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Effects of bosentan on nondigital ulcers in patients with systemic sclerosis

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Conflicts of interest
None declared.

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Summary

Background Bosentan is an oral dual endothelin receptor antagonist, which has been shown to be efficacious for preventing new digital ulcers in patients with systemic sclerosis (SSc) in two high-quality randomized controlled trials. However, its efficacy for nondigital ulcers in SSc remains unknown.

Objectives To evaluate the efficacy of bosentan on nondigital ulcers in patients with SSc.

Methods Bosentan was administered to five patients with SSc with pulmonary arterial hypertension, who also had nondigital ulcers refractory to conventional treatments. The efficacy of bosentan on nondigital ulcers and its association with clinical features of ulcers were analysed.

Results The nondigital ulcers refractory to conventional treatments were significantly improved by the administration of bosentan in cases surrounded with severe cyanosis. In contrast, nondigital ulcers without cyanosis were still refractory to bosentan therapy.

Conclusions Bosentan may be efficacious for accelerating the healing of nondigital ulcers with severe cyanosis, suggesting that nondigital ulcers caused by severely impaired peripheral circulation are highly responsive to this treatment.

Systemic sclerosis (SSc) is a multisystem autoimmune disorder characterized by vascular injuries and fibrosis of skin and certain internal organs.[1] In addition to life-threatening organ involvement, skin ulcers are important complications severely affecting the quality of life and activity of daily living in SSc.[2]

A recent advance in the therapeutic field of SSc is the emergence of a dual endothelin (ET) receptor antagonist, bosentan. Two high-quality randomized controlled trials demonstrated that bosentan prevents the development of new digital ulcers in SSc.[3,4] Although these studies failed to prove the efficacy of bosentan for accelerating the healing of digital ulcers, its efficacy against skin ulcers on areas other than digits has not been well evaluated.[5] Therefore, we evaluated the effect of bosentan on nondigital ulcers in five patients with SSc with pulmonary arterial hypertension (PAH).

Patients and methods

Among patients with SSc starting bosentan for PAH between July 2009 and September 2010, five patients with refractory nondigital ulcers were selected. PAH was diagnosed by ultrasound cardiography and/or right ventricular catheterization. Patients were treated with bosentan 125 mg daily for the first 4 weeks and 250 mg daily afterwards. Concomitant medications were not substantially modified during the follow-up period. Ulcer healing was considered to be complete when total re-epithelialization was observed.

Results

Patient information, the clinical features of nondigital ulcers, and the efficacy of bosentan therapy are summarized in Table 1. Healing of nondigital ulcers was seen in three of five patients. All of the three ulcers that were completely healed after the administration of bosentan were surrounded by severe cyanosis, while the other two ulcers refractory to bosentan were not accompanied by cyanosis (Fig. 1). In patients 2 and 5 with nondigital ulcers highly responsive to bosentan therapy, imaging studies revealed multiple organic narrowing of arteries and poor peripheral circulation in the affected legs before the administration of bosentan (Fig. 2). Bosentan did not affect any immunological or inflammatory parameters, including serum immunoglobulin levels, autoantibody titres, and C-reactive protein levels. The clinical courses are described below.

Patient 1

In July 2009, a 45-year-old woman with a 15-year history of systemic lupus erythematosus (SLE) and SSc overlap syndrome was treated with bosentan for PAH. Six months previously, a

Table 1 Clinical features and results of bosentan therapy

Patient	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age (years)	45	80	69	62	49
Disease duration (years)	15	4	15	42	25
Classification	Diffuse	Limited	Diffuse	Diffuse	Diffuse
Specific antibodies	Topo-I, U1RNP	ACA	Topo-I	U1RNP	Topo-I
Location of ulcers or gangrene	L heel	L foot	Both heels	R ankle	L heel
Cyanosis surrounding ulcers	+	+	–	–	+
Macrovascular involvement confirmed by imaging studies	ND	+	ND	ND	+
Healing of ulcer by bosentan	+	+	–	–	+
Treatment duration before complete epithelialization (months)	4	15	NA	NA	6
Attenuation of cyanosis by bosentan	+	+	NA	NA	+
Concomitant medications	Tocopherol nicotinate, ethyl icosapentate, beraprost sodium, alprostadil, algatroban	Tocopherol nicotinate, beraprost sodium, sarpogrelate, cilostazol, alprostadil, algatroban	Sarpogrelate, limaprost alfadex, alprostadil, algatroban	Beraprost sodium, limaprost alfadex, alprostadil, algatroban	Tocopherol nicotinate, limaprost alfadex, alprostadil, algatroban
Risk factors of atherosclerosis	–	Well-controlled HT	–	Well-controlled HT	–

Topo-I, antitopoisomerase I antibody; ACA, anticentromere antibody; U1RNP, anti-U1RNP antibody; ND, not determined; NA, not applicable; HT, hypertension.

refractory ulcer appeared on her left heel surrounded by an area of cyanosis (Fig. 1a). Eventually, the ulcer enlarged up to 25 × 15 mm even in the summer season (Fig. 1b). After starting bosentan, the cyanosis improved and the ulcer became shallow, finally resulting in complete re-epithelialization in 4 months (Fig. 1c).

Patient 2

In August 2009, an 80-year-old women with a 4-year history of SSc was treated with bosentan for PAH. Just after the diagnosis of SSc, she had had digital ulcers and gangrene on bilateral toes, finally resulting in distal foot ulceration. Computed tomography angiography revealed multiple stenoses of the left anterior tibial artery (Fig. 2a) due to macrovascular involvement associated with SSc. When bosentan was administered, the ulcers were covered with thick necrotic tissue and granulation tissue was not seen (Fig. 1d). Thereafter, however, necrotic tissues were gradually replaced with granulation tissue (Fig. 1e) and the ulcer finally healed completely in 15 months (Fig. 1f).

Patient 3

In October 2009, a 69-year-old women with a 15-year history of SLE and SSc overlap syndrome was treated with bosentan for PAH. Refractory ulcers appeared on bilateral heels 10 years previously and had never healed afterwards. During a 22-month bosentan therapy, the ulcers had never shown any healing at all (Fig. 1g–j).

Patient 4

In January 2010, a 62-year-old woman with a 42-year history of SSc began to be treated with bosentan for PAH. She had had a refractory ulcer on the right lateral malleolus for more than 25 years. Despite bosentan treatment, her ulcer did not show any healing at all during a 16-month follow-up period (Fig. 1k, l).

Patient 5

In September 2010, a 49-year-old woman with a 25-year history of SLE and SSc overlap syndrome started to be treated with bosentan for PAH. Two months previously, a refractory ulcer appeared on the left heel surrounded by an area of cyanosis. Magnetic resonance angiography showed multiple stenoses of anterior tibial and peroneal arteries due to macrovascular involvement associated with SSc (Fig. 2b). After the initiation of bosentan, her ulcer stopped enlarging and began to heal, leading to complete re-epithelialization in 6 months (Fig. 1m, n). In addition to healing of the ulcer, the cyanosis greatly improved.

Discussion

The present study demonstrated that cyanosis surrounding ulcers may serve as a useful clinical sign to choose nondigital ulcers highly responsive to bosentan. Skin ulcers in SSc occur due to a

