in various diseases and improve pathological conditions.

Levy *et al.* (10, 11) performed 3–6 courses of IVIG therapy in 5 limited cutaneous SSc (lcSSc) and 10 dcSSC patients, in whom a single course was comprised of IVIG administration at 400 mg/kg/day for 5 consecutive days monthly, and achieved improvements in the modified Rodnan skin thickness score (MRSS). Nacci *et al.* (12) administered IVIG at 2 g/kg per month for 6 months to 5 lcSSC and 2 dcSSC patients with severe joint involvement, and observed improvements in the MRSS, joint pain, tenderness, hand function, and quality of life (QOL). Ihn *et al.* (13) and Asano *et al.* (14) administered a single course of IVIG therapy comprised of IVIG administration at 400 mg/kg/day for 5 consecutive days to 5 dcSSc patients, and observed an effect from 2 weeks after the initiation of administration. They observed improvements in the MRSS in all patients at 12 weeks, and improvements continued thereafter in 4 patients.

Since the collagen-metabolising function could be destroyed by an immunological mechanism in the pathology of SSc, the normalisation of immune function is considered important, and IVIG treatment is expected to show efficacy for SSc. IVIG does not excessively inhibit immunity and is not categorised as immunosuppressors such as oral steroids and cyclosporine.

To evaluate the efficacy and safety of a single-course administration of IVIG for skin sclerosis in dcSSc, we performed the first randomised, double-blind, placebo-controlled, multicentre trial (DBT) in which we examined the effect of IVIG for dcSSc of 17 medical institutions in Japan and subsequent long-term observational and readministration studies.

Methods

The study protocols were approved by the Institutional Review Board of each participating institutions, and the trials were carried out in accordance with the Declaration of Helsinki and Good Clinical Practice in 17 medical institutions in Japan. DBT was registered in ClinicalTrials.gov (number NCT 00348296).

Study design

The outline of the study design is shown in Figure 1. In DBT, to exclude subjects in whom the disease was improved by drugs administered before this study, the MRSS was determined at provisional registration, 6 weeks after provisional registration, and at definitive registration, and subjects with no change (within 2 points) or exacerbation (a 3-point or more increase) over the 12-week period from provisional registration were included in definitive registration. Subjects received an intravenous infusion of IVIG (Venoglobulin-IH®, Japan Blood Products Organisation, Tokyo, Japan) or indistinguishable placebo at 400mg (8 mL)/kg/day for 5 consecutive days (a single course). The corticosteroid (at a dose exceeding 15 mg/day as prednisolone) and disease-modifying drugs were not allowed throughout the clinical trial period.

To observe persistence of the effect, responder subjects in whom a 5-point or more improvement in the MRSS was noted 12 weeks after investigational drug administration were subjected to the long-term observational study to observe the condition, and subjects with less than a 5-point improvement in the MRSS were subjected to the readministration study in which IVIG (a single course) was administered. To assure the data in DBT, a 12-week data fixation period was set before IVIG readministration.

Patients

The conditions required for provisional registration were an age of 16 years or older at the time of obtaining informed consent and dcSSc with a 20-point or higher MRSS regardless of gender. Any of the following patients who did not respond to corticosteroids and/or other disease modifying drugs adequately could not be treated with corticosteroids and/or other disease modifying drugs due to complications, and lost the suitable treatment period with corticosteroids and/or other disease modifying drugs judging from the symptoms and history of the disease were selected.

Patients complicated by severe hepatic, renal, and cardiac disorders and malignant tumours, with a past medical history of cerebral infraction or its symptoms, and previously diagnosed with IgA deficiency were excluded on provisional registration.

Efficacy assessment

The primary endpoint in DBT was set at MRSS changes 12 weeks after administration or at discontinuation from that at definitive registration. The MRSS

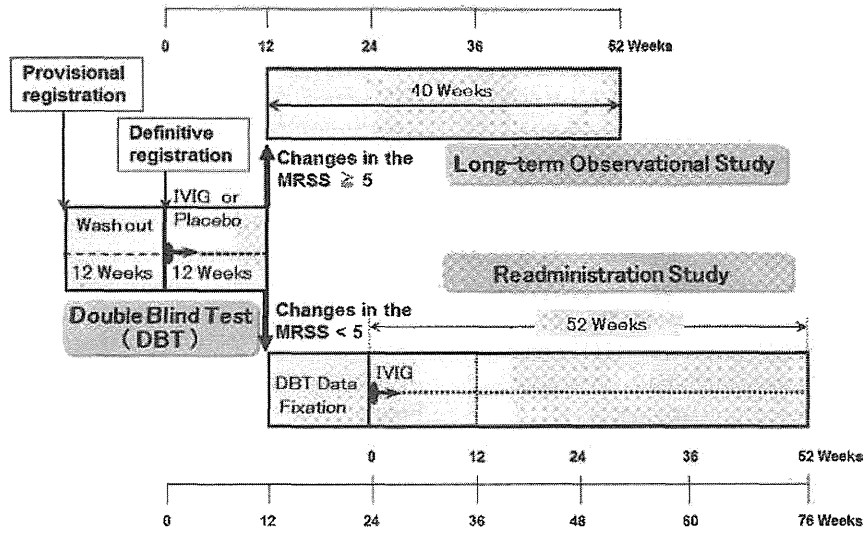

Fig. 1. Outline of the study design.
IVIG or placebo were administered to subjects after a definitive registration. MRSS changes 12 weeks after administration or at discontinuation from that at definitive registration were assessed. Subjects with a 5-point or more improvement in the MRSS were observed in the long-term observational study. IVIG was readministered to subjects with less than a 5-point improvement in the MRSS after DBT data fixation in the readministration study.

has been used as a standard evaluation item in SSc related clinical studies (3-7). To unify MRSS assessment criteria, raters were gathered and trained before study initiation. The number of MRSS raters was limited to two in each institution, and they rated the same patients as much as possible.

The following items were selected for the secondary endpoint of efficacy: dermal fibrotic thickness, joint range of motion (hand, elbow, and knee), oral aperture, hand extension, hand flexion, health assessment questionnaire, respiratory function (%VC, %DLco), and interstitial pneumonia. Skin samples biopsied from the extensor side of the forearm were blinded by a third party, and dermal thickness was measured by the same measurer.

Statistical analyses

Statistical analyses in DBT were independently performed, and those of the long-term observational and readministration studies were integrated with those in DBT. All statistical analyses were performed based on Intent-To-Treat, *i.e.* when baseline and subsequent at least one observation data were present, all data of the allocated patient were included in analysis.

DBT

For the bias of demographics or baseline between groups, measured values and rank data were analysed employing the matched paired ranked Wilcoxon test, and categorical data were analysed employing Fisher's exact test. Regarding the primary endpoint, MRSS changes 12 weeks after administration or at discontinuation from that at definitive registration were compared between groups using the Wilcoxon signed-rank test. In addition, changes in secondary endpoints after administration were similarly analysed.

Long-term observational and readministration studies

MRSS changes in DBT were integrated with results of the long-term observational or readministration study, and the repeated measurement analysis (covariance structure type: compound symmetry) combining between-group comparison, time-point, and interaction in the model was performed regarding the score before administration as a covariance and the comparison between the groups to used LSD (Least square difference) in each time-point. Results were presented as the LS-mean±SEM.

Table I. Baseline demographic data.

	IVIG n=31	Placebo n=31	*p*-value[a]
Female	24 (77.4)	24 (77.4)	1.0000
Age (year)	54.3 ± 12.1	53.8 ± 11.0	0.9775
Disease duration (year)	6.05 ± 7.41	5.76 ± 6.32	0.9888
MRSS	29.2 ± 6.0	27.8 ± 6.4	0.2676
History of corticosteroids at enrolment	23 (74.2)	24 (77.4)	1.0000
History of disease modifying drugs at screening	17 (54.8)	12 (38.7)	0.3087
Anti-topoisomerase I antibody	17 (54.8)	18 (58.1)	1.0000
Anti-U1-RNP antibody	2 (6.5)	4 (12.9)	0.6713
Anti-centromere antibody	5 (16.1)	4 (12.9)	1.0000

Values are mean±SD for continuous variables and n (%) for categorical variables.
[a]*p*-values for categorical variables were calculated with Fisher's exact tests, and *p*-values for continuous variables were calculated with *t*-tests.

Results

Patient population

In DBT, 71 patients signed informed consent, and 64 were registered and randomly allocated to IVIG or P groups. The investigational drug was administered to 63 subjects, and 59 subjects completed DBT and progressed to the next study (long-term observational study: 20, readministration study: 39). In the readministration study, 36 subjects were treated with IVIG.

DBT

i. Demographic characteristics

Table I shows patient backgrounds. No bias was noted in backgrounds between the groups (allocation factors: gender, with or without corticosteroid treatment, and the median MRSS on definitive registration).

ii. Primary endpoint

Figure 2 shows changes in the MRSS. MRSS changes (mean±SD) 12 weeks after administration or at discontinuation were -3.3±4.2 and -4.2±4.6 in IVIG and P groups, respectively, with no significant differences between the groups.

iii. Secondary endpoints

Percentage changes in dermal fibrotic thickness (mean±SD) were -2.23±34.48 (n=21) and 7.51±25.55 (n=22) in IVIG and placebo groups, respectively, showing that thickness decreased more in the IVIG group than that in the P group, but this decrease was not statistically significant. No significant difference was noted in any other secondary endpoint between the groups.

iv. Safety evaluation in DBT

Adverse drug reactions were noted in 32.3% (10/31) and 12.5% (4/32) of IVIG and P groups, respectively, and abnormal changes in laboratory test values were noted in 25.8% (8/31) and 12.5% (4/32), respectively. The main adverse drug reactions of IVIG were fever and elevations in CRP and ALT.

