Impaired IL-17 Signaling Pathway Contributes to the Increased Collagen Expression in Scleroderma Fibroblasts

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Among IL-17 families, IL-17A and IL-17F share amino acid sequence similarity and bind to IL-17R type A. IL-17 signaling is implicated in the pathogenesis of various autoimmune diseases, but its role in the regulatory mechanism of extracellular matrix expression and its contribution to the phenotype of systemic sclerosis (SSc) both remain to be elucidated. This study revealed that IL-17A expression was significantly increased in the involved skin and sera of SSc patients, whereas the IL-17F levels did not increase. In contrast, the expression of IL-17R type A in SSc fibroblasts significantly decreased in comparison with that in normal fibroblasts, due to the intrinsic TGF-β1 activation in these cell types. Moreover, IL-17A, not IL-17F, reduced the protein expression of α1(I) collagen and connective tissue growth factor. miR-129-5p, one of the downregulated microRNAs in SSc fibroblasts, increased due to IL-17A and mediated the α1(I) collagen reduction. These results suggest that IL-17A signaling, not IL-17F, has an antifibrogenic effect via the upregulation of miR-129-5p and the downregulation of connective tissue growth factor and α1(I) collagen. IL-17A signaling is suppressed due to the downregulation of the receptor by the intrinsic activation of TGF-β1 in SSc fibroblasts, which may amplify the increased collagen accumulation and fibrosis characteristic of SSc. Increased IL-17A levels in the sera and involved skin of SSc may be due to negative feedback. Clarifying the novel regulatory mechanisms of fibrosis by the cytokine network consisting of TGF-β and IL-17A may lead to a new therapeutic approach for this disease. *The Journal of Immunology*, 2012, 188: 3573–3583.

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 unclear, it includes inflammation, autoimmune attack, and vascular damage, leading to the activation of fibroblasts and abnormal accumulation of extracellular matrix (ECM) (1, 2). Therefore, the abnormal SSc fibroblasts responsible for fibrosis may develop from a subset of cells that have escaped from normal control mechanisms (3, 4).

Although the mechanism of fibroblast activation in SSc is unknown, many of the characteristics of SSc fibroblasts resemble those of healthy fibroblasts stimulated by TGF-β1 (5, 6). The principal effect of TGF-β1 on mesenchymal cells is the stimulation of ECM deposition. Dermal fibroblasts from affected SSc skin cultured in vitro display an increased transcription of various collagens, mainly type I collagen, which consists of α1(I) and α2(I) collagen (7–10), whereas the blockade of TGF-β1 signaling with anti–TGF-β1–neutralizing Abs abolishes the increased expression of collagen mRNA in cultured SSc fibroblasts (11). However, the TGF-β1 levels in the culture media of SSc fibroblasts do not increase in comparison with those of normal fibroblasts (11), thus suggesting that the activation of fibroblasts in SSc may be a result of the intrinsic TGF-β1 activation. This is supported by recent findings: 1) although the transcriptional activity of the collagen gene in SSc fibroblasts is constitutively higher than that in normal fibroblasts, the responsiveness to ectopic TGF-β1 has been reported to decrease in SSc cells (9); and 2) the phosphorylated levels and DNA-binding activity of Smad3, a mediator of TGF-β1 signaling, are constitutively upregulated in SSc fibroblasts (12). In addition, other cytokines, including connective tissue growth factor (CTGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), IL-1, IL-6, or IL-7 are also reported to be involved in the pathogenesis of this disease (13–17). Therefore, clarifying the cytokine network mediating fibroblast activation as well as the inflammation, autoimmune attack, and vascular damage of SSc is important to understand the molecular mechanism of this disease and to develop new therapeutic strategies.

IL-17 is cytokine family of six members (A–F). Among them, IL-17A is the founding member of the IL-17 family and shares an amino acid sequence identity of ~50% with IL-17F. IL-17A and IL-17F are homodimeric cytokines produced by the Th17 T cell lineage and have similar biological activities, including induction of cytokines and chemokines involved in inflammatory responses (18–21). In contrast, the IL-17R family consists of five members: RA, RB, RC, RD, and RE. IL-17A and IL-17F have been shown to bind IL-

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Abbreviations used in this article: Ct, threshold cycle; CTGF, connective tissue growth factor; ECM, extracellular matrix; IGF, insulin-like growth factor; IL-17RA, IL-17R type A; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; PM/DM, polymyositis/dermatomyositis; siRNA, small interfering RNA; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; UTR, untranslated region.

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17R type A (IL-17RA; also referred to as IL-17R), and IL-17RA is required for the biological activity of IL-17A and IL-17F (22–27).

IL-17A and IL-17F are implicated in a variety of autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, asthma, and psoriasis (26–32). In addition, several studies suggest IL-17A is involved in the pathogenesis of SSc. For example, Murata et al. (33) reported the serum levels of IL-17A to be higher in SSc patients than those in healthy controls, inversely correlating with the severity of skin sclerosis. Furthermore, peripheral blood T cells from SSc patients are reported to produce increased levels of IL-17A (34). However, the IL-17A expression levels in SSc sera may be still controversial, because Grourh et al. (35) concluded that the serum IL-17A levels decrease in SSc patients. In contrast, IL-17A stimulates cell proliferation in SSc fibroblasts and induces production of adhesion molecules or IL-1 in endothelial cells in vitro (34). Taken together, the exact role of the IL-17 signaling pathway in the pathogenesis of this disease has not been fully elucidated. To clarify this point, we compared the expression pattern of IL-17F and IL-17RA as well as IL-17A between normal healthy subjects and SSc patient, and investigated the involvement of IL-17 signaling pathway in the regulation of ECM expression in SSc.

Materials and Methods

Reagents

Recombinant human IL-17A, IL-17F, PDGF, IGF-1, and Ab for IL-17A (MAB3171) or IL-17RA (AF177) were obtained from R&D Systems (Minneapolis, MN). Actinomycin D, DMSO, and GAPDH Ab (G9545) were purchased from Sigma-Aldrich (St. Louis, MO). Smad3 Ab was from Santa Cruz Biotechnology (Santa Cruz, CA). The Ab for type I collagen (1301-01) or CTGF (ab6992) was from Southern Biotechnology Associates (Birmingham, AL) or Abcam (Cambridge, U.K.), respectively.

Patients

Serum samples were obtained from 20 patients with SSc (5 males and 15 females; age range 28–84 y, mean 58.3 y). All patients with SSc were grouped according to the classification system proposed by LeRoy et al. (36): 10 patients had diffuse cutaneous SSc, and 10 patients had limited cutaneous SSc. All patients fulfilled the criteria proposed by the American College of Rheumatology (37). Ten patients with systemic lupus erythematosus (SLE) and 10 patients with polymyositis/dermatomyositis (PM/DM) were also included in this study. Control serum samples were also collected from 10 healthy age- and sex-matched volunteers. The clinical and laboratory data reported in this study were obtained at the time of serum sampling. Skin biopsy specimens of SSc patients were obtained from lesional skin. Control skin samples were obtained from routinely discarded skin of healthy human subjects undergoing skin grafts. The samples were fixed in formalin immediately after removal and then were embedded in paraffin.

Institutional review board approval and written informed consent were obtained before patients and healthy volunteers were entered into this study according to the Declaration of Helsinki.

The measurement of serum IL-17A and IL-17F concentrations

The serum IL-17A and IL-17F levels were measured with specific ELISA kits (R&D Systems and BioLegend, San Diego, CA, respectively). Briefly, mAb for IL-17A or IL-17F was precoated onto microtiter wells. Aliquots of serum were added to each well, followed by peroxidase-conjugated Abs to IL-17A or IL-17F. Color was developed with hydrogen peroxide and tetramethylbenzidine peroxidase, and the absorbance at 450 nm was measured. Wavelength correction was performed by absorbance at 540 nm. The concentration of IL-17A or IL-17F in each sample was determined by interpolation from a standard curve.

Cell cultures

Human dermal fibroblasts were obtained by skin biopsies from the affected areas (dorsal forearm) of five patients with diffuse cutaneous SSc and <2 y of skin thickening as described previously (38). Control fibroblasts were obtained by skin biopsies from five healthy donors. Control donors were each matched with SSc patients for age, sex, and biopsy site. Institutional review board approval and written informed consent were obtained according to the Declaration of Helsinki. Primary explant cultures were established in 25-cm^2 culture flasks in modified Eagle's medium supplemented with 10% FCS and Antibiotic-Antimycotic (Invitrogen, Carlsbad, CA). Monolayer cultures independently isolated from different individuals were maintained at 37°C in 5% CO_2 in air. Fibroblasts between the third and sixth subpassages were used for experiments. The cells were serum-starved for 12–24 h before experiments.

Cell lysis and immunoblotting

Fibroblasts were cultured until they were confluent, and then the medium was removed. The remaining cells were washed twice with cold PBS and lysed in Denaturing Cell Extraction Buffer (Biosource International, Camarillo, CA). Aliquots of the cell lysates (normalized for protein concentrations) were separated by electrophoresis on 10% $NaDodSO_4$–polyacrylamide gels and transferred onto polyvinylidene difluoride filters. The polyvinylidene difluoride filters were then incubated with Ab against IL-17RA, type I collagen, CTGF, or GAPDH. The filters were incubated with secondary Ab, and the immunoreactive bands were visualized using an ECL system (Amersham Biosciences, Arlington Heights, IL) according to the manufacturer's recommendations. The densities of the bands were measured using the Quantity One 1D analysis software version 4.6.6 on a ChemiDoc XRS System (Bio-Rad, Hercules, CA).

Immunohistochemistry

Wax-embedded sections (4-μm thick) were dewaxed in xylene and rehydrated in graded alcohols. For the immunostaining of IL-17A, Ags were retrieved by incubation with citrate buffer (pH 6) for 9 min with a microwave. The endogenous peroxidase activity was inhibited with a solution of 0.3% hydrogen peroxide in methyl alcohol, after which the sections were blocked with 5% donkey serum for 20 min and then reacted with Ab for IL-17A (1:100) for 48 h at 4°C. After excess Ab was washed out with PBS, the samples were incubated with HRP-labeled anti-mouse Ab (Nichirei, Tokyo, Japan) for 30 min at 37°C.

For the immunostaining of IL-17RA, Ags were retrieved by incubation with citrate buffer (pH 9) for 9 min with a microwave. Ab for IL-17RA (1:100) and HRP-labeled anti-goat Ab (Nichirei) were used.

The reaction was visualized using the diaminobendine substrate system (Dojin, Kumamoto, Japan). Slides were counterstained with Mayer's hematoxylin and examined under a light microscope (Olympus BX50; Olympus, Tokyo, Japan).

Intradermal treatment with bleomycin

Bleomycin (Nippon Kayaku, Tokyo, Japan) was dissolved in PBS at a concentration of 1 mg/ml and sterilized by filtration. Bleomycin (300 μg) or PBS was injected intradermally into the shaved back of 6-wk-old C57BL/6 mice daily for 4 wk, as described previously (39, 40). The back skin was removed on the next day after the final injection, fixed in 10% formalin solution, and embedded in paraffin.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from the culture cells with ISOGEN (Nippon Gene, Tokyo, Japan) or from paraffin-embedded sections with the RNeasy FFPE kit (Qiagen, Valencia, CA), respectively.

First-strand cDNA was synthesized using the RT^2 First Strand Kit (SABiosciences, Frederick, MD) for PCR array (SABiosciences). cDNA was mixed with RT^2 SYBR Green/ROX qPCR Master Mix (SABiosciences), and the mixture was added to a 96-well Extracellular Matrix and Adhesion Molecules PCR Array (SA Biosciences) that included primer pairs for 84 human RNAs. PCR was performed on Takara Thermal Cycler Dice (TP800) following the manufacturer's protocol (Takara Bio, Shiga, Japan). The threshold cycle (Ct) for each RNA was extracted using the Thermal Cycler Dice Real Time System version 2.10B (Takara Bio). The raw Ct was normalized using the values of housekeeping genes.

