was diagnosed through an upper gastrointestinal endoscope based on the Frequency Scale for Symptoms of GERD and the Los Angeles classification (Los Angeles classification grade M or greater was diagnosed as reflux esophagitis).[18,19]

Statistics

P-values were calculated with the Student's *t*-test or χ^2 analysis. Error bars represent standard errors of the mean.

RESULTS

The 8 SSc patients with LSc among 220 SSc patients (3.6%) are summarized in Table 1. Their mean age was 45.3 ± 3.8 years, and the male/female ratio was 2:6. The disease types of LSc were as follows: plaque type (n = 5, including 3 cases with generalized morphea type), guttate type (n = 2), and linear type (n = 1). LSc lesions were mostly distributed bilaterally and symmetrically. The chest was the most common affected site (n = 5), followed by the abdomen (n = 5). All cases were diagnosed as having SSc within 5 years before or after the development of LSc. In three cases (37.5%; cases 1–3), the onset of LSc preceded the first clinical manifestation of SSc. In case 1, sclerodactyly occurred 10 months after the appearance of LSc lesions. In case 2, anticentromere antibody was negative when the LSc lesions appeared; sclerodactyly and anticentromere antibody were identified 4 years after the appearance of LSc lesions. In case 3, Raynaud's phenomenon appeared 4 years after the appearance of LSc lesions, sclerodactyly appeared 5 years after the appearance of LSc lesions, and anti-RNA polymerase III antibody was identified 5 years after the appearance of LSc lesions. However, we have not determined whether the anti-RNA polymerase III antibody was positive. Additionally, three SSc patients with LSc (37.5%) had past medical histories with allergic diseases and/or autoimmune disorders. We confirmed that all cases of SSc with LSc were defined as SSc based on the new American College of Rheumatology/European League Against Rheumatism classification criteria (2013). According to this criteria, patients with a total score of ≥9 were classified as having definite SSc (Table 1).[20]

We compared the clinical and laboratory features between SSc patients with LSc (3.6%, 8/220) and without LSc (96.4%, 212/220) (Table 2). The onset age for SSc patients with LSc was significantly lower than for those without LSc (45.3 ± 3.8 years vs 56.2 ± 0.9 years, $P < 0.05$). Sex and types of disease ratios were comparable between the two groups. Negative antinuclear antibody (25% vs 5.2%, $P < 0.05$) and positive anti-RNA polymerase III antibody (25% vs 4.2%, $P < 0.05$) were significantly prevalent in SSc patients with LSc. The positivity of anticentromere antibody tended to be prevalent in SSc patients without LSc (12.5% vs 45.2%, $P = 0.067$). No significant differences in the frequency of interstitial lung disease, reflux esophagitis, and pulmonary artery hypertension were detected.

DISCUSSION

The frequency of SSc with LSc in SSc patients has varied in previous studies; for example, 5.7% of 106 SSc patients had SSc with LSc in a US study,[11] and in Japanese studies 4.9% of 349 SSc patients had SSc with LSc and 6.7% of 135 patients had it.[12,13] Consistent with these previous analyses, 3.6% of 220 SSc patients had SSc complicated with LSc in the present study.

LSc generally appears at any age, and onset at 20–50 years old is common, with the exception of the linear type, which often develops in childhood.[5,21] The estimated incidence of LSc is 0.4–2.7 per 100 000 individuals, and the female-to-male ratio is 2.4 to 4.2:1.[21–25] The peak age for SSc onset is 35–55 years old, and it rarely develops in children and men younger than 35 years old.[1–4,26] Our study suggests that young age might be a characteristic of SSc with LSc.

Table 2. Demographic and clinical characteristics of SSc patients with or without LSc

		SSc with LSc (n = 8, 3.6%)	SSc without LSc (n = 212, 96.4%)	*P*-value
Onset age (years; mean ± SE)		45.3 ± 3.8	56.2 ± 0.9	<0.05
Sex (M:F) (%)		2:6 (25.0:75.0)	27:185 (12.7:87.3)	0.314
Type (dcSSc:lcSSc) (%)		4:4 (50:50)	60:152 (28.3:71.7)	0.185
Antibody	ANA (%)	87.5 (7/8)	94.8 (201/212)	0.371
	Negative (%)	25 (2/8)	5.2 (11/212)	<0.05
	Topo I (%)	37.5 (3/8)	22.2 (47/212)	0.31
	RNP (%)	12.5 (1/8)	13.7 (29/212)	0.924
	Centromere (%)	12.5 (1/8)	45.2 (96/212)	0.067
	RNAP (%)	25 (2/8)	4.2 (9/212)	<0.05
Complication	ILD (%)	37.5 (3/8)	41.5 (88/212)	0.238
	RE (%)	62.5 (5/8)	45.2 (96/212)	0.337
	PAH (%)	0 (0/8)	6.6 (14/212)	0.453

ANA, antinuclear antibody; centromere, anticentromere antibody; dcSSc, diffuse cutaneous type of SSc; ILD, interstitial lung disease; lcSSc, limited cutaneous type of SSc; LSc, localized scleroderma; negative, no antibodies detected; PAH, pulmonary artery hypertension; RE, Reflux esophagitis; RNP, ribonucleoprotein; RNAP, anti-RNA polymerase III antibody; SE, standard error; SSc, systemic scleroderma; Topo I, anti-topoisomerase I antibody.

Several autoantibodies have been detected in patients with LSc, including anti-single-stranded DNA, anti-histone, and anti-topoisomerase IIα antibodies.[27–29] These antibodies are found at a much lower frequency in patients with SSc.[30] In contrast, SSc-related autoantibodies, such as anti-topoisomerase I antibody, anticentromere antibody, and anti-RNA polymerase III antibody, are generally not detected in LSc.[30] In this study, 75% (6/8) of SSc patients with LSc were positive for SSc-related autoantibodies, suggesting that SSC with LSc might be intrinsically SSc.

With regard to the association between LSc and SSc, some believe that LSc is merely the skin involvement of SSc because it is observed in approximately 4% of SSc patients, despite LSc being a rare disease.[13] In contrast, others believe that LSc cannot be deemed the skin involvement of SSc because the progression of SSc and LSc do not correspond with each other.[31] It has been reported that esophageal and pulmonary lesions were found in 21% of patients with LSc,[32] and that immune abnormalities and internal organ involvements were significantly more prevalent among juvenile LSc patients,[33,34] suggesting that LSc may be a variant of SSc.[32–36] In our all cases, diagnoses of SSc were established within 5 years before or after the appearance of LSc, and almost all cases had SSc-related autoantibodies. This suggests that SSc with LSc might be intrinsically SSc and that LSc lesions might be the skin involvement of SSc in SSc with LSc patients.

The awareness of SSc with LSc is essential to establish an early diagnosis and to start early treatment. In addition, the characteristics of SSc with LSc, including young age and positivity of SSc-related autoantibodies, may help physicians to identify LSc patients at risk for developing SSc. We recognize that our study was limited by the small number of cases of SSc with LSc cases, so future studies should involve a larger group of patients.

CONFLICT OF INTEREST: The authors declare there are no conflicts of interest.

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Original Article

Global DNA hypomethylation and hypoxia-induced expression of the ten eleven translocation (TET) family, TET1, in scleroderma fibroblasts

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Abstract: The precise mechanisms of tissue fibrosis have not yet been elucidated in systemic sclerosis (SSc). However, studies of the regulation of DNA methylation, the most widely studied epigenetic mechanism, have confirmed the involvement of the TET family proteins, recently identified DNA demethylases, in the pathogenesis of SSc. The mRNA levels of TET family members were compared in normal and SSc fibroblasts. The effects of hypoxia and siRNA specific to HIF-1α on TET expression were also examined. Global methylation status was analysed by LUMA. The presence of 5-hydroxymethylcytosine (5hmC) in SSc was examined by immunohistochemistry. The level of TET1 mRNA in SSc fibroblasts was elevated by 1.68 fold compared with that of normal fibroblasts, but the expression levels of TET2 and TET3 were comparable between both cell types. The expression levels of DNMT1 and DNMT3B mRNA have a tendency to elevate in SSc fibroblasts. Among TET family members, the expression of TET1 was exclusively induced by hypoxia via HIF-1α-independent pathways in SSc fibroblasts, but not in normal fibroblasts. The methylation level was decreased in SSc fibroblasts relative to normal fibroblasts, and 5hmC was present in dermal fibroblasts of skin sections from patients with SSc. TET1 expression in SSc fibroblasts was abnormally regulated in the hypoxic environment and accompanied by global DNA hypomethylation, suggesting the involvement of aberrant DNA methylation in the pathogenesis of SSc.

Key words: DNA methylation – epigenetics – fibroblasts – systemic sclerosis – ten eleven translocation

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Introduction

Systemic sclerosis is a complex and heterogeneous autoimmune disease, which may involve vascular, immunological and fibrotic processes (1,2). Although the pathogenesis of SSc remains unknown, recent studies have indicated that epigenetic mechanisms such as DNA methylation and histone modifications may mediate the fibrotic manifestations of SSc (3–5). DNA methylation is the most widely studied epigenetic mechanism catalysed by DNA methyltransferases (DNMTs). It refers to the covalent attachment of a methyl group to the C5 position of cytosine residues to form 5-methylcytosine (mC) in CpG dinucleotide sequences that are called CpG islands (6). When DNA is in its methylated state, transcription is repressed through decreased binding of transcription factors and increased binding of methyl-CpG-binding domain (MBD) proteins. DNA methylation can also alter the chromatin structure and lead to the formation of a co-repressor complex. In contrast, in its unmethylated state, DNA is more permissive to transcription (7).

Another DNA modification, cytosine hydroxymethylation (hmC), is under the spotlight since the identification of the ten eleven translocation (TET) family proteins that convert 5mC to 5hmC (8). This newly discovered conversion of 5mC to 5hmC by TET proteins is so far the most important and consistent mechanism underlying the active demethylation of DNA (9). In addition to its role as an intermediate in DNA demethylation, 5hmC has been proposed to have gene regulatory functions involved in processes such as development, pluripotency and regulation of RNA splicing, albeit its role is not completely understood (10). All TET family members possess 5mC oxidation activity, but their expression levels vary depending on the cell type and tissue (11). As the roles of TET family proteins and 5hmC have been not been elucidated in dermal fibroblasts, we examined the possible involvement of TET family members in the fibrotic phenotype of SSc fibroblasts in this study.

