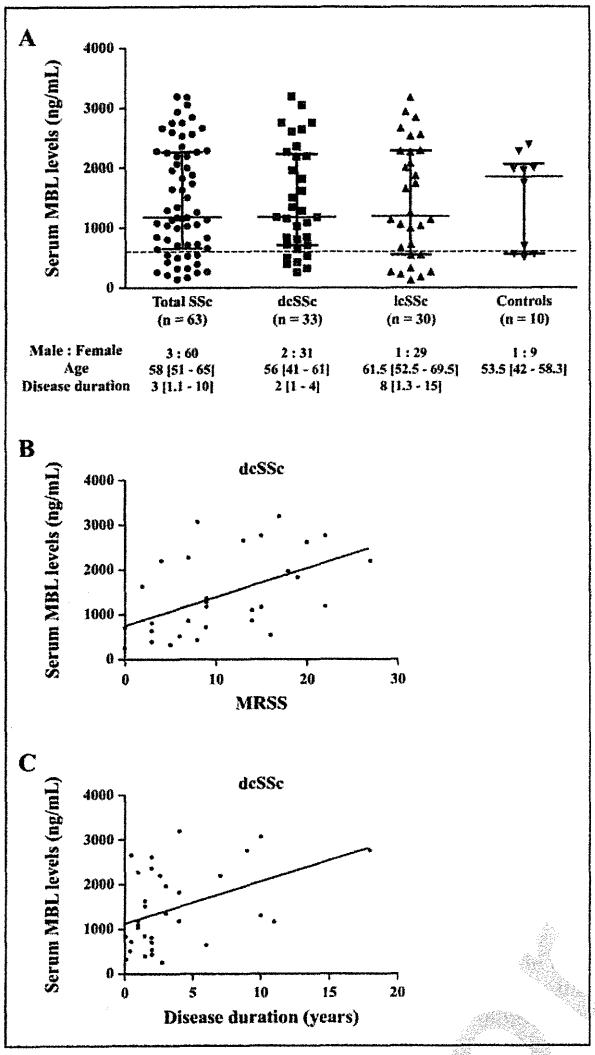

Figure 1. Serum MBL levels and their clinical correlation in patients with systemic sclerosis. A) Serum MBL levels were determined by a specific ELISA. The horizontal bars indicate median and 25-75 percentiles. SSc, systemic sclerosis; dcSSc, diffuse cutaneous SSc; lcSSc, limited cutaneous SSc. "median (years) (25-75 percentiles)" of age and disease duration in each group is shown below the graph. **B)** A significant positive correlation was found between serum MBL levels and modified Rodnan total skin thickness score (MRSS) in dcSSc patients ($r = 0.51$, $P<0.005$, by Spearman's rank correlation test). The solid line represents the regression line. **C.** A significant positive correlation was found between serum MBL levels and disease duration in dcSSc patients ($r = 0.42$, $P <0.01$, by Spearman's rank correlation test). The solid line represents the regression line.

of enrolled patients is small, the present data support the canonical idea that innate immune responses through pattern recognition receptors contribute to the developmental process of autoimmune diseases. ■

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SHORT COMMUNICATION

Serum resistin levels: a possible correlation with pulmonary vascular involvement in patients with systemic sclerosis

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Abstract Our latest studies demonstrated the potential role of adipocytokines, including adiponectin, visfatin, retinol binding protein-4, and apelin, in the pathogenesis of systemic sclerosis (SSc). Given that resistin is another member of adipocytokines with pro-inflammatory and pro-angiogenic properties, we measured serum resistin levels by enzyme-linked immunosorbent assay in 52 SSc and 19 control subjects and evaluated their clinical correlation. Since serum resistin levels greatly and inversely correlated with estimated glomerular filtration rate in SSc patients with renal dysfunction [$r = -0.78$, $p < 0.05$ ($n = 9$)], we evaluated the clinical correlation of serum resistin levels in SSc patients with normal renal function ($n = 43$). Although serum resistin levels were comparable between diffuse cutaneous SSc ($n = 22$), limited cutaneous SSc ($n = 21$), and control subjects ($n = 19$) [median (25–75 percentiles); 18.7 ng/ml (13.3–48.0), 23.3 ng/ml (12.9–54.1), and 22.9 ng/ml (9.4–36.7), respectively], the prevalence of elevated right ventricular systolic pressure (RVSP) was significantly higher in SSc patients with elevated serum resistin levels than in those with normal levels [67 % (4/6) vs. 16 % (6/37), $p < 0.05$], and serum resistin levels were significantly increased in SSc patients with elevated RVSP ($n = 10$) as compared to those with normal RVSP ($n = 33$) [52.1 ng/ml (20.8–117.5) vs. 18.5 ng/ml (12.2–46.2), $p < 0.05$]. Thus, serum resistin levels may serve as a useful marker for pulmonary vascular involvement in SSc, suggesting a possible contribution of resistin to the pathogenesis of pulmonary arterial hypertension associated with SSc.

Keywords Systemic sclerosis · Resistin · Pulmonary arterial hypertension · Renal dysfunction

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Introduction

Systemic sclerosis (SSc) is a multisystem autoimmune disease characterized by vascular injuries and fibrosis of skin and certain internal organs. Although the pathogenesis of SSc still remains elusive, our latest studies demonstrated a possible contribution of adipocytokines to the pathological process of this disorder [1].

Resistin is a member of adipocytokines, originally discovered in a search for adipocyte-derived molecules linking obesity and insulin-resistance diabetes [2]. In addition to adipocytes, resistin is produced by inflammatory cells and has been shown to possess a pro-inflammatory nature [3] and to be potentially involved in the pathological process of various inflammatory diseases. For instance, serum resistin levels positively correlate with C-reactive protein levels in rheumatoid arthritis [4] and with disease severity in psoriasis [5]. Furthermore, serum resistin levels predict anti-inflammatory effect of glucocorticoids in asthma [6] and the responder to IVIG in Kawasaki's disease [7]. Moreover, serum resistin levels serve as a surrogate marker for tuberculosis treatment as well as an early prognostic biomarker for monitoring its disease onset [8]. In addition to a pro-inflammatory property, increasing evidence has indicated that resistin triggers proliferative response in vascular smooth muscle cells [9] and induces angiogenic response in

endothelial cells [10]. Supporting this, a latest study demonstrated the independent relationship between circulating resistin levels and endothelial activation, which is defined as a summation of z-score of circulating biomarkers for atherosclerosis (E-selectin, vascular cell adhesion molecule-1, intercellular cell adhesion molecule-1, and monocytes chemoattractant protein-1), in rheumatoid arthritis [11]. According to these clinical and experimental data, resistin has been believed to be closely associated with the developmental process of a certain subset of inflammatory and/or vascular diseases.