For quantitative real-time PCR, first-strand cDNA was synthesized by PrimeScript RT reagent Kit (Takara Bio) with both oligo(dT) primer and random hexamers. GAPDH primer was purchased from SABiosciences, and primer sets for IL-17A, IL-17F, IL-17RA, CTGF, Smad3, TGF-β1, and TGF-β receptors I, II, and III were from Takara Bio. All primer sets were prevalidated to generate single amplicons. One microliter diluted first-strand cDNA product was used for amplification in a 25-μl reaction solution containing 12.5 μl SYBR Premix Ex Taq II (Takara Bio) and 1 μl each primer. RNA (not reverse transcribed) was used as a negative control. DNA was amplified for 40 cycles of denaturation for 5 s at 95°C and annealing for 30 s at 60°C, with the Takara Thermal Cycler Dice (TP800; Takara Bio). The data generated from each PCR reaction were analyzed

using Thermal Cycler Dice Real Time System version 2.10B (Takara Bio). The specificity of reactions was determined by melting curve analysis. The relative expression of each gene of interest and GAPDH were calculated by the standard curve method. At least three independent experiments were performed for each gene of interest.

microRNA isolation, reverse transcription, and PCR analysis of microRNA expression

microRNA isolation from total RNA was performed using the RT^2 qPCR-Grade miRNA Isolation Kit (SABiosciences). For RT^2 Profiler PCR Array (SABiosciences), microRNAs were reverse-transcribed into first-strand cDNA using the RT^2 miRNA First Strand Kit (SABiosciences). The cDNA was mixed with RT^2 SYBR Green/ROX qPCR Master Mix (SABiosciences), and the mixture was added to a 96-well RT^2 miRNA PCR Array (SABiosciences) that included primer pairs for 88 human microRNAs. PCR was performed on Takara Thermal Cycler Dice (TP800; Takara Bio) following the manufacturer's protocol. The Ct for each microRNA was extracted using Thermal Cycler Dice Real Time System version 2.10B (Takara Bio). The raw Ct was normalized using the values of small RNA housekeeping genes.

For quantitative real-time PCR, the Mir-X miRNA First-Strand Synthesis Kit (Takara Bio) was used to synthesize first-strand cDNA. The sequence of miR-129-5p primer was designed based on the miRBase (http://www.mirbase.org): 5′-CTTTTTGCGGTCTGGGCTTGC-3′. PCR was performed on Takara Thermal Cycler Dice (TP800; Takara Bio) following the manufacturer's protocol. DNA was amplified for 40 cycles of denaturation for 5 s at 95°C and annealing for 20 s at 60°C. The data generated from each PCR reaction were analyzed using the Thermal Cycler Dice Real Time System version 2.10B (Takara Bio). The transcript levels of miR-129-5p were normalized to U6.

Transient transfection

Small interfering RNAs (siRNAs) against TGF-β1, Smad3, or IL-17RA were purchased from Santa Cruz Biotechnology. microRNA mimic and miScript Target Protector for miR-129p-5p were from Qiagen. Lipofectamine RNAiMAX (Invitrogen) was used as the transfection reagent. For reverse transfection, siRNAs, microRNA mimics, and miScript Target protectors (6 pmol) mixed with the transfection reagent were added when the cells were plated, followed by incubation for either 12 h (for real-time PCR) or 120 h (for immunoblotting) at 37°C in 5% CO_2. Control experiments showed transcript levels for target genes of siRNA to be reduced by >80%, and the expression of microRNAs was increased at least 5-fold by the transfection of mimics (data not shown).

Statistical analysis

The statistical analysis was carried out with a Mann–Whitney *U* test for the comparison of the means and Fisher's exact probability test for the analysis of the frequency. The *p* values of <0.05 were considered to be significant.

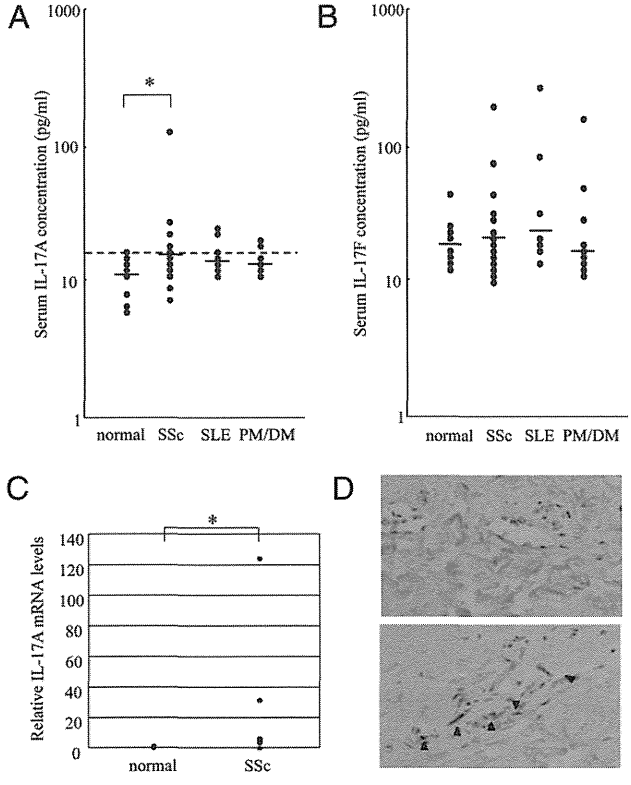

FIGURE 1. Serum IL-17A and IL-17F levels in patients with SSc, SLE, and PM/DM. Serum samples were obtained from patients with SSc (n = 20), SLE (n = 10), PM/DM (n = 10), and from healthy donors (n = 10). The y-axis is a logarithmic scale, which indicates serum levels of IL-17A (**A**) and IL-17F (**B**) determined by an ELISA kit as described in the *Materials and Methods*. Bars show the means. In (A), the horizontal dotted line indicates the cutoff levels set at the 99th percentile of the healthy controls. *$p < 0.05$ as compared with the values in samples from healthy controls. (**C**) Total RNA was extracted from skin tissue derived from 10 patients with SSc and 7 healthy donors, and then the quantitative real-time PCR was performed to determine mRNA expression of IL-17A and GAPDH. The highest value in samples from healthy controls was set at 1. *$p < 0.05$ as compared with the values in normal skin. (**D**) Paraffin sections were subjected to immunohistochemical analysis for IL-17A as described in *Materials and Methods*. *Upper panel*, Human normal skin, original magnification ×100; *lower panel*, SSc skin, original magnification ×100. A representative result of three normal and SSc involved skin samples is shown.

Table I. Correlation of serum IL-17A levels with the clinical and serological features in patients with SSc

	Patients with Elevated IL-17A Levels (n = 9)	Patients with Normal IL-17A Levels (n = 11)
Mean age at onset (y)	56.3	60.5
Duration of disease (y)	5.3	3.9
Type (diffuse:limited)	3:6	7:4
MRSS (point)	7.4	16.5
Clinical features		
Pitting scars	77.8*	27.3
Ulcers	44.4	36.4
Nailfold bleeding	44.4	54.5
Raynaud's phenomenon	77.8	81.8
Telangiectasia	33.3	9.1
Contracture of phalanges	33.3	63.6
Calcinosis	0	9.1
Diffuse pigmentation	0	36.4
Short SF	44.4	63.6
Sicca symptoms	11.1	27.3
Organ involvement		
Pulmonary fibrosis		
Mean percent VC	94.2	93.5
Mean percent DLco	83.8	78.1
Pulmonary hypertension	0	0
Esophagus	0	27.3
Heart	22.2	18.2
Kidney	0	0
Joint	22.2	0
Thrombosis	0	0
ANA specificity		
Anti-topo I	55.6	36.4
Anti-centromere	33.3	18.2
Anti-U1 RNP	33.3	9.1
Overlap	22.2	9.1
Others	22.2	44.5

Unless indicated, values are percentages.

*$p < 0.05$ versus patients with normal IL-17A levels using Fisher's exact probability test.

ANA, anti-nuclear Abs; Anti-centromere, anti-centromere Ab; Anti-topo I, anti-topoisomerase I Ab; Anti-U1 RNP, anti-U1 RNP Ab; DLco, diffusing capacity of lung carbon monoxide; MRSS, modified Rodman skin thickness score; Others, positive for other Abs than anti-topoisomerase I, centromere, and U1 RNP; Overlap, positive for more than one autoantibody among anti-topoisomerase I, centromere, and U1 RNP; SF, sublingual frenulum; VC, vital capacity.

Results

Expression of IL-17A and -17F levels in sera and involved skin of patients with SSc

As an initial experiment, we measured the serum levels of IL-17A and IL-17F in SSc patients by an ELISA. Fig. 1A shows the mean serum IL-17A levels in SSc patients to be higher than those of healthy controls as well as other collagen diseases including SLE and PM/DM, and there was a significant difference in the values between SSc patients and healthy donors (mean ± SE: 20.0 ± 5.4 versus 11.2 ± 1.1 mg/dl; $p < 0.05$). Increased serum concentrations of IL-17A were found in 9 out of the 20 (45%) SSc patients, when the cutoff value was set at the 99th percentile of the healthy controls. In contrast, there was no significant difference in the serum IL-17F levels among healthy donors, SSc patients, and patients with other collagen diseases (Fig. 1B). In addition, no correlation was observed between the serum concentrations of IL-17A and IL-17F in each patient.