Materials and methods

Cell culture and hypoxia culture conditions

Human dermal fibroblast culture was established from skin biopsies from dorsal forearm of either healthy donors or patients with SSc, upon informed consent and in compliance with the Institutional Review Board of Gunma University. The study was conducted according to the Declaration of Helsinki Principles. Fibroblasts were expanded by the explant culture method and then were grown in Dulbecco's modified Eagle's medium (DMEM, Nissui Seiyaku, Tokyo, Japan) supplemented with 10% foetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO_2 humidified incubator. All experiments were conducted between 4

and 6 passages. Hypoxic experiments were performed in an N_2–CO_2–O_2 incubator (APMW-36, ASTEC, Fukuoka, Japan). Human dermal fibroblasts in serum-free medium were incubated under hypoxic condition (1% O_2, 5% CO_2 and 94% N_2) for 24 h, and total RNA was extracted.

TGF-β stimulation

Normal and SSc fibroblasts were incubated in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, either treated or untreated with 10 ng/ml TGF-β for 24 h, and then, the cells were harvested.

Quantitative real-time RT-PCR analysis

Total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan). One microgram of total RNA was reverse transcribed with random hexamers using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA). Real-time PCR assays were performed using the 7300 Real-Time PCR system (Applied Biosystems). To amplify the target gene, TaqMan Gene Expression Assays (Hs00286756_m1 for TET1, Hs00758658_m1 for TET2, Hs00379125_m1 for TET3, Hs00154749_m1 for DNMT1, Hs00171876_m1 for DNMT3B, Hs00164004_m1 for collagen, type I, α1 (COL1A1) and Hs00171558_m1 for tissue inhibitor of metalloproteinase 1 (TIMP1), Applied Biosystems) were used. All samples were analysed in parallel for gene expression of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Hs99999905_m1) as an internal control. As hypoxia may affect the level of GAPDH (9), 18S rRNA (Hs99999901_s1) was used as an internal control in hypoxic experiments. Cycling condition consisted of an initial incubation at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative change in the levels of genes of interest was determined by the $2^{-\Delta\Delta C_T}$ method.

Suppression of hypoxia-inducible factor (HIF)-1α by siRNA

Oligonucleotides specific to HIF-1α (5′-UGUGAGUUCGCAUC UUGAUTT-3′ and 5′-AUCAAGAUGCGAACUCACATT -3′) were obtained from Japan Bio Services (Saitama, Japan). For annealing, 20 μM of oligonucleotides was incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) for 2 min at 95°C and then was gradually cooled to room temperature. Cells were transfected with the annealed oligonucleotides at the concentration of 0.2 μM using Lipofectamine 2000 reagent (Invitrogen) according to the method described previously (12). After 24 h, the cells were exposed to the hypoxic condition described above for another 24 h, and then, the cells were harvested. To remove the effect of serum, cells were serum-starved for the last 24 h.

Western blot analysis

The total cellular protein was extracted in extraction buffer (RIPA buffer, protease inhibitor, phosphatase inhibitor, 5 mM EDTA; Thermo, Rockford, IL, USA). Protein concentration was determined by Bradford protein assay (Bio-Rad, Hercules, CA, USA). Equal amounts (10 μg) of total protein samples were separated by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk in Tris-buffered saline containing 0.05% Tween 20 for 1 h at room temperature, followed by incubation with anti-HIF-1α monoclonal antibody (1:250; BD Biosciences, San Jose, CA, USA) in blocking solution at 4°C for 12 h. To control for protein loading, blots were probed for β-actin expression using monoclonal anti-β-actin antibody (Sigma, Saint Louis, MO, USA). After incubation with peroxidase-conjugated goat anti-mouse immunoglobulins (1:2000; Dako, Carpinteria, CA, USA) in blocking solution for 1 h at room temperature, the signals were visualized using the ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK).

Genomic DNA extraction and measurement of global DNA methylation

Genomic DNA was isolated from normal and SSc fibroblasts, using a DNeasy blood & Tissue Kit (Qiagen Valencia, CA). A Luminometric-based assay (LUMA) was performed according to the methods described by Karimi et al. Briefly, 300 ng of genomic DNA was digested for 4 h by *Hpa*II + *Eco*RI or *Msp*I + *Eco*RI in 2 separate reactions, which were set up in a 96-well plate format. Then, 15 μl annealing buffer was added to the digestion product, and samples were analysed with a PyroMark Q24 system (Qiagen). Methylation was estimated from the 1 – (*Hpa*II/*Eco*RI)/(*Msp*I/*Eco*RI) ratios.

Immunohistochemistry

Sections (5-μm thick) were deparaffinized with Hemo-De (Falma, Tokyo, Japan) and rehydrated through a graded series of ethanol. Epitope retrieval was performed in Target Retrieval Solution (Dako) with a pressure cooker (1.2 kg/cm^2) at 121°C for 10 min. Endogenous peroxidase was blocked by incubation in peroxidase blocking solution (Dako) for 5 min, followed by incubation with Protein Block Serum-Free (Dako) for 10 min. The sections were then incubated overnight at 4°C with 5-hydroxymethylcytidine rabbit pAb (diluted 1:1000 in Protein Block Serum-Free; Active-motif, Carlsbad, CA, USA), followed by incubation for 1 h with HRP-conjugated secondary antibody solution (Histofine Simple Stain MAXPO(R), Nichirei Biosciences). Normal rabbit IgG (1 μg/ml, diluted in Protein Block Serum-Free, Santa Cruz) was used for the negative control sections. Diaminobenzidine (EnVision + Kit/HRP (DAB), Dako) was used for visualization before counterstaining with haematoxylin.

Immunofluorescence staining

Fibroblasts were plated onto culture slide (BD Bioscience) and incubated at 37°C 24 h. The cells were fixed in 4% paraformaldehyde for 60 min at room temperature and then permeabilized and blocked in blocking buffer (0.1% TritonX, 5% goat serum, 3% skim milk in TBST) for 60 min at room temperature. Cells were incubated overnight at 4°C with TET1 rabbit antibody (diluted 1:1000 in blocking buffer, GeneTex, Irvine, CA, USA), followed by incubation with Alexa fluor 488 goat anti-rabbit (diluted 1:250 in PBS; Life Technologies, Carlsbad, CA, USA) for 1 h at room temperature and then mounted in ProLong Gold (Life Technologies).

Statistics

P values were calculated using Mann–Whitney U-test, Welch's t-test or Student's t-test as appropriate. *P* values <0.05 were considered significant. Error bars represent standard deviation, and numbers of experiments (n) are as indicated.

Results

Expression of TET1 is elevated in SSc fibroblasts

We examined the expression levels of the TET family members in dermal fibroblasts from healthy control and patients with SSc (normal and SSc fibroblasts, respectively). Fibroblasts from skin lesions generally exhibit the SSc phenotype in the early stage of

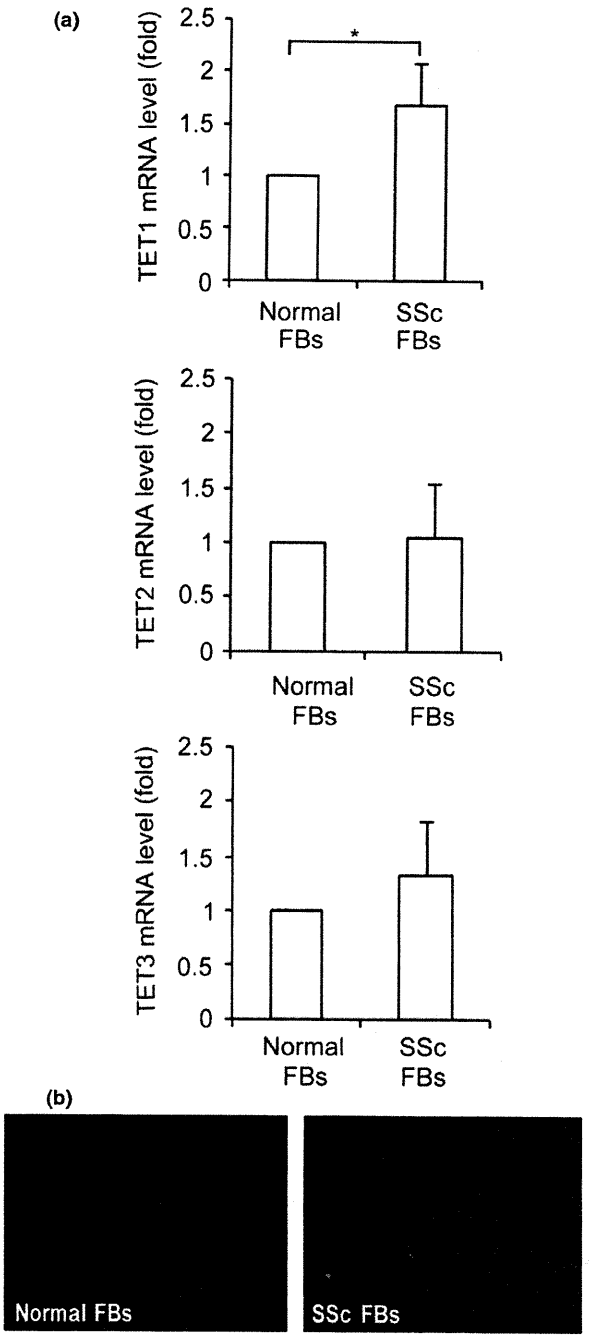

Figure 1. The mRNA expression of TET family members in normal and SSc fibroblasts. (a) In normal and systemic sclerosis (SSc) fibroblasts (FBs), mRNA levels of TET family members were determined by quantitative RT-PCR. To verify the SSc phenotype in SSc FBs, the higher expression levels of both COL1A1 and TIMP1 mRNA were confirmed before the analysis. Values are normalized relative to normal FBs (arbitrarily set as 1) and means ± SD of five independent cell lines are shown. (n = 5) *P < 0.01 (Mann–Whitney U-test) versus normal FBs. (b) Immunofluorescence staining was performed using antibody against TET1. TET1 was overexpressed in SSc fibroblasts than normal fibroblasts.

in vitro culture; however, not all cell lines from patients show the phenotype (13). Thus, we confirmed 5 cell pairs, in which SSc fibroblasts expressed higher levels of both COL1A1 and TIMP1 mRNA than normal fibroblasts (1.76 ± 0.55 fold (P = 0.015) and 2.92 ± 1.85 fold (P = 0.049), respectively). The level of TET1 mRNA in SSc fibroblasts was elevated by 1.68 fold compared with that of normal fibroblasts, whereas the expression levels of TET2 and TET3 mRNA were comparable between SSc and normal fibroblasts (Fig. 1a). Additionally, in immunofluorescence staining, TET1 was overexpressed in SSc fibroblasts than normal fibroblasts (Fig. 1b).

