and 8 men) had diffuse cutaneous SSc (dSSc). The mean±SD age of the patients was 47±16 years (dSSc; 48±18, lSSc; 45±17) and the disease duration of patients with dSSc and lSSc was 8.5±9.5 and 3.1±2.5 years, respectively. None of the patients was treated with corticosteroid or other immunosuppressive therapy at the evaluation. Antinuclear antibody (Ab) was determined by indirect immunofluorescence using HEp-2 cells and specificities were further assessed by ELISA and immunoprecipitation. Anti-topoisomerase I Ab was positive for 41 (34 dSSc and 7 lSSc), anticentromere Ab for 37 (2 dSSc and 35 lSSc), anti-U1RNP Ab for 2 (all lSSc), anti-U3RNP Ab for 1 (dSSc), anti-RNA polymerases I and III Ab for 7 (all dSSc) and Th/To Ab for 1 (lSSc). The remaining two patients were negative for autoantibodies. Twenty age- and sex-matched healthy Japanese individuals (17 women and 3 men; age 48±15 years) were used as normal controls. In a retrospective longitudinal analysis we examined serum samples from 10 patients with SSc (5 dSSc and 5 lSSc).

Clinical assessment

Complete medical histories, physical examinations and laboratory tests including vital capacity (VC) and diffusion capacity for carbon monoxide (Dlco) were conducted for all patients. When the Dlco and VC were <75% and <80%, respectively, of the predicted normal values, they were considered to be abnormal. Skin score was measured by the modified Rodnan total skin thickness score (TSS).[28] Organ involvement was defined by rheumatologists, neurologists, nephrologists and radiologists as described previously[24–26]: pulmonary fibrosis=bibasilar fibrosis on chest radiography and high-resolution CT; oesophagus=hypomotility shown by barium radiography; joints=inflammatory polyarthralgias or arthritis; heart=pericarditis, congestive heart failure or arrhythmias requiring treatment; kidney=malignant hypertension and rapidly progressive renal failure with no other explanation; muscle=proximal muscle weakness and elevated serum creatine kinase.

IL-27R expression in sclerotic skin

Immunohistochemistry for IL-27R was performed as previously described.[29] Skin tissues were obtained from the forearms of 10 patients with dSSc (5 women and 5 men; median age 42 years, range 28–53) and 5 normal controls. Sections were incubated with a primary monoclonal Ab to human IL-27R (10 μg/ml; Abcam, Cambridge, Massachusetts, USA). Concentration matched monoclonal mouse immunoglobulin G (IgG) (Abcam) was used as isotype control staining. The reaction products were visualised using diaminobenzidine (Dako, Carpinteria, California, USA) with methyl green as a counterstain. Each section was examined independently by two investigators (AY and SS) in a blinded manner. Expression levels of IL-27R and IL-27 were also analysed using a real-time PCR quantification method as described previously.[6] Each sample was tested in duplicate.

ELISAs for serum IL-27, interferon γ, IL-4, IL-10, IL-17 and hyaluronan levels

ELISAs for serum levels of IL-27 (ID Labs, London, Ontario, Canada), interferon γ (R&D Systems, Minneapolis, Minnesota, USA), IL-4 (R&D Systems), IL-10 (Immunotech, Munster, Germany), IL-17 (BioSource, Fleurus, Belgium) and hyaluronan (Echelon Biosciences, Salt Lake City, Utah, USA) were performed as described using specific ELISAs.[4 5 30 31] Each sample was tested in duplicate.

Monocyte, T cell and B cell purification and stimulation

Heparinised blood samples were obtained from 25 patients with dSSc (21 women and 4 men; median age 47 years, range 21–68) and 10 healthy individuals. Peripheral blood monocytes, CD4 T cells or B cells were enriched with each isolation kit using AutoMACS isolator (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively. A total of >99% of these cells were CD14, CD4 or CD19 (data not shown). To analyse mRNA expression of IL-27R, total RNA was isolated from CD4 T cells and B cells with RNeasy spin columns (Qiagen, Crawley, UK). Purified monocytes, CD4 T cells or B cells (1×10^5, respectively) were cultured in RPMI 1640 containing 10% heat-inactivated fetal calf serum (Gibco Life Technologies, Paisley, UK). Cells were serum-starved for 12 h and then stimulated with 0.2 ng/ml human recombinant IL-27 (rIL-27; R&D Systems) with or without 50 ng/ml low molecular weight hyaluronan (15–40 kDa; R&D Systems) or LPS (Sigma-Aldrich, St Louis, Missouri, USA). rIL-27, hyaluronan and LPS were dissolved in phosphate-buffered saline (PBS). Monocytes or T cells were cultured for 72 h and IL-27 or IL-17 concentrations in the culture medium were measured by ELISA. In addition, B cells were cultured for 8 days and IgG concentrations in the culture medium were measured by ELISA (Bethyl Laboratories, Montgomery, Texas, USA). Each sample was performed in triplicate.

Fibroblast proliferation and collagen synthesis with IL-27 stimulation

Human dermal fibroblasts were obtained by skin biopsy from the forearms of six patients with dSSc (3 women and 3 men; median age 41 years, range 28–53) and six healthy individuals. Primary explant cultures were established.[32] Fibroblasts were serum-starved for 12 h and then cultured for 24 h with or without rIL-27 (0.2 ng/ml) and/or rIL-17 (0.2 ng/ml; R&D Systems). Expression of IL-27R was analysed using western blot assay.[33] The protein was subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred onto membranes for immunoblotting. These membranes were incubated for 1 h at room temperature with 1:200 dilutions of the anti-IL-27R Ab (Santa Cruz Biotechnology, Santa Cruz, California, USA). We processed anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) Ab (Cell Signaling Technology, Beverly, Massachusetts, USA) as an indicator of the amounts of protein loaded. The intensities of IL-27R band and GAPDH band in each lane were quantified with an automated gel digitising system (Un-Scan-It; Silk Scientific, Orem, Utah, USA). Proliferation of cultured dermal fibroblasts was quantified by a colorimetric 5-bromo-2-deoxyuridine (BrdU) cell proliferation ELISA kit (Roche Applied Science, Indianapolis, Indiana, USA). Type I collagen, the major fibre-forming collagen of the skin, is the product of the proα1 (I) collagen type I α1 (COL1A1) and proα2 (I) collagen (COL1A2) genes.[34] Therefore, to assess the collagen synthesis activity in fibroblasts, COL1A1 and COL1A2 were analysed using real-time PCR. Type 1 collagen protein levels were also assessed using a specific ELISA kit (Applied Cell Biotechnologies, Yokohama, Japan). Each sample was performed in triplicate.

Statistical analysis

The Statview III program (Abacus Concepts, Berkeley, California, USA) was used for statistical analyses. Statistical analysis was performed using the Mann–Whitney U test for determining the level of significance of differences between sample means, the Fisher exact probability test for comparison of frequencies and the Bonferroni test for multiple comparisons. Spearman rank

correlation coefficient was used to examine the relationship between two continuous variables. A p value <0.05 was considered statistically significant.

RESULTS

Serum IL-27 levels in SSc

The levels of IL-27 in serum samples from patients with SSc and controls were assessed by ELISA (figure 1). Serum IL-27 levels were significantly elevated in patients with SSc (median 74.1 pg/ml (range 21.5–188.8)) compared with controls (median 41.8 pg/ml (range 28.7–63.6); p<0.005). For the SSc subgroups, IL-27 levels in both patients with dSSc (median 83.0 pg/ml (range 37.1–188.8)) and those with lSSc (median 65.3 pg/ml (range 21.5–112.1)) were elevated compared with those in controls (p<0.001 and p<0.01, respectively). Furthermore, serum IL-27 levels were significantly elevated in patients with dSSc relative to those with lSSc (p<0.05). Values higher than the mean +2SD (62.9 pg/ml) of the control serum samples were found in 58% (53/91) of all patients with SSc, in 71% (32/45) of patients with dSSc and in 46% (21/46) of patients with lSSc. By contrast, only 5% (1/20) of controls had elevated IL-27 levels.

Clinical features of patients with SSc with IL-27 overproduction

We assessed the clinical features of patients with SSc with increased IL-27 production compared with patients with SSc with normal IL-27 production (table 1). The duration of disease was significantly shorter (p<0.01) in patients with SSc with increased IL-27 production (3.0±3.2 years, n=52) than in those with normal IL-27 production (7.2±8.1 years, n=39). Moreover, the retrospective longitudinal analysis in this study showed a tendency for serum IL-27 levels to be attenuated during the follow-up period (figure 2).

In addition, as shown in table 1, patients with SSc with elevated serum levels of IL-27 had significantly higher modified Rodnan TSS points (total SSc, p<0.05; dSSc, p<0.01; lSSc,

Table 1 Clinical and laboratory features of patients with SSc

Characteristic	Elevated IL-27 (n=52)	Normal IL-27 (n=39)
Sex, M/F	6/46	4/35
Age at onset, mean±SD (years)	47±14	46±17
Disease duration, mean±SD (years)	3.0±3.2**	7.2±8.1
Disease pattern, number with dSSc/lSSc	32/20*	13/26
Clinical features		
Modified Rodnan TSS, mean±SD (points)	16.9±11.2*	11.9±8.6
Pitting scar/ulcer	60	41
Organ involvement		
Lungs		
Pulmonary fibrosis	67**	31
%VC, mean±SD	85.1±23.4*	97.6±22.3
%Dlco, mean±SD	56.5±17.6*	66.7±18.2
Oesophagus	63	62
Heart	21	15
Kidneys	4	5
Joints	23	15
Muscles	21	15
Laboratory findings		
Serum IgG, mean±SD (mg/dl)	1847±539*	1604±428
Serum IgM, mean±SD (mg/dl)	197±125	182±74
CRP, mean±SD (mg/dl)	0.27±0.38	0.21±0.17
Serum hyaluronan, mean±SD (ng/ml)	283.9±161.5*	228.7±129.6
Serum IFN-γ, mean±SD (pg/ml)	12.7±3.3	11.9±2.4
Serum IL-4, mean±SD (pg/ml)	67.5±5.9	56.6±7.8
Serum IL-10, mean±SD (pg/ml)	42.1±6.2	45.3±8.1
Serum IL-17, mean±SD (pg/ml)	26.9±8.1**	14.3±5.8

Values are percentages unless stated otherwise.
*p<0.05, **p<0.01 vs patients with SSc with normal IL-27 levels.
CRP, C reactive protein; Dlco, diffusion capacity for carbon monoxide; dSSc, diffuse cutaneous SSc; IFN-γ, interferon-γ; IL, interleukin; lSSc, limited cutaneous SSc; SSc, systemic sclerosis; TSS, total skin thickness score; VC, vital capacity.