Given that autoreactive inflammation and altered angiogenesis are sequential central events leading to fibroblast activation in SSc, resistin may be involved in the pathological process of this disorder. Therefore, we herein investigated serum resistin levels and their association with clinical symptoms in SSc.

Materials and methods

Patients

Serum samples, frozen at −80 °C until assayed, were obtained from 52 SSc patients [49 women, 3 men; age, median (25–75 percentiles): 58 years (49.5–64.3); disease duration, 2.5 years (1–9.3); body mass index, 21.1 kg/m^2 (19.2–23.1)] and 19 healthy individuals [17 women, 2 men; age, 55 years (44.5–61.5); body mass index, 20.2 kg/m^2 (19.7–22.5)] after getting written informed consent and institutional approval (University of Tokyo Graduate School of Medicine). Patients treated with corticosteroids or other immunosuppressants prior to their first visits were excluded. Patients were grouped by the LeRoy's classification system [12]: 26 patients with limited cutaneous SSc (lcSSc) [26 women; age, 59.5 years (52–66.5); disease duration, 8 years (1.6–15); body mass index, 20.0 kg/m^2 (19.1–22.8)] and 26 with diffuse cutaneous SSc (dcSSc) [23 women, 3 men; age, 55.5 years (41.3–60.8); disease duration, 2 years (1.5–3.8); body mass index, 21.7 kg/m^2 (19.8–23.8)]. All dcSSc and 23 lcSSc patients fulfilled the criteria proposed by the American College of Rheumatology [13]. Three lcSSc patients not meeting these criteria had sclerodactyly and at least two other features of SSc, such as calcinosis, Raynaud's phenomenon, esophageal dysfunction, and telangiectasia.

The measurement of serum resistin levels

Specific enzyme-linked immunosorbent assay kits were used to measure serum resistin levels (R&D Systems, Minneapolis, MN, USA). Briefly, polystyrene 96-well plates coated with antibodies against resistin were incubated with 100 μl of 5-fold diluted serum at room temperature for 2 h. Then, the wells were washed and incubated at room temperature for 2 h with horseradish peroxidase-conjugated antibodies against resistin. Next, the wells were washed again, added with tetramethylbenzidine, and incubated at room temperature for 30 min. Finally, sulfuric acid was added to terminate the reaction, and the absorbance at 450 nm was measured. Serum resistin levels were calculated using standard curve. According to manufacturer's instruction, the inter- and intra-assay coefficients of variation are 8.4 and 4.7 %, respectively, and lower detection limit is 0.16 ng/ml.

Clinical assessments

Disease onset was defined as the first clinical event of SSc other than Raynaud's phenomenon. Disease duration was defined as the interval between the onset and the time of blood sampling. The clinical and laboratory data were obtained when the blood samples were drawn. Skin score was measured using modified Rodnan total skin thickness score (MRSS). The degree of interstitial lung disease (ILD) was evaluated by the percentage of predicted vital capacity (%VC) and the percentage of predicted diffusion lung capacity for carbon monoxide (%DLco) on pulmonary function test. Elevated right ventricular systolic pressure (RVSP) was defined as 35 mmHg or more on echocardiogram. Scleroderma renal crisis (SRC) was defined as malignant hypertension and/or rapidly progressive renal failure. Estimated glomerular filtration rate (eGFR) was calculated from routine creatinine measurements using the Modification of Diet in Renal Disease equation.

Statistical analysis

The statistical analysis was carried out with a Kruskal–Wallis test and a Steel–Dwass' test for multiple comparison, with Mann–Whitney U test for two-group comparison, and with Fisher's exact probability test for the analysis of frequency. Correlations with clinical data were assessed by Spearman's rank correlation coefficient. Statistical significance was defined as a p value of <0.05.

Results

Serum resistin levels in SSc

Elevated serum resistin levels are significantly associated with the prevalence of chronic kidney disease, which is defined as an eGFR of <60 mL/min/1.73 m^2, in the general Japanese population [14]. In this study, two patients with

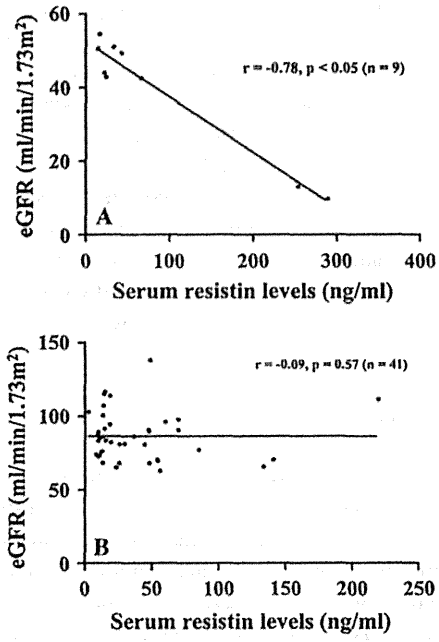

Fig. 1 Correlation of serum resistin levels with eGFR in SSc patients with renal dysfunction and in those with normal renal function. SSc patients were classified into two groups: patients with renal dysfunction (**a** eGFR < 60 min/ml/1.73 m^2) and those retaining normal renal function (**b** eGFR ≥ 60 min/ml/1.73 m^2). The correlation of serum resistin levels with eGFR was evaluated in these two groups. The *solid line* represents the regression line

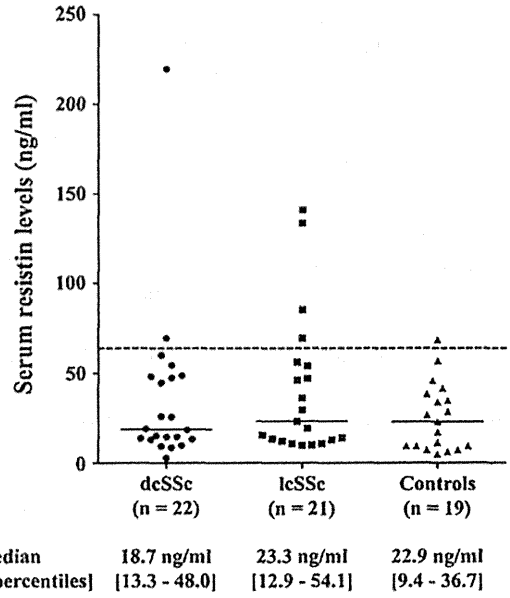

Fig. 2 Concentrations of resistin in sera from SSc patients with normal renal function and healthy individuals. Serum resistin levels were determined by a specific ELISA in healthy controls and dcSSc and lcSSc patients with normal renal function. The *dashed line* indicates the cut-off value (63.0 ng/ml; mean + 2SD of healthy controls). The *horizontal bars* indicate the median value in each group. Elevated serum levels of resistin were found in 2 of 22 dcSSc patients and 4 of 21 lcSSc patients