Hypoxia upregulates TET1 mRNA expression in SSc fibroblasts via HIF-1α-independent mechanisms

As tissue hypoxia due to peripheral vasculopathy is a characteristic feature of SSc and might contribute to the progression of the disease (14), we examined the effect of hypoxia on the expression of TET family members in fibroblasts. Normal and SSc fibroblasts were exposed to hypoxic conditions (1% oxygen for 24 h), and the expression levels of mRNA were examined by quantitative RT-PCR. As shown in Fig. 2a, hypoxia treatment increased TET1 expression by 1.32 fold in SSc fibroblasts, but not in normal fibroblasts (P < 0.05). Hypoxic conditions did not affect the expression levels of TET2 and TET3 in either normal or SSc fibroblasts (Fig. 2a). To examine the involvement of HIF-1α in the hypoxia-induced TET1 expression, the expression of HIF-1α was suppressed using siRNA under hypoxic conditions (Fig. 2b). Suppression of HIF-1α did not restore the elevated expression of TET1 (Fig. 2b), suggesting hypoxia-induced TET1 expression was HIF-1α-independent in SSc fibroblasts.

To investigate whether TET1 mRNA expression in fibroblasts is dependent on TGF-β

Hypoxia is known to induce TGF-β and the pro-fibrotic effects are mainly mediated by TGF-β. We analysed whether the hypoxia-induced regulation of TET1 is dependent on TGF-β. When TGF-β was added to SSc fibroblasts under hypoxic condition, the expression of TET1 did not change (Figure S1). Therefore, we presume that the hypoxia-induced regulation of TET1 is not dependent on TGF-β. On the other hand, as shown in Figure S1, when TGF-β was added to normal fibroblasts under hypoxic condition, the expression of TET1 increased.

Global DNA methylation was decreased in SSc fibroblasts

The recent discovery that the three members of the TET protein family can convert 5mC into 5hmC has provided a potential mechanism for DNA demethylation. In this context, we compared the level of global DNA methylation in SSc fibroblasts with that in normal fibroblasts using LUMA. As shown in Fig. 3a, the methylation level was significantly decreased in SSc fibroblasts relative to normal fibroblasts (P < 0.05). The oxidation of pre-existing 5mC by the TET protein family is thought to be the only route for the synthesis of genomic 5hmC. Immunohistochemically, the presence of 5hmC was detected in spindle-shaped cells in sclerotic dermis of patients with SSc (Fig. 3b), suggesting that TET1 may catalyse the oxidation of 5mC in dermal fibroblasts of patients with SSc.

Expression of DNMT1 and DNMT3B has a tendency to elevate in SSc fibroblasts

We examined the expression levels of the DNMTs in normal and SSc fibroblasts. The levels of DNMT1 and DNMT3B mRNA in SSc fibroblasts have a tendency to elevate in SSc fibroblasts compared with normal fibroblasts (Fig. 4).

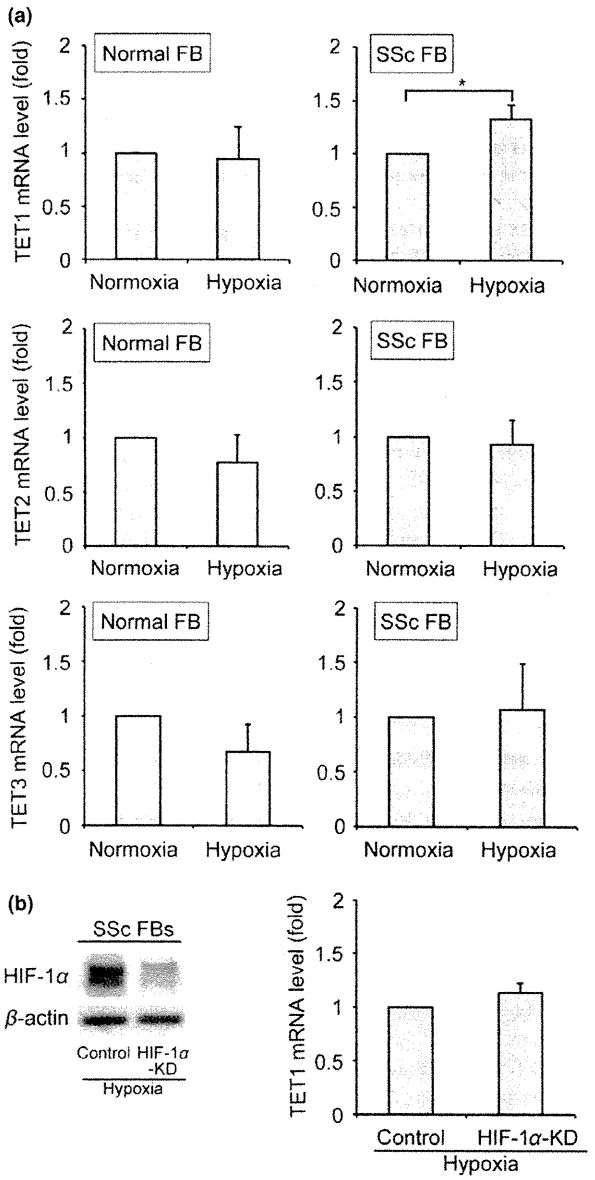

Figure 2. Hypoxia-induced TET1 expression in SSc fibroblasts via HIF-1α-independent mechanisms. (a) Normal and SSc fibroblasts were exposed to hypoxic conditions in 1% oxygen for 24 h, and mRNA levels of TET family members were determined by quantitative RT-PCR. Values are normalized relative to normoxia (arbitrarily set as 1), and means + SD of three independent experiments are shown. ($n = 3$) $^*P < 0.05$ (Welch's t-test) versus normoxia. (b) SSc fibroblasts were transfected with either 0.2 μM HIF-1α siRNA (HIF-1α-KD) or a corresponding concentration of MOCK siRNA (control) on the day of cell seeding. Twenty-four hours later, cells were serum-starved and exposed to the hypoxic conditions for another 24 h, and then, the cells were harvested. The level of HIF-1α protein was determined by Western blot. We used β-actin as an internal control. Representative data of three independent experiments are shown. mRNA levels of TET1 were determined by quantitative RT-PCR. Means + SD of three independent experiments are shown, with values normalized to control (arbitrarily set at 1).

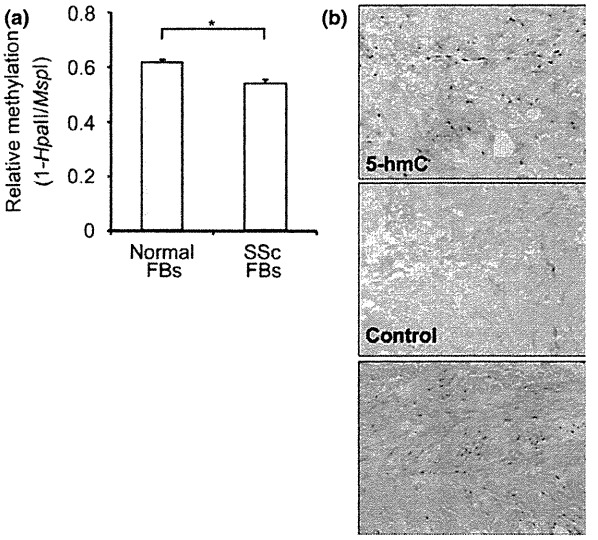

Figure 3. Global DNA methylation in normal and SSc fibroblasts and immunodetection of 5hmC in the skin from patients with SSc. (a) Genomic DNA was isolated from normal and SSc fibroblasts, and then, 300 ng of DNA was analysed by a Luminometric-based assay (LUMA). Methylation was estimated from the 1 – (*HpaII*/*EcoRI*)/(*MspI*/*EcoRI*) ratios. Means +/- SD of three independent cell lines are shown. ($n = 3$) $^*P < 0.05$ (Student's t-test) (b) immunodetection of 5hmC in the skin from patients with SSc. Sections (5-μm thick) were stained with either the specific antibody against 5hmC or normal rabbit IgG (control). The nuclei were stained with haematoxylin. The corresponding haematoxylin–eosin-stained section is shown in the lower panel. Original magnification is 400-fold.

Discussion

DNA methylation is the most widely studied epigenetic mechanism in autoimmune diseases (6), and aberrant methylation patterns have been demonstrated in peripheral lymphocytes or dermal fibroblasts from patients with SSc (3,15–18). In SSc fibroblasts, there was a hypermethylation of CpG islands in the Fli1 promoter, which is a transcription factor that inhibits collagen synthesis (3). The reduced expression of Fli1 increases collagen production, resulting in the tissue fibrosis characteristic of the disease (19,20). On the other hand, $CD4^+$ T cells' DNA from patients with SSc, like that of systemic lupus erythematosus (SLE) patients, was significantly hypomethylated (15). In $CD4^+$ T cells, regulatory elements of the genes, such as CD40L and CD70, which were up-regulated in patients with SSc, were also found to be demethylated (16,17). As the hypomethylation status has received attention in other autoimmune diseases such as SLE and rheumatoid arthritis (RA) (6), the importance of demethylation mechanisms has been recognized.