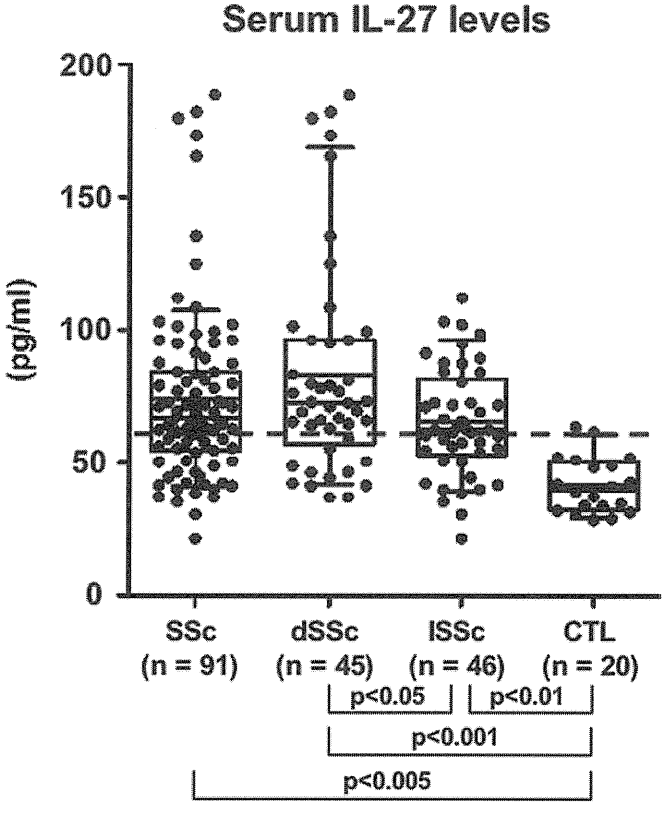

Figure 1 Serum levels of interleukin 27 (IL-27) in patients with systemic sclerosis (SSc), diffuse cutaneous SSc (dSSc) or limited cutaneous SSc (lSSc) and healthy controls (CTL). Serum IL-27 levels were determined by a specific ELISA. Data are presented as box plots, where the lines inside the boxes indicate the medians, the boxes represent the 25th and 75th percentiles and the lines outside the boxes represent the 10th and 90th percentiles. Broken line indicates the cut-off value (mean +2SD of healthy control samples).

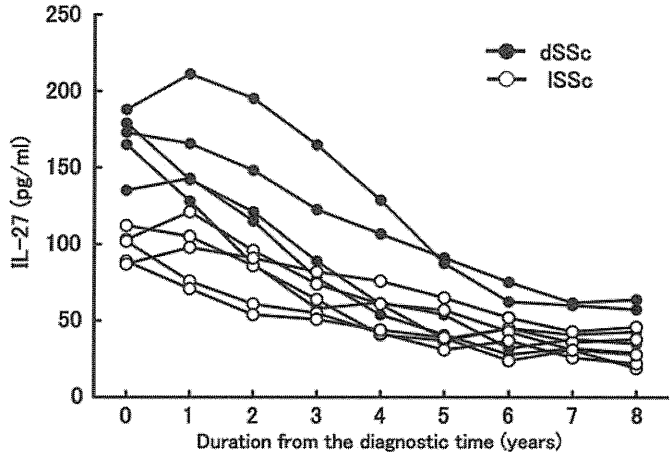

Figure 2 Serial changes in serum interleukin 27 (IL-27) levels during the follow-up period in 10 patients with systemic sclerosis (SSc) patients (5 with diffuse cutaneous SSc (dSSc) and 5 with limited cutaneous SSc (lSSc)). This retrospective longitudinal analysis showed the tendency for serum IL-27 levels to be attenuated during the follow-up period.

p<0.05), higher frequency of dSSc (p<0.01), decreased %VC (total SSc, p<0.05; dSSc, p<0.01; lSSc, p<0.05), decreased %Dlco (total SSc, p<0.05; dSSc, p<0.01; lSSc, p<0.05), elevated levels of serum hyaluronan (total SSc; p<0.05, dSSc; p<0.01, lSSc; p<0.05) and more frequent involvement of pulmonary fibrosis (total SSc, p<0.01; dSSc, p<0.01; lSSc, p<0.01) than those with normal levels. Serum IL-27 levels also correlated inversely with %VC (total SSc: r=−0.29, p<0.005; dSSc: r=−0.34, p<0.001; lSSc: r=−0.28, p<0.005) or %Dlco (total SSc: r=−0.32, p<0.005; dSSc: r=−0.36, p<0.001; lSSc: r=−0.29, p<0.005; figure 3). Furthermore, serum IL-27 levels correlated positively with the modified Rodnan TSS (total SSc: r=0.36, p<0.0005; dSSc: r=0.44, p<0.001; lSSc: r=0.31, p<0.005) and serum hyaluronan levels (total SSc: r=0.45, p<0.0001; dSSc: r=0.47, p<0.0001; lSSc: r=0.44, p<0.0001; figure 3). However, serum IL-27 levels did not correlate with any other clinical parameters.

With regard to correlation of serum IL-27 levels with immunological parameters, patients with SSc with elevated IL-27 levels had significantly higher frequency of elevated levels of serum IgG (total SSc, p<0.05; dSSc, p<0.05; lSSc, p<0.05) and IL-17 (total SSc, p<0.01; dSSc, p<0.01; lSSc, p<0.01) than those with normal levels (table 1). Moreover, serum IL-27 levels correlated positively with levels of serum IgG (total SSc: r=0.43, p<0.0001; dSSc: r=0.42, p<0.0001; lSSc: r=0.45, p<0.0001) and IL-17 (total SSc: r=0.39, p<0.0005; dSSc: r=0.41, p<0.0001; lSSc: r=0.35, p<0.0005; figure 3).

IL-27 production by cultured SSc monocytes

Unstimulated dSSc monocytes produced higher levels of IL-27 relative to normal monocytes (p<0.05, figure 4A). When monocytes were stimulated with LPS or hyaluronan, IL-27 production by both dSSc and normal monocytes was significantly increased (p<0.05). In addition, IL-27 production by LPS- or hyaluronan-stimulated dSSc monocytes was higher than those of normal monocytes (p<0.01). Similar results were obtained using lSSc samples (data not shown). The minimal effective dose of hyaluronan was 50 μg/ml.

Effect of IL-27 on SSc T cells and B cells

The levels of IL-27R expression on dSSc B cells and CD4 T cells were 3.6- and 3.1-fold higher than those observed in healthy controls (p<0.005 and p<0.001, respectively; figure 4B). In the absence of IL-27, the production of IgG by hyaluronan-treated dSSc B cells was higher than that of hyaluronan-stimulated healthy B cells (p<0.005, figure 4C). Healthy B cells treated with hyaluronan and IL-27 decreased production of IgG compared with those treated with hyaluronan alone (24% decrease, p<0.01), while dSSc B cells treated with hyaluronan and IL-27 increased production of IgG compared with those treated with hyaluronan alone (31% increase, p<0.01; figure 4C). Similar to IgG production by B cells, IL-27 treatment reduced IL-17 production by hyaluronan-treated healthy CD4 T cells (41% decrease, p<0.01), while dSSc CD4 T cells treated with both hyaluronan and IL-27 increased production of IL-17 (38% increase, p<0.01; figure 4D). Similar results were obtained using lSSc samples (data not shown).

IL-27R expression in the fibrotic skin

IL-27R expression was only faintly detected in the cell membrane and cytoplasm of healthy skin fibroblasts (figure 5A). In contrast, patients with dSSc had higher membrane and cytoplasmic expression of IL-27R in skin fibroblasts (figure 5A). While IL-27R expression was also detected in keratinocytes, endothelial cells and perivascular infiltrated and/or resident mononuclear cells, the expression levels of IL-27R in these cells were not different between dSSc and normal skin. IL-27R expression in sclerotic skin of patients with lSSc was similar to that of patients with dSSc (data not shown). Furthermore, IL-27R expression levels of total skin extract were confirmed using the real-time PCR quantification method (p<0.05, figure 5B). We also assessed IL-27 expression levels in skin samples. IL-27 expression levels in patients with dSSc were significantly higher than those in healthy controls (p<0.01, figure 5B).

Fibroblast proliferation and collagen synthesis with IL-27 stimulation

Stimulation of dSSc or healthy fibroblasts with rIL-17 increased expression of IL-27R compared with PBS alone (p<0.05, figure 5C). Similarly, stimulation of fibroblasts with a combination of rIL-17 and rIL-27 increased IL-27R expression compared with PBS alone (p<0.05). Furthermore, the expression levels of IL-27R in dSSc fibroblasts treated with both rIL-17 and rIL-27

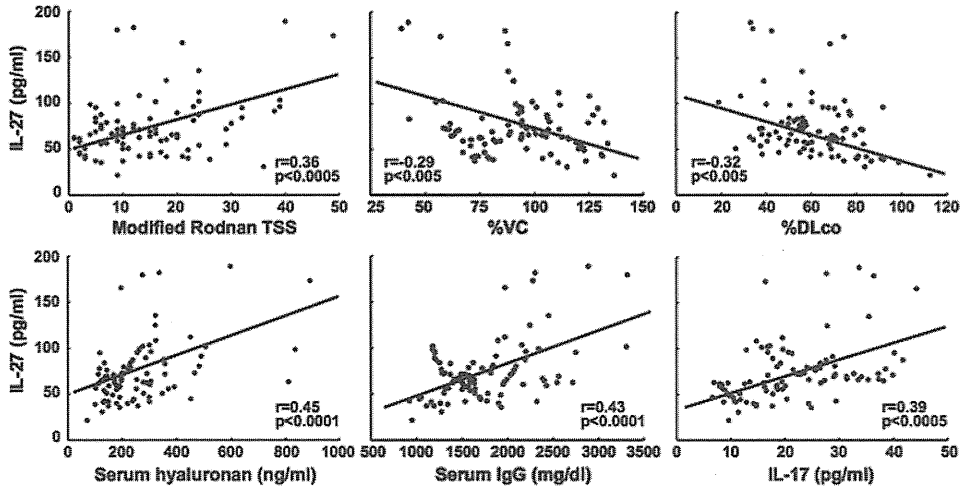

Figure 3 Correlations of serum interleukin 27 (IL-27) levels with modified Rodnan total skin thickness score (TSS), percentage vital capacity (%VC), percentage diffusion capacity for carbon monoxide (%Dlco) and serum levels of hyaluronan, IgG and IL-27 in patients with systemic sclerosis. Serum IL-27, hyaluronan and IL-17 levels were determined by specific ELISAs.

