Decreased ratio of circulatory vascular endothelial growth factor to endostatin in patients with systemic sclerosis – association with pulmonary involvement

B. Dziankowska-Bartkowiak[1], E. Waszczykowska[1], E. Dziankowska-Zaboroszczyk[2], J.E. de Graft-Johnson[3,6], A. Zalewska[4], M. Łuczyńska[5], D. Nowak[3]

[1]Division of Immunodermatology, [2]Department of Social and Preventive Medicine, [3]Department of Clinical Physiology, [4]Department of Dermatology, [5]Department of Cell-to-Cell Communication, [6]Divison of Medical Studies in English Medical University of Lodz.

Abstract

Objective

Vascular endothelial growth factor (VEGF) and endostatin appear to be involved in development of systemic sclerosis (SSc). We undertook this study to determine ratios of serum concentrations of VEGF to endostatin in SSc patients, healthy controls, assessments between cytokines, and lung-diffusing capacity (DLCO) as lung injury measurements related to interstitial lung disease (ILD).

Materials and methods

Serum VEGF and endostatin levels were measured with ELISA in 28 SSc patients (16 with lcSSc) and 20-matched healthy volunteers, evaluating correlation and balance. DLCO was corrected for hemoglobin, alveolar volume, and determined with a single breath technique.

Results

SSc serum concentrations (median; range) of endostatin were higher than controls (107.2; 13.6-261.2 vs. 77.8; 18.0-110.4 ng/ml, $p < 0.05$); VEGF levels did not differ (151.2; 4.5-836.4 vs. 286.4; 23.7-708.5 pg/ml, $p < 0.05$). Ratios of VEGF to endostatin were 2.6 and 3.6 times lower ($p < 0.05$) in SSc and dcSSc in comparison to healthy subjects. There were significant negative correlations between VEGF, endostatin in SSc ($r = -0.51$), and controls ($r = -0.57$). SSc with ILD ($n = 20$) had similar concentrations of VEGF, endostatin, and ratios of VEGF to endostatin compared to SSc alone. No correlations were seen between DLCO, VEGF, endostatin and their ratios in the whole SSc group. Negative correlations were noted between DLCO and VEGF ($r = -0.82$), with DLCO and the ratio of VEGF to endostatin (-0.62) in lcSSc with ILD ($n = 10$).

Conclusions

Decreased ratios of VEGF to endostatin may reflect imbalances between serum angiogenic, and anti-angiogenic activity in SSc, explaining impaired neoangiogenesis.

Key words

Systemic sclerosis, vascular endothelial growth factor, endostatin, interstitial lung disease.

Bożena Dziankowska-Bartkowiak, MD, PhD; Elżbieta Waszczykowska, MD, PhD; Elżbieta Dziankowska-Zaboroszczyk, MS; Jeffrey E. de Graft-Johnson, BS; Anna Zalewska, MD, PhD; Maria Łuczyńska, MD, PhD; Dariusz Nowak, MD, PhD.

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Please address correspondence and reprint requests to: D. Nowak MD, PhD, Department of Clinical Physiology, Medical University of Lodz, Mazowiecka 6/8, 92-215 Lodz, Poland.
E-mail: dnowak@zdn.am.lodz.pl

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Introduction

Systemic sclerosis (SSc) belongs to a group of connective tissue diseases characterized by fibrosis of the skin and internal organs including the digestive system, lungs, heart, kidneys and joints (1). There is variability and unpredictability in the course of SSc as well as the degree of organ involvement. SSc is generally divided into two forms, limited cutaneous (lcSSc) and diffuse cutaneous (dcSSc) systemic sclerosis. After skin and esophagus, lungs are the third most frequently involved in the course of SSc. Interstitial lung disease (ILD) leading to pulmonary fibrosis and consequently to severe respiratory insufficiency is frequently observed in patients with SSc (2). Recent studies have shown that angiogenic factors such as vascular endothelial growth factor (VEGF) and endostatin may be involved in the process of pulmonary fibrosis (3-5) including those related to SSc (6, 7).