The tight regulation of DNA methylation patterns is important to ensure proper embryogenesis and prevent the development of disease. However, no direct DNA demethylase had been identified until the recent finding that the TET1 protein can catalyse the conversion of 5mC into 5hmC, being a potential mechanism for active demethylation (8,21). To our knowledge, this is the first report showing the global hypomethylation in SSc fibroblasts, consistent with the observed TET1 overexpression. It should be noted that 5hmC was present in fibroblasts of the skin sections from patients with SSc. A number of cancer cells also display aberrant DNA methylation patterns including both global hypomethylation of the genome- and promoter-specific hypermethylation (22). Global hypomethylation is believed to create genomic instability, whereas hypermethylation of CpG islands at gene promoters can lead to undesirable silencing of genes (22). Taken together with the hypermethylation status of the Fli1 gene promoter in SSc

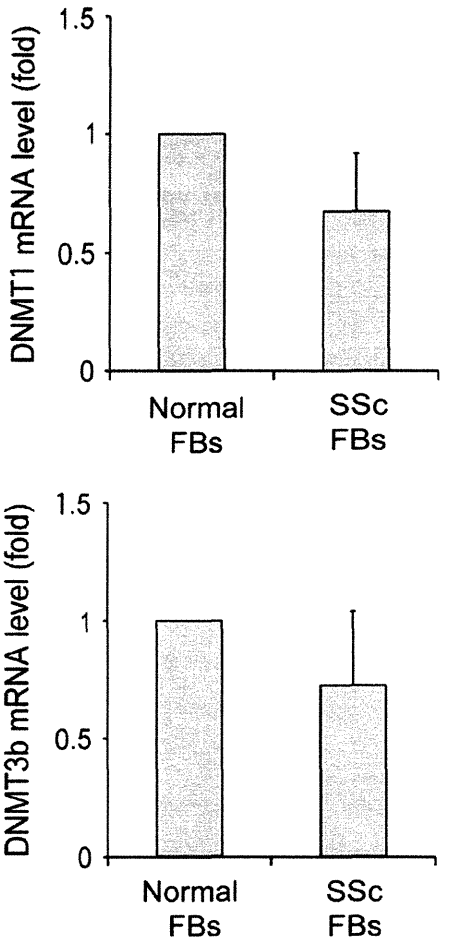

Figure 4. The mRNA expression of DNMTs in normal and SSc fibroblasts. In normal and SSc fibroblasts, mRNA levels of DNMTs were determined by quantitative RT-PCR. Values are normalized relative to normal fibroblasts (arbitrarily set as 1), and means + SD of three independent experiments are shown. (n = 3).

fibroblasts (3), the methylation status of SSc fibroblasts observed in this study should be important in shedding light on the relationship between aberrant DNA methylation and the fibrotic phenotype of SSc fibroblasts.

The expression levels of TET family members (TET1-3) are different depending on the cell type and tissue, suggesting that these proteins may play non-overlapping biological functions in a developmentally regulated and tissue-specific manner (9). All members show 5mC oxidation activity, and 5hmC can be further oxidized by TET proteins to produce 5-formylcytosine (fC) and 5-carboxylcytosine (caC)(9). In our study, SSc fibroblasts expressed a higher level of TET1, but comparable levels of TET2 and TET3 to normal fibroblasts. The levels of DNMT1 and DNMT3B mRNA in SSc fibroblasts have a tendency to elevate in SSc fibroblasts compared with normal fibroblasts. In addition, TET1 expression was exclusively responsive to hypoxic conditions in SSc fibroblasts, indicating the specific involvement of TET1 in the cutaneous fibrosis in patients with SSc. To clarify the role of TET1 in the regulation of epigenetic mechanisms, 5hmC as well as 5fC and 5caC should be detected in the genomic DNA of SSc fibroblasts in future studies.

Physiological mechanisms to overcome tissue hypoxia are impaired and dysregulated in SSc. Namely, microangiopathy with impaired angiogenesis and excessive accumulation of extracellular matrix may cause severe tissue hypoxia in SSc. Hypoxia could stimulate the production and accumulation of extracellular matrix, resulting in a vicious circle of hypoxia and fibrosis (14). While hypoxia has a wide range of molecular effects on cells through both HIF-1α-dependent as well as HIF-1α-independent manners (23), the response to hypoxia in SSc might be driven by HIF-1α-independent pathways (10). On the other hand, epigenetic modifications respond to endogenous and exogenous stimuli, as well as environmental signals (24), and hypoxia can cause chromatin alteration including DNA methylation (25). The finding in this study that TET1 was upregulated in SSc fibroblasts but not in normal fibroblasts under hypoxic conditions through HIF-1α-independent mechanisms suggests that epigenetic alteration through catalysation by TET1 in the hypoxic condition might contribute to the pathogenesis of SSc.

The exact aetiology of SSc remains unknown; however, the role of epigenetic modifications caused by environmental factors has been intensively studied in relation to the pathogenesis of this disease (4). Epigenetics accounts for chromatin-based mechanisms important in the regulation of gene expression that do not involve changes to the DNA sequence *per se*. One of the most intriguing characteristics of epigenetics is the reversibility of its modifications. Through controlling DNA methylation, novel therapies could emerge in the near future. TET1 can be a new target to better understand the involvement of epigenetic modifications in the pathogenesis of SSc and to develop new therapeutic strategies.

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Conflicts of interest

The authors have declared no conflicting interests.

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Supporting Information

Additional supporting data may be found in the supplementary information of this article.

Figure S1. To investigate whether TET1 mRNA expression in fibroblasts is dependent on TGF-β

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Sildenafil attenuates the fibrotic phenotype of skin fibroblasts in patients with systemic sclerosis☆

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ABSTRACT

Systemic sclerosis (SSc) is a multi-organ fibrotic disease that affects the skin and various internal organs. Therapeutic strategies for tissue fibrosis have not been established; however, aberrantly activated fibroblasts in affected lesions are key targets for modulating fibrosis. Recently, increased intracellular cyclic GMP (cGMP) levels were demonstrated to improve fibrosis levels in various diseases. The purpose of this study was to assess the anti-fibrotic properties of cGMP in cultured fibroblasts from patients with SSc. The phosphodiesterase (PDE) 5 inhibitor sildenafil increased the intracellular cGMP levels in skin fibroblasts in a dose-dependent manner. Sildenafil treatment also significantly decreased the expression of several pro-fibrotic factors that were upregulated by TGF-β1 treatment in SSc skin fibroblasts. These inhibitory effects occurred via non-canonical TGF-β signaling. Our findings revealed that sildenafil might be a novel strategy to treat tissue fibrosis and vasculopathy in SSc.

1. Introduction

Systemic sclerosis (SSc) is a connective tissue disease that is characterized by inflammation, vasculopathy and fibrosis [1–4]. Tissue fibrosis directly contributes to increased mortality and decreased quality of life [5,6]. However, an effective therapy for tissue fibrosis has not been established. Aberrantly activated fibroblasts in areas affected by SSc, which play a central role in the production and remodeling of collagen and other extracellular matrix (ECM) components, are thought to be key therapeutic targets [1].

Skin fibrosis is one of the most characteristic SSc traits, and transforming growth factor-β (TGF-β) is a main pro-fibrotic mediator in skin fibrosis [1,2,7–10]. Raynaud's phenomenon, which is caused by endovascular damage and decreased blood flow in the digital vein, precedes skin fibrosis in most cases [2,11], suggesting that vasculopathy triggers or worsens skin fibrosis; thus, treating vasculopathy might have the simultaneous effect of ameliorating fibrosis [12]. Nitric oxide (NO) is a potent vasodilator that is endogenously synthesized from L-arginine in the presence of NO synthase (NOS) [13,14]. Some of the key biological actions of NO, including vasodilation, are mediated by the downstream signaling molecules soluble guanylate cyclase (sGC) and cyclic GMP (cGMP) [14], which has led to an experimental focus on the vasodilative effects of the NO/sGC/cGMP pathway [15–17]. cGMP is a key mediator in NO/sGC/cGMP signaling, and its action is mediated by cGMP-dependent protein kinase (PKG) and cGMP-gated channels [14]. Cyclic nucleotide phosphodiesterases (PDEs) are a group of enzymes that degrade the phosphodiester bond in cAMP and cGMP to a linear, inactive form [9]. There are at least 11 PDE isoforms (PDEs 1 to 11), and each isoform has a different affinity for cAMP and cGMP [18]. PDE5 is a cGMP-specific enzyme with 3 isoforms (PDE5A-C) [19]. PDE5 is abundant in vascular smooth muscle cells, and PDE5 inhibition has a potent vasodilatory effect. Therefore, various PDE5 inhibitors such as sildenafil and tadalafil have been developed and have demonstrated excellent outcomes for the treatment of SSc-related pulmonary arterial hypertension (PAH) and digital ulcers [15–18]. Sildenafil binds to the active catalytic site of PDE in a competitive manner and blocks the hydrolyzing activity of PDE5 and to a lesser extent, PDE1 and PDE6 [20].

It has also recently been observed that NO/sGC/cGMP signaling exerts anti-fibrotic actions [21–26] and that sGC stimulation reportedly reverses the fibrotic phenotype in SSc and other fibrotic disorders in vivo and in vitro by increasing intracellular cGMP levels [12,27,28]. PDE5 inhibitors also reportedly have anti-fibrotic effects in models of various fibrotic disorders [26,29,30]. These results suggest that PDE5 inhibitors might exhibit pleiotropic effects on vasculopathy and fibrosis related to SSc. The purpose of this study was to assess the anti-fibrotic properties of cGMP in cultured skin fibroblasts from patients with SSc.