SRC had the first and second highest serum resistin levels (289.1 and 253.7 ng/ml), suggesting that renal dysfunction affects serum resistin levels in SSc patients as well as in general population. Therefore, we classified SSc patients into two groups according to eGFR and evaluated the correlation of serum resistin levels with eGFR in each group. Expectedly, serum resistin levels inversely correlated with eGFR in SSc patients with eGFR < 60 min/ml/1.73 m^2 ($r = -0.78$, $p < 0.05$; Fig. 1a), while not in SSc patients with eGFR ≥ 60 min/ml/1.73 m^2 ($r = -0.09$; Fig. 1b), indicating that renal dysfunction affects serum resistin levels in SSc patients with eGFR < 60 min/ml/1.73 m^2. Therefore, we excluded 9 SSc patients with eGFR < 60 ml/min/1.73 m^2 (4 dcSSc and 5 lcSSc patients) in the following analyses.

When we evaluated serum resistin levels in dcSSc, lcSSc, and control subjects, serum resistin levels were comparable (Fig. 2). Given that dcSSc is characterized by extensive fibrosis in skin and lung, these results suggest that resistin is barely associated with the process of pathological fibrosis in SSc. Consistently, serum resistin levels did not correlate with the values of MRSS ($r = 0.15$), %VC ($r = -0.02$), and %DLco ($r = -0.12$) in total SSc patients.

Clinical correlation of serum resistin levels in SSc

We next investigated the association of serum resistin levels with clinical features related to SSc vasculopathy. To this end, we set the cut-off value at 63.0 ng/ml (mean + 2SD) based on the data of normal controls and classified SSc patients into two groups: SSc patients with elevated serum resistin levels and those with normal levels. As shown in Table 1, there was no significant difference between these two groups in terms of sex, age, body mass index, disease duration, and the frequency of dcSSc. Regarding the frequency of cutaneous vascular manifestations, including pitting scars, Raynaud's phenomenon, telangiectasia, and nailfold bleeding, there was no significant difference between these two groups. As for organ involvement associated with proliferative obliterative vasculopathy, the frequency of elevated RVSP was significantly greater in patients with increased serum resistin levels than in those with normal levels (67 vs. 16 %, $p < 0.05$), while there was a trend toward the high prevalence of digital ulcers in patients with elevated serum resistin levels as compared to those with normal levels (50 vs. 16 %, $p = 0.09$). Furthermore, serum resistin levels were significantly increased in patients with elevated RVSP than in those with normal

Table 1 Correlation of serum resistin levels with clinical vascular symptoms in SSc patients with normal renal function

	Patients with elevated resistin levels ($n = 6$)	Patients with normal resistin levels ($n = 37$)
Sex, no. male : no. female	0:6	3:34
Age (years)	52 (48.3–58)	56 (48–63)
Body mass index	21.4 (19.1–22.2)	21.6 (19.2–23.2)
Disease duration (years)	1.5 (0.4–4.3)	3 (1.5–10)
dcSSc: lcSSc	2:4	20:17
Cutaneous vascular symptoms		
Pitting scars	33	26
Raynaud's phenomenon	100	93
Telangiectasia	60	38
Nailfold bleeding	67	61
Organ involvements associated with proliferative vasculopathy		
Digital ulcers	50	16
Elevated RVSP	67*	16

Statistical analysis was carried out with Fisher's exact probability test

dcSSc diffuse cutaneous SSc, *lcSSc* limited cutaneous SSc, *RVSP* right ventricular systolic pressure

* $p < 0.05$

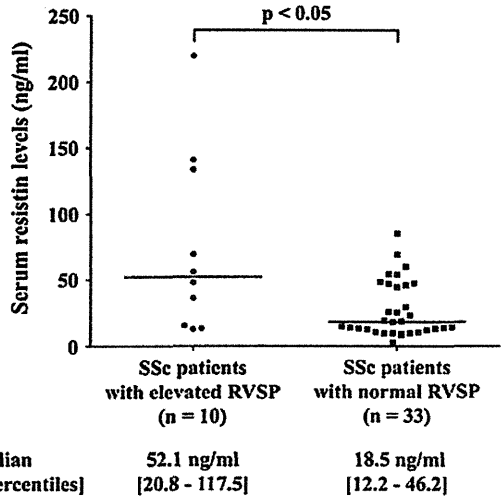

Fig. 3 Comparison of serum resistin levels between SSc patients with elevated RVSP and those with normal RVSP. SSc patients were divided into 2 groups: SSc patients with elevated RVSP and those with normal RVSP. Serum resistin levels were determined by a specific ELISA. The *horizontal bars* indicate the median value in each group

RVSP (Fig. 3). These results suggest that elevation of resistin levels is associated with the developmental process of proliferative obliterative vasculopathy, especially pulmonary arterial involvement leading to pulmonary arterial hypertension (PAH). We also evaluated the association of serum resistin levels with high-sensitive C-reactive protein and erythrocyte sedimentation rate in SSc patients because serum resistin levels positively correlate with the degree of inflammation in pathological conditions [4], but failed to detect any correlation (data not shown).

