these LAP-injected skin sites showed a significant decrease compared with the control PBS injection (Fig. 3a), (LAP: 3.89 ± 0.42 $\mu g/100\ \mu l$ vs PBS: 4.49 ± 0.28 $\mu g/100\ \mu l$, $P = 0.03$).

LAP injection reduces TGF-β production induced by BLM

To examine the effects of TGF-β1 activation and production in BLM-induced scleroderma, TGF-β1 protein in the injected skin sites was measured by ELISA. As a recent study has demonstrated that a local injection of BLM increased the amount of total (active/latent forms) TGF-β1 protein in a mouse strain-dependent manner (14), we focused on the biologically active form of TGF-β1 protein. Antigen-specific ELISA without acid pretreatment, enabling protein determination of the constitutively active form of TGF-β1, revealed that co-injection of BLM and LAP for 1 week significantly reduced the amount of active TGF-β1 protein compared with levels in skin sites treated with BLM with or without an additional control injection of PBS (Fig. 3b) (LAP: 114.83 ± 48.25 pg/ml vs PBS: 226.02 ± 36.2 pg/ml, $P = 0.0285$).

Time course of expression of fibrogenic cytokines and collagen α1(I) mRNA

Transcriptional levels of TGF-β1 and CTGF, both of which are important fibrogenic cytokines in skin sclerosis, were analysed by a real-time PCR assay. The steady-state expression of TGF-β1 mRNA tended to decrease during co-injection of BLM and LAP for 4 weeks, but almost remained unchanged in the LAP- and PBS-treated groups, implying post-translational regulation of TGF-β1. In contrast, expression levels of CTGF and collagen α1(I) mRNA were downregulated transiently after 2 weeks of co-injection of BLM and LAP (Fig. 3c,d, respectively) and then recovered to steady-state levels 4 weeks after the co-injection. These results suggest that (i) there are different regulatory pathways for TGF-β1 and CTGF in BLM-induced scleroderma and (ii) post-transcriptional upregulation may be predominant in the TGF-β1 system elicited by BLM.

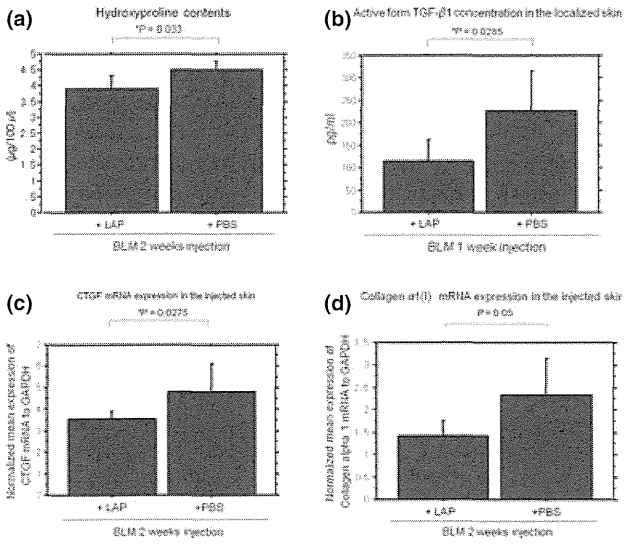

Figure 3. (a) The hydroxyproline content of injected skin sites. The hydroxyproline content of local skin sites co-injected with bleomycin (BLM) and latency-associated peptide (LAP) for 2 weeks is significantly decreased. (LAP, 3.89 ± 0.42 $\mu g/100\ \mu l$ vs PBS, 4.49 ± 0.28 $\mu g/100\ \mu l$, $P = 0.03$). (b) Concentration of active form of transforming growth factor-beta1 (TGF-β1) in local skin injection sites. In the local skin injection sites, constitutively active TGF-β1 protein revealed that co-injection of BLM and LAP for 1 week significantly reduced the amounts of active TGF-β1 protein compared with that of a control group receiving PBS. (LAP: 114.83 ± 48.25 pg/ml vs PBS: 226.02 ± 36.2 pg/ml, $P = 0.0285$). (c) connective tissue growth factor (CTGF) mRNA expression in local skin injection sites after treatment. After 2 weeks of BLM/LAP treatment, the CTGF mRNA expression in the local skin injection sites was downregulated compared with the PBS-injected control group. (d) Collagen α1(I) mRNA expression in local skin injection sites after treatment. Collagen α1(I) mRNA tended to be downregulated compared with PBS-injected control group.

Discussion

Despite various therapeutic approach using murine models (15–18), therapies for human scleroderma are often questionable and still provide inconsistent outcomes, particularly in cases with skin sclerotic phenotype alone. TGF-β increases the synthesis of ECM proteins, modulates cell-matrix adhesion protein receptors and regulates the proteins that can modify the ECM by proteolytic action. TGF-β increases TGF-βreceptor (TGF-βR) levels in fibroblasts, and the maintenance of increased TGF-β production may lead to the progressive deposition of ECM, resulting in fibrosis. In SSc fibroblasts, TGF-β and its receptor levels are elevated significantly. Moreover, TGF-β type I and II receptor levels correlated with elevated collagen α2(I) and mRNA and promoter activity (19). TGF-β1 thus plays a key role via autocrine signalling in the pathogenesis of scleroderma. Molecularly based strategies targeting TGF-β1 and related molecules have recently been explored with the aim of improving the safety and efficacy of symptom-specific treatment of the disease. Against this background, neutralization of *in vivo* TGF-β1 action is now considered a plausible approach in several animal models of fibrotic and/or sclerotic disease, although the clinical trials utilizing anti-TGF-β1 antibody (CAT-192) for human scleroderma failed to show efficacy (9).

Transforming growth factor-beta activity is controlled predominantly through activation of latent molecule. LAP constructs N-terminal remnant of the TGF-β precursor that is the indispensable molecule of activation steps of TGF-β. To convert to the mature form of TGF-β, the large latent complex is cleaved by acidic or alkaline conditions, heat treatment, certain enzyme glycosidase, plasmin and interacts as some kind of ligands. In dermal fibroblasts, several membrane proteins such as thrombospondin-1 (TSP-1) and integrin $\alpha v\beta 5$ catalyse the activation of latent TGF-β in the local microenvironment (20). The activation step of latent TGF-β is schematically shown in Fig. 4. Activated TGF-β is a potent fibrogenic cytokine, known to induce collagen synthesis by fibroblast *in vivo* and *in vitro*. Because of the critical role of TGF-β activity, LAP plays a pivotal role in regulating the effect of TGF-β1 and may be one of the therapeutic interventions of scleroderma. In this study, we attempted to examine the therapeutic effects of local administration of recombinant LAP in the mouse model of BLM-induced scleroderma. Combined with our previous series of investigations (4) and the molecular specificity of LAP, the results demonstrate that *in vivo* TGF-β1 inactivation by the local administration of LAP can produce a therapeutic benefit in scleroderma. This effect was associated with decrease of collagen contents and TGF-β1 concentration and downregulation of mRNA levels of CTGF and collagen α1(I) in the skin tissues. These results corresponded to the previous report of local LAP administration for murine Scl-GvHD (11), in which transcriptional activity of fibrogenic cytokines such as CTGF and TGF-β1 was decreased by LAP treatment as well as collagen synthesis. In addition, neutralization of TGF-β by recombinant LAP on imma-

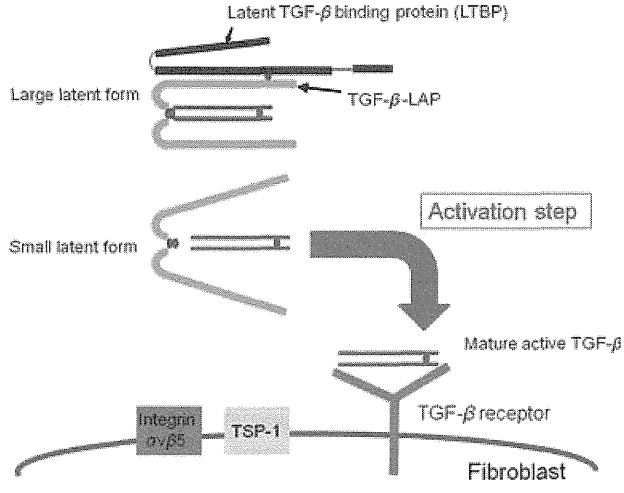

Figure 4. Schematic representation of the activation step of latent transforming growth factor-beta (TGF-β). In the activation steps of TGF-β, latency-associated peptide is cleaved by acidic or alkaline conditions, heat treatment, certain enzyme glycosidase, plasmin and any other environments. In dermal fibroblasts, several membrane proteins such as thrombospondin-1 (TSP-1) and integrin $\alpha v\beta 5$ catalyse the activation of latent TGF-β.

ture dendritic cells enhances T-cell proliferation and increases the production of IFN-γ (21), which may shift Th2 cytokine balance to Th1 response. On the other hand, independently of TGF-β, LAP blocks inflammation by inhibiting chemotaxis of monocytes and producing IL-10 (22), which may have a role. Recently, the pathogenesis of innate immunity in BLM-induced scleroderma is shown (23). BLM induced hyaluronan production and BLM itself enhances an intrinsic signal of skin and lung fibrosis, which is regulated by CD19 via TLR2/TLR4 signalling. Boswell et al. (24) demonstrated that a LAP-TGF-β expression on $CD4^+$ T-cell induced by novel peptide Glypican-3 (GPC_{81-95}) suppress TLR4 ligand-induced TNF-α production. In our study, LAP may contribute to suppress the inflammation and fibrosis via interference of TLR signalling pathway.

In the post-onset experiments, however, the LAP treatment did not exhibit reverse effects after 4 weeks of pretreatment with BLM alone. It is therefore likely that the biological action of active TGF-β1 and chemotaxis in inflammatory phase are indispensable in BLM-induced skin sclerosis, particularly in the early stages, and also hovers at the boundary between inflammatory and sclerotic responses. From the clinical perspective, the temporal administration of recombinant LAP awaits further investigation regarding disease stage-specific treatment in human scleroderma.

Moreover, TGF-β1-dependent skin fibrosis becomes persistent after simultaneous or subsequent injection of recombinant CTGF (25). Likewise, our data also demonstrated that upon co-injection of BLM and LAP, a transient decrease in active TGF-β1 protein expression preceded that of CTGF mRNA expression and paralleled that of collagen α1(I) mRNA. The molecular association between TGF-β1 and CTGF is strengthened by evidence that CTGF enhanced TGF-β1-induced phosphorylation and nuclear translocation of Smad 2/3 (26). Specifically, Smad 3 is a key intercellular signal transduction molecule downstream of BLM-induced TGF-β1 action on skin fibroblasts, as supported by studies in Smad 3-knockout mice (5). *In vitro* studies using fibroblasts derived from either Smad 3- or Smad 2-null embryos have revealed a complex picture of TGF-β1 transcriptional regulation, indicating distinct mechanisms of TGF-β1 gene regulation by Smad 2, Smad 3 or both. In addition, a TGF-β1 response element is located within the promoter region of CTGF gene, notwithstanding the fact that the promoter activity is independent of Smad phosphorylation. A series of these complex interactions may potentiate the stratified signalling cascade between TGF-β1 and CTGF, indicating that CTGF represents a downstream regulatory molecule in the TGF-β1 cascade (27). On this basis, the transient CTGF downregulation preceded by a decrease in active TGF-β1 protein expression may inevitably be involved in attenuating the presclerotic inflammation induced by LAP treatment, and also in shifting to the later sclerotic phenotype. Our present data are in agreement with the recently discussed scenario for the TGF-β1 and CTGF interrelationship in scleroderma; that is, TGF-β1 acts as a trigger initiating presclerotic inflammation, to some extent with the orchestration of the subsequently developed sclerotic phenotype, and the resultant induction of CTGF maintains the sclerotic phenotype. Once dermal sclerosis is established – or goes even beyond the biological boundary between inflammatory and sclerotic responses – downregulation of CTGF transcription and/or pre-existing inflammation may be driven in the local fibroblasts.