2. Materials and methods

2.1. Cell culture

Healthy skin fibroblasts were purchased from Takara Bio (Otsu, Japan) and Kurabo (Tokyo, Japan). SSc skin fibroblasts were obtained

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from skin biopsies of the affected areas of 5 patients with SSc. All of the patients fulfilled the criteria for SSc, as proposed by the American College of Rheumatology (Subcommittee for Scleroderma, Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee, 1980), and had received no medical interventions such as corticosteroids or immunosuppressive agents. The profiles of SSc patients enrolled in this study are summarized in Table 1. This study was approved by the Tokyo Women's Medical University ethics committee. We informed all of the participants of the content of this study, and written consent was obtained. Fibroblasts were cultured as previously described [31]. The fibroblasts used were at passages 3–5.

2.2. Immunofluorescence staining

Skin fibroblasts were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 at room temperature, and then washed three times with phosphate-buffered saline (PBS). Nonspecific binding in cells was blocked with a blocking solution (Dako, Glostrup, Denmark), and then the slides were incubated with FITC-conjugated anti-αSMA antibodies (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. After washing three times with PBS, the chamber slides were mounted and assessed by immunofluorescence microscopy (Keyence, Osaka, Japan).

2.3. Measurement of intracellular cGMP levels

Skin fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA) in the presence or absence of sildenafil. Cell lysates were centrifuged at 2000 rpm at 4 °C, and the supernatants were collected to measure the intracellular cGMP levels using a commercially available enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA).

2.4. RNA extraction and quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Cultured skin fibroblasts were incubated with serum-free DMEM for 24 h. After serum starvation, fibroblasts were treated with the specific PDE5 inhibitor sildenafil (kindly provided by Pfizer, New York, NY, USA) for 1 h prior to the administration of 10 ng/ml TGF-β1 (R&D Systems, Minneapolis, MN, USA). Total RNA was extracted using an RNA extraction kit (Life Technologies) according to the manufacturer's instructions. Equal amounts of total RNA were reverse-transcribed to generate cDNA using a commercially available cDNA synthesis kit (Life Technologies). Real-time PCR analysis was performed using TaqMan Gene Expression Assays (Life Technologies) for *COL1A1*, *COL1A2*, *CTGF*, *ACTA2* and *GAPDH* mRNA. *GAPDH* mRNA was used as a loading control. Relative mRNA expression levels were calculated using the delta delta Ct method.

2.5. Western blotting

Cells were preincubated with 100 μM sildenafil for 1 h and then treated with 10 ng/ml TGF-β1. Cell lysates were collected with lysis buffer (Life Technologies) containing a phosphatase/proteinase inhibitor cocktail (Thermo Scientific, Rockford, IL, USA), and the supernatants were clarified by centrifugation. Protein concentrations were measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific), and the samples were boiled with loading buffer at 70 °C for 10 min. Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 8–12% Tris-glycine gels (Life Technologies), and proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. The primary antibodies used in this study included anti-phospho-p38, anti-p38, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-Smad3, anti-Smad3 (Cell Signaling Technology, Danvers, MA, USA), anti-type I collagen (Southern Biotech, Birmingham, AL, USA), anti-CTGF, anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-αSMA antibodies (Sigma-Aldrich). Immunodetection was accomplished by incubating the membranes with HRP-conjugated secondary antibodies (Medical and Biological Laboratories, Nagoya, Japan). The samples were visualized using a Chemiluminescence Blotting kit (WAKO, Osaka, Japan). All of the bands were imaged, and the blot density was measured using ImageJ software (NIH).

Table 1
Profile of SSc patients who were enrolled in the study. Scl-70 = anti-Scl-70 antibodies.

Patient	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age (yrs)	72	58	59	36	34
Gender	Female	Female	Female	Female	Female
Disease duration (ms)	31	12	35	6	12
Disease subset	Diffuse	Diffuse	Diffuse	Diffuse	Diffuse
Autoantibody	Scl-70	Scl-70	Scl-70	Scl-70	Scl-70

2.6. Statistical analysis

The results are expressed as the mean ± standard deviation (SD). Measurements were performed in triplicate for quantitative RT-PCR or in duplicate for EIA. Significant differences between the treatments were calculated by one-way analysis of variance (ANOVA) followed by Student's *t*-test for comparisons between groups. The data were analyzed using JMP software (version 10). p values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Sildenafil treatment increased intracellular cGMP levels

Sildenafil reportedly has a relatively high affinity for phosphodiesterase (PDE) 1A and PDE5A [20]. Immunohistochemistry was performed to detect PDE1A and PDE5A expression in human skin fibroblasts, and as expected, both healthy and SSc fibroblasts expressed PDE1A and PDE5A (data not shown).

Next, we examined the basal intracellular cGMP levels in healthy and SSc fibroblasts and observed that the basal intracellular cGMP levels were significantly higher in SSc fibroblasts (Fig. 1A). Furthermore, to investigate the effects of sildenafil on cGMP concentrations, we measured dose- and time-dependent changes in the intracellular cGMP levels in the presence or absence of sildenafil. Sildenafil (100 μM) significantly increased the intracellular cGMP levels in SSc skin fibroblasts compared with untreated fibroblasts ($p < 0.05$), and the cGMP levels were reduced in a time-dependent manner (Fig. 1B). These effects were not observed

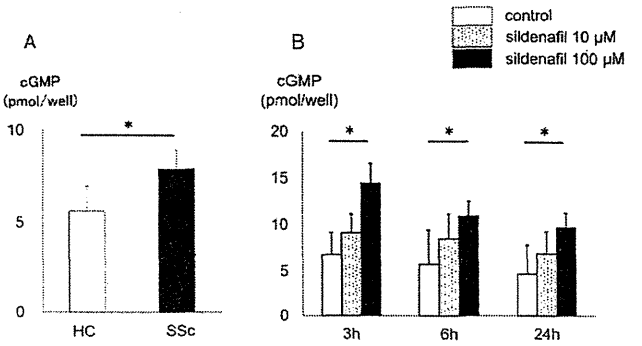

Fig. 1. Intracellular cGMP levels in skin fibroblasts. (A) Basal cGMP levels in healthy skin. fibroblasts (HC) and SSc skin fibroblasts. (B) SSc skin fibroblasts were stimulated. with 10 and 100 μM sildenafil for 3, 6 or 24 h. Representative data from three. independent experiments are shown. * = $p < 0.05$.

in healthy controls who had been treated with sildenafil (data not shown).

3.2. Sildenafil attenuated the profibrotic phenotype of SSc skin fibroblasts

To evaluate the anti-fibrotic potency of sildenafil, we measured the expression of several fibrotic factors in skin fibroblasts using quantitative real-time RT-PCR analysis. Unexpectedly, sildenafil treatment did not decrease the expression of fibrotic factors in healthy or SSc fibroblasts (data not shown). Next, we assessed the effects of sildenafil on the expression of fibrotic factors in the presence of TGF-β1. Fibroblasts were pretreated with various concentrations of sildenafil 1 h prior to the addition of 10 ng/ml TGF-β1. TGF-β1 significantly increased the expression of *COL1A1*, *COL1A2* and *CTGF* mRNA compared with untreated controls at 48 and 72 h after treatment ($p < 0.05$; Figs. 2, 3), and 100 μM sildenafil significantly downregulated *COL1A1*, *COL1A2* and *CTGF* mRNA in SSc fibroblasts that had been treated with TGF-β1 ($p < 0.05$; Fig. 3). Intriguingly, the anti-fibrotic effects of sildenafil did not influence skin fibroblasts that had been derived from healthy individuals at 48 or 72 h (Fig. 2).

αSMA staining is a marker for myofibroblasts and activated fibroblasts in SSc patients [1]. To further examine the effects of sildenafil on the pro-fibrotic properties of skin fibroblasts, we investigated the expression of αSMA protein and its encoding gene *ACTA2*. Sildenafil strongly downregulated *ACTA2* mRNA expression in both healthy and SSc skin fibroblasts ($p < 0.05$; Fig. 4A). Immunohistochemical analysis of αSMA indicated that TGF-β1-driven αSMA overexpression was suppressed by 100 μM sildenafil (Fig. 4B).

Sildenafil also significantly suppressed TGF-β1-driven type I collagen, CTGF and αSMA protein expression 72 h after treatment, as detected by Western blotting ($p < 0.05$; Fig. 5).

3.3. Sildenafil blocked non-canonical TGF-β signaling

TGF-β signal transduction is divided into two pathways: the Smad cascade and the non-Smad cascade [9]. Both Smad- and non-Smad-dependent TGF-β pathways have been extensively investigated in SSc skin fibroblasts, and both are associated with fibrosis. The MAPK cascade, which includes p-38 and ERK1/2, is involved in the latter pathway, and their blockade can inhibit fibrosis in SSc fibroblasts [32,33]. To clarify the inhibitory action of sildenafil in TGF-β signaling, SSc fibroblasts were incubated with sildenafil in the presence of TGF-β1, and cell lysates were collected for immunoblotting. Sildenafil did not influence Smad 3 phosphorylation (Fig. 6A). In the non-Smad cascade, sildenafil significantly reduced TGF-β1-induced p38-MAP kinase and ERK1/2 phosphorylation ($p < 0.05$; Figs. 6B and 6C). These results suggest that sildenafil suppresses the non-Smad signaling pathway.

4. Discussion

In this study, we demonstrated the anti-fibrotic effects of sildenafil on cultured SSc fibroblasts. Sildenafil is a PDE5 inhibitor and a standard therapeutic agent for pulmonary arterial hypertension due to its strong vasodilation effects. Endothelial cell (EC) dysfunction is responsible for various vascular diseases including SSc [2,9,30]. ECs regulate vascular tone by balancing vasoconstrictors (e.g., ET-1) and vasodilators (e.g., NO) [13,14,34,35]. Because EC impairment in SSc causes ET-1 overexpression and reduced NO production, which leads to endothelial damage in SSc, improving the imbalance using endothelin receptor antagonists (ERAs) and PDE5 inhibitors can be effective [15–18,35]. Vascular abnormalities also result in chronic exposure to hypoxia and tissue ischemia, which leads to tissue fibrosis [1,2,8,36]. ET-1 overexpression has also been demonstrated in lesioned areas of SSc skin fibroblasts, which suggests that common pathogenic factors exist in fibroblasts and ECs and that their blockade might have simultaneous effects. In our opinion, the data from this study demonstrate for the first time that PDE5 inhibition can potently reverse the fibrotic phenotype of skin fibroblasts obtained from lesioned areas of patients with SSc by interfering with the pro-fibrotic effects of TGF-β. Furthermore, sildenafil treatment blocked the Smad-independent MAPK pathway, which was consistent with a previous report [37].