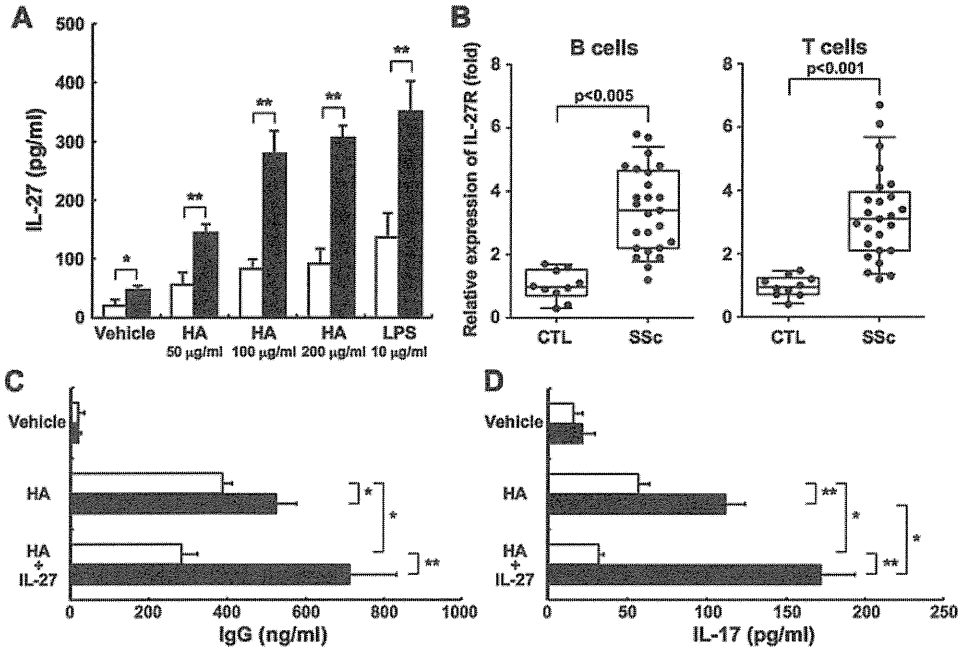

Figure 4 (A) Interleukin 27 (IL-27) production by monocytes, (B) expression of IL-27R on B cells and CD4 T cells and production of (C) IgG and (D) IL-17 by B cells and CD4 T cells, respectively. Monocytes, B cells and CD4 T cells were obtained from peripheral blood of 25 patients with diffuse cutaneous systemic sclerosis (SSc) and 10 healthy controls (CTL). For production of IL-27, purified monocytes (1×10^5) were stimulated with hyaluronan (HA) or lipopolysaccharide (LPS). IL-27R mRNA expression was analysed using real-time PCR. For production of IgG and IL-17, purified B cells (1×10^5) and CD4 T cells (1×10^5) were stimulated by HA with or without rIL-27. Culture supernatants were analysed by ELISA to determine the amount of IL-27, IgG and IL-17. In (A), (C) and (D), bars show the mean and SD. In (B), the data are presented as box plots where the lines inside the boxes indicate the medians, the boxes represent the 25th and 75th percentiles and the lines outside the boxes represent the 10th and 90th percentiles. *p<0.01, **p<0.005.

were higher than those of rIL-17-treated fibroblasts (p<0.05). However, only rIL-27 stimulation did not affect expression of IL-27R. The levels of IL-27R expression on SSc fibroblasts were higher than those of healthy controls in each group (p<0.05). The proliferative effect of IL-27 was not observed on healthy fibroblasts but was detectable with 0.2 ng/ml IL-27 on SSc fibroblasts (p<0.01, figure 5D). Similarly, IL-27 had a significant effect on collagen synthesis in fibroblasts. In healthy fibroblasts, 1 ng/ml IL-27 significantly increased COL1A1 and COL1A2 expression and type I collagen production compared with vehicle alone (p<0.05). In SSc fibroblasts, 0.1 ng/ml IL-27 also significantly increased COL1A1 and COL1A2 expression and type I collagen production compared with vehicle alone (p<0.05, figure 5D).

DISCUSSION

This study is the first to show that IL-27 levels are elevated in serum samples from patients with SSc compared with normal controls (figure 1). We also showed that IL-27 levels correlated positively with modified Rodnan TSS (figure 3 and table 1). Furthermore, elevation of IL-27 levels was accompanied by the presence of pulmonary fibrosis and decreased %VC and %Dlco, indicating that IL-27 levels correlated with the severity of lung fibrosis. Furthermore, retrospective longitudinal analysis showed that there was a tendency for serum IL-27 levels to be attenuated during the follow-up period (figures 2 and 3 and table 1). Collectively, these results may suggest that overproduction of IL-27 has an important role in the pathogenesis of SSc, especially in the earlier phase of the disease.

IL-27 is mainly secreted from activated antigen-presenting cells such as monocytes by stimulation with their TLR.[11–13 35] LPS is an exogenous ligand for TLR4 which strongly induces IL-27 secretion from monocytes (figure 4A), as previously described.[14 15] Recently, many studies have identified various endogenous ligands for TLR4, such as hyaluronan which regulates inflammatory responses.[6 36] In our study, hyaluronan treatment enhanced IL-27 production by both healthy and SSc monocytes (figure 4A). Recent studies have shown that, in patients with SSc, monocyte activation may be maintained by one or more enhancing signals such as IL-6 and TNFα which are increased in patients with SSc[37] and are known to trigger the activation of TLR4 expression.[38] This may explain why IL-27 production by SSc monocytes was higher than by healthy monocytes (figure 4A). Thus, IL-27 production is strongly induced by hyaluronan stimulation in SSc monocytes compared with healthy monocytes.

IL-27R is expressed by a wider range of cells, especially activated CD4 T cells and B cells, which may explain the pleiotropic role of IL-27.[35] To date, IL-27R expression in SSc T cells and B cells remains unknown, although T cell and B cell activation is detected in patients with SSc.[39 40] This study is the first to indicate that T cells and B cells in patients with SSc had higher expression levels of IL-27R than in healthy controls (figure 4B). Currently, the role of IL-27 in various models of inflammatory disease is still controversial. In some disease models such as allergic asthma and experimental autoimmune encephalomyelitis, IL-27 has been found to suppress the inflammation[20 22 41] but, in other disease models such as concanavalin A-induced hepatitis and dextran sulphate sodium- or oxazorolone-induced colitis, IL-27 has been found to exacerbate inflammation.[42–44] In this study, serum IL-27 levels correlated with serum levels of IgG and IL-17 in patients with SSc (figure 3 and table 1). Furthermore, IL-27 stimulation strongly induced IgG and IL-17 production by

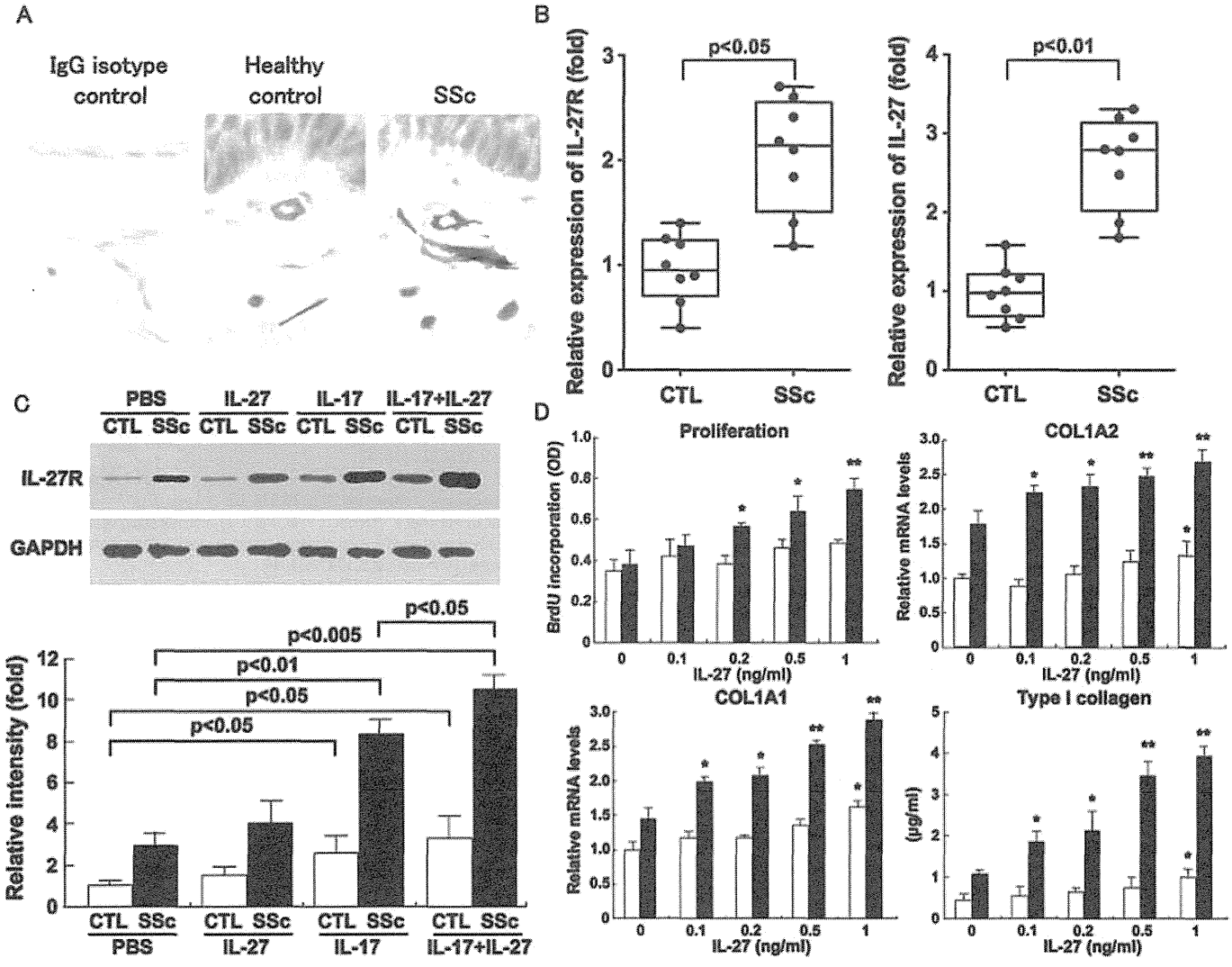

Figure 5 (A) Immunohistochemical analysis of fibrotic skin using interleukin 27 receptor (IL-27R) mouse monoclonal antibody compared with concentration-matched mouse monoclonal antibody IgG isotype control (CTL). (B) IL-27R and IL-27 mRNA expression of healthy control (n=8) and systemic sclerosis (SSc) (n=8) skin analysed using real-time PCR. (C) Western blot analysis of IL-27R protein in stimulated fibroblasts with or without rIL-27 and/or rIL-17. Fibroblasts were grown to confluence and culture medium was replaced with serum-free modified Eagle's medium. (D) After further incubation for 24 h, fibroblasts were stimulated with each concentration of rIL-27 for 24 h. After incubation, collagen type I α1 (COL1A1) and COL1A2 mRNA expression and type I collagen protein production were analysed by real-time PCR and ELISA, respectively. In proliferation assay, after 24 h incubation, 5-bromo-2-deoxyuridine (BrdU) (10 μm) was added to each well and incubated for 24 h. BrdU incorporation in proliferating cells was quantified by ELISA. In (B), data are presented as box plots, where the lines inside the boxes indicate the medians, the boxes represent the 25th and 75th percentiles and the lines outside the boxes represent the 10th and 90th percentiles. In (C) and (D), bars show the mean and SD. *p<0.05, **p<0.01 vs each fibroblast cultured with vehicle alone. Original magnification ×100.

B cells and T cells in patients with SSc (figure 4C,D). Thus, IL-27 may have a role in immunological abnormalities associated with SSc.