The mammalian LAP has four distinct isoforms, LAP1-4, having a propensity to associate non-covalently with specific TGF-β family members, TGF-β1-3, in humans (28). Given that subcutaneous injection of all three TGF-β isoforms can elicit the skin fibrosis, the therapeutic cocktail utilizing selective LAP isoforms may thus inhibit the isoform-specific action of TGF-β1, contributing to the antisclerotic effects without adverse reactions. Moreover, cell surface molecules such as integrin $\alpha v\beta 5$ and thrombospondin-1 capable of activating the latent form of TGF-β are potential therapeutic targets (20). Myofibroblasts activate latent TGF-β as a function of their contractile activity and extracellular domain stiffness may be a new approach to control the mechanism of fibrosis (29).

Several lines of evidence have shed light on local mast cell function in scleroderma pathology. In human scleroderma, numbers of mast cells in skin lesions, both involved and uninvolved, are increased (30). Mast cells are one of the major sources of TGF-β in SSc, and degranulation of dermal mast cells is an important mechanism of TGF-β secretion (12). TGF-β can also recruit mast cells into the dermis (31). After injection of BLM into mouse skin, infiltrating mast cells increased not only in the early oedematous skin but also in the sclerotic skin and reached a peak at 2–3 weeks after the injection (32). The co-injection of LAP significantly inhibited the BLM-induced mast cell infiltration, whereas the opposite action of TGF-β1 raised a variety of inhibitory effects on steady-state levels of cell surface markers, degranulation and responsiveness to IgE-mediated activation in mast cells. Shiota et al. (33) showed the lesional skin of scleroderma mainly contains connective tissue-type mast cells, by which a synthetic chymase-specific inhibitor can prevent the establishment of skin fibrosis in tight-skin (Tsk) mice, another reliable scleroderma mouse model. Walker et al. (34) reported that ketotifen, an inhibitor of mast cell degeneration, decreased

skin fibrosis in Tsk mice. Such characteristic responses of the local mast cells differ considerably from the time-dependent regulation of active TGF-β1 protein and CTGF mRNA, and more critically, our recent observations suggest that BLM-induced skin sclerosis was demonstrable in both the mast cell-deficient strain of WBB6F1-W/Wv mice, much the same as in control mice (35). These observations concerning local mast cell biology may provide insight into the as yet uncharacterized action of BLM in the skin, as well as the underlying conclusion that mast cells may not be the principal players in the establishment of BLM-induced scleroderma.

In conclusion, in this study, we demonstrated the therapeutic potential of *in vivo* LAP administration in the scleroderma model mouse, making a focus on skin sclerosis. Our data warrant further detailed investigations to clarify the disease stage-specific action of this multifactorial cytokine in scleroderma, particularly with respect to an adequate period of LAP administration to the lesional skin. In future studies, it will be necessary to determine the LAP effect on lung fibrosis and investigate its pathogenesis of auto-immune systems.

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Author contributions

T. Nakamura-Wakatsuki performed the research and T. Yamamoto designed the research study. T. Nakamura-Wakatsuki, N. Oyama and T. Yamamoto analysed the data and wrote the paper. All authors substantially contributed to the research study and were involved in drafting the article or revising it critically, and all authors approved the submitted and final versions.

Conflicts of interest

The authors have declared no conflicting interests.

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ORIGINAL ARTICLE

Dynamics of serum angiopoietin-2 levels correlate with efficacy of intravenous pulse cyclophosphamide therapy for interstitial lung disease associated with systemic sclerosis

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Abstract
Objective Angiopoietin-2 (Ang2) regulates the transition between vascular quiescence and angiogenesis in a context-dependent manner. In systemic sclerosis (SSc), serum Ang2 levels correlate with its disease activity. Therefore, we investigated the clinical significance of monitoring serum Ang2 levels during intravenous pulse cyclophosphamide (IVCY) therapy in SSc patients with interstitial lung disease (ILD).
Methods Serum Ang2 levels were determined by a specific enzyme-linked immunosorbent assay in seven SSc patients treated with IVCY and 20 healthy controls. In the patient group, serum samples were drawn the day before each IVCY therapy.
Results Serum Ang2 levels tended to be higher in SSc patients before IVCY than in healthy controls and significantly correlated with KL-6, surfactant protein D, erythrocyte sedimentation rate, and C-reactive protein in SSc patients with ILD. In sera drawn before the last IVCY, Ang2 levels were significantly decreased compared with initial levels. Notably, Δ serum Ang2 levels between baseline and after the first IVCY significantly correlated with Δ ILD score between before and after the entire IVCY therapy ($r = 0.90$, $p < 0.01$).
Conclusion Monitoring Ang2 levels during IVCY treatment may be useful to evaluate and predict the efficacy of this treatment for SSc-ILD.

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Keywords Angiopoietin-2 · Interstitial lung disease · Intravenous pulse cyclophosphamide · Systemic sclerosis

Introduction

Systemic sclerosis (SSc) is a multisystem autoimmune disorder characterized by vascular injuries and fibrosis in the skin and internal organs [1]. Although the pathogenesis of SSc remains unknown, increasing evidence suggests that angiopathies, including endothelial cell (EC) activation and damage and vascular morphological changes, appear to precede the development of fibrosis. Extensive studies have demonstrated that altered angiogenesis is one cause of angiopathies in SSc [2, 3].

Angiopoietins are ligands for the endothelium-specific tyrosine kinase Tie2 receptor and comprise four structurally related proteins, termed angiopoietin-1 (Ang1), -2 (Ang2), -3, and -4 [4]. Among them, the roles of Ang1 and Ang2 in Tie2 signaling have been extensively studied. Of note, the interaction of Tie2 with Ang1 and Ang2 exerts the dual effects on vascular quiescence and angiogenesis in a context-dependent manner [5]. In quiescent vessels, Ang1 released from mural cells induces transassociation of Tie2 at EC–EC contacts, which activates angiostatic signaling to maintain vascular quiescence. On the other hand, once vascular endothelial growth factor (VEGF) is released from ischemic tissues, detachment of mural cells from ECs and disruption of EC–EC adhesions occur. Under this situation, Tie2 is anchored to extracellular-matrix-bound Ang1 and activates angiogenic signaling, thereby promoting angiogenesis cooperatively with VEGF. Likewise, Ang2 and VEGF coordinately regulate endothelial behavior. In the presence of VEGF, Ang2 enables EC migration and proliferation and the sprouting of new blood vessels, whereas

the presence of Ang2 leads to EC death and vessel regression if the activity of endogenous VEGF is inhibited [4, 6]. Thus, Ang/Tie2 signaling and VEGF work in concert to organize the complex processes of angiogenesis and vascular remodeling.

Consistent with the pivotal roles of angiopoietins in angiostatic and angiogenic processes, serum levels of Ang1 and Ang2 have been shown to be associated with the pathological events of tumor progression [7] and vascular diseases, including Crohn's disease [8, 9], ulcerative colitis [10], and systemic lupus erythematosus [11]. In SSc, serum levels of Ang1 are significantly decreased and those of Ang2 significantly elevated compared with healthy controls [12]. Furthermore, serum Ang2 levels correlate with the modified Rodnan total skin thickness score, the European Scleroderma Study Group (EScSG) disease activity index score, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) while inversely correlating with the percentage of predicted diffusion lung capacity for carbon monoxide (%DLco). Moreover, serum Ang2 levels are significantly higher in SSc patients with more advanced capillary damage than in those with less severe microangiopathy [12]. These data indicate that serum Ang2 levels may serve as a useful marker to evaluate SSc disease severity and activity. However, there has been no report regarding the clinical significance of monitoring serum Ang2 levels to evaluate the efficacy of any SSc treatment. Therefore, as an initial step to address this issue, we focused on interstitial lung disease (ILD) associated with SSc, which is at least partially caused by microvascular damage, and evaluated the association between the dynamics of serum Ang2 levels and the clinical efficacy of intravenous pulse cyclophosphamide (IVCY) therapy for SSc-ILD.

Materials and methods

Patients

Serum samples, frozen at −80 °C until assayed, were obtained from seven SSc patients who underwent IVCY therapy against ILD during October 2009 and September 2010 at our hospital (all women; mean age 57.4 ± 15.5 years; mean disease duration 7.5 ± 10.1 years) and 20 healthy individuals (all women; mean age 50.9 ± 9.2 years) after getting informed consent and institutional approval (University of Tokyo Graduate School of Medicine). Prednisone was also administered orally in all patients. In patient 6, 20 mg/day was started when the first IVCY was administered. In patient 4, the pre-existing dose (9 mg/day) was continued through the entire IVCY therapy because the patient did not agree to a dose increase. In the other five patients, dosage was increased up to 20 or 30 mg/day a few weeks prior to first IVCY therapy administration. In patients, serum samples were collected before each IVCY. Patient demographics and concurrent treatments are shown in Table 1. All patients were diagnosed with diffuse cutaneous SSc by LeRoy's classification system [13].

Serum Ang2, KL-6, and surfactant protein D measurement

Specific enzyme-linked immunosorbent assay (ELISA) kits were used to measure serum Ang2 levels (R&D Systems, Minneapolis, MN, USA). Briefly, polystyrene 96-well plates coated with antibodies against Ang2 were incubated with 100 μl of fivefold diluted serum at room temperature for 2 h. Then, the wells were washed and incubated at room temperature for 2 h with horseradish-peroxidase-conjugated antibodies against Ang2. The wells were washed again, tetramethylbenzidine added, and they were incubated at room temperature for 30 min. Finally, sulfuric acid (H_2SO_4) was added to terminate the reaction, and absorbance at 450 nm was measured. Serum Ang2 levels were calculated using standard curve. Serum levels of KL-6 and surfactant protein D (SP-D) were measured, as described previously [14, 15].

Evaluation of ILD

During treatment, IVCY efficacy was evaluated subjectively by the degree of dry cough and dyspnea and objectively by pulmonary function test and chest computed tomography (CT). Two independent readers scored ground-glass opacity (GGO) (ground-glass score) and honeycombing (fibrosis score), as reported by Kazerooni et al. [16], on a scale of 0–5 in the three lobes of both lungs, as follows: 0, no GGO; 1, involving <5 % of the lobe; 2, involving 5–24 % of the lobe; 3, involving 25–49 % of the lobe; 4, involving 50–75 % of the lobe; 5, involving >75 % of the lobe. For ground glass score: 0, no interstitial disease; 1, septal thickening without honeycombing; 2, honeycombing involving up to 25 % of the lobe; 3, honeycombing involving 25–49 % of the lobe; 4, honeycombing involving 50–75 % of the lobe; 5, honeycombing involving >75 % of the lobe for interstitial score. Each observer assessed the extent of involvement in each of three defined regions: above aortic arch, between arch and inferior pulmonary veins, and between inferior pulmonary veins and lung base. The mean estimate of the two readers was used to define interstitial and ground-glass score for each lobe. We used the summation of overall interstitial and ground-glass scores as total ILD score.