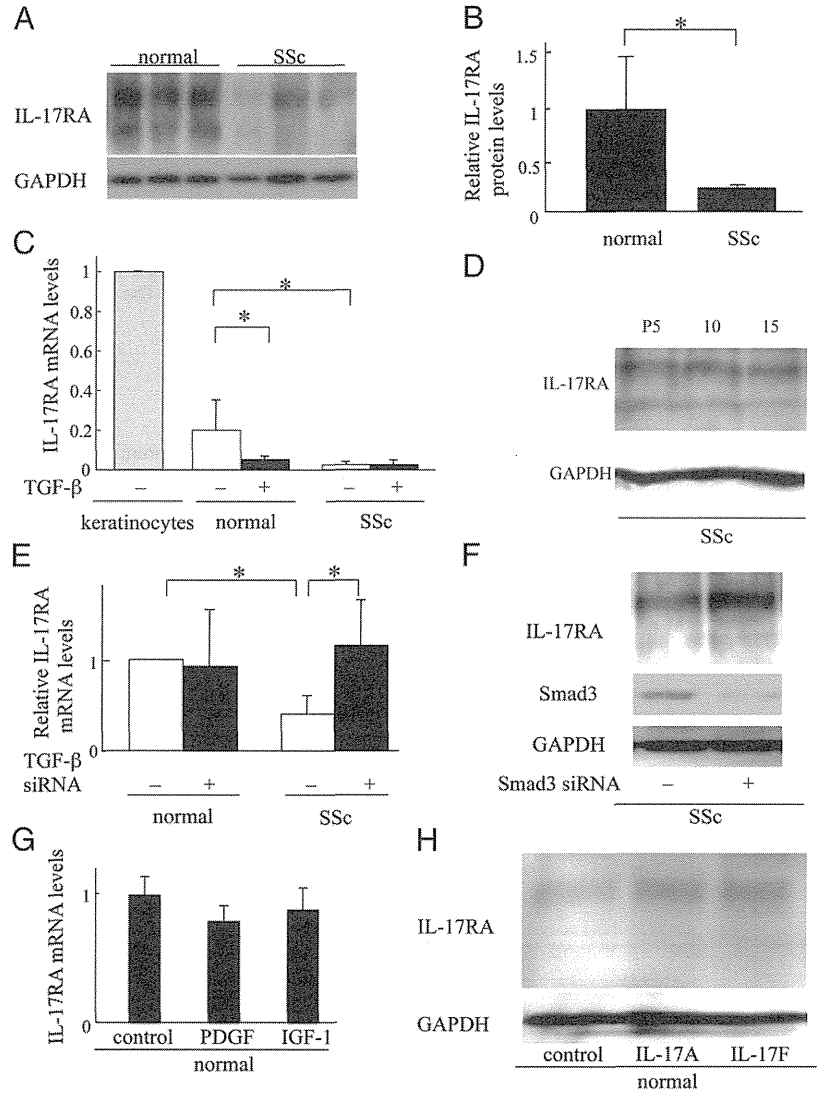

FIGURE 2. The IL-17RA expression in SSc fibroblasts in vitro. (**A** and **B**) Comparison of IL-17RA protein expression between normal and SSc fibroblasts. Human dermal fibroblasts from healthy donors and SSc patients were cultured independently under the same conditions until they were confluent and then serum-starved for 24 h. Cell lysates were subjected to immunoblotting with Ab for IL-17RA. GAPDH was used as the control. The representative results for three normal and SSc fibroblasts are shown (A). The protein levels of IL-17RA quantitated by scanning densitometry and corrected for the levels of GAPDH in the same samples are shown relative to those in normal fibroblasts (1.0). Data are expressed as the mean ± SE of three independent experiments. *$p < 0.05$ as compared with the values in normal fibroblasts (B). (**C**) Human dermal fibroblasts from five healthy donors and five SSc patients were incubated with or without TGF-β1 (2 ng/ml) for 12 h and total RNA was extracted. Quantitative real-time PCR determining mRNA expression of IL-17RA and GAPDH was performed. The value in normal human keratinocytes was set at 1. Data are expressed as the mean ± SE of five independent experiments. *$p < 0.05$ as compared with the values in untreated normal fibroblasts. (**D**) Lysates were obtained from cultured SSc dermal fibroblasts at passages 5, 10, and 15. Cell lysates were subjected to immunoblotting with Ab for IL-17RA. GAPDH was used as a control. (**E**) The effects of the TGF-β1 siRNA on the expression levels of IL-17RA were investigated by real-time PCR as described in (C). Fibroblasts were transfected with the TGF-β1 siRNA (black bars) or control siRNA (white bars). The values in normal fibroblasts transfected with control siRNA were set at 1. Data are expressed as the mean ± SE of three independent experiments. *$p < 0.05$ as compared with the values in fibroblasts transfected with control siRNA. (**F**) SSc fibroblasts were transfected with control or Smad3 siRNA as described in *Materials and Methods*. Cell lysates were subjected to immunoblotting with the Ab for IL-17RA, Smad3, and GAPDH. (**G**) Normal fibroblasts were incubated in the absence or presence of PDGF or IGF-1 (250 ng/ml) for 12 h. Quantitative real-time PCR was performed to evaluate the mRNA levels of IL-17RA. The mean value in untreated fibroblasts was set at 1. Data are expressed as the mean ± SE of three independent experiments. (**H**) Normal dermal fibroblasts were incubated in the presence or absence of IL-17A or IL-17F (250 ng/ml) for 24 h, and cell lysates were subjected to immunoblotting with Ab for IL-17RA.

Table I shows the association of the serum IL-17A levels with the clinical and laboratory features in SSc patients. Patients with elevated IL-17A levels had a significantly higher prevalence of pitting scars than those with normal levels (77.8 versus 27.3%; $p < 0.05$). Consistent with previous report (33), the modified Rodman skin thickness score (MRSS) tended to be lower in patients with elevated IL-17A than those without (7.4 versus 16.5), although we could not find statistical significance, probably because of the small number of patients. There were no statistically significant differences in other clinical or laboratory features between the patients with and without increased IL-17A levels: for example, patients with elevated IL-17A levels tended to have more than one of three major autoantibodies (anti-topoisomerase I, centromere, and U1 RNP Ab) compared with those without (22.2 versus 9.1%). In contrast, patients with normal IL-17A levels tended to have other autoantibodies than the three major Abs in comparison with those with elevated values (44.5 versus 22.2%). However, no statistically significant differences were observed. Therefore, the increased serum IL-17A concentration detected only in SSc patients indicates that the cytokine may play some role in the pathogenesis of this disease.

Then, to determine whether IL-17A expression is also increased in the involved skin of SSc in vivo, total RNA was extracted from skin tissue derived from 10 patients with SSc and 7 healthy controls, and real-time PCR was performed. IL-17A mRNA expression in SSc involved skin tissue was also more significantly elevated than that in normal controls ($p < 0.05$; Fig. 1C), whereas IL-17F mRNA was not detected in the skin (not shown). Immunohistochemical staining also revealed the expression of IL-17A in the infiltrated lymphocytes in the SSc dermis, but not in normal skin (Fig. 1D). Therefore, the IL-17A expression increased in the sera and the involved skin of SSc patients.

IL-17RA protein and mRNA expression in SSc fibroblasts

Next, we examined the expression of IL-17R (IL-17RA) in SSc fibroblasts. Although IL-17RA is known to be ubiquitously distributed in many tissues (25), expression of the receptor in dermal fibroblast has not yet been examined. As shown in Fig. 2A and 2B, immunoblotting revealed that the protein expression of IL-17RA in cultured SSc fibroblasts was significantly decreased in comparison with that in normal fibroblasts in vitro. In addition, we found cultured normal dermal fibroblasts express IL-17RA mRNA to the extent of 20% of skin keratinocytes, a positive control that is reported to express the receptor abundantly (41), and the expression of IL-17RA mRNA was also downregulated in all five SSc fibroblasts ($p < 0.05$; Fig. 2C). Therefore, the decreased IL-17RA protein synthesis may result from the downregulation of IL-17A mRNA in SSc fibroblasts. The downregulation of IL-17RA in SSc cells was maintained until at least passage 15 in vitro (Fig. 2D). In contrast, ectopic TGF-β1 stimulation significantly downregulated the IL-17RA expression in normal fibroblasts, but not in SSc fibroblasts (Fig. 2C). Furthermore, transfection with TGF-β1 siRNA into SSc fibroblasts normalized the downregulation of IL-17RA, but not in normal cells (Fig. 2E). These results indicate that the downregulated IL-17RA expression in SSc fibroblasts may be caused by the intrinsic TGF-β activation in these cell types as described in the introductory paragraphs. In addition, when Smad3 was knocked down by the transfection of Smad3 siRNA, the reduced IL-17RA expression in SSc fibroblasts was also recovered (Fig. 2F), thus suggesting that the effect of TGF-β1 on IL-17RA is

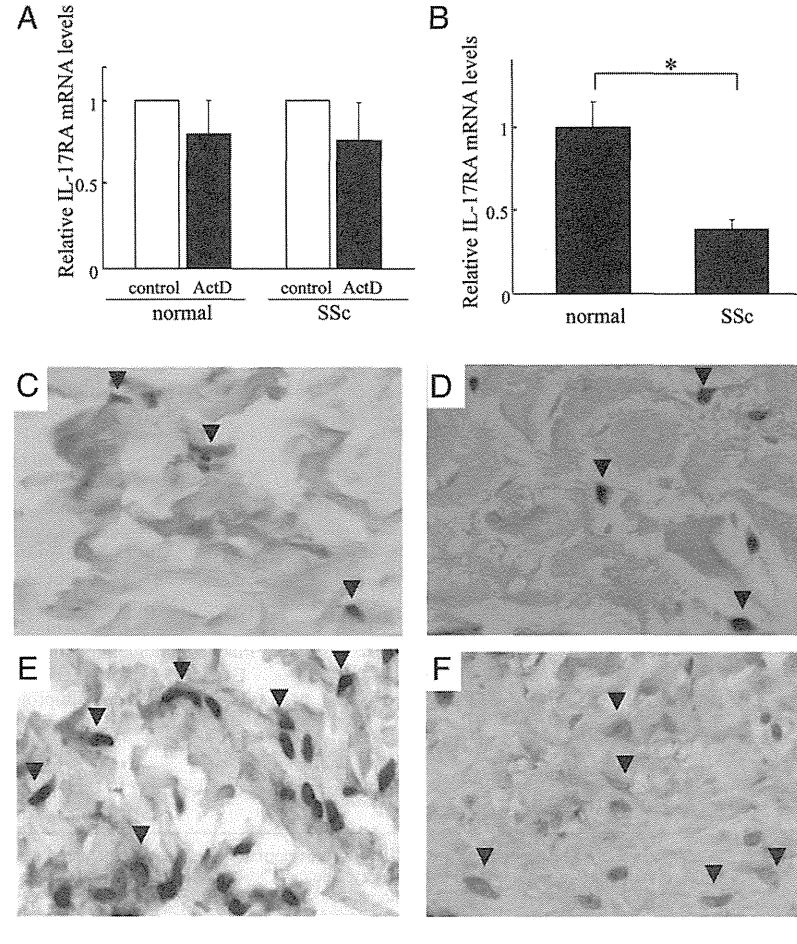

FIGURE 3. The mechanism of IL-17RA downregulation in SSc fibroblasts. (**A**) Normal and SSc dermal fibroblasts were serum-starved for 24 h and incubated in the absence or presence of 2.5 ng/μl actinomycin D for 12 h. Quantitative real-time PCR was performed to evaluate the levels of IL-17RA mRNA. The values in untreated fibroblasts were set at 1 (white bars). Data are expressed as the mean ± SE of three independent experiments. (**B**) Quantitative real-time PCR was performed to determine mRNA expression of IL-17RA and GAPDH using total RNA obtained from SSc involved skin and normal skin. *$p < 0.05$, as compared with the values in normal skins (1.0). (**C–F**) Paraffin sections were subjected to immunohistochemical analysis for IL-17RA as described in *Materials and Methods*. Human normal skin, original magnification ×400 (C), SSc skin, original magnification ×400 (D), mice skin treated with PBS, original magnification ×400 (E), and mice skin treated with bleomycin, original magnification ×400 (F). Fibroblasts are indicated by black arrows.

Table II. The expression profiles of extracellular matrix-related genes in the presence or absence of IL-17A or -17F in the PCR array

Symbol	Name	Fold Change
Upregulated genes by IL-17A		
ITGB2	Integrin, β_2	58.32
MMP16	Matrix metalloproteinase 16	21.5
VCAM1	VCAM 1	6.76
TIMP3	Tissue inhibitor of metalloproteinase 3	5.68
ITGA8	Integrin, α8	3.35
CLEC3B	C-type lectin domain family 3, member B	3.26
ITGA4	Integrin, α4	2.28
KAL1	Kallmann syndrome 1 sequence	2.21
NCAM1	Neural cell adhesion molecule 1	2.08
SPARC	Secreted protein, acidic, cysteine-rich	2.04
COL16A1	Collagen, type XVI, α1	2.01
Downregulated genes by IL-17A		
ITGA2	Integrin, α2	0.04
SPP1	Secreted phosphoprotein 1	0.11
ITGAM	Integrin, αM	0.24
ITGA5	Integrin, α5	0.25
SELP	Selectin P	0.29
ITGB3	Integrin, β3	0.3
LAMB3	Laminin, β3	0.34
ITGB4	Integrin, β4	0.39
ITGAL	Integrin, αL	0.41
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	0.42
ITGA3	Integrin, α3	0.43
CTGF	Connective tissue growth factor	0.44
VTN	Vitronectin	0.44
ITGA6	Integrin, α6	0.48
Upregulated genes by IL-17F		
MMP12	Matrix metalloproteinase 12	224.1
MMP16	Matrix metalloproteinase 16	93.57
ITGB2	Integrin, β_2	39.07
TIMP3	Tissue inhibitor of metalloproteinase 3	38.27
CLEC3B	C-type lectin domain family 3, member B	19
KAL1	Kallmann syndrome 1 sequence	11.54
ITGA5	Integrin, α5	6.36
COL6A2	Collagen, type VI, α2	5.42
ECM1	Extracellular matrix protein 1	2.96
VCAM1	VCAM 1	2.94
ITGA7	Integrin, α7	2.6
SPARC	Secreted protein, acidic, cysteine-rich	2.55
MMP3	Matrix metalloproteinase 3	2.19
NCAM1	Neural cell adhesion molecule 1	2.07
ITGA4	Integrin, α7	2.01
Downregulated genes by IL-17F		
ITGAM	Integrin, αM	0.01
MMP9	Matrix metalloproteinase 9	0.01
CTNND2	Catenin (cadherin-associated protein), δ2	0.01
VTN	Vitronectin	0.01
ADAMTS8	ADAM metallopeptidase with thrombospondin type 1 motif, 8	0.04
MMP7	Matrix metalloproteinase 7	0.06
ADAMTS13	ADAM metallopeptidase with thrombospondin type 1 motif, 13	0.06
MMP10	Matrix metalloproteinase 10	0.07
SELP	Selectin P	0.07
MMP13	Matrix metalloproteinase 13	0.08
SELE	Selectin E	0.08
LAMA1	Laminin, α1	0.11
HAS1	Hyaluronan synthase 1	0.12
MMP15	Matrix metalloproteinase 15	0.13
ITGAL	Integrin, αL	0.14
MMP8	Matrix metalloproteinase 8	0.15
ITGB3	Integrin, β3	0.19
PECAM1	Platelet/endothelial cell adhesion molecule 1	0.21
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	0.23
MMP11	Matrix metalloproteinase 11	0.29
SPP1	Secreted phosphoprotein 1	0.31
LAMA3	Laminin, α3	0.32
CNTN1	Contactin 1	0.36
ITGA3	Integrin, α3	0.37
ITGB4	Integrin, β4	0.38