A recent study has shown that increased IL-27R expression is also detected in activated fibroblasts.[17] In our study, IL-27R expression was significantly higher in SSc fibroblasts than in healthy fibroblasts (figure 5A–C). In patients with SSc, fibroblast activation is induced by several cytokines including IL-17, which may explain the higher expression of IL-27R in SSc fibroblasts.[45 46] In addition, ligation of IL-27 to IL-27R expressed by fibroblasts is likely to facilitate further fibroblast activation.[17] Indeed, in this study rIL-17 stimulation increased IL-27R expression on SSc fibroblasts which was further enhanced by rIL-27 costimulation (figure 5B). Furthermore, IL-27 expression was increased in the skin of patients with SSc (figure 5A) and rIL-27 strongly increased proliferation and collagen synthesis of SSc fibroblasts (figure 5D). Thus, IL-27 may have a role in the development of SSc by amplifying collagen synthesis and increasing fibroblast proliferation.

Although further studies are required to clarify the role of IL-27 in the development of SSc, it may be a useful serological marker for disease severity and a new therapeutic target in SSc.

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Competing interests None.

Patient consent Obtained.

Ethics approval *This study was conducted with the approval of the Nagasaki University Hospital.*

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Original article

Association between nail-fold capillary findings and disease activity in dermatomyositis

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Abstract

Objective. Although findings of nail-fold capillary changes and reduced red blood cell velocity in SSc patients are well established, studies in adult-onset DM patients are scarce. Our objective was to assess the changes and red blood cell velocity in finger nail-fold capillaries using nail-fold video capillaroscopy (NVC) in patients with adult-onset DM.

Methods. This study included 50 patients with adult-onset DM and 20 healthy subjects. A semi-quantitative rating scale was used to score capillaroscopy changes. Red blood cell velocity was evaluated using frame-to-frame determination of the position of capillary plasma gaps.

Results. Thirty-seven (74%) patients showed the scleroderma NVC pattern. Patients with the scleroderma pattern exhibited elevated serum creatine kinase levels more frequently and increased visual analogue scale of muscle disease activity. Scores of loss of capillaries were associated with muscle and global disease activity, whereas scores of haemorrhages were associated with skin disease activity. However, NVC findings were not significantly associated with lung involvement. The scores of irregularly enlarged capillaries, haemorrhages and loss of capillaries were reduced after stabilization of disease activity by treatment. The mean red blood cell velocity was not significantly reduced in DM patients compared with healthy controls and was not changed by treatment.

Conclusion. Our results suggest that changes in nail-fold capillaries reflect disease activity in DM. Furthermore, the differences found in red blood cell velocity may reflect somewhat distinct microcirculation injuries in DM and SSc.

Key words: Capillaroscopy, Dermatomyositis, Disease activity, Red blood cell velocity, Systemic sclerosis.

CLINICAL

Introduction

DM is an autoimmune connective tissue disease in which characteristic patterns of inflammatory injury occur in striated muscle. PM is a similar disease, but DM can be distinguished from PM via the presence of cutaneous features such as heliotrope rash or Gottron's papule/sign. Interstitial pneumonia and internal malignancy are examples of organ complications that affect the prognosis of DM. Pulmonary involvement is frequently seen in patients with anti-aminoacyl tRNA synthetase (ARS) antibodies [1] or anti-clinically amyopathic DM (CADM)-140 antibody [2]. In contrast, a recent study has reported that autoantibodies reactive with 155 kDa (and 140 kDa) nuclear proteins (anti-p155 antibody/anti-155/140 antibody) are associated with malignancy in patients with DM [3, 4]. DM patients with anti-Mi-2 antibodies typically present with relatively mild symptoms and have a good prognosis [5].

A vast amount of well-documented data exist regarding nail-fold capillaroscopy and nail-fold video capillaroscopy (NVC) in the diagnosis and follow-up of microvascular damage in patients with RP or scleroderma spectrum disorder (SSD), SSc and its related diseases [6–11]. Furthermore, NVC changes are just as prevalent and prominent in DM as in SSD [12, 13]. However, such findings

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are not frequently detected in patients with other connective tissue diseases. Video recordings of blood capillary flow also allow measurement of capillary red blood cell velocity. We recently reported that red blood cell velocity is remarkably reduced in SSc patients compared with healthy individuals [14]. Additionally, reduced peripheral blood flow has been demonstrated using laser-Doppler perfusion imaging [15, 16].

Despite numerous reports regarding NVC findings in SSc, there are relatively few NVC examinations in DM, and this is especially true for adult-onset DM [17–20] compared with JDM [21–23]. In addition, the clinical relevance of NVC findings in DM has not yet been established, as there are inconsistencies among reports, and quantitative analyses have not yet been adequately assessed. Furthermore, red blood cell velocity has not been evaluated, except for our previous report of a small cohort [14] of patients with DM. In this study, we assessed changes in nail-fold capillaroscopy and red blood cell velocity in patients with DM.

Materials and methods

Patients and clinical assessment

Fifty Japanese patients with DM [39 females and 11 males; mean (S.D.) age, 54.7 (14.8) years] who visited Kanazawa University Hospital between 7 October 2007 and 31 July 2010 were included in this study. Forty patients fulfilled the criteria of Bohan and Peter [24, 25], while the remaining 10 did not fulfil the criteria, but fulfilled Sontheimer's criteria [26], due to the absence of clinical muscle symptoms and presence of subsistent clinical skin eruptions. No patients met the criteria for other rheumatic diseases such as SSc. Mean disease duration was 3.9 (5.1) years. At the time of evaluation, 72% of the patients were already receiving oral predonisolone (PSL) therapy and 26% of the patients were treated with immunosuppressive drugs, including CYC, CSA and tacrolimus, in addition to PSL. A total of 20 healthy subjects [15 females and 5 males; mean (S.D.) age, 51.4 (17.2) years] were also evaluated.

Complete medical histories, physical examinations and laboratory tests were conducted for all patients during the first visit, with limited evaluations during follow-up visits. The patients were diagnosed as having interstitial lung disease (ILD) according to the results of chest radiography, chest CT and pulmonary function testing. Serum Krebs von den Lungen-6 (KL-6) levels as a serum marker of ILD were determined by ELISA as described previously [27]. Presence of internal malignancy was carefully examined using CT, gastrointestinal fibrescope, gallium scintigraphy and other procedures according to need. The local ethics committee (Kanazawa University Hospital, Ishikawa, Japan) approved this study protocol. Informed consent was obtained from each patient.

Immunoprecipitation assays were performed to identify autoantibodies using extracts of the leukaemia cell line K562, as previously described [2]. Using this method, 25.5% of patients were positive for anti-ARS antibody, 18.6% were positive for anti-155/140 antibody and 9.3% were positive for anti-Mi-2 antibody. Anti-SS-A antibody was detected in two patients and anti-NOR 90 antibody, anti-SRP antibody, anti-U1 RNP antibody and anti-Wa antibody were detected in one patient each.

To assess disease activity based on individual organ systems, the Myositis Disease Activity Assessment VAS (MYOACT) portion of the Myositis Disease Activity Tool [28] was used. The MYOACT assessment utilizes separate 100-mm visual analogue scales (VASs) to gauge the physician's evaluation of disease activity in several discrete domains. Involvement of all non-muscle organ systems (constitutional, cardiac, pulmonary, gastrointestinal, skeletal and cutaneous) was also evaluated using the composite extra-skeletal muscle VAS score. An additional VAS measure, the global VAS score, was used to rate overall disease activity.

NVC pattern classification

We assessed NVC findings using a video capillaroscopy system (CP-1000; Chunichi Denshi, Nagoya, Japan), as previously reported [14]. Classification of NVC patterns was performed according to the criteria of Maricq and colleagues [29, 30]. Diagnostic capillaroscopy patterns were grouped into the following categories, as previously described [31]: (i) normal pattern: homogeneous capillary distribution in the nail-fold plexus without capillary loss (normal medium density: linear 30 capillaries per 5 mm) and no morphological alterations; (ii) scleroderma pattern (defined according to Maricq and colleagues [29, 30], with modifications according to Bergman *et al.* [32]): two or more of the following abnormalities: enlarged capillaries, haemorrhages (more than two punctate haemorrhages per finger, or confluent haemorrhage areas), disorganization of the normal capillary distribution, moderate or extensive capillary loss (i.e. avascular areas) and tortuous, crossed and/or ramified capillaries; and (iii) non-specific pattern: lack of complete scleroderma pattern criteria. Since it was often difficult to distinguish between the normal and non-specific patterns, these patterns were combined into a normal/non-specific group in this study.

The scleroderma patterns were further classified as previously reported [33], as follows: (i) early NVC pattern: few enlarged/giant capillaries, few capillary haemorrhages, relatively well-preserved capillary distribution and no evident loss of capillaries; (ii) active NVC pattern: frequent giant capillaries, frequent capillary haemorrhages, moderate loss of capillaries with some avascular areas, mild disorganization of the capillary architecture and absent or ramified capillaries; and (iii) late NVC pattern: irregular capillary enlargement, few or absent giant capillaries, absence of haemorrhages, severe loss of capillaries with large avascular areas, severe disorganization of the normal capillary array and frequent ramified/bushy capillaries.

Scoring of NVC findings

A semi-quantitative rating scale to score six capillary parameters (irregularly enlarged capillaries, giant capillaries, haemorrhages, loss of capillaries, disorganization of the

vascular array and capillary ramifications) was adopted (0 = no changes, 1 = <33% capillary alterations/reduction, 2 = 33–66% capillary alterations/reduction, 3 = >66% capillary alterations/reduction, per linear mm) according to previous studies [11].

Measurement of red blood cell velocity

We measured red blood cell velocity using the video capillaroscopy system, as previously reported [34]. Mean blood cell velocity was calculated by averaging the results from three capillaries, excluding giant or ramified capillaries, in each ring finger. The examination was performed blindly by the same operator, without knowledge of patients' clinical conditions or characteristics. Red blood cell speed in nail-fold capillaries was evaluated by frame-by-frame analysis of video data, as described previously [35]: displacement of the edge of the plasma gap was measured on a frame-to-frame basis as it moved in the capillary limb. The velocity was then calculated by relating this displacement to the framing rate (fixed at 30 frames/s) [35].

Statistical analysis

Statistical analyses were performed using JMP® 7.01 Statistical Discovery Software (SAS institute, Cary, NC, USA). Chi-squared test and *t*-test were used for comparing the frequency and the mean value, respectively. Spearman's rank correlation coefficient was used to examine the relationship between two continuous variables. Bonferroni correction was performed for multiple comparisons. A $P < 0.05$ after Bonferroni correction was considered indicative of statistical significance. All data are shown as mean (S.D.), unless otherwise indicated.

Results

NVC pattern classification

Among patients with DM, 74% displayed the NVC scleroderma pattern. Among these patients, the early NVC pattern was observed in 12 (24.0%) patients, the active NVC pattern was noted in 23 (46.0%) patients and the late NVC pattern was recognized in 2 (4.0%) patients. None of the 20 normal control subjects demonstrated a scleroderma pattern.