Table 1 Patient information

Patient no./sex/age in years	Disease duration (years)	Number of IVCY therapies performed	Ang2 at baseline (pg/mL)	Ang2 after the first IVCY (pg/mL)	Ang2 at the last IVCY (pg/mL)	ILD score 1 year before treatment	ILD score at baseline	ILD score at follow-up period	%VC at baseline	%VC at follow-up period	%DLco at baseline	%DLco at follow-up period	Disease-modifying drugs at baseline	Immunosuppressant after IVCY therapy
1/F/57	7	6	893	991	615	8	14	13	77.3	88.3	56.2	62.2	PSL	AZP
2/F/57	4	6	1,586	1,142	920	8	13	10	64.4	73.5	56.5	62.2	PSL	AZP
3/F/62	8	4	1,234	1,046	725	5	6	6	127.7	126.3	96.1	94.1	PSL	None[b]
4/F/67	5	6	1,428	1,779	1,222	14	21	21	61.4	57.8	49.8	38.9	PSL	AZP
5/F/48	7	5	2,573	2,945	1,723	12	29	30	54.2	57.2	18.6	15.3	PSL, Bos	None[c]
6/F/63	5	3	2,995	1,434	1,380	7	18	14	111.8	103.4	69.2	70.8	PSL	AZP
7/F/57	14	5	2,308	1,176	824	28	28	23	46.6	53.3	ND[a]	24.1	PSL, Bos	AZP

IVCY intravenous pulse cyclophosphamide, *ILD* interstitial lung disease, *%VC* percentage of predicted vital capacity, *%DLco* percentage of predicted diffusion lung capacity for carbon monoxide, *PSL* prednisone, *Bos* bosentan, *AZP* azathioprine

[a] %DLco of patient 7 at baseline was unmeasurable due to low %VC

[b] Patient 3 was not administered AZP after IVCY therapy due to hepatitis B virus infection

[c] Patient 5 was not administered AZP after IVCY therapy because rituximab treatment was scheduled against highly active and refractory ILD

Statistical analysis

Statistical analysis was performed with Welch's *t* test to compare means between SSc patients and healthy controls and with paired *t* test between before and after treatment results. Statistical significance was defined as p value <0.05.

Results

Serum Ang2 levels in SSc patients and healthy controls before IVCY therapy administration

The difference in serum Ang2 levels between SSc patients before IVCY therapy and healthy controls was not statistically significant, but Ang2 levels in patients tended to be higher than those of healthy controls (1,860.04 ± 773.21 vs. 1,526.51 ± 403.61 pg/ml, $p = 0.31$; Fig. 1). As shown in Table 1, three patients (5, 6, and 7) exhibited initial Ang2 levels >2,000 pg/ml. Among them, patients 5 and 7 had much more severe baseline ILD scores than the other patients, with relatively lower Ang2 levels. Patients 5 and 6 showed marked ILD deterioration characterized by more than twofold increase in ILD score in the last year before treatment initiation. Although the pathological process of SSc was modified in these patients due to the immunosuppressive treatments, there was a trend that serum Ang2

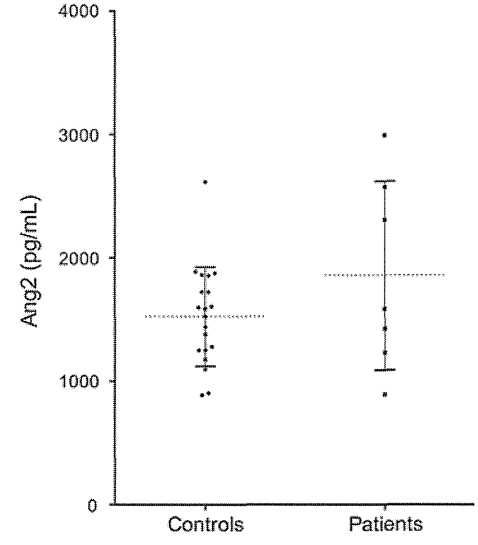

Fig. 1 Serum angiopoietin-2 (Ang2) levels in systemic sclerosis (SSc) patients and healthy controls determined using a specific enzyme-linked immunosorbent assay (ELISA). The difference in serum Ang2 levels between SSc patients before intravenous pulse cyclophosphamide (IVCY) therapy and healthy controls was not statistically significant [1,860.04 ± 773.21 pg/ml ($n = 7$) vs. 1,526.51 ± 403.61 pg/ml ($n = 20$), $p = 0.31$; Welch's *t* test]. *Dotted lines* indicate mean value in each group. *Error bars* (one standard deviation on either side of the mean value) are shown with *horizontal solid bars*

levels correlated with SSc-ILD severity and activity, which is consistent with the previous report [12].

Correlation of serum Ang2 levels with KL-6, SP-D, ESR, and CRP in patients with SSc-ILD

To further confirm the notion described above, we evaluated the correlation of serum Ang2 levels with serum KL-6 and SP-D levels, established markers for inflammatory and fibrotic lung disorders, including SSc-ILD [14, 15], in SSc-ILD patients during IVCY therapy. As shown in Fig. 2, serum Ang2 levels significantly correlated with serum KL-6 and SP-D levels [$r = 0.47$ ($n = 27$, $p < 0.01$) and $r = 0.43$ ($n = 26$, $p < 0.05$), respectively]. Furthermore, consistent with a previous report [12], there were significant correlations between serum Ang2 levels and inflammatory markers, such as ESR and CRP [$r = 0.64$ ($n = 35$, $p < 0.0001$) and $r = 0.74$ ($n = 35$, $p < 0.0001$), respectively]. Taken together, these results strongly support our hypothesis that serum Ang2 levels reflect SSc-ILD severity and activity.

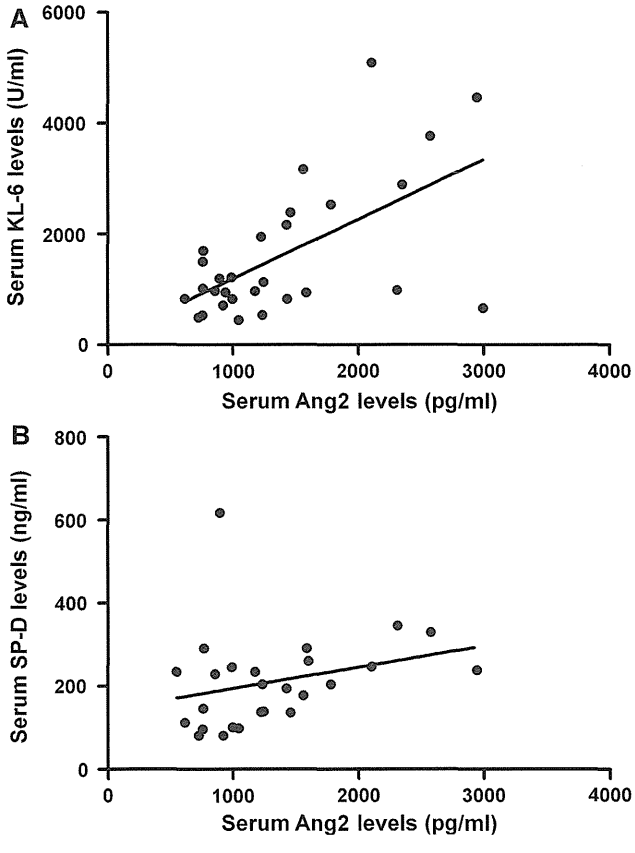

Fig. 2 Correlation of serum angiopoietin 2 (Ang2) levels with serum KL-6 and surfacant protein D (SP-D) levels in patients with systemic sclerosis interstitial lung disease (SSc-ILD) during intravenous pulse cyclophosphamide (IVCY). Serum Ang2 levels significantly correlated with serum KL-6 and SP-D levels [$r = 0.47$ ($n = 27$, $p < 0.01$) and $r = 0.43$ ($n = 26$, $p < 0.05$) by Spearman's rank correlation test, respectively]. *Solid line* represents regression line

Evaluation of the link between dynamics of serum Ang2 levels and efficacy of IVCY therapy against SSc-ILD

Throughout IVCY therapy, serum Ang2 levels in SSc patients showed statistically significant decreases at the last pulse compared with baseline levels (1,058.43 ± 398.69 vs. 1,860.04 ± 773.21 pg/ml, $p = 0.0089$; Fig. 3a). To

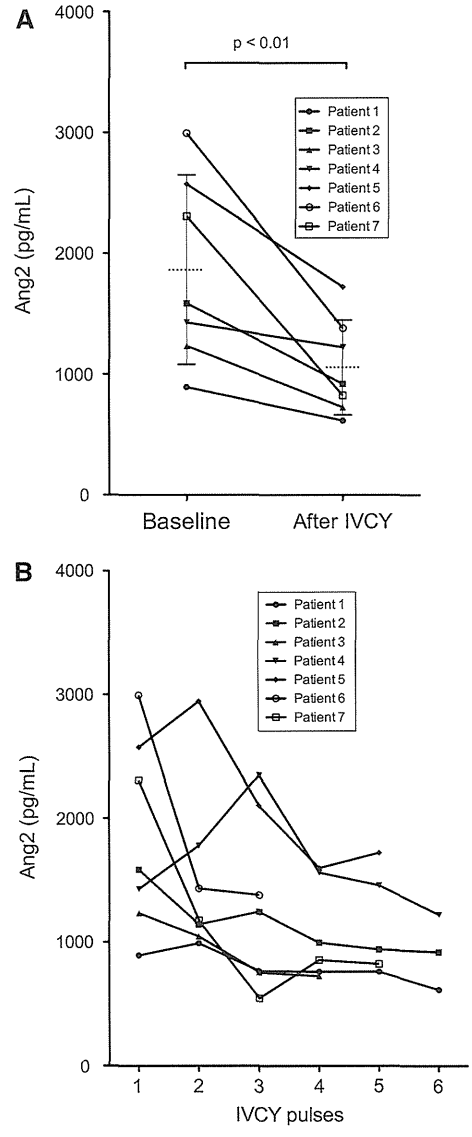

Fig. 3 Time course of serum angiopoietin 2 (Ang2) levels in patients with systemic sclerosis with interstitial lung disease (SSc-ILD) throughout intravenous pulse cyclophosphamide (IVCY) therapy. **a** Serum Ang2 levels at the last IVCY therapy were significantly lower than those at baseline [1,058.43 ± 398.69 pg/ml ($n = 7$) vs. 1,860.04 ± 773.21 pg/ml ($n = 7$), $p = 0.0089$; paired t test). *Dotted lines* indicate mean value in each group. *Error bars* (one standard deviation on either side of the mean value) are indicated by *horizontal solid bars*. **b** Time course of serum Ang2 levels in each patient shown in Table 1. Serum samples were collected the day before each IVCY therapy

further assess the association between the dynamics of serum Ang2 levels and the clinical course of SSc-ILD with IVCY treatment, we classified SSc patients into three groups based on change in serum Ang2 levels (Fig. 3b): patients in whom levels increased >300 pg/ml after the first IVCY therapy (patients 4 and 5), patients in whom levels decreased ≥50 % after the first IVCY therapy (patients 6 and 7; 52 % and 49 % decrease, respectively), and patients in whom levels did not meet these two criteria (patients 1, 2, and 3).

Two patients (patients 4 and 5) showed increased serum Ang2 levels >300 pg/ml after the first IVCY therapy (Fig. 3b). The efficacy of IVCY therapy against ILD in these patients was relatively limited when evaluated by chest CT images and pulmonary function test (Table 1). Of note, both patients experienced ILD exacerbation during the treatment and/or the follow-up period, which was characterized by significant deterioration of subjective symptoms (i.e. dyspnea and dry cough) and >15 % decrease in %DLco compared with baseline levels. By contrast, in two patients with serum Ang2 levels decreased ≥50 % after the first IVCY therapy (patients 6 and 7; Fig. 3b), subjective symptoms, such as dry cough and dyspnea, were markedly improved during the first and second IVCY therapies, along with substantial decrease in ILD score after IVCY treatment compared with baseline levels (Table 1). In the other three patients (patients 1, 2, and 3), serum Ang2 levels were moderately decreased throughout the entire IVCY therapy period (Fig. 3b). Their ILD activity was moderate and stabilized by IVCY treatment according to subjective symptoms, pulmonary function test, and ILD score (Table 1).