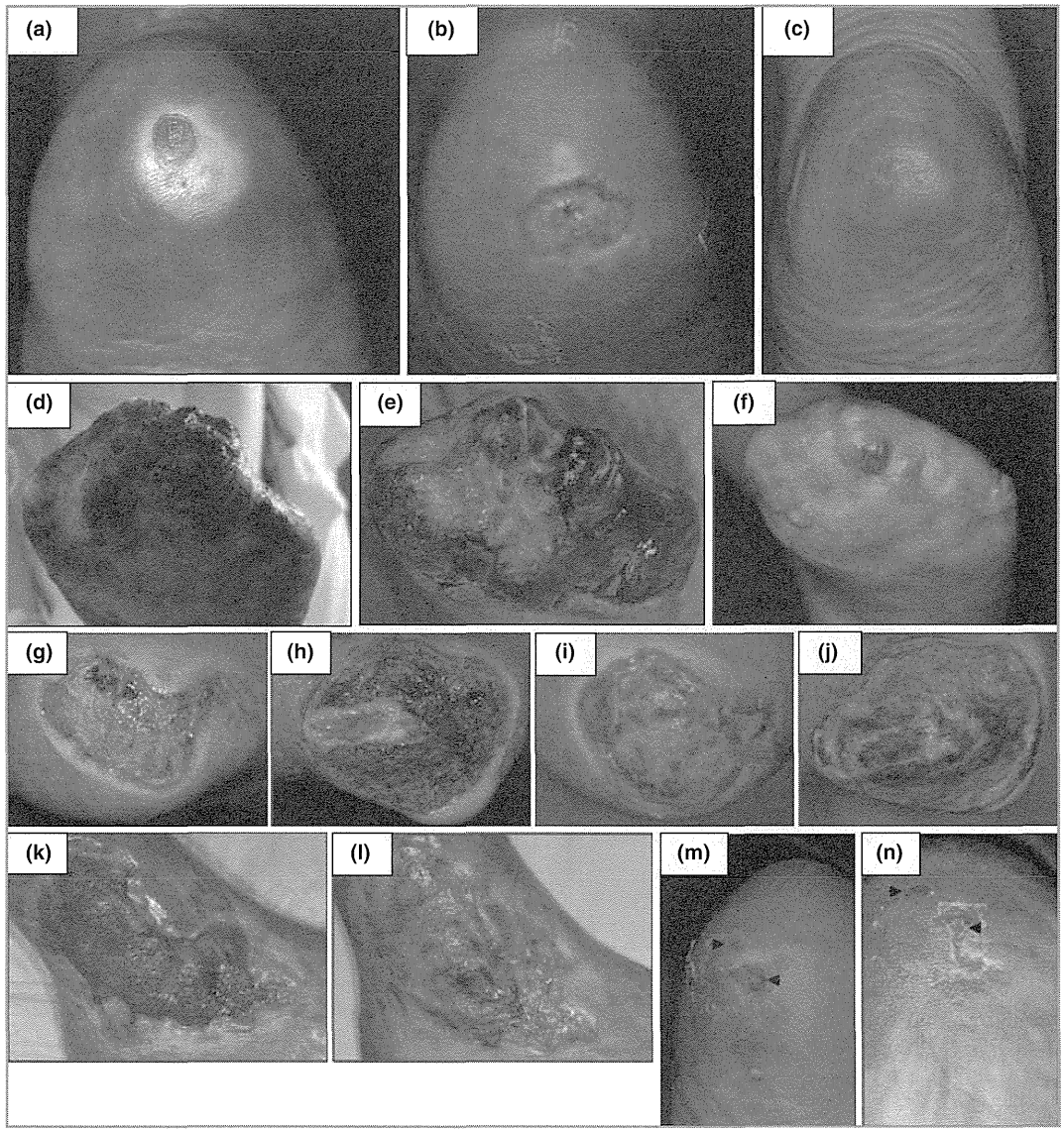

Fig 1. Clinical course of skin ulcers. Patient 1. (a) An ulcer developed on the left heel, surrounded by an area of cyanosis. (b) Clinical appearance of the ulcer before the administration of bosentan. (c) After starting bosentan, the ulcer healed completely in 4 months. Patient 2. (d) Clinical appearance of an ulcer before bosentan therapy. All of the toes had dropped off, resulting in extensive ulceration of the left foot. (e) One month after the administration of bosentan, necrotic tissues started to be replaced gradually with granulation tissues. (f) Fifteen months after the administration of bosentan, the ulcer finally healed completely. Patient 3. Before the administration of bosentan, there were hen's egg-sized ulcers on the right heel (g) and left heel (h). Ten months after the administration of bosentan, the ulcers on the right heel (i) and left heel (j) did not show any improvement at all and were covered with thick necrotic tissues. Patient 4. (k) Before bosentan therapy, a clenched hand-sized ulcer was located on the right lateral malleolus. Necrotic tissues were removed by debridement after hospitalization. The skin around the ulcer was atrophic without any cyanotic change. (l) Four months after the administration of bosentan, the ulcer did not show any healing at all and was covered with fixed necrotic tissues. Patient 5. (m) Before bosentan treatment, an ulcer was located on the left heel surrounded by an area of cyanosis. (n) Six months after the administration of bosentan, complete re-epithelialization was seen. Corresponding areas are labelled with arrowheads in the clinical images.

variety of reasons, including impaired peripheral circulation caused by microangiopathy and/or macrovasculopathy, microinjuries, bacterial infection, calcinosis, and atrophy due to extensive fibrosis. Once skin ulcers develop, disorganized angiogenesis leading to impaired peripheral circulation and altered extracellular matrix remodelling are major factors decelerating wound healing, but the contribution of these factors to impaired wound healing differs between individual patients. Our data suggest that bosentan is useful for the treatment of nondigital ulcers associated with severely impaired peripheral circulation, especially with macrovasculopathy. Consistent with this, we previously demonstrated that bosentan may improve severe cyanosis associated with macrovasculopathy through reversing organic vascular change in a certain subset of SSc.[6]

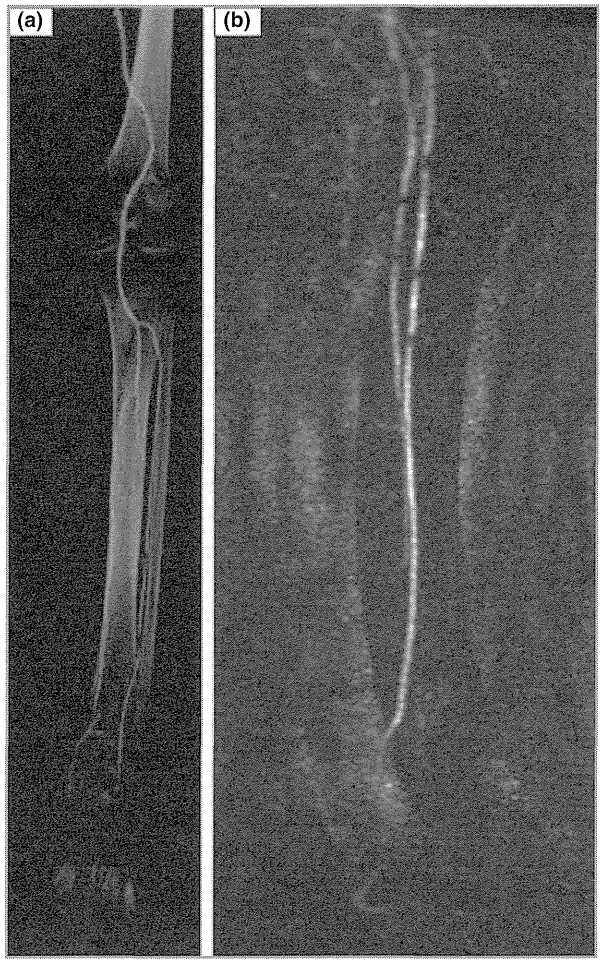

Fig 2. Imaging studies in patients 2 and 5. (a) In patient 2, computed tomography angiography revealed multiple stenoses of the left anterior tibial artery. (b) In patient 5, magnetic resonance angiography showed multiple stenoses of anterior tibial and peroneal arteries in the lower left leg.

Contrary to the present observation, bosentan failed to accelerate the healing of digital ulcers in randomized controlled trials.[3,4] This discrepancy may be attributable to the differential roles of ET-1 in organic vascular change and wound healing. ET-1 exerts proliferative, profibrotic and proinflammatory effects by acting on fibroblasts,[7,8] vascular smooth muscle cells,[9] endothelial cells[10] and macrophages,[11] leading to the development of organic vascular change in SSc. Given that plasma ET-1 levels are elevated in limited cutaneous SSc with PAH and diffuse cutaneous SSc,[12] it is plausible that bosentan reverses organic vascular change and improves peripheral circulation in a certain subset of SSc.[6] On the other hand, ET-1 mediates a significant portion of the antimigratory and antiproliferative actions of transforming growth factor (TGF)-β on endothelial cells in an autocrine manner, promoting the maturation of newly formed blood vessels.[13] Furthermore, ET-1 and TGF-β1 coordinately enable quiescent dermal fibroblasts to reactivate migration and proliferation programmes during the late proliferation phase of wound healing.[14] Consistent with this, bosentan prevents the acceleration of wound closure induced by overexpression of TGF-β1 in animal models.[15] Taken together, bosentan potentially accelerates the healing of skin ulcers closely associated with impaired peripheral circulation, even though it partially inhibits the process of wound healing. Further studies are ongoing in our department to confirm these findings in a large number of skin ulcers associated with SSc, both digital and nondigital.

What's already known about this topic?

- Two high-quality randomized controlled trials demonstrated that bosentan prevents the development of new digital ulcers in systemic sclerosis (SSc).
- Although these studies failed to prove the efficacy of bosentan for accelerating the healing of digital ulcers in SSc, its efficacy against skin ulcers on areas other than digits has not been well evaluated.

What does this study add?

- Nondigital ulcers refractory to conventional treatments were significantly improved by the administration of bosentan in cases surrounded with severe cyanosis.
- Bosentan may be efficacious for accelerating the healing of nondigital ulcers with severe cyanosis, suggesting that nondigital ulcers caused by severely impaired peripheral circulation are highly responsive to this treatment.

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Concise report

Investigation of prognostic factors for skin sclerosis and lung function in Japanese patients with early systemic sclerosis: a multicentre prospective observational study

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Abstract

Objective. To clarify the clinical course of SSc in Japanese patients with early-onset disease. It is well known that ethnic variations exist in the clinical features and severity of SSc. However, neither the clinical course nor prognostic factors have been thoroughly investigated in the Japanese population.

Methods. Ninety-three Japanese patients of early-onset SSc (disease duration: <3 years) with diffuse skin sclerosis and/or interstitial lung disease were registered in a multi-centre observational study. All patients had a physical examination with laboratory tests at their first visit and at each of the three subsequent years. Factors that could predict the severity of skin sclerosis and lung involvement were examined statistically by multiple regression analysis.

Results. Two patients died from SSc-related myocardial involvement and four patients died from other complications during the 3-year study. Among various clinical data assessed, the initial modified Rodnan total skin thickness score (MRSS) and maximal oral aperture were associated positively and negatively with MRSS at Year 3, respectively. Additionally, initial ESR tended to be associated with final MRSS. Pulmonary vital capacity (VC) in the third year was significantly associated with initial %VC. Furthermore, patients with anti-topo I antibody tended to show reduced %VC at Year 3.

Conclusions. Several possible prognostic factors for skin sclerosis and lung function were detected in Japanese patients with early SSc. Further longitudinal studies of larger populations will be needed to confirm these findings.

Key words: systemic sclerosis, scleroderma, prognostic factor, skin sclerosis, interstitial lung diseases, treatment

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Introduction

SSc is a CTD characterized by tissue fibrosis in the skin and internal organs. Interstitial lung diseases (ILDs) develop in more than half of SSc patients and are one of the major SSc-related causes of death [1, 2]. The natural course of skin sclerosis and internal organ involvement and identification of prognostic factors have been extensively reported in Europe and the USA [3–6]. However, there are some racial differences in the clinical and laboratory features of SSc [7]. For example, the severity of skin sclerosis is modest in Japanese patients [8]. Furthermore, pulmonary arterial hypertension and renal crisis are rare in Japanese SSc patients [9]. Furthermore, racial differences are found in the distribution of SSc-related serum ANAs [10]. The frequency of anti-RNA polymerases antibody (Ab) is lower in the Japanese population than in US or European patient populations [9]. However, there have been no multiple-centre prospective studies concerning the clinical features of SSc in Japanese individuals.

