Increased Alveolar Concentration of Nitric Oxide Is Related to Serum-induced Lung Fibroblast Proliferation in Patients with Systemic Sclerosis

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ABSTRACT. ***Objective.*** Lung inflammation is present in patients with systemic sclerosis (SSc) and interstitial lung disease (ILD), but the mechanisms linking inflammatory and fibrotic processes in ILD are unknown. Our aim was to investigate whether alveolar inflammation, reflected by increased alveolar concentration of exhaled nitric oxide (C_ANO), is related to the ability of serum from patients with SSc to induce pulmonary fibroblast proliferation (PFP) and myofibroblast conversion.

Methods. C_ANO was measured in all subjects (37 patients with SSc and 10 healthy controls) whose sera were used to stimulate PFP (assessed by BrdU labeling index) and myofibroblast conversion (detected by α-smooth muscle actin expression). The PFP index in patients with SSc was compared to control values, and between patients with SSc who had elevated (> 4.3 ppb) and normal (≤ 4.3 ppb) C_ANO values.

Results. Both C_ANO and the PFP index were significantly greater in patients with SSc compared to controls. In patients with SSc, the PFP index was directly related to C_ANO levels ($r = 0.48$; $p = 0.002$). The median PFP index was significantly higher in patients with SSc who had elevated C_ANO (> 4.3 ppb; n = 25, median 1.1, range 0.98–1.23) than in patients with SSc who had normal C_ANO (≤ 4.3 ppb; n = 12, median 0.93, range 0.82–1.08; $p = 0.01$). Similarly, myofibroblast conversion induced by SSc serum was significantly greater in patients with C_ANO > 4.3 ppb than in patients whose C_ANO was ≤ 4.3 ppb ($p < 0.001$) and controls ($p < 0.001$).

Conclusion. Alveolar inflammation reflected by increased nitric oxide production was related to serum-induced PFP and myofibroblast conversion, linking the active alveolitis process to cell proliferation and lung fibrosis in patients with SSc. (J Rheumatol First Release July 1 2010; doi:10.3899/jrheum.090915)

Key Indexing Terms:
SYSTEMIC SCLEROSIS MYOFIBROBLAST ALVEOLITIS
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Systemic sclerosis (SSc) is a connective tissue disease of unknown origin, characterized by microvascular endothelial damage, inflammation, and progressive fibrosis. The latter usually takes place in the skin but also involves multiple internal organs[1,2]. Lung involvement, including pulmonary hypertension and interstitial lung disease (ILD), is now the main cause of death in endstage disease[3]. Although the pathogenesis of ILD remains largely unknown, it is now accepted that endothelial cell damage and immune-inflammatory processes are the primary events leading to proliferation of fibroblast and its phenotypic switch to myofibroblast, the latter producing high amounts of collagen that eventually lead to lung fibrosis in SSc[4,5].

Nitric oxide (NO), an important intracellular mediator, is both a powerful endogenous vasodilator and a sensitive biomarker of inflammation[6]. In patients with SSc, inducible NO synthase (NOS) is highly expressed as a result of lung inflammation, which explains why exhaled NO could be increased when active alveolitis is present in patients with SSc[7].

Lung parenchymal inflammation causes release into the bloodstream of several cytokines or chemokines that have both proinflammatory and proliferative effects. Serum from patients with SSc contains biological mediators that have the ability to induce fibroblast proliferation or myofibroblast conversion[8,9]. Studies have shown that proinflammatory

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cytokines known to be involved in fibroblast proliferation *in vitro* induce a high expression of NOS and a high amount of NO[10].

Because metabolites of NO in serum were influenced by many factors such as a nitrite-rich diet, the amount of NO directly collected in exhaled air resulting from lung inflammatory activity could more accurately reflect active alveolitis[11]. Several studies have reported that the total exhaled NO level was increased with SSc lung involvement[12,13] and its association with alveolitis was documented by bronchoalveolar lavage (BAL) fluid cell count[7]. Using a new method, partitioned exhaled NO measurement, to separately assess NO originating from conducting airways (NO maximal bronchial flow rate: JawNO) and alveoli (NO alveolar concentration: C_ANO), we[14] and others[15] have reported that C_ANO was related to the severity of ILD in patients with SSc.