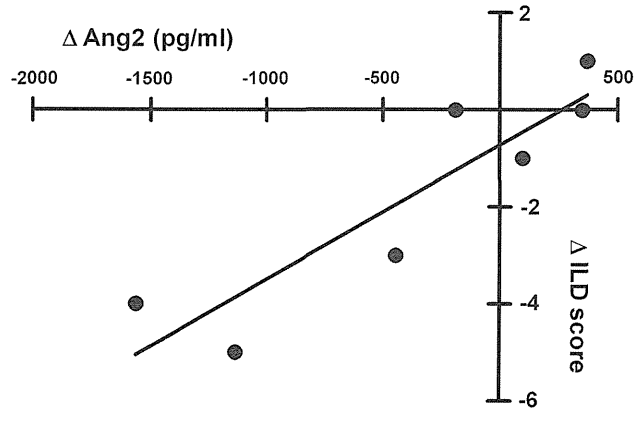

Fig. 4 The correlation of Δ serum Ang2 levels between baseline and after the first IVCY with Δ ILD score between before and after the whole IVCY. Significant correlation was found between Δ serum Ang2 levels (between baseline and after the first IVCY) and Δ ILD score (between before and after the whole IVCY) in SSc-ILD [$r = 0.90$ ($n = 7$, $p < 0.01$), by Spearman's rank correlation test]. The *solid line* represents the regression line

These observations suggest that Δ serum Ang2 levels between baseline and after the first IVCY treatment reflect the efficacy of the entire IVCY therapy against SSc-ILD. Supporting this idea, Δ serum Ang2 levels between baseline and after the first IVCY therapy significantly correlated with Δ ILD score between before and after the entire IVCY therapy ($r = 0.90$, $p < 0.01$; Fig. 4). The Δ serum Ang2 levels between baseline and before the last IVCY therapy failed to significantly correlate with Δ ILD score between before and after the entire treatment ($r = 0.54$, $p = 0.24$).

Discussion

Consistent with the notion that serum Ang2 levels correlate with severity and activity of various vascular diseases, including SSc, we found that serum Ang2 levels tended to be higher in SSc patients before IVCY therapy than in healthy controls and significantly correlated with KL-6, SP-D, ESR, and CRP in SSc-ILD. More importantly, the dynamics of serum Ang2 levels reflected the IVCY efficacy against SSc-ILD. In two patients with SSc-ILD, in whom IVCY treatment dramatically and rapidly attenuated dry cough and dyspnea during the first and second therapies, serum Ang2 levels were markedly decreased, especially after the first therapy. In contrast, two patients, who experienced ILD exacerbation during the follow-up period, showed the increase in serum Ang2 levels after the first IVCY therapy, even though levels were finally decreased at the last IVCY therapy. These results indicate that the dynamics of serum Ang2 levels after the first IVCY therapy may reflect the sensitivity of pathological vascular events associated with SSc-ILD to IVCY treatment. A rapid decrease in serum Ang2 levels may reflect the high sensitivity of pathological endothelial damage to IVCY treatment, whereas the increase in serum Ang2 levels even after IVCY therapy administration may represent the highly active pathological vascular event refractory to IVCY treatment. Consistently, in the other three patients, who had moderately active ILD stabilized after IVCY treatment, serum Ang2 levels were uniformly decreased during the treatment period, suggesting that their vascular damage was sensitive to treatment. Supporting this idea, Δ serum Ang2 levels between baseline and after the first IVCY treatment significantly correlated with ΔILD score between before and after the IVCY course. Although IVCY is the first-line treatment for SSc-ILD, there is a certain subset of SSc-ILD patients refractory to IVCY treatment. Our observations indicate that monitoring serum Ang2 levels during IVCY treatment may be useful to distinguish patients with SSc-ILD refractory to IVCY from those sensitive to the treatment.

Although the detailed pathological process leading to activation of lung fibroblasts in SSc remains unknown, mounting data demonstrate that endothelial damage is involved in the mechanism responsible for activation of SSc lung fibroblasts. One factor causing endothelial damage in SSc is anti-endothelial-cell (EC) antibody, which is present in sera of 22–86 % SSc patients [17] and has been shown to induce apoptosis of ECs in vivo and in vitro [18, 19]. Wusirika et al. [20] disclosed that 42 of 45 SSc patients with ILD possess anti-EC antibody, whereas no SSc patient without ILD, or healthy controls, have this antibody, suggesting the possible causal relationship of anti-EC antibody with the development of SSc-ILD. Laplante et al. [21] demonstrated that medium conditioned by apoptotic ECs promotes myofibroblastic differentiation and prevents apoptosis of lung fibroblasts in vitro. Furthermore, SSc lung fibroblasts are much more sensitive to medium conditioned by apoptotic ECs than are normal lung fibroblasts. Moreover, EC apoptosis is a primary pathogenic event in pulmonary fibrosis in a chicken model of SSc, UCD-200 [22], and the number of apoptotic ECs, which is much higher in SSc lung tissue with ILD than in normal lung tissue, inversely correlates with values of pulmonary function test, including percentage of predicted vital capacity, in SSc patients [23]. Collectively, these previous data indicate that vascular damage may promote fibroblast activation in SSc lung tissue. Supporting this idea, a wealth of evidence has revealed that IVCY exerts its efficacy for SSc-ILD at least partially by ameliorating vascular injuries [24, 25]. Most importantly, IVCY increases the number of circulating endothelial progenitor cells (EPCs) in SSc patients [26]. In humans, given that EPCs are mobilized and recruited to the damaged lesions in acute lung injury, and given that therapeutic application of EPCs promotes remodeling of the lung and heart in an animal model with pulmonary hypertension, the clinical benefit of IVCY treatment observed in a subset of SSc patients may result from remodeling of lung injuries through EPC mobilization. Consistently, this study revealed that the dynamics of a serum marker for vascular damage, Ang2, reflects the efficacy of IVCY treatment for SSc-ILD.

Michalska-Jakubus et al. [12] assessed the clinical significance of serum Ang2 levels in SSc patients. Their findings are as follows: (1) serum Ang2 levels are significantly increased in SSc patients compared with healthy controls; (2) serum Ang2 levels positively correlate with modified Rodnan total skin thickness score and inflammatory markers, including ESR and CRP, while inversely with %DLco, (3) patients with active digital ulcers have significantly higher serum levels of Ang2 than patients without fingertip ulceration, and (4) serum Ang2 levels significantly correlate with the EScSG activity index. More importantly, those authors carried out a multivariate regression analysis and demonstrated that serum Ang2 levels are independently associated with EScSG activity index and ESR, and inversely with the presence of digital ulcers. These results together suggest that vasculopathy associated with the elevation of serum Ang2 levels is closely linked with the mechanism of inflammatory process underlying high SSc disease activity but not to the development of digital ulcers. Also, elevation of serum Ang2 levels in SSc patients with digital ulcers is due to high disease activity rather than the ulcers themselves. In our study, we failed to detect statistically significant elevation of serum Ang2 levels in SSc patients. This is partly due to the smaller number of samples in our than in previous studies. Alternatively, a relatively higher dose of prednisone in our study may explain this discrepancy. In our study, five of seven patients were treated with prednisone at the dose of >20 mg/day when the first serum samples were obtained, whereas all of SSc patients were administered low-dose prednisone (5–10 mg/day) in the previous study. Therefore, an anti-inflammatory effect of prednisone may affect the levels of serum Ang2 much greater in our study than in the previous one. Additionally, some confounding factors, rather than the effect of baseline treatment, potentially affect the statistical significance. In contrast, regarding inflammatory markers such as ESR and CRP, the significant correlations with serum Ang2 levels were reproduced in SSc-ILD patients during IVCY therapy. Taken together with the significant correlation of serum Ang2 levels with KL-6 and SP-D, our data suggest that IVCY therapy improves SSc-ILD by ameliorating inflammation and/or vasculopathy, which coordinately contribute to the complicated pathological process of SSc-ILD by interacting with each other. Supporting the link between Ang2 and pulmonary injuries, serum Ang2 levels reflect the severity of interstitial pulmonary damage in patients with acute respiratory distress syndrome [27, 28].

In previous reports, Ang2 is shown to be a leading serum marker reflecting the severity and activity of various vascular diseases, including Crohn's disease [8, 9], ulcerative colitis [10], and systemic lupus erythematosus [11]. As in these diseases, vascular involvement plays a central role in the pathogenesis of SSc. As supported by the study reported here, serum markers of vascular damage appear to be useful to evaluate and predict SSc disease activity. Therefore, the combination of several serum markers reflecting vascular damage, including Ang2, may be further useful to evaluate and/or predict SSc severity and activity and its specific involvement in organs, such as ILD. This project is ongoing in our laboratory.

In summary, we herein report the first study regarding the potential of monitoring serum Ang2 levels to evaluate and predict IVCY treatment efficacy for SSc-ILD. As this

is still a preliminary hypothesis, further studies are necessary to evaluate its accuracy in the large number of cases.

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Conflict of interest None.

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ORIGINAL ARTICLE

Discoidin Domain Receptor 2–microRNA 196a–Mediated Negative Feedback against Excess Type I Collagen Expression Is Impaired in Scleroderma Dermal Fibroblasts

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Systemic sclerosis (SSc) is characterized by excess collagen deposition in the skin, due to intrinsic transforming growth factor-β (TGF-β) activation. We tried to determine the expression and the role of discoidin domain receptor 2 (DDR2) in SSc. The expression of DDR2 mRNA and protein was significantly decreased in SSc dermal fibroblasts, which was recovered by knocking down TGF-β. The knockdown of DDR2 in normal fibroblasts induced microRNA-196a expression, which led to type I collagen downregulation, indicating that DDR2 itself has a negative effect on microRNA-196a expression and inducible effect on collagen expression. In SSc fibroblasts, however, the DDR2 knockdown did not affect TGF-β signaling and microRNA-196a expression. The microRNA-196a levels were significantly decreased in normal fibroblasts treated with TGF-β and in SSc fibroblasts. Taken together our data indicate that, in SSc fibroblasts, intrinsic TGF-β stimulation induces type I collagen expression, and also downregulates DDR2 expression. This probably acts as a negative feedback mechanism against excess collagen expression, as a decreased DDR2 expression is supposed to stimulate the microRNA-196a expression and further change the collagen expression. However, in SSc fibroblasts the microRNA-196a expression was downregulated by TGF-β signaling. DDR2–microRNA-196a pathway may be a previously unreported negative feedback system, and its impairment may be involved in the pathogenesis of SSc.

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INTRODUCTION

Systemic sclerosis (SSc) or scleroderma is an acquired disorder characterized by the activation of fibroblasts and subsequent excess accumulation of extracellular matrix, resulting in tissue fibrosis of the skin and internal organs (Korn, 1989; Mauch and Kreig, 1990). Although the mechanism of fibroblast activation in SSc is presently unknown, many of the characteristics of SSc fibroblasts resemble those of healthy fibroblasts stimulated by transforming growth factor-β1 (TGF-β1; Leroy *et al.*, 1989; Massagué, 1990): Cultured fibroblasts obtained from affected SSc skin overproduce various collagens, mainly type I collagen consisting of the α1(I) and α2(I) chains (LeRoy, 1974; Jimenez *et al.*, 1986; Kikuchi *et al.*, 1992; Hitraya and Jiménez, 1996), thus indicating that the activation of dermal fibroblasts in SSc may be a result of stimulation by "autocrine" TGF-β signaling. This notion is supported by our findings that (1) the phosphorylation levels and DNA-binding activity of Smad3, a downstream mediator of TGF-β, is constitutively upregulated in SSc fibroblasts (Asano *et al.*, 2004b), and (2) the blockade of TGF-β signaling with an anti-TGF-β-neutralizing antibody abolished the increased expression of human α2(I) collagen mRNA in SSc fibroblasts (Ihn *et al.*, 2001).

The discoidin domain receptors (DDR1 and DDR2) are transmembrane receptors belonging to the receptor tyrosine kinase family (Shrivastava *et al.*, 1997; Vogel *et al.*, 2006). DDRs consist of three components: an extracellular discoidin domain, a transmembrane region, and an intracellular kinase domain (Leitinger, 2003). DDR1 is expressed in epithelial cells and can bind collagen types I through V and VIII, which results in autophosphorylation of the receptor, whereas DDR2 is mainly detected in mesenchymal cells and responds to collagen types I, II, III, and X (Leitinger and Kwan, 2006; Ichikawa *et al.*, 2007; Carafoli *et al.*, 2009; Klatt *et al.*, 2009). Both DDRs are thought to regulate extracellular matrix remodeling, as well as many cellular activities including cell proliferation, migration, or adhesion (Wall *et al.*, 2005; Vogel *et al.*, 2006).