Discussion

This study was undertaken to assess the clinical significance of serum resistin levels and speculate the contribution of this adipocytokine to the developmental process of SSc. Consistent with a previous report [14], serum resistin levels were extensively elevated in SSc patients with SRC and inversely correlated with eGFR in SSc patient with eGFR < 60 min/ml/1.73 m^2. Therefore, we excluded SSc patients with eGFR < 60 ml/min/1.73 m^2 and analyzed the clinical correlation of serum resistin levels. Under this situation, serum resistin levels were comparable among dcSSc, lcSSc, and control subjects, suggesting that resistin plays little role in the pathological fibrosis of SSc. Consistently, serum resistin levels did not correlate with the values of MRSS, %VC, and %DLco in total SSc. Therefore, we speculated that resistin is associated with disease processes other than fibrosis, such as angiogenesis and altered immune activation. Supporting this, the frequency of elevated RVSP was significantly greater in SSc patients with elevated serum resistin levels than in those with normal levels, and SSc patients with elevated RVSP had serum resistin levels much higher than those with normal RVSP. Furthermore, the prevalence of digital ulcers tended to be higher in SSc patients with elevated serum resistin levels than in those with normal levels. On the other hand, serum resistin levels did not correlate with systemic inflammatory markers. Taken together, the present findings indicate that resistin may contribute to aberrant angiogenesis associated with proliferative obliterative vasculopathy in SSc, especially pulmonary vascular involvement leading to PAH.

Evidence has revealed the potential role of resistin in the development of pathological vascular conditions. Incubation of ECs with resistin results in an increase in ET-1 expression, while not affecting nitrogen oxide production

[15], suggesting that resistin is associated with dysregulated vasoconstriction under certain pathological conditions. In addition to a prominent vasoconstrictive effect, ET-1 has a potent mitogenic action on fibroblasts and vascular smooth muscle cells. Furthermore, ET-1 promotes the differentiation of quiescent fibroblasts into myofibroblasts and prolongs its survival by preventing apoptosis. Moreover, ET-1 triggers the pathological inflammation by modulating the expression of cell adhesion molecules on endothelial cells and subsequently promoting T cell infiltration and by acting on macrophages and promoting the production of free radical and cytokines, including interleukin-8, monocyte chemoattractant protein-1, and transforming growth factor-β [16]. Given these proliferative, profibrotic, and proinflammatory effects of ET-1, resistin appears to play its pathological role in the development of proliferative vasculopathy, which is histopathologically characterized by prominent intimal proliferation further leading to an impaired blood flow in tissues [17], associated with SSc at least partially through the up-regulation of endothelial ET-1 production. Consistently, similar to the present data, plasma ET-1 levels are increased in lcSSc patients with PAH and dcSSc patients, in whom digital ulcers as a result of severe proliferative obliterative vasculopathy are frequently seen, compared with normal controls and lcSSc patients without PAH [18].

Supporting previous reports in the field of nephrology [19, 20], serum resistin levels were inversely and greatly correlated with eGFR in SSc patients with renal dysfunction, while not in those retaining normal renal function. This clinical observation has been believed to be attributable to reduced renal clearance of resistin [21]. However, a couple of clinical studies have raised the possibility that the elevation of serum resistin levels reflects not only reduced renal clearance but also the potential role of resistin in the development of chronic kidney disease [14, 19]. Although it still remains unknown how resistin is involved in the pathogenesis of chronic kidney disease, its pro-angiogenic property may be related to the development of SRC, which is caused by the proliferative obliterative vasculopathy as well as PAH and digital ulcers associated with SSc.

In summary, we herein reported the first study demonstrating the close association of serum resistin levels with the development of proliferative obliterative vasculopathy in SSc, especially pulmonary vascular involvement leading to PAH. Although further studies to determine the source of resistin explaining its significant association with SSc vasculopathy are required in the future, this study supports the previous data regarding the pro-angiogenic property of resistin and definitely provides us the first clue to further expand the studies regarding the role of resistin in the mechanism responsible for the development of SSc vasculopathy.

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Conflict of interest The authors have declared no conflict of interest.

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INVESTIGATIVE REPORT

Serum Angiopoietin-like Protein 3 Levels: Possible Correlation with Progressive Skin Sclerosis, Digital Ulcers and Pulmonary Vascular Involvement in Patients with Systemic Sclerosis

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Angiopoietin-like protein 3 (ANGPTL3), which is part of a family of secreted glycoproteins that are structurally similar to angiopoietins, is principally expressed in the liver and is involved in lipid metabolism and angiogenesis. The aim of this study was to determine the clinical significance of serum ANGPTL3 levels, measured with a specific enzyme-linked immunosorbent assay, in patients with systemic sclerosis. Serum ANGPTL3 levels correlated positively with skin score in diffuse cutaneous systemic sclerosis with a disease duration ≤6 years. Furthermore, the prevalence of digital ulcers was significantly higher in patients with elevated serum ANGPTL3 levels than in other patients. Moreover, among patients excluding diffuse cutaneous systemic sclerosis with disease duration ≤6 years, serum ANGPTL3 levels correlated positively with estimated right ventricular systolic pressure. In conclusion, ANGPTL3 may contribute to the development of progressive skin sclerosis and proliferative obliterative vasculopathy, such as digital ulcers and pulmonary vascular involvement leading to pulmonary arterial hypertension, in systemic sclerosis. ***Key words: systemic sclerosis; angiopoietin-like protein 3; angiogenesis; skin sclerosis; digital ulcers; pulmonary arterial hypertension.***

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Systemic sclerosis (SSc) is a multisystem autoimmune disease characterized by initial vascular injuries and resultant fibrosis of the skin and certain internal organs. Although fibroblast activation is the final consequence of SSc, autoimmune attacks and vascular injuries prior to the onset of the fibrotic response appear to play central roles in the pathogenesis of this complicated disorder (1).

A family of secreted glycoproteins structurally similar to angiopoietins was identified and designated as angiopoietin-like proteins (ANGPTLs), which consist of an N-terminal coiled-coil domain and C-terminal fibrinogen-like domain. Despite the structural similarity to angiopoietins, ANGPTLs do not bind to endothelial tyrosine kinase receptors Tie1 and Tie2, suggesting that these molecules are functionally different from angiopoietins (2). Angiopoietin-like protein 3 (ANGPTL3), a member of the ANGPTLs, is expressed principally in the liver during development and in adults (3). ANGPTL3 is involved in lipid metabolism and angiogenesis in a domain-dependent manner. The N-terminal coiled-coin domain regulates serum lipid levels by inhibiting lipolysis of triglyceride-rich lipoproteins in mice (4, 5), while the C-terminal fibrinogen-like domain induces endothelial cell adhesion and migration and *in vivo* angiogenesis by interacting with endothelial integrin αVβ3 (6). In humans, serum ANGPTL3 levels correlate positively with the intima-media thickness of carotid and femoral arteries in healthy subjects, but not with triglyceride, low-density lipoprotein, high-density lipoprotein, and total cholesterol levels. More importantly, serum ANGPTL3 levels still show a positive association with arterial wall thickness after adjustment for other possible confounding variables, suggesting that the angiogenic property of ANGPTL3 explains its association with arterial wall thickness (7). Thus, unlike animal models, the role of ANGPTL3 in lipid metabolism is controversial in humans, but it definitely contributes to the development of atherosclerosis, probably by activating angiogenesis.

