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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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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.

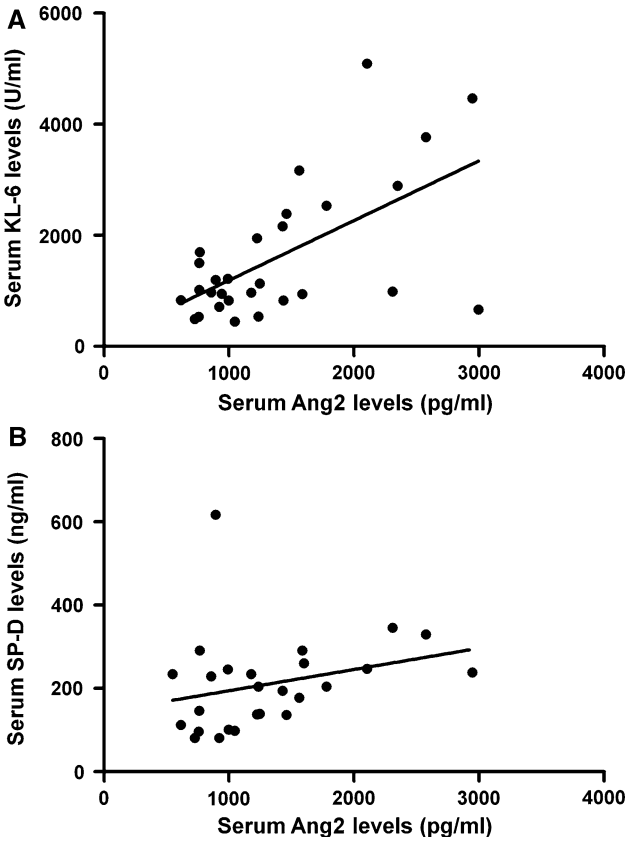

Fig. 2 Correlation of serum angiopoietin 2 (Ang2) levels with serum KL-6 and surfacant protein D (SP-D) levels in patients with systemic sclerosis interstitial lung disease (SSc-ILD) during intravenous pulse cyclophosphamide (IVCY). Serum Ang2 levels significantly correlated with serum KL-6 and SP-D 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

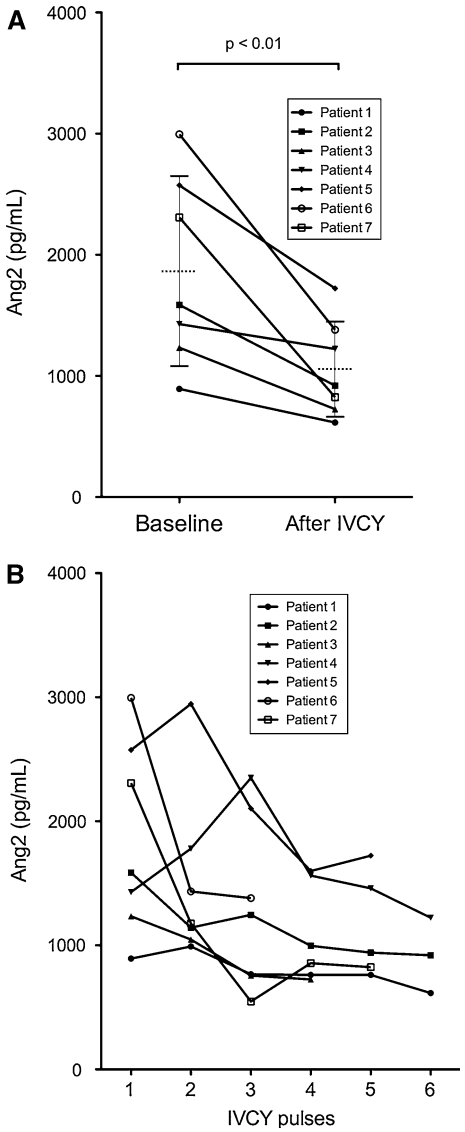

Fig. 3 Time course of serum angiopoietin 2 (Ang2) levels in patients with systemic sclerosis with interstitial lung disease (SSc-ILD) throughout intravenous pulse cyclophosphamide (IVCY) therapy. **a** Serum Ang2 levels at the last IVCY therapy were significantly lower than those at baseline [1,058.43 ± 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).

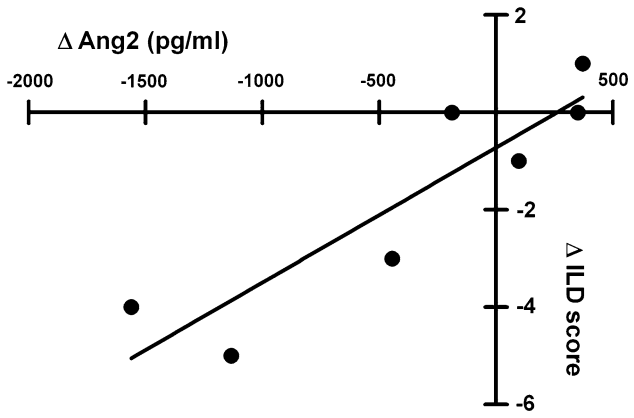

Fig. 4 The correlation of Δ serum Ang2 levels between baseline and after the first IVCY with Δ ILD score between before and after the whole IVCY. Significant correlation was found between Δ serum Ang2 levels (between baseline and after the first IVCY) and Δ ILD score (between before and after the whole 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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