Figure 3. CTSB expression was up-regulated in dermal vasculatures of SSc patients compared to those in controls. CTSB expression levels in dermal vasculatures were determined by immunohistochemistry in skin section from 8 healthy control subjects (A, B) and 8 SSc patients (C, D). Representative results are shown. Original magnification was ×200 (A, C) and ×400 (B, D). Analysis of CTSB expression levels in vessel walls is included in Table 2.

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Endothelial Fli1 deficiency is associated with the up-regulation of CTSB in dermal blood vessels in animal models

Finally, we further sought the mechanism by which CTSB is up-regulated in SSc dermal blood vessels. We previously demonstrated that the deficiency of transcription factor Fli1 in endothelial cells is potentially associated with the development of SSc vasculopathy [22]. Therefore, we carried out immunostaining for CTSB using skin sections from Fli1$^{+/-}$ mice and wild type mice. As expected, CTSB expression was much higher in blood vessels of Fli1$^{+/-}$ mice than in those of wild type mice (**Fig. 5A and 5B**), suggesting that Fli1 regulates the expression of CTSB in ECs. To further confirm this notion *in vitro*, we looked at the effect of Fli1 gene silencing on the mRNA levels of CTSB gene in HDMECs. As shown in **Fig. 5C**, ~50% knockdown of Fli1 resulted in the significant increase of CTSB mRNA levels (34% increase, P<0.05). Collectively, these results indicate that Fli1 deficiency is at least partially involved in the mechanism of CTSB up-regulation in SSc vasculature.

Discussion

This study was undertaken to clarify the contribution of CTSB, a proteolytic enzyme related to fibrosis and angiogenesis, in the pathogenesis of SSc. As an initial step to address this issue, we investigated the serum pro-CTSB levels and their association with clinical features in SSc. As expected, serum pro-CTSB levels were significantly increased in lcSSc and tended to be elevated in dcSSc compared to healthy controls. In lcSSc, we failed to detect any

correlation of serum CTSB levels with clinical features, suggesting that CTSB elevation is associated with the development of lcSSc, but not with any specific clinical features. In contrast, in dcSSc, there was a strong positive correlation between serum pro-CTSB levels and disease duration (r = 0.50, P<0.01). Importantly, serum pro-CTSB levels were significantly elevated in late-stage dcSSc compared with healthy controls and dcSSc patients with elevated pro-CTSB levels had a significantly higher prevalence of digital ulcers than those with normal levels. Given that digital ulcers in dcSSc are closely associated with macrovascular involvements caused by proliferative vasculopathy [32], we also evaluated the association of serum pro-CTSB levels with other vascular symptoms associated with proliferative vasculopathy, such as elevated RVSP and scleroderma renal crisis, but did not see any correlation. Collectively, these results suggest that elevation of CTSB contributes to the pathological process leading to SSc vasculopathy, especially to digital ulcers in dcSSc.

Although the pathogenesis of SSc vasculopathy still remains unknown, we recently demonstrated that endothelial Fli1 deficiency is potentially associated with the development of vascular changes characteristic for SSc [22,37]. Endothelial cell-specific Fli1 knockout (Fli1 ECKO) mice reproduce the pathological and morphological features of SSc vasculopathy, such as stenosis of arterioles, dilation capillaries, and increased vascular permeability. Gene silencing of Fli1 in HDMECs results in the down-regulation of molecules regulating EC-EC interaction, including PECAM-1 and VE-Cadherin, and those regulating EC-pericyte interaction, including VE-Cadherin, S1P$_1$, and platelet-derived growth factor-B, and in the up-regulation of
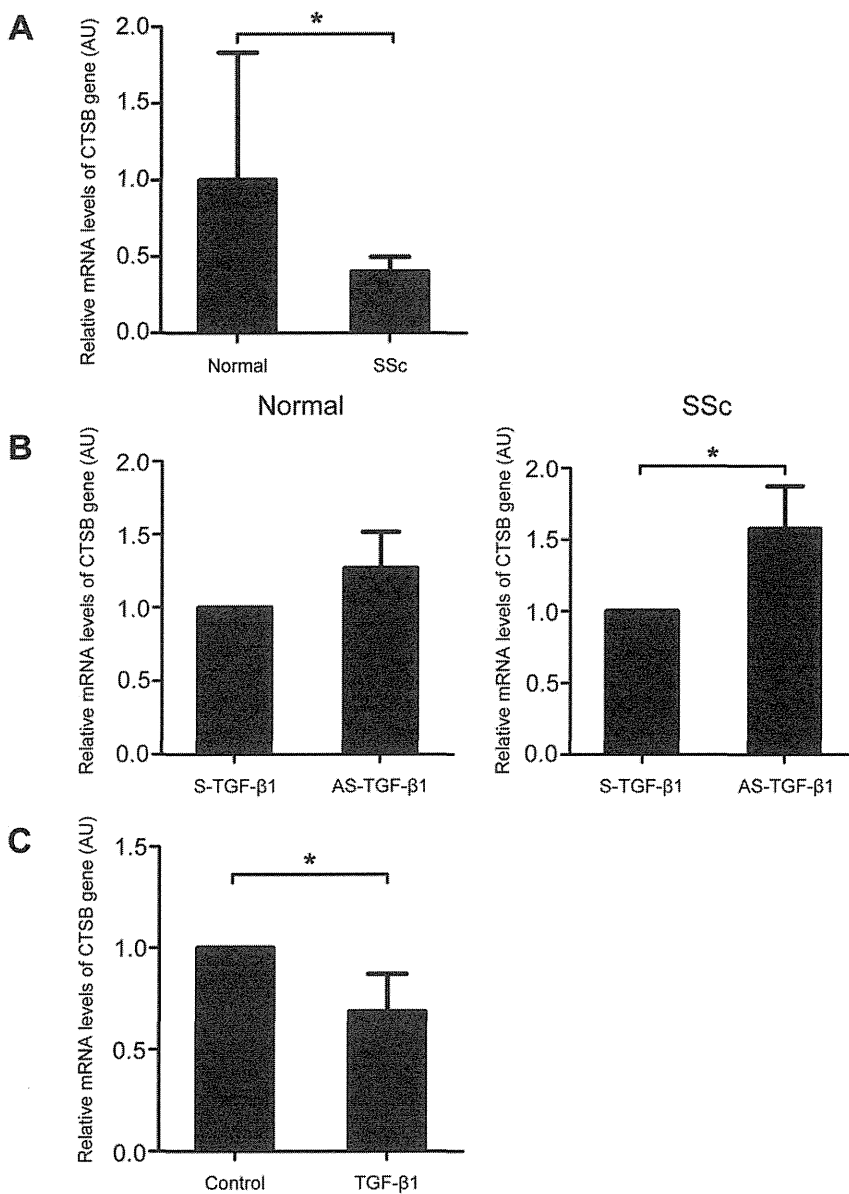
Figure 4. CTSB mRNA expression in SSc dermal fibroblasts was down-regulated due to constitutively activated TGF-β signaling. mRNA levels of CTSB gene were determined by real-time PCR in confluent quiescent dermal fibroblasts from 9 SSc patients and 5 healthy controls (A), in confluent quiescent dermal fibroblasts from 4 SSc patients and 4 healthy controls treated with a TGF-β1 antisense oligonucleotide (GAGGGCGGCATGGGGAGG; AS-TGF-β1), which overlaps the promoter and transcriptional start site of the TGF-β1 gene, or a TGF-β1 sense oligonucleotide (S-TGF-β1) as a control for 48 hours (B), and in normal dermal fibroblasts stimulated with recombinant human TGF-β1 (PeproTech, Rocky Hill, NJ, USA) at 10 ng/ml for 24 hours (C). In real-time PCR, the mRNA levels of target genes were normalized to the levels of human 18S rRNA gene. Results of controls or relative value compared with the controls are expressed as means ± SD. Statistical analysis was carried out with a 2-tailed unpaired (A) or paired (B, C) t-test. *P<0.05.
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MMP9 promoting the degradation of vascular basement membrane (vBM). Furthermore, Fli1 deficiency promotes endothelial proliferation and survival [38], probably linked to the development of arteriolar stenosis, which is similar to proliferative vasculopathy in SSc, in Fli1 ECKO mice. As shown in the present study, Fli1 gene silencing up-regulated the expression of CTSB in HDMECs and Fli1^{+/−} mice exhibited high expression levels of CTSB in dermal vasculature. These results indicate that the elevation of endothelial CTSB expression is included in the gene program triggered by Fli1 deficiency in SSc. Given that CTSB is proteolytic enzyme expressed at high levels in vasculature during vBM

degradation associated with tumor angiogenesis [7], CTSB promotes the degradation of vBM together with other proteolytic enzyme such as MMP9 in SSc [39]. Consistent with this notion, we and others demonstrated that components of dermal vBM are altered in SSc [22,40,41]. Thus, proteolytic activity of CTSB may be associated with the mechanism responsible for vascular fragility in SSc.