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Abbreviations: DDR2, discoidin domain receptor 2; miRNA, microRNA; siRNA, small interfering RNA; SSc, systemic sclerosis; TGF-β, transforming growth factor-β

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Although DDRs are known to be receptors of collagens as described above, the role of DDRs in SSc has not yet been well investigated. Therefore, this study was undertaken to clarify the expression pattern of DDRs in SSc and their role in the pathogenesis of this disease.

RESULTS

Expression levels of DDR2 in SSc skin

As an initial experiment, we compared the transcript levels of DDR1 and DDR2 between normal and SSc skin *in vivo*. Total RNA was extracted from the skin derived from 10 patients with SSc (5 diffuse cutaneous SSc and 5 limited cutaneous SSc) and 5 healthy controls, and quantitative real-time PCR was performed. The relative transcript levels of DDR1 in SSc skin were not altered compared with normal skin (Figure 1a, left). On the other hand, the DDR2 expression levels were significantly lower in SSc skin than those in control skin (Figure 1a, right).

Next, cultured human dermal fibroblasts obtained from normal and SSc skin were incubated under the same

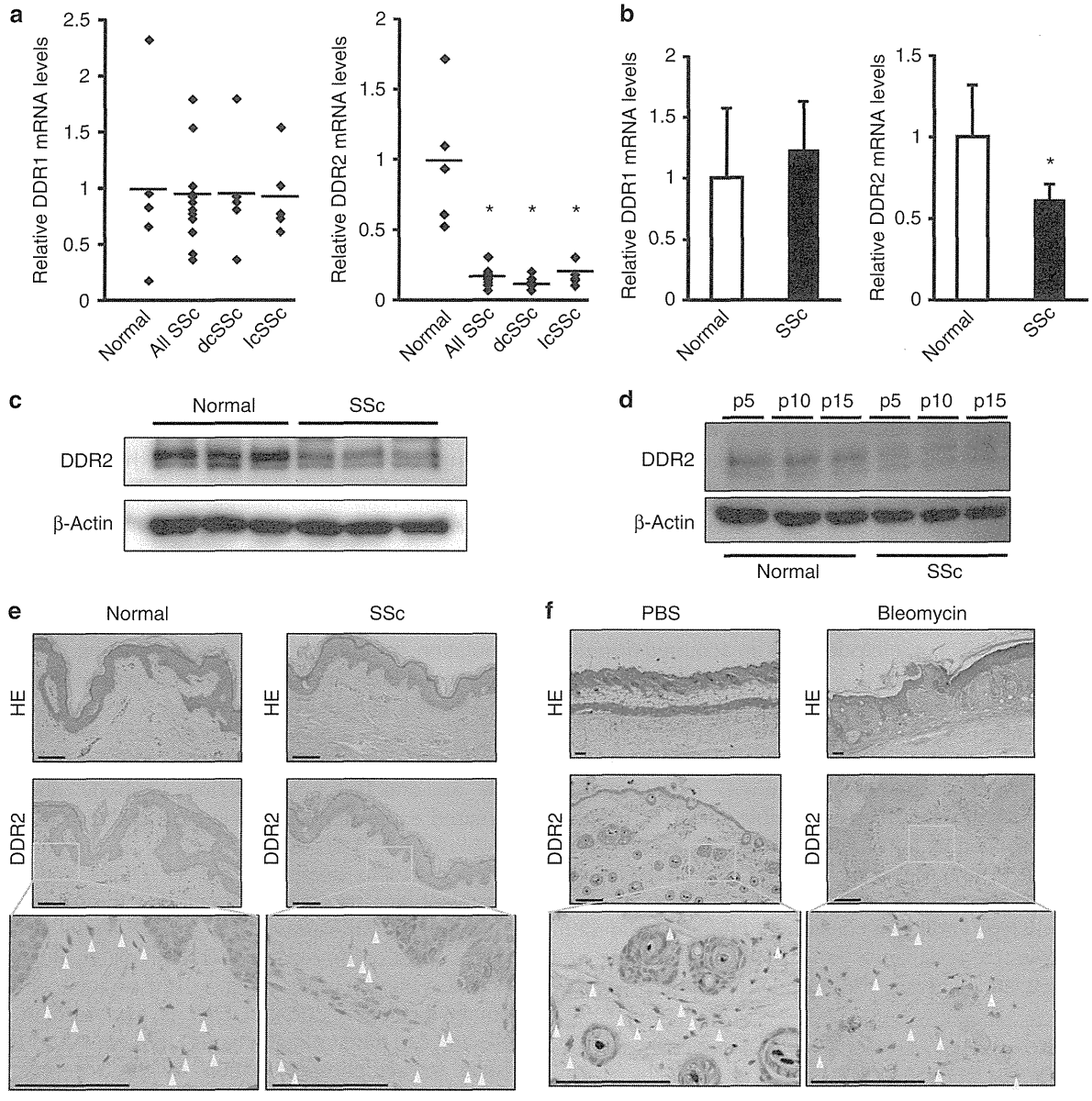

Figure 1. The discoidin domain receptor 2 (DDR2) expression in normal and systemic sclerosis (SSc) skin. (**a**) The mRNA levels of DDR1 and DDR2 in skin tissues from five controls, five limited cutaneous SSc (lcSSc) patients, and five diffuse cutaneous SSc (dcSSc) patients. Horizontal lines show means. $^*P<0.05$ compared with values in control skin (1.0). (**b**) The mRNA levels of DDR1 and DDR2 in normal and SSc-cultured fibroblasts ($n=5$). (**c**) Lysates from normal and SSc-cultured dermal fibroblasts were subjected to immunoblotting with antibody against DDR2 or β-actin. Three representative results for five normal and SSc fibroblasts are shown. (**d**) Lysates were obtained from cultured normal and SSc fibroblasts at passages 5, 10, and 15 (p5, p10, and p15), and subjected to immunoblotting ($n=3$). (**e**, **f**) Hematoxylin and eosin (HE) and DDR2 staining of (**e**) normal and SSc skin or (**f**) mouse skin treated with phosphate-buffered saline (PBS) or bleomycin ($n=3$). DDR2 was stained brown. Arrowheads indicate dermal fibroblasts. Bar = 100 μm.

conditions, and their mRNA expression was analyzed. The expression of DDR2 mRNA was also downregulated in SSc fibroblasts, whereas DDR1 mRNA did not decrease (Figure 1b), which was consistent with the *in vivo* results (Figure 1a).

Immunoblotting revealed that the amounts of DDR2 protein in the cell lysates from SSc-cultured fibroblasts were also decreased compared with those in normal fibroblasts (Figure 1c): The downregulation of DDR2 in SSc cells was maintained until at least passage 15 *in vitro* (Figure 1d). Furthermore, the *in vivo* DDR2 protein expression in SSc skin was examined by immunostaining. Hematoxylin and eosin staining showed that SSc skin has dermal fibrosis with an increased number of thickened collagen bundles (Figure 1e). The DDR2 expression was strongly detected in spindle-shaped fibroblasts in normal skin, but not in SSc fibroblasts in spite of similar staining levels in the epidermis. Taken together, DDR2 protein and mRNA expression were significantly downregulated in SSc fibroblasts both *in vivo* and *in vitro*.

To further investigate the DDR2 expression pattern *in vivo*, paraffin-embedded sections from the skin of bleomycin-treated mouse model were stained for DDR2. Weaker DDR2 staining of fibroblasts was found in the bleomycin-induced thickened skin (Figure 1f) compared with control skin treated with phosphate-buffered saline. These results confirmed that DDR2 expression decreased in dermal fibroblasts under fibrotic conditions.

Regulatory mechanisms for DDR2 in SSc fibroblasts

The steady-state level of mRNA can be affected by the level of gene transcription and/or the stability of mRNA. To determine whether the decrease of DDR2 mRNA in SSc fibroblasts takes place at the transcriptional level or post-transcriptional level, *de novo* mRNA synthesis was inhibited by treatment with actinomycin D, an RNA synthesis inhibitor, in normal and SSc fibroblasts (Asano *et al.*, 2004a; Mimura *et al.*, 2005). As shown in Figure 2a, after actinomycin D treatment, the DDR2 mRNA levels decreased because of mRNA degradation in both cells. The rate of decrease in the DDR2 mRNA was similar between normal and SSc fibroblasts for at least 12 hours, indicating that the stability of DDR2 mRNA did not decrease in SSc fibroblasts. Taken together, DDR2 expression likely decreased because of the inactivated gene transcription, and not because of the decreased mRNA stability, in SSc dermal fibroblasts.

We tried to clarify the mechanism that mediates decreased expression of DDR2 in SSc fibroblasts. To examine the possibility that the downregulated DDR2 synthesis in SSc fibroblasts is due to the stimulation of autocrine TGF-β signaling seen in these cells as described above (Ihn *et al.*, 2001), we investigated the effect of exogenous TGF-β1 stimulation in normal and SSc fibroblasts. The expression of DDR2 mRNA was downregulated by TGF-β1 in both normal (Figure 2b, left) and SSc (Figure 2b, right) fibroblasts. In addition, immunoblotting revealed that the amount of DDR2 protein was reduced by exogenous TGF-β1 in normal and SSc fibroblasts (Figure 2c).

In contrast, when the TGF-β1 expression was downregulated by TGF-β1 small interfering RNA (siRNA), the α1(I) and α2(I) collagen expression also decreased in SSc fibroblasts (Figure 2d), owing to the presence of autocrine TGF-β signaling (Ihn *et al.*, 2001), but not in normal fibroblasts. On the other hand, the expression of DDR2 was not significantly affected by TGF-β siRNA in normal fibroblasts (Figure 2e, left), although the downregulated DDR2 protein levels in SSc fibroblasts were recovered by TGF-β1 siRNA (Figure 2e, right). These results indicate that the downregulated expression of DDR2 in SSc fibroblasts is a result of stimulation by autocrine TGF-β signaling.

The function of DDR2 in the regulation of type I collagen in dermal fibroblasts

As described above, DDR2 is known to be a tyrosine kinase receptor of collagens. Immunoprecipitation experiments were performed to evaluate the phosphorylation state of DDR2 in normal and SSc fibroblasts. Cell lysates were prepared from normal fibroblasts in the presence or absence of TGF-β1 and SSc fibroblasts. As shown in Figure 3a, the DDR2 phosphorylation levels were not different between normal and SSc fibroblasts. We therefore expected that the change in expression levels, and not a change in phosphorylation, may have a role in the pathogenesis of SSc. Accordingly, normal and SSc fibroblasts were transfected with control or DDR2 siRNA. In normal fibroblasts transfected with DDR2 siRNA, the DDR2 protein expression was knocked down and type I collagen synthesis was also downregulated (Figure 3b, left). However, the levels of α2(I) collagen mRNA in normal fibroblasts did not significantly decrease by DDR2 siRNA (Figure 3b, right). In SSc fibroblasts, the protein and mRNA levels of collagen were not significantly affected by DDR2 siRNA (Figure 3c).

We also overexpressed DDR2 by lentiviral transfection in normal fibroblasts. As expected, the DDR2 overexpression induced type I collagen protein expression in a dose-dependent manner, but the empty vector did not (Figure 3d and e). Similarly, the expression of type I collagen increased by DDR2 overexpression in SSc fibroblasts (Figure 3f). Therefore, the exogenous DDR2 overexpression itself seems to have an inducible effect on collagen expression.