A mixture of equal amounts of mRNAs from three normal fibroblasts was prepared in the presence or absence of IL-17A or -17F, and mRNA expression profile was evaluated using PCR array. The raw Ct was normalized using the mean value of five housekeeping genes. Fold change ($1/2^{\text{the raw Ct of each mRNA} - \text{Ct of housekeeping genes}}$) is shown.

Smad dependent. In contrast, other important mediators of fibrosis, such as PDGF and IGF-1, did not have any significant effects on IL-17RA expression (Fig. 2G). Furthermore, neither exogenous IL-17A nor IL-17F downregulated IL-17RA (Fig. 2H), indicating that IL-17RA may be specifically regulated by the stimulation of TGF-β1.

The steady-state level of mRNA can be affected by the level of gene transcription and/or the stability of mRNA. De novo mRNA synthesis was blocked by an RNA synthesis inhibitor, actinomycin D, in normal and SSc fibroblasts to determine whether the decrease of IL-17RA mRNA in SSc fibroblasts takes place at the transcriptional level or posttranscriptional level. Fig. 3A shows that 12 h of actinomycin D treatment decreased the IL-17RA mRNA levels due to mRNA degradation in both cells. The rate of IL-17RA mRNA decrease was similar between the normal and SSc fibroblasts, indicating that the stability of IL-17RA mRNA was not downregulated in SSc fibroblasts. Taken together, the IL-17RA expression most likely decreased at a transcriptional level in SSc dermal fibroblasts due to TGF-β signaling.

Moreover, IL-17RA mRNA expression was examined in vivo. Real-time PCR using total RNA extracted from skin tissue revealed IL-17RA mRNA expression in SSc skin tissue was significantly lower than that in the normal controls (Fig. 3B), which is consistent with the in vitro results (Fig. 2A–C). Furthermore, we found mild inverse correlation between MRSS and IL-17RA mRNA levels of the skin in SSc patients, although not statistically significant ($r = -0.61$; $p = 0.28$).

We also performed immunohistochemical staining using paraffin-embedded skin sections derived from SSc patients and healthy donors to evaluate protein expression of IL-17RA in vivo. The expression of IL-17RA was detected strongly in normal human dermal fibroblasts, but hardly detected in SSc fibroblasts between the thickened collagen bundles (Fig. 3C, 3D). Negative controls using isotype control Ab showed little or no background staining (data not shown). In addition, to further investigate the IL-17RA expression pattern in vivo, paraffin-embedded sections derived from the mouse fibrotic skin model induced by bleomycin treatment were stained for IL-17RA. Spindle-shaped fibroblasts positive for IL-17RA staining were detected in control mouse skin treated with PBS, whereas they were not detected in bleomycin-induced thickened skin (Fig. 3E, 3F). Therefore, both in vitro and in vivo, the expression of IL-17RA decreased under fibrotic conditions.

PCR array analysis of ECM-related genes in the presence or absence of IL-17

Next, the effect of IL-17 signaling pathway on ECM expression and the involvement of IL-17 signaling pathway in the pathogenesis of SSc were examined.

A PCR array analysis, consisting of 84 ECM-related genes, was conducted to identify any differences in the expression pattern of ECM genes in IL-17A– or IL-17F–treated fibroblasts. Normal human dermal fibroblasts were cultured until they were confluent, stimulated with IL-17A or IL-17F for 12 h, and total RNA was extracted. When a 2-fold difference in the ΔΔCt method was considered significant, 11 of the 84 genes were upregulated and 14 genes were downregulated in IL-17A–treated fibroblasts in comparison with untreated cells, whereas 15 genes were upregulated and 24 genes were downregulated in IL-17F–treated fibroblasts (Table II: complete dataset available at the Gene Expression Omnibus microarray data repository, http://www.ncbi.nlm.nih.gov/geo/, accession number GSE33581). Human α1(I) collagen gene expression was slightly decreased by IL-17A (0.80-fold increase in ΔΔCt method) or -17F (0.70-fold increase), but the difference was not significant. In addition, other matrix genes, such as fibronectin or tenascin-C, were not significantly altered by IL-17A or -17F. However, we found that the expression of CTGF mRNA, which is known to induce tissue fibrosis and has also been implicated in the pathogenesis of SSc (13), significantly decreased by the treatment with IL-17A (0.44-fold) in comparison with untreated cells by the array, but not with IL-17F (0.58-fold). This result was confirmed by real-time PCR, which showed the mean transcript levels of CTGF to significantly decrease by the treatment with IL-17A as compared with untreated cells and IL-17F

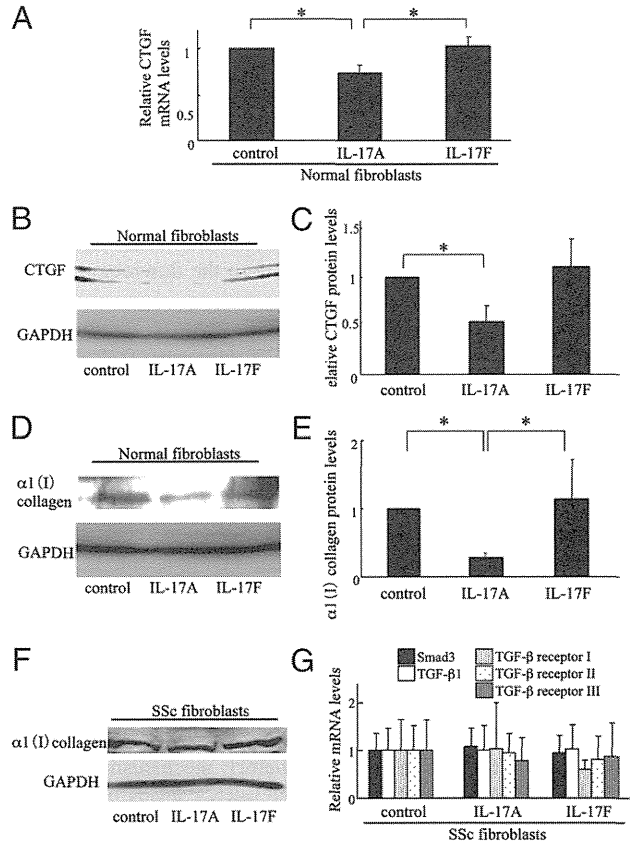

FIGURE 4. The effect of IL-17A and IL-17F on the ECM expression in normal fibroblasts. (**A**) Human dermal normal fibroblasts were cultured until they were confluent and then serum-starved for additional 24 h. The cells were incubated in the presence or absence of IL-17A or IL-17F (250 ng/ml) for 12 h, and the quantitative real-time PCR was performed to evaluate mRNA levels of CTGF and GAPDH. The mean relative transcript levels of CTGF normalized to GAPDH in the same samples are shown relative to the levels in untreated cells (1.0). Data are expressed as the mean ± SE of three independent experiments. *$p < 0.05$ as compared with the values in normal fibroblasts treated with IL-17A. (**B** and **C**) Human dermal fibroblasts were incubated in the presence or absence of IL-17A or IL-17F for 24 h, and immunoblotting was performed using Ab for CTGF or GAPDH (B). The levels of CTGF proteins quantitated by scanning densitometry are shown, as described in Fig. 2B. *$p < 0.05$ as compared with the values in IL-17A–treated fibroblasts (C). Normal cells were incubated in the presence or absence of IL-17A or IL-17F (250 ng/ml) for 24 h, and immunoblotting was performed (**D**). The levels of α1(I) collagen quantitated by scanning densitometry are shown, as described in Fig. 2B. *$p < 0.05$ as compared with the values in IL-17A–treated fibroblasts (**E**). SSc fibroblasts were incubated in the presence or absence of IL-17A or IL-17F (250 ng/ml) for 24 h (**F**), and immunoblotting was performed as described in Fig. 4D (**G**) Normal fibroblasts were incubated in the absence or presence of IL-17A or -17F (250 ng/ml) for 12 h. The quantitative real-time PCR was performed to evaluate the mRNA levels of Smad3, TGF-β1, and TGF-β receptor types I, II, and III. The mean values in untreated fibroblasts were set at 1. Data are expressed as the mean ± SE of five independent experiments.

treated cells (Fig. 4A). Immunoblotting also showed IL-17A downregulated protein expression of CTGF (Fig. 4B, 4C). Therefore, IL-17A may have an antifibrogenic effect via the downregulation of CTGF, but IL-17F may not.

In contrast, immunoblotting revealed that the protein synthesis of α1(I) collagen was significantly decreased by treatment with IL-17A in comparison with untreated cells ($p < 0.05$), but not with IL-17F, in normal fibroblasts (Fig. 4D, 4E). Considering the array result, IL-17A is thought to decrease α1(I) collagen protein expression posttranscriptionally without altering the mRNA level in normal fibroblasts.

Taken together, IL-17A may have an inhibitory effect on the expression of α1(I) collagen and CTGF, and the suppression of IL-17A signaling due to downregulation of IL-17RA by TGF-β1 signaling in SSc fibroblasts contributes to the increased collagen accumulation and fibrosis characteristic of SSc as described in the introductory paragraphs. To support this notion, in SSc fibroblasts, the protein expression of α1(I) collagen was not altered by treatment with IL-17A and IL-17F as compared with untreated cells (Fig. 4F), probably due to the downregulation of IL-17RA.

The decreased expression of CTGF upon stimulation with IL-17A suggests the possibility that not only does TGF-β inhibit IL-17 signaling in fibroblasts, but also IL-17 may inhibit TGF-β signaling, because CTGF is one of the downstream targets of TGF-β1. However, the array data indicated that IL-17A or -17F did not affect the expression of other targets of TGF-β such as fibronectin, tenascin-C, or matrix metalloproteinase-1 (MMP-1) (Table II) (42–44). In addition, the expression levels of TGF-β–related molecules including Smad3, TGF-β1, and TGF-β receptors I, II, and III were not affected by IL-17A and -17F (Fig. 4G). Therefore, IL-17 is not likely to inhibit TGF-β signaling.

microRNA may be involved in the regulation of collagen expression by IL-17A

Lastly, we tried to clarify the regulatory mechanisms of α1(I) collagen protein without changing the mRNA levels by IL-17A.