In addition to vBM degradation, CTSB modulates angiogenesis via the generation of endostatin from type XVIII collagen and the suppression of endothelial VEGF production [9,42]. Given that Fli1 downregulation activates angiogenic process,
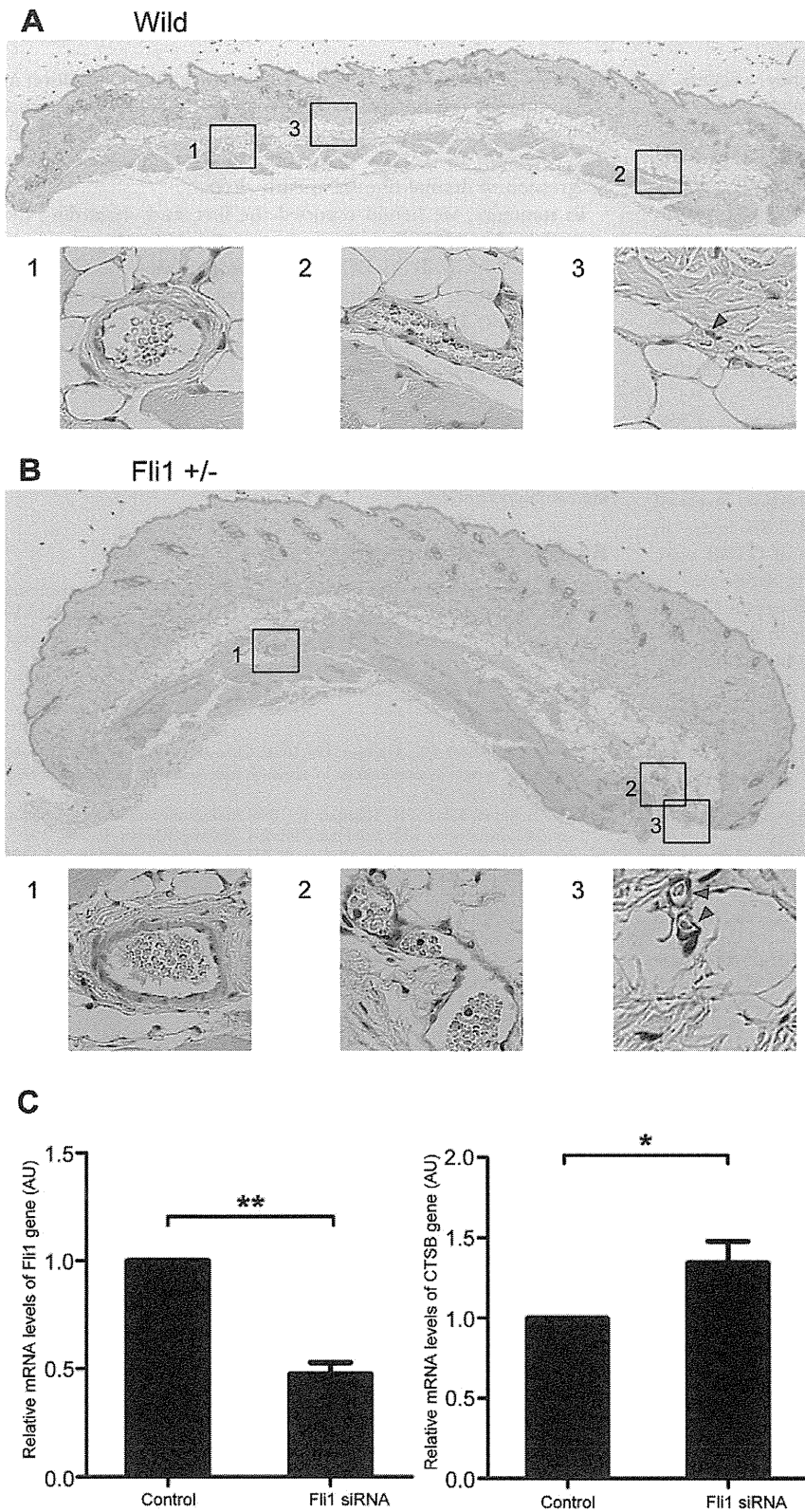
A   Wild

B   Fli1 +/-

C

Figure 5. CTSB expression was up-regulated in dermal vasculature of Fli1$^{+/-}$ mice and in Fli1 siRNA-treated HDMECs.
Immunodetection of CTSB proteins in the skin sections of 3 month-old wild type (A) and Fli1$^{+/-}$ (B) mice (original magnification was ×40) by Vectastain ABC kit according to the manufacturer's instruction. Insets (original magnification was ×40) depict representative arterioles (panel 1), venules (panel 2), and capillaries (panel 3; red arrowheads), respectively. Representative results in 5 wild type and 5 Fli1$^{+/-}$ mice are shown. (C) HDMECs were seeded shortly before transfection. The cells were transfected with 10 nM of Fli1 and scrambled non-silencing siRNA (Santa Cruz) using HiPerfect transfection reagent (Qiagen, Valencia, CA, USA) for 72 hours. Cells were then serum starved for the last 24 hours. mRNA levels of Fli1 and CTSB genes were examined by quantitative 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 ± SD of 3 independent experiments. Statistical analysis was carried out with a 2-tailed paired t-test. *P<0.05, **P<0.005.
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CTSB up-regulation followed by Fli1 deficiency triggers a negative feedback control of angiogenesis through endostatin generation and VEGF suppression. Angiogenesis is a dynamic process regulated by various pro-angiogenic and angiostatic cytokines and growth factors, which are normally tightly regulated both spatially and temporally. Therefore, the constitutive up-regulation of CTSB in SSc vasculature itself means impaired vascular homeostasis in this disease. Although the role of CTSB in SSc vasculopathy still remains unknown, the high prevalence of digital ulcers in dcSSc patients with elevated serum pro-CTSB levels implies the significant role of CTSB in the pathological vascular changes resulting in digital ulcers. Further studies are currently on going in our laboratory.

Although serum pro-CTSB levels were decreased in early dcSSc compared with lcSSc, CTSB signals in vasculature were comparable between early dcSSc and lcSSc. A plausible explanation for this observation is that the expression levels of CTSB in dermal fibroblasts affect serum pro-CTSB levels in dcSSc. Consistent with our hypothesis, the levels of CTSB were decreased in dermal fibroblasts derived from early dcSSc due to autocrine TGF-β stimulation. Given that TGF-β appears to be sequestered in lesional skin of early dcSSc, but not in that of late-stage dcSSc and lcSSc, and stimulate dermal fibroblasts [36], the

dynamics of serum pro-CTSB levels along with disease duration in dcSSc may be attributable to CTSB levels from lesional dermal fibroblasts. Given that a prominent proteolytic activity of CTSB, the decrease in CTSB expression may contribute to the progression of dermal fibrosis in early dcSSc.

In summary, we herein reported the first study regarding the potential role of CTSB in the pathogenesis of SSc. Up-regulated expression of CTSB in ECs and downregulation of CTSB in dermal fibroblasts may contribute to pathological angiogenesis and fibrosis in SSc. This study provides a new idea that the members of cathepsin family as well as MMPs have pivotal roles in the pathogenesis of SSc.

Acknowledgments

Author Contributions

Conceived and designed the experiments: YA. Performed the experiments: SN YA. Analyzed the data: SN YA KA NA T. Taniguchi T. Takahashi YI T. Toyama HS KY. Wrote the paper: SN YA YT MS TK. Final approval: SS.

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Augmented production of soluble CD93 in patients with systemic sclerosis and clinical association with severity of skin sclerosis

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Summary

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Background The cell surface protein CD93, expressed on endothelial and myeloid cells, mediates phagocytosis, inflammation and cell adhesion. A soluble form of CD93 (sCD93) is released during inflammation.

Objectives To determine the serum sCD93 level and its association with clinical parameters in patients with systemic sclerosis (SSc).

Methods Serum sCD93 levels were examined by enzyme-linked immunosorbent assay in 59 patients with SSc, 24 patients with systemic lupus erythematosus and 47 healthy individuals. The expression of CD93 in skin tissues was examined immunohistochemically. In a retrospective longitudinal study, sera from 11 patients with SSc were analysed.

Results Serum sCD93 levels were increased in patients with SSc compared with healthy individuals (P < 0·001). Patients with diffuse cutaneous SSc showed greater levels of sCD93 than those with limited cutaneous SSc (P < 0·01) or systemic lupus erythematosus (P < 0·01). Serum sCD93 levels correlated positively with the severity of skin sclerosis. Strong CD93 immunostaining was observed on endothelial cells in lesional skin tissues. In the longitudinal study, sCD93 levels decreased in parallel with improvement in skin sclerosis.

Conclusions Serum sCD93 levels are increased in patients with SSc and correlate with the severity and activity of skin sclerosis. CD93 may contribute to the development of skin fibrosis in SSc.

Systemic sclerosis (SSc) is a heterogeneous disorder characterized by excessive fibrosis and microvascular damage of the skin and various internal organs. Although the pathogenesis of SSc remains unclear, various immunological abnormalities have been detected.[1] Autoantibodies are found in more than 90% of patients, and these autoantibodies react to various intracellular components, such as DNA topoisomerase I, centromeres and RNA polymerases.[2] Increasing evidence suggests that cytokines or growth factors regulate SSc induction by stimulating the synthesis of extracellular matrix components, which may injure endothelial cells and modulate leucocyte function.[1] In early SSc skin lesions, mononuclear cell infiltration is first seen around small vessels in the dermis.[3,4] The degree of mononuclear cell infiltration correlates with both the degree and progression of skin thickening.[5] Thus, these cells are potential candidates for releasing cytokines, chemokines or growth factors, which play a crucial part in the initiation and development of fibrosis in SSc.

CD93 is a transmembrane glycoprotein that is expressed on monocytes, neutrophils, platelets and endothelial cells, but not in tissue macrophages.[6–8] CD93 was originally characterized as one of several supposed complement protein 1q receptors and was designated C1qRp.[9,10] Although CD93-deficient mice show no severe developmental abnormalities, these animals do exhibit impaired phagocytic removal of apoptotic cells,[9] demonstrating a critical role of CD93 in the clearance of dying cells. A soluble form of CD93 (sCD93) was recently identified in human plasma.[11] sCD93 production is enhanced in inflammation,[12] and sCD93 concentration is increased in the synovial fluid of patients with rheumatoid arthritis.[13] We hypothesized that sCD93 may play a role in the pathogenesis of SSc. In the present study, we examined the serum sCD93 level in patients with SSc and evaluated the results with respect to clinical features. In addition, we undertook a retrospective longitudinal study of sCD93 levels in some of these patients to determine changes in sCD93 levels over time.