Association between scleroderma pattern and clinical/laboratory findings

We examined the association between NVC changes and clinical or laboratory features. Patients with the scleroderma pattern had shorter disease duration than patients with the normal NVC pattern, but the difference was not significant (Table 1). The frequencies of muscle weakness, Gottron's sign and heliotrope rash were higher in patients with the scleroderma pattern than in patients with the normal NVC pattern, but these differences were not significant. In addition, patients with the scleroderma pattern displayed internal malignancies more frequently than patients without the pattern, although this difference was not significant. The presence or duration of RP was not associated with scleroderma pattern. Examination of laboratory findings revealed that patients with the scleroderma pattern displayed elevated serum creatine kinase (CK) levels more frequently than patients without the scleroderma pattern ($P < 0.01$). Although patients with anti-155/140 antibody had the scleroderma pattern more frequently compared with patients without the antibody, the difference was not statistically significant. The frequency of oral PSL and CSA use was lower in patients with the scleroderma pattern than in patients without it, but the difference was not significant. Other than these results, there were no associations between the NVC scleroderma pattern and clinical or laboratory features in patients with DM. Thus, the scleroderma pattern was most associated with elevated serum CK levels in patients with DM.

Table 1 Association between scleroderma pattern and clinical or laboratory findings

Clinical or laboratory finding	Scleroderma pattern ($n = 37$)	Normal/ non-specific pattern ($n = 13$)	*P*-value
Age, mean (S.D.), years	55.9 (14.5)	51.3 (16.0)	1.0
Sex (male : female)	6 : 31	5 : 8	0.30
Disease duration, mean (S.D.), months	37.0 (62.0)	75.8 (50.7)	0.14
Symptoms, %			
Muscle weakness	48.6	30.8	1.0
Gottron's sign	54.1	38.5	0.30
Heliotrope rash	24.3	7.7	0.60
ILDs	43.2	53.8	1.0
Internal malignancy	24.3	7.7	0.22
RP	21.6	15.4	1.0
Laboratory findings, %			
Elevated CK	40.5	0	0.0061**
Elevated KL-6	37.8	61.5	0.14
Autoantibodies, %			
Anti-ARS antibody	29.7	15.4	0.93
Anti-155/140 antibody	24.3	0	0.15
Anti-Mi-2 antibody	5.4	15.4	0.75
Medications, %			
PSL	62.2	100	0.090
CYC	2.7	0	1.0
CSA	8.1	53.8	0.090
Tacrolimus	2.7	0	1.0
MTX	0	7.7	1.0
IVIG	2.7	15.4	1.0

**$P < 0.01$.

Association between scleroderma pattern and disease activity

To assess disease activity based on individual organ systems, the MYOACT portion of the Myositis Disease Activity Assessment Tool was used. The VAS scales of muscle disease activity were significantly higher in patients with the scleroderma pattern than in patients

without it ($P < 0.01$, Table 2). We examined six aspects of global extra-skeletal muscle disease activity. The total global extra-skeletal muscle disease activity, constitutional disease activity, cutaneous disease activity and skeletal disease activity were higher in patients with the scleroderma pattern than in patients without it, although these differences were not significant. Pulmonary activity was comparable between patients with the scleroderma pattern and patients with the normal/non-specific pattern. Global disease activity, defined as muscle disease activity merged with global extra-skeletal muscle disease activity, was higher in patients with the scleroderma pattern than in patients without it, but the difference was not significant. Thus, the scleroderma pattern was significantly associated with muscle disease activity.

Association between the score of NVC changes and disease activity scales

Among six capillaroscopic parameters, the score of capillary ramifications was excluded in this analysis, since the number of patients with this abnormality was small (12%). The scores for loss of capillaries were significantly associated with the scales of muscle disease activity ($r = 0.34$; $P < 0.05$) and global disease activity ($r = 0.37$; $P < 0.05$, Table 3). On the other hand, the scores of haemorrhages were significantly associated with the scales of cutaneous disease activity ($r = 0.41$; $P < 0.01$, Table 3). However, no parameters were associated with pulmonary disease activity. Thus, there are some specific associations between the scores of NVC changes and muscle, cutaneous and global disease activity scales.

Table 2 Association between scleroderma pattern and myositis disease activity scale

Myositis disease activity scale	Scleroderma pattern ($n = 37$)	Normal/ non-specific pattern ($n = 13$)	*P*-value
Muscle disease activity	20.7 (24.7)	4.6 (7.8)	0.0030*
Global extra-skeletal muscle disease activity	13.7 (7.7)	7.9 (6.7)	0.060
Constitutional disease activity	18.2 (17.0)	6.2 (8.7)	0.29
Cutaneous disease activity	37.0 (23.5)	18.2 (17.8)	0.17
Skeletal disease activity	3.5 (9.2)	0.8 (2.8)	1.0
Gastrointestinal disease activity	2.4 (11.6)	2.3 (6.0)	1.0
Pulmonary disease activity	17.8 (24.2)	20.0 (24.5)	1.0
Cardiac disease activity	3.2 (6.7)	0	1.0
Global disease activity	23.9 (16.6)	11.4 (11.9)	0.054

*$P < 0.01$. Data are shown as mean (S.D.).

Red blood cell velocity

Mean red blood cell velocity was 0.800 (0.164) mm/s in healthy volunteers (Fig. 1). Patients with DM had a reduced blood velocity of 0.663 (0.204) mm/s, which was 82.9% that of healthy controls; however, this difference was not statistically significant. In addition, the blood velocity values were not significantly different between NVC patterns (early, active, late and normal/non-specific). No significant association between blood velocity and clinical features was found in patients with DM (data not shown). Although there are several patients who showed reduced red blood cell velocity, no specific clinical features were detected in these patients. Thus, red blood cell velocity was not significantly changed in patients with DM.

NVC changes during the longitudinal study

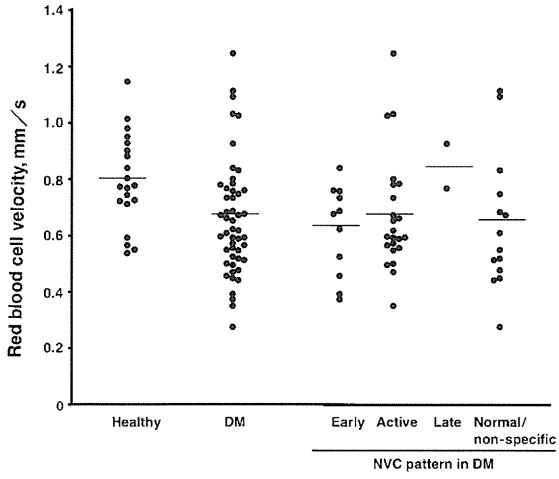
Twelve patients who had a first visit due to active DM were followed up until the disease was stabilized by treatment with immunosuppressive agents, a period of time that averaged 9.2 (8.4) months. The profile of these patients is shown in Table 4. The mean scale of global disease activity was 33.3 (16.1) at their first visit, which was significantly reduced to 11.9 (9.4) after stabilization by treatment ($P < 0.001$). Of six capillaroscopic parameters, the score of capillary ramifications was excluded from this analysis, since only one patient had this abnormality. The scores of irregularly enlarged capillaries [1.33 (0.89) $\rightarrow$0.17 (0.58) mm/s], haemorrhages [1.83 (1.19)$\rightarrow$0.17 (0.39) mm/s] and loss of capillaries [0.58 (0.72)$\rightarrow$0.08 (0.29) mm/s] were significantly reduced after stabilization of disease ($P < 0.01$), whereas the scores of giant capillaries [1.92 (0.67)$\rightarrow$1.17 (0.39) mm/s] and disorganization of the vascular array [1.17 (1.19)$\rightarrow$0.75 (0.87) mm/s] were not significantly changed. Five representative cases are shown in Fig. 2. These pictures demonstrate that irregularly enlarged capillaries, haemorrhages and loss of capillaries are reduced or disappear, and gradually regenerate, after stabilization of disease activity. In contrast, red blood cell

Table 3 Association between the score of NVC change and disease activity scale

Myositis disease activity scale	Irregularly enlarged capillaries, *r*	Giant capillaries, *r*	Haemorrhages, *r*	Loss of capillaries, *r*	Disorganization of the vascular array, *r*
Muscle disease activity	0.08	0.11	0.21	0.34*	0.28
Cutaneous disease activity	0.29	0.22	0.41**	0.21	0.30
Pulmonary disease activity	0.03	0.16	−0.15	0.003	0.04
Global disease activity	0.15	0.26	0.19	0.37*	0.33

*$P < 0.05$, **$P < 0.01$.

FIG. 1 Red blood cell velocity in patients with DM and in healthy controls. In patients with DM, the data were also shown dependent on the NVC pattern. Red blood cell velocity was evaluated using frame-to-frame determination of the position of a plasma gap in the capillary. The short bar indicates the mean value in each group.



velocity was not significantly changed by treatment [0.739 (0.149)→0.653 (0.161) mm/s]. Thus, the NCV abnormalities, especially irregularly enlarged capillaries, and haemorrhages and loss of capillaries likely reflect therapeutic effects in patients with DM.

Discussion

Using a video capillaroscopy system, we assessed morphological change and red blood cell velocity in the nail-fold capillaries of DM patients. The NVC scleroderma pattern was frequently detected in patients with DM, and was associated with disease activity, especially muscle disease activity. Among various NVC findings, loss of capillaries was significantly associated with muscle and global disease activities. In addition, haemorrhage was significantly associated with cutaneous disease activity. Findings of irregularly enlarged capillaries, haemorrhage and loss of capillaries were decreased after stabilization of disease activity by treatment. Red blood cell velocity was not significantly reduced in patients with DM compared with normal controls and was not changed by treatment.

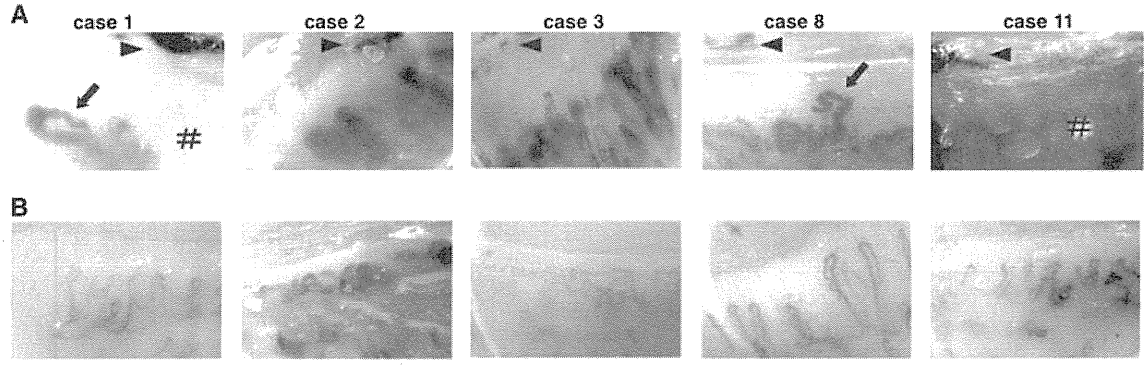
Although information regarding nail-fold capillary changes and red cell velocity had been previously available for SSc, such information was not fully established for adult DM. In our study, the NVC scleroderma pattern was found in 74% of DM patients, which was slightly lower than, but comparable to, what we previously reported in SSc patients (84%) [14]. Previous studies have reported significant positive correlation between cutaneous blood flow measured by laser Doppler imaging, and disease severity in adult patients with DM [36]. In contrast, reduced cutaneous blood flow detected by laser Doppler imaging has been reported in patients with