Given that aberrant activation of angiogenesis underlies the pathogenesis of vasculopathy and subsequent fibroblast activation in SSc, ANGPTL3 may be involved in the mechanism responsible for the pathological process in this disorder. In an initial study of this issue, we investigated serum ANGPTL3 levels and their association with clinical features in SSc.

MATERIALS AND METHODS

Patients

Serum samples, frozen at –80°C until assayed, were obtained from 51 patients with SSc (48 women, 3 men; age, median [25–75 percentile]: 59 years [51.5–66.5]; disease duration, 3 years [2–10]) and 18 healthy individuals (16 women, 2 men; age, 54.5 years [48.3–61.8]), who visited our department between July 2009 and June 2011. Patients treated with corticosteroids or other immu-

nosuppressants prior to their first visit were excluded. There were no significant differences in age and disease duration between men and women (age 59 [26–66] vs. 59 years [51.3–67], $p=0.66$, Mann–Whitney U test; disease duration, 1 year [1–2] vs. 3.5 years [2–10], $p=0.066$, Mann–Whitney U test). Patients were grouped by the LeRoy's classification system (8): 24 patients with limited cutaneous SSc (lcSSc) (all women; age, 65 years [56.5–70.3]; disease duration, 8 years [2.8–15]) and 27 with diffuse cutaneous SSc (dcSSc) (24 women, 3 men; age 57 years [46–61]; disease duration, 2 years [2–4]). dcSSc patients were significantly younger than lcSSc patients ($p=0.0073$, Mann–Whitney U test) and disease duration was much shorter in dcSSc patients than in lcSSc patients ($p=0.036$, Mann–Whitney U test). All dcSSc and 22 lcSSc patients fulfilled the criteria proposed by the American College of Rheumatology (9). Two lcSSc patients not meeting these criteria had sclerodactyly and at least 2 other features of SSc, such as calcinosis, Raynaud's phenomenon, oesophageal dysfunction, and telangiectasia. The study was performed according to the Declaration of Helsinki and approved by the ethics committee of the University of Tokyo Graduate School of Medicine. Written informed consent was obtained from all patients and healthy controls.

Measurement of serum ANGPTL3 levels

Specific enzyme-linked immunosorbent assay kits were used to measure serum ANGPTL3 levels (R&D Systems, Minneapolis, USA). Briefly, polystyrene 96-well plates coated with antibodies against ANGPTL3 were incubated with 100 µl 50-fold diluted serum at room temperature for 2 h. The wells were then washed and incubated at room temperature for 2 h with horseradish peroxidase conjugated antibodies against ANGPTL3. Next, the wells were washed again, added with tetramethylbenzidine, and incubated at room temperature for 30 min. Finally, sulphuric acid was added to terminate the reaction and the absorbance at 450 nm was measured. Serum ANGPTL3 levels were calculated using standard curve.

Clinical assessment

Disease onset was defined as the first clinical event of SSc other than Raynaud's phenomenon. Disease duration was defined as the interval between onset and time of blood sampling. The clinical and laboratory data were obtained when the blood samples were drawn. Skin score was measured using modified Rodnan total skin thickness score (MRSS) (10). The degree of interstitial lung disease (ILD) was evaluated by the percentage of predicted vital capacity (%VC) and the percentage of predicted diffusion lung capacity for carbon monoxide (%DLco) on pulmonary function test. Ground-glass opacity on chest computed tomography was scored by 2 independent readers, as previously reported (11) and the mean estimate of the 2 readers was used as ground glass score. Elevated right ventricular systolic pressure (RVSP) was defined as 35 mmHg or more on echocardiogram. Scleroderma renal crisis (SRC) was defined as malignant hypertension and/or rapidly progressive renal failure.

Statistical analysis

The statistical analysis used for each experiment is described in the figure legends and Results section. Statistical significance was defined as $p<0.05$.

RESULTS

Serum ANGPTL3 levels in patients with systemic sclerosis

No significant difference was found between serum ANGPTL3 levels in SSc patients and control subjects (median [25–75 percentiles]; 2.24 ng/ml [0.21–5.30] vs. 2.69 ng/ml [2.12–3.27], $p=0.51$, Welch's t-test, respectively). There was also no significant difference in serum ANGPTL3 levels among healthy controls, dcSSc (2.12 ng/ml [0.21–3.60]), and lcSSc patients (3.16 ng/ml [0.56–5.87]) ($p=0.42$ by Kruskal-Wallis test) (Fig. 1). However, close examination of the distribution of serum ANGPTL3 levels in SSc patients, revealed 2 subgroups of SSc patients: those with quite low levels and those with highly elevated levels of serum ANGPTL3. This observation suggests that serum ANGPTL3 levels are linked to certain clinical features in patients with SSc.

Serum ANGPTL3 levels correlated positively with MRSS in dcSSc patients with disease duration ≤6 years

In order to investigate the clinical association of serum ANGPTL3 levels, we initially studied the correlation of serum ANGPTL3 levels with dermal and pulmonary fibrotic markers, including MRSS, %VC, and %DLco, because activation of angiogenic process is potentially linked to the initiation and progression of fibrosis in SSc. Since severe organ damage occurs within 5–6 years of disease onset in most cases of dcSSc, and progression to severe skin thickening seldom occurs afterwards (12), the correlation of serum ANGPTL3 levels with MRSS was also assessed in dcSSc patients with disease duration ≤6 years in addition to dcSSc and lcSSc patient groups. As