We hypothesized that microRNA is involved in this process, because microRNAs usually inhibit translation of their target genes and do not cause degradation of the target transcript. We focused on miR-129-5p as the regulator of α1(I) collagen according to microRNA target gene predictions using the TargetScan (version 5.1; http://www.targetscan.org/) and Miranda (http://www.microrna.org/microrna/home.do), which are the leading programs in the field (45). Fig. 5A shows that the mean transcript levels of miR-129-5p were significantly increased by the treatment with IL-17A in comparison with untreated cells ($p < 0.05$), but not by IL-17F, which is consistent with the effect of IL-17A or -17F on α1(I) collagen protein expression (Fig. 4D).

Furthermore, because the construct of α1(I) collagen 3′ untranslated regions (UTR) for reporter assay was not available, to determine direct interaction between miR-129-5p with α1(I) collagen 3′ UTR, we used microRNA mimic and miScript Target Protector (Qiagen), single-stranded, modified RNAs designed to specifically interfere with their interaction, while leaving the regulation of other targets by the same miR-129-5p unaffected (http://www.qiagen.com/products/miscripttargetprotectors.aspx) (46). In the presence of the control protector, the protein levels of α1(I) collagen were downregulated by the overexpression of miR-129-5p mimic in normal fibroblasts (Fig. 5B), confirming the possibility that α1(I) collagen is the target of miR-129-5p. In contrast, the protector specific to miR-129-5p inhibited the miR-129-5p mimic-mediated downregulation of α1(I) collagen (Fig. 5B),

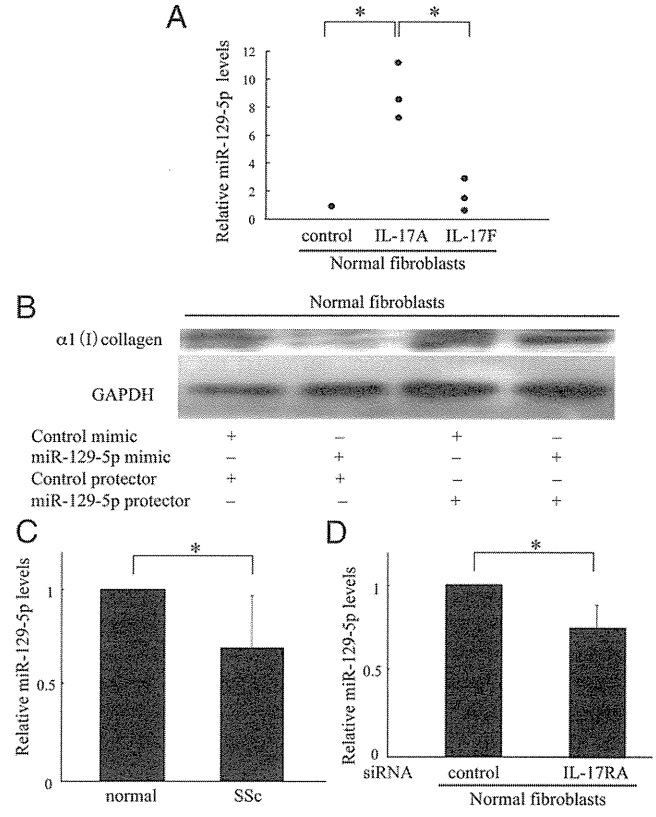

FIGURE 5. The effect of IL-17A on the expression of miR-129-5p. (**A**) Normal human dermal fibroblasts were incubated in the presence or absence of IL-17A or IL-17F (250 ng/ml) for 12 h, and microRNAs were extracted. Relative miR-129-5p expression levels (normalized with U6) were determined by quantitative real-time PCR. Data are shown on the ordinate ($n = 3$). The values in untreated cells were set at 1. *$p < 0.05$ as compared with the values in IL-17A–treated cells. (**B**) Normal dermal fibroblasts were transfected with control or miR-129-5p mimic in the presence of control or 129-5p–specific microRNA protector as described in *Materials and Methods*. The cells were harvested after 120 h. The cell lysates were subjected to immunoblotting. (**C**) Normal and SSc dermal fibroblasts were incubated under the same conditions, and microRNAs were extracted. Relative miR-129-5p expression levels (normalized with U6) were determined by quantitative real-time PCR. The values in normal fibroblasts were set at 1. Data are expressed as the mean ± SE of three independent experiments. (**D**) Normal fibroblasts were transfected with the IL-17RA siRNA or control siRNA. Relative miR-129-5p expression levels (normalized with U6) were determined by quantitative real-time PCR. The values in cells transfected with control siRNA were set at 1. Data are expressed as the mean ± SE of three independent experiments. *$p < 0.05$ as compared with the value in normal fibroblasts.

thus suggesting the direct interaction between miR-129-5p and α1(I) collagen 3′ UTR.

Therefore, IL-17A may regulate α1(I) collagen protein expression via miR-129-5p. Interestingly, a microRNA PCR array, consisting of 88 microRNAs involved in human cell differentiation and development, revealed that several microRNAs were downregulated in SSc fibroblasts compared with normal fibroblasts (Table III) and that miR-129-5p was one of the microRNAs downregulated in SSc fibroblasts (0.22-fold change in ΔΔCt method: the complete dataset is available at the Gene Expression Omnibus microarray data repository, http://www. ncbi.nlm.nih.gov/geo/, accession number GSE34142). Real-time PCR with specific primer for miR-129-5p confirmed the downregulation of miR-129-5p in SSc fibroblasts (Fig. 5C). These results suggest that IL-17A inhibits α1(I) collagen synthesis by the induction of

Table III. Summary of downregulated microRNAs in SSc dermal fibroblasts by a microarray analysis

Gene Name	Fold Change
miR-10a	<0.01
miR-142-3p	<0.01
miR-146a	<0.01
miR-196a	<0.01
miR-206	0.05
miR-185	0.05
miR-146b-5p	0.07
miR-208a	0.09
miR-192	0.10
miR-15b	0.13
let-7c	0.13
miR-195	0.16
miR-126	0.16
miR-124	0.18
miR-16	0.18
miR-99a	0.20
miR-210	0.20
miR-370	0.20
miR-100	0.21
miR-128	0.22
miR-129-5p	0.22
miR-488	0.23
miR-93	0.24
miR-125a-5p	0.25
miR-21	0.28
miR-142-5p	0.28
miR-10b	0.28
miR-155	0.28
let-7a	0.32
miR-17	0.32
let-7g	0.33

A mixture of equal amounts of microRNAs from five normal fibroblasts or five SSc fibroblasts was prepared, and microRNA expression profile in each cell type was evaluated using PCR array. The raw Ct was normalized using the mean value of four housekeeping genes. Fold-change ($1/2^{\text{the raw Ct of each microRNA} - \text{Ct of small RNA housekeeping genes}}$) is shown.

miR-129-5p in normal dermal fibroblasts, but the signal is suppressed by the downregulation of IL-17RA in SSc, which leads to the downregulation of miR-129-5p and upregulation of α1(I) collagen. To support the hypothesis, miR-129-5p levels in normal fibroblasts were decreased by the transfection of IL-17RA siRNA (Figs. 5D, 6).

Discussion

This study demonstrated the role of the IL-17 signaling pathway in ECM regulation and its contribution to the SSc phenotype by three major findings.

As described in the introduction to this article, the serum IL-17A levels in SSc patients remain controversial, and their clinical significance remains to be elucidated. Additionally, to our knowledge, this is the first report determining serum IL-17F levels in SSc patients. This study showed that SSc patients had significantly higher serum IL-17A levels, but not IL-17F, and that patients with high serum IL-17A levels tend to have pitting scars. Furthermore, skin sclerosis tends to be less severe in patients with elevated IL-17A than those without, thus suggesting the antifibrotic effect of IL-17A. However, the number of serum samples from SSc patients in the current study was too limited to yield definite conclusions. Larger studies are thus called for in the future.

Second, we found that the expression of IL-17RA in SSc fibroblasts significantly decreased in comparison with that in normal fibroblasts at the transcriptional level in vitro and in vivo. As described in the introduction, there is thought to be an intrinsic TGF-β activation in SSc fibroblasts. The current results may indicate that TGF-β1 has an inhibitory effect on the IL-17 signaling via the suppression of its receptor, the IL-17 signaling pathway is suppressed due to the downregulation of IL-17RA in SSc, and the elevation of serum IL-17A levels in SSc patients results from a negative-feedback mechanism.

This study also examined the effect of ectopic IL-17A and IL-17F on ECM expression in human normal dermal fibroblasts. Although IL-17A is thought to stimulate proliferative activity of fibroblasts as described in the introduction (34), the exact role of the IL-17 signaling pathway in the regulation of ECM remains to be fully elucidated. We focused on CTGF and α1(I) collagen genes, based on the changes in the PCR array of ECM-related genes by IL-17 in vitro. IL-17A, but not IL-17F, reduced protein expression of CTGF and α1(I) collagen. microRNA analysis revealed miR-129-5p, which may be a negative regulator of α1(I) collagen, was decreased in SSc fibroblasts, but upregulated in normal cells stimulated by IL-17A. microRNAs, short RNA molecules on average only 22 nt long, are posttranscriptional regulators that bind to complementary sequences in the 3′ UTRs of mRNAs, leading to gene silencing. Recent research in this field

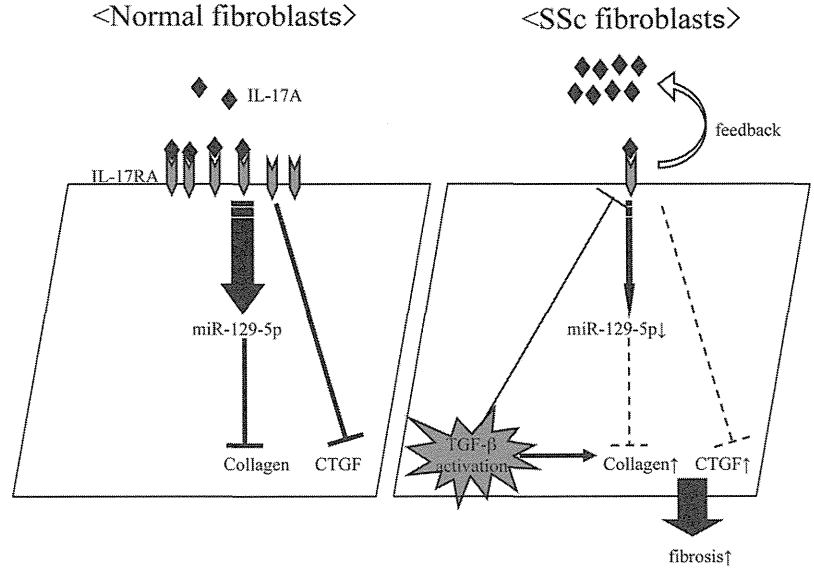

FIGURE 6. Model of IL-17 signaling in SSc fibroblasts. The IL-17A signaling pathway has an antifibrogenic effect; ectopic IL-17A stimulation in normal fibroblasts decreases CTGF expression and downregulates collagen expression via miR-129-5p. In SSc fibroblasts, the intrinsic activation of TGF-β1 stimulates the collagen production. At the same time, TGF-β1 inhibits IL-17A signaling by the downregulation of the receptor, which also contributes to the excess collagen accumulation and tissue fibrosis. The induction of CTGF by the suppression of the IL-17A signal may also further the fibrosis. In contrast, the increased IL-17A levels in the sera and the involved skin of SSc are probably due to a negative-feedback mechanism in this disease.

indicates that microRNAs play a role in angiogenesis as well as immune responses or carcinogenesis in vivo (47–51). The current study suggests that microRNAs are also involved in the regulation of ECM.