Although the sGC stimulator and the PDE5 inhibitor demonstrated similar activity to increase intracellular cGMP levels, there are some differences in their chemical properties. First, the sGC stimulator is NO-independent, whereas the PDE5 inhibitor is ineffective when NO levels are low and requires an NO donor, which often causes unfavorable

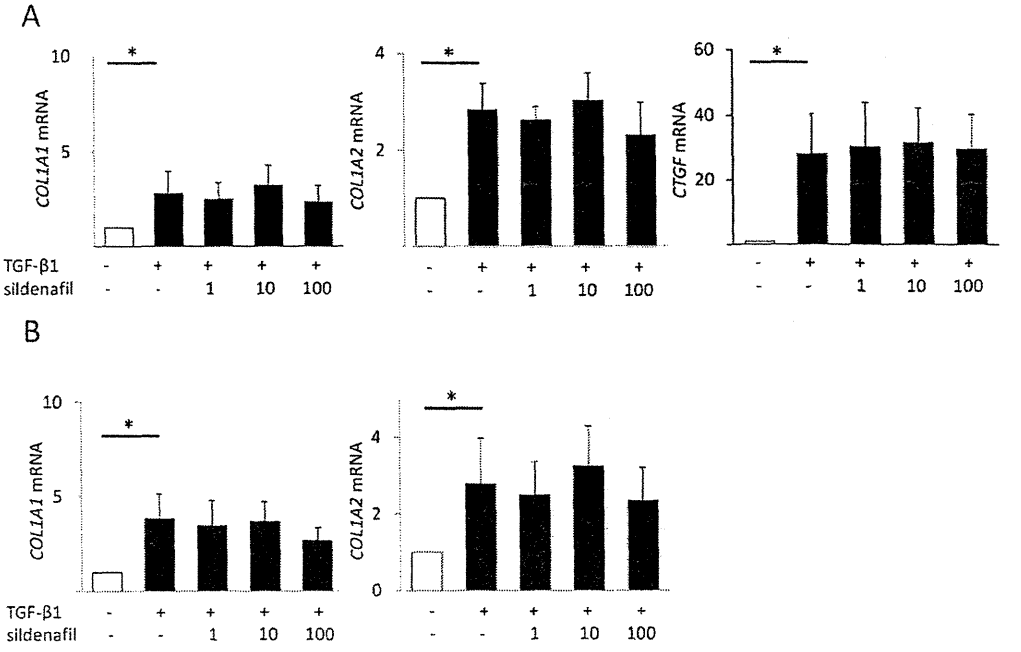

Fig. 2. Effects of sildenafil on healthy skin fibroblasts (n = 3). Healthy human skin fibroblasts were treated with 1–100 μM sildenafil in the presence of 10 ng/ml TGF-β1. for 48 (A) or 72 h (B). *COL1A1*, *COL1A2* and *CTGF* mRNA levels were measured by quantitative RT-PCR. Representative data from three independent experiments are shown. * = $p < 0.05$.

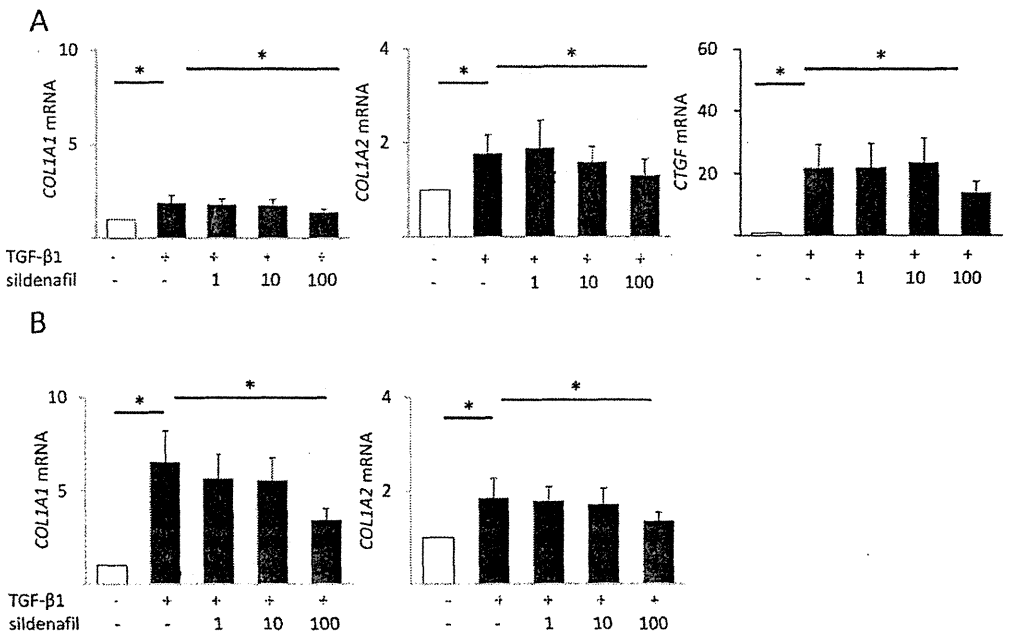

Fig. 3. Sildenafil attenuated the expression of pro-fibrotic factors in SSc skin fibroblasts. (n = 5). (A–B) Changes in the level of pro-fibrotic genes in SSc skin fibroblasts. SSc skin. fibroblasts were treated with 1–100 μM sildenafil in the presence of 10 ng/ml TGF-β1 for 48. or 72 h (B). The expression of *COL1A1*, *COL1A2* and *CTGF* mRNA was measured using. quantitative RT-PCR. Representative data from three independent experiments are shown. * = $p < 0.05$.

adverse effects. We demonstrated that basal intracellular cGMP levels in SSc skin fibroblasts are significantly higher than those in healthy skin fibroblasts, which is in agreement with a previous report [38]. This finding appears to conflict with our result that increased intracellular cGMP levels might have an anti-fibrotic effect. One hypothesis is that the expression of inducible NO synthase (iNOS) might be upregulated in the lesioned areas of SSc skin fibroblasts, thus resulting in increased intracellular cGMP levels [39]. In contrast, NO and its metabolites (e.g., peroxynitrate) cause oxidative stress and DNA damage, and they convert fibroblasts to a myofibroblast-like phenotype with fibrotic activity [40,41]. Second, cGMP is also produced by the natriuretic peptide (NP)/particulate guanylyl cyclase (pGC) pathway, but sGC stimulators do not activate that pathway [42]. However, the contribution of the NP/pGC pathway to cGMP production is unknown, given that the levels of serum natriuretic peptides such as BNP in SSc patients tend to be higher than those in healthy individuals, and PDE5 inhibitors might be more potent cGMP-induction agents than sGC stimulators [43]. In light of these points, the combination of a sGC stimulator and PDE5 inhibitor for fibrosis treatment might have additive effects, and this treatment paradigm will be a challenge for future research.

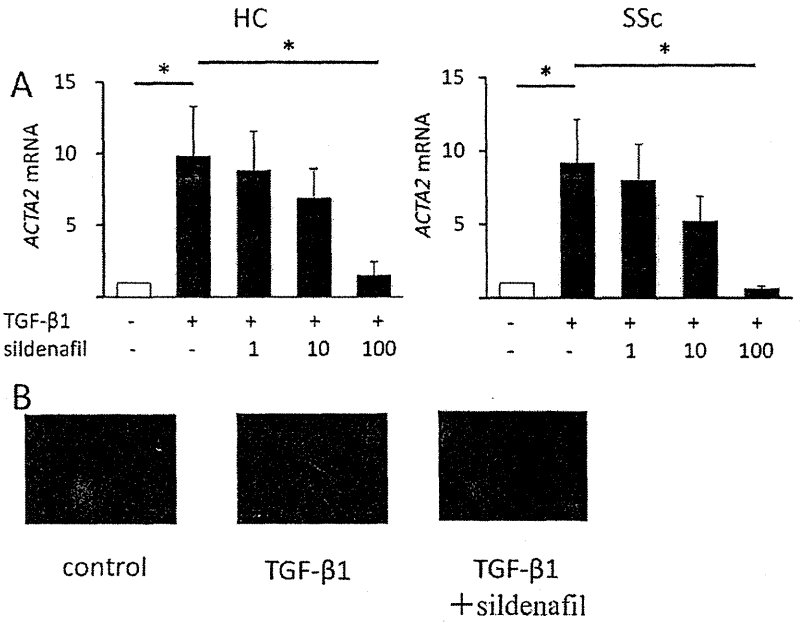

Fig. 4. Effects of sildenafil on αSMA expression in healthy (n = 3) and SSc (n = 5) skin. fibroblasts. Fibroblasts were treated with 1–100 μM sildenafil in the presence of 10 ng/ml. TGF-β1 for 72 h, and αSMA (*ACTA2*) expression was assessed using quantitative. RT-PCR (A) and immunofluorescence (B). For immunofluorescence, 100 μM sildenafil. was added in the presence or absence of 10 ng/ml TGF-β1. Representative data from. three independent experiments are shown. * = $p < 0.05$.