The association between DDR2 and microRNAs (miRNAs) in normal and SSc fibroblasts

Finally, to clarify the regulatory mechanism(s) by which DDR2 affects type I collagen expression, we determined whether DDR2 regulates collagen expression via TGF-β1 signaling. In normal fibroblasts, Smad2 and Smad3 were not phosphorylated (Figure 4a). When DDR2 was knocked down by the siRNA, total and phosphorylated levels of Smad2/3 were not affected (Figure 4a). In contrast, although Smad2 and Smad3 were constitutively phosphorylated in SSc fibroblasts (Asano *et al.*, 2004b), DDR2 siRNA did not alter the phosphorylation state (Figure 4a). In addition, the mRNA levels of TGF-β1, TGF-β receptor I, TGF-β receptor II, endoglin, thrombospondin 1, integrin β3, or integrin β5 were not influenced by DDR2 siRNA (Figure 4b). In addition, DDR2 overexpression did not change the levels of phosphorylated Smad2/3 and TGF-β-related molecules (Figure 4c and d). Next, considering that

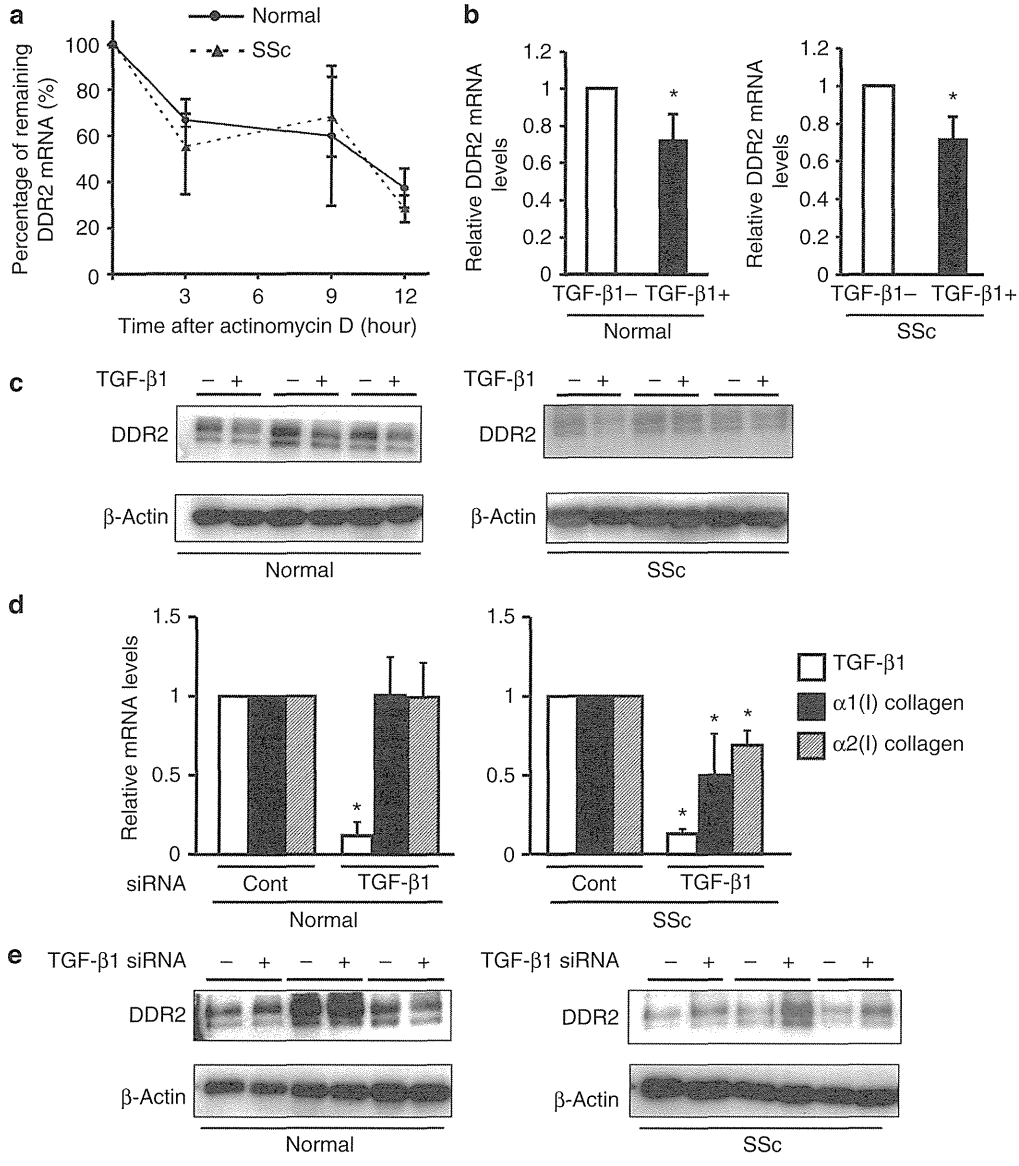

Figure 2. Decreased discoidin domain receptor 2 (DDR2) synthesis is induced by stimulation with transforming growth factor-β (TGF-β). (**a**) Normal and systemic sclerosis (SSc) fibroblasts were incubated for 3, 9, and 12 hours after treatment with 2.5 μg ml^{-1} actinomycin D. DDR2 mRNA expression was analyzed by real-time PCR and normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH; $n=3$). The values in untreated fibroblasts were set at 100%. (**b**, **c**) Normal and SSc fibroblasts were incubated in the presence or absence of 2 ng ml^{-1} TGF-β1 for 24 hours. (**b**) DDR2 mRNA levels were determined by real-time PCR ($n=5$). (**c**) Protein was analyzed by immunoblotting. (**d**, **e**) Normal and SSc fibroblasts were transfected with control or TGF-β1 small interfering RNA (siRNA) for 96 hours. (**d**) The mRNA levels of TGF-β1 and collagen were quantified by real-time PCR ($n=5$). (**e**) The expression of DDR2 was analyzed by immunoblotting. *$P<0.05$ compared with controls.

DDR2 regulates collagen protein expression without altering mRNA levels, we hypothesized that DDR2 regulates type I collagen expression via miRNAs, because miRNAs usually inhibit the translation of their target genes and do not cause degradation of the target transcript. According to the miRNA target gene predictions using MiRanda (August 2010 Release, http://www.microrna.org/), TargetScan (version 5.1, http://www.targetscan.org/), and PicTar (http://pictar.mdc-berlin.de/), we found several miRNAs, including miR-29, 196a, or let-7, as putative regulators of α1(I) and α2(I) collagen. DDR2 knockdown by siRNA (Figure 4a) upregulated only the expression of miR-196a significantly in normal fibroblasts among these candidates (Figure 4e). In SSc fibroblasts, however, the miR-196a expression was not affected by DDR2 siRNA (Figure 4e). In contrast, DDR2 overexpression (Figure 4c) decreased the miR-196a expression in both normal and SSc fibroblasts to a similar extent (Figure 4f).

We then confirmed the association of miR-196a with type I collagen expression in normal and SSc fibroblasts. We used miRNA mimics and miScript Target Protectors, single-stranded, modified RNAs designed to specifically interfere with the interaction between miR-196a and the α1(I)/α2(I)

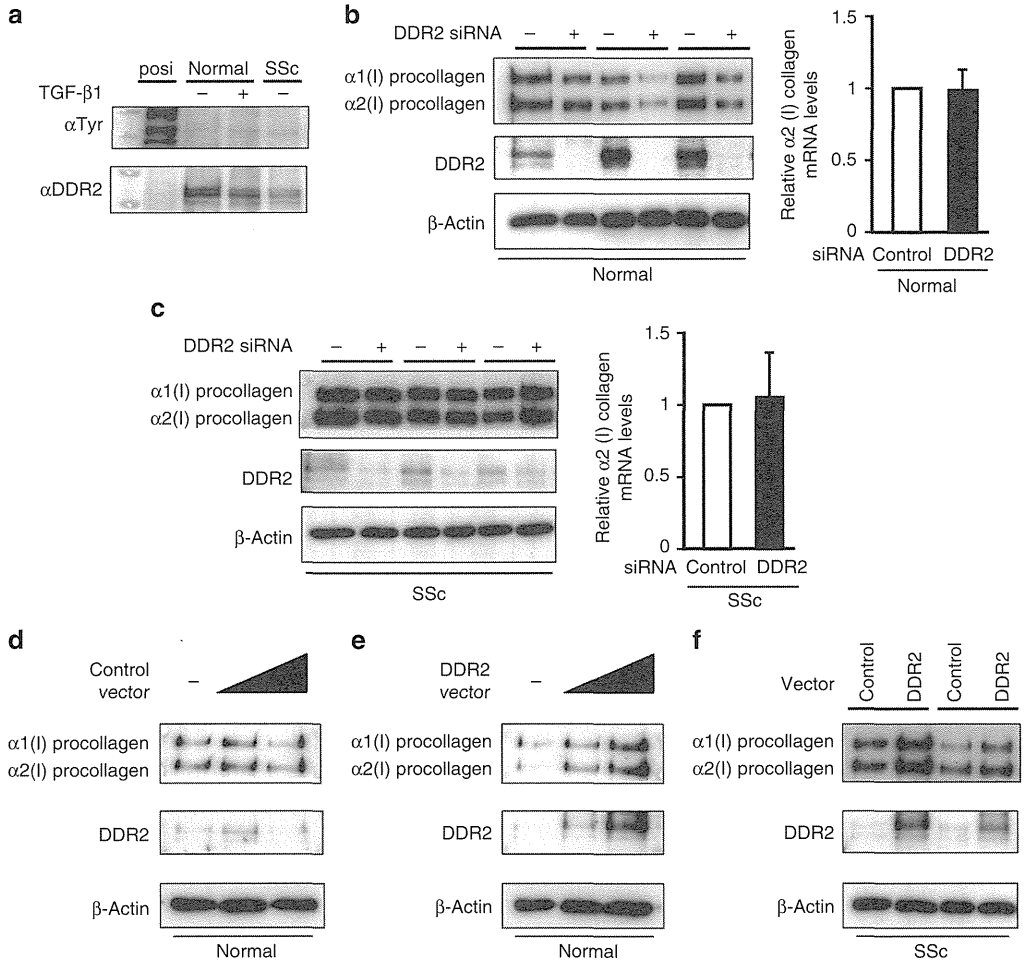

Figure 3. The effect of discoidin domain receptor 2 (DDR2) on the expression of type I collagen. (**a**) DDR2 was immunoprecipitated and the phosphorylated DDR2 was detected by immunoblotting using 4G10 antibody. EGF-stimulated A431 cell lysates were used as a positive control ($n=3$). (**b**, **c**) Normal (**b**) and systemic sclerosis (SSc; **c**) fibroblasts were transfected with the indicated small interfering RNA (siRNA). Lysates were subjected to immunoblotting. Three representative results for five normal and SSc fibroblasts are shown (left). The relative amounts of α2(I) collagen transcripts were determined by real-time PCR ($n=5$, right). (**d**, **e**) Normal fibroblasts were treated with increasing amounts (0, 2, and 5 μl) of virus-containing medium with a (**d**) control or (**e**) DDR2 expression vector. Protein was analyzed by immunoblotting ($n=3$). (**f**) SSc fibroblasts were transduced with the control vector or DDR2 vector for 96 hours, and then the cell lysates were subjected to immunoblotting.

collagen 3′-untranslated region (http://www.qiagen.com/products/miscripttargetprotectors.aspx) (Long and Lahiri, 2011). The transfection of miRNA mimic specific for miR-196a reduced type I collagen expression, and this effect was inhibited by the protectors in both normal (Figure 5a) and SSc (Figure 5b) fibroblasts. These results indicate that miR-196a can regulate the collagen expression.