Patients and methods

Patients

The study protocol was approved by the University of Tokyo and University of Tokyo Hospital (Tokyo, Japan), and informed consent was obtained from all patients. Serum samples were obtained from 59 Japanese patients with SSc [53 women and six men; 10–77 years of age (mean, 53 years)]. All patients fulfilled the criteria for SSc proposed by the American College of Rheumatology.[14] Patients were grouped according to the classification system proposed by LeRoy et al.[15] Twenty-nine patients had limited cutaneous SSc (lcSSc) and 30 had diffuse cutaneous SSc (dcSSc). Antitopoisomerase I antibodies (Abs) were present in 23 patients, anticentromere Abs in 25, anti-RNA polymerases I and III Abs in six, anti-U1RNP Abs in two, and anti-U3RNP Abs in two, and one patient was negative for these Abs. The mean ± SD disease duration was 3·5 ± 4·4 (range, 0·2–23) years. Duration was calculated from the time of onset of the first clinical event (other than the Raynaud phenomenon) that was a clear manifestation of SSc. None of the patients had received corticosteroids or other immunosuppressants. Twenty-four patients with systemic lupus erythematosus (SLE) who fulfilled the American College of Rheumatology criteria[16] acted as disease controls. Forty-seven age- and sex-matched healthy Japanese individuals were included in the study as healthy controls.

In a retrospective longitudinal study, we analysed serum samples from 11 of the 59 patients with SSc who could be followed longitudinally (all women; six with dcSSc and five with lcSSc). Two samples from each patient were obtained at the initial visit and the last visit. These patients were aged 26–73 (mean, 55) years. The mean ± SD follow-up period was 2·1 ± 0·9 (range, 0·5–3·5) years with two time points. The mean ± SD disease duration was 4·6 ± 7·1 (range, 0·2–23) years. Antitopoisomerase I Abs, anticentromere Abs, anti-RNA polymerases I and III Abs and anti-U3RNP Abs were present in three, five, two and one of these patients, respectively.

Fresh venous blood samples were centrifuged shortly after clot formation. All samples were stored at −70 °C until use.

Clinical assessment

Complete medical histories, physical examinations and laboratory tests were conducted for all patients during their first visit. Organ system involvement was defined as described;[17,18] lung: bibasilar fibrosis on chest radiography and high-resolution computed tomography; oesophagus: hypomotility shown by barium radiography; heart: pericarditis, congestive heart failure or arrhythmias requiring treatment; kidney: malignant hypertension and rapidly progressive renal failure with no other explanation; joint: inflammatory polyarthralgias or arthritis; and muscle: proximal muscle weakness and elevated serum creatine kinase levels. Pulmonary fibrosis was defined as bibasilar interstitial fibrosis on chest high-resolution computed tomography. In addition, a pulmonary function test, including vital capacity

(VC) and diffusion capacity for carbon monoxide (DLco), was evaluated to examine the severity of pulmonary fibrosis. When the DLco and VC were < 70% and < 80% of predicted normal values, respectively, they were considered abnormal.

Patients with SSc who were smokers or who had other respiratory disorders that might have affected %DLco or %VC were excluded from this study. Pulmonary artery pressure was estimated by Doppler echocardiogram. The modified Rodnan total skin score (TSS) was measured by summing skin thickness measurements determined by palpation on a scale of 0–3 in 17 body areas.[19]

Measurement of serum sCD93

Fresh venous blood samples were drawn into pyrogen-free blood collection tubes without additives, immediately immersed in melting ice and allowed to clot for 1 h before centrifugation. All serum samples were stored at −70 °C until use. The serum sCD93 level was measured with a specific enzyme-linked immunosorbent assay kit (eBioscience, San Diego, CA, U.S.A.), according to the manufacturer's protocol. Each sample was tested in duplicate. The detection limit of this assay was 3·3 ng mL^{-1}.

Immunohistochemistry

Skin biopsy samples were taken from the dorsal aspect of the mid-forearm of eight female patients with dcSSc and eight healthy female volunteers. CD93 immunostaining in skin tissue was determined with a rabbit antihuman CD93 polyclonal Ab (Sigma-Aldrich, St Louis, MO, U.S.A.). Rabbit IgG (Sigma-Aldrich) was used as a control for nonspecific CD93 staining.

Statistical analysis

Data are presented as mean ± SD values. The Kruskal–Wallis test was used to compare sCD93 levels between groups, Fisher's exact probability test was used to compare frequencies, and Bonferroni's test was used for multiple comparisons. Spearman's rank correlation coefficient was used to examine the relationship between two continuous variables. A probability $P < 0.05$ was considered significant.

Results

Serum sCD93 level in patients with systemic sclerosis

The mean serum sCD93 level at first visit was significantly greater in patients with SSc (394·1 ± 228·0 ng mL^{-1}) than in healthy individuals (224·0 ± 107·4 ng mL^{-1}; $P < 0.001$) (Fig. 1). Similarly, the mean serum sCD93 level was significantly greater in patients with SLE (348·8 ± 162·2 ng mL^{-1}) than in healthy individuals ($P < 0.01$). With respect to the SSc subgroups, sCD93 levels in patients with dcSSc (475·8 ± 242·4 ng mL^{-1}) and in those with lcSSc (307·8 ± 172·2 ng mL^{-1}) were significantly increased compared with those in
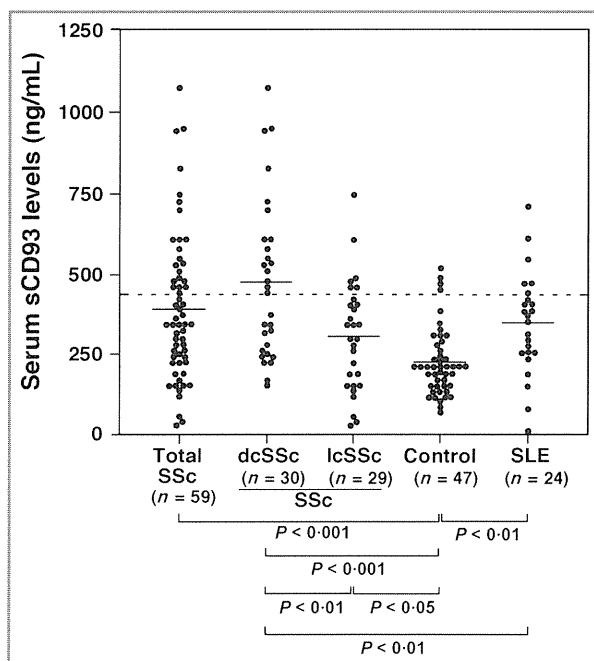
Fig 1. Serum soluble CD93 (sCD93) level in patients with systemic sclerosis (SSc), diffuse cutaneous SSc (dcSSc), limited cutaneous SSc (lcSSc) or systemic lupus erythematosus (SLE) and in healthy individuals (control). The serum sCD93 level was determined by a specific enzyme-linked immunosorbent assay. Horizontal lines indicate the mean value in each group. The dashed line indicates cut-off value (mean + 2 SD of the values from healthy individuals).

healthy individuals (P < 0·001 and P < 0·05, respectively). In addition, the serum sCD93 level was significantly increased in patients with dcSSc compared with those with lcSSc (P < 0·01) or SLE (P < 0·01).

Clinical correlation with serum sCD93 level

Clinical and laboratory parameters obtained at the first evaluation were compared between patients with SSc and with increased sCD93 levels and those with normal sCD93 levels. Values greater than the mean + 2 SD (438·8 ng mL^{-1}) of control serum samples were considered increased in this study. An increased sCD93 level was observed in 37% (22/59) of all patients with SSc, 53% (16/30) of patients with dcSSc and 21% (6/29) of patients with lcSSc. Patients with SSc and increased sCD93 levels more frequently had dcSSc than those with normal sCD93 levels (73% vs. 38%; P < 0·01) (Table 1). Consistent with the association of higher sCD93 levels with dcSSc, patients with SSc and increased sCD93 levels showed significantly greater modified Rodnan TSS values than those with normal sCD93 levels (15·0 ± 9·6 vs. 9·6 ± 8·8; P < 0·05). Furthermore, sCD93 levels correlated positively with modified Rodnan TSS value (P < 0·01, r = 0·35) (Fig. 2a). Thus, sCD93 levels correlated with the severity of skin sclerosis in SSc.

Patients with SSc and elevated serum sCD93 levels had shorter disease durations than those with normal sCD93 levels

Table 1 Clinical and laboratory findings in patients with systemic sclerosis (SSc) showing an elevated serum soluble CD93 (sCD93) level on initial visit

	Elevated sCD93 n = 22	Normal sCD93 n = 37
Age at onset (years), mean ± SD	51 ± 20	53 ± 14
Male:Female	3:19	3:34
Duration (years), mean ± SD	2·4 ± 2·7*	4·2 ± 5·1
TSS, mean ± SD	15·0 ± 9·6*	9·6 ± 8·8
Clinical features, %		
dcSSc	73**	38
lcSSc	27**	62
Pitting scars/ulcers	36	32
Contracture of phalanges	59	38
Diffuse pigmentation	55	32
Telangiectasia	36	38
Organ involvement, %		
Pulmonary fibrosis	59	38
Decreased, %VC	14	22
Decreased, %DLco	64	51
Pulmonary hypertension	9	3
Oesophagus	73	76
Heart	18	8
Kidney	5	3
Joint	41	25
Muscle	27	16
Laboratory findings, %		
Antitopoisomerase I Ab	45	35
Anticentromere Ab	27	51
Increased IgG	23	22
Increased ESR	45	32
Increased CRP	23	11

Unless otherwise stated, values are expressed as a percentage. TSS, modified Rodnan total skin score; dcSSc, diffuse cutaneous SSc; lcSSc, limited cutaneous SSc; VC, vital capacity; DLco, diffusion capacity for carbon monoxide; Ab, antibody; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein. *P < 0·05, **P < 0·01 vs. patients with SSc and normal serum sCD93 level.

(2·4 ± 2·7 years vs. 4·2 ± 5·1 years; P < 0·05). Moreover, patients with SSc and a disease duration of < 6 years showed significantly increased serum sCD93 levels compared with those with a disease duration of ≥ 6 years (P < 0·001) (Fig. 2b). Thus, increased serum sCD93 levels were associated with shorter disease duration in patients with SSc.

Immunohistochemical staining for CD93 in skin from patients with systemic sclerosis

CD93 in the skin was assessed by immunohistochemical analysis. In skin from healthy individuals, faint CD93 immunostaining was observed in vascular endothelial cells (Fig. 3). In contrast, in lesional skin from patients with SSc, endothelial cells showed strong CD93 immunostaining. Thus, augmented
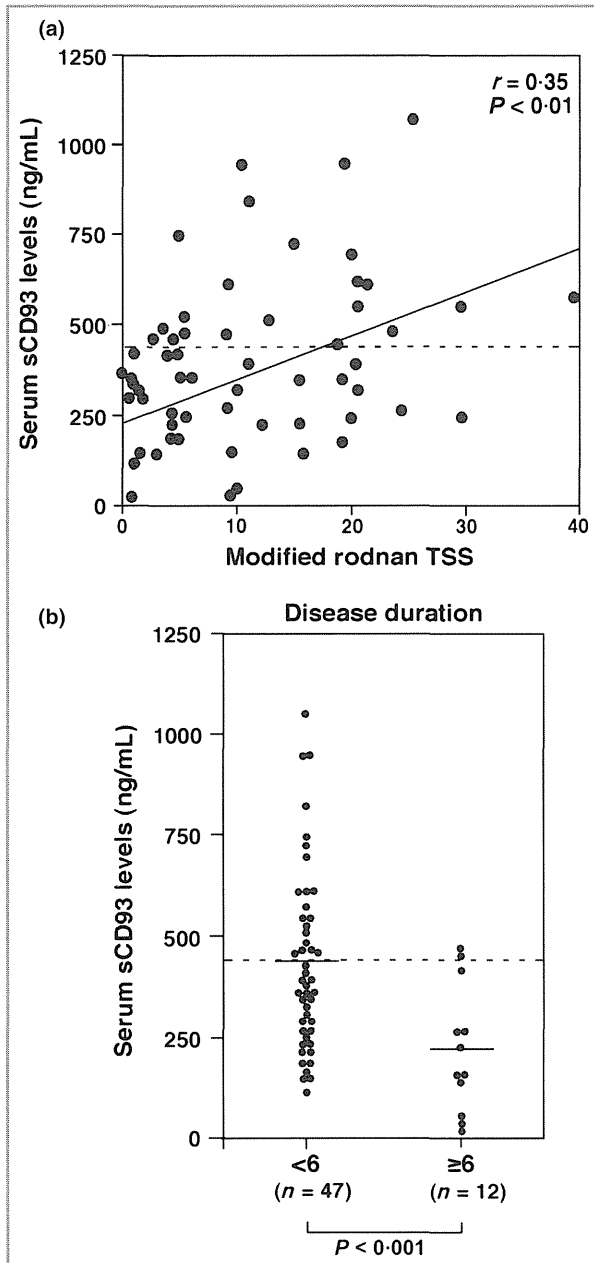
Fig 2. (a) Correlation of soluble CD93 (sCD93) level in serum with modified Rodnan total skin score (TSS) value in patients with systemic sclerosis (SSc). (b) Correlation of disease duration (< 6 and ≥ 6 years) with serum sCD93 level in patients with SSc. Serum sCD93 level was determined by a specific enzyme-linked immunosorbent assay. The dashed lines indicate cut-off values.

CD93 immunostaining was found in lesional skin from patients with SSc.

Longitudinal study of serum sCD93 level

To assess changes in serum sCD93 levels over time, serum samples from 11 patients with SSc (six dcSSc and five lcSSc) were analysed twice over a period of 2·1 years. None of these patients had received any treatment at their first visit. During
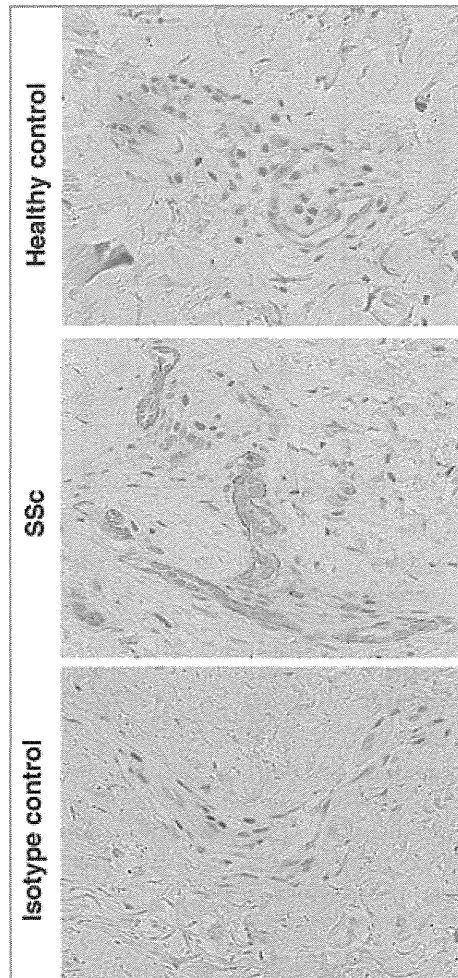

Fig 3. Representative CD93 expression in normal skin tissues and lesional skin tissues from patients with diffuse cutaneous systemic sclerosis (SSc). Staining with control polyclonal rabbit IgG in lesional skin tissue from patients with SSc is shown as a negative control. Original magnification ×250.

the follow-up period, one of the 11 patients exhibited sub-acute deterioration of interstitial pneumonitis and received cyclophosphamide pulse therapy. After the initial visit, four patients received treatment with low-dose steroids. Three patients showed increased sCD93 levels at the first visit, but levels decreased to the normal range thereafter (Fig. 4). Notably, the mean sCD93 level and TSS values were significantly decreased during the follow-up period (P < 0·05 for both). Thus, serum sCD93 levels were generally decreased during the follow-up period. Steroid or immunosuppressive therapy might have affected this change.

Discussion

This is the first report of increased serum sCD93 levels in patients with SSc. Although the sCD93 level was increased in patients with dcSSc, as well as in those with lcSSc, patients with dcSSc had a greater serum sCD93 level compared with those with lcSSc (Fig. 1). An increased sCD93 level was
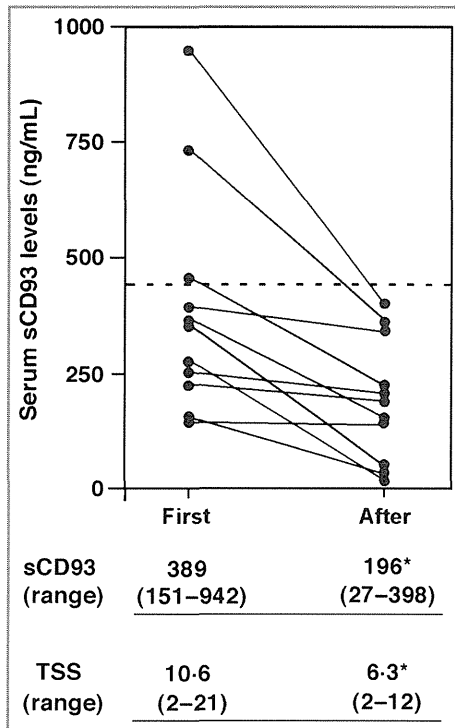
Fig 4. Changes in soluble CD93 (sCD93) level in serum, mean sCD93 level and modified Rodnan total skin score (TSS) value during the follow-up period in patients with systemic sclerosis. The serum sCD93 level was determined by a specific enzyme-linked immunosorbent assay. The dashed line indicates cut-off value. *P < 0·05 vs. sCD93 level or TSS value at first visit.

consistently associated with greater severity of skin fibrosis (Fig. 2a and Table 1). sCD93 levels were significantly greater in patients with early-phase SSc than in those with late-phase SSc (Fig. 2b). Furthermore, strong CD93 immunostaining was observed in vascular endothelial cells in sclerotic skin from patients with SSc, but only weak immunostaining was observed in the skin of healthy individuals (Fig. 3). In the longitudinal study, sCD93 levels generally decreased in parallel with improvement of skin sclerosis (Fig. 4). These results suggest that sCD93 may play an important role in the development of skin sclerosis in patients with SSc.

The Raynaud phenomenon is a characteristic feature of SSc, and occurs in > 95% of patients with SSc.[15] Ischaemia–reperfusion injury after the Raynaud phenomenon can generate reactive oxygen species that cause apoptotic cell death, leading to vascular endothelial cell damage.[20,21] CD93 expression in endothelial cells is upregulated after transient cerebral ischaemia in mice.[22] In the present study, vascular endothelial cells in patients with SSc showed stronger CD93 immunostaining than those in healthy individuals, suggesting that ischaemia–reperfusion injury is likely to contribute to the enhanced expression of CD93 in patients with SSc. In addition, *in vitro* stimulation with tumour necrosis factor-α (TNF-α) or lipopolysaccharide triggers CD93 shedding from human monocytes.[11] Consistently, serum TNF-α levels are significantly increased in patients with SSc compared with healthy individuals.[23] The serum level of

hyaluronan, which activates Toll-like receptor (TLR) 2 and TLR4, is also increased in patients with SSc and is associated with the severity of skin sclerosis.[24] Furthermore, hyaluronan expression in the dermis is enhanced in a mouse model of SSc.[25] Therefore, it is possible that the increased expression of TLR4 ligand or TNF-α in patients with SSc enhances CD93 shedding, thereby increasing the serum sCD93 concentration. Further studies are needed to determine how CD93 is released into the circulation during SSc pathogenesis.

SSc is considered to be a T-helper (Th) 2–dominant autoimmune disease because serum levels of interleukin (IL)-4 and IL-13, representative Th2 cytokines, are significantly increased in patients with SSc compared with healthy individuals.[26–28] In contrast, the serum level of IL-12, a Th1-inducing cytokine, is lower in the early phase of SSc than in healthy individuals but becomes greater in the late phase of SSc,[29] suggesting that a shift from the Th2 to the Th1 response may be associated with improvement of SSc. Previous studies have suggested that IL-6 promotes Th2 differentiation and simultaneously inhibits Th1 polarization.[30,31] Consistently, the serum level of IL-6 is increased in patients with SSc and correlates with the severity of skin fibrosis,[32] whereas IL-6 deficiency attenuates the development of skin fibrosis in a mouse model of SSc.[33] It has recently been shown that sCD93 enhances TLR4-stimulated IL-6 production in human monocytes in *vitro*,[13] whereas sCD93 increases the sensitivity of human monocytes to TLR4 stimulation.[13] Consistent with these findings, the serum sCD93 level correlated positively with the severity of skin sclerosis, and the sCD93 level decreased in parallel with improvement of skin sclerosis. Thus, increased sCD93 is likely to promote Th2 polarization via IL-6 production, leading to the development of skin fibrosis in SSc.

There are several potential limitations of the present study. Firstly, the population was relatively small; a larger study is essential to confirm our present results. Secondly, because all patients with SSc and healthy individuals in the present study were Japanese, additional studies are needed to verify our results in other ethnic groups. Thirdly, the precise mechanism by which CD93 contributes to the development of skin sclerosis in SSc has not been clarified. None the less, given that no therapy has proven effective in suppressing or improving skin sclerosis in SSc to date, our findings suggest that CD93 inhibition could be a possible treatment for patients with SSc who have severe skin sclerosis. In addition, measurement of serum sCD93 in patients with early SSc may offer an important means for further evaluation of SSc disease severity.

What's already known about this topic?

- Systemic sclerosis (SSc) is a generalized connective tissue disorder characterized by sclerotic and vascular changes in the skin and various internal organs.
- CD93 has been shown to play a critical role in the clearance of dying cells.
- It remains unknown whether CD93 plays a role in autoimmune diseases.

What does this study add?

- Serum levels of a soluble form of CD93 (sCD93) were found to be increased in patients with SSc, and therefore correlate with severity of skin sclerosis.
- Strong CD93 immunostaining was observed on endothelial cells in lesional skin tissues from patients with SSc.
- sCD93 levels decrease in parallel with improvement in skin sclerosis.
- CD93 may contribute to the development of skin fibrosis in SSc.

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Constitutive activation of c-Abl/protein kinase C-δ/Fli1 pathway in dermal fibroblasts derived from patients with localized scleroderma

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Summary

Background A noncanonical pathway of transforming growth factor-β signalling, the c-Abl/protein kinase C-δ (PKC-δ)/Friend leukemia virus integration 1 (Fli1) axis, is a powerful regulator of collagen synthesis in dermal fibroblasts.

Objectives To investigate the significance of the c-Abl/PKC-δ/Fli1 pathway for the establishment of the profibrotic phenotype in lesional dermal fibroblasts from patients with localized scleroderma (LSc).

Methods The activation status of the c-Abl/PKC-δ/Fli1 pathway was evaluated by immunoblotting and chromatin immunoprecipitation using cultured dermal fibroblasts from patients with LSc and closely matched healthy controls and by immunostaining on skin sections. The effects of a platelet-derived growth factor receptor inhibitor AG1296 and gene silencing of c-Abl on the expression levels of type I collagen were evaluated by immunoblotting.

Results The phosphorylation levels of Fli1 at threonine 312 were increased, while the total Fli1 levels and the binding of Fli1 to the COL1A2 promoter were decreased, in cultured LSc fibroblasts compared with cultured normal fibroblasts. Furthermore, in cultured LSc fibroblasts, the expression levels of c-Abl were elevated compared with cultured normal fibroblasts and PKC-δ was preferentially localized in the nucleus. These findings were also confirmed in vivo by immunohistochemistry using skin sections. Moreover, gene silencing of c-Abl, but not AG1296, significantly suppressed the expression of type I collagen in cultured LSc fibroblasts.

Conclusions Constitutive activation of the c-Abl/PKC-δ/Fli1 pathway at least partially contributes to the establishment of the profibrotic phenotype in LSc dermal fibroblasts, which provides a novel molecular basis to explain the efficacy of imatinib against skin sclerosis in a certain subset of LSc.

Localized scleroderma (LSc) is a connective tissue disorder affecting a limited area of the skin, subcutaneous tissue, and underlying muscle and bone. While elevated collagen synthesis by lesional dermal fibroblasts is one of the common characteristics of LSc and systemic sclerosis (SSc),[1,2] the absence of vasculopathy, acrosclerosis and visceral involvement differentiates LSc from SSc. Evidence-based treatment options of LSc are limited secondary to the rarity of the disease and the lack of universally used validated outcome measures.[3]

Transforming growth factor (TGF)-β is generally considered to play a major role in the pathogenesis of fibrotic diseases, including LSc.[1,4] Although the Smad signalling pathway is a powerful regulator of various biological effects of TGF-β, a

growing body of evidence has drawn much attention to the noncanonical signalling pathways as the potential therapeutic targets of pathological TGF-β signalling.[5] Importantly, a newly identified noncanonical pathway, the c-Abl/protein kinase C-δ (PKC-δ)/Friend leukemia virus integration 1 (Fli1) axis, is a positive regulator of collagen synthesis.[6] Fli1 is a member of the Ets transcription factor family, which is highly expressed in endothelial cells (ECs) and haematopoietic cells.[7] Despite relatively low expression levels, Fli1 functions as a potent repressor of genes encoding type I collagen[8,9] and CCN2[10] in dermal fibroblasts. The association of Fli1 transcriptional activity with its post-translational modification has been well studied in the human α2(I) collagen (COL1A2) gene. Activated

PKC-δ directly phosphorylates Fli1 at threonine 312 in the nucleus,[11] which subsequently induces Fli1 acetylation at lysine 380 by p300/CREB-binding protein-associated factor (phosphorylation-acetylation cascade),[9] resulting in the dissociation of Fli1 from the COL1A2 promoter.[12] As nuclear translocation of activated PKC-δ is tightly regulated by c-Abl tyrosine kinase,[6] TGF-β-dependent activation of c-Abl triggers the phosphorylation-acetylation cascade, leading to the robust increase of COL1A2 gene expression by attenuating Fli1 transcriptional activity. Given that gene silencing of Fli1 is enough to increase the mRNA levels of the COL1A2 gene to the levels comparable with those achieved by TGF-β stimulation,[10] this noncanonical pathway plays a pivotal role in the regulation of the fibrotic gene programme in dermal fibroblasts.

During the last 5 years, the efficacy of imatinib mesylate, a selective tyrosine kinase inhibitor of c-Abl, platelet-derived growth factor (PDGF) receptor (PDGFR) and c-kit,[13] against skin sclerosis in SSc has been well studied in a few clinical studies.[14–17] Although the conclusion is still controversial, imatinib exerted a great therapeutic effect on skin sclerosis in a group of selected patients with SSc.[14–18] Importantly, we recently demonstrated that the c-Abl/PKC-δ/Fli1 pathway is constitutively activated in SSc dermal fibroblasts and the blockade of this pathway by imatinib results in the upregulation of Fli1 protein via increasing its protein stability, indicating that imatinib may elicit an antifibrotic effect on SSc dermal fibroblasts at least partially by reversing transcriptional repressor activity of Fli1.[6] Based on this background, a recent case report[19] demonstrating a great effect of imatinib on skin sclerosis associated with LSc stimulated our interest to investigate the significance of the c-Abl/PKC-δ/Fli1 pathway in the establishment of the profibrotic phenotype in LSc dermal fibroblasts.

Materials and methods

Reagents

Anti-Fli1 antibody for immunoblotting and anti-PKC-δ antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-Fli1 antibody for immunohistochemistry was obtained from R&D Systems (Minneapolis, MN, U.S.A). Antibodies against c-Abl and phospho-c-Abl (Tyr245) were bought from Cell Signaling (Danvers, MA, U.S.A.). Antibodies for β-actin and type I collagen were from Sigma-Aldrich (St Louis, MO, U.S.A.) and Southern Biotech (Birmingham, AL, U.S.A.), respectively. The polyclonal rabbit antiphospho-Fli1 (Thr312)-specific antibody was generated as described previously.[11]

Cell culture

Human dermal fibroblasts were obtained by skin biopsy from the affected areas, which developed within 3 years, of five patients with LSc. Patient information is summarized in Table 1. All of the patients were diagnosed by clinical appearance and histological findings, and none had any other collagen disease, including SSc. Control fibroblasts were obtained

Table 1 Patient information

Patients	Age (years)	Sex	Disease duration	Previous treatments
Cell culture				
Patient 1	14	M	2 months	None
Patient 2	45	F	8 months	None
Patient 3	9	M	1 year	None
Patient 4	5	F	3 years	None
Patient 5	48	F	1 year	None
Immunohistochemistry				
Patient 1	63	M	10 years	None
Patient 2	55	F	2 years	None
Patient 3	37	F	4 months	None
Patient 4	30	M	1 year	None
Patient 5	37	F	2 years	None

by skin biopsy from five age-, sex- and site-matched healthy donors. Institutional approval (University of Tokyo Graduate School of Medicine) and informed consent were obtained from all subjects. Fibroblasts were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 2 mmol L^{-1} L-glutamine and antibiotic–antimycotic solution. These cells were individually maintained as monolayers at 37 °C in 95% air, 5% CO_2. All studies used cells from passage numbers 3–6.

Immunoblotting

Confluent quiescent fibroblasts were serum starved for 48 h. After harvesting, cells were divided into two samples, which were used for whole cell lysates and nuclear extracts, respectively. Whole cell lysates[20] and nuclear extracts[21] were prepared as described previously. Cell supernatants were collected after incubation of confluent fibroblasts for 72 h in serum-free medium. The amount of samples mounted for immunoblotting was normalized by total cell number. Samples were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotting. Bands were detected using enhanced chemiluminescent techniques (Thermo Scientific, Rockford, IL, U.S.A.). According to a series of pilot experiments, anti-Fli1 antibody and antiphospho-Fli1 (Thr312)-specific antibody work much better in immunoblotting using nuclear extracts and whole cell lysates, respectively.

Immunostaining

Immunocytochemistry was carried out to determine the subcellular localization of PKC-δ in normal and LSc fibroblasts, as described previously.[22] Anti-PKC-δ antibody and secondary antibody conjugated with fluorescein isothiocyanate were used at the concentration of 1 : 50 and 1 : 100, respectively. 4′,6-Diamidino-2-phenylindole was used for nuclear staining. Slides were blinded, and 10 random fields were examined using a fluorescence microscope. For immunohistochemistry, Vectastain ABC kit (Vector Laboratories, Burlingame, CA,

U.S.A.) was used according to the manufacturer's instructions. Skin samples from five patients with LSc and five closely matched healthy controls were used for the analysis. Patient information is summarized in Table 1.

Treatment with small-molecule kinase inhibitor

Cells were grown to confluence and then incubated for 48 h in serum-free medium. Cells were treated with dimethyl sulphoxide or PDGFR inhibitor AG1296 (Calbiochem, Merck, Darmstadt, Germany) for the last 24 h at the concentration of 10, 30 and 100 µmol L^{-1}. AG1296 selectively inhibits phosphorylation of both human PDGF α- and β-receptors and their downstream signalling in a dose-dependent manner.[23-25] Incubation with AG1296 at 25 µmol L^{-1} for 24 h effectively inhibited activated tyrosine phosphorylation of PDGF β-receptors in human foreskin fibroblasts.[26] Furthermore, AG1296 (10 µmol L^{-1} for 24 h) reduced the phospho-PDGFR protein expression by 70%, Akt activity by 80%, and extracellular signal-regulated kinase 1/2 activity by 50% in human skin fibroblasts stimulated with thrombin-activated platelets.[27]

Inhibition of protein expression by small interfering RNA

For the inhibition of Fli1 expression using small interfering RNA (siRNA) oligos, dermal fibroblasts were seeded shortly before transfection. The cells were transfected with 10 nmol L^{-1} c-Abl siRNA (Santa Cruz Biotechnology), or the corresponding concentration of scrambled nonsilencing siRNA (Santa Cruz Biotechnology) for 48 h using HiPerFect Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Cells were then serum starved for 48 h.

RNA isolation and real-time reverse transcription–polymerase chain reaction

One microgram of RNA isolated from cells using Tri reagent (MRC Inc., Cincinnati, OH, U.S.A.) was reverse transcribed in 20 µL of reaction volume using iScript cDNA Synthesis kits (Bio-Rad, Hercules, CA, U.S.A.). Real-time reverse transcription–polymerase chain reaction (PCR) was carried out using Fast SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, U.S.A.) on ABI Prism 7000 (Applied Biosystems) in triplicate. The mRNA levels of COL1A2, c-Abl and Fli1 genes were normalized to those of human 18S rRNA gene. The sequences of primers were obtained from previous publications (COL1A2[28] forward 5′-GATGTTGAACTTGTTGCTGAGG-3′ and reverse 5′-TCTTTCCCCATTCATTTGTCTT-3′; c-Abl[29] forward 5′-CCCAACCTTTTCGTTGCACTGT-3′ and reverse 5′-CGGCTCTCGGAGGAGACGTAGA-3′; Fli1[6] forward 5′-GGATGGCAAGGAACTGTGTAA-3′ and reverse 5′-GGTTGTATAGGCCAGCAG-3′; 18S rRNA[30] forward 5′-CGCCGCTAGAGGTGAAAT TC-3′ and reverse 5′-TTGGCAAATGCTTTCGCTC-3′). PCR conditions were 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s, 60 °C for 30 s. Dissociation analysis for each primer pair and reaction was performed to verify specific amplification.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was carried out using EpiQuik ChIP kit (Epigentek, Farmingdale, NY, U.S.A.). Briefly, cells were treated with 1% formaldehyde for 10 min. The cross-linked chromatin was then prepared and sonicated to an average size of 300–500 bp. The DNA fragments were immunoprecipitated with polyclonal anti-Fli1 antibody or IgG isotype control antibody at room temperature. After reversal of cross-linking, the immunoprecipitated chromatin was amplified by PCR amplification of specific regions of the COL1A2 genomic locus. The primers were as follows: COL1A2/F-404, 5′-CTGGACAGCTCCTGCTTTGAT-3′; COL1A2/R-233, 5′-CTTT CAAGGGGAAACTCTGACTC-3′. The amplified DNA products were analysed by agarose gel electrophoresis.

Statistical analysis

Statistical analysis was carried out with unpaired or paired two-tailed t-test for two-group comparison. Statistical significance was defined as $P < 0.05$.

Results

Constitutive activation of c-Abl/protein kinase C-δ/Fli1 pathway in localized scleroderma dermal fibroblasts

As an initial experiment, we confirmed that LSc dermal fibroblasts used in this study maintain their profibrotic phenotype in in vitro culture system (Fig. 1a). Therefore, a series of in vitro experiments was carried out using these cells. In order to investigate the activation status of the c-Abl/PKC-δ/Fli1 pathway, we initially compared the phosphorylation levels of Fli1 at threonine 312 between LSc fibroblasts and closely matched normal fibroblasts. To this end, the phosphorylation levels and the total levels of Fli1 were determined by immunoblotting using whole cell lysates and nuclear extracts, respectively. As shown in Figure 1b, the phosphorylation levels of Fli1 were increased, while the total Fli1 levels were decreased, in LSc fibroblasts compared with normal fibroblasts. The phosphorylation levels of Fli1 normalized by total Fli1 levels were much more elevated in LSc fibroblasts than in normal fibroblasts. Given that protein stability of Fli1 decreases following its phosphorylation at threonine 312,[9,11] these results suggest that the expression levels of Fli1 are decreased via the reduction of its protein stability in LSc fibroblasts. Supporting this idea, there was no significant difference in mRNA levels of the Fli1 gene between normal and LSc fibroblasts (Fig. 1c). As the phosphorylation of Fli1 decreases its DNA binding ability as well as its protein stability,[9,11] which together attenuate the transcriptional activity of Fli1, the levels of functional Fli1 theoretically decrease to a greater extent in LSc fibroblasts. Consistently, the occupancy of the COL1A2 promoter by Fli1 was markedly decreased in LSc fibroblasts compared with normal fibroblasts (Fig. 1d).

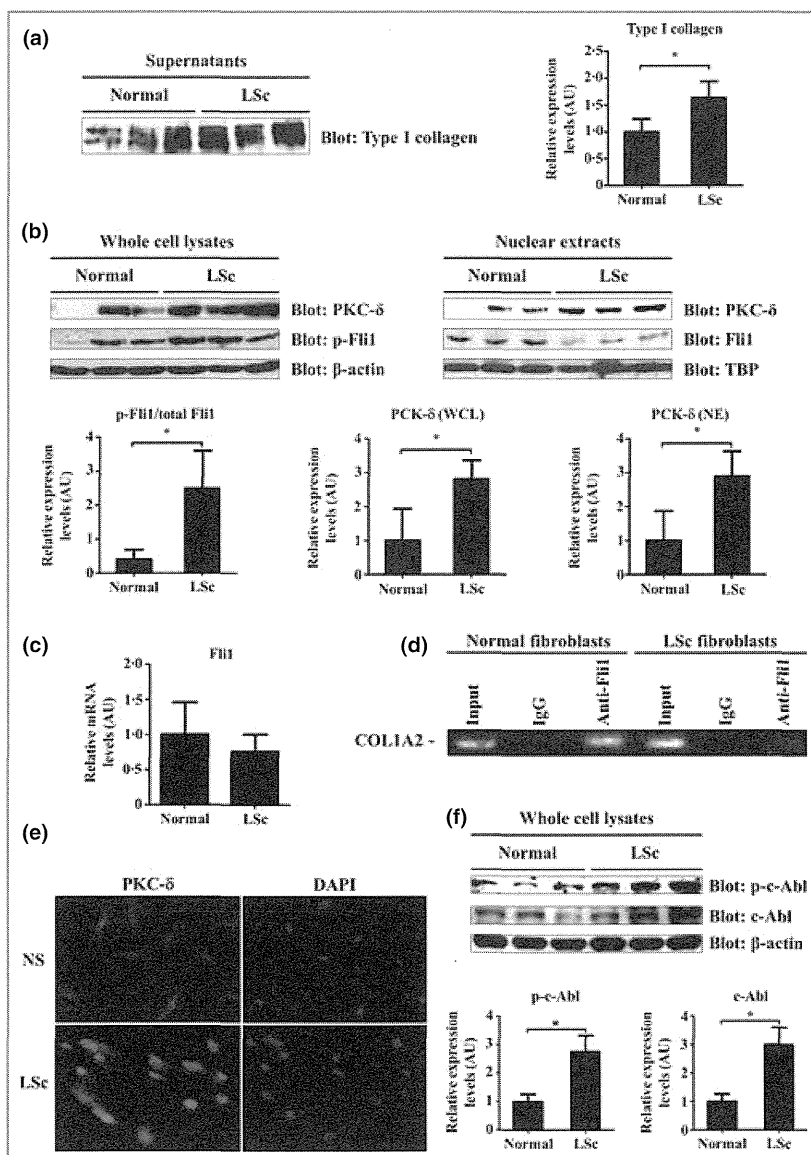We next examined the activation status of PKC-δ in LSc fibroblasts. As PKC-δ is upregulated and translocated into the

Fig 1. Constitutive activation of the c-Abl/protein kinase C-δ (PKC-δ)/Fli1 pathway in cultured dermal fibroblasts from patients with localized scleroderma (LSc). (a) Type I collagen protein levels were analysed in cell supernatants of normal and LSc fibroblasts collected after 72 h incubation in serum-free medium. The amount of samples mounted for immunoblotting was normalized by the total cell number. Results of densitometric analysis are shown in the right panel. (b) Dermal fibroblasts were grown to confluence and then serum-starved for 48 h. Cells were harvested and divided into two samples. Each sample was used for the preparation of whole cell lysates (WCL) and nuclear extracts (NE), respectively. The levels of total PKC-δ and phospho-Fli1 (p-Fli1) (Thr312) were determined by immunoblotting using WCL (left panels), while the levels of nuclear PKC-δ and total Fli1 were analysed by immunoblotting using NE (right panels). β-actin and TATA-binding protein (TBP) were used as loading controls for WCL and NE, respectively. Representative blots are shown. The results of densitometric analyses are also shown. (c) mRNA levels of Fli1 gene were determined by real-time reverse transcription–polymerase chain reaction in normal and LSc dermal fibroblasts. (d) Chromatin was isolated from cultured dermal fibroblasts and immunoprecipitated using anti-Fli1 antibody or IgG isotype control antibody. After isolation of bound DNA, polymerase chain reaction amplification was carried out using human COL1A2 promoter-specific primers. Input DNA was taken from each sample before addition of an antibody. The bands show one representative of three independent experiments. (e) Subcellular localization of PKC-δ was examined by immunocytochemistry in cultured normal fibroblasts (NS) and LSc fibroblasts. Nuclear staining was also performed by using 4′,6-diamidino-2-phenylindole (DAPI). Original magnification × 40. (f) The levels of total c-Abl and phospho-c-Abl (p-c-Abl) (Tyr245) were determined by immunoblotting using WCL in normal and LSc fibroblasts. The results of densitometric analyses are also shown. AU, arbitrary units. Results are shown as mean ± SD. *P < 0·05.

nucleus by TGF-β stimulation in dermal fibroblasts,[31,32] we assessed the expression levels and subcellular localization of PKC-δ in LSc fibroblasts, which are at least partially activated by autocrine TGF-β stimulation.[1] The expression levels of PKC-δ were much more increased in LSc fibroblasts than in normal fibroblasts as shown by immunoblotting using whole

cell lysates (left panels of Fig. 1b). Furthermore, nuclear translocation of PKC-δ was greatly promoted in LSc fibroblasts compared with normal fibroblasts as shown by immunoblotting using nuclear extracts (right panels of Fig. 1b) and by immunocytochemistry (Fig. 1e). Given that c-Abl activation promotes nuclear localization of PKC-δ in dermal fibroblasts,[6] we also looked at the expression levels and phosphorylation levels of c-Abl in LSc fibroblasts. As shown in Figure 1f, the expression levels and the phosphorylation levels of c-Abl were highly elevated in LSc fibroblasts compared with normal fibroblasts. Collectively, these results indicate that the c-Abl/PKC-δ/Fli1 pathway is constitutively activated in LSc fibroblasts, which leads to the decrease in functional Fli1 protein levels via its accelerated degradation and loss of DNA binding capacity.

Comparison of the expression levels of c-Abl, protein kinase C-δ and Fli1 proteins in skin sections derived from patients with localized scleroderma and closely matched healthy controls

To evaluate further the expression levels of c-Abl, PKC-δ and Fli1 proteins and their localization in LSc lesional skin tissues, immunohistochemistry was carried out using skin samples taken from five patients with LSc and five closely matched healthy controls. Representative pictures are shown in Figure 2a–h. Generally, PKC-δ was localized in both nucleus and cytoplasm, while c-Abl and Fli1 proteins were detected in cytoplasm and nucleus, respectively. Our results showed that c-Abl was preferentially detected in cytoplasm in LSc fibroblasts (Fig. 2b), while mostly below detectable levels in normal fibroblasts (Fig. 2a). Regarding PKC-δ, cytoplasmic staining was stronger than nuclear staining in normal fibroblasts (Fig. 2c), whereas diffuse staining of nucleus and cytoplasm was seen in LSc fibroblasts (Fig. 2d) (images at higher magnification are shown in Figure S1; see Supporting Information). As for Fli1, the expression was downregulated in LSc fibroblasts (Fig. 2f) compared with normal fibroblasts (Fig. 2e). As Fli1 expression is markedly decreased in ECs of patients with SSc,[33] we also investigated Fli1 expression levels in dermal vessels. Notably, Fli1 expression levels in LSc ECs did not differ from those in normal ECs (Fig. 2g,h). These results indicate that constitutive activation of the c-Abl/PKC-δ/Fli1 pathway occurs *in vivo* in LSc lesional dermal fibroblasts. Fli1 expression levels in LSc ECs were not attenuated, which is consistent with the idea that patients with LSc lack EC activation characteristic for SSc.

Gene silencing of c-Abl, but not a platelet-derived growth factor receptor inhibitor, significantly decreased type I collage protein levels and mRNA levels of *COL1A2* gene in localized scleroderma dermal fibroblasts

Imatinib mesylate targets c-Abl and PDGFR pathways, both of which are implicated in the pathogenesis of fibrotic diseases. To determine which pathway is more likely to be involved in the mechanism underlying the potential therapeutic effect of
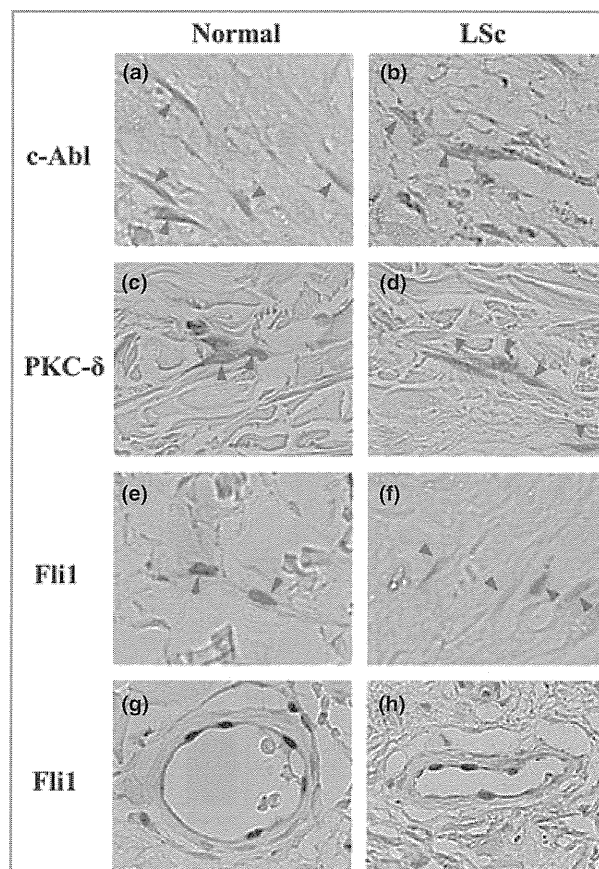

Fig 2. The expression levels of c-Abl, protein kinase C-δ (PKC-δ) and Fli1 proteins in skin sections between patients with localized scleroderma (LSc) and healthy controls. Expression levels of c-Abl (a, b), PKC-δ (c, d) and Fli1 (e, f) in dermal fibroblasts and those of Fli1 in endothelial cells (g, h) were determined by immunohistochemistry in skin sections from healthy controls (a, c, e, g) and from patients with LSc (b, d, f, h). Dermal fibroblasts are indicated by arrowheads. The most optimal concentration of antibodies, exposure time to diaminobenzidine and antigen retrieval protocol were different between each antibody, which is responsible for the variations of background staining. Original magnification ×400.

imatinib on LSc, we examined the mRNA levels of the *COL1A2* gene in dermal fibroblasts with c-Abl gene silencing or chemical PDGFR inhibition. While AG1296, which effectively inhibits PDGF signalling in human dermal fibroblasts,[26,27] did not affect the mRNA levels of the *COL1A2* gene either in LSc fibroblasts or in normal fibroblasts (Fig. 3a), c-Abl knockdown (approximately 80%, upper panels in Fig. 3b) significantly reduced the mRNA levels of the *COL1A2* gene only in LSc fibroblasts (29% reduction, $P < 0.05$, right lower panel in Fig. 3b), but not at all in normal fibroblasts (left lower panel in Fig. 3b). The effect of c-Abl gene silencing on the expression of type I collagen protein was also confirmed by immunoblotting in normal and LSc fibroblasts (Fig. 3c). These results suggest that imatinib exerts its antifibrotic effect against LSc fibroblasts largely by inhibiting the constitutively activated c-Abl/PKC-δ/Fli1 pathway.

Fig 3. The effects of c-Abl gene silencing and a chemical platelet-derived growth factor receptor (PDGFR) inhibition on mRNA levels of the COL1A2 gene and type I collagen protein levels in normal and localized scleroderma (LSc) fibroblasts. (a) Quiescent normal and LSc dermal fibroblasts were left untreated or treated with the indicated amount of PDGFR inhibitor (AG1296) for 24 h. The mRNA levels of the COL1A2 gene were examined by real-time reverse transcription–polymerase chain reaction (RT-PCR). DMSO, dimethyl sulphoxide (control). (b) Normal and LSc dermal fibroblasts were transiently transfected with 10 nmol L^{-1} c-Abl or scrambled control small interfering RNA (siRNA) oligos and cultured for 96 h with serum starvation for the last 48 h. mRNA levels of the c-Abl gene (upper panels) and COL1A2 gene (lower panels) were determined by real-time RT-PCR. (c) The protein levels of type I collagen were also determined by immunoblotting under the same conditions. AU, arbitrary units. Values are the mean ± SD of three separate experiments using three different LSc fibroblast cultures and three different normal fibroblast cultures. Statistical significance of differences was determined by paired two-tailed t-test. *P < 0·05.
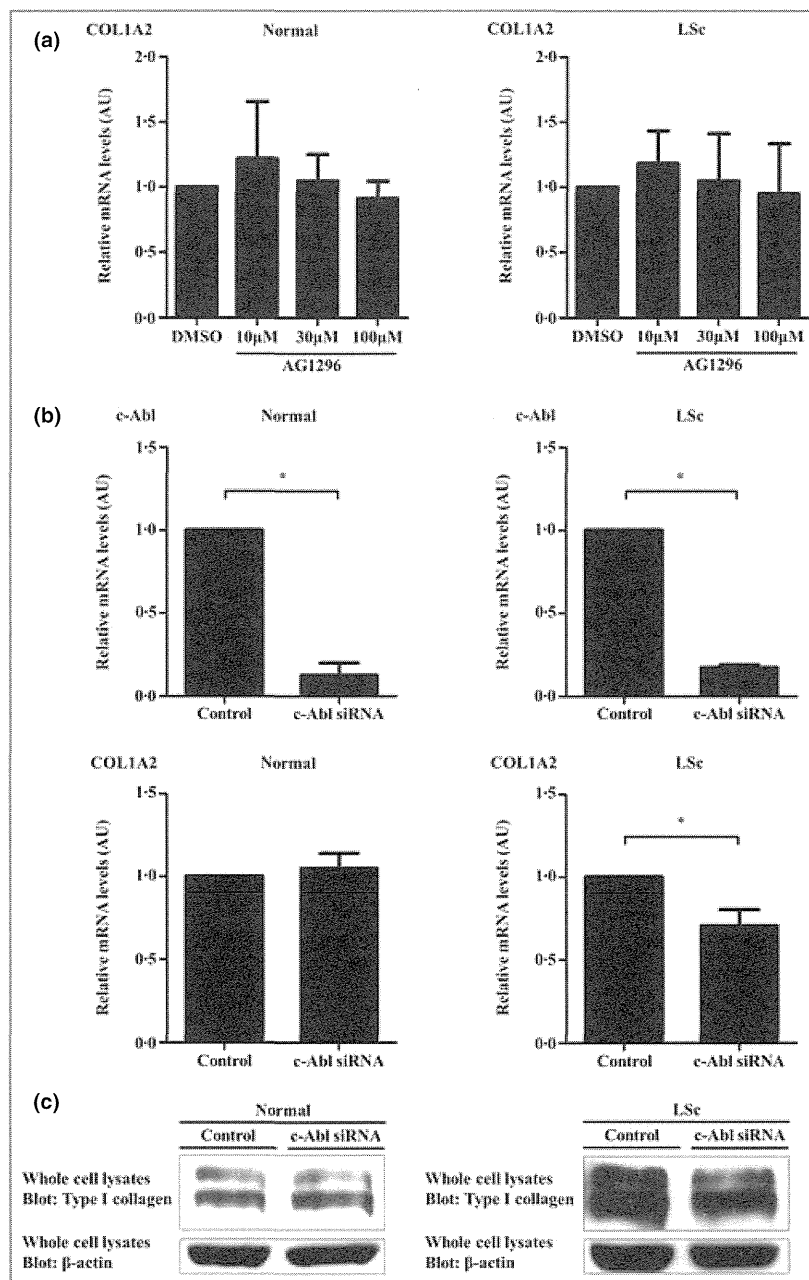
Discussion