Our study has some limitations. First, IL-17RA mRNA levels of the skin tend to be inversely correlated with MRSS in SSc patients, suggesting the antifibrotic effect of IL-17 signaling. However, we could not find statistical significance, probably due to the small number of the patients. Second, previous reports suggest that the binding affinity of IL-17A to the receptor is higher than that of IL-17F (52, 53). Thus, the difference in the affinity may explain the different effect of IL-17A and -17F on ECM expression, but we could not prove this hypothesis in this study. In addition, IL-17A shares the closest amino acid sequence identity and the receptor RA only with IL-17F, but not with the other IL-17 family. Therefore, we did not determine whether the other four members of the IL-17 family have a similar effect on ECM expression to IL-17A. These points should be clarified in a future study.

In conclusion, the IL-17 signaling pathway has antifibrogenic effects via the upregulation of miR-129-5p and downregulation of α1(I) collagen. In SSc fibroblasts, the intrinsic activation of TGF-β1 stimulates the collagen production. At the same time, TGF-β1 inhibits IL-17 signaling by the downregulation of the receptor, which also contributes to the excess collagen accumulation and tissue fibrosis (Fig. 6). The induction of CTGF by the suppression of IL-17 signal may also further the fibrosis. In contrast, increased IL-17 levels in the sera and involved skin of SSc may be due to a negative-feedback mechanism. These effects were likely to be specific for IL-17A. A larger study to clarify the novel regulatory mechanisms of fibrosis by the cytokine network consisted of TGF-β1 and IL-17A may lead to the development of new therapeutic approaches for this disease.

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Disclosures

The authors have no financial conflicts of interest.

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Original article

microRNA-92a expression in the sera and dermal fibroblasts increases in patients with scleroderma

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Abstract

Objectives. microRNAs (miRNAs) play a part in various cellular activities. However, the role of miRNA in SSc is not fully understood. This study investigated the expression and role of miR-92a in SSc patients and evaluated the possibility that miR-92a is involved in the pathogenesis of this disease.

Methods. Serum samples were obtained from 61 SSc patients. mRNAs were purified from serum and levels of miR-92a and miR-135 were measured with quantitative real-time PCR. miR-92a expression in dermal fibroblasts was also determined by quantitative real-time PCR. Immunoblotting was performed to detect MMP-1 protein.

Results. The median serum levels of miR-92a, not miR-135, were significantly higher in SSc patients than normal subjects. The constitutive up-regulated miR-92a expression was also found in cultured dermal fibroblasts from SSc skin, which was decreased by the transfection with siRNA of TGF-β. Furthermore, the forced overexpression of miR-92a in normal dermal fibroblasts using miR-92a mimic resulted in the down-regulation of MMP-1 expression.

Conclusion. The increase of miR-92a in SSc may be due to the stimulation of intrinsic TGF-β activation seen in this disease. There is also a possibility that MMP-1 is the target of miR-92a and that increased miR-92a expression therefore plays a role in excessive collagen accumulation in SSc via the down-regulation of MMP-1. Clarifying the role of miRNAs in SSc may result in a better understanding of this disease and the development of new therapeutic approaches.

Key words: collagen disease, PCR, integrin.

SCIENCE

Introduction

SSc is an autoimmune disorder characterized by cutaneous and visceral fibrosis. Such fibrosis may result from the activation of fibroblasts triggered by vascular dysfunction, immune dysregulation and inflammation, but its mechanism is still unknown [1, 2]. However, many characteristics of the activated fibroblasts from SSc skin resemble those of healthy fibroblasts stimulated by exogenous TGF-β1, one of the most potent stimulatory cytokines of extracellular matrix production [3, 4]. SSc fibroblasts produce excessive amounts of various collagens [4, 5], while the blockade of TGF-β1 signalling normalizes the increased collagen expression in these cells [6], suggesting that the activation of dermal fibroblasts in SSc may result from the intrinsic activation of TGF-β1 signalling.

We previously reported that αvβ5 and αvβ3 integrins are overexpressed in SSc fibroblasts, which cause the activation of TGF-β1 signalling [7–10]. Integrin overexpression is thought to be the most upstream event in the process of the self-activation of SSc fibroblasts. However, the factors that mediate integrin overexpression in SSc have yet to be clarified.

We focused on microRNA (miRNA) in investigating integrin overexpression. miRNAs, short ribonucleic acid molecules an average of only 22 nucleotides long, are post-transcriptional regulators that bind to complementary sequences in the three prime untranslated regions (3′-UTRs) of mRNAs, leading to gene silencing. Among

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them, miR-92a can bind to the 3′-UTR of the integrin αv subunit based on miRNA target gene predictions using TargetScan (version 5.1; http://www.targetscan.org/), a bioinformatics program [11].

Many studies have shown serum miRNA levels to be a useful biomarker for diagnosis, prognosis and therapy, especially in various malignant tumors. Therefore this study investigated the serum levels of miR-92a in SSc patients and tried to evaluate the possibility that miR-92a is involved in the pathogenesis of this disease.

Materials and methods

Clinical assessment and patients' material

Serum samples were collected from 61 patients with SSc (12 males and 49 females; age range 29–85 years). All patients were grouped according to the classification system designed by LeRoy *et al.* [12]: twenty-three patients had dcSSc and 38 patients had lcSSc, as previously described [13]. All patients fulfilled the criteria proposed by the ACR [14]. Clinical and laboratory data shown in this study were recorded at the time of serum sampling. The patients were assessed for the presence of gastrointestinal, pulmonary, cardiac or renal involvement, as previously described [13]. Control serum samples were obtained from 18 healthy age- and sex-matched volunteers. Seven patients with DM, 7 patients with SLE and 12 patients with scleroderma spectrum disorder (SSD), who did not fulfill the ACR criteria of SSc, but who were considered to be at risk for developing SSc in the future based on the criteria described below, were also included in this study [15–17]. The study was approved by the Ethics Review Committee at Kumamoto University (No. 177). Written informed consent was obtained before the patients and healthy volunteers were entered into this study according to the Declaration of Helsinki. All serum samples were stored at −80°C prior to use.

miRNA extraction

miRNA isolation from cultured cells was performed using the RT^2 qPCR-Grade miRNA Isolation Kit (SA Bioscience, Frederick, MD, USA). miRNA was isolated from serum samples using an miRNeasy RNA Isolation Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions with minor modifications [18–22]. Briefly, 100 μl of serum was supplemented with 5 μl of 5 fmol/μl synthetic non-human miRNA (*Caenorhabditis elegans* miR-39; Takara Bio Inc., Shiga, Japan) as controls, providing an internal reference for normalization of technical variations between samples. After Qiazol solution (1 ml) was added and mixed by vortexing, samples were incubated at room temperature for 5 min. Aqueous and organic phase separation was achieved through the addition of chloroform. The aqueous phase was applied to an RNeasy spin column and an RNeasy MinElute spin column. miRNA was eluted from the column with nuclease-free water.

Quantitative real-time PCR

miRNAs from cultured cells were reverse transcribed into first strand cDNA using an RT^2 miRNA First Strand Kit (SABiosciences). For quantitative real-time PCR, primers for miR-92a or U6 (SABiosciences) and templates were mixed with the SYBR Premix Ex TaqII (Takara Bio, Inc.). DNA was amplified for 40 cycles of denaturation for 5 seconds at 95°C and annealing for 30 seconds at 60°C. Data generated from each PCR were analyzed using a Thermal Cycler Dice Real Time System v2.10B (Takara Bio, Inc.). The specificity of the reactions was determined using a melting curve analysis. Transcript levels were normalized to U6.

For cDNA synthesis from serum miRNA, we used mir-X miRNA First Strand Synthesis and SYBR qRT-PCR Kit (Takara Bio, Inc.) [22]. Primers and templates mixed with the SYBR Premix were used for quantitative real-time PCR with a Takara Thermal Cycler Dice (TP800). The primer sequence of hsa-miR-92a, hsa-miR-135 and cel-miR-39 was designed based on miRBase (http://www.mirbase.org). The primer sets were pre-validated to generate single amplicons. DNA amplification was 40 cycles of denaturation for 5 s at 95°C and annealing for 20 s at 60°C. The relative fold changes of hsa-miR-92a, hsa-miR-135 and cel-miR-39 were calculated using the standard curve method.

ANAs

ANAs were detected by IIF using HEp-2 cells as the substrate, as previously described [17]. Antibody for topo I, ACA or U1 RNP was determined using a MESACUP TEST Kit for each antibody (MBL, Nagano, Japan) [23].

Diagnostic method of SSD using the point system

For the diagnosis of SSD, a total score was obtained as the sum of the following five factors [15–17]:

(i) Extent of skin sclerosis: 10 points for truncal sclerosis, 5 points for skin sclerosis limited to the extremities and face, 3 points for sclerodactylia alone and 1 point for swollen fingers.
(ii) Pulmonary changes: 4 points for pulmonary fibrosis accompanied by decreased vital capacity (<80%) and 2 points for pulmonary fibrosis accompanied by normal vital capacity (⩾80%).
(iii) ANA: 5 points for positive anti-Topo I, 3 points for positive ACA or anti-U1 RNP antibody, 2 points for anti-nucleolar antibody and 1 point for other positive ANA.
(iv) Pattern of RP: 3 points for triphasic (pale, purple, red), 2 points for biphasic (two of the above colours) and bilateral, and 1 point for biphasic and hemilateral, or monophasic (pale or purple only) and bilateral.
(v) Nailfold bleeding (NFB): 2 points for NFB in three or more fingers, and 1 point for NFB in one or two fingers.

A score of 9 or more points is consistent with SSc and a score of 5–8 points is consistent with SSD.

Cell culture

Human dermal fibroblasts were obtained by skin biopsy from the affected areas (dorsal forearm) of patients with dcSSc and <2 years of skin thickening, as previously described [7–10]. Control fibroblasts were obtained by skin biopsies from healthy donors. Control donors were matched with SSc patients for age, sex and biopsy site. Institutional review board approval and written informed consent were obtained according to the Declaration of Helsinki. Primary explant cultures were established in 25-cm^2 culture flasks in modified Eagle's medium supplemented with 10% fetal calf serum and antibiotic–antimycotic (Invitrogen, Carlsbad, CA, USA). Monolayer cultures independently isolated from different individuals were maintained at 37°C in 5% CO_2 in air. Fibroblasts between the third and six subpassages were used for the experiments. The cells were serum starved for 12–24 h before experiments.

Transient transfection

TGF-β siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). miR-92a mimic was obtained from Qiagen. Control siRNA or control miRNA mimic was also obtained. Lipofectamine RNAiMAX (Invitrogen) was used as the transfection reagent. For reverse transfection, siRNAs or miRNA mimics mixed with transfection reagent were added when cells were plated, followed by incubation for 48 h (for real-time PCR) or 72 h (for Immunoblotting) at 37°C in 5% CO_2. Control experiments showed transcript levels for targets of siRNA to be reduced by >80%, and the expression of miRNA to be increased by at least 5-fold by the transfection of mimic (data not shown).

Cell lysis and immunoblotting

Fibroblasts were washed with cold PBS twice and lysed in Denaturing Cell Extraction Buffer (BIOSOURCE, Camarillo, CA, USA). Aliquots of cell lysates (normalized for protein concentrations) were subjected to electrophoresis on 10% SDS–polyacrylamide gels and transferred onto polyvinylidene fluoride filters. The filters were blocked and incubated with antibody against MMP-1 (Chemicon, Temecula, CA, USA) or β-actin (Sigma, St Louis, MO, USA). The filters were incubated with secondary antibody, and the immunoreactive bands were visualized using an ECL system (Amersham Biosciences, Arlington Heights, IL, USA) according to the manufacturer's recommendations.

Statistical analysis

The statistical analysis was carried out with Mann–Whitney U test for the comparison of medians and Fisher's exact probability test for the analysis of frequency. Correlations were assessed by Pearson's correlation coefficient. The Bonferroni correction was used for multiple comparisons: $P < 0.0071$ was considered to be significant for the comparison of serum miR-92a levels. $P < 0.0020$ or 0.0062 was considered to be significant for the correlation analysis of serum miR-92a levels with the clinical features of patients with SSc or SSD, respectively.