TABLE 4 The profile of patients followed until the stabilization of disease activity

Case	Age	Sex	Duration, months	Muscle weakness	ILD	Internal malignancy	RP	Serum CK level, IU/l	Autoantibodies	Initial therapy	Follow-up period, months	Therapy at the second point (inactive)
1	40	Female	2	++	−	−	−	6942	Mi-2	PSL 50 mg, mPSL pulse, CYC, IVIG	15	Rinderon 1 mg, tacrolimus 5 mg
2	42	Male	1	++	−	−	−	4795	Mi-2	PSL 50 mg, mPSL pulse, tacrolimus 3 mg	3	Rinderon 2.5 mg, tacrolimus 3 mg
3	44	Female	3	+	−	+	−	9388		Rinderon 100 mg × 3 days, PSL 30 mg	1	PSL 20 mg
4	51	Female	12	+	+	−	+	345	ARS (Jo-1)	PSL 30 mg	2	PSL 20 mg, tacrolimus 2 mg
5	53	Female	14	−	−	−	−	100	negative	PSL 20 mg	6	PSL 7.5 mg
6	57	Female	3	−	−	−	−	63	U1	PSL 20 mg	5	PSL 10 mg
7	59	Female	9	−	+	−	−	64	Wa	PSL 45 mg, mPSL pulse	3	PSL 25 mg
8	61	Female	30	+	+	+	−	49	ARS (Jo-1)	PSL 50 mg, CSA 750 mg	9	PSL 20 mg, tacrolimus 3 mg
9	61	Male	18	+	+	−	−	8261	ARS (Jo-1)	PSL 50 mg	1.5	PSL 35 mg
10	70	Male	8	+	−	+	−	1700	155/140	PSL 50 mg, mPSL pulse	6	PSL 18 mg
11	73	Female	4	+	+	−	−	126	CADM140	PSL 50 mg, CYC, tacrolimus 2 mg	4	PSL 30 mg, tacrolimus 3 mg
12	82	Female	3	+	−	+	−	169	155/140	PSL 20 mg	2	PSL 15 mg

mPSL: methylprednisolone.

FIG. 2 Representative NVC images at (**A**) base line and (**B**) after treatment, demonstrating how changes can be followed after stabilization of the disease activity in patients with DM. ◄: haemorrhages; ✎: irregularly enlarged capillaries; #: loss of capillaries.



PM/DM [19]. However, as far as we know, red blood cell velocity using video capillaroscopy has not been assessed by other groups. We previously reported that patients with SSc showed a 63% decrease in red blood cell velocity compared with healthy controls [34]. In that study, DM patients included as disease controls exhibited slightly but not significantly reduced red blood cell velocities compared with healthy controls. In this study, we confirmed that result in a larger DM population, and assessed the association with clinical features. Although the NVC findings in DM are indistinguishable from those in SSc [12], our findings indicate that the reduction in red blood cell velocity is more modest in DM patients than that in SSc patients. This may reflect somewhat different microcirculation injuries in DM *vs* SSc.

Our findings indicate that NVC changes are significantly associated with disease activity in patients with DM. Patients with the scleroderma pattern had elevated serum CK levels more frequently and had higher VAS scales of muscle disease activity than patients without the scleroderma pattern. Patients with scleroderma pattern also showed skin symptoms more frequently and elevated cutaneous disease VAS scales compared with patients without scleroderma pattern, although these differences were not significant. On the other hand, the frequency and disease activity of interstitial pneumonia was comparable between patients with the scleroderma pattern and patients without it. Since interstitial pneumonia is often retractable, our findings may at least partly reflect the difficulty of evaluating lung activity. Thus, the current study suggests that NVC change is associated with disease activity, especially muscle disease activity.

Our study identified several disparities between DM patients who displayed the scleroderma pattern and those who did not. For example, scleroderma pattern DM patients had shorter disease duration than DM patients without the scleroderma pattern, although the difference was not significant. Furthermore, patients without scleroderma pattern were receiving PSL and CSA more frequently than patients with scleroderma pattern, although these difference were not significant. These findings likely reflect the fact that patients with short disease duration tend to have active disease, whereas most patients with long disease duration are stable with treatment. In fact, the clinical features at their active phase (before treatment) were not significantly different between patients treated with PSL or CSA and patients not receiving treatment (data not shown). In addition, DM patients with the scleroderma pattern had internal malignancies more frequently than DM patients without the scleroderma pattern, although the difference was not significant. Consistent with this, anti-155/140 autoantibody, which is commonly detected in DM patients with internal malignancy, tended to be more frequently detected in DM patients with the scleroderma pattern than in patients without it. Since DM patients with either anti-155/140 antibody or internal malignancy typically exhibit cutaneous eruption and myositis without lung involvement [3, 4], such associations may be due to the cutaneous and muscle disease activity in these patients.

Importantly, NVC changes were improved by disease stabilization in DM patients during the follow-up period. Among NVC changes, irregularly enlarged capillaries, haemorrhages and loss of capillaries were significantly reduced after stabilization of disease activity (Fig. 2). Therefore, monitoring these changes will likely be useful in evaluating disease activity and therapeutic efficacy. On the other hand, it has been reported that capillary loss is associated with progression of SSc and generally of the microvascular damage in secondary Raynaud's syndrome, at least in SSc [37–39]. In SSc patients, giant capillaries and haemorrhages were not considered critically important in the evaluation of SSc microangiopathy, as these abnormalities are evident only in the early stages of the disease, and then disappear or become rare in the advanced stages [33, 40]. Thus, our study demonstrates that the significance of each NVC change is different in some degree between DM and SSc.

A previous study of adult-onset PM and DM patients found that RP, arthritis and pulmonary involvement were

associated with increased numbers of enlarged capillary loops and more severe avascular lesions [18]. In that study, the severity of the observed abnormalities did not correlate with the occurrence of malignancy or active myositis, but tended to decline with prolonged disease remission [18]. In a recent study including 53 adult patients with inflammatory myopathy, disease activity and severity were both significantly associated with alterations in capillary morphology [20]. Furthermore, marked abnormalities of capillaries were significantly associated with the involvement of internal malignancy or ILD. There are also some reports regarding NVC findings in JDM. One study found that NVC abnormalities are associated with skin involvement in patients with JDM [22]. Another study demonstrated that capillary loss was associated with skin involvement in JDM [21]. A prospective study involving 13 JDM patients demonstrated that capillary dropout was most frequently correlated with disease activity [23]. Longer duration of untreated disease and severe skin lesions were associated with capillary reduction in JDM [41]. Regarding associations with autoantibody, anti-Jo-1 antibody was associated with reduced capillary density [17]. Thus, our findings show some discrepancies with previous findings in patients with inflammatory myopathy. The main cause is likely due to the heterogeneity of inflammatory myopathy, and this makes the case for studying adult DM exclusively. Most previous papers have assessed inflammatory myopathy, both DM and PM, whereas our study is restricted to DM. For example, it has been reported that microhaemorrhages and capillary enlargement appear to represent the characteristic NVC pattern in patients with DM, but not in those with PM [20]. Ethnic differences also affect the results. Nonetheless, our results in DM, along with previous findings in inflammatory myopathy, are at least consistent in finding that general disease activity and severity are associated with prominent morphological changes.

Thus, our findings, together with previous reports, indicate that NVC findings are useful for diagnostic purposes, as well as for assessment of disease activity and response to treatment in patients with DM. Nonetheless, this study has some limitations. Although DM is rare, the number of patients analysed was not large and the study was restricted to Japanese individuals. Furthermore, most patients were already stable due to treatment at the time of evaluation. Therefore, further prospective multicentre studies using larger patient populations will be needed to confirm our results.

Rheumatology key message

- Morphological variation of nail-fold capillaries but not red blood cell velocity is associated with disease activity of DM.

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Serum Levels of Galectin-3: Possible Association with Fibrosis, Aberrant Angiogenesis, and Immune Activation in Patients with Systemic Sclerosis

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ABSTRACT. ***Objective.*** Galectin-3 is a multifunctional protein implicated in a variety of biological processes including fibrosis, angiogenesis, and immune activation, all of which are associated with the development of systemic sclerosis (SSc). We investigated the clinical significance of serum galectin-3 levels in SSc.

Methods. Serum galectin-3 levels were determined by a specific ELISA in 58 patients with SSc and 19 healthy controls.

Results. Serum galectin-3 levels were significantly lower in patients with diffuse cutaneous SSc (dcSSc) than in controls (3.29 ± 3.27 ng/ml vs 4.91 ± 2.67 ng/ml, respectively; $p < 0.05$), while being comparable between limited cutaneous SSc (3.70 ± 2.39 ng/ml) and healthy controls. In dcSSc, serum galectin-3 levels significantly correlated with total skin score ($r = 0.45$, $p < 0.05$). Serum galectin-3 levels were significantly decreased in early dcSSc (disease duration < 1 year; 1.64 ± 1.74 ng/ml; $p < 0.05$), but not in mid-stage dcSSc (1 to 6 years; 3.22 ± 3.16 ng/ml) or late-stage dcSSc (> 6 years; 4.86 ± 4.10 ng/ml), compared with controls. Serum galectin-3 levels were higher in SSc patients with both digital ulcers (DU) and elevated right ventricular systolic pressure (RVSP) than in those without each symptom (DU: 5.44 ± 3.74 ng/ml vs 2.99 ± 2.36 ng/ml, $p < 0.05$; elevated RVSP: 4.44 ± 3.14 ng/ml vs 2.82 ± 2.64 ng/ml, $p < 0.05$).

Conclusion. Galectin-3 may be related to the developmental process of skin sclerosis in dcSSc and of DU and pulmonary vascular involvements in total SSc. (First Release Jan 15 2012; J Rheumatol 2012;39:539–44; doi:10.3899/jrheum.110755)

Key Indexing Terms:

SYSTEMIC SCLEROSIS | GALECTIN-3 | SKIN FIBROSIS
PULMONARY ARTERIAL HYPERTENSION | DIGITAL ULCER

Systemic sclerosis (SSc) is a multisystem autoimmune disease characterized by initial vascular injuries and resultant fibrosis of skin and certain internal organs[1]. Although the pathogenesis of SSc remains unknown, an increasing number of growth factors, cytokines, and other molecules have been shown to be involved in the complex network of signaling pathways driving aberrant immune activation, dysregulated angiogenesis, and deposition of extracellular matrix throughout the course of this complex disorder.