Patients with dcSSc were reported to have elevated serum VEGF levels that correlated with the occurrence of lung fibrosis and reduction of vital lung capacity (6). Also elevated serum concentrations of endostatin were found in SSc patients; the highest concentrations were noted in patients with abnormal changes in chest X-ray (7). On the other hand, Distler *et al*. showed somewhat conflicting data (8). They observed increased VEGF serum levels in SSc patients while endostatin concentrations in healthy subjects presented no dissimilarity. Moreover, neither circulating VEGF nor endostatin correlated with lung diffusing capacity for carbon monoxide (DLCO) in SSc patients (8). In contrast, plasma VEGF levels were not elevated in patients with interstitial pneumonia comparing to healthy controls, however, they correlated with increased fibrotic score and a decreased forced vital capacity over six months of observation (3). This is in accordance with Meyer *et al*. who reported normal circulating VEGF in patients with idiopathic pulmonary fibrosis, in the presence of decreasing levels of cytokine in bronchoalveolar lavage fluid (4).

We re-evaluated changes in circulating VEGF and endostatin levels as well as the ratios of VEGF to endostatin levels in patients with SSc with respect to occurrences of interstitial lung disease and a decrease in DLCO. Furthermore, mutual associations between serum VEGF and endostatin were analyzed in SSc patients and healthy age- and sex-matched controls.

Materials and methods

The study was conducted at the Department of Dermatology, Medical University of Lodz (patient recruitment, clinical characteristics, determination of serum endostatin and VEGF levels) and at the Department of Experimental and Clinical Physiology (lung function measurement, serum samples preparation, pregnancy test performance) from September 2004 to March 2005. 28 patients with SSc, in accordance of the American College of Rheumatology (ACR) (9), and 20 age- and sex-matched healthy controls (Table I) participated in the study. Candidates were required to fulfil the following inclusion criteria: (1) age ≥ 18 years, (2) negative pregnancy test (women of childbearing age), and (3) disease duration ≥ 1 year. The exclusion criteria were as follows (1) cigarette smoking; (2) pregnancy; (3) active alcohol or drug abuse; (4) presence of any coexistent chronic disease not related to SSc pathology and (5) history of any infectious disease within 3 months prior to the study. Healthy control subjects were free of any medication and fulfilled all above listed inclusion and exclusion criteria except those relating to SSc.

The disease classifications as limited cutaneous SSc (lcSSc) or diffuse cutaneous SSc (dcSSc) were based on the criteria of Le Roy *et al*. (10). Interstitial lung disease (ILD) was considered present if (a) haemoglobin- and alveolar volume-corrected lung diffusion capacity for carbon monoxide (DLCO) was < 80% of predicted (n = 20 patients) and at least one of the following signs and or symptoms were noted; (b) bilateral reticulo-nodular shadowing predominantly involving the lower lobes of the chest X-ray (n = 13) and or high-resolution computed tomography (n = 7)

(11), (c) bilateral fine, and mid-to-late inspiratory crackles on clinical chest examination (n = 20). High-resolution computed tomography and chest X-rays were performed within a 6 month period prior to recruitment of patients. These patients (one with lcSSc and two with dcSSc) presented with a right ventricular systolic pressure of > 35 mmHg as assessed with Doppler echocardiography. Also secondary pulmonary hypertension could then be concluded due to a predicted FVC of < 60%. ILD, musculoskeletal dysfunction (muscle weakness, arthralgia, and flexion contractures), esophageal dismotility (evaluated with esophageal scintigraphy and or manometry), cardiac disease (conduction disturbances, arrhythmias and or echocardiographic abnormalities), and renal disease (elevated arterial blood pressure, elevated serum creatinine, proteinuria and abnormal urinalysis) were noted in patients 20, 22, 21, 15 and 1, respectively. The number of involved organs in patients ranged from 0 (n = 1) to 4 (n = 10) (Table I). Patients did not present with fingertip ulcers or digital amputations, although six patients were seen to experienced healed fingertip ulcers (1 with dcSSc and 5 with lcSSc). Scl70 -, U3RNP-, ANA-non specific-, and RNP-autoantibodies were detected in patients 15, 6, 6 and 1 with SSc, respectively. Two patients presented with Scl70- and ACA-, while another patient presented with Scl-70- and Ku-autoantibodies. Concomitant treatment of SSc patients included calcium channel blockers (nifedipine 10 mg daily) and vitamin E (400 mg daily). All patients with dcSSc (n = 12), were on cyclophosphamide 50 mg daily and or prednisone 15-20 mg daily (treatment duration, median 10 months; range 7-129 months) due to ILD (n = 8) and musculoskeletal involvement (n = 4). The Ethics Committee of the Medical University of Lodz approved protocol in this study, also all subjects gave informed consent in participation of this study.