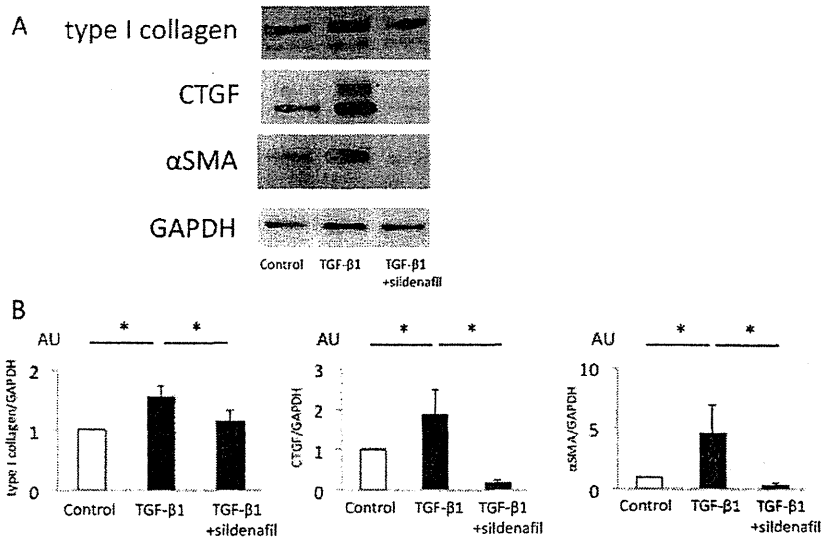

Fig. 5. Western blotting analysis of pro-fibrotic proteins. SSc skin fibroblasts. were incubated with 100 μM sildenafil with or without 10 ng/ml TGF-β1 for 72 h, and. equal amounts of cell lysate were immunoblotted for type I collagen, CTGF, αSMA and GAPDH. Representative images are shown in (A), and blot density was. measured (B). Representative data from three independent experiments are shown. * = $p < 0.05$.

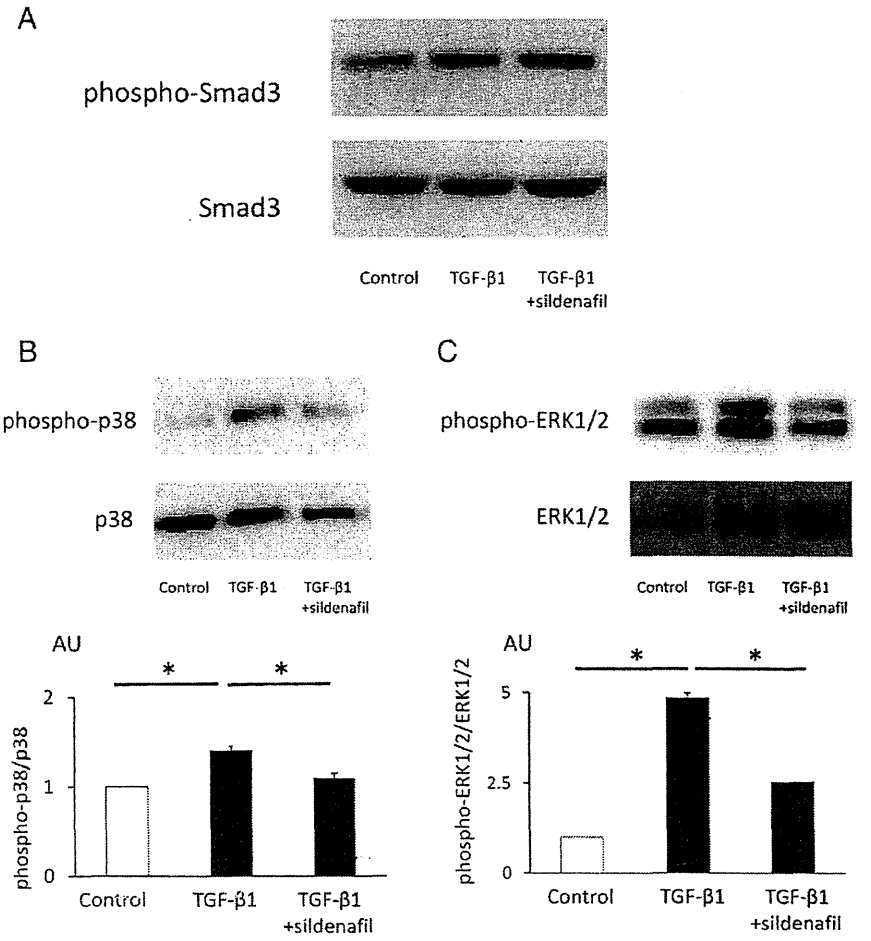

Fig. 6. The anti-fibrotic effects of sildenafil on SSc skin fibroblasts (n = 3) were mediated. via non-canonical TGF-β signaling. SSc skin fibroblasts were incubated with 100 μM. sildenafil 1 h prior to the administration of 10 ng/ml TGF-β1 for 60 min, and cell. lysates were collected for Western blotting. Phospho-Smad3/Smad 3 expression (A). The phospho-p38/p38 (B) and phospho-ERK1/2/ERK1/2 (C) ratios were quantified. Representative data from three independent experiments are shown. * = $p < 0.05$.

Recently, anti-fibrotic mechanisms of activated PKG that are unrelated to TGF-β signaling have been demonstrated. PDE5 inhibition and subsequent PKG phosphorylation reportedly suppress G protein coupled receptor (GPCR) pathways by activating regulator of G protein signaling (RGS) in mice [2,23,24,44]. Inhibiting fibrotic GPCR stimulants such as ET-1 via RGS might contribute to the anti-fibrotic effects of the PDE5 inhibitor, which requires further examination.

The sildenafil concentrations used in this study were much higher than the clinically relevant dose. High sildenafil concentrations inhibit PDE1 and PDE5 activity [45], and we demonstrated that PDE1A was expressed in skin fibroblasts. However, PDE1A has a higher affinity for cGMP than cAMP [45], and the intracellular cAMP levels, as measured using an EIA kit (Cayman Chemical), after sildenafil treatment were not higher than the control group (data not shown). Although these data did not fully demonstrate that sildenafil had little influence on intracellular cAMP levels, the anti-fibrotic effects of sildenafil might be primarily due to increased cGMP levels.

In conclusion, we demonstrated anti-fibrotic effects of the PDE5 inhibitor sildenafil and a relationship between intracellular cGMP levels and TGF-β signaling. Sildenafil reduced the expression of type I collagen, CTGF and αSMA at the mRNA and protein levels in SSc skin fibroblasts after TGF-β1 stimulation. The phenomenon of higher basal intracellular cGMP levels in SSc fibroblasts compared with control fibroblasts might explain the response of SSc fibroblasts to sildenafil. We also determined that the anti-fibrotic effects of sildenafil were exerted through non-canonical Smad signaling. The PDE5 inhibitor sildenafil might be a novel treatment for improving tissue fibrosis in addition to improving vasculopathy. The anti-fibrotic activity of sildenafil should be warranted by the further research using in vivo models and clinical trials.

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実地医家が活用すべき治療の進歩とその実際を実践する

膠原病に伴う間質性肺炎の治療

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膠原病に伴う間質性肺炎の治療

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はじめに

膠原病に間質性肺炎を合併することはよく知られている．そのため，膠原病と診断された症例では，間質性肺炎の合併がないかどうか検索する．一方，間質性肺炎が先行して呼吸器内科での精査の結果，膠原病の診断に至ることもある．近年，特発性間質性肺炎と診断された症例の中に膠原病にみられる自己抗体が出現することが報告され，それらの症例では，間質性肺炎に対して副腎皮質ステロイド薬が有効であることが多い[1]．自己抗体が陽性であっても膠原病と診断できるかどうかは臨床症状や他の検査結果による．

間質性肺病変を合併する膠原病は，全身性強皮症 systemic sclerosis (SSc)，多発性筋炎 polymyositis (PM)，皮膚筋炎 dermatomyositis (DM)，血管炎，関節リウマチ rheumatoid arthritis (RA)，シェーグレン症候群 Sjögren's syndrome (SS) である．全身性エリテマトーデス systemic lupus erthematosus (SLE) においてもまれに間質性肺病変を合併するとされるが疾患活動性として問題になることはほとんどない．上記の間質性肺病変をしばしば合併する膠原病に関して，その診断に有用な血清学的指標と治療方法に関して解説する．

全身性強皮症(SSc)

SSc は，日本では2万人程度の患者がいると推定されている．男女比は1：8～10と女性に多い．初発症状は，レイノー現象などの血管障害が多い．その後，皮膚が硬くなることに気づく．多臓器の線維化および血管障害が主症状である．皮膚の線維化だけでなく，肺の線維化が生じ，間質性肺病変の合併が生命予後に関与する．60～70% の症例に間質性肺炎を合併し，免疫抑制療法の適応となる．その間質性肺炎の発症を予測する因子として，抗核抗体がある．90% 以上の症例で抗核抗体が陽性となり，その多くの対応抗原が同定されている．それらの自己抗体の種類を**表1**に示す．それぞれの自己抗体は，SSc の臨床症状とよく関連する．抗U1-RNP抗体は，SLEやPMの合併がみられる．また，抗 Ku 抗体は，PM との重複症候群に認められる．

表1　全身性強皮症と疾患関連マーカー

自己抗体	臨床症状
抗 topoisomerase Ⅰ抗体（抗 Scl-70 抗体）	びまん性皮膚硬化，間質性肺病変
抗 RNA polymerase Ⅰ/Ⅲ抗体	びまん性皮膚硬化，強皮症腎
抗 U3-RNP 抗体	びまん性皮膚硬化，筋炎，下部腸管病変
抗 U1-RNP 抗体	関節炎，発熱，白血球減少症，肺高血圧症 皮膚硬化はびまん性と限局性がほぼ同頻度
抗セントロメア抗体	限局性皮膚硬化，CREST 症候群，肺高血圧症
抗 Th/To 抗体	限局性皮膚硬化，間質性肺病変
抗 Ku 抗体	限局性皮膚硬化，筋炎

間質性肺炎は，抗 Scl-70 抗体や抗 Th/To 抗体陽性の SSc に高頻度である．間質性肺炎は線維化の病態であり，皮膚硬化が重症であるび

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■ 間質性肺炎を合併する膠原病は，SSc，炎症性筋疾患，血管炎，RA，SS が多い．

■ SSc では，治療にシクロホスファミドを中心とした寛解導入治療と，経口免疫抑制薬（カルシニューリン阻害薬，アザチオプリンなど）による維持療法が必要である．

表 2　全身性強皮症の間質性肺炎に対する治療方法

1）プレドニゾロン(5 mg)　6～8 錠(0.8 mg/体重(kg))分 3
　1ヵ月後より，週に 10% ずつの減量を行い，3～5ヵ月後には，10 mg/日まで減量して維持する．
2）シクロホスファミド 0.4 g/体表面積(m^2)を 500 ml の生食あるいはソリタ®-T3 に混和して点滴静注する．4 週間隔で 3 回投与する．
3）タクロリムス 2～3 mg/日
　血中濃度(トラフ)は，5～10 ng/ml とする．
　あるいは，
　シクロスポリン 125～200 mg/日
　血中濃度(トラフ)は，100～150 ng/ml とする．
　あるいは，
　アザチオプリン 50～100 mg/日
1），2)の併用量を行い，エンドキサン終了後より 3) を加える．

まん皮膚硬化型に合併することが多いとされる．一方，抗 Th/To 抗体の症例では，限局皮膚硬化型であるが，間質性肺炎を高頻度(60% 以上)に合併する．抗 RNA polymerase Ⅲ抗体陽性の SSc では，皮膚硬化はびまん性に広がり，難治性で高度の皮膚硬化を呈するが，間質性肺炎は軽度であることが多い．これらのことより，肺の線維化は必ずしも皮膚硬化の重症度と相関しないことがわかってきた．

間質性肺炎の初発症状としては，乾性咳，労作時呼吸困難であり，早期の病変は，胸部の高解像度 CT (computed tomography) にて診断される．間質性肺炎に対する治療としては，副腎皮質ステロイド薬，免疫抑制薬（シクロホスファミド，アザチオプリン，シクロスポリン-A，タクロリムス，ミコフェノール酸モフェチル）の有効性が報告されている．われわれは，中等量のステロイドとシクロホスファミドを併用し[2]，後療法として，タクロリムス（プログラフ®）の併用を行う．すべての間質性肺炎に免疫抑制療法を行うわけではない．自覚症状が数ヵ月の単位で悪化し，高解像度 CT での間質陰影の進行，あるいは血清 KL-6 や SP-D の上昇がみられる症例が治療の適応となると考える．処方例を**表 2** に記載する．

多発性筋炎（PM），皮膚筋炎（DM）

PM，DM 患者は，わが国では 1 万 7 千人程度と推定されている．男女比は，1：3～4 と女性に多い．PM は皮膚症状がなく，近位筋に炎症が認められ，筋力の低下が生じる．骨格筋の病理検査からは，非壊死性筋線維に浸潤する T 細胞は CD8 陽性細胞で，筋内に遊走している．これらの T 細胞は自己の筋細胞に対して HLA class Ⅰ抗原拘束性の細胞傷害能を有する．これらの浸潤している T 細胞の T 細胞受容体は単一であることより，なんらかの自己抗原に反応して集積していることが推測される．一方，DM は，特徴的な皮膚症状を呈する筋炎疾患である．皮膚症状としてはヘリオトロープ疹とゴットロン徴候が特徴的である．DM の筋炎所見では，主に B 細胞と CD4 陽性の T 細胞浸潤が有意で，血管周囲にみられる．これらの病理所見からは，PM と DM の筋炎所見では異なった病態を呈していることがわかる．PM, DM に対して疾患関連マーカーとして種々の自己抗体がある(**表 3**)．他の膠原病と異なり，これらの多くは抗核抗体ではなく，抗細胞質抗体であ

■ 炎症性筋疾患では，2種類の間質性肺炎の合併が認められる．抗MDA5抗体陽性の急性間質性肺炎と抗ARS抗体陽性の慢性間質性肺炎である．
■ 抗MDA5抗体陽性の急速進行型の間質性肺炎には強力な3剤併用の免疫抑制療法が必要である．
■ 抗ARS抗体陽性の間質性肺炎では，副腎皮質ステロイド薬とカルシニューリン阻害薬の2剤併用療法が有効である．

る．

近年，無筋症性皮膚筋炎amyopathic dermatomyositis（ADM）あるいはclinically amyopathic dermatomyositis（CADM）という，筋炎症状はないが特徴的なDMの皮膚症状を呈する疾患があることがわかってきた．このADM/CADMの特徴は，急速に進行する間質性肺炎を高頻度に合併することである．**表3**に示したように，抗MDA5（melanoma differentiation-associated gene 5）抗体は，ADM/CADM症例に特異的にみられる自己抗体である[3]．抗MDA5抗体陽性のADM/CADM症例においては，80％以上に急性間質性肺炎acute interstitial pneumonia（AIP）がみられる．このAIPの重症度とは，血清フェリチン値がよく相関することがわかってきた[4]．ADM/CADMに合併したAIPが進行すると免疫抑制療法に抵抗性で致命率が高いことがわかっていた．そのため，現在では，HRCT所見での肺病変が軽度でも血清フェリチン値が500 ng/m*l*以上であれば，早期から強力な免疫抑制療法を行うことが推奨される．当院では，シクロホスファミド（0.5～0.6 g/体表面積（m^2））を2～3週間に1度，点滴静注する．同時に副腎皮質ステロイドとしてプレドニゾロン（PSL）換算で，0.8～1 mg/体重（kg）の内服と，シクロスポリンの3 mg/体重（kg）の内服を併用する．日和見感染症に留意しながら，フェリチン値が正常域に低下するまで，シクロホスファミドを継続する．

PM，DMにみられる抗アミノアシルtRNA合成酵素aminoacyl tRNA synthetase（ARS）抗体は，現在，6種類以上の種類が報告されている．これらのなかで最も頻度が高いものが，抗Jo-1抗体である．これらの抗ARS抗体陽性の炎症性筋疾患は，亜急性あるいは慢性に進行する間質性肺炎を高頻度（80％以上）に合併する．しかし，これらの間質性肺炎は，副腎皮質ステロイド薬などの免疫抑制療法が有効であり，生命予後は比較的良好である．この抗ARS抗体を有する疾患群は，筋炎，関節炎と間質性肺炎を同時に合併することが特徴である．特に，抗PL-12抗体陽性では筋炎症状に乏しく間質性肺炎だけがみられ，特発性間質性肺炎と診断されている症例に認められることがある．治療は，副腎皮質ステロイド薬を0.6～0.8 mg/体重（kg）とタクロリムス3～6 mgの併用を行う．タクロリムスは血中濃度をモニターして，トラフが5～10 mg/m*l*になるように調整する．

表3　多発性筋炎，皮膚筋炎と疾患関連マーカー

自己抗体	臨床症状
抗アミノアシルtRNA合成酵素（ARS）抗体 抗Jo-1抗体 抗PL-7抗体 抗PL-12抗体 抗OJ抗体 抗EJ抗体 抗KS抗体	間質性肺炎（亜急性あるいは慢性の進行）
抗MDA5抗体	筋症状が軽度の皮膚筋炎，急性間質性肺炎
抗SRP抗体	ステロイド抵抗性の筋症
抗Mi-2抗体	皮膚筋炎，治療反応性良好
抗TIF-1γ抗体	悪性腫瘍を合併する皮膚筋炎

血管炎

血管炎の疾患関連マーカーは，抗好中球細胞質抗体 anti-neutrophil cytoplasmic antibody（ANCA）が知られている．ANCA には2種類の対応抗原があり，それぞれ MPO（myeloperoxidase）-ANCA と PR3（proteinase 3）-ANCA とされている．これらの ANCA が認められる血管炎を ANCA 関連血管炎と呼ぶ．これらの血管炎には，その臨床症状により，顕微鏡的多発血管炎 microscopic polyangiitis（MPA），多発血管炎性肉芽腫症 granulomatosis with polyangiitis（GPA），好酸球性多発血管炎性肉芽腫症 eosinophilic granulomotosis with polyangiitis（EGPA）がある．GPA，EGPA は，以前は，Wegener 肉芽腫症，Churg-Strauss 症候群と呼ばれていた．MPA には，高頻度に間質性肺炎および肺胞出血が高頻度に合併する．また，GPA，EGPA には，肺に肉芽腫性血管炎病変を合併する．治療は，副腎皮質ステロイドを PSL 換算で0.6～1 mg/体重（kg）にて開始し2～4週間後に徐々に漸減する．同時に，シクロホスファミド静注療法を0.5 g/体表面積（m^2）の量で月に1回行い，3～6ヵ月継続する．シクロホスファミドが終了後は，アザチオプリン50～100 mg にて維持療法を行い，副腎皮質ステロイドを10 mg 以下に減量する．

関節リウマチ（RA）

RA は，手指や足趾などの小関節を中心に多発性の関節炎を主症状とする．診断に用いられる疾患関連マーカーは，リウマトイド因子（RF）と抗 CCP 抗体である．ともに感度，特異度は80％以上と種々の報告があり，有用性は高い．リウマチ肺と呼ばれる RA に合併する間質性肺炎は，HRCT 上，蜂巣肺を呈する usual interstitial pneumonia（UIP）パターンを呈することが多く，その進行は非常に緩徐である．合併する頻度は報告により違いがあるが，19～33％とされている[5]．間質性肺炎を合併する症例では RF が著明に高値であり，自己免疫の関与が示唆される．慢性的な経過をとる症例が多いが，肺炎などの感染症を契機に急性増悪することもあり，定期的な HRCT での検査が必要である．また，器質化肺炎 organizing pneumonia（OP）を合併する．免疫抑制療法を行っているため感染性肺炎が問題となることが多いが，浸潤影を呈し，抗菌薬治療が無効な病態に OP がある．OP の治療は副腎皮質ステロイド薬であり，感染性肺炎とは大きく異なるので，それらの鑑別は重要である．

シェーグレン症候群（SS）

RA に次いで患者数が多い膠原病は SS であると推定される．男女比が1：14程度で圧倒的に女性に多い疾患である．SS の疾患関連マーカーは，抗 SS-A（Ro）抗体と抗 SS-B（La）抗体である．特徴的な血液検査所見としては，高ガンマグロブリン血症，抗核抗体陽性，RF 陽性がしばしば認められる．臨床症状としては，乾燥症状が共通してみられる所見である．特に涙と唾液の減少が特徴的である．これらは，自