We expected that the constitutively downregulated DDR2 in SSc fibroblasts would cause an overexpression of miR-196a, and that the miR-196a levels would be elevated in SSc cells compared with normal cells. However, unlike normal fibroblasts, the expression of miR-196a in SSc fibroblasts was not upregulated by DDR2 siRNA, as shown above (Figure 4e). Furthermore, miR-196a levels in SSc skin were significantly lower than normal skin both *in vivo* and *in vitro* (Figure 5c and d). Therefore, the negative effect of DDR2 on miR-196a expression may be specific to normal fibroblasts, and it may be impaired in SSc fibroblasts. The miR-196a expression was downregulated by exogenous TGF-β1 stimulation in normal fibroblasts (Figure 5e), whereas it was recovered by TGF-β1 siRNA in SSc fibroblasts (Figure 5f). Therefore, in SSc fibroblasts, the stimulation of autocrine TGF-β signaling constitutively downregulates miR-196a, resulting in the unresponsiveness of miR-196a to DDR2.

DISCUSSION

This study is the first to demonstrate the role of DDR2 in type I collagen expression in dermal fibroblast and to elucidate its contribution to the pathogenesis of SSc by three major findings.

First, we demonstrated a constitutive downregulation of DDR2 protein and mRNA in cultured SSc fibroblasts *in vitro* and *in vivo*. On the other hand, there was no significant difference in the DDR1 expression between normal and SSc

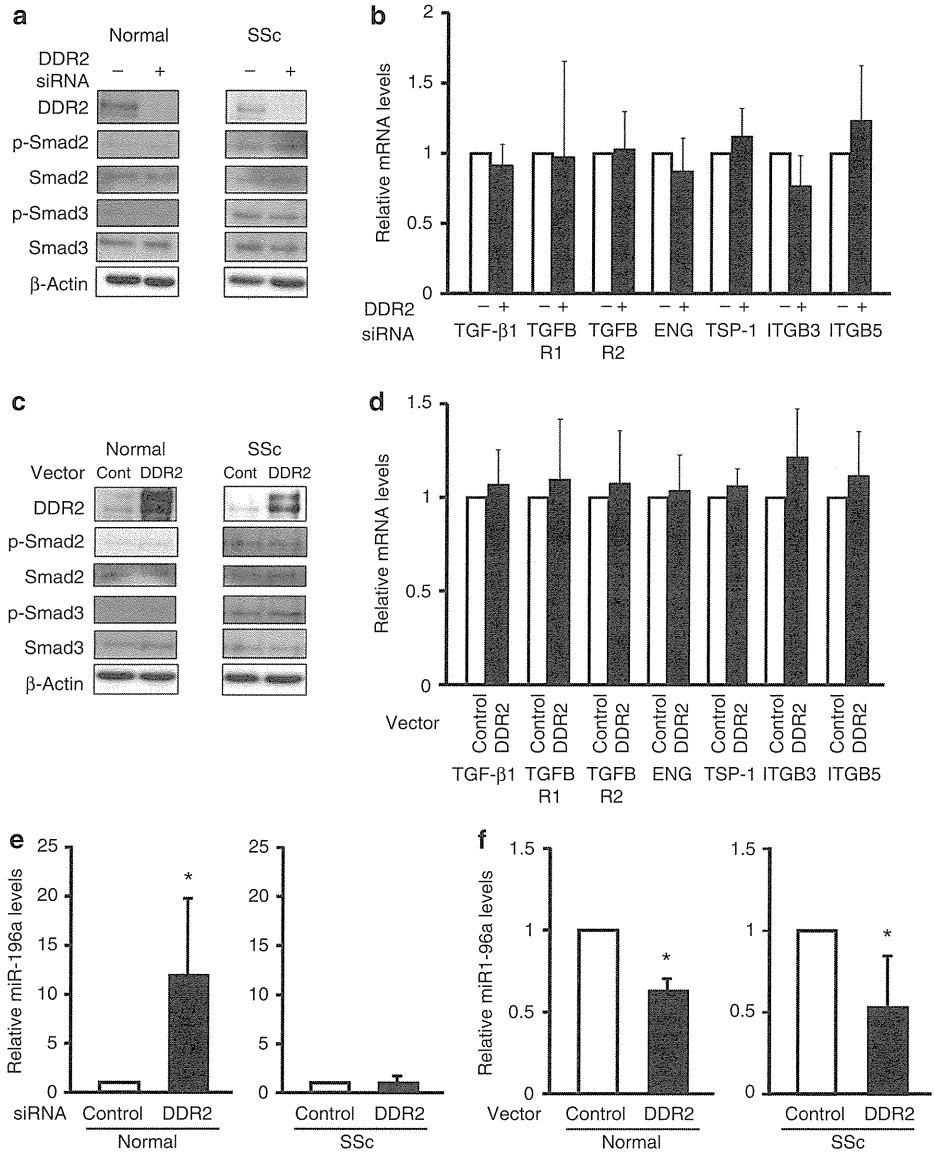

Figure 4. Regulation of microRNA-196a (miR-196a) by discoidin domain receptor 2 (DDR2). (**a**, **b**) Fibroblasts were transfected with control or DDR2 small interfering RNA (siRNA). Cell lysates were subjected to immunoblotting. (**a**) A representative result from five independent experiments is shown. (**b**) The mRNA levels of transforming growth factor-β1 (TGF-β1), TGF-β receptor I (TGFBR1), TGF-β receptor II (TGFBR2), endoglin (ENG), thrombospondin-1 (TSP-1), integrin β3 (ITGB3), and integrin β5 (ITGB5) were determined by real-time PCR ($n=5$). (**c**, **d**) Fibroblasts were treated with control vector or DDR2 expression vector. Lysates were subjected to immunoblotting. (**c**) A representative result from five independent experiments is shown. (**d**) The mRNA levels of indicated molecules were determined by real-time PCR ($n=3$). (**e**, **f**) Fibroblasts were transfected with (**e**) siRNAs or (**f**) expression vectors. The miR-196a levels were determined by real-time PCR ($n=5$). $^*P<0.05$ compared with cells treated with controls (1.0).

fibroblasts. As described above, DDR1 is expressed in epithelial cells, whereas DDR2 is mainly found in mesenchymal cells including fibroblasts and it has a higher binding specificity for type I collagen than DDR1 (Leitinger and Kwan, 2006; Ichikawa *et al.*, 2007; Carafoli *et al.*, 2009; Klatt *et al.*, 2009). Considering that collagen type I is the most abundant extracellular matrix protein found in SSc skin (Mauch and Kreig, 1990), DDR2 may be more important in the pathogenesis of the disease than DDR1.

Second, as described in the Introduction, numerous publications have indicated that there is an autocrine activation of TGF-β signaling in SSc fibroblasts. Our results suggest that such autocrine TGF-β signaling may play a major role in the downregulation of DDR2 in SSc dermal fibroblasts.

Finally, we tried to determine the function of DDR2 in SSc fibroblasts. We found that the overexpression of DDR2 leads to type I collagen induction via the downregulation of miR-196a, whereas the knockdown of DDR2 leads to the downregulation of collagen expression via the upregulation of miR-196a in normal fibroblasts, without affecting TGF-β signaling. Thus, DDR2 may affect the miR-196a expression

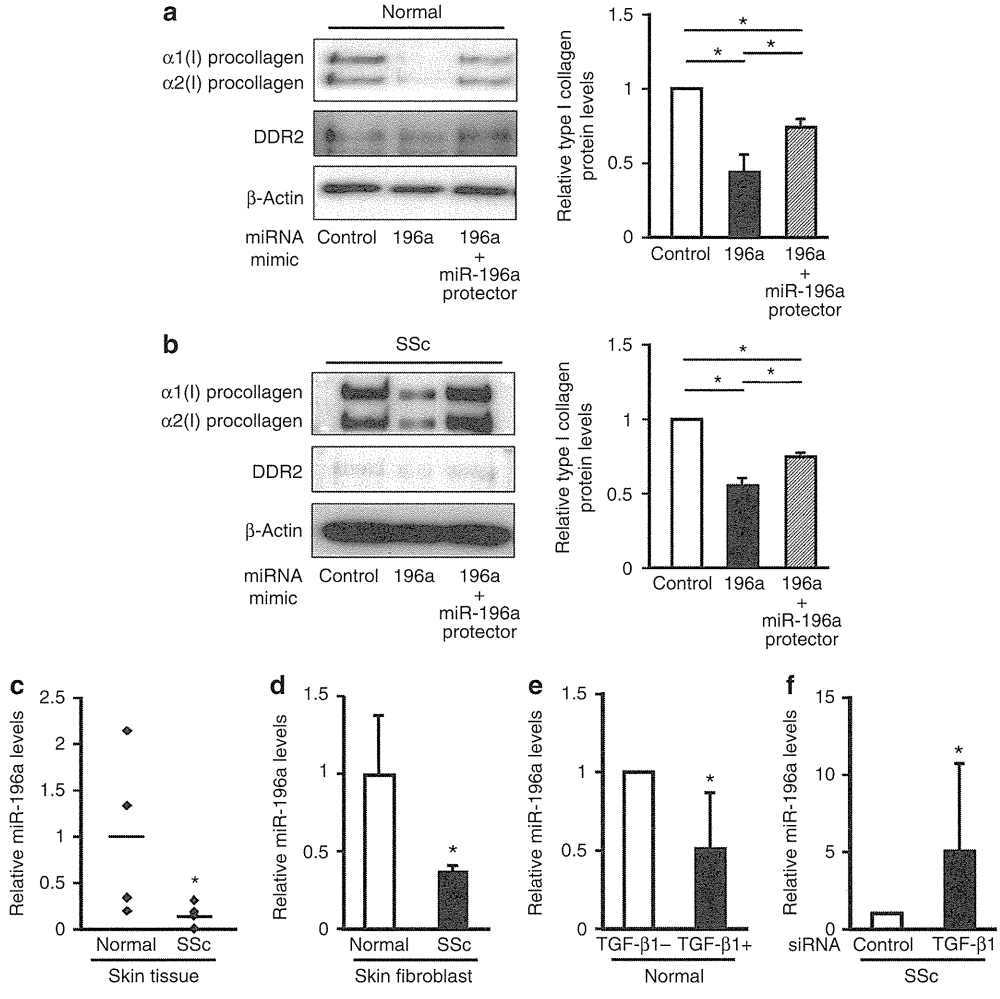

Figure 5. The microRNA-196a (miR-196a) expression in normal and systemic sclerosis (SSc) fibroblasts. (**a**, **b**) Normal (**a**) and SSc (**b**) fibroblasts were transfected with control or miR-196a mimic in the presence or absence of miScript Target protector for 96 hours. Lysates were subjected to immunoblotting. Type I procollagen protein levels quantified by scanning densitometry and corrected for β-actin levels in the same samples are shown ($n=3$, right). (**c**) Relative miR-196a expressions in tissues from normal and SSc skin ($n=4$). (**d**) Relative miR-196a expressions in normal and SSc-cultured dermal fibroblasts ($n=4$). (**e**) Normal fibroblasts were stimulated with 2 ng ml^{-1} transforming growth factor-β1 (TGF-β1) for 24 hours. Relative miR-196a expressions were determined by real-time PCR ($n=4$). (**f**) SSc fibroblasts were transfected with control small interfering RNA (siRNA) or TGF-β1 siRNA for 96 hours. Then, relative miR-196a levels were determined by PCR ($n=4$). *$P<0.05$ compared with controls.

directly, not via TGF-β signaling. The miRNAs, which are short ribonucleic acid molecules only 22 nucleotides long on average, bind to the 3′-untranslated regions of target mRNAs and lead to gene silencing. Recent vigorous research efforts in this field indicated that miRNAs play a role in the pathogenesis of numerous disorders (Chen and Gorski, 2008; Kuehbacher *et al.*, 2008; Lu and Liston, 2009; Davidson-Moncada *et al.*, 2010; Furer *et al.*, 2010). Our study suggests that miRNAs are also involved in the regulatory mechanisms of extracellular matrix protein and tissue fibrosis.