Results

hsa-miR-92a is present in serum

No previous study has demonstrated the expression of hsa-92a in cell-free body fluid. To confirm that the miRNA is actually detectable in human serum, total miRNA was isolated from the serum of healthy individuals and the presence of miR-92a was determined by quantitative real-time PCR using a primer set specific for miR-92a (Fig. 1). hsa-miR-92a was amplified, and C_t values were increased by the serial dilution of the miRNA. Therefore hsa-miR-92a is likely to be present in the serum and quantitative using our method.

Serum levels of hsa-miR-92a

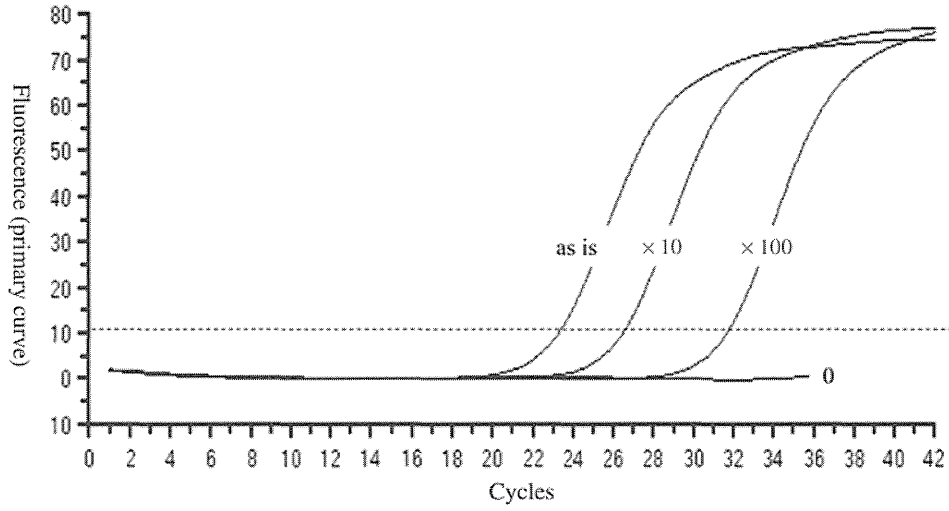
Fig. 2 depicts the serum miR-92a levels in 61 SSc patients. Seven DM patients, 7 SLE patients and 12 SSD patients who did not fulfill the criteria of SSc but were thought likely to develop SSc in the future were also included in the study [15–17].

The median serum miR-92a levels were significantly higher in SSc patients than in normal control subjects ($P < 0.00001$ by Mann–Whitney U test). Classification of SSc patients into lcSSc and dcSSc as described in the Materials and methods section revealed that the median serum levels in both lcSSc and dcSSc patients were significantly increased in comparison with those in normal subjects ($P < 0.00001$), whereas we could not find a significant difference in the values between dcSSc and lcSSc patients.

On the other hand, the median miR-92a levels in the 7 DM patients, 7 SLE patients and 12 SSD patients were slightly elevated in comparison with those in normal subjects, but not significantly. miR-92a levels in SSD patients were significantly lower than those in SSc ($P < 0.003$). Taken together, the serum miR-92a levels tended to be increased only in SSc patients. When the cut-off value was set at the mean (2 S.D.) of the normal subjects, increased serum levels of miR-92a were found in 29 of the 61 (47.5%) SSc patients and 3 of the 12 (25.0%) SSD patients.

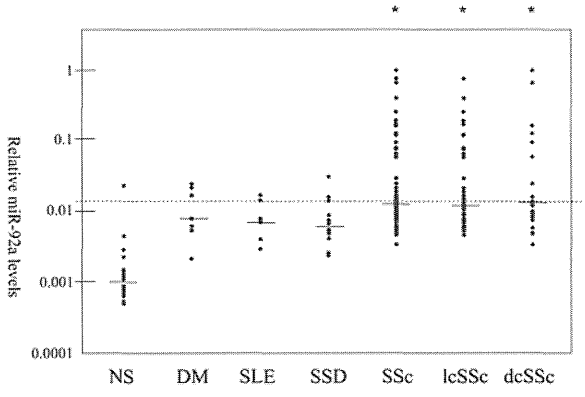
We also determined the serum levels of miR-135, another putative regulator of integrin αv based on TargetScan. As shown in Fig. 3, miR-135 was also found in the serum, but was detected in only 3 of 18 normal subjects (16.7%), 3 of 12 SSD patients (25.0%) and 5 of 61 SSc patients (8.2%). The median serum miR-135 levels were not different among SSc patients, SSD patients and normal subjects, and there was no correlation between serum miR-92a levels and miR-135 levels by Pearson's correlation coefficient in both normal subjects and patients with SSc or SSD. Therefore miR-92a seems to be specifically increased in the sera of SSc patients.

FIG. 1 hsa-miR-92a is detected in serum.



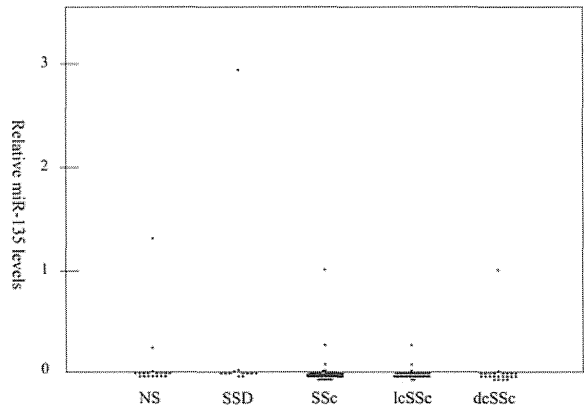
Serial dilution of cDNA (as is, 10-fold dilution, 100-fold dilution and 0) synthesized from serum miRNA was amplified by PCR with the hsa-miR-92a primer. Amplification curves of gene-specific transcripts are shown to figure the exponential increase of fluorescence. The horizontal dotted line indicates the threshold.

FIG. 2 Expression of miR-92a in the sera of patients with DM, SLE, SSD or SSc, and of normal subjects.



miR-92a levels measured as described in the Materials and methods section are shown on the ordinate. The maximal value in SSc patients was set at 1. The horizontal dotted line indicates the cut-off level. Bars show medians. *P*-values were determined using a Mann–Whitney U test. **P*<0.05 as compared with the value in normal subjects.

FIG. 3 Serum levels of miR-135 in patients with SSD or SSc, and in normal subjects.



The maximal value in SSc patients was set at 1, as described in Fig. 2.

Correlation of serum miR-92a levels with clinical and laboratory features in patients with SSc or SSD

Table 1 represents the association of serum miR-92a levels with the clinical features of patients with SSc or SSD. We found that SSc patients with increased miR-92a levels (above the cut-off value) tended to have telangiectasia at a lower frequency than those with normal levels (10.3% *vs* 31.3%, $P < 0.05$ by Fisher's exact probability test), but the difference was insignificant when $P < 0.0020$ was considered to be significant according to the Bonferroni correction. There was no statistically significant difference in terms of other clinical or laboratory features, including skin score or pulmonary fibrosis between SSc patients with and without increased miR-92a levels.

In SSD patients, the points calculated by the diagnostic method of SSD were not correlated with the levels of miR-92a ($r = -0.39$, $P = 0.19$) by Pearson's correlation coefficient. We could not find a significant difference between SSD patients with and without increased

TABLE 1 Correlation of serum miR-92a levels with clinical and serological features in patients with SSc or SSD

	Serum miR-92			
	SSc patients with increased miR-92a levels ($n = 29$)	SSc patients with normal miR-92a levels ($n = 32$)	SSD patients with increased miR-92a levels ($n = 3$)	SSD patients with normal miR-92a levels ($n = 9$)
Age at the serum sampling mean (S.D.) years	76.4 (22.1)	60.1 (12.3)	53.7 (12.4)	58.3 (12.5)
Duration of disease mean (S.D.) years	5.4 (7.9)	4.9 (8.0)		
Type (diffuse:limited)	10:19	13:19		
MRSS mean score (S.D.)	11.6 (11.0)	10.6 (8.6)		
Point mean score (S.D.)			5.3 (0.6)	6.4 (1.3)
Clinical features				
Pitting scars/ulcers	30.0	29.2		
NFB	52.2	52.2	33.3	66.6
RP	88.0	88.9	66.7	66.7
Telangiectasia	10.3*	31.3		
Contracture of phalanges	77.3	96.0		
Calcinosis	0.0	0.0		
Diffuse pigmentation	38.9	42.1		
Short SF	63.2	86.4		
Sicca symptoms	61.1	57.1		
Organ involvement				
Pulmonary fibrosis	46.2	39.3	33.3	22.2
%VC mean % (S.D.)	103.7 (17.6)	93.4 (19.8)		
%DLco mean % (S.D.)	85.6 (20.7)	80.0 (25.1)		
Pulmonary hypertension	29.8	29.8		
Oesophagus	22.7	23.1		
Heart	32.0	48.1		
Kidney	0.0	3.6		
Joint	70.6	41.7		
ANA specificity				
Topo I	21.4	25.8	66.7	33.3
ACA	32.1	35.5	33.3	44.4
U1 RNP	10.7	12.9	33.3	0

Unless indicated, values are percentages. The points were calculated by the diagnostic method of SSD as described in the Materials and methods section. MRSS: modified Rodnan total skin thickness score; SF: sublingual frenulum; VC: vital capacity; DLco: diffusion capacity for carbon monoxide; U1 RNP: anti-U1 RNP antibody. *$P < 0.05$ *vs* patients with normal 92a levels using Fisher's exact probability test.

miR-92a levels in the frequency of clinical features such as NFB or RP. Therefore the serum miR-92a levels are not likely to be a specific marker for clinical manifestations of SSD.

Functional studies of miR-92a

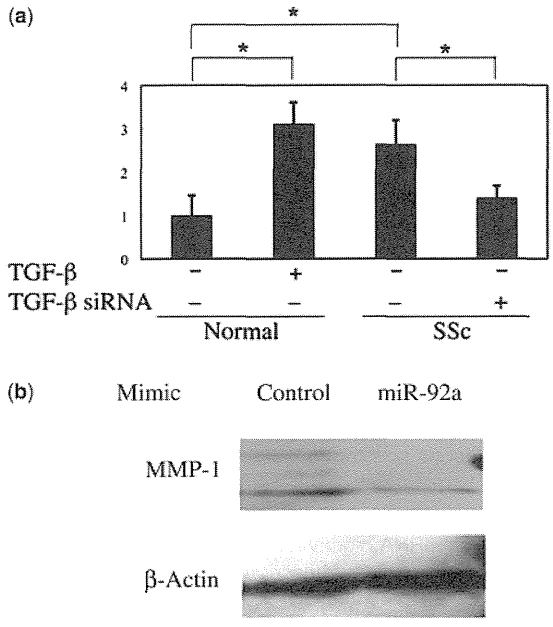
Lastly, we tried to clarify upstream pathways of the increased miR-92a in SSc. miR-92a expression was significantly increased in normal cultured dermal fibroblasts in the presence of TGF-β and SSc fibroblasts in comparison with untreated normal fibroblasts ($P < 0.05$, Fig. 4a). Inhibition of TGF-β signalling by specific siRNA in SSc fibroblasts down-regulated miR-92a expression ($P < 0.05$; Fig. 4a). Constitutively, up-regulated miR-92a expression in SSc fibroblasts is consistent with increased serum miR-92a in SSc patients, indicating the possibility that dermal fibroblasts are one of the cellular sources of serum miR-92a. The normalization of miR-92a levels by TGF-β siRNA in SSc fibroblasts suggests that an increase of miR-92a is due to stimulation by intrinsic TGF-β activation seen in this cell type, as described in the Introduction section.