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Lectins are carbohydrate-binding proteins with a selective affinity for specific oligosaccharides[2]. Galectins are a growing family of ß-galactoside-binding animal lectins, 15 members of which have been identified to date[3]. Among them, galectin-3 has a unique structure whereby its single polypeptide chain forms 2 structurally distinct domains, such as the C-terminal carbohydrate recognition domain and the atypical N-terminal domain[2]. While the C-terminal domain is responsible for lectin activity of galectin-3, the N-terminal participates in multimer formation and secretion of galectin-3 and in oligosaccharide binding[4,5,6]. In normal adults, galectin-3 has been detected mainly in epithelial cells and myeloid cells, including the epithelium of gastrointestinal and respiratory tracts, renal distal tubules, activated macrophages, eosinophils, neutrophils, and mast cells[3]. In addition, galectin-3 displays pathological expression in many tumors, such as thyroid, pancreatic, and colon cancers[7]. Although galectin-3 is predominantly located in the cytoplasm, it has also been found on the cell surface, within the extracellular matrix, and in the nucleus, suggesting the pleiotropic roles of galectin-3 depending on its localization[3]. Intracellular galectin-3 inhibits apoptosis, regulates the cell cycle, and participates in the nuclear

splicing of pre-messenger RNA[8,9,10,11]. Its extracellular functions encompass cell to cell and cell to matrix adhesions[12,13,14], angiogenesis[15], fibrosis[16,17,18,19], and the activation of various immune cells, such as macrophages, neutrophils, mast cells and lymphocytes[20,21,22,23,24]. Consistent with its various roles, the levels of soluble and/or cellular galectin-3 are increased in autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, and Behçet's disease, and correlate with their disease activities[25,26,27].

This situation led us to focus on the role of galectin-3 in the mechanism responsible for the development of SSc. As an initial step, we measured the serum levels of galectin-3 in patients with SSc and evaluated their association with clinical features.

MATERIALS AND METHODS

Patients. Serum samples, frozen at –80°C until assayed, were obtained from 58 consecutive patients with SSc (56 women, 2 men; mean age 57.0 ± 14.2 yrs; mean disease duration 7.9 ± 10.2 yrs) who presented to the dermatology department of Tokyo University Hospital, a tertiary academic center in Japan, and 19 healthy individuals (18 women, 1 man; mean age 56.0 ± 10.0 yrs) without history or family history of SSc. All subjects gave informed consent, and institutional approval was obtained (University of Tokyo Graduate School of Medicine). Patients were excluded if they had been treated with corticosteroids or other immunosuppressants against clinical symptoms associated with SSc prior to their first visits. Patients were grouped by LeRoy's classification system[1]: 29 with limited cutaneous SSc (lcSSc) and 29 with diffuse cutaneous SSc (dcSSc). All patients with dcSSc and 26 of the patients with lcSSc fulfilled the criteria proposed by the American College of Rheumatology[28]. Three patients with lcSSc not meeting these criteria had sclerodactyly and at least 2 other features of SSc, such as calcinosis, Raynaud's phenomenon, esophageal dysfunction, and telangiectasia. All patients but those with skin sclerosis clinically limited to distal to hands underwent skin biopsy and were histologically diagnosed as having SSc.

Measurement of serum galectin-3 levels. Specific ELISA kits were used to measure serum galectin-3 levels (R&D Systems, Minneapolis, MN, USA). Briefly, polystyrene 96-well plates coated with antigalectin-3 antibodies were incubated with 100 μl of serum at room temperature for 2 h. Then the wells were washed and incubated at room temperature for 2 h with horseradish peroxidase-conjugated antigalectin-3 antibodies. Next, the wells were washed again, added to tetramethylbenzidine, and incubated at room temperature for 30 min. Finally, H_2SO_4 was added to terminate the reaction and the absorbance at 450 nm was measured. Serum galectin-3 levels were calculated using the standard curve.

Clinical assessments. The clinical and laboratory data were obtained when the blood samples were drawn. Patients were evaluated for the presence of esophageal, cardiac, renal, joint, and muscle involvements as described[29,30]. Skin score was measured using modified Rodnan total skin thickness score (mRSS)[31]. The degree of interstitial lung disease (ILD) was evaluated by the percentage of predicted vital capacity (%VC) and the percentage of predicted %DLCO on pulmonary function test. Elevated right ventricular systolic pressure (RVSP) was defined as ≥ 35 mm Hg on echocardiogram. Disease onset was defined as the first clinical event of SSc other than Raynaud's phenomenon. Disease duration was defined as the interval between disease onset and the time the blood samples were drawn.

Statistical analysis. Statistical analysis was carried out with the Mann-Whitney U test for 2-group comparisons, with a Kruskal-Wallis test and a Steel-Dwass test for multiple comparisons, 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 < 0.05.

RESULTS

Serum galectin-3 levels in SSc. Serum galectin-3 levels in patients with SSc were significantly lower than those in healthy individuals (3.50 ± 2.85 vs 4.91 ± 2.67 ng/ml, respectively; p = 0.035, 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 galectin-3 levels in these subgroups. As shown in Figure 1, serum galectin-3 levels were significantly lower in patients with dcSSc (3.29 ± 3.27 ng/ml) than in healthy controls ($p < 0.05$, Kruskal-Wallis test and Steel-Dwass test), while there was no significant difference in serum galectin-3 levels between patients with lcSSc (3.70 ± 2.39 ng/ml) and controls ($p > 0.05$, Kruskal-Wallis test and Steel-Dwass test).

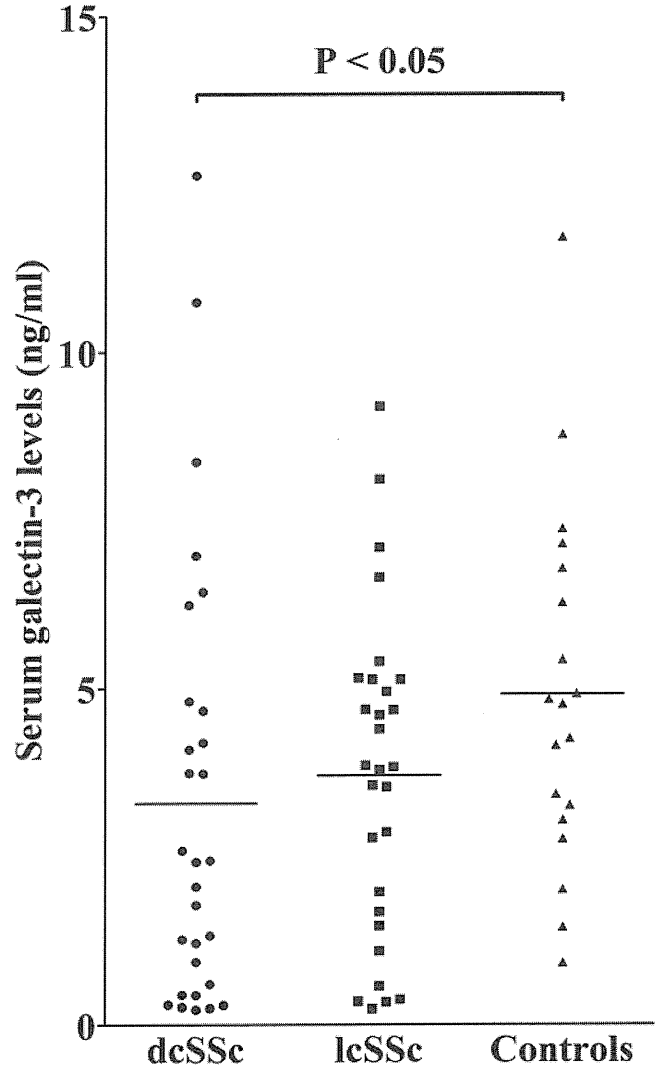

Figure 1. Serum galectin-3 levels in patients with diffuse cutaneous systemic sclerosis (dcSSc), limited cutaneous SSc (lcSSc), and healthy individuals. Serum galectin-3 levels were determined by a specific ELISA. Bars indicate the mean value in each group. Statistical analysis by Kruskal-Wallis test and Steel-Dwass test for multiple comparisons.

Collectively, the decrease in serum galectin-3 levels may be associated with some aspects of the disease process in dcSSc.

Serum galectin-3 levels correlate with the severity of skin sclerosis, but not ILD, in dcSSc. Since dcSSc is characterized by progressive skin sclerosis and ILD, we next focused on this patient group and evaluated the association of serum galectin-3 levels with fibrotic response in skin and lung. To this end, we looked at the correlation of serum galectin-3 levels with mRSS, %VC, and %DLCO in dcSSc.

A significant positive correlation was found between serum galectin-3 levels and mRSS in patients with dcSSc ($r = 0.45$, $p < 0.05$; Figure 2). In contrast, neither %VC nor %DLCO showed a significant correlation with serum galectin-3 levels in dcSSc (data not shown). Given a pro-fibrotic effect of galectin-3 in various pathological fibrotic conditions[16,17,18,19], these results suggest that galectin-3 is associated with the development of dermal fibrosis, but not ILD, in dcSSc.

Decrease in serum galectin-3 levels and initiation of dermal fibrotic responses in dcSSc. As described, serum galectin-3 levels correlated positively with mRSS in dcSSc. However, this result appears to be contradictory to the finding that serum galectin-3 levels are relatively decreased in patients with dcSSc compared with patients with lcSSc. One of the plausible explanations for this observation is that reduction of galectin-3 levels may largely contribute to the initiation of fibrosis in the early stage of dcSSc, but not afterward. To assess this hypothesis, we classified patients with dcSSc into 3 subgroups according to their disease duration, that is, early dcSSc (disease duration < 1 year), mid-stage dcSSc (disease duration 1 to 6 years), and late-stage dcSSc (disease duration > 6 years), and evaluated the correlation of serum galectin-3 levels with disease duration (Figure 3). Supporting our hypothesis, serum galectin-3 levels were decreased in patients with early dcSSc (1.64 ± 1.74 ng/ml) compared with healthy controls ($p < 0.05$, Kruskal-Wallis test and Steel-Dwass test), while there was no significant difference between patients with mid-stage dcSSc (3.22 ± 3.16 ng/ml) or late-stage dcSSc (4.86 ± 4.10 ng/ml) and controls ($p > 0.05$, Kruskal-Wallis test and Steel-Dwass test). Therefore, the reduction of galectin-3 may contribute to the initiation of dermal fibrosis in early dcSSc, probably modulating immune responses.