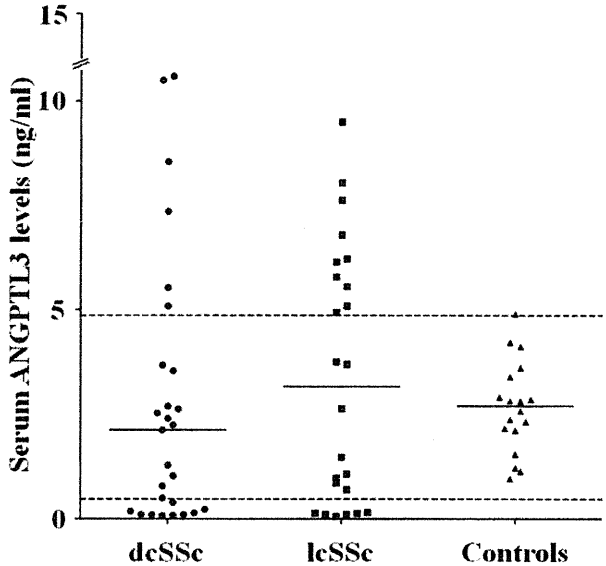

Fig. 1. Serum angiopoietin-like protein 3 (ANGPTL3) levels in systemic sclerosis (SSc) and healthy controls. Serum ANGPTL3 levels were determined by a specific enzyme-linked immunoassay (ELISA). Multiple comparison was carried out with a Kruskal–Wallis test. Since serum ANGPTL3 levels were normally distributed in healthy controls ($p=0.76$, Shapiro–Wilk test), but not in dcSSc patients ($p=0.0001$, Shapiro-Wilk test) and lcSSc patients ($p=0.012$, Shapiro-Wilk test), horizontal bars indicate median for each group. The dotted lines represent mean ± 2 SD of serum AGPTL3 levels in healthy controls, which were used as cut-off values in the following analyses.

shown in Fig. 2A, serum ANGPTL3 levels correlated significantly with MRSS in dcSSc patients with disease duration ≤6 years ($r=0.53$, $p=0.022$; Spearman's rank correlation coefficient), but not in dcSSc and lcSSc patients ($r=0.39$, $p=0.06$, and $r=0.38$, $p=0.11$, respectively). Given that there was no correlation between serum ANGPTL3 levels and MRSS in dcSSc with disease duration >6 years ($r=-0.31$, $p=0.56$), serum ANGPTL3 levels may reflect the activation of angiogenic process during the development of skin sclerosis in dcSSc. On the other hand, regarding %VC and %DLco, no significant correlation with serum ANGPTL3 levels was detected in these 3 groups. We also evaluated the association of serum ANGPTL3 levels with ground glass score in these 3 groups because ground glass opacity reflects inflammation and vasculopathy associated with ILD better than %VC and %DLco, but did not see any significant correlation. Collectively, ANGPTL3 is potentially involved in the mechanism underlying skin sclerosis, but not ILD, in dcSSc.

Clinical features of SSc patients with elevated serum ANGPTL3 levels

We next investigated the association of serum ANGPTL3 levels with clinical features related to SSc vasculopathy. Since there was a subset of SSc patients with quite high serum ANGPTL3 levels, we first set the cut-off value at 4.86 ng/ml (mean +2 SD) of healthy controls) and classified SSc patients into 2 groups; patients with elevated serum ANGPTL3 levels (31% of SSc patients; 6 of 27 dcSSc patients and 10 of 24 lcSSc patients) and the other patients. Patient information and the prevalence of clinical features associated with SSc vasculopathy in these 2 groups are shown in the left-hand columns of Table I. There was no significant difference between these 2 groups in terms of sex, age, disease duration, and the frequency of dcSSc. Regarding the frequency of cutaneous vascular manifestations, including Raynaud's phenomenon, nail-fold bleeding, telangiectasia, and pitting scars, there was no significant difference between these 2 groups. As for organ involvements associated with proliferative obliterative vasculopathy, the frequency of digital ulcers was significantly greater in patients with increased serum ANGPTL3 levels than in the other patients (40 % vs. 11%, $p=0.048$), while the frequencies of SRC and elevated RVSP were comparable between these 2 groups. Thus, the increase in serum ANGPTL3 levels is associated with the development of digital ulcers, which are attributable to intimal proliferation and luminal narrowing or occlusion of small digital arteries

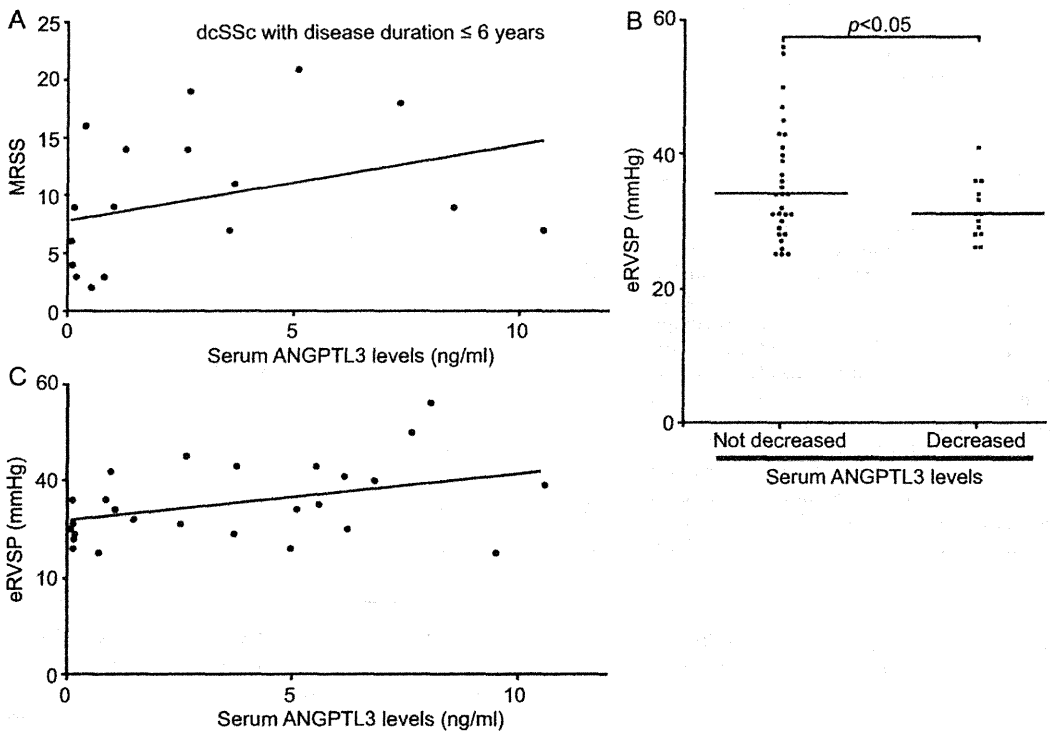

Fig. 2. Clinical correlation of serum angiopoietin-like protein 3 (ANGPTL3) levels in systemic sclerosis (SSc). Serum ANGPTL3 levels correlated with modified Rodnan total skin thickness score (MRSS) in dcSSc patients with disease duration ≤6 years (A; $r=0.53$, $p=0.022$, Spearman's rank correlation coefficient) and with right ventricular systolic pressure (RVSP) in SSc patients excluding dcSSc with disease duration ≤6 years (C; $r=0.39$, $p=0.044$, Spearman's rank correlation coefficient). The solid line represents the regression line. The values of RVSP were significantly higher in SSc patients with normal and elevated serum ANGPTL3 levels than in those with descreased levels. (B; $p=0.036$, Welch's t-test). Since serum ANGPTL3 levels were normally distributed in SSc patients with decreased serum ANGPTL3 levels ($p=0.02$, Shapiro-Wilk test), but not in patients with normal and elevated ANGPTL3 levels ($p=0.54$, Shapiro-Wilk test), median was shown as horizontal bars.

Table I. *Clinical features of systemic sclerosis (SSc) patients with increased or decreased serum angiopoietin-like protein 3 (ANGPTL3), levels*

	Serum ANGPTL3 levels			
	Increase		Decrease	
	Yes (n=16)	No (n=35)	Yes (n=15)	No (n=36)
Sex, male:female, n	0:16	4:31	3:12	1:35
Age, years, median [25–75 percentiles]	58 [54.5–68]	59 [48.8–65]	59 [54–66.5]	58.5 [48.8–65]
Disease duration, years, median [25–75 percentiles]	6 [1.9–16]	3 [1.5–10]	2.6 [1.8–7]	4 [1.5–10.8]
dcSSc:lcSSc, n	6:10	21:14	9:6	18:18
Cutaneous vascular symptoms, % (n)				
Raynaud's phenomenon	88 (14/16)	88 (30/34)	80 (12/15)	91 (32/35)
Nail-fold bleeding	80 (12/15)	76 (25/33)	71 (10/14)	79 (27/34)
Telangiectasia	46 (6/13)	48 (13/27)	33 (4/12)	54 (15/28)
Pitting scars	38 (5/13)	38 (13/34)	27 (4/15)	44 (14/32)
Organ involvements associated with proliferative vasculopathy, % (n)				
Digital ulcers	40* (6/15)	11 (4/35)	13 (2/15)	26 (9/35)
Scleroderma renal crisis	6 (1/16)	6 (2/35)	7 (1/15)	6 (2/36)
Elevated RVSP	44 (7/16)	26 (9/35)	20 (3/15)	39 (14/36)

dcSSc: diffuse cutaneous SSc; lcSSc: limited cutaneous SSc; RVSP: right ventricular systolic pressure. Statistical analysis was carried out with Fisher's exact probability test. *p=0.048 (Yes compered with No).

and larger arteries (the palmar arch and radial and ulnar arteries) (13), in SSc patients.

Clinical features of patients with systemic sclerosis with decreased serum ANGPTL3 levels

The other feature of serum ANGPTL3 levels in patients with SSc was the presence of another patient subset with quite low serum ANGPTL3 levels. Therefore, we also set another cut-off value at 0.47 ng/ml (mean –2 SD of healthy controls) and classified SSc patients into 2 groups; patients with decreased serum ANGPTL3 levels (29% of SSc patients; 9 of 27 dcSSc patients and 6 of 24 lcSSc patients) and the other patients. Similar analyses were carried out between these 2 groups (right-hand columns in Table I). There was no significant difference in patients' backgrounds (sex, age, disease duration, and frequency of dcSSc). In contrast to the former analysis, we did not detect any clinical features associated with SSc vasculopathy in patients with decreased serum ANGPTL3 levels. However, when the RVSP values were evaluated, they were found to be significantly higher in SSc patients with normal and elevated serum ANGPTL3 levels than in those with decreased levels (34 mmHg [29–41.3] vs. 31 mmHg [28.3–33.8], p=0.036; Fig. 2B). Collectively, these results suggest that decreased serum ANGPTL3 levels serve as a marker for SSc patients at low risk of pulmonary vascular involvement leading to pulmonary arterial hypertension (PAH).

Serum ANGPTL3 levels correlated positively with right ventricular systolic pressure in SSc patient group excluding dcSSc patients with disease duration ≤6 years

As described above, RVSP values were significantly higher in SSc patients with normal and elevated serum ANGPTL3 levels than in those with decreased levels, while there was no significant correlation between serum ANGPTL3 levels and RVSP (r=0.19, p=0.22). Given that serum ANGPTL3 levels correlate with MRSS in dcSSc patients with disease duration ≤6 years, this may affect the relationship between serum ANGPTL3 levels and RVSP. Therefore, we re-evaluated the association of serum ANGPTL3 levels with RVSP in SSc patient group excluding dcSSc patients with disease duration ≤6 years. As expected, serum ANGPTL3 levels positively correlated with RVSP in this patient group (r=0.39, p=0.044; Spearman's rank correlation coefficient) (Fig. 2C), but not in dcSSc patients with disease duration ≤6 years (r=–0.19, p=0.42; Spearman's rank correlation coefficient). These results suggest the potential association of ANGPTL3 with the mechanism responsible for the development of pulmonary vascular involvement leading to PAH in SSc.

DISCUSSION

This study assessed the clinical significance of serum ANGPTL3 levels and enabled speculation about the contribution of this molecule to the developmental process in SSc. Although serum ANGPTL3 levels were comparable among dcSSc, lcSSc and healthy controls, some SSc patients had highly elevated or quite low serum ANGPTL3 levels, suggesting that altered expression of ANGPTL3 contributes to certain pathological process in SSc. Supporting this idea, serum ANGPTL3 levels showed a positive correlation with MRSS in dcSSc patients with disease duration ≤6 years. Furthermore, digital ulcers were seen in SSc patients with elevated serum ANGPTL3 levels much more frequently than in the other patients. Moreover, in the SSc patient group excluding dcSSc patients with disease duration

≤6 years, serum ANGPTL3 levels positively correlated with the values of RVSP. Collectively, the increased expression of ANGPTL3 may play a role in the mechanism responsible for the initiation and progression of skin sclerosis and the development of proliferative obliterative vasculopathy, such as digital ulcers and pulmonary vascular involvement leading to PAH in SSc.

The significant role of ANGPTL3 in lipid metabolism has clearly been shown in animal models. Consistent with the *in vitro* data that ANGPTL3 inhibits the activity of lipoprotein lipase (14), lower plasma triglyceride levels in *Angptl3*-deficient mice are reversed to normal levels by introducing the functioning *Angptl3* gene (4, 14) or by intravenous administration of recombinant human ANGPTL3 protein (4, 15). Furthermore, the development of atherosclerosis in apolipoprotein E knockout mice is suppressed, along with the reduction in triglyceride levels by introducing a recessive mutation of the *Angptl3* gene homozygously (16). Consistent with these findings in animal models, serum ANGPTL3 levels correlate with the thickness of arterial walls in humans. However, serum ANGPTL3 levels do not correlate with triglyceride, high-density lipoprotein, low-density lipoprotein, and total cholesterol levels. Most importantly, the positive association of serum ANGPTL3 levels with intima-media thickness of carotid and femoral arteries is independent of lipids and other possible confounding variables, such as age, sex, smoking, body mass index, systolic blood pressure, plasma glucose, and insulin resistance index (7). Supporting this notion, the association between the *Angptl3* gene polymorphisms and coronary plaque area is independent of lipids and other classical risk factors in survivors of myocardial infarction (17). Collectively, in contrast to animal models, serum ANGPTL3 levels have a significant association with atherosclerotic vascular changes independent of plasma lipid levels in humans. Although the lipid-independent mechanism by which ANGPTL3 promotes atherosclerosis is totally unknown, the direct effect of ANGPTL3 on endothelial cells potentially explains this phenomenon. Camenisch et al. (6) demonstrated that integrin αVβ3 serves as a receptor for ANGPTL3 on endothelial cells and this interaction promotes endothelial cell adhesion and migration. Furthermore, the author revealed that ANGPTL3 induces angiogenesis to a similar extent to that caused by vascular endothelial growth factor (VEGF)-A in rat corneal assay. Given that adventitial angiogenesis induced by the transduction of adenovirus encoding VEGF-A, VEGF-D and VEGF-DΔNΔC correlates positively with the intimal hyperplasia in animal models (18), the angiogenic property of ANGPTL3 may explain the positive association of its serum levels with arterial wall thickness. Thus, ANGPTL3 potentially causes intimal hyperplasia in concert with other pro-angiogenic factors in various vascular diseases.

Vasculopathy associated with SSc is generally classified into 2 subgroups; destructive vasculopathy and proliferative obliterative vasculopathy (19). Destructive vasculopathy is characterized by progressive loss of capillaries, which is clinically related to pitting scars. In contrast, proliferative obliterative vasculopathy is characterized by proliferation of vascular cells resulting in intimal hyperplasia, intimal fibrosis, and luminal narrowing or occlusion of arteries, which is associated with the development of digital ulcers, PAH and SRC. As shown in the present study, the increase in serum ANGPTL3 levels is closely linked to the development of digital ulcers and elevated RVSP. Given that ANGPTL3 is potentially involved in the mechanism by which thickening of arterial walls develops in humans (7), the current data strongly suggest that ANGPTL3 plays a role in the pathogenesis of SSc vasculopathy, especially proliferative obliterative vasculopathy.

In order to evaluate the association of serum ANGPTL3 levels with pulmonary arterial involvement leading to PAH in SSc, we used RVSP measured by echocardiography. However, it has been reported that RVSP lacks specificity for the diagnosis of PAH in SSc due to the influence of left-heart disease and ILD associated with this disease (20). Therefore, in order to better evaluate the potential role of ANGPTL3 in SSc-PAH, it is necessary to assess the correlation of serum ANGPTL3 levels with mean pulmonary arterial pressure (mPAP) measured by right heart catheterization. Given the relatively low correlation of RVSP with mPAP in SSc, serum ANGPTL3 levels may have a much better correlation with mPAP than RVSP.

It is generally accepted that a certain subset of pro-angiogenic factors serves as a biomarker of disease activity and severity in SSc. For instance, Michalska-Jakubus et al. (21) demonstrated that serum levels of angiopoietin-2, a potent pro-angiogenic factor exerting its biological effect through Tie1 and Tie2 tyrosine kinase receptors, correlate positively with MRSS and inversely with %DLco and are significantly elevated in intermediate/late SSc compared with early SSc. More importantly, serum angiopoietin-2 levels are independently associated with the European Scleroderma Study Group disease activity index score in multivariate regression analysis. Another study by Dunne et al. (22), in which platelet-free plasma samples from relatively severe patients were used, also demonstrated the contribution of the angiopoietin-1/angiopoietin-2/Tie2 system to ongoing vasculopathy in SSc by the following data: (*i*) soluble angiopoietin-1, angiopoietin-2 and Tie2 levels and the ratios of angiopoietin-2/angiopoietin-1 and angiopoietin-2/Tie2 are higher in dcSSc and lcSSc than in healthy controls; (*ii*) angiopoietin-2 levels correlate with RVSP and %DLco in lcSSc; and (*iii*) angiopoietin-2/Tie2 ratio shows a positive association with disease activity in both dcSSc and lcSSc. These results

indicate that the activation of angiogenesis occurs throughout the disease course and is closely related to the activity and severity of SSc. In the present study, serum ANGPTL3 levels correlated with MRSS in dcSSc with disease duration ≤6 years, but not in dcSSc with disease duration >6 years and lcSSc. As for ILD, serum ANGPTL3 levels were associated with neither %VC nor %DLco in the 3 subgroups. These results suggest that ANGPTL3 plays a role in the constitutive activation of angiogenesis, which leads to fibroblast activation, together with other pro-angiogenic factors especially during the progressive stage of skin sclerosis in dcSSc.

In summary, we report here the first study demonstrating the close association of serum ANGPTL3 levels with the development of progressive skin sclerosis and proliferative obliterative vasculopathy, such as digital ulcers and pulmonary vascular involvement leading to PAH, in SSc. However, the present conclusion regarding pulmonary vascular involvement is still preliminary, because RVSP measured by echocardiography lacks specificity for the diagnosis of PAH in SSc due to the influence of left-heart disease and ILD (20). Furthermore, we cannot deny the possibility that the elevation of serum ANGPTL3 levels in SSc is an epiphenomenon because the correlation of ANGPTL3 mRNA levels in dcSSc skin with MRSS was not confirmed due to undetectably low levels of ANGPTL3 expression (data not shown). Regardless of the limitations, this study supports previous data regarding the pro-angiogenic property of ANGPTL3 and provides a useful clue to further understanding of the mechanism responsible for the development of skin sclerosis and vasculopathy in SSc.

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