We also evaluated the consequences of up-regulated miR-92a in SSc fibroblasts. Forced overexpression of miR-92a in normal fibroblasts using miR-92a mimic resulted in the down-regulation of MMP-1 expression (Fig. 4b), suggesting that MMP-1 is the target of miR-92a and that increased miR-92a expression plays a role in the excessive collagen accumulation in SSc fibroblasts via the down-regulation of MMP-1.

Discussion

Over 1000 miRNAs are thought to be present in the human genome, and they may target ~60% of mammalian genes [24]. It has been revealed by recent research that miRNAs

FIG. 4 miR-92a expression is regulated by TGF-β signalling in SSc fibroblasts.



(**a**) Levels of miR-92a in normal fibroblasts in the presence or absence of TGF-β and in SSc fibroblasts with or without TGF-β siRNA ($n = 3$). The value in normal fibroblasts transfected with control siRNA without TGF-β stimulation was set at 1. *$P < 0.05$. (**b**) Normal human fibroblasts at a density of 2×10^4 cells/well in 24-well culture plates were transfected with control miRNA mimic or miR-92a mimic for 72 h. Cell lysates were subjected to immunoblotting using MMP-1 antibody.

play a role in various cellular activities, including the immune response, carcinogenesis and angiogenesis *in vivo* [25, 26]. Serum miRNA levels have attracted attention for their usefulness in the diagnosis and follow-up of tumors.

We expected that serum miRNA could also be useful as a marker in collagen disease and that serum miR-92a levels are decreased in SSc patients because miR-92a is a putative down-regulator of integrin, which is up-regulated in SSc. Contrary to expectations, however, median serum miR-92a levels were observed to be significantly higher in SSc patients than in normal subjects. We theorize that such an increase of miR-92a in SSc may be caused by intrinsic activation of TGF-β, and may be induced as a negative feedback mechanism against the overexpressed integrin. Another possibility is that increased miR-92a may contribute to the pathogenesis of this disease by regulating other targets. The overexpression of miR-92a results in the down-regulation of MMP-1. MMP-1 down-regulation in SSc has been reported and it may further the excess accumulation of collagen in this disease by decreasing collagen degradation [27], indicating that the overexpression of miR-92a plays a role in the pathogenesis of SSc through the down-regulation of MMP-1. Since MMP-1 is not predicted to be a target of miR-92a by the bioinformatics program described above, miR-92a may regulate MMP-1 expression indirectly via other targets.

We found that high levels of miR-92a are associated with SSc, whereas moderately raised levels are associated with connective tissue diseases in general. Serum miR-135 was not up-regulated in SSc patients. Therefore serum miR-92a levels may be specifically increased in SSc patients in comparison with normal subjects but a sensitivity of 47.5% is unlikely to be clinically useful. Serum miR-92a levels may have the potential to be used alongside other parameters in diagnostic algorithms.

In this study the serum samples of SSc patients were taken from those who easily fulfill the clinical criteria for this disease. Before the test can be used in a clinical setting, it needs to be examined longitudinally in a cohort of patients in whom the disease is in its early stages. The concept of SSD was originally proposed by Maricq *et al.* [15, 16] to unify typical SSc, early forms of SSc and closely related disorders, including MCTD. Following this, Ihn *et al.* [17] redefined SSD as occurring in patients who did not fulfill the criteria of SSc but were thought likely to develop SSc in the future, and proposed a new method using a points system to diagnose SSD patients earlier and follow-up carefully against the development of SSc. In this study we found a significant difference in the miR-92a levels between SSD and SSc patients. Serum levels of miR-92a levels may be useful for the differentiation of SSc from SSD, and serial time-course measurement of miR-92a levels in SSD patients may lead to early detection of developing SSc.

We also found that the prevalence of telangiectasia in SSc patients with increased miR-92a levels tended to be lower than in those with normal levels. Vascular damage is one of the earliest events in SSc and, as described above, this damage may contribute to the fibroblast activation and tissue fibrosis seen in this disease. Although the mechanisms of the decreased frequency of telangiectasia by increased miR-92a are unknown, there is a possibility that MMP-1 is involved in the formation of telangiectasia and increased miR-92a may regulate telangiectasia formation negatively via the down-regulation of MMP-1. Clarifying the role of miRNAs in SSc may help to achieve a better understanding of this disease and lead to the development of new therapeutic approaches.

Rheumatology key messages

- Median serum miR-92a levels were higher in scleroderma patients than in normal subjects.
- miR-92a was constitutively overexpressed in cultured dermal fibroblasts from SSc skin.
- The forced overexpression of miR-92a mimics in normal dermal fibroblasts down-regulated MMP-1 expression.

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Original article

Clinical characteristics and survival of Japanese patients with connective tissue disease and pulmonary arterial hypertension: a single-centre cohort

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Abstract

Objective. To clarify the characteristics, survival and predictors of mortality in Japanese patients with pulmonary arterial hypertension (PAH) associated with CTD.

Methods. This single-centre cohort study enrolled 70 consecutive patients with PAH-CTD who visited a tertiary referral centre in Japan between 1970 and 1990 ($n = 30$, historical group) and between 2000 and 2009 ($n = 40$, recent group). Baseline clinical features, haemodynamic parameters and ANA profiles were recorded. The Cox proportional hazards regression model was used to determine independent factors associated with an increased risk of mortality.

Results. MCTD and SLE were the major underlying CTDs, comprising 43% and 29% of PAH patients respectively, whereas SSc was less common (19%). Anti-U1RNP antibody was the most prevalent ANA (61%). The cumulative survival rate was significantly better in the recent group in comparison with the historical group (76% *vs* 26% at 3 years; $P < 0.001$). When both groups were combined, World Health Organization functional class III or IV at diagnosis was identified as an independent predictor of mortality, whereas modern PAH drug use was associated with a favourable outcome.

Conclusion. The major PAH-CTD population in Japan suffers from MCTD or SLE with anti-U1RNP antibody, in contrast to PAH-CTD patients in the USA and Europe. Modern PAH treatment has improved survival rates, but long-term outcomes are still unsatisfactory. Independent predictors of mortality indicate that early diagnosis and the prompt use of PAH drugs should improve survival.

Key words: pulmonary arterial hypertension, mixed connective tissue disease, systemic lupus erythematosus, scleroderma, anti-U1RNP antibody.

SCIENCE

Introduction

Pulmonary arterial hypertension (PAH) is an intractable condition that in patients with CTDs has progressive debilitating symptoms and a poor prognosis [1–3]. In cohort studies conducted in the USA and Europe, the majority of patients with PAH associated with CTD (PAH-CTD) had SSc [4–7], and the most prevalent serum ANA was an ACA [8–12]. However, in our clinical experience, SLE and MCTD are fairly common in Japanese patients with PAH-CTD. In fact, a multicentre survey conducted in Japan in the late 1980s revealed a high prevalence of MCTD and serum anti-U1RNP antibodies in CTD patients with pulmonary hypertension (PH) [13], although that study did not require right heart catheterization for the diagnosis of PH, and did not exclude patients with PH due to left-sided heart disease, interstitial lung disease (ILD) or chronic thromboembolism. Therefore,

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Japanese patients with PAH-CTD have not been characterized in detail.

The prognosis for patients with PAH-CTD was very poor before 2000, when non-specific vasodilators were the sole treatment option: the 1-year survival rate in patients with SSc and PAH was only 45% [1]. Over the past decade, PAH-specific vasodilative agents, including prostanoids, endothelin receptor antagonists and phosphodiesterase 5 inhibitors, have become available for clinical use. Consequently, several studies that primarily enrolled SSc patients reported improvement in short-term survival: the 1-year survival rate was >80% [4, 5, 10, 12, 14–16]. In Japan, beraprost and i.v. epoprostenol have been available since 1999, and bosentan and sildenafil were introduced to clinical practice in 2005. However, few data are available on the improvement in survival of Japanese patients with PAH-CTD in the modern treatment era.

In this study we evaluated the clinical characteristics and prognosis of patients with PAH-CTD who were diagnosed and monitored at a tertiary PH referral centre in Japan. By comparing databases covering 1970–90 (pre-PAH drug era) and 2000–09 (modern treatment era), we also investigated the impact of PAH drugs on survival.

Materials and methods

Study population

All the incident cases of PAH-CTD consecutively diagnosed at the specialized PH clinic of Keio University Hospital were included in this study: 30 patients were diagnosed with PAH between 1970 and 1990 (historical group); 40 patients were diagnosed between 2000 and 2009 (recent group). Patients in whom PAH was diagnosed between 1991 and 1999 were excluded because complete clinical data were not available. PAH was diagnosed based on the following criteria: (i) a mean pulmonary arterial pressure (mPAP) $\geqslant$25 mmHg at rest by right heart catheterization [17]; (ii) exclusion of left-sided heart disease, determined by pulmonary capillary wedge pressure $\leqslant$15 mmHg by right heart catheterization; (iii) exclusion of advanced ILD, determined by forced vital capacity predicted <70% [18]; and (iv) exclusion of chronic thromboembolism, determined by contrast-enhanced CT scan and ventilation/perfusion scan with or without pulmonary angiography. The study was approved by the Keio University Institutional Review Board, and clinical information was obtained after the patients had given written informed consent.

The clinical diagnoses of SLE and SSc were made according to the ACR preliminary classification criteria [19, 20]. MCTD was diagnosed according to the criteria proposed by Kasukawa *et al.* [21], as having all of the following: RP or swollen fingers/hands, a positive anti-U1RNP antibody, and overlapping features of at least two diseases among SLE, SSc and polymyositis. SS was diagnosed according to the revised criteria proposed by the American–European Consensus Group [22]. SS patients without any apparent CTDs were regarded as having primary SS.

Data collection

A complete medical history, physical examination, laboratory evaluations and right heart catheterization were performed for each patient at the time of PAH diagnosis, with more limited evaluations during follow-up. Except in SSc patients, the onset of CTD was defined as the first symptom attributable to CTD; in SSc patients, disease onset was defined as the first non-RP manifestation. The follow-up clinical and laboratory information was prospectively recorded in the database. Data collected at the time of PAH diagnosis included age, sex, underlying CTD, RP, ILD, pericardial effusion, renal disorder, World Health Organization-functional class (WHO-FC) and haemodynamic parameters. ILD was defined as bibasilar reticular pattern of linear or lineonodular densities most pronounced in basilar portions of the lungs in chest radiographs and/or CT scans [20]. Pericardial effusion was detected by echocardiography. Renal disorder was defined as persistent proteinuria >0.5 g/day and/or the presence of cellular casts [19]. All treatment regimens used for PAH were recorded, and included PAH drugs (beraprost, epoprostenol, bosentan and sildenafil) and immunosuppressive treatment, which consisted of CSs (>0.5 mg/kg prednisolone equivalent) in combination with or without AZA, CYC or MMF.

ANA profiles

A serum sample was obtained from each patient at the time of PAH diagnosis and stored at −20°C. Serum ANA profiles were identified by IIF on HeLa cell chromosomal spreads (anticentromere), radioimmunoassay (anti-double-stranded DNA), RNA immunoprecipitation (anti-U1RNP, anti-Sm, anti-SSA and anti-SSB) and protein immunoprecipitation (anti-topo I) [23].

Statistical analysis

Continuous variables were compared with the Mann–Whitney U-test. Categorical variables were compared with the chi-square test. For a 2×4 contingency table, significant differences (overall $P < 0.05$) were further analysed by pairwise comparisons. Survival analysis was performed using the Kaplan–Meier method, and the survival rates of the two groups were compared using the log-rank test. The Cox proportional hazards regression model was used to determine factors associated with an increased risk of mortality at 1, 3 and 5 years after PAH diagnosis. Variables selected by univariate analysis were further subjected to multivariate analysis. The results are presented as a hazard ratio (HR) with 95% CI. Statistical analysis was performed using SPSS 17.0 statistical software (SPSS; Chicago, IL, USA).