Elevated serum galectin-3 levels and development of digital ulcer and pulmonary vascular involvement in SSc. To further investigate the association of serum galectin-3 levels with clinical manifestations of SSc other than skin fibrosis and ILD, we classified patients with SSc into 2 groups according to the presence or absence of each organ involvement and compared serum galectin-3 levels between these 2 groups (Table 1). Among various organ involvements, digital ulcer (DU) and elevated RVSP were associated with elevated serum galectin-3 levels in SSc. Serum galectin-3 levels were higher in SSc patients with DU than in those without (5.44 ± 3.74 vs 2.99 ± 2.36 ng/ml; $p = 0.033$). Further, patients with elevated RVSP showed higher galectin-3 levels than those without (4.44 ± 3.14 vs 2.82 ± 2.64 ng/ml; $p = 0.033$). These results indicate that the increase of galectin-3 may be involved in the mechanism responsible for the development of DU and pul-

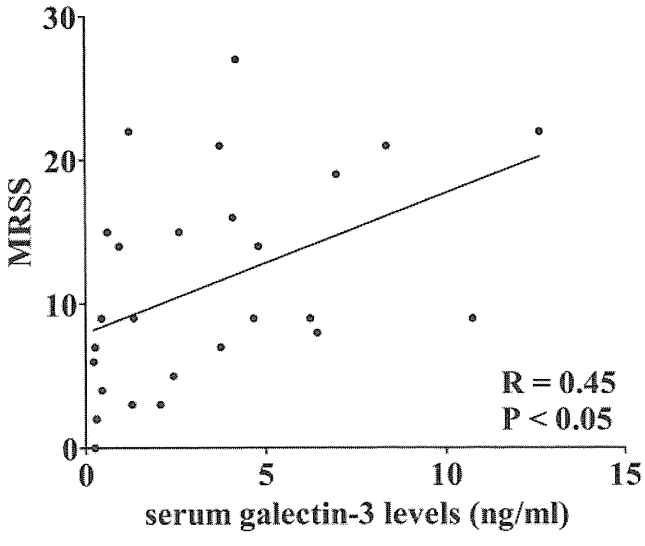

Figure 2. Correlation of serum galectin-3 levels with modified Rodnan total skin thickness score (MRSS) in patients with diffuse cutaneous systemic sclerosis (dcSSc). A significant positive correlation was found between serum galectin-3 levels and MRSS in patients with dcSSc ($r = 0.45$, $p < 0.05$, Spearman's rank correlation test). Solid line represents the regression line.

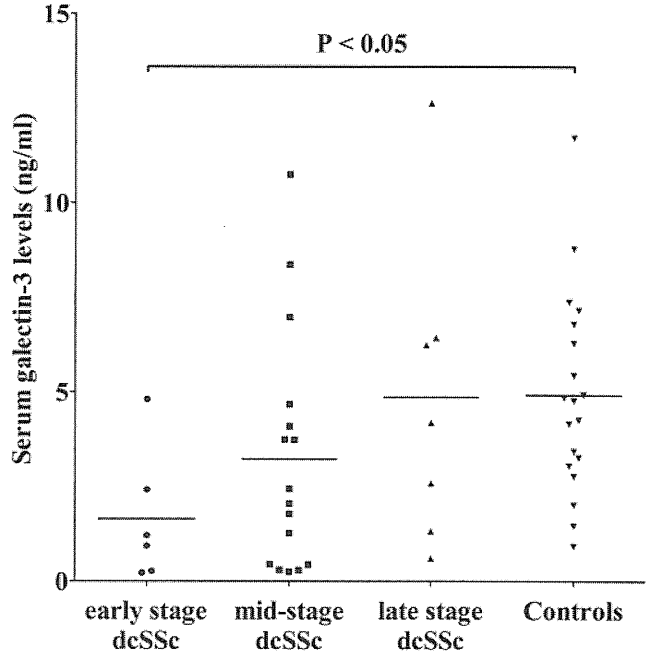

Figure 3. Serum galectin-3 levels in patients with diffuse cutaneous systemic sclerosis (dcSSc) classified into 3 subgroups based on disease duration < 1 year, disease duration 1 to 6 years, and disease duration > 6 years. Serum galectin-3 levels were determined by a specific ELISA. Horizontal bars indicate the mean value in each group. Statistical analysis by Kruskal-Wallis test and Steel-Dwass test for multiple comparisons.

Table 1. Associations of serum galectin-3 levels with organ involvement.

Organ Involvement	Status	Mean ± SD ng/ml	p
Digital ulcers	+	5.44 ± 3.74	0.033
	–	2.99 ± 2.36	
Elevated RVSP	+	4.44 ± 3.14	0.033
	–	2.82 ± 2.64	
Esophageal dysfunction	+	3.48 ± 3.15	0.908
	–	3.46 ± 2.51	
Heart involvement	+	2.58 ± 2.24	0.538
	–	3.43 ± 2.91	
SSc renal crisis	+	3.72 ± 2.70	0.483
	–	3.49 ± 2.93	
Arthralgia/arthritis	+	3.00 ± 3.00	0.293
	–	4.06 ± 3.31	
Muscle involvement	+	2.58 ± 2.24	0.538
	–	3.43 ± 2.91	

RVSP: right ventricular systolic pressure; SSc: systemic sclerosis.

monary vascular involvement leading to pulmonary arterial hypertension in SSc.

DISCUSSION

Although the pathogenesis of SSc is still unknown, experimental data from patient cells and sera continue to provide us with clues to the mechanism responsible for the development of fibrosis, vasculopathy, and autoimmunity in this complicated disorder. Recent studies demonstrating pleiotropic effects of galectin-3 on fibrosis, angiogenesis, and immune activation led us to investigate if this cytokine is involved in the pathological events in SSc. As an initial step to address this issue, we investigated serum galectin-3 levels and their associations with clinical features in SSc. Serum galectin-3 levels were significantly lower in patients with dcSSc than in controls, while there was no significant difference in serum galectin-3 levels between patients with lcSSc and controls. Serum galectin-3 levels were decreased in patients with early-stage dcSSc compared with controls, while mid-stage and late-stage patients had serum galectin-3 levels similar to those in controls. Together with the finding that serum galectin-3 levels did not correlate with %VC or %DLCO, but with mRSS in dcSSc, galectin-3 may be involved in the development of dermal, but not pulmonary, fibrotic response in dcSSc, probably through its profibrotic and immunomodulating effects. On the other hand, elevated serum galectin-3 levels were linked to the development of DU and pulmonary vascular involvement in total SSc. Therefore, galectin-3 appears to play some roles in the pathological events relevant to vasculopathy as well as dermal fibrosis in SSc.

A series of *in vivo* and *in vitro* studies demonstrated that galectin-3 plays a pivotal role in the developmental process of certain pathological fibrotic conditions. A study by Henderson, *et al*[16] demonstrated that galectin-3 is upregulated in established human fibrotic liver disease and is temporally and spatially related to the induction and resolution of experimental hepatic fibrosis. Further, RNA interference-mediated silencing of the galectin-3 gene inhibits myofibroblastic differentiation of hepatic stellate cells, and galectin-3-null mice are resistant to the development of CCL_4-induced hepatic fibrosis largely due to the lack of transforming growth factor-ß (TGF-ß)-induced transactivation of hepatic stellate cells. Similarly, Henderson, *et al*[16] also revealed that galectin-3 expression and secretion by macrophages promotes renal myofibroblast accumulation/activation and fibrosis in a mouse model of progressive renal fibrosis. These data suggest that galectin-3 is required for TGF-ß-mediated myofibroblast activation and matrix production in certain types of pathological fibrosis. Supporting this idea, serum levels of galectin-3 reflect the severity of fibrotic conditions in liver and heart diseases[32,33,34,35,36], and bronchoalveolar lavage fluid levels of galectin-3 correlate with the severity of pulmonary fibrosis[37] in humans. As for the association of galectin-3 with dermal fibrosis, a recent study by Dvoránková, *et al*[19] demonstrated that galectin-3 induces the expression of α-smooth muscle actin, a marker of myofibroblasts, suggesting that galectin-3 exerts a profibrotic effect by promoting the activation of dermal fibroblasts into matrix-secreting myofibroblasts. With our current data that serum galectin-3 levels positively and significantly correlate with mRSS but with neither %VC nor %DLCO in dcSSc, galectin-3 may be associated with the development of dermal fibrosis, but not ILD. To confirm this, we are currently investigating the significance of galectin-3 for the establishment of myofibroblastic phenotype in SSc dermal fibroblasts.

A growing body of evidence demonstrated that galectin-3 is one of the important factors skewing Th1/Th2 polarization of the Th cell response in various pathological conditions. For instance, galectin-3 induces selective downregulation of interleukin 5 (IL-5) gene expression in several cell types, including eosinophils, T cell lines, and antigen-specific T cells[38]. Consistently, treatment of chronic asthmatic mice with gene therapy using plasmid encoding galectin-3 leads to an improvement in Th2 allergic inflammation[39]. On the other hand, galectin-3 deficiency reduces the development of experimental autoimmune encephalomyelitis[40], where Th1 and Th17 cells are thought to be responsible for the inflammatory demyelination, by decreasing interferon-γ and IL-17 production but expanding populations of Th2 cells and inducible regulatory T cells. Thus, galectin-3 promotes the pathological Th1/Th2 polarization in a context-dependent manner.

In the case of SSc, serum galectin-3 levels were decreased in its early stage, but reversed up to normal levels afterward. The dynamics of galectin-3 along with disease course implies that galectin-3 acts as a factor promoting the Th1 polarization in SSc according to the canonical Th1/Th2 paradigm in this disorder. In the early stage of dcSSc with progressive skin sclerosis, serum levels of IL-6 and IL-10 are significantly elevated, while levels decreased to normal in the late stage of dcSSc with the improvement of skin sclerosis[41]. Another Th2

cytokine, IL-4, has normal levels in the early stage of dcSSc, but is decreased as well in the late stage of dcSSc. In contrast, serum levels of IL-12, a key Th1 cytokine, are decreased in the early stage of dcSSc, then gradually increase in parallel with disease duration and finally reach significantly higher levels than normal controls in the late stage of dcSSc with the resolution of skin sclerosis[42].

Of note, the maximal levels of serum IL-12 throughout the disease course correlate inversely with mortality in early dcSSc with diffuse cutaneous involvement. Thus, immune polarization in SSc generally shifts from Th2 to Th1 in parallel with disease duration, while the sustained Th2 immune polarization closely associates with exacerbation of the disease. Therefore, the decrease in galectin-3 levels may contribute to the initiation of the disease by decelerating the Th1 polarization of the Th cell response in concert with various cytokines in early stage SSc.

Another important pathological aspect of galectin-3 is to exert a potent proangiogenic effect in concert with various proangiogenic factors, including vascular endothelial growth factor and basic fibroblast growth factor[15,43]. In our study, serum galectin-3 levels were significantly elevated in patients with DU or elevated RVSP compared to those without, in total SSc. Given that macrovascular involvement, which is characterized by intimal thickening, is closely associated with severe skin ulcers, galectin-3 may be linked to the development of proliferative vasculopathy, such as pulmonary vascular involvement leading to pulmonary arterial hypertension and macrovascular involvement, in total SSc. Although the detailed mechanism of proliferative vasculopathy remains unknown, proliferation of endothelial cells and vascular smooth muscle cells due to aberrant activation of angiogenesis may play a pivotal role in this pathological process[44]. Increasing evidence revealed that angiogenic factors can be placed in 2 categories: the factors inducing proliferation and differentiation of endothelial cells (e.g., basic and acid fibroblast growth factor, vascular endothelial growth factor, and TGF-α), and the factors inducing differentiation but not proliferation of endothelial cells (e.g., angiogenin, TGF-ß, and tumor necrosis factor-α)[45]. Although it appears to belong to the latter category[15], galectin-3 may accelerate the activation of pathological angiogenesis leading to proliferative vasculopathy in concert with various proangiogenic factors promoting endothelial proliferation.

We have reported the first study, to our knowledge, regarding the clinical significance of serum galectin-3 levels in SSc. A series of analyses indicate that galectin-3 may be associated with the pathological events relevant to the development of skin fibrosis and proliferative vasculopathy in SSc. Our study supports previous findings that galectin-3 may be involved in the mechanism responsible for the development of autoimmune diseases.

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