Lung function measurement

Lung function was measured between 9 a.m. - 11 a.m. with Master-Laboratory Screen (Jaeger Toennies, Wuerzburg, Germany) according to American Thoracic Standards (13, 14). This involved measurement of forced vital capacity (FVC), forced expiratory volume in the first second (FEV_1), single breathe carbon monoxide diffusing capacity (DLCO) corrected for hemoglobin level (determined within one preceding week), and alveolar volume. All results were expressed as a percentage of the predicted value (15).

Table I. Characteristics of systemic sclerosis (SSc) patients and healthy controls.

Parameter/ Symptom	Patients with systemic sclerosis			Healthy controls
	Whole group	Limited cutaneous SSc	Diffuse cutaneous SSc	
Number	28	16	12	20
Age (yrs)	47.5 (18-70)	47 (27-70)	48 (18-64)	46 (25-64)
Sex F/M	22/6	15/1	7/5	15/5
Disease duration (yrs)	3.5 (1-17)	4 (1-7)*	2 (1-10)	- - -
Raynaud's duration (yrs)	8 (2-46)	15 (3-46)*	6 (2-30)	- - -
No. of organs involved	3 (0-4)	3 (0-4)	4 (1-4)	0
Patients with ILD	20	10	10	0
m. Rodnan skin score	23 (12-53)	15 (12-24)*	44 (14-53)	- - -
DLCO %	70.9 (28.3-101.4)**	79.05 (28.3-101.4)**	66.6 (37.7-98.1)**	102 (91-110)
FVC %	74 (43-114)**	77.5 (53-114)**	74 (43-98)**	100 (90-120)
FEV_1%	62 (31-115)**	62 (31-115)**	62 (39-79)**	99 (90-109)

ILD: interstitial lung disease; DLCO: carbon monoxide diffusing capacity; FVC: forced vital capacity; FEV_1: forced expiratory volume in the first second; % of predicted. *: $p< 0.05$ vs. dcSSc; **: $p< 0.05$ vs. healthy subjects.

Measurement of endostatin and vascular endothelial growth factor in serum

Blood was collected in the morning (before lung function measurement) into pyrogen-free tubes, incubated for 30 min at room temperature, centrifuged for 15 min (1500xg at 4°C) and then obtained serum was stored at –20°C for not more than 3 weeks until endostatin and VEGF measurement were ready. The concentrations of endostatin and VEGF in serum specimens were measured with ELISA sandwich kits manufactured by Oncogene Research Products (Boston, MA, USA) and Quantikine R&D Systems Inc. (Minneapolis, MN, USA), respectively. The concentrations of VEGF and endostatin were assayed in duplicates and individual results were expressed in pg/ml and ng/ml on the basis of standard curves generated with specific standards provided by the manufacturer. The reproducibility of the assay was confirmed by repeated measurements in the same subject (3 SSc patients and 3 healthy controls) within two consecutive days. The lower detection limit was 9.0 pg/ml for VEGF and 1.95 ng/ml for endostatin.

Additional measurements and diagnostic techniques used for patient clinical and laboratory characteristics were previously described (16).

Statistical analysis

Data are expressed as median and range. The differences between the groups were computed with Mann-Whitney U test. For serum specimens that gave negative VEGF readings (n = 3, from SSc patients) the concentration of this cytokine was assumed to be half of the detection limit – 4.5 pg/ml. Correlation coefficients were calculated by the Spearman test, and a p value of < 0.05 was considered significant.

Results

Median serum endostatin concentrations were almost 1.4-times higher ($p < 0.05$) in SSc patients than in healthy controls. Both subgroups of SSc patients i.e. lcSSc and dcSSc also revealed elevated serum endostatin concentrations in comparison to the control group (Table II). Although, median VEGF levels in the serum of SSc patients were 1.9-times lower than that in healthy controls, it did not reach

statistical significance, perhaps due to high inter-individual variability. The ratio of circulating VEGF to endostatin levels were markedly lower ($p < 0.05$) in SSc patients (2.6-times) and also in patients with dcSSc (3.6-times) than that in healthy controls.