Long-term observational and readministration studies

i. Long-term observational study

The results of repeated measurement analysis of MRSS changes are shown in Figure 3. A significant difference was noted only in the evaluation week. The MRSS rapidly decreased from -4.9±1.2 (LS-mean±SEM) at one week after administration to -9.2±1.2 at 8 weeks, and this reduction was maintained at 52 weeks (-9.7±1.2) in the IVIG group. In the P group, although the reduction was slower than that in the IVIG group, the MRSS decreased from -3.9±1.0 at one week after administration to -7.6±1.0 at 8 weeks, and this reduction was maintained at 52 weeks (-11.7±1.1).

ii. Readministration study

The results of repeated measurement analysis of MRSS changes are shown

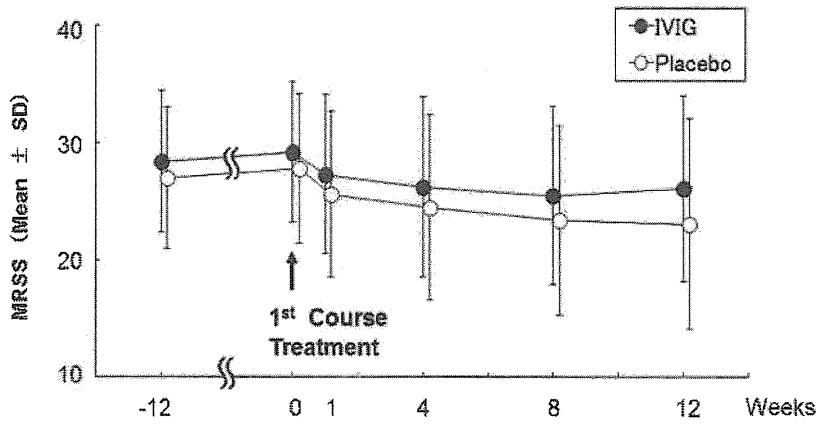

Fig. 2. Course of the MRSS in DBT.
Between IVIG and placebo groups, MRSS changes were not significantly different at any time during the 12-week trial.

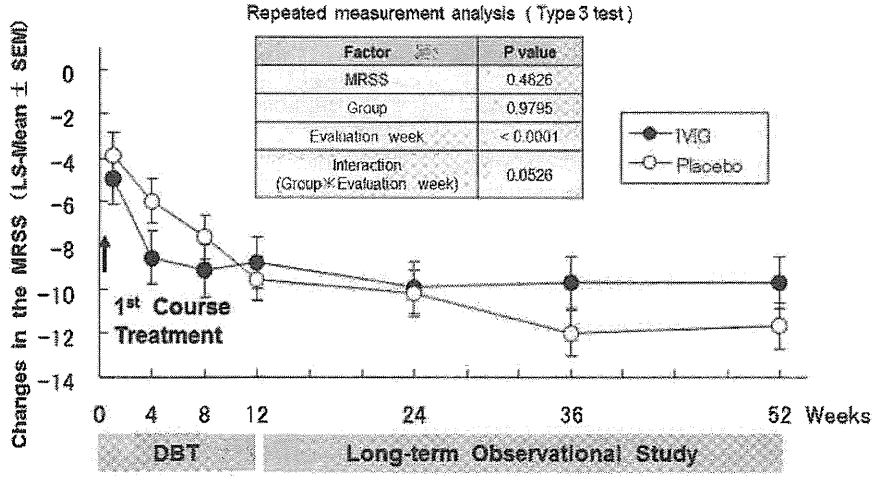

Fig. 3. Changes in the MRSS in the long-term observational study.
Subjects with a 5-point or more improvement in the MRSS in DBT were observed. The repeated measurement analysis combining between-group comparison, time-point, and interaction in the model was performed regarding the score before administration as a covariance and the comparison between the groups to used LSD in each time-point. No significant difference was noted in the MRSS change between the groups.

in Figure 4. Significant differences were noted in the evaluation week and interaction (group and evaluation week). Almost no change was noted in the MRSS in the first course in either group, but the second course of IVIG administration (24 weeks after the first administration) decreased the score from -1.4±1.0 (LS-mean±SEM) to -5.7±1.0 at 32 weeks, and the score continuously decreased until 60 weeks in the IVIG → IVIG (GG) group. In the P → IVIG (PG) group, the score decreased from -1.3±1.0 to -5.0±1.0 at 32 weeks, but no further decrease was noted. At 60 weeks, scores (LS-mean±SEM) were -8.3±1.0 and -4.1±1.1 in GG and PG groups, respectively, showing a significant decrease (LSD difference: p=0.0040; 95% confidence interval for the difference: -7.1 ~ -1.4), and this is the reason for the significant difference observed in interaction.

iii Safety evaluation in the readministration study

In the readministration study in which IVIG was administered, the incidences of adverse drug reactions were 38.9% (7/18) and 31.6% (6/19) in GG and PG groups, respectively. The incidences of abnormal changes in laboratory test values were 5.6% (1/18) and 15.8% (3/19), respectively, showing that the incidence was not markedly increased by the second course of treatment.

Discussion

No significant difference was noted in the primary endpoint, MRSS change, between IVIG and P groups, but significant improvements in the MRSS were noted in the GG group over those in the PG group in the readministration study, suggesting that the efficacy of a single course of administration is insufficient for patients with this disease requiring IVIG, but readministration (multiple courses) may decrease MRSS. In addition, dermal fibrotic thickness generally tended to improve in the IVIG group in DBT, and this tendency was marked in patients confirmed to be responders based on the MRSS at 12 weeks.

We expected IVIG to exhibit an effect after a single course similar to that for other autoimmune diseases, based on reports from Ihn *et al.* (13) and Asano *et al.* (14), but no efficacy was observed in this placebo-controlled study.

The pharmacological actions of IVIG on cells of patients with SSc and experimental SSc models have been reported. In a report in which IVIG was administered to tight skin mice twice a week for 4 weeks (total dose: 2 g/kg), collagen expression and type I collagen gene expression in skin tissue decreased, and TGF-β1 and IL-4 production by splenocytes significantly decreased, showing that IVIG improved these parameters involved in skin fibrosis (15). Skin fibrosis accompanied by skin collagen production was shown to be caused in a mouse model of skin fibrosis induced by subcutaneous administration of bleomycin (9), and it has been reported that IVIG inhibited collagen production by inhibiting macrophage accumulation in skin fibrotic lesions and MCP-1 and TGF-β production in macrophages and monocytes involved in the activation of fibroblasts (16). Furthermore, it has been confirmed by functional analysis of human skin fibroblasts that type I procollagen, TGF-β receptors, and α-SMA involved in fibrosis were more strongly expressed in skin fibroblasts of dcSSc patients than those in healthy subjects, whereas MMP-1, which destroys fibrotic regions, was not expressed; however,

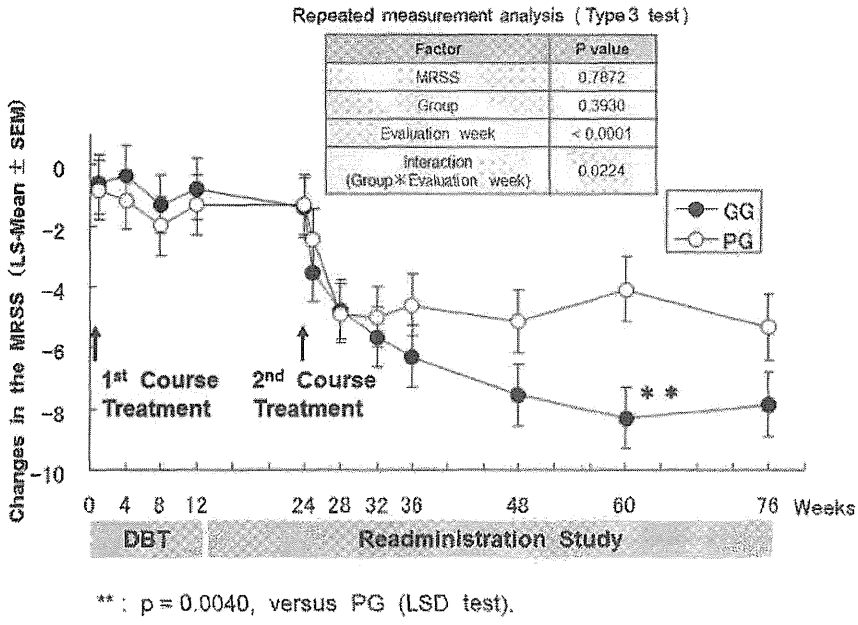

Fig. 4. Changes in the MRSS in the readministration study.
Subjects with less than a 5-point improvement in the MRSS in DBT were enforced in the 2nd course treatment. The repeated measurement analysis combining between-group comparison, time-point, and interaction in the model was performed regarding the score before administration as a covariance and the comparison between the groups to used LSD in each time-point.

the expressions of all factors were improved to normal levels 12 weeks after a single-course administration of IVIG (14).

Based on the above, it was suggested that IVIG inhibits fibrosis by acting on immune function. Although we did not have a chance to measure cytokines, it is assumed that IVIG exhibits its inhibitory effect at the cytokine level.

Only a few clinical studies on the efficacy of IVIG for skin sclerosis in dcSSc have been performed, and these were pilot studies. We performed first DBT and subsequent long-term studies of IVIG in dcSSc patients, which is very significant. Since the cause of dcSSc is complex and markedly heterogeneous, it may be difficult to demonstrate the efficacy of drugs by a comparative study. In other countries, multiple-course administration of IVIG for SSc, such as the administration of a single course/month for 6 months, was performed (10-12). Since two-course administration of IVIG exhibited an MRSS-improving effect in the readministration study, further investigation with multiple-course treatment including the timing of treatment is necessary to demonstrate the usefulness of IVIG.

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Original article

Decreased cathepsin V expression due to Fli1 deficiency contributes to the development of dermal fibrosis and proliferative vasculopathy in systemic sclerosis

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Abstract

Objectives. Cathepsin V (CTSV) is a proteolytic enzyme potentially modulating angiogenic processes, collagen degradation and keratinocyte differentiation. We aimed to investigate the clinical association of serum CTSV levels and the mechanism by which CTSV expression is altered in SSc.

Methods. Serum CTSV levels were determined by ELISA in 51 SSc and 18 healthy subjects. CTSV expression was evaluated by immunostaining in SSc and normal skin and by RT–real-time PCR in normal and SSc dermal fibroblasts, normal dermal fibroblasts treated with TGF-β1 or Fli1 siRNA and human dermal microvascular endothelial cells (ECs) treated with Fli1 siRNA.

Results. Serum CTSV levels were significantly lower in dcSSc and lcSSc patients than in healthy controls. In early-stage dcSSc, serum CTSV levels were remarkably and uniformly decreased compared with healthy controls. The decrease in serum CTSV levels in mid- and late-stage dcSSc and in lcSSc was linked to the development of proliferative vasculopathy. CTSV expression was decreased in microvascular ECs, pericytes/vascular smooth muscle cells and keratinocytes of dcSSc and lcSSc skin and in dermal fibroblasts of dcSSc skin compared with control skin. Consistently, CTSV expression was decreased in cultured dermal fibroblasts from early-stage dcSSc. Furthermore, mRNA levels of the *CTSV* gene were significantly decreased in normal fibroblasts treated with TGF-β1 or Fli1 siRNA and in human dermal microvascular ECs treated with Fli1 siRNA.

Conclusion. Loss of CTSV expression may contribute to the development of fibrosis, vasculopathy and the altered phenotype of keratinocytes in SSc.

Key words: systemic sclerosis, cathepsin V, angiogenesis, fibrosis, keratinocytes, Fli1.

SCIENCE

Introduction

SSc is a multisystem autoimmune disease characterized by initial vascular injuries and resultant fibrosis of skin and certain internal organs. Although the pathogenesis of SSc still remains unknown, microangiopathies probably caused by aberrant activation of angiogenesis and endothelial cell (EC) damage appear to precede the development of fibrosis [1, 2]. The resultant fibrosis following microangiopathies is characterized by excessive deposition of extracellular matrix (ECM) [3, 4], which is regulated by the balance between ECM production by a variety of cells and ECM degradation by proteolytic enzymes, including MMPs [3]. The critical role of MMPs in fibrosis associated with SSc is represented by the dynamic change of *MMP1* gene expression along with disease

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duration in diffuse cutaneous subtype. mRNA levels of the *MMP1* gene are significantly higher in SSc dermal fibroblasts from patients with disease duration of <1 year, while being significantly lower in those from patients with disease duration of 2–4 years, than in normal dermal fibroblasts. Furthermore, mRNA levels of the *MMP1* gene were comparable between SSc dermal fibroblasts from patients with disease duration of >6 years and normal dermal fibroblasts [5]. On the other hand, MMPs are potentially implicated in physiological and pathological angiogenesis in SSc [2]. For instance, MMP-12-mediated cleavage of urokinase-type plasminogen activator receptor may impair capillary formation [6]. While the role of several MMPs in SSc has been extensively studied, our latest study demonstrated the potential role of cathepsin B, a member of the cathespin proteolytic enzyme family, in the pathogenesis of SSc [7].

Most cathepsins are papain-like cysteine proteases mainly localized in endosomes and lysosomes, but also function extracellularly and inside cells such as in secretary vesicles, the cytosol and the nucleus [8]. This proteolytic enzyme family comprises 11 members in human, cathepsins B, C, F, H, K, L, O, S, V, W and X [4]. Notably, cathepsin V (CTSV; also known as cathepsin L2) generates potent anti-angiogenic mediators, including endostatin and angiostatin, by cleavage of collagen XVIII and plasminogen, respectively. Furthermore, deficiency in murine cathepsin L (CTSL), the orthologue of human CTSV, induces fibrosis in myocardium probably due to the lack of collagenolytic activity of murine CTSL. Based on these backgrounds, we hypothesized that CTSV may be associated with the development of vasculopathy and tissue fibrosis in SSc. To address this issue, we herein investigated the clinical association of serum CTSV levels, the expression of CTSV protein in lesional skin and the mechanism regulating CTSV expression in SSc.

Methods

Patients

Serum samples, frozen at −80°C until assayed, were obtained from 51 SSc patients [49 women, 2 men; age, median (25th–75th percentile): 59 (51–66.5) years; disease duration, 2.5 (1.4–9.5) years; modified Rodnan total skin thickness score (MRSS), 7 (2.3–14); prevalence of interstitial lung disease, 45%; percentage of predicted vital capacity (%VC), 101 (86.3–118.9)%; percentage of predicted diffusion lung capacity for carbon monoxide (%DL_{CO}), 79 (68.0–97.6)%] and 18 healthy individuals [17 women, 1 man; age 55 (51–59) years]. Patients who had been treated with CSs or other immunosuppressants before their first visit were excluded. Patients were grouped by the LeRoy classification system [9]: 28 with dcSSc and 23 with lcSSc. All dcSSc patients and 20 lcSSc patients fulfilled the criteria proposed by the ACR [10]. Three lcSSc patients not meeting these criteria had sclerodactyly and at least two other of the following features: calcinosis, RP, oesophageal dysmotility 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.

Measurement of serum CTSV levels

Specific ELISA kits were used to measure serum CTSV levels (R & D Systems, Minneapolis, MN, USA). Briefly, polystyrene cups coated with anti-CTSV antibodies were incubated with 50 μl of 2-fold diluted serum at room temperature for 2 h. Then, the cups were washed and incubated at room temperature for 2 h with horseradish peroxidase-conjugated anti-CTSV antibodies. Next, the wells were washed again, tetramethylbenzidine was added and they were incubated at room temperature for 30 min. Finally, sulphuric acid was added to terminate the reaction and absorbance at 450 nm was measured. Serum CTSV levels were calculated using a standard curve.

Clinical assessment

Clinical and laboratory data were obtained when the blood samples were drawn. Disease duration was defined as the interval between the onset defined as the first clinical event of SSc other than RP and the time the blood samples were drawn. Clinical symptoms were evaluated as described previously [11–14]. Details of the assessments are briefly summarized in the footnote to Table 1.

Immunohistochemistry

Immunohistochemistry with Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) was performed on formalin-fixed, paraffin-embedded tissue sections using anti-human CTSV antibody (R & D Systems). Skin samples were obtained from forearms of six SSc patients (three dcSSc and three lcSSc) and six healthy controls closely matched for age and sex. Disease duration was <1 year in all patients.

Cell cultures

Human dermal microvascular ECs (HDMECs) and human dermal fibroblasts were prepared and maintained as described previously [15, 16].

Gene silencing of *Fli1* and treatment with TGF-β1

These experiments were performed as described previously [7, 17]. The details of each experiment are described in figure legends.

RNA isolation and RT—real-time PCR

RNA isolation and RT-real-time PCR were carried out as described previously [16]. The sequences of CTSV [18], Fli1 [19] and 18S rRNA [20] primers have been previously reported.

Statistical analysis

The statistical analysis carried out in each experiment is described in figure legends or results. Statistical significance was defined as $P < 0.05$.

TABLE 1 Correlation of serum CTSV levels with clinical features in dcSSc patients

Cutaneous vascular symptoms and organ involvement	Total dcSSc		Mid- and late-stage dcSSc	
	Decreased CTSV levels ($n=8$)	Normal CTSV levels ($n=20$)	Persistently low CTSV levels ($n=10$)	Increasing CTSV levels ($n=10$)
Nail-fold bleeding	75	67	67	67
RP	88	83	90	78
Telangiectasia	40	44	29	63
Digital ulcers	43	11	50	10
Elevated RVSP	25	15	30	11
Scleroderma renal crisis	0	10	20	0
Organ involvement associated with proliferative obliterative vasculopathy	50	35	80*	20

Values are percentages. Elevated RVSP was defined as $\geqslant 35$ mmHg on echocardiogram. SRC was defined as malignant hypertension and/or rapidly progressive renal failure. Organ involvement associated with proliferative obliterative vasculopathy includes digital ulcers, elevated RVSP and scleroderma renal crisis. Statistical analysis was carried out with Fisher's exact probability test. *$P < 0.05$.

Results

Serum CTSV levels in SSc patients

Serum CTSV levels in SSc patients were significantly lower than those in healthy individuals [111.9 (98.0–137.3) ng/ml *vs* 165.6 (153.9–183.1) ng/ml; $P < 0.05$, Mann–Whitney U-test]. When we classified patients into dcSSc and lcSSc, serum CTSV levels were significantly lower in both of these groups [113.8 (102.1–158.1) ng/ml and 100.7 (94.4–119.5) ng/ml, respectively] than in healthy controls (Kruskal–Wallis test, $P < 0.0001$; Steel–Dwass test, $P < 0.05$ for each; Fig. 1A). We also evaluated the relationship between serum CTSV levels and autoantibodies, but the presence or absence of autoantibodies, including anti-topo I antibody ($n = 17$) and ACA ($n = 19$), did not affect serum CTSV levels (data not shown). Collectively, the decrease in serum CTSV levels may be associated with some aspects of the disease process in SSc.

Serum CTSV levels were decreased in early-stage dcSSc, but not in mid- or late-stage dcSSc

Since there were no significant correlations between serum CTSV levels and MRSS, %VC or $\%DL_{CO}$ in total SSc ($P = 0.11$, -0.11 and 0.05, respectively), we evaluated the clinical significance of serum CTSV levels in each of the subgroups, dcSSc and lcSSc, in the following analyses.

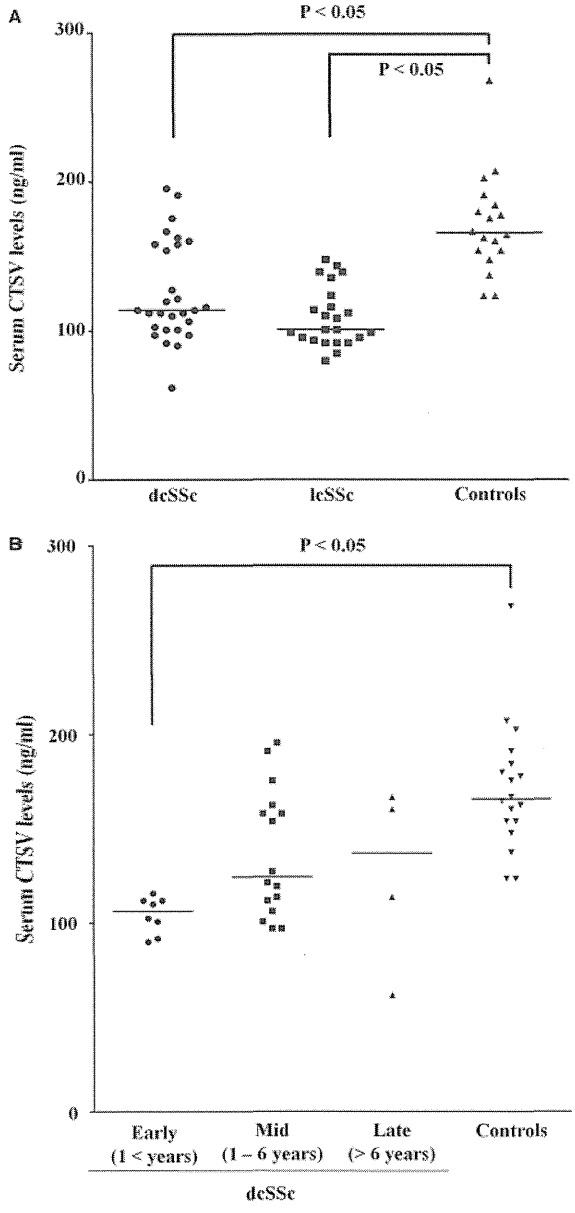
According to the dynamics of *MMP1* gene expression along with disease duration in dcSSc [5], we classified dcSSc patients into three subgroups, such as early-stage dcSSc (disease duration <1 year), mid-stage dcSSc (disease duration 1–6 years) and late-stage dcSSc (disease duration >6 years), and evaluated serum CTSV levels among these groups. As shown in Fig. 1B, serum CTSV levels were uniformly and markedly decreased in early-stage dcSSc patients [106.3 (98.5–111.9) ng/ml] compared with healthy controls (Kruskal–Wallis test, $P < 0.0005$; Steel–Dwass test, $P < 0.05$). Importantly, all the early-stage dcSSc patients had serum CTSV levels lower than the minimum serum CTSV value in healthy controls. On the other hand, serum CTSV levels were almost comparable between healthy controls and mid- or late-stage dcSSc [124.4 (110.4–159.1) ng/ml and 137.0 (100.8–161.8) ng/ml, respectively]. These results suggest that loss of proteolytic activity due to CTSV down-regulation is involved in the initiation of fibrotic process in dcSSc.

Clinical correlation of serum CTSV levels in dcSSc

We next looked at the association of serum CTSV levels with dermal and pulmonary fibrotic parameters. Regarding MRSS, we failed to detect any correlation with serum CSTV levels in total dcSSc [$r = 0.16$ ($P = 0.46$); Spearman's rank correlation test]. Given that skin sclerosis usually progresses mostly up to 6 years after the disease onset in dcSSc, and starts to regress afterwards, we also evaluated the correlation in dcSSc with disease duration of <6 years, but did not see any correlation [$r = 0.22$ ($P = 0.35$)]. Furthermore, we failed to detect any correlation of serum CTSV levels with %VC or $\%DL_{CO}$ in total dcSSc [$r = -0.25$ ($P = 0.22$) and $r = -0.21$ ($P = 0.31$), respectively] and in dcSSc with disease duration of <6 years [$r = -0.22$ ($P = 0.32$) and $r = -0.13$ ($P = 0.58$), respectively]. Collectively, these results suggest that serum CTSV levels do not reflect the severity of fibrotic response in dcSSc.

Given that CTSV is associated with the angiogenic process, we classified dcSSc patients into two groups based on the cut-off value (103.4 ng/ml; mean $-$ 2 S.D. of healthy controls), such as dcSSc patients with decreased serum CTSV levels and those with normal CTSV levels, and compared the prevalence of vascular clinical symptoms. As shown in the left columns of Table 1, the reduction in serum CTSV levels did not increase the prevalence of any vascular clinical symptoms.

FIG. 1 Concentration of CTSV in sera from patients with SSc.



Serum CTSV levels were measured by a specific ELISA. The levels were compared among dcSSc, lcSSc and control subjects (**A**) and among early-, mid-, late-stage dcSSc and control subjects (**B**). The horizontal bars indicate the median value in each group.

Since serum CTSV levels were uniformly decreased in early-stage dcSSc, further analyses were carried out without this group. Looking closely at the distribution of serum CTSV levels in mid- and late-stage dcSSc, the patients appeared to consist of two subgroups: patients with persistently decreased serum CTSV levels and those with serum CTSV levels increasing along with disease duration. Therefore, we classified mid- and late-stage dcSSc patients into two groups using a cut-off value of 123.6 ng/ml (mean + 2 S.D. of early-stage dcSSc) and compared the prevalence of vascular complications. As shown in the right columns of Table 1, we failed to detect any correlation of serum CTSV levels with cutaneous vascular manifestations, including RP, nail-fold bleeding and telangiectasia, and with organ involvement associated with proliferative obliterative vasculopathy, including digital ulcers, scleroderma renal crisis (SRC) and elevated right ventricular systolic pressure (RVSP). However, when we looked at the percentage of patients with at least one organ involvement associated with proliferative obliterative vasculopathy, it was significantly increased in dcSSc patients with persistently low serum CTSV levels compared with those with increasing serum CTSV levels (80% *vs* 20%, $P < 0.05$). These results suggest that the persistent decrease in CTSV expression is potentially associated with the development of proliferative obliterative vasculopathy in mid- and late-stage dcSSc.

Clinical association of serum CTSV levels in lcSSc

In lcSSc, there were no correlations between serum CTSV levels and disease duration or MRSS [$r = 0.17$ ($P = 0.44$) and $r = -0.09$ ($P = 0.76$), respectively]. For further analyses, we classified lcSSc patients into two groups based on the cut-off value (103.4 ng/ml; mean − 2 S.D. of healthy controls): lcSSc patients with decreased serum CTSV levels and those with normal CTSV levels. As shown in Table 2, the prevalence of elevated RVSP was significantly higher in lcSSc patients with decreased serum CTSV levels than in those with normal levels (75% *vs* 18%, $P < 0.05$; Fisher's exact test). Supporting this finding, serum CTSV levels tended to correlate with $\%DL_{CO}$ ($r = 0.38$, $P = 0.07$), but not %VC ($r = 0.07$), in lcSSc. In contrast, there was no significant difference in the prevalence of digital ulcers and SRC between these two groups. Regarding cutaneous vascular symptoms we also failed to detect any significant difference. Collectively, these results suggest that decreased serum CTSV levels are associated with the development of pulmonary vascular injuries leading to pulmonary arterial hypertension in lcSSc.

Expression levels of CTSV protein in skin sections derived from SSc patients and healthy controls

To further confirm the reduction of CTSV protein and investigate its origin in SSc skin tissues, immunohistochemistry was carried out using skin samples from six healthy controls and three dcSSc and three lcSSc patients with disease duration of <1 year. Clinical information and the results are summarized in Table 3. In normal skin sections, CTSV protein was detected in keratinocytes (Fig. 2A and 2B), dermal fibroblasts (Fig. 2C) and blood vessels, including ECs and pericytes (PCs)/vascular smooth muscle cells (vSMCs), especially at high levels in ECs (Fig. 2D and 2E). In the epidermis, CTSV was mainly restricted to the stratum granulosum and stratum corneum (Fig. 2A) as previously reported [21]. In contrast, CTSV was moderately but diffusely expressed in epidermis

in all SSc skin sections (Fig. 2F and 2G). Regarding fibroblasts, CTSV expression was decreased in dcSSc compared with healthy controls (Fig. 2H), while being comparable between lcSSc and healthy controls. Furthermore, CTSV expression was uniformly and markedly reduced in blood vessels (Fig. 2I and 2J). These results suggest that low serum CTSV levels in SSc patients reflect the decreased expression of CTSV in various cell types.

CTSV down-regulation in SSc dermal fibroblasts and normal dermal fibroblasts treated with TGF-β1 or Fli1 siRNA

We next evaluated the mRNA levels of the *CTSV* gene in cultured dermal fibroblasts from healthy controls and early-stage dcSSc patients. As shown in Fig. 3A, SSc fibroblasts expressed significantly lower mRNA levels of the *CTSV* gene than normal fibroblasts. Since SSc dermal fibroblasts are constitutively activated by the stimulation of autocrine TGF-β [15, 22–24], we asked whether TGF-β1 stimulation suppresses the expression of the *CTSV* gene in normal dermal fibroblasts. As expected, TGF-β1 stimulation significantly suppressed the mRNA expression of the *CTSV* gene in normal fibroblasts (Fig. 3B). Fli1 down-regulation due to epigenetic suppression, as well as autocrine TGF-β stimulation, is another important feature of SSc fibroblasts [25, 26]. Therefore, we further sought whether gene silencing of *Fli1* affects the mRNA levels of the *CTSV* gene in normal fibroblasts. As shown in Fig. 3C and 3D, a sufficient knockdown of Fli1 significantly decreased the expression of CTSV mRNA in normal fibroblasts. Collectively, CTSV expression is decreased in lesional dermal fibroblasts of early-stage dcSSc at least partially due to autocrine TGF-β stimulation and Fli1 deficiency.

Table 2 Correlation of serum CTSV levels with clinical features in patients with lcSSc

Cutaneous vascular symptoms and organ involvement	lcSSc with decreased CTSV levels ($n = 12$)	lcSSc with normal CTSV levels ($n = 11$)
Nail-fold bleeding	55	64
RP	90	82
Telangiectasia	63	22
Digital ulcers	9	29
Elevated RVSP	75*	18
Scleroderma renal crisis	10	9

Values are percentages. Statistical analysis was carried out with Fisher's exact probability test. *$P < 0.05$.

Gene silencing of *Fli1* down-regulated CTSV expression in HDMECs

We further investigated the mechanism by which CTSV is down-regulated in SSc dermal blood vessels. Since we previously demonstrated that the deficiency of Fli1 in ECs is potentially associated with the development of SSc vasculopathy [16], we looked at the effect of *Fli1* gene silencing on the mRNA levels of the *CTSV* gene in HDMECs. As shown in Fig. 3E and 3F, ~50% knockdown of Fli1 resulted in significant reduction of CTSV mRNA levels (58% decrease, $P < 0.05$). Collectively, these results indicate that Fli1 deficiency is at least partially involved in the mechanism of CTSV down-regulation in SSc vasculature.

Discussion

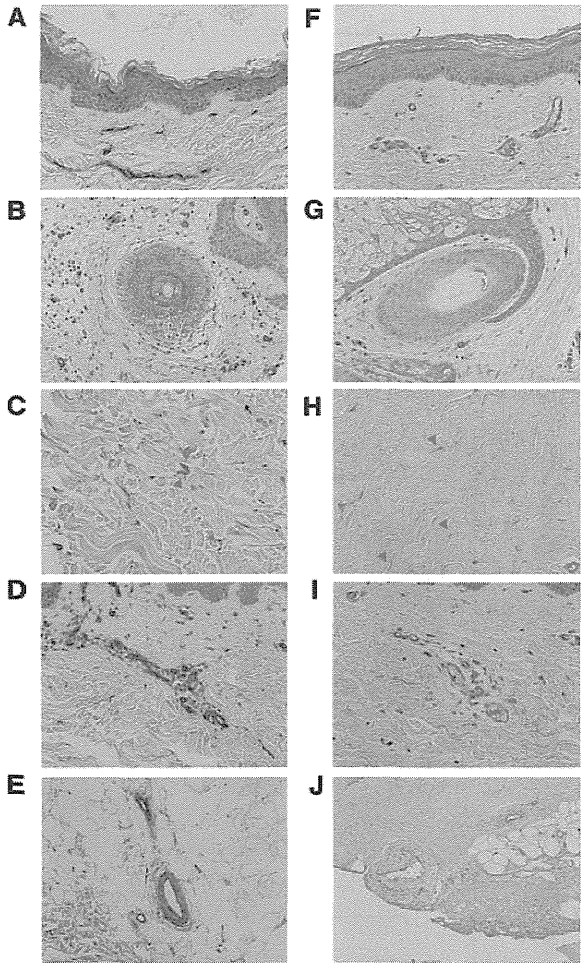
This article was undertaken to investigate the role of CTSV in the developmental process of fibrosis and vasculopathy associated with SSc. As expected, serum CTSV levels in SSc patients were significantly lower than those in healthy controls. In dcSSc, similar to the dynamics of *MMP1* gene expression along with disease duration [5], serum CTSV levels were uniformly decreased in its early stage and

Table 3 CTSV levels in skin sections from SSc patients and healthy controls

Samples	Age, years/sex	Duration, years	dcSSc/lcSSc	Fibroblasts	ECs
NS1	65F			+	+++
SSc1	61F	1	dcSSc	+/−	++
NS2	63F			+	+++
SSc2	64F	0.5	dcSSc	+	++
NS3	64M			++	+++
SSc3	59M	0.5	dcSSc	+/−	++
NS4	56F			+	+++
SSc4	52F	1	lcSSc	+	++
NS5	55F			+	+++
SSc5	51F	0.2	lcSSc	+	++
NS6	59F			+/−	+++
SSc6	58F	1	lcSSc	+/−	++

We used the following grading system: −: no staining; +: slight staining; ++: moderate staining; +++: strong staining. NS: normal skin; F: female; M: male.

FIG. 2 Immunohistochemical analyses for CTSV expression in skin sections from SSc patients and healthy controls.



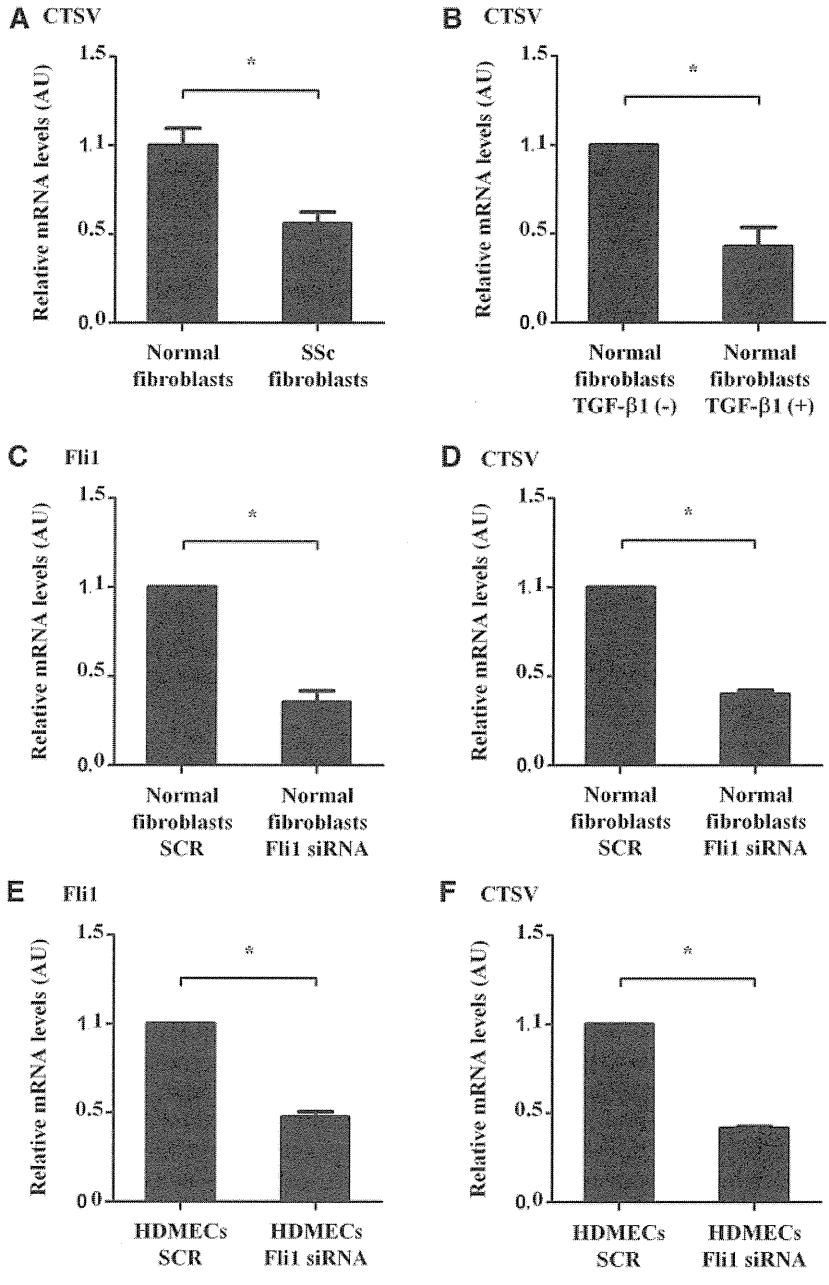
Skin sections from healthy controls (**A**–**E**) and SSc patients (**F**–**J**) were subjected to immunohistochemistry with anti-CTSV antibody. Representative results in the epidermis (**A**, **F**), hair follicles (**B**, **G**), fibroblasts (shown with arrow heads; **C**, **H**), dermal microvessels (**D**, **I**) and subcutaneous muscular vessels (**E**, **J**) are shown. In skin sections of patients with SSc, CTSV expression was markedly reduced in all of these cell types. Original magnification ×400 (**A**–**D**, **F**–**I**). Original magnification ×200 (**E**, **J**).

gradually increased along with disease duration in ~50% of patients. Consistently, dermal fibroblasts from early-stage dcSSc patients expressed mRNA of the *CTSV* gene at lower levels than those from healthy controls. Given that the lack of CTSV results in fibrosis due to the lack of its collagenolytic activity, CTSV down-regulation is potentially associated with the initiation of dermal fibrotic response in dcSSc. On the other hand, in mid- and late-stage dcSSc, patients with persistently low CTSV levels had a significantly higher prevalence of proliferative obliterative vasculopathy than those with increasing CTSV levels. Taken together with the angiostatic role of CTSV and the low CTSV expression in SSc dermal blood vessels, constitutive down-regulation of CTSV may contribute to the development of proliferative obliterative vasculopathy in dcSSc. Supporting these findings in dcSSc, the following observations were made in lcSSc patients: (i) CTSV expression levels in SSc dermal fibroblasts are similar to those in dermal fibroblasts of control skin and disease duration did not affect serum CTSV levels; (ii) reduced levels of serum CTSV are linked to the high prevalence of elevated RVSP. Collectively, these results suggest that CTSV deficiency contributes to the initiation of extensive fibrosis in dcSSc and proliferative vasculopathy in total SSc.

The potential protective effect of CTSV against tissue fibrosis has been shown in animal models. Null mice of CTSL, the orthologue of human CTSV, develop cardiac fibrosis via reduced collagenolytic activity, suggesting that murine CTSL is one of the collagenolytic enzymes regulating the homeostasis of ECM in the heart [27]. Given that type I collagen is the major ECM component in both SSc skin and myocardial fibrosis [27], the down-regulation of CTSV in SSc fibroblasts may contribute to the development of skin fibrosis in SSc. Previous reports demonstrated that the decelerated ECM degradation in SSc is largely attributed to the up-regulated expression of tissue inhibitor of metalloproteinase-1, a potent inhibitor of MMP1, in dermal fibroblasts [5] and the presence of anti-MMP1 neutralizing antibodies in sera [28]. The down-regulation of CTSV may work in concert with these factors to establish the pathological fibrosis in SSc.

Although the role of CTSV in the regulation of endothelial behaviour still remains unknown, this molecule has a potential to regulate vascular quiescence and angiogenesis through the production of potent anti-angiogenic factors, including angiostatin and endostain. Angiostatin is a circulating 38-kDa protein identical to plasminogen fragment, while endostatin is a 20-kDa C-terminal fragment of collagen XVIII, which is a component of almost all epithelial and endothelial basement membranes (BMs) [29–31]. CTSV is one of the enzymes mediating the production of angiostatin and endostatin from original proteins [32, 33]. Angiostatin and endostatin gave promising results in cancer therapy trials [34] as well as preclinical arthritis studies [35], suggesting the pathological reduction of these factors in various diseases with vascular involvements. In various animal models, hypoxia suppresses the expression of these molecules [36–38], indicating their important roles as critical regulators of physiological angiogenesis. Although the expression of CTSV has not been well studied in human diseases, the down-regulation of CTSV may contribute to the activation of pro-angiogenic responses through the decreased production of angiostatin and endostatin in SSc. Consistently, Distler *et al.* [39] reported that there is a trend towards a decrease in serum endostatin levels in SSc patients compared with healthy controls and that serum endostatin levels are significantly reduced in SSc patients with giant

FIG. 3 CTSV levels in SSc dermal fibroblasts and in activated normal dermal fibroblasts and HDMECs.



mRNA levels of *CTSV* gene were determined by RT-real-time PCR in confluent quiescent dermal fibroblasts from five SSc patients (disease duration <1 year) and five healthy controls (**A**) and in normal dermal fibroblasts treated with or without recombinant human TGF-β1 (PeproTech, Rocky Hill, NJ, USA) at 10 ng/ml for 24 h (**B**). Normal dermal fibroblasts were treated with scrambled RNA (SCR) or Fli1 siRNA and mRNA levels of *Fli1* (**C**) or *CTSV* (**D**) genes were determined by RT-real-time PCR. HDMECs were transfected with SCR or Fli1 siRNA and evaluated for mRNA levels of *Fli1* (**E**) or *CTSV* (**F**) genes by RT-real-time PCR. For siRNA experiments, cells were seeded shortly before transfection. The cells were transfected with 10 nM of Fli1 siRNA (Santa Cruz Biothechnology, Santa Cruz, CA, USA) or scrambled non-silencing RNA (Santa Cruz Biothechnology, Santa Cruz, CA, USA) using HiPerfect transfection reagent (Qiagen, Valencia, CA, USA) for 72 h. Cells were then serum starved for the last 24 h. mRNA levels of *Fli1* and *CTSV* genes were examined by RT-real-time PCR and normalized to the levels of human *18S rRNA* gene. Results of controls or relative value compared with the controls are expressed as means (S.D.) of three independent experiments. Statistical analysis was carried out with a two-tailed paired *t*-test. $^*P < 0.05$.

capillaries on capillaroscopy compared with those without. Given that the dilation of nail-fold capillaries is one of the most prominent clinical symptoms reflecting pathologically activated angiogenesis in SSc, the reduction in CTSV production may promote pro-angiogenic response via decreased endostatin action. In this study, we did not see the association of serum CTSV levels with nail-fold capillary changes because capillaroscopy was unavailable at our facility. Instead, we looked closely at the association of serum CTSV levels with vascular symptoms and revealed the possible association of low serum CTSV levels with the development of proliferative obliterative vasculopathy. Given that dilation of nail-fold capillaries, as well as proliferative obliterative vasculopathy, is characterized by proliferation of ECs and PCs/vSMCs, the activation of angiogenic process due to CTSV down-regulation may contribute to the development of clinical symptoms associated with proliferative change of vasculature.

Our previous studies demonstrated that endothelial Fli1 deficiency is potentially associated with the development of vascular changes, especially proliferative vasculopathy, in SSc [16, 25]. EC-specific *Fli1* knockout (Fli1 ECKO) mice reproduce the histopathological features of SSc vasculopathy, such as stenosis of arterioles and dilation capillaries. Gene silencing of *Fli1* in HDMECs results in the down-regulation of molecules regulating EC–EC interaction, including platelet endothelial cell adhesion molecule (PECAM)-1 and vascular endothelial (VE)-cadherin, and those regulating EC–PC interaction, including VE-cadherin, $S1P_1$ and platelet-derived growth factor-B, and in the up-regulation of MMP9 promoting the degradation of vascular BM. Furthermore, Fli1 deficiency promotes endothelial proliferation and survival [40], probably linked to the development of arteriolar stenosis and dilation capillaries, which are similar to proliferative vasculopathy in SSc, in Fli1 ECKO mice. As shown in the present study, *Fli1* gene silencing down-regulated the expression of CTSV mRNA in HDMECs. These results indicate that the reduction of endothelial CTSV expression is included in the gene programme triggered by Fli1 deficiency. Given that Fli1 deficiency activates a series of angiogenic gene programmes, down-regulation of CTSV may further promote this process by decreasing the production of angiostatin and endostatin. These present observations further support the idea that Fli1 deficiency plays a pivotal role in the development of SSc vasculopathy.

Another interesting observation in this study was the altered expression of CTSV in SSc keratinocytes. Given that collagen XVIII is a component of epithelial BM as well as endothelial BM [29], CTSV may affect keratinocyte behaviour through the production of endostatin, whose constitutive overexpression in keratinocytes results in the promotion of keratinocyte terminal differentiation in animal models [41]. Importantly, a recent study demonstrated the impairment of terminal differentiation of keratinocytes in SSc [42, 43]. Relevant to this observation, CTSL null mice show hair loss that can be rescued by keratinocyte-specific expression of CTSL [44]. Con sidering that hair loss frequently occurs in lesional skin of SSc patients, the impaired CTSV expression in keratinocytes may contribute to the altered epidermal phenotype in SSc.

In summary, we herein report the first study of the clinical significance of CTSV in SSc. The present data suggest the possible contribution of CTSV to the development of vasculopathy, fibrosis and altered epidermal phenotype in SSc. Further cell-based studies are required to confirm this notion in the future.

Rheumatology key messages

- Decreased serum CTSV levels may be associated with progressive fibrosis and proliferative vasculopathy in SSc.
- Altered CTSV expression in fibroblasts, ECs and keratinocytes may contribute to SSc clinical symptoms.

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Clinical vignette

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Ochronotic arthropathy of the spine limited to the thoracic section

A 63-year-old non-obese woman was referred for longstanding osteoarthritis (OA) mainly involving her hips, knees and spine. Radiographs of hips, knees and cervical and lumbar spine (performed 1 year before) confirmed diffuse OA and showed evidence of chondrocalcinosis in the knees.

Skin and eye examination revealed grey-black pigmentation of auricles and scleras. The patient reported dark urine in her sanitary towels and greyish cerumen.

X-ray examination showed a typical picture of ochronotic arthropathy in the thoracic spine (Fig. 1, left) characterized by wafer-like calcifications, narrowing of the disc spaces and increased vertebral radiolucency [1]. In the lumbar spine only OA was detected (Fig. 1, right).

Ochronosis is a feature of alkaptonuria, an uncommon autosomal recessive disease causing an accumulation of homogentisic acid and subsequent abnormal pigmentation of several tissues and secretions (urine, cerumen and sweat).

Ochronotic arthropathy is characterized by premature onset of OA in the large joints often associated with pyrophosphate and apatite crystal deposition [1]. The lumbar spine usually presents pathognomonic radiographic features [1] that, unusually, were detected only in the thoracic spine in our patient. Clinical history, physical examination and a simple X-ray examination were sufficient to formulate the right diagnosis.

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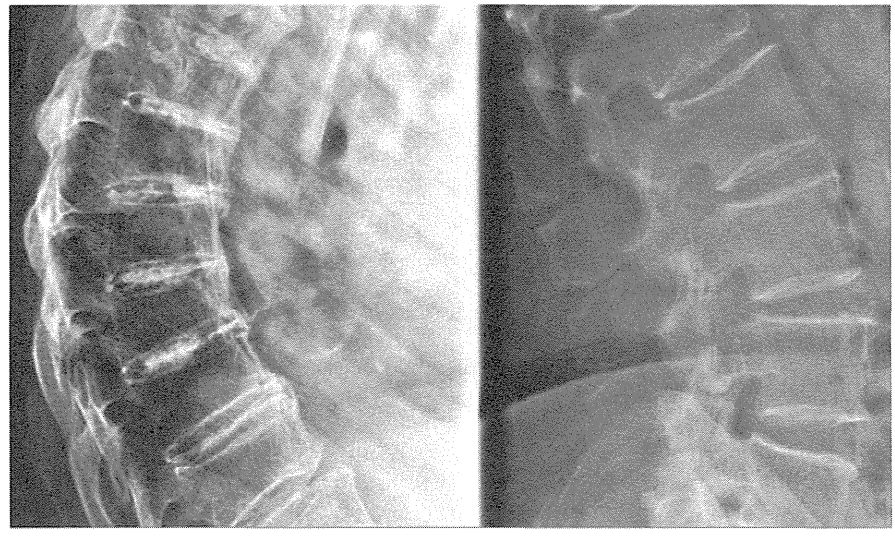
FIG. 1 Dorsal spine involvement in ochronosis.



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ORIGINAL ARTICLE

Serum chemokine levels as prognostic markers in patients with early systemic sclerosis: a multicenter, prospective, observational study

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Abstract

Objective To assess the utility of serum chemokine levels as a prognostic indicator of disease progression in systemic sclerosis (SSc) patients with early onset disease.

Methods Seventy Japanese patients with early onset SSc presenting with diffuse skin sclerosis and/or interstitial lung disease were registered in a multicenter, observational study. Concentrations of CCL2, CCL5, CXCL8, CXCL9, and CXCL10 in serum samples from all patients were measured using cytometric beads array. In 33 patients, chemokine levels were measured each year for 4 years. The ability of baseline chemokine levels to predict changes in clinical features were evaluated statistically by multiple regression analysis.

Results At their first visit, serum levels of CCL2, CCL5, CXCL8, CXCL9, and CXCL10 were significantly elevated in patients with SSc compared with healthy controls. There were significant associations between CCL2 and CXCL8 levels and between CXCL9 and CXCL10 levels in patients. The initial serum CXCL8 levels were significantly associated with the HAQ-DI at the fourth year while the %VC of baseline tended to be negatively associated with HAQ-DI at the fourth year. Initial chemokine levels were not associated with other clinical features including skin thickness score and the respiratory function.

Conclusion Serum CXCL8 level may serve as a prognostic indicator of the physical dysfunction in SSc. Further

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longitudinal studies of larger populations are needed to confirm these findings.

Keywords Chemokine · CXCL8 · HAQ · Serum marker · Systemic sclerosis

Introduction

Systemic sclerosis (SSc) is a connective tissue disease characterized by tissue fibrosis in the skin and internal organs [1, 2]. Interstitial lung disease (ILD) develops in more than half of SSc patients and is one of the major SSc-related causes of death. Additionally, joint contracture due to extensive skin sclerosis and/or severe internal organ involvement results in impaired physical function.

SSc patients exhibit increased numbers and activation of monocytes/macrophages and T cells in the circulation and tissues [3, 4]. Infiltration of these cells into the skin or internal organs of SSc patients may promote endothelial damage and fibrosis, most likely through the production of soluble mediators including cytokines and chemokines. Several reports have shown chemokine abnormalities in SSc that might explain the altered accumulation of effector leukocyte subsets in affected tissues [5, 6].

In most patients, severe organ involvement occurs within the first 3 years of disease and skin sclerosis seldom progresses after 5 or 6 years [7, 8]. Thus, predicting disease progression is particularly important for SSc patients at their first visit; however, except for SSc-related autoantibodies [9], there are no definitive serum biomarkers available to estimate disease progression. We hypothesized that some chemokines may be related to underlying biologic process which is ongoing and which will change clinical features over time.

In the present study, we focused on five major chemokines (CCL2, CCL5, CXCL8, CXCL9, and CXCL10), since these chemokines have been reported to be elevated and are associated with disease activity in other connective tissue diseases such as systemic lupus erythematosus [10, 11]. We sought to determine if baseline serum chemokines levels could predict the progress of symptoms in early SSc patients diagnosed with disease no more than 3 years prior to the onset of the study. To this end, serum levels of five chemokines were evaluated in early SSc patients at baseline and every year over the course of 4 years.

Materials and methods

Patients

Patients were grouped according to the degree of skin involvement based upon the classification system proposed by LeRoy et al. [diffuse cutaneous SSc (dcSSc) versus limited cutaneous SSc (lcSSc), 12]. In this study, 70 Japanese patients with early SSc (disease duration defined by the period from the first symptom including Raynaud's phenomenon attributable to SSc to our first assessment $\leq$3 years) who had dcSSc and/or ILD were registered at nine major scleroderma centers in Japan (Gunma University Hospital, Kanazawa University Hospital, Keio University Hospital, Kitasato University Hospital, Kumamoto University Hospital, Nagasaki University Hospital, Tokyo University Hospital, Tokyo Women's Medical University Hospital, Tsukuba University Hospital). Patients with other inflammatory, infectious, or malignant diseases were not included in this study.

Among the patients, 39 patients had dcSSc with ILD, 23 patients had dcSSc without ILD, and 8 patients had lcSSc with ILD. Forty-seven patients were female and twenty-three patients were male. The median age was 53 years (range, 14–76 years). The median disease duration was 12 months (range, 1–36 months). All patients fulfilled the criteria for SSc proposed by the American College of Rheumatology [13]. With respect to the specificity of anti-nuclear antibodies (Abs) in the serum, 41 patients were positive for anti-topoisomerase I Ab and 8 patients were positive for anticentromere Ab. Fifteen age- and gender-matched healthy persons [11 females and 4 males, median age 49 years (range, 20-65 years)] were also included as normal controls in this study.

Among 70 patients, 33 patients could be followed every year for 4 years. Twenty-one patients had dcSSc with ILD, nine patients had dcSSc without ILD, and nine patients had lcSSc with ILD. Twenty-one patients were female and twelve patients were male. The median age was 55 years (range, 14–75 years). The median disease duration was 19 months (range, 1–36 months). With respect to the specificity of anti-nuclear Abs, 23 patients were positive for anti-topoisomerase I Ab and one patient was positive for anticentromere Ab. The ethical committee at each center approved all protocols and informed consent was obtained from all patients.

Clinical assessments

Patients had a physical examination and laboratory tests were performed at their first visit and at each subsequent year for 4 years. The degree of skin involvement was determined according to the modified Rodnan total skin thickness score (MRSS), as described elsewhere [14]. Organ system involvement was defined as described previously [15] with some modifications: ILD = bibasilar interstitial fibrosis or ground-glass shadow on computed tomogram (CT); pulmonary arterial hypertension (PAH) = clinical evidence of pulmonary hypertension and

elevated right ventricular systolic pressure (>45 mmHg) documented by echocardiography in the absence of severe pulmonary interstitial fibrosis; esophagus = apparent dysphagia, reflux symptoms, or hypomotility shown by barium radiography; heart = pericarditis, congestive heart failure, or arrhythmias requiring treatment; kidney = malignant hypertension and rapidly progressive renal failure unexplained by certain diseases other than SSc; joint = inflammatory polyarthralgias or arthritis; and muscle = proximal muscle weakness and elevated serum creatine kinase. A health assessment questionnaire-disability index (HAQ-DI) modified for Japanese patients [16] including digital ulcer, pitting scar, maximal oral aperture (the maximum vertical length of opened mouth), and skin pigmentation/depigmentation was also evaluated. Erythrocyte sedimentation rate (ESR) and pulmonary function, including vital capacity (VC) and diffusion capacity for carbon monoxide (DLco), were also tested.

Serum cytokine and chemokine assays

Fresh venous blood samples were taken from 70 patients and 15 healthy controls at their first visit (baseline). In 33 patients, blood samples were also taken at each subsequent year for 4 years. Samples were centrifuged shortly after clot formation. All serum samples were stored at −70 °C prior to use in assays. Serum levels of CCL2/monocyte chemoattractant protein-1 (MCP-1), CCL5/RANTES (regulated upon activation, normally expressed in T cells, and secreted), CXCL8/interleukin 8 (IL-8), CXCL9/monokine induced by interferon γ (MIG), and CXCL10/interferon γ-inducible protein-10 (IP-10) were measured by cytometric beads array (BD PharMingen, San Diego, CA) using a FACScan flow cytometer (BD PharMingen). Limit of detection was as follows; CCL2 2.7, CCL5 1.0, CXCL8 0.2, CXCL9 2.5, CXCL10 2.8 pg/ml.

Statistical analysis

JMP® Statistical Discovery Software (SAS Institute, Cary, NC) was used for analysis. Since the Shapiro–Wilk test did not indicate that serum chemokine concentration showed normal distribution, the data were converted to logarithm so that the data exhibited normal distribution. Then statistical analyses were performed using the Student's t test for the comparison of sample levels between two groups. The Pearson product-moment correlation coefficient was used to examine the relationship between two continuous variables. Potential prognostic factors for estimating subsequent MRSS, %VC, and HAQ-DI were statistically examined by multiple regression analysis. A P value <0.05 was considered statistically significant. All values are expressed as the median (range) otherwise indicated.

Results

Baseline serum chemokine levels were elevated in SSc patients

Serum samples taken from normal controls ($n = 15$) and all patients ($n = 70$) at their first visit were assayed and CCL2 levels were found to be significantly increased in SSc patients compared with healthy controls ($P < 0.0001$, Fig. 1). Similarly, the levels of serum CCL5, CXCL8, CXCL9, and CXCL10 were also significantly elevated in the SSc patients ($P < 0.0001$, respectively, Fig. 1). In this regard, serum levels of CCL2 were significantly associated with levels of CXCL8 in patients ($r = 0.76$, $P < 0.0001$). Similarly, serum CXCL9 levels were significantly associated with serum CXCL10 levels in patients ($r = 0.58$, $P < 0.0001$). Other combinations of chemokines were not significantly associated with each other.

At the initial visit, there was a significant association between serum CCL2 levels and HAQ-DI in patients with SSc ($r = 0.41$, $P = 0.010$). There was also a significant association between serum CXCL8 levels and HAQ-DI ($r = 0.40$, $P = 0.020$). However, there were no statistically significant differences in the chemokine levels between dcSSc and lcSSc patients or between patients with ILD and patients without ILD. Moreover, no significant correlations were found between the levels of any of the chemokines measured and any other clinical or laboratory findings. Steroid treatment did not significantly affect the levels of these chemokines [steroid (+) vs. steroid (−) (median (range) pg/ml); CCL2 131.0 (41.8–3806.6) vs. 167.7 (48.3–872.7); CCL5 7902.9 (144.7–27071.9) vs. 7276.5 (1482.2–25752.4); CXCL8 18.7 (<0.2–12166.9) vs. 14.0 (<0.2–6705.1); CXCL9 104.9 (4.2–1177.3) vs. 94.3 (7.5–813.4); CXCL10 156.6 (3.5–1554.5) vs. 201.3 (5.0–651.1)].

Longitudinal change of clinical features

To assess progression of SSc over time, thirty-three patients were followed-up every year for 4 years (Table 1). To assess the degree of skin involvement in patients, MRSS values were calculated, and %VC and %DLco were used to assess lung involvement. HAQ-DI was also obtained in order to evaluate the functional abilities of the patients. For the patient population as a whole, the median MRSS value decreased from 16 to 10 during the first year. The median MRSS was 7 at the end of year 2, 9 at the end year 3, and 8 at the end year 4. Median values for %VC did not significantly change during the 4-year evaluation period. In this regard, the %VC was 96 at first visit, 92 at the end of the first year, 96 at the end of the second year, 94 at the end of the third year, and 91 at the end of the fourth

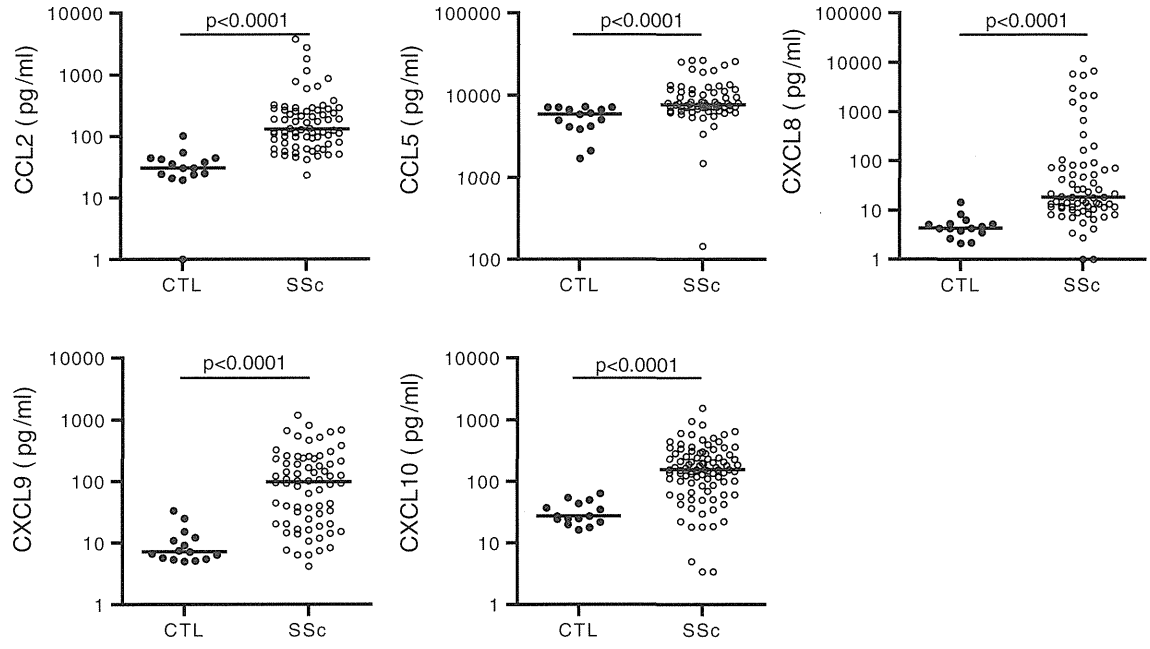

Fig. 1 Serum chemokine levels in early SSc patients (SSc) and healthy controls (CTL). The *horizontal bar* in each group indicates the median value

Table 1 The course of clinical and laboratory features in patients with SSc

	Baseline	1 year follow-up	2 year follow-up	3 year follow-up	4 year follow-up
MRSS	16 (2–39), $n = 33$	10 (0–38), $n = 33$	7 (1–25), $n = 33$	9 (1–25), $n = 33$	8 (0–29), $n = 33$
HAQ-DI	0.125 (0–1.5), $n = 3$	0.125 (0–1.75), $n = 33$	0.375 (0–2.5), $n = 33$	0.125 (0–1.875), $n = 33$	0.25 (0–1.75), $n = 33$
%VC	96 (53–144), $n = 27$	92 (62–120), $n = 20$	96 (61–144), $n = 20$	94 (56–137), $n = 22$	91 (58–136), $n = 24$
%DLco	70 (41–113), $n = 27$	68 (43–104), $n = 19$	70 (44–96), $n = 19$	69 (28–119), $n = 22$	63 (21–107), $n = 25$
ILD	24 (73), $n = 33$	24 (73), $n = 33$	25 (76), $n = 33$	28 (85), $n = 33$	28 (85), $n = 33$
PAH	0 (0), $n = 33$	1 (3.7), $n = 27$	1 (3.6), $n = 28$	0 (0), $n = 29$	0 (0), $n = 27$
Renal crisis	0 (0), $n = 33$	2 (6.1), $n = 33$	0 (0), $n = 33$	1 (3.0), $n = 33$	1 (3.0), $n = 33$
Corticosteroid therapy	23 (70), $n = 33$	28 (85), $n = 33$	31 (94), $n = 33$	31 (94), $n = 33$	28 (85), $n = 33$
Cyclophosphamide therapy	3 (9), $n = 33$	5 (15), $n = 33$	3 (9), $n = 33$	3 (9), $n = 33$	4 (12), $n = 33$
Other immunosuppressive agents therapy	1 (3), $n = 33$	1 (3), $n = 33$	4 (12), $n = 33$	4 (12), $n = 33$	5 (15), $n = 33$

Values are represented as median (range) or as number of positive cases with percentage within parentheses, in total patients in whom those data are available

year. Similarly, median values for %DLco did not significantly change during the 4-year evaluation period. Specifically, the %DLco was 70 at first visit, 68 at the end of the first year, 70 at the end of the second year, 69 at the end of the third year, and 63 at the end of the fourth year. The median HAQ-DI was 0.125 at the first visit, 0.125 at the end of year 1, 0.375 at the end of year 2, 0.125 at the end of year 3, and 0.25 at the end of year 4. The frequency of patients with ILD determined by CT was unchanged during the evaluation period. The development of PAH and renal crisis was rare during the course of the study. Orally administered prednisolone (~20 mg/day) use was common at baseline and subsequent years (70–94 %).

Longitudinal change of chemokine levels

The yearly changes in serum chemokine levels for each case are shown in Fig. 2. The dotted horizontal lines

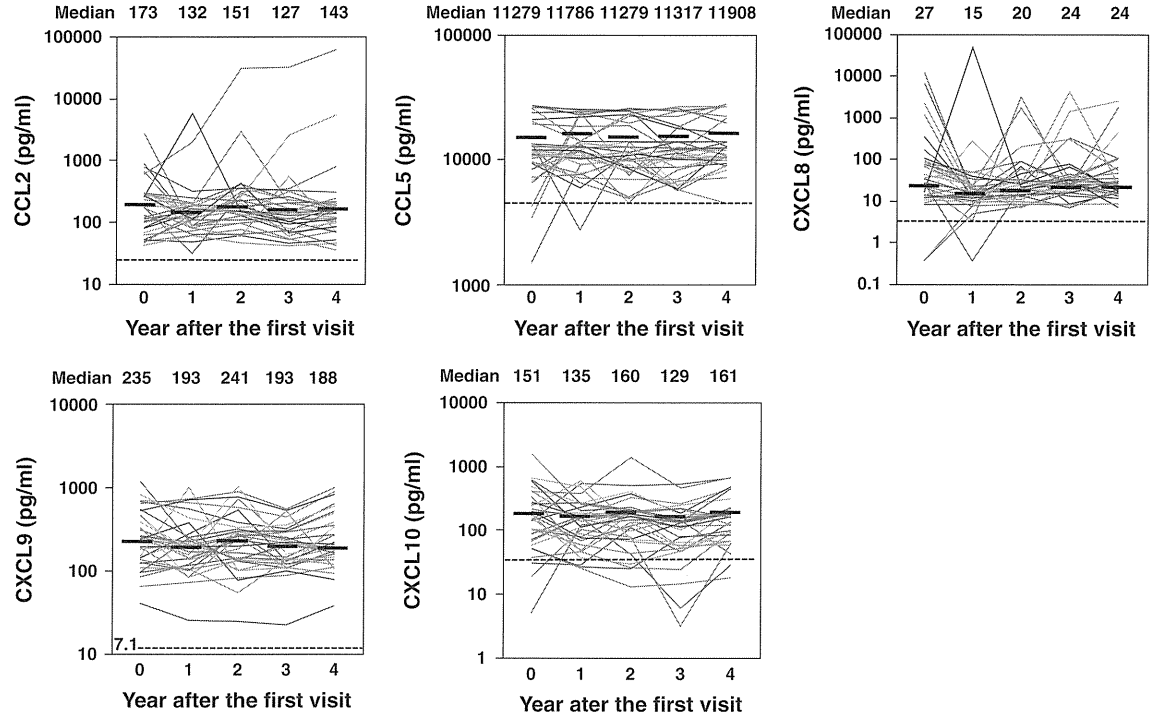

Fig. 2 Longitudinal change of serum chemokine levels in each patient during the 4 years of the study. The *horizontal dotted line* indicates the median value in healthy controls. The *horizontal bar* at each time point indicates the median value

indicate median values of healthy controls. Overall, the levels of each chemokine in the serum showed considerable variations in each patient; however, the median values of each of the five chemokines measured did not change significantly over time (Fig. 2). Furthermore, the variation in chemokine levels over time was not significantly associated with the variation of MRSS, %VC, %DLco, and HAQ-DI during the 4 years course of the study (data not shown).

Association between each chemokine level and clinical features of SSc

Next, we evaluated if baseline serum chemokine levels are associated with baseline and subsequent clinical features of SSc by univariate analysis. Baseline serum levels of CCL2 and CXCL8 were significantly associated with HAQ-DI values at baseline and subsequently every year for 4 years (Table 2). However, serum levels of CCL2 and CXCL8 did not significantly associated with other clinical features including skin thickness score and lung function. No significant associations between serum levels of CCL5, CXCL9, or CXCL10 and subsequent clinical features were found. These data indicate that serum levels of CCL2 and CXCL8 may be useful biomarkers for estimating the subsequent progression of physical disability.

Association between the level of each chemokine and clinical features

Finally, we utilized multiple regression analysis to evaluate the ability of serum chemokine levels to predict clinical or laboratory factors such as MRSS, %VC, and HAQ-DI of patients 4 years after the first visit. Selected variables were as follows: each chemokine level, anti-topoisomerase I Ab, anticentromere Ab, MRSS, %VC, %DLco, presence of ILD, HAQ-DI, ESR, corticosteroid treatment, and cyclophosphamide treatment at the first visit. We performed step-wise multiple regression analyses that specified the α level for either adding or removing a regression as 0.15. As a result, the multiple regression equation predicting the HAQ-DI of 4 year follow-up $= -0.24 + \log_{10}$ [serum CXCL8 levels (pg/ml)] of baseline $+ -0.0061 \times$ %VC of baseline ($R^2 = 0.41$, root mean square error $= 0.36$, $P = 0.0016$, Table 3). Using our equation, we found that the HAQ-DI value at the fourth year was significantly associated with the CXCL8 level of baseline ($P = 0.0016$). Additionally, the HAQ-DI value of 4 year follow-up tended to be negatively associated with %VC of baseline ($P = 0.086$). ESR, MRSS, %VC, or %DLco at the fourth year was not significantly associated with any chemokine levels or clinical factors of baseline.