This study was undertaken to investigate if the c-Abl/PKC-δ/Fli1 pathway is associated with the establishment of profibrotic phenotype in LSc dermal fibroblasts. A series of in vitro experiments demonstrated the following findings: (i) the elevation of total c-Abl and phospho-c-Abl levels in LSc fibroblasts, (ii) the increased expression and the preferential nuclear localization of PKC-δ in LSc fibroblasts, and (iii) the increase in Fli1 phosphorylation levels as well as the decrease in total Fli1 levels and Fli1 occupancy of the COL1A2 promoter in LSc fibroblasts. Given that sequential activation of c-Abl and PKC-δ triggers the phosphorylation-acetylation cascade of Fli1, eventually resulting in its degradation,[6,9,11] these findings indicate that the c-Abl/PKC-δ/Fli1 pathway is constitutively activated in LSc dermal fibroblasts. Importantly, this notion was confirmed in vivo by immunohistochemistry with lesional skin of patients with LSc, where the elevation of total c-Abl, nuclear translocation of PKC-δ and the decrease in total Fli1 expression were detected in dermal fibroblasts. Finally, another set of data revealed the significant suppression of COL1A2 gene expression by the knockdown of c-Abl, but not by the inhibition of PGDFR, in LSc fibroblasts. To our best knowledge, this is the first study which provides the molecular basis explaining the potential efficacy of imatinib against skin sclerosis in LSc.

As shown in the previous and present studies, LSc dermal fibroblasts are constitutively activated and produce excessive levels of extracellular matrix, including type I collagen.[1] Simi-

larly to SSc dermal fibroblasts, the upregulation of integrin αVβ5 contributes to the establishment of autocrine TGF-β signalling in LSc dermal fibroblasts by the activation of latent TGF-β via integrin-mediated myofibroblast contraction.[1,34–36] As TGF-β is a main stimulus triggering the activation of the c-Abl/PKC-δ/Fli1 pathway,[6] it is plausible that this signalling pathway is constitutively activated in LSc fibroblasts. In terms of a therapeutic targeting of pathological TGF-β signalling, the identification of abnormally activated signalling pathways in certain pathological conditions is beneficial because the blockade of TGF-β by neutralizing antibody or latency-associated peptide potentially induces undesirable adverse effects by targeting its physiologically indispensable effects on various cell types. Therefore, the present observation that c-Abl, but not PDGFR, contributes to the constitutive activation of LSc dermal fibroblasts provides a useful clue to develop further the therapeutic strategy based on the clinical observation that imatinib improves skin fibrosis in a certain subset of LSc.[19] Accordingly, emerging tyrosine kinase inhibitors targeting c-Abl may be potential candidates for new treatment options of LSc.

In addition to its central role regulating type I collagen gene expression in dermal fibroblasts, Fli1 functions as a pivotal regulator of the angiogenic process in ECs.[37,38] In SSc skin, Fli1 levels are constitutively downregulated in these cell types, especially through the epigenetic mechanism in dermal fibroblasts,[33,39] suggesting that Fli1 is one of the genetic factors in SSc. Gene silencing of Fli1 activates ECs as well as dermal fibroblasts in vitro[9,40] and a series of Fli1 mutant mice reproduces the histopathological features of SSc skin, including collagen deposition and abnormal vascular structure.[12,40] Collectively, Fli1 deficiency can be one of the markers reflecting SSc-like activation of fibroblasts and ECs. In the present study, Fli1 was deficient in dermal fibroblasts, but not in ECs, in lesional skin of LSc. This suggests that LSc lacks endothelial activation characteristic for SSc, which clearly explains the clinical difference between these two diseases, namely, vascular involvement associated with SSc is totally absent in LSc. As vascular alteration is closely linked to the fibroblast activation in SSc,[41] the process leading to fibroblast activation is likely to be quite different between SSc and LSc. Thus, the different expression profile of Fli1 proteins provides a new molecular basis to understand further the distinct pathological process between LSc and SSc.

In summary, a new noncanonical TGF-β signalling pathway, c-Abl/PKC-δ/Fli1, is constitutively activated in LSc dermal fibroblasts as well as SSc dermal fibroblasts. Tyrosine kinase inhibitors against c-Abl, including imatinib, theoretically have a large potential to reverse the profibrotic phenotype of LSc dermal fibroblasts. To clarify this point, further basic and clinical studies are required in the future.

What's already known about this topic?

- A newly identified noncanonical pathway of transforming growth factor-β signalling, the c-Abl/protein kinase C-δ (PKC-δ)/Friend leukemia virus integration 1 (Fli1) axis, is

a powerful regulator of collagen synthesis, which is constitutively activated in systemic sclerosis dermal fibroblasts.
- A recent case report demonstrated a great effect of imatinib, a selective tyrosine kinase inhibitor of c-Abl and platelet-derived growth factor receptor, on skin sclerosis associated with localized scleroderma (LSc).

What does this study add?

- Constitutive activation of the c-Abl/PKC-δ/Fli1 pathway at least partially contributes to the establishment of the profibrotic phenotype in LSc dermal fibroblasts, which provides a novel molecular basis to explain the efficacy of imatinib against skin sclerosis in a certain subset of LSc.

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Supporting Information

Additional Supporting Information may be found in the online version of the article:

Fig S1. Higher magnification images of Figure 2c,d.

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Matrix Pathobiology

Increased Accumulation of Extracellular Thrombospondin-2 Due to Low Degradation Activity Stimulates Type I Collagen Expression in Scleroderma Fibroblasts

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The aim of the present study was to determine the expression and role of thrombospondin-2 (TSP-2) in systemic sclerosis (SSc). Both TSP-2 mRNA levels and protein synthesis in cell lysates were significantly lower in cultured SSc fibroblasts than in normal fibroblasts; however, the TSP-2 protein that accumulated in the conditioned medium of SSc fibroblasts was up-regulated, compared with that of normal fibroblasts, because of an increase in the half-life of the protein. *In vivo* serum TSP-2 levels were higher in SSc patients than in healthy control subjects, and SSc patients with elevated serum TSP-2 levels tended to have pitting scars and/or ulcers. TSP-2 knockdown resulted in the down-regulation of type I collagen expression and the up-regulation of miR-7, one of the miRNAs with an inhibitory effect on collagen expression. Expression levels of miR-7 were also up-regulated in SSc dermal fibroblasts both *in vivo* and *in vitro*. Taken together, these findings suggest that the increased extracellular TSP-2 deposition in SSc fibroblasts may contribute to tissue fibrosis by inducing collagen expression. Down-regulation of intracellular TSP-2 synthesis and the subsequent miR-7 up-regulation in SSc fibroblasts may be due to a negative feedback mechanism that prevents increased extracellular TSP-2 deposition and/or tissue fibrosis. Thus, TSP-2 may play an important role in the maintenance of fibrosis and

angiopathy in patients with SSc. *(Am J Pathol 2012, 180: 703–714; DOI: 10.1016/j.ajpath.2011.10.030)*

Systemic sclerosis (SSc), or scleroderma, is an acquired disorder that typically results in fibrosis of the skin and internal organs. Although the pathogenesis of this disease is still unclear, inflammation, autoimmune attack, and vascular damage are involved, leading to activation of fibroblasts and excess deposition of extracellular matrix (ECM).[1,2] Thus, abnormal SSc fibroblasts responsible for fibrosis may develop from a subset of cells that have escaped normal control mechanisms.[3,4]

Although the mechanism of fibroblast activation in SSc is currently unknown, many of the characteristics of SSc fibroblasts resemble those of healthy fibroblasts stimulated by transforming growth factor beta-1 (TGF-β1).[5,6] The principal effect of TGF-β1 on mesenchymal cells is its stimulation of ECM deposition. Fibroblasts derived from SSc skin cultured *in vitro* produce excessive amounts of various collagens, mainly type I collagen, which consists of α1(I) and α2(I) collagen,[7,8] suggesting that the activation of dermal fibroblasts in SSc may be a result of stimulation by TGF-β signaling.

Recently, thrombospondin (TSP) has attracted attention because of its involvement in TGF-β signaling, regulation of ECM expression, and angiogenesis. The TSP family consists of five members.[9] Among these, TSP-1 and cartilage oligomeric matrix protein (COMP; TSP-5) have been implicated in the pathogenesis of SSc; levels of TSP-1 are higher in fibroblasts and sera of SSc patients