Patients with lcSSc and also those with dcSSc did not differ from healthy subjects in respect to VEGF serum concentrations (Table II). Analysis of results obtained for SSc patients with clinically evident ILD revealed the similar differences, such as higher endostatin concentrations and the tendency to decrease VEGF in comparison to healthy controls (Table III). Median concentration of endostatin (118.0; 13.6-233.0 ng/ml) and VEGF (131.8; 4.5-836.4 pg/ml) in serum of SSC patients with ILD (n = 20) did not differ significantly ($p > 0.05$) from those found in SSc patients without ILD (87.6; 32.4-261.2 ng/ml, 151.3; 4.5-326.6 pg/ml, n = 8), respectively. Observation presented insignificant correlation between DLCO and serum concentrations of endostatin and VEGF; these insignificant correlations were also evident in the whole SSc group, lcSSc and dcSSc subgroups (Table. IV). Negative correlations between DLCO and serum VEGF were noted only in lcSSc patients with ILD ($r = -0.82$, $p < 0.01$). VEGF and endostatin ratios correlated with DLCO in SSc patients without ILD ($r = 0.78$, $p < 0.02$, $n = 8$) and in lcSSc patients with ILD ($r = -0.62$, $p < 0.05$). Significant negative correlations between VEGF and endostatin serum levels were noted in healthy controls ($r = -0.57$ $p < 0.01$), the whole SSc group ($r = -0.51$, $p < 0.01$) and the dcSSc subgroup ($r = -0.73$, $p < 0.01$) (Fig. 1). VEGF and endostatin did not significantly correlate ($p > 0.05$) with other clinical features; disease duration ($r = 0.20$, $r = -0.03$), Raynaud's duration ($r = 0.24$, $r = -0.09$), number of involved organs ($r = 0.14$, $r = 0.09$), and skin score ($r = 0.22$, $r = -0.19$) in SSc patients, respectively. Similarly, no significant associations between these angiogenic factors and clinical variables were found in SSc patients with ILD (data not shown).

Table II. Vascular endothelial growth factor (VEGF) and endostatin serum levels in patients with systemic sclerosis and healthy controls.

Parameter	Patients with systemic sclerosis			Healthy controls
	Whole group	lcSSc	dcSSc	
VEGF (pg/ml)	151.2 (4.5-836,4)	180.7 (4.5-836.4)	134.3 (4.5-404.9)	286.4(23.7-708.5)
Endostatin (ng/ml)	107.2(13.6-261.2)*	101.4(13.6-209.6)*	124.2(18.0-261.2)*	77.8 (18.0-110.4)
VEGF/endostatin ratio ($x10^3$)	1.29 (0.02-16.17)*	2.49 (0.02-16.17)	0.95 (0.02-15.00)*	3.41(0.21-19.90)

lcSSc: limited cutaneous systemic sclerosis; dcSSc: diffuse cutaneous systemic sclerosis, results expressed as median (range).
* - $p < 0.05$ vs. healthy controls.

Table III. Comparison of vascular endothelial growth factor (VEGF) and endostatin serum levels between SSc patients with interstitial lung disease and the control group.

Parameter	Systemic sclerosis patients with interstitial lung disease			Healthy controls
	Whole group	lcSSc	dcSSc	
VEGF (pg/ml)	131.8 (4.5-836.4)	119.6 (4.5-836.4)	134.3 (4.5-404.9)	286.4 (23.7-708.5)
Endostatin (ng/ml)	118.0 (13.6-233.0)*	110.2 (13.6-209.6)*	124.2 (18.0-233.6)*	77.8 (18.0-110.4)
VEGF/endostatin ratio ($x10^3$)	1.20 (0.02-16.17)	2.81 (0.02-16.17)	0.95 (0.03-15.00)	3.41(0.21-19.90)

lcSSc: limited cutaneous systemic sclerosis; dcSSc: diffuse cutaneous systemic sclerosis. * - $p < 0.05$ vs. healthy controls.

Table IV. Correlations (r) between carbon monoxide diffusing capacity and serum concentrations of vascular endothelial growth factor (VEGF) and endostatin in patients with systemic sclerosis.

Serum parameter	Carbon monoxide diffusing capacity (% of predicted)		
	Whole SSc group	lcSSc	dcSSc
VEGF	-0.23 (-0.31)	-0.40 (-0.82)*	-0.09 (0.05)
Endostatin	-0.28 (-0.05)	-0.46 (0.24)	-0.03 (-0.26)
VEGF/endostatin ratio	-0.07 (-0.20)	-0.18 (-0.62)†	0.06 (0.26)

SSc: systemic sclerosis; lcSSc: limited cutaneous systemic sclerosis, dcSSc: diffuse cutaneous systemic sclerosis.
In parentheses r calculated only for patients with interstitial lung disease. †: $p < 0.05$; *: $p < 0.01$.

Discussion

The novel aspect of this study was the finding of decreased ratios of circulatory VEGF to endostatin levels in SSc patients. These were the consequence of elevated serum endostatin concentrations and tendency of decreased circulatory VEGF in SSC patients as compared to healthy controls. Lowest VEGF to endostatin ratios were noted in dcSSc patients, while lcSSc patients only exhibited tendencies of lower ratios, as compared to that of healthy subjects.

SSc patients were all treated with nifedypine and vitamin E. It was found that administration of nifedipine did not change the circulatory VEGF levels in SSC patients (17). However, we uncovered no supporting literature concerning the effects of vitamin E on serum VEGF and endostatin concentrations. In contrast, patients with dcSSc also received long-term treatment with cyclophosphamide and or prednisone. *In vitro* studies showed that corticosteroids could attenuate cytokine-enhanced VEGF production by human airway smooth muscle cells (18). In patients with polymialgia rheumatica, a

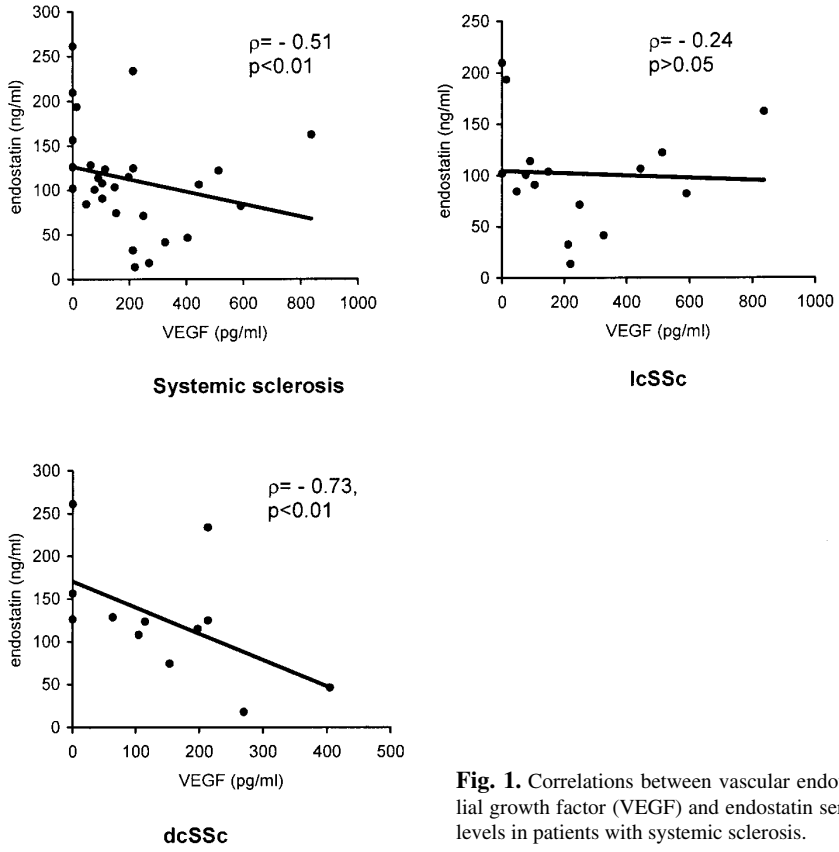

Fig. 1. Correlations between vascular endothelial growth factor (VEGF) and endostatin serum levels in patients with systemic sclerosis.

6-month prescribed amount of steroids resulted in a significant decrease in serum VEGF levels (19). Combined treatment with prednisone and bucillamine or salazosulfapyridine caused a remarkable decrease in circulating VEGF and increase in serum endostatin concentrations in patients with rheumatoid arthritis (20). Six-month treatment with combination of low-dose oral cyclophosphamide and methotrexate decreased serum VEGF concentration in women with metastatic breast cancer (21). This may suggest that low VEGF to endostatin ratios in patients with dcSSc could be in part the consequence of treatment with cyclophosphamide and or prednisone. Subset analysis of SSc patients with clinically evident ILD, revealed similar results, as well as no significant difference in analyzed parameters between SSc patients presenting with or without ILD. These findings suggest that lung involvement does not contribute to changes of circulatory VEGF and endostatin in the course of SSc. Conversely, negative correlations between DLCO and circulatory VEGF and VEGF to endostatin ratios in lcSSc patients with ILD, suggests that alterations in plasma levels of these cytokines may reflect lung damage. Despite the increased severity of lung damage in dcSSc, patients with ILD did not express any correlations between DLCO and VEGF and endostatin. Modulating effects in treatment with methotrexate and prednisone on serum levels of these cytokines may aid in explaining the lack of correlations.

Negative correlations between serum VEGF and endostatin concentrations were observed in both healthy subjects and SSc patients. This is consistent with previous observations in healthy volunteers (22) and therefore, suggests the occurrence of negative feedback regulatory mechanisms of circulatory VEGF activity in patients with SSc and healthy controls. However, lower VEGF to endostatin ratios in SSc patients implicates higher contributions of endostatin to regulation of serum VEGF activity in this group, in contrast to normal volunteers.

Recent study presented by Distler *et al.* demonstrated normal endostatin and increased circulatory VEGF levels in SSc patients (8). Moreover, patients with dcSSc had higher VEGF concentrations than patients with lcSSc. This study involved an elderly population of healthy controls and SSc patients (median age higher by 13 years than that of our patients, however median DLCO% was almost the same) that did not receive steroids or any disease-modifying anti-rheumatic drugs (particularly patients with dcSSc). Although, significant differences were not observed between patients presenting with early and or late stages of the disease (8), previous authors reported negative correlations between circulating VEGF levels and disease duration, as well as higher VEGF levels in patients displaying SSc for less than 5 years in comparison to patients with longer disease duration, and no effect of disease formation (dcSSc and lcSSc) on the concentration of this cytokine (17). We also did not observe any differences between lcSSc and dcSSc in patients in respect to VEGF and endostatin serum concentrations. Treadmill exercise increased circulatory endostatin and decreased VEGF in healthy subjects by 1.7 and 2.1 times, respectively (22). Younger subjects including those with SSc could also partake in higher every day physical activity. Therefore, different ages of studied populations as well as disease duration and treatment with cyclophosphamide and or prednisone are acceptable explanations concerning differences between our findings and results obtained by Distler *et al.* (8). Investigation of subjects with lower age, finding increased circulatory levels of endostatin in SSc patients by Hebbar and coworkers (7) are consistent with our findings. The consequence of decreased VEGF to endostatin ratios are perhaps due to the reduction of endothelial cell growth and increased cell apoptosis (23). This may explain the occurrence of ischemic changes of internal organs and skin in the course of SSc and conceivably contribute somewhat to the development of pulmonary and systemic hypertension in SSc patients.

Interestingly enough, SSc patients who were treated with cyclophosphamide and or prednisone had lower VEGF to endostatin ratios than patients never treated with disease-modifying anti-rheumatic drugs. Although, the effect of disease form cannot be excluded, it seems that disturbances in VEGF to endostatin ratios could be augmented by treatment with cyclophosphamide and or prednisone.

This is in accordance with results of our previous findings showing failure of this cure to normalize elevated whole blood chemiluminescence and hydrogen peroxide exhalation in patients with SSc (16, 24). This may reflect limited efficacy of cyclophosphamide and prednisone in the treatment of SSc. In conclusion, patients with SSc had decreased circulatory VEGF to endostatin ratios that may have reflected the imbalance between serum angiogenic and anti-angiogenic activity. The lowest VEGF to endostatin ratios were in dcSSc patients on long term-treatment with cyclophosphamide and or prednisone. Serial determinations of VEGF to endostatin ratios in serum and bronchoalveloar lavage fluid (or induced sputum) of SSc patients with or without ILD and before and after introduction of treatment with prednisone and other disease-modifying anti-rheumatic drugs are essential in clarifying the origin of this angiogenic and anti-angiogenic imbalance.

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