In most patients, severe organ involvement occurs within the first 3 years of disease and skin sclerosis seldom progresses after 5 or 6 years [3, 11]. Therefore, predicting disease progression is particularly important for SSc patients at their first visit. In the present study, we aimed to determine if any initial clinical or laboratory features were associated with subsequent disease severity in Japanese SSc patients with a short disease duration of <3 years.

Materials and methods

Patients

Patients were grouped according to the degree of skin involvement, based on the classification system proposed by LeRoy *et al.* (dcSSc *vs* lcSSc) [12]. In this study, 93 Japanese patients with early SSc (disease duration: <3 years) who had dcSSc or ILD were registered at 12 major scleroderma centres in Japan (Atami Hospital, International University of Health and Welfare; Gunma University Hospital; Kanazawa University Hospital; Keio University Hospital; Kitasato University Hospital; Kumamoto University Hospital; Nagasaki University Hospital; Nagoya University Hospital; Sapporo Medical University Hospital; Tokyo University Hospital; Tokyo Women's Medical University Hospital; Tsukuba University Hospital).

Among these patients, two died from SSc-related myocardial involvement and four died from complications (ANCA-associated vasculitis, sepsis, thrombotic thrombocytopenic purpura and uterine cancer, respectively) during the 3-year study. Therefore, 87 patients (49 patients had dcSSc with ILD, 27 patients had dcSSc without ILD and 11 patients had lcSSc with ILD) were followed for 3 years. Sixty-four were females and 23 were males; the median (range) age was 50 (3–74) years. All patients fulfilled the criteria for SSc proposed by the ACR [13]. The median (range) disease duration (the period from the development of any symptoms excluding RP to our first assessment) of patients was 20 (1–35) months. With respect to ANA, 56 patients were positive for anti-topo I Ab and 7 patients were positive for ACA. Medical ethics committee of Kanazawa University approved the study. In addition, this study was approved by the ethics committees of International University of Health and Welfare, Gunma University, Keio University, Kitasato University, Kumamoto University, Nagasaki University, Nagoya University, Sapporo Medical University, Tokyo University, Tokyo Women's Medical University and Tsukuba University. Informed consent was obtained from all patients.

Clinical assessments

Patients had a physical examination and laboratory tests performed at their first visit and at each subsequent year for 3 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 high-resolution CT (HRCT); 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; oesophagus = apparent dysphasia, 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. An HAQ modified for Japanese patients [16], digital ulcer, pitting scar, maximal oral aperture (the maximum vertical length of opened mouth) and skin pigmentation/depigmentation were also evaluated. ESR and pulmonary function, including vital capacity (VC) and diffusion capacity for carbon monoxide (DL_{CO}) were also tested.

Statistical analysis

JMP Statistically Discovery Software (SAS institute, Cary, NC, USA) was used for analysis. Potential prognostic factors for the severity of skin sclerosis and lung function were statistically examined by multiple regression analysis. A $P < 0.05$ was considered to be statistically significant. All values are expressed as the median (range).

Results

The clinical course of SSc in Japanese patients

To provide a comprehensive evaluation of the clinical features of SSc in Japanese patients, we analysed clinical data as well as laboratory test results from 87 patients with short disease duration (Table 1). To assess the degree of skin involvement in patients, MRSS values were calculated; VC and DL_{CO} percentages were used to assess lung involvement. For the patient population as a whole, the median (range) MRSS decreased from 17 (2–42) to 12 (0–41) during the first year. The median (range) MRSS

was 12 (0–41) at the end of Year 2 and 10 (0–47) at the end Year 3. Median (range) values for %VC did not significantly change during the 3-year evaluation period: 95 (49–144) at first visit, 93 (26–137) at the end of the first year, 95 (49–144) at the end of the second year and 92 (51–137) at the end of the third year. Similarly, median values for % DL_{CO} did not significantly change during the 3 years.

The frequency of patients with ILD or PAH was stable during the evaluation period. Similarly, the number of patients with oesophageal or joint involvement, pitting scar or skin pigmentation/depigmentation did not vary significantly over time. The value of HAQ and maximal oral aperture did not significantly change during the course. The median (range) value of ESR was 18 (2–95) mm/h at the first visit, then it reduced to 16 (2–84), 13 (1–63) and 12 (0.5–122) mm/h, during the subsequent 3 years. Oral prednisolone (~20 mg/day) use was common, with 56 patients starting to take this drug after the first visit and 70 patients having taken it by the end of Year 3. Two patients developed renal crisis during the course of the study (data not shown). Patients with digital ulcer or heart or muscle involvement were rare during the course (fewer than 10 patients, data not shown).

Prognostic factors of the progress of skin sclerosis

Next, we evaluated clinical or laboratory factors presenting at first visit that could predict the severity of skin sclerosis of 3 years later. Investigated factors were as follows: age, gender, disease duration, anti-topo I Ab, ACA, MRSS at the first visit, %VC, $\%DL_{CO}$, existence of each organ involvement (ILD, PAH, oesophagus, joint), pitting scar, skin pigmentation/depigmentation, HAQ, maximal oral aperture, ESR, CS treatment and cyclophosphamide treatment. Cases that have any missing data were excluded and thereby 80 patients were analysed. We performed multiple regression using stepwise way that specified the α-level for either adding or removing a regression as 0.20 (Table 2). As a result, the multiple regression equation predicting MRSS at the third year = 17.11 + 0.35 × MRSS at the first visit + −0.26 × maximal oral aperture + 0.042 × ESR ($R^2 = 0.63$, $P < 0.0001$). Thus, MRSS at the third year was significantly associated with MRSS at first visit ($P < 0.001$) and was negatively associated with initial maximal oral aperture at first visit ($P < 0.01$). Additionally, initial ESR tended to be associated with final MRSS ($P = 0.17$).

Prognostic factors of lung function

We similarly assessed the prognostic factors of impaired lung function to estimate ILD severity. Here, we used %VC as representative markers of lung function. Cases that have any missing data including %VC at the third year were excluded and thereby 58 patients were analysed. We performed multiple regression in a stepped manner that specified the α-level for either adding or removing a regression as 0.20 (Table 3). As a result, the multiple regression equation predicting %VC at the third

Table 2 Factors predicting MRSS at the third year determined by multiple regression analysis

	Estimate	Standard error	*P*-value
Intercept	17.11	4.88	<0.01
MRSS at the first visit	0.35	0.089	<0.001
Maximal oral aperture	−0.26	0.075	<0.01
ESR	0.042	0.043	0.17

The multiple regression equations predicting MRSS at the third year are as follows; MRSS at the third year = 17.11 + 0.35 × MRSS at the first visit + −0.26 × maximal oral aperture + 0.042 × ESR. R^2 (determination coefficient) = 0.63; Root mean square error = 4.73; $P < 0.0001$.

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

	First visit	Year 1	Year 2	Year 3
MRSS	17 (2–42); *n* = 87	12 (0–41); *n* = 84	12 (0–41); *n* = 84	10 (0–47); *n* = 87
%VC	95 (49–144); *n* = 70	93 (26–137); *n* = 55	95 (49–144); *n* = 57	92 (51–137); *n* = 60
$\%DL_{CO}$	70 (11–113); *n* = 70	68 (10–105); *n* = 55	69 (11–96); *n* = 57	68 (10–120); *n* = 60
ILD	54 (62); *n* = 87	47 (64); *n* = 73	47 (64); *n* = 73	46 (63); *n* = 73
PAH	9 (10); *n* = 87	9 (12); *n* = 76	8 (11); *n* = 72	11 (13); *n* = 84
Oesophagus	33 (37); *n* = 87	26 (34); *n* = 77	35 (48); *n* = 73	34 (40); *n* = 85
Joint	20 (23); *n* = 86	14 (18); *n* = 77	9 (12); *n* = 73	17 (20); *n* = 84
Pitting scar	27 (33); *n* = 87	29 (38); *n* = 76	35 (48); *n* = 73	33 (38); *n* = 86
Pigmentation/depigmentation	54 (62); *n* = 87	49 (64); *n* = 77	41 (57); *n* = 72	50 (60); *n* = 84
HAQ	0.08 (0–2); *n* = 83	0.125 (0–1.75); *n* = 74	0.25 (0–2.5); *n* = 73	0.125 (0–2.25); *n* = 83
Maximal oral aperture	45 (18–70); *n* = 87	45 (28–65); *n* = 75	46 (25–67); *n* = 72	45 (10–67); *n* = 83
ESR	18 (2–95); *n* = 80	16 (2–84); *n* = 61	13 (1–63); *n* = 52	12 (0.5–122); *n* = 57
CS	56 (64); *n* = 87	61 (82); *n* = 74	64 (86); *n* = 74	70 (80); *n* = 87
Cyclophosphamide	11 (13); *n* = 87	14 (19); *n* = 75	8 (12); *n* = 68	9 (10); *n* = 87

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.

TABLE 3 Factors predicting %VC at the third year determined by multiple regression analysis

	Estimate	Standard error	*P*-value
Intercept	10.94	8.54	0.20
%VC at the first visit	0.85	0.09	<0.0001
Anti-topo I Ab (+)	2.32	1.64	0.19

The multiple regression equations predicting %VC at the third year are as follows: %VC at the third year = 10.94 + 0.85 × %VC at the first visit + anti-topo I Ab ('+' → −2.32, '−' → 2.32). $R^2 = 0.70$; Root mean square error = 12.00; $P < 0.0001$.

year = 10.94 + 0.85 × %VC at the first visit + anti-topo I Ab ('+' → −2.32, '−' → 2.32) ($R^2 = 0.70$, $P < 0.0001$). Thus, %VC at the third year was significantly associated with the value of %VC at first visit ($P < 0.0001$). In addition, %VC at the third year tended to be lower in patients with anti-topoisomerase I Ab ($P = 0.19$).

Discussion

To our knowledge, this study is the first multiple-centre, longitudinal prospective study to investigate the clinical course of Japanese patients. For this study, 87 patients with early-onset SSc (<3 years) were followed over 3 years. Median MRSS was reduced 5 points during the first year, and continued to decrease through the third year. This trend was similar to that identified in our previous, single-centre prospective observational study of Japanese SSc patients [17]. Although the reason for the prominent first-year reduction in MRSS in our current study is unknown, our previous single-centre study [17] indicated that the dose of oral CS was related to the decrease of MRSS. However, in this multi-centre observational study we could not perform a similar analysis of prednisolone dose in patients at each centre. In addition, other therapies including cyclophosphamide were also used in a part of patients in our observational study. Previous large studies demonstrated that MRSS naturally reduced during the disease course and time was a significant predictor of MRSS [3–6]. Therefore, the effect of CS therapy for MRSS remains unclear from our data. Since it has been suggested that CS therapy can induce renal crisis, high doses of CSs have not been recommended for the treatment of SSc [18]. However, renal crisis is not as common in Japanese patients [9], and only two patients (one had been taking low-dose CS, whereas the other had not) developed renal crisis during the course of our study.

The main aim of this study was to define the prognostic factors of skin sclerosis and ILD. The multiple regression equation was defined to predict the MRSS at the third year among multiple factors presenting at the first visit. MRSS at the first visit was significantly correlated with MRSS at the third year in all patients. Maximal oral aperture was correlated inversely with MRSS in the third year. Thus, the current skin sclerosis likely reflects the extent of skin sclerosis of 3 years later independent of other organ's involvement or treatment. Additionally, ESR tended to be associated with final MRSS. The presence of autoantibodies such as anti-topo I Ab and ACA was not shown to have value as a prognostic indicator of MRSS. However, this may be due to population bias in our study, since most patients were positive for anti-topo I Ab and negative for ACA.

The current study revealed that %VC and $\%DL_{CO}$ remained nearly constant or slightly reduced during the 3-year period. Since patients with progressive ILD received immunosuppressive treatment, including cyclophosphamide therapy in the participating facilities, this may have affected the stabilization of lung function in our cases. The frequency of ILD detected by HRCT was not increased during the course of the study, indicating ILD is usually detected early in the disease course and rarely develops later. In consistent with generally stable course of %VC, %VC at their first visit highly associated with the %VC at the third year in all patients with or without treatment. Patients with anti-topo I Ab tended to show reduced %VC at the third year. Although these findings are not surprising, we first confirmed them in Japanese patients.

Our study has some limitations. The population is not large and the follow-up period is not long. This is an observational study and therefore the treatment is heterogeneous. In addition, other parameters including CRP could not be analysed due to the lack of data. We should also include disease activity variables [19] and disease severity scale [20] in our future study. Further longitudinal studies in a larger population will be needed to clarify the natural course and prognostic factors in Japanese SSc patients.

Rheumatology key messages

- Initial ESR tended to be associated with skin score at Year 3 in Japanese scleroderma patients.
- Japanese scleroderma patients with anti-topo I Ab tended to show reduced %VC at the third year.

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CONCISE REPORT

Quantification of circulating endothelial progenitor cells in systemic sclerosis: a direct comparison of protocols

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► Additional data are published online only. To view the files please visit the journal online (http://ard.bmj.com)

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ABSTRACT

Background It has been proposed that dysfunctional endothelial progenitor cells (EPCs) play a role in pathogenic vasculopathy in systemic sclerosis (SSc). However, there is some debate as to whether the EPC count is reduced in SSc. The European League Against Rheumatism Scleroderma Trials and Research (EUSTAR) group recently proposed recommendations for evaluating EPCs.

Objective To validate the proposed EUSTAR recommendations by a side-by-side comparison of methods for quantifying EPCs.

Methods Peripheral blood samples were obtained from 11 patients with SSc and 11 age-matched healthy controls. EPCs were simultaneously quantified by two methods: flow cytometry combined with immunomagnetic CD34+ cell enrichment or rosette-based lineage-negative (Lin−) cell enrichment. EPCs, defined as CD34+CD133+VEGFR2+ cells, were counted with and without fluorosphere calibration.

Results EPC counts measured with fluorosphere calibration correlated well with each other, regardless of the enrichment procedure used. In contrast, EPC counts from protocols that did not use fluorospheres correlated poorly with results from other protocols.

Conclusions The EUSTAR recommendations are valid when they are combined with fluorosphere calibration.

INTRODUCTION

In adults, new blood vessels are formed by at least two mechanisms: endothelial sprouting from pre-existing endothelial cells (angiogenesis) and the peripheral recruitment of bone marrow-derived endothelial progenitor cells (EPCs) (vasculogenesis). Over the last decade, EPCs have emerged as crucial regulators of vascular healing and remodelling, homing in on injury sites and working in concert with mature endothelial cells.[1] The role of EPCs in the vascular pathogenesis of connective tissue disease has therefore attracted considerable attention, both as potential biomarkers for vascular manifestations and as novel therapeutic targets. It has been shown that EPC counts are altered in patients with systemic sclerosis (SSc), rheumatoid arthritis, systemic lupus erythematosus and vasculitis.[2] However, research findings have varied. Since our report of reduced EPC counts in patients with SSc,[3] some researchers subsequently confirmed our finding[4 5] but others found an increased number of EPCs in patients with SSc.[6–9] These contradictory results may result from differences in the protocols used for quantifying EPCs. In an effort to standardise EPC research, the European League Against Rheumatism Scleroderma Trials and Research (EUSTAR) recently proposed recommendations on EPC detection methods.[10] However, these recommendations have not yet been validated. In this study we examined the reproducibility of the different EPC quantification protocols that would conform to EUSTAR recommendations by examining samples from patients with SSc and healthy controls.

METHODS

Patients and controls

The study included 11 women with SSc who were followed at Keio University Hospital. All patients fulfilled the American College of Rheumatology preliminary classification criteria for SSc.[11] Patients taking an immunosuppressant, statin or >10 mg/day prednisolone were excluded. Eleven healthy women age-matched with the patients with SSc were recruited from the hospital staff. All subjects were free of diabetes, hypertension or dyslipidaemia and none of the subjects smoked. The age at evaluation was similar between the patients with SSc and the healthy controls (mean±SD 60.5±14.7 years and 56.8±14.6 years). Six were classified as having diffuse cutaneous SSc. The disease duration from the onset of non-Raynaud's symptoms ranged from 12 to 262 months.

EPC quantification

Heparinised peripheral blood samples (60 ml) were taken from a forearm vein while the subject was at rest. The samples were taken in the morning and immediately transported to the laboratory where each sample was divided equally into two tubes. EPCs were quantified by flow cytometric analysis of partially enriched progenitor cells according to two methods, as published previously[3 8]: CD34+ cells by a magnetic-activated cell sorter (MACS) technique (MACS method)[3] or lineage-negative (Lin^-) cells by a rosette-based technique (rosette method).[8] The viability marker 7-amino actinomycin D (7AAD) was used in a multiparameter flow cytometer. These methods conform to the EUSTAR recommendations for flow cytometry (see online supplement).[10] EPCs were identified by the co-expression of CD34, CD133 and vascular endothelial growth factor receptor type 2 (VEGFR2) and were expressed as the number per 10^6 CD34+ or Lin− cells, counted based on the acquisitioned events on a flow cytometer. In addition, the absolute number of EPCs in 1 ml peripheral blood was calculated using FlowCount polystyrene fluorospheres as an internal calibrator.

Table 1 Correlation coefficient between different methods of EPC quantification in 11 patients with SSc and 11 healthy controls

Method for EPC quantification	MACS/7AAD−/fluorosphere+ (/1 ml PB)	MACS/7AAD+/fluorosphere+ (/1 ml PB)	MACS/7AAD+/fluorosphere− (/10^6 CD34+ cells)	Rosette/7AAD+/fluorosphere+ (/1 ml PB)	Rosette/7AAD+/fluorosphere− (/10^6 Lin− cells)
MACS/7AAD−/fluorosphere+ (/1 ml PB)		0.95†	0.17	0.73†	0.24
MACS/7AAD+/fluorosphere+ (/1 ml PB)	–		0.28	0.81†	0.26
MACS/7AAD+/fluorosphere− (/10^6 CD34+ cells)	–	–		0.30	0.11
Rosette/7AAD+/fluorosphere+ (/1 ml PB)	–	–	–		0.40
Rosette/7AAD+/fluorosphere− (/10^6 Lin− cells)	–	–	–	–	

†p<0.01.
EPC, endothelial progenitor cell; Lin−, lineage-negative; MACS, magnetic-activated cell sorter; PB, peripheral blood; SSc, systemic sclerosis.

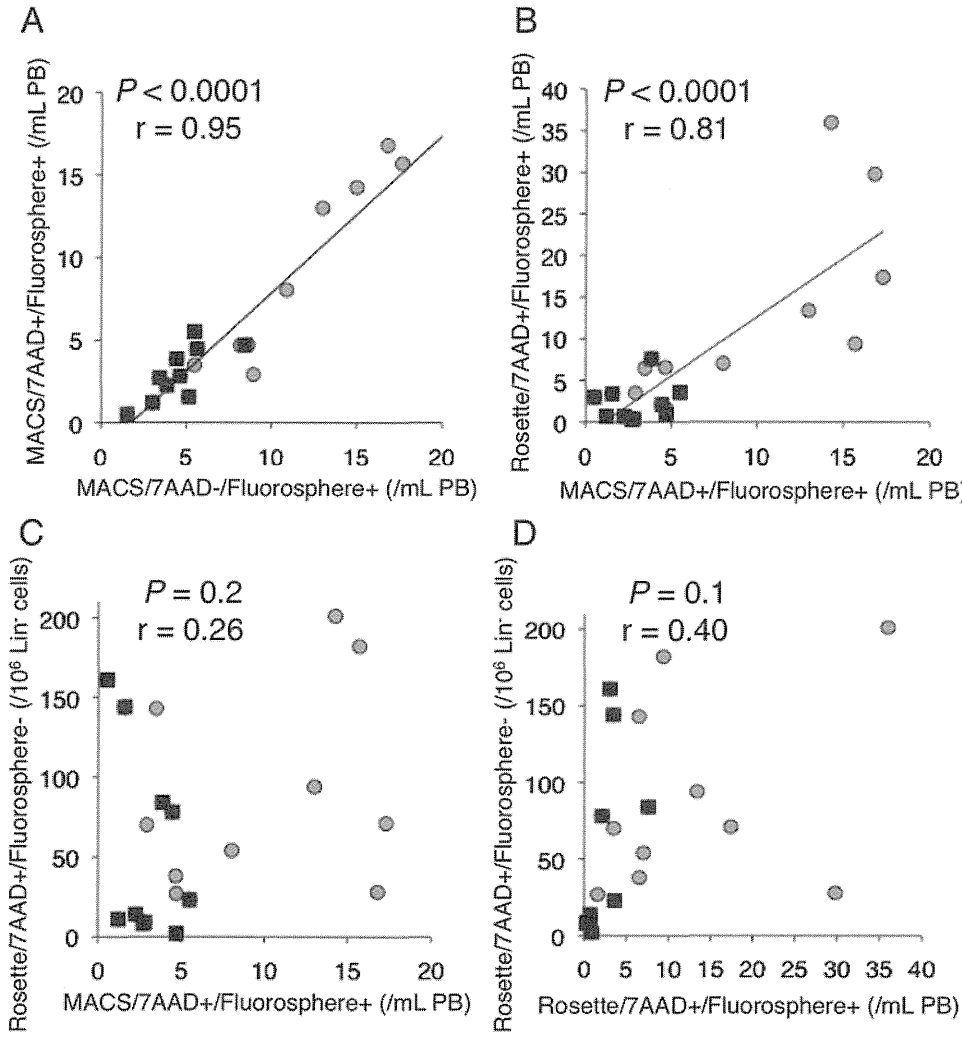

Figure 1 Correlations between endothelial progenitor cells (EPC) counts measured simultaneously by different protocols in 11 patients with systemic sclerosis (SSc, squares) and 11 healthy controls (circles). (A) Correlation between the magnetic-activated cell sorter (MACS) method with fluorosphere calibration, with and without 7-amino actinomycin D (7AAD) staining. (B) Correlation between the MACS and rosette methods with 7AAD staining and fluorosphere calibration. (C) Correlation between the MACS method with 7AAD staining and fluorosphere calibration, and the rosette method with 7AAD staining and no fluorosphere calibration. (D) Correlation between the rosette method with 7AAD staining, with and without fluorosphere calibration. PB, peripheral blood.

All procedures were performed by an experienced flow cytometry operator who was blinded to the sample identity.

Analysis of contaminating cell types in the Lin− cell fraction

The enriched Lin^- cell fraction was incubated with a combination of fluorescently labelled monoclonal antibodies anti-CD3, anti-CD14, anti-CD15, anti-CD41, antiglycophorin A (Beckman-Coulter, Fullerton, CA, USA) and anti-CD19 (BD Biosciences, San Diego, CA, USA). A flow cytometer was used to detect fluorescent cell staining. Dead cells were excluded by scatter analysis and by 7AAD staining.

Statistical analysis

The correlation coefficient (r) was determined using a single regression model. Comparisons between two groups were tested for statistical significance using the Mann–Whitney U test.

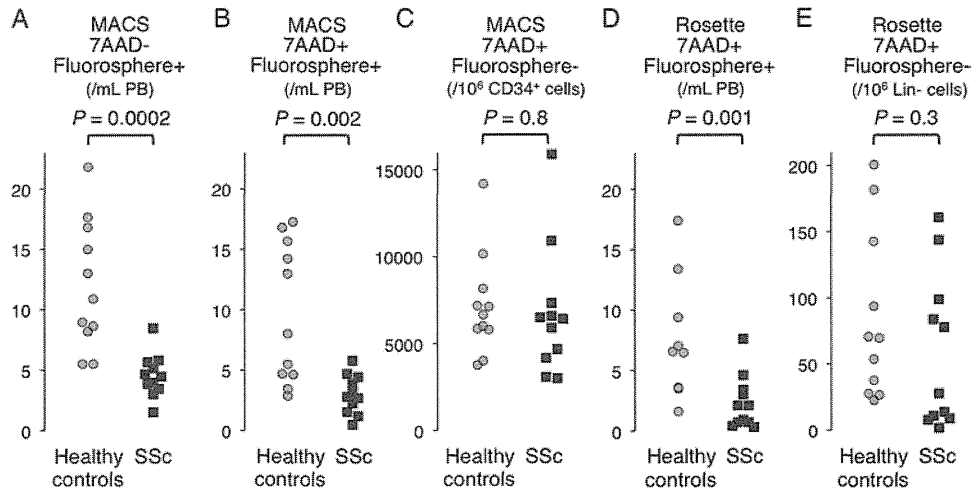

Figure 2 Comparison of endothelial progenitor cells (EPC) counts measured in 11 patients with systemic sclerosis (SSc) and 11 healthy controls using five different protocols. (A) Magnetic-activated cell sorter (MACS) method without 7-amino actinomycin D (7AAD) staining and with fluorosphere calibration. (B) MACS method with 7AAD staining and fluorosphere calibration. (C) MACS method with 7AAD staining but without fluorosphere calibration. (D) Rosette method with 7AAD staining and fluorosphere calibration. (E) Rosette method with 7AAD staining but without fluorosphere calibration. PB, peripheral blood, HC, healthy controls.

RESULTS

Before beginning data collection we analysed more than 100 samples from patients with SSc and healthy individuals as a practice session. The coefficient of variation for four repeated measures of EPCs on five samples was 9–18% and 19–31% for the original MACS and rosette methods, respectively. Representative flow cytometric analyses using different protocols are shown in figures S1–3 in the online supplement. Nearly all the EPCs detected by these protocols were confirmed to be negative for CD14 but weakly positive for CD45.

EPCs were measured simultaneously in 11 patients with SSc and 11 healthy controls using five different protocols: (1) MACS method without 7AAD staining but with fluorosphere calibration (only method that did not conform to EUSTAR recommendations); (2) MACS method with both 7AAD staining and fluorosphere calibration; (3) MACS method with 7AAD staining but without fluorosphere calibration; (4) rosette method with both 7AAD staining and fluorosphere calibration; and (5) rosette method with 7AAD staining but without fluorosphere calibration. The correlation coefficients between the EPC counts obtained by these protocols are summarised in table 1. EPC counts obtained by the MACS method with and without 7AAD staining correlated well when fluorospheres were used (figure 1A; r=0.95, p<0.0001). Interestingly, there was a statistically significant correlation between EPC counts obtained by the MACS and rosette methods when combined with fluorosphere calibration (figure 1B; r=0.81, p<0.0001). In contrast, protocols that did not use fluorosphere calibration correlated poorly with other protocols, regardless of the method of progenitor cell enrichment used (figure 1C,D).

We further examined the purity of CD34+ or Lin– cells in the enriched fractions. CD34+ cells in the enriched fraction ranged from 4% to 49% after viable cells were selected by gating with scatter analysis and 7AAD staining. To evaluate the contaminating cells in the Lin⁻ cell fractions, multiparameter flow cytometry was performed using the gate setting used for EPC measurement (see figure S4 in online supplement). While CD3+ T cells, CD14+ monocytes, CD15+ granulocyte/monocytes and CD19+ B cells accounted for <6% of the cells in the Lin– fraction, the predominant cell populations consisted of CD41+ platelets and glycophorin A+ erythrocytes. When all the cells with lineage markers were excluded, the percentage of Lin– cells in the enriched fraction ranged from 8% to 28%.

When EPC counts between patients with SSc and healthy controls were compared (figure 2), protocols using fluorosphere quantification consistently detected reduced numbers of EPCs in SSc. In contrast, protocols that did not use fluorospheres did not find any difference between the two groups.

DISCUSSION

This is the first study to directly compare protocols for quantifying EPCs. Our findings indicate that the EUSTAR recommendations are valid when combined with an accurate quantification technique such as the use of fluorospheres as an internal calibrator. In previous studies EPCs were quantified using a variety of strategies and were expressed as a proportion (%) in mononuclear cells[4 7 9] or the absolute number in peripheral blood[3 5 6] or in 10^6 Lin– cells,[8] but our results indicate that the quantification strategy strongly affects the consistency of the results. The inter-method concordance was very poor when CD34+ or Lin– cell counts, based on acquisitioned events by a flow cytometer, were used as a reference. This is probably because CD34+ or Lin– cells are rare cell populations that comprise less than 1% of circulating mononuclear cells. The purity of the enriched fractions varied greatly between samples even when an intensive gating protocol was used. Under these circumstances, introducing a fluorosphere technique significantly and substantially improved the reproducibility of the results. Thus, incorporating this critical issue into the EUSTAR recommendations should allow better comparison between studies.

In this study we observed a reduced number of EPCs in patients with SSc. However, the number of subjects was too small to draw a conclusion. To answer the central question whether EPCs are increased or reduced in patients with SSc, large-scale studies that conform to EUSTAR recommendations and use an accurate calibration protocol are needed. These studies should include patients with other connective tissue

diseases and should analyse potential correlations between EPC counts and clinical features of SSc.

The definition of EPCs is currently much debated.[12–14] Cells originally identified as EPCs in various assays are now known to be heterogeneous, and nearly all of the cells are members of the haematopoietic lineage and display pro-angiogenic properties. Non-haematopoietic circulating cells with a clonal proliferative potential and postnatal vasculogenic activity, termed endothelial colony-forming cells, are considered true EPCs, but these cells are detected only in long-term culture and are presumed to be very rare (<1 per 10^6 circulating mononuclear cells).[12] This makes it challenging to identify EPCs by flow cytometry. Nevertheless, CD34+CD133+VEGFR2+ cells have become widely used as a means of measuring putative circulating EPCs,[15] although a direct link between CD34+CD133+VEGFR2+ cells and the 'true EPCs' detected in culture is still missing. In fact, the majority of CD34+CD133+VEGFR2+ cells detected as EPCs in this study were apparently CD45+ haematopoietic progenitors. Because several different types of blood cells have been implicated as EPCs, further study is required to determine the exact role of each of these cell types in the pathogenic processes of SSc.

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Ethics approval This study was approved by the relevant ethical committees.

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

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Association of *UBE2L3* polymorphisms with diffuse cutaneous systemic sclerosis in a Japanese population

Susceptibility genes to systemic sclerosis (SSc) are substantially shared by other autoimmune diseases.[1] *UBE2L3*, encoding a ubiquitin-conjugating enzyme, was associated with systemic lupus erythematosus (SLE)[2 3] and rheumatoid arthritis (RA).[4] In this study, we examined whether *UBE2L3* is associated with SSc.

A case–control association study was performed on 391 Japanese patients and 1010 healthy controls recruited at Kanazawa University, the University of Tokyo, Institute of Rheumatology, Tokyo Women's Medical University and Sagamihara Hospital, National Hospital Organisation. All patients and controls were unrelated Japanese. All patients fulfilled the criteria proposed by the American College of Rheumatology[5] and were classified as having diffuse cutaneous (dc) or limited cutaneous (lc) SSc according to the classification by LeRoy *et al*.[6] One hundred and eighty-seven patients were classified as dcSSc and 201 as lcSSc. One hundred and fourteen patients were positive for antitopoisomerase I antibody (ATA) and 122 for anticentromere antibody (ACA). One hundred and seventy-seven patients were classified as having interstitial lung disease based on high resolution CT. This study was reviewed and approved by the ethics committees of each participating institute. Informed consent was provided by all subjects.

The whole *UBE2L3* gene is encompassed by one linkage disequilibrium block. Therefore, rs131654 and rs2298428, located upstream and downstream of *UBE2L3* and both associated with SLE in Chinese,[3] were genotyped using the TaqMan genotyping assay (Applied Biosystems, Foster City, California, USA). Association analyses were conducted by χ^2 test using 2×2 contingency tables. To correct for multiple testing, the significance level was set at $p<0.125$ to achieve a false discovery rate <0.05. This study had >80% detection power for the genotype with OR >1.55 (rs131654) and >1.76 (rs2298428) under the recessive model.

Although significant association was not detected in the whole SSc group, the rs131654T/T genotype was significantly increased in dcSSc and ATA positive subsets and the rs2298428T/T genotype was significantly increased in dcSSc, when compared with healthy controls (table 1). Comparison under the allele model showed only a marginal association of rs2298428T with ATA (p=0.017, OR 1.40, 95% CI 1.06 to 1.85). These single-nucleotide polymorphisms (SNPs) were in linkage disequilibrium (D′=0.94, r^2=0.44), and logistic regression analysis showed that the association was not attributable to either one of the SNPs (data not shown).

These results suggested that *UBE2L3* is a shared susceptibility gene for SLE, RA and dcSSc. To our knowledge, this is the first study to suggest the association of *UBE2L3* with SSc. Although the functional role of *UBE2L3* in autoimmune diseases remains unclear, it is notable that polymorphisms in other ubiquitin-related genes such as *TNFAIP3* and *TNIP1* have also been associated with SLE, RA and SSc.[7 8] These molecules are considered to play a role in NF-κB regulation. Of particular interest, *TNFAIP3* and *TNIP1* have also been shown to be more strongly associated with dcSSc or ATA positive subsets.[7 8] Thus, our findings suggest that *UBE2L3* may also contribute to the genetic background of SSc through ubiquitination modification, which might play a substantial role in the pathogenesis of dcSSc. At this point, our findings are preliminary, and will need independent replication in larger populations, both in the Japanese and Caucasians.

Table 1 Association of *UBE2L3* single nucleotide polymorphisms with systemic sclerosis in a Japanese population

Genotype frequency*					Risk allele frequency†	Recessive association‡ OR (95% CI)	p Value
rs131654	T/T	T/G	G/G	Total			
All SSc	118 (30.2)	174 (44.5)	99 (25.3)	391	52.4	1.28 (0.99 to 1.66)	0.061
dcSSc §	65 (34.8)	70 (37.4)	52 (27.8)	187	53.5	1.58 (1.13 to 2.20)	0.0069
lcSSc §	53 (26.4)	102 (50.8)	46 (22.9)	201	51.7	1.06 (0.75 to 1.50)	0.74
ATA+	43 (37.7)	42 (36.8)	29 (25.4)	114	56.1	1.79 (1.20 to 2.68)	0.0042
ACA+	35 (28.7)	66 (54.1)	21 (17.2)	122	55.7	1.19 (0.79 to 1.81)	0.41
ILD	56 (31.6)	77 (43.5)	44 (24.9)	177	53.4	1.37 (0.97 to 1.94)	0.075
Controls¶	255 (25.2)	517 (51.2)	238 (23.6)	1010	50.8	Referent	
rs2298428	T/T	T/C	C/C	Total			
All SSc	56 (14.3)	177 (45.3)	158 (40.4)	391	37.0	1.35 (0.96 to 1.91)	0.084
dcSSc§	33 (17.7)	78 (41.7)	76 (40.6)	187	38.5	1.74 (1.14 to 2.64)	0.010
lcSSc§	23 (11.4)	97 (48.3)	81 (40.3)	201	35.6	1.05 (0.65 to 1.69)	0.85
ATA+	20 (17.5)	56 (49.1)	38 (33.3)	114	42.1	1.72 (1.03 to 2.89)	0.039
ACA+	18 (14.8)	61 (50.0)	43 (35.3)	122	39.8	1.40 (0.82 to 2.40)	0.22
ILD	29 (16.4)	81 (45.8)	67 (37.9)	177	39.3	1.59 (1.02 to 2.47)	0.040
Controls¶	111 (11.0)	468 (46.3)	431 (42.7)	1010	34.2	Referent	

*The numbers of the subjects with each genotype and percentages are shown.
†T allele represents the risk allele for both single nucleotide polymorphisms. The frequencies are shown in percentages.
‡The OR, 95% CI and p values were calculated by χ^2-analysis using 2×2 contingency tables under the recessive model for the T allele. The significance level to achieve false discovery rate <0.05 was $p<0.0125$.
§Classification by skin involvement was unavailable in three patients.
¶Genotype frequencies in the controls were not deviated from Hardy-Weinberg equilibrium (p=0.44 in rs131654, p=0.34 in rs2298428).
ATA, antitopoisomearase I antibody; ACA, anticentromere antibody; dc, diffuse cutaneous; ILD, interstitial lung disease; lc, limited cutaneous; SSc, systemic sclerosis.

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Contributors NH and AK performed genotyping and statistical analysis, and wrote the manuscript. II and MK performed genotyping and statistical analysis. MH, MF, HF, ST, TS, KT, SS and YK recruited patients and collected clinical information. NT conceived and designed this study, performed statistical analysis and wrote the manuscript. All authors read and approved the manuscript.

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A Possible Contribution of Altered Cathepsin B Expression to the Development of Skin Sclerosis and Vasculopathy in Systemic Sclerosis

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Abstract

Cathepsin B (CTSB) is a proteolytic enzyme potentially modulating angiogenic processes and extracellular matrix remodeling. While matrix metalloproteinases are shown to be implicated in tissue fibrosis and vasculopathy associated with systemic sclerosis (SSc), the role of cathepsins in this disease has not been well studied. The aim of this study is to evaluate the roles of CTSB in SSc. Serum pro-CTSB levels were determined by enzyme-linked immunosorbent assay in 55 SSc patients and 19 normal controls. Since the deficiency of transcription factor Fli1 in endothelial cells is potentially associated with the development of SSc vasculopathy, cutaneous CTSB expression was evaluated by immunostaining in $Fli1^{+/-}$ and wild type mice as well as in SSc and control subjects. The effects of Fli1 gene silencing and transforming growth factor-β (TGF-β) on CTSB expression were determined by real-time PCR in human dermal microvascular endothelial cells (HDMECs) and dermal fibroblasts, respectively. Serum pro-CTSB levels were significantly higher in limited cutaneous SSc (lcSSc) and late-stage diffuse cutaneous SSc (dcSSc) patients than in healthy controls. In dcSSc, patients with increased serum pro-CTSB levels showed a significantly higher frequency of digital ulcers than those with normal levels. CTSB expression in dermal blood vessels was increased in $Fli1^{+/-}$ mice compared with wild type mice and in SSc patients compared with healthy controls. Consistently, Fli1 gene silencing increased CTSB expression in HDMECs. In cultured dermal fibroblasts from early dcSSc, CTSB expression was decreased compared with normal fibroblasts and significantly reversed by TGF-β1 antisense oligonucleotide. In conclusion, up-regulation of endothelial CTSB due to Fli1 deficiency may contribute to the development of SSc vasculopathy, especially digital ulcers, while reduced expression of CTSB in lesional dermal fibroblasts is likely to be associated with skin sclerosis in early dcSSc.

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Introduction

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 still remains unknown, an increasing number of growth factors, cytokines, and other molecules have been shown to be involved in the orchestrated complex network of signaling pathways driving aberrant immune activation, dysregulated angiogenesis, and deposition of extracellular matrix (ECM) throughout the course of this complex disorder [2,3].

Cathepsins are a family of proteases mostly consisting of papain-like cysteine proteases, which are mainly localized in endosomes and lysosomes [4]. However, cathepsins also function extracellularly and are involved in various biological processes, including ECM degradation, angiogenesis, and tumor invasion [4,5]. Some of the cathepsins (B, H, L, and C) are constitutively expressed in all cell types and tissues, whereas others are present in specific cell types (cathepsins S, V, X, O, K, F, and W) [5]. While matrix metalloproteinases (MMPs) are shown to be implicated in tissue fibrosis and vasculopathy associated with SSc, the role of cathepsins in this disease has not been well studied.

Among the member of cathepsin family, the roles of CTSB have been well studied in fibrosis and angiogenesis. In a murine model of liver fibrosis caused by CCl_4, CTSB expression increases in hepatic stellate cells and its inactivation mitigates CCl_4-induced inflammation, hepatic stellate cell activation, and collagen deposition [6]. Regarding angiogenesis, murine CTSB in vasculature is remarkably up-regulated during the degradation of vascular basement membrane associated with tumor angiogenesis [7]. In glioma cell lines, CTSB knockdown inhibits tumor-induced angiogenesis by modulating the expression of vascular endothelial growth factor (VEGF) [8]. In contrast to these observations, CTSB also has the capacity to suppress pro-angiogenic response, probably as a negative feedback control, by

increasing the generation of endostatin, an endogenous angiogenesis inhibitor derived from the breakdown of type XVIII collagen, while decreasing VEGF expression [9]. Importantly, serum endostatin levels are increased in SSc patients and associated with the presence of skin sclerosis, giant capillaries in nailfold capillaroscopy, cardiovascular changes, and pulmonary vascular involvement [10–14], suggesting that CTSB contributes to the pathological processes associated with fibrosis and vasculopathy at least partially via modulating endostatin production.

Based on these backgrounds, in order to clarify the role of CTSB in the development of SSc, we herein investigated the association of serum pro-CTSB levels with clinical features of SSc and also examined the possible mechanism responsible for the altered expression of CTSB in this disease.

Materials and Methods

Ethics Statement

The study protocol was reviewed and approved by the Ethical Committee of the Faculty of Medicine, University of Tokyo. Serum samples, skin tissue, and dermal fibroblasts were obtained from systemic sclerosis patients and healthy individuals after getting written informed consent. Human dermal microvascular endothelial cells (HDMECs) were purchased from Takara Bio (Shiga, Japan). All animal work was reviewed and approved by Animal Research Committee of the Faculty of Medicine, University of Tokyo.

Patients

Serum samples, frozen at −80°C until assayed, were obtained from 55 SSc patients (52 women and 3 men, including 27 diffuse cutaneous SSc [dcSSc] and 28 limited cutaneous SSc [lcSSc] according to LeRoy's classification [15]) and 19 healthy individuals (18 women, one man). Patients treated with corticosteroids or other immunosuppressants were excluded. All patients fulfilled the American College of Rheumatology criteria [16] except for 4 lcSSc patients who had sclerodactyly and at least two other features of CREST syndrome.

The measurement of serum pro-CTSB levels

Specific enzyme-linked immunosorbent assay kits were used to measure serum pro-CTSB levels (R & D Systems, Minneapolis, MN, USA) according to the manufacturer's instruction.

Clinical assessment

The clinical and laboratory data were obtained when the blood samples were drawn. Clinical symptoms were evaluated as described previously [17–20]. The details of assessments are briefly summarized in the legends of **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 CTSB antibody (R & D Systems) or anti-mouse CTSB antibody (Santa Cruz, Santa Cruz, CA, USA). Skin samples were obtained from forearms of 8 SSc patients and 8 closely matched healthy controls and from the back of 3-month-old mice.

Cell cultures

HDMECs and human dermal fibroblasts were prepared and maintained as described previously [21,22].

Table 1. Correlation of serum pro-cathepsin B levels with clinical features in patients with dcSSc and lcSSc.

dc/lc SSc	dcSSc		lcSSc	
Serum pro-CTSB levels	**Elevated**	**Normal**	**Elevated**	**Normal**
The number of patients	**n = 4**	**n = 23**	**n = 8**	**n = 20**
Age of onset (years old)	54.5±16.8	47.7±16.5	53.9±18.8	51.8±15.5
Disease duration (years)	6.3±3.9	2.7±2.8	9.4±10.0	12.2±13.1
Clinical features				
MRSS	9.8±9.1	11.7±7.5	3.0±1.7	4.8±5.1
Nailfold bleeding	50	71	63	68
Pitting scars	50	26	50	29
Digital ulcers	75*	9	0	29
Telangiectasia	25	47	60	50
Raynaud's phenomenon	75	86	88	88
Contracture of phalanges	25	57	40	54
Calcinosis	25	0	17	17
Organ involvement				
ILD	75	65	13	25
Decreased %DLco	25	30	50	28
Decreased %VC	0	19	13	15
Elevated RVSP	50	17	29	32
Esophagus	25	60	38	51
Heart	25	0	0	2
Kidney	25	4	25	7
Muscle	25	5	17	6

Unless noted otherwise, values are percentages. dcSSc, diffuse cutaneous systemic sclerosis; lcSSc, limited cutaneous systemic sclerosis; MRSS, modified Rodnan total skin thickness score; DLco, diffuse capacity for carbon monoxide; VC, vital capacity; RVSP; right ventricular systolic pressure. Patients were evaluated for the presence of esophageal, pulmonary, cardiac, renal, or muscle involvements, as follows. Esophagus hypomotility was defined as distal esophageal hypomotility on barium-contrast radiography. Interstitial lung disease (ILD) was defined as bibasilar interstitial fibrosis on chest radiographs, and in patients with no abnormalities on chest radiographs early ILD was defined as alveolitis on high-resolution computer tomography. Elevated right ventricular systolic pressure (RVSP) was defined as 35 mmHg or more on echocardiogram. Cardiac involvement was defined as any of the following: symptomatic pericarditis, clinical evidence of left ventricular congestive heart failure, or arrhythmias requiring treatment. Scleroderma renal crisis was defined as malignant hypertension and/or rapidly progressive renal failure. Skeletal muscle involvement was defined as proximal muscle weakness and elevated serum creatine kinase levels, plus abnormal electromyographic findings consistent with myopathy and/or histopathologic changes in inflammatory myopathy. Disease onset was defined as the first clinical event of SSc other than Raynaud's phenomenon. Disease duration was defined as the interval between the onset and the time the blood samples were drawn. Statistical analysis was carried out with Fisher's exact probability test.
*$P<0.05$.
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Gene silencing of Fli1 and the treatment with TGF-β1 or TGF-β1 antisense oligonucleotide

These experiments were performed as described previously [21,23]. The details of each experiment are described in figure legends.

RNA isolation and quantitative real-time PCR

RNA isolation and quantitative real-time PCR were carried out as described previously [22]. The sequences of CTSB [24], Fli1 [25] and 18S rRNA [26] primers were previously reported.

Fli1 heterozygous mice

Fli1 heterozygous mice with C57BL/6J background were provided from Prof. Maria Trojanowska (Boston University School of Medicine, Arthritis Center, Boston, MA, USA) [27].

Statistical analysis

The statistical analysis carried out in each experiment is described in figure legends or "Results". Statistical significance was defined as a P value of <0.05.

Results

Serum pro-CTSB levels were significantly increased in SSc patients compared to healthy controls

Serum pro-CTSB levels in SSc patients were significantly higher than those in healthy individuals (62.2±30.7 versus 44.4±18.7 ng/ml; $P<0.05$). Since the expression profiles of certain growth factors and cytokines can be quite different between dcSScand lcSSc, we also evaluated serum pro-CTSB levels in these subgroups. As shown in **Fig. 1**, serum pro-CTSB levels were significantly higher in lcSSc patients (66.2±32.3 ng/ml) than in healthy controls ($P<0.05$), while there was a trend toward the elevation in dcSSc patients (58.1±28.9 ng/ml) compared with healthy controls that did not reach significance. Collectively, the increase in serum pro-CTSB levels may be associated with some aspects of disease process in SSc.

Clinical association of serum pro-CTSB levels in lcSSc

Since lcSSc patients showed significantly higher serum pro-CTSB levels compared to healthy controls, we next classified lcSSc patients into two groups based on the cut-off value (81.8 ng/ml, normal mean+2SD), such as lcSSc patients with increased serum pro-CTSB levels and those with normal levels, and assessed the correlation of serum pro-CTSB levels with clinical features (right columns in **Table 1**). However, we failed to detect the correlation of serum pro-CTSB levels with any clinical features, suggesting that the increase in CTSB is not associated with any specific pathological process leading to each clinical feature in lcSSc.

Serum pro-CTSB levels were significantly increased in late-stage dcSSc patients compared to early-stage dcSSc patients or healthy controls

We next focused on dcSSc patients because serum pro-CTSB levels tended to be increased in this subgroup compared with healthy controls. Since dcSSc is characterized by progressive skin sclerosis and ILD, we evaluated the association of serum pro-CTSB levels with parameters reflecting the degree of fibrosis in skin and lung, such as modified Rodnan total skin thickness score (MRSS), %VC, and %DLco. Despite its pro-fibrotic effect, none of these three parameters correlated with serum pro-CTSB levels in dcSSc ($r=0.08$, 0.009, and 0.03, respectively). According to previous reports, the expression profile of proteolytic enzymes can be altered along with the disease duration in dcSSc. For example, although the mRNA levels of MMP1 gene in SSc dermal fibroblasts from patients with disease duration of <1 year are significantly higher than those in normal dermal fibroblasts, SSc dermal fibroblasts from patients with disease duration of 2–4 years show low mRNA levels of MMP1 gene compared with normal dermal fibroblasts. Furthermore, the mRNA levels of MMP1 gene in SSc dermal fibroblasts from patients with disease duration of more than 6 years were comparable to those in normal dermal fibroblasts [28]. Therefore, we classified dcSSc patients into 3

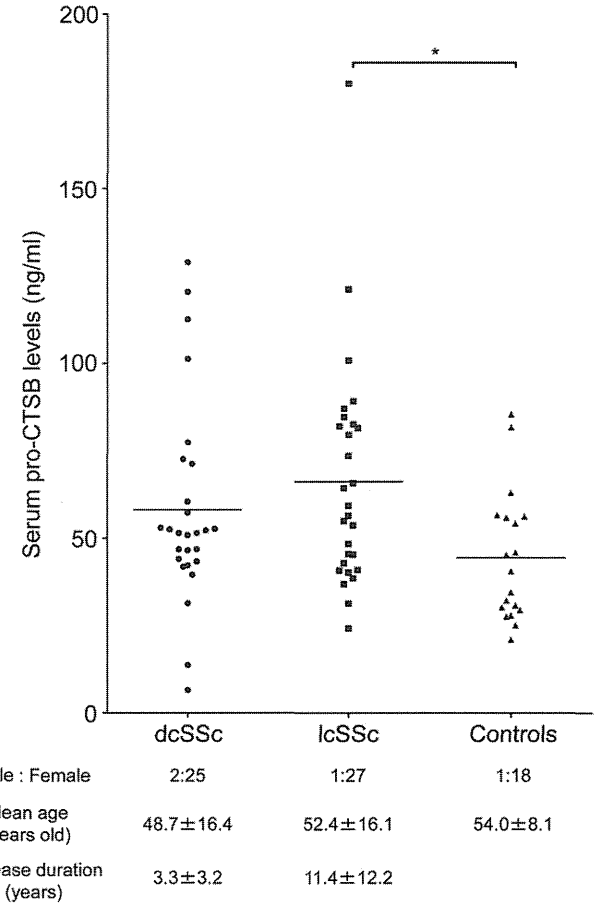

Figure 1. Serum pro-CTSB levels in patients with dcSSc, lcSSc, and healthy individuals. Serum pro-CTSB levels were determined by a specific ELISA. Bars indicate the mean value in each group. Statistical analysis was carried out with a Kruskal-Wallis test and a Steel-Dwass' test for multiple comparison. *$P<0.05$.
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subgroups based on disease duration, such as early-stage dcSSc (disease duration of <1 year), mid-stage dcSSc (disease duration of 1 to 6 years), and late-stage dcSSc (disease duration of >6 years), and evaluated the correlation of serum pro-CTSB levels with disease duration. As shown in **Fig. 2**, serum pro-CTSB levels were increased in late-stage dcSSc patients (86.4±33.6 ng/ml) compared with early-stage dcSSc patients (35.7±21.3 ng/ml) and healthy individuals ($P<0.05$ for each), while there was no significant difference between early-stage dcSSc or mid-stage dcSSc patients (57.7±23.1 ng/ml) and healthy individuals. Consistently, there was a strong positive correlation between serum pro-CTSB levels and disease duration in dcSSc patients ($r=0.50$, $P<0.01$, Spearman's rank correlation coefficient). Thus, serum pro-CTSB levels gradually increased along with disease duration in dcSSc, suggesting that CTSB may be linked to certain clinical features which develop or get worse in the late stage of dcSSc. Alternatively, downregulation of CTSB in early dcSSc compared with late-stage dcSSc or lcSSc may reflect the extensively activated fibrotic response in early dcSSc.

Elevated serum pro-CTSB levels were associated with the development of digital ulcers in dcSSc

To further investigate the association of serum pro-CTSB levels with clinical manifestations in dcSSc other than skin fibrosis and

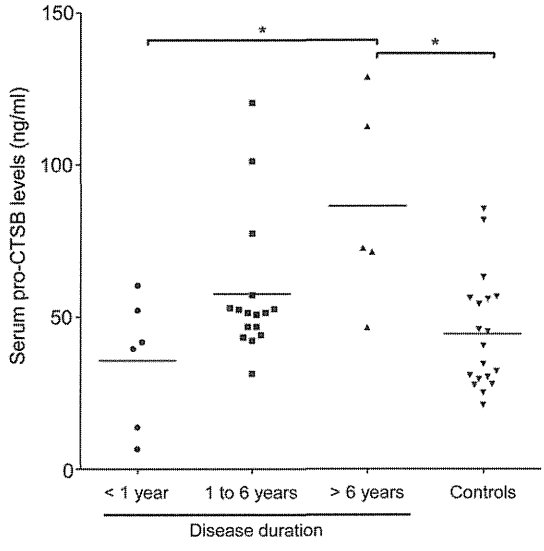

Figure 2. Serum pro-CTSB levels in dcSSc patients further classified into subgroups based on disease duration. dcSSc patients were divided into 3 subgroups: those with disease duration of <1 years, those with disease duration of 1 to 6 years, and those with disease duration of >6 years. Serum pro-CTSB levels were determined by a specific ELISA. The horizontal bars indicate the mean value in each group. Statistical analysis was carried out with a Kruskal-Wallis test and a Steel-Dwass' test for multiple comparison. *P<0.05.
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Table 2. Cathepsin B levels in dermal vasculature in normal and systemic sclerosis skin.

Samples	Age/sex	Duration (years)	dcSSc/lcSSc	Signal intensity
NS1	65F			−
SSc1	61F	1	dcSSc	+
NS2	63F			+
SSc2	64F	0.5	dcSSc	+++
NS3	40F			+
SSc3	43F	2.6	dcSSc	+++
NS4	55F			−
SSc4	56F	1.5	dcSSc	++
NS5	64M			++
SSc5	59M	0.5	dcSSc	++
NS6	56F			+
SSc6	52F	1	lcSSc	++
NS7	55F			+
SSc7	51F	0.2	lcSSc	++
NS8	59F			+
SSc8	58F	1	lcSSc	++

NS, normal skin; SSc, systemic sclerosis; dcSSc, diffuse cutaneous systemic sclerosis; lcSSc, limited cutaneous systemic sclerosis. We used the following grading system: −, no staining; +, slight staining; ++, moderate staining; +++, strong staining.
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ILD, we classified dcSSc patients into 2 groups according to the cut-off value and analyzed (the left column in Table 1). There was no significant difference between these two groups in terms of sex, age, and disease duration. The frequency of digital ulcers was significantly higher in patients with increased serum pro-CTSB levels than in those with normal levels (75% versus 8.7%, $P = 0.013$). Although digital ulcers in dcSSc patients are closely related to macrovascular involvements resulting from proliferative vasculopathy [29], there was no significance difference in the prevalence of elevated right ventricular systolic pressure (RVSP) and scleroderma renal crisis, which are also caused by proliferative vasculopathy [30,31], between these two groups [32]. Regarding other clinical features, we failed to detect any correlation with serum pro-CTSB levels. Collectively, these results suggest that elevation of CTSB contributes to the pathological process associated with digital ulcers in dcSSc.

Comparison of the CTSB expression in skin sections derived from SSc patients and healthy controls

As described above, CTSB is potentially associated with the disease process of SSc, especially fibrosis and vasculopathy. To further confirm this notion, immunohistochemistry was carried out using skin samples from 5 dcSSc and 3 lcSSc patients and 8 healthy controls. Clinical information and the results were summarized in Table 2. In normal skin sections, CTSB staining was especially strong in small blood vessels consisting of endothelial cells (ECs) and pericytes/vascular smooth muscle cells compared to other cell types (**Fig. 3A and 3B**). Although a similar predominant distribution of CTSB in blood vessels was observed in SSc skin sections, the signals were much stronger than those in normal skin sections (**Fig. 3C and 3D**). Importantly, there was no remarkable difference in CTSB signals between dcSSc and lcSSc. These results suggest that CTSB is up-regulated in dermal blood vessels in SSc and plays some roles in the developmental process of SSc vasculopathy.

CTSB was down-regulated in SSc dermal fibroblasts due to the constitutive activation of TGF-β signaling

Although the CTSB expression in blood vessels was similar between dcSSc and lcSSc, serum pro-CTSB levels were decreased in early dcSSc compared with late-stage dcSSc and lcSSc. Given that lesional dermal fibroblasts are extensively activated in early dcSSc, the dynamics of serum pro-CTSB levels along with disease duration in dcSSc may be linked to the activation status of SSc dermal fibroblasts. Since CTSB signals in dermal fibroblasts were below the detectable levels in immunohistochemistry (**Fig. 3**), we next investigated the mRNA levels of CTSB gene in cultured normal and SSc dermal fibroblasts. As shown in **Fig. 4A**, SSc fibroblasts expressed significantly lower mRNA levels of CTSB gene than normal fibroblasts. Since SSc dermal fibroblasts are constitutively activated by the stimulation of autocrine TGF-β [21,33–35], we asked if CTSB down-regulation depends on autocrine TGF-β stimulation in SSc fibroblasts. To this end, we employed TGF-β1 antisense oligonucleotide, which effectively blocks endogenous TGF-β production in dermal fibroblasts [33–35]. As expected, TGF-β1 antisense oligonucleotide, but not TGF-β1 sense oligonucleotide, significantly increased the mRNA levels of CTSB gene in SSc fibroblasts (**Fig. 4B**). Furthermore, TGF-β1 stimulation significantly suppressed the mRNA expression of CTSB gene in normal fibroblasts (**Fig. 4C**). Collectively, CTSB expression is decreased in lesional dermal fibroblasts of early dcSSc as a result of autocrine TGF-β stimulation. Given the implication of TGF-β in the pathogenesis of early dcSSc, but not late-stage dcSSc [36], CTSB produced by dermal fibroblasts may affect serum pro-CTSB levels in dcSSc throughout the disease course.