Thus, DDR2 itself has an inducible effect on collagen expression in normal fibroblasts. In SSc fibroblasts, autocrine TGF-β stimulation induces type I collagen expression, and also downregulates DDR2 expression. This probably acts as a negative feedback mechanism against excess collagen expression, as a decreased DDR2 expression is supposed to stimulate the miR-196a expression and further change the collagen expression. However, in SSc fibroblasts the miR-196a expression was downregulated by TGF-β signaling, which releases collagen expression and promotes tissue fibrosis. Thus, our data showed that the DDR2–miR-196a–mediated negative feedback mechanism was impaired in SSc fibroblasts: the constitutive downregulation of downstream miR-196a by TGF-β signaling causes an unresponsiveness of miR-196a to the upstream DDR2 (Figure 6). Multiple negative feedback systems for TGF-β signaling have been shown to be impaired in SSc fibroblasts (Asano *et al.*, 2004b; Jinnin *et al.*, 2007). For example, c-Ski/SnoN is induced by exogenous TGF-β stimulation, which attenuates the effect of TGF-β on collagen expression in normal fibroblasts. However, in SSc fibroblasts, forced overexpression of c-Ski/SnoN does not affect the excess collagen

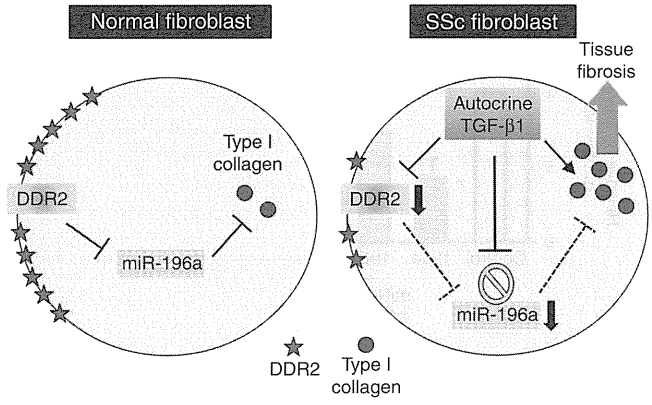

Figure 6. A hypothetical model of the role of discoidin domain receptor 2 (DDR2) in systemic sclerosis (SSc) fibroblasts. DDR2 has a negative effect on microRNA-196a (miR-196a) expression and an inducible effect on collagen expression in normal fibroblasts. In SSc fibroblasts, autocrine transforming growth factor-β (TGF-β) stimulation induces collagen expression, and also downregulates DDR2 expression as a negative feedback mechanism to protect against excess collagen expression. However, the DDR2–miR-196a–mediated negative feedback is impaired: the constitutive downregulation of miR-196a by TGF-β causes an unresponsiveness of the miRNA to DDR2, leading to tissue fibrosis.

expression (Jinnin *et al.*, 2007). The DDR2–miR-196a pathway may be a new negative feedback system that protects against TGF-β–mediated excess collagen accumulation, and its impairment may be involved in the pathogenesis of SSc. Further studies are needed to confirm the direct interaction between miR-196a and collagen using a 3′-untranslated region reporter assay.

In summary, we demonstrated that an impairment of the DDR2–miRNA–mediated regulation of type I collagen in SSc fibroblasts may play an important role in the maintenance of fibrosis. Investigations of the overall regulatory mechanisms of fibrosis by DDR2 and miRNAs may lead to new therapeutic approaches for this disease.

MATERIALS AND METHODS

Reagents

The antibodies against DDR2 and type I collagen were purchased from R&D Systems (Minneapolis, MN) and Southern Biotechnologies (Birmingham, AL), respectively. The antibodies against β-actin, Smad2/3, and phosphorylated Smad2/3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for Smad2/3 and phosphorylated Smad2 were from Cell Signaling (Danvers, MA). The anti-phosphotyrosine antibody 4G10 and EGF-stimulated A431 cell lysates were from Upstate Biotechnology (Lake Placid, NY). Recombinant human TGF-β1 was obtained from R&D Systems. Actinomycin D was purchased from Sigma (St Louis, MO).

Patient materials

Skin samples were obtained from five diffuse cutaneous SSc patients and five limited cutaneous SSc patients (LeRoy *et al.*, 1988). Control skin samples were obtained from routinely discarded skin of healthy human subjects undergoing skin grafting. Immediately after removal, skin samples were fixed in formalin and embedded in paraffin (Ihn *et al.*, 1996). 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 Principles.

Cell culture

Human dermal fibroblasts were obtained by skin biopsies from the affected areas (dorsal forearm) of 5 diffuse cutaneous SSc patients who had <2 years of skin thickening (Ihn *et al.*, 1997). Control fibroblasts were obtained by skin biopsies from 5 healthy donors. Institutional review board approval and written informed consent were obtained according to the Declaration of Helsinki Principles.

Analysis of RNA and miRNA

Total RNA from formalin-fixed, paraffin-embedded tissue sections was extracted with RNeasy FFPE Kit (QIAGEN, Hilden, Germany). Total RNA from cultured cells was extracted using ISOGEN (Nippon Gene, Tokyo, Japan). First-strand complementary DNA was synthesized by the PrimeScript RT reagent Kit (Takara, Otsu, Japan). Quantitative real-time PCR used primers and templates mixed with the SYBR Premix Ex Taq II Kit (Takara). Primer sets for DDR1 (PPH19940E, designed against exon 16), DDR2 (PPH20974A, designed against exon 19), TGF-β1 (PPH00508A, against exon 2), and glyceraldehyde-3-phosphate dehydrogenase (PPH00150E, against exon 9) were purchased from SABiosciences (Frederick, MD). Primer sets for α1(I) collagen (sense 5′-CCCGGGTTTCAGA GACAACTTC-3′, antisense 5′-TCCACATGCTTTATTCCAGCAATC-3′), α2(I) collagen (sense 5′-GAGGGCAACAGCAGGTTCACTTA-3′, antisense 5′-TCAGCACCACCGATGTCCA-3′), TGF-β receptor I (sense 5′-AACCCTGCCTAGTGCAAGTTACAA-3′, antisense 5′-GAC TAACAAATGTGCTGACCCAAAG-3′), TGF-β receptor II (sense 5′-AACCCTGCCTAGTGCAAGTTACAA-3′, antisense 5′-GACTAAC AAATGTGCTGACCCAAAG-3′), endoglin (sense 5′-TTGAACAT CATCAGCCCTGACC-3′, antisense 5′-CGTGTGCGAGTAGATGTAC CAGAG-3′), thrombospondin 1 (sense 5′-GGAGACAAAGACTG GCTTCTGGAC-3′, antisense 5′-GGCCACTGCAGGTGATGAGTA A-3′), integrin β3 (sense 5′-ACTGTGTCATCAAATGTGCGGTTA-3′, antisense 5′-GCTCTTGCCAAAGCCAGGTC-3′), and integrin β5 (sense 5′-GTTTCAGAGCGAGCGATCCAG-3′, antisense 5′-CACAG TGCCATTGTAGGATTTGTTG-3′) were from Takara. DNA was amplified for 40 cycles of denaturation for 5 seconds at 95 °C and annealing for 30 seconds at 60 °C. The transcript levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase.

The miRNA isolation from total RNA was performed using RT^2 qPCR-Grade miRNA Isolation Kit (SABioscience). For quantitative real-time PCR, primers for miR-196a or U6 (SABioscience) and templates were mixed with the RT^2 Real-Time PCR master mix (SABiosciences). DNA was amplified for 40 cycles of denaturation for 5 seconds at 95 °C and annealing for 30 seconds at 60 °C. The transcript levels of miR-196a were normalized to those of U6.

Cell lysis and immunoblotting

Fibroblasts were lysed in Denaturing Cell Extraction Buffer (BIOSOURCE, Camarillo, CA). Aliquots of the cell lysates (normalized for protein concentrations) were subjected to electrophoresis on SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride filters. These filters were then incubated with primary antibodies.

Immunoprecipitation

Cell lysates were prepared from normal fibroblasts in the presence or absence of TGF-β1 (2 ng ml^{-1}) for 24 hours and SSc fibroblasts on 15 cm dishes. Cells were lysed in Pierce IP lysis buffer (Thermo, Rockford, IL) with the Halt phosphatase inhibitor cocktail (Thermo). The lysates were precleared with 20 μl of Protein A/G Plus Agarose (Santa Cruz) at 4 °C for 1 hour. For DDR2 immunoprecipitation, the lysates were incubated with an anti-DDR2 antibody (2 μg) and 40 μl of Protein A/G Plus Agarose overnight at 4 °C. The immunoprecipitated proteins were washed 5 times with Pierce buffer. Agarose-bound proteins were extracted by a 5-minute incubation in sample buffer at 95 °C. The sample was then assessed by immunoblotting. Phosphorylated DDR2 was detected using 4G10. The same membrane was stripped and reprobed with anti-DDR2 antibody. EGF-stimulated A431 cell lysates were used as positive control (Gill and Lazar, 1981).

Immunohistochemistry

Wax-embedded sections (4 μm thick) were dewaxed in xylene and rehydrated in graded alcohols. Antigens were retrieved by incubation with an antigen retrieval solution (pH 9; Nichirei, Tokyo, Japan) for 10 minutes with an autoclave apparatus at 121 °C. The endogenous peroxidase activity was inhibited, after which the sections were blocked with 10% rabbit blood serum for 20 minutes and then reacted with the antibody against DDR2 (15 μg ml^{-1}) overnight at 4 °C. After the excess antibody was washed off with phosphate-buffered saline, sections were incubated with horseradish peroxidase–labeled rabbit anti-goat antibody (Nichirei) for 60 minutes at 20 °C. The reaction was visualized by the diaminobenzidine substrate system (Dojin, Kumamoto, Japan). Slides were counterstained with Mayer's hematoxylin.

Intradermal treatment with bleomycin

Bleomycin (Nippon Kayaku, Tokyo, Japan) was dissolved in phosphate-buffered saline at a concentration of 1 mg ml^{-1} and sterilized by filtration (Yamamoto *et al.*, 1999; Tanaka *et al.*, 2010). Bleomycin or phosphate-buffered saline (100 μl) was injected intradermally into the shaved backs of 6-week-old C57BL/6 mice (CLEA, Tokyo, Japan) daily for 4 weeks. The back skin samples were removed on the day after the final injection, fixed in 10% formalin solution, and embedded in paraffin. This protocol was approved by the Committee on the Animal Research at Kumamoto University.

Transient transfection

The siRNA against TGF-β1 (sc-37191; mixture of three siRNA duplexes: GACACCAACUAUUGCUUCAtt, CUGUCUGCACUAU UCCUUUtt, and GAACACUACUGUAGUUAGAtt) or DDR2 (sc-39922, mixture of three siRNA duplexes: GUAUGAGAGUG GAGCUUUAtt, CAUCCAGGCUGAUACGAAAtt, and CACUCCAU CUGGACAUUUAtt) was purchased from Santa Cruz Biotechnology. The miRNA mimics and miScript target protectors were purchased from QIAGEN. For reverse transfection, siRNA, miRNA mimics, or miScript target protectors mixed with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) were added when 3×10^4 cells per well were plated in 24-well culture dishes, followed by incubation for 24–96 hours at 37 °C in 5% CO_2. Control experiments showed a >80% transfection efficiency (data not shown).

Lentiviral gene transfer

The constructs containing full-length human DDR2 complementary DNA were provided by Libo Yao (Fourth Military Medical University, China) (Su *et al.*, 2009). CSII-EF-RfA, pCMV-VSV-G-RSV-Rev, and pHIVgp, necessary for lentiviral gene expression, were kindly donated by Hiroyuki Miyoshi (RIKEN, Wako, Japan). The DDR2 complementary DNAs were cloned into CSII-EF-RfA (Tahara-Hanaoka *et al.*, 2002). A lentiviral vector–mediated gene transfer was performed as described (Tahara-Hanaoka *et al.*, 2002).

Statistical analysis

The data were expressed as the means ± SD of at least three independent experiments. The statistical analysis was carried out using Mann–Whitney *U* test. The *P*-values of <0.05 were considered to be significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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