Although studies have reported the profibrotic effect induced by SSc serum in murine and human dermal fibroblasts[9,16], the links between the ability of serum from patients with SSc to induce lung fibroblast proliferation, the early launching process of lung fibrosis, and the importance of pulmonary inflammation, reflected by high alveolar output of NO, have not been fully described. We therefore investigated whether alveolar inflammation, quantified by increased C_ANO, is related to the ability of serum to induce pulmonary fibroblast proliferation (PFP) and myofibroblast conversion in patients with SSc.

MATERIALS AND METHODS

Subjects. Between November 2006 and 2008, all consecutive patients seen in our academic hospital over 18 years old who fulfilled the American College of Rheumatology SSc criteria[17] were eligible for and enrolled into this prospective study. Patients who had upper airway infections less than 3 months before, history of smoking less than 1 month, pulmonary arterial hypertension (PAH, defined as systolic pulmonary artery pressure > 40 mm Hg, estimated by echocardiogram), or immunosuppressive, corticosteroid or NO donor therapy were excluded. Hence, 37 inpatients and outpatients with SSc (33 women, mean age 55 yrs, range 47–63.5) were included. Twenty-eight patients had the limited form and 9 had the diffuse form of SSc, according to LeRoy's subset classification[18]. The median disease duration was 10.4 years (range 4.9–18.7 yrs). Because endothelial dysfunction related to PAH in patients with SSc might decrease the exhaled NO output[19], we excluded patients likely to have PAH in order to make sure that the increased C_ANO in our study was due only to alveolar inflammation.

Clinical features and blood samples were collected from all 37 patients within a week of measurement of partitioned exhaled NO. Pulmonary function tests (PFT) and chest high-resolution computed tomography (HRCT) were performed within 1 month of blood sampling. Blood sampling and measurement of partitioned exhaled NO were performed on the fast day after 72 h of low-nitrate meals. Blood samples were centrifuged after 1 h of coagulation at room temperature and serum was collected and stored at –80˚C until use. We simultaneously collected serum of 10 age and sex-matched nonsmoking healthy subjects (8 women, mean age 53.5 yrs, range 42.5–57.0) for control. The study was approved by the local ethics committee and all patients provided written consent.

Exhaled NO measurement. Exhaled NO was measured in all 37 patients with SSc, using a chemiluminescent NO analyzer (Seres, EndoNO 8000; Aix-en-Provence, France), according to the validated method for online measurement of exhaled NO concentration in adults by the American Thoracic Society/European Respiratory Society (ATS/ERS) recommendation[20]. After full inspiration from room air with ambient NO levels < 20 ppb, the subject exhaled against positive pressure that was constantly kept between 5 cm H_2O (lower limit) and 20 cm H_2O (upper limit) to generate exhalation flow rates (V'_E) of 50, 100, 150, 200, and 250 ml/s ($FE_{NO50-250}$). For each V'_E, the elimination rate of NO (V'_{NO}) was calculated ($V'_{NO} = V'_E \times F_ENO$)[21,22]. F_ENO is inversely related to V'_E, while V'_{NO} varies directly as a function of V'_E. At the flow rate ≥ 50 ml/s, the latter relationship is linear and can be expressed as $V'_{NO} = V'_E \times F_ENO = C_ANO \times V'_E + J'awNO$. For each patient and control subject, the R^2 values of the relationship between F_ENO and V'_E were calculated. We have reported that the C_ANO cutoff value of 4.3 ppb accurately defined early impairment of DLCO (< 80% of predicted value) and the presence of ILD on lung HRCT scans[23]. We used this cutoff to separate patients with high and low levels of C_ANO.

Lung function measurement. This measure, including total lung capacity (TLC), forced vital capacity (FVC), DLCO, and alveolar volume (VA) was performed (MasterScreen® Body, VIASYS Healthcare GmbH, Hoechberg, Germany) according to ATS/ERS recommendations[24]. Results were expressed as percentage of predicted values.

Pulmonary CT scanning. HRCT of the lungs was performed in all patients. ILD was considered present if lesions such as ground-glass attenuation, lobular septal thickening, and subpleural honeycomb changes were demonstrated on chest HRCT.

Cell culture and proliferation assays. Primary human lung fibroblasts from healthy subjects (PromoCell®, Heidelberg, Germany) were used between the fourth and eighth passages with Dulbecco modified Eagle's medium (DMEM; Thermo Scientific, Waltham, MA, USA) complemented with antibiotics (100 IU/ml penicillin, 100 IU/ml streptomycin, and 0.25 μg/ml amphotericin B). Chemicals were provided by Sigma-Aldrich, St. Louis, MO, USA, unless otherwise stated.

Cell proliferation was determined by the colorimetric cell proliferation Biotrak ELISA method (GE Healthcare Europe GmbH, Orsay, France) based on the measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporation during DNA synthesis of proliferating cells.

Primary human normal pulmonary fibroblasts (4×10^3/well) were seeded in 96-well plates (Nunclon™ Delta, Nunc, Hørsholm, Denmark), starved of fetal bovine serum for 24 h, and then incubated in DMEM (10%) with serum from patients with SSc or healthy subjects for 72 h. Cells were then subjected to BrdU incorporation for 2 h. Detection procedure was performed according to manufacturer's instructions. Serum was used in quadruplicate. Results were accepted when the coefficients of variation were < 10%.

Immunofluorescence staining for α-smooth muscle actin (α-SMA). Normal human lung fibroblasts were seeded (3×10^4/well) on cover slips placed on a 24-well plate in serum-free DMEM for 24 h. After being treated with sera as described, cells were fixed with 3% paraformaldehyde/PBS, permeabilized with 0.1% Triton X-100, and incubated sequentially with mouse anti-α-SMA IgG antibody (1:100; DakoCytomation, Glostrup, Denmark) and FITC goat anti–mouse IgG antibody (1:200; Molecular Probes, Invitrogen, Burlingame, CA, USA). Cell nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI) Vectashield (Vector Labs, Burlingame, CA, USA). Expression of α-SMA was visualized by confocal microscopy and evaluated semiquantitatively by coding every visual field (250×) into 0, +1, +2, or +3 according to its density. Ten optical fields were counted for 1 slide and each group contained at least 5 sera used in duplicate.

Western blot analysis for α-smooth muscle actin. Primary human lung fibroblasts were seeded on 25-cm^2 flasks (1.5×10^5 cells/flask) and treated with human sera as described. Cell extracts, protein measurement, and Western blot techniques were as described[25] using primary mouse anti-α-SMA IgG antibody (1:1000) and horseradish peroxidase-associated goat anti–mouse IgG antibody (1:15,000; Santa Cruz Biotechnology,

Heidelberg, Germany). Immunostained bands were visualized using the enhanced chemiluminescent kits (Amersham, Orsay, France). Samples were normalized to ß-tubulin and quantified by densitometry.

Statistical analysis. Data were analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA). Categorical data were described using percentage, and comparisons were performed by chi-squared test with Yates correction. Continuous data were summarized using median and range (first and third quartiles). Comparisons were done with the Mann-Whitney U test unless otherwise specified. Spearman's test was used to study the relationship between PFP assessed by the BrdU labeling index and disease measurements. Multivariate linear regression analysis was also performed. All reported p values were 2-sided and deemed significant when < 0.05.

RESULTS

Demographic, clinical, and functional characteristics as well as the serum ability to induce PFP of 37 patients with SSc and 10 healthy controls are reported in Table 1. There was no difference in age and sex between the groups of patients and controls. Among patients with SSc, 16 had ILD on lung HRCT, 9 had restrictive respiratory syndrome (defined as TLC < 80% of predicted value), and 11 had severe gas exchange abnormalities (defined as DLCO < 60% of predicted value). C_ANO was significantly ($p < 0.001$) higher in patients with SSc (median 5.77 ppb; range 3.85-9.46) compared with controls (2.38 ppb; range 2.14-3.15). Twenty-five out of 37 patients with SSc had a level of C_ANO higher than 4.3 ppb (Table 2 summarizes the characteristics of the patients with SSc according to C_ANO levels).

Lung fibroblast proliferation induced by serum from patients with SSc and controls. The median of the PFP index induced by sera from patients with SSc (n = 37, median 1.04, range 0.93-1.19) was significantly higher than that induced by sera from controls (n = 10, median 0.82, range 0.72-1.00; p = 0.007; Table 1).

The proliferative effect of serum from patients with $C_ANO > 4.3$ ppb (n = 25, median 1.1, range 0.98-1.23) was significantly stronger than that from patients with $C_ANO \leq 4.3$ ppb (n = 12, median 0.93, range 0.82-1.08; p = 0.01; Table 2, Figure 1). Interestingly, there was no significant difference in the PFP index between sera from patients with $C_ANO \leq 4.3$ ppb and those from controls (median 0.82, range 0.72-1.0; p = 0.3).

To identify other factors that could be associated with serum ability to induce PFP, we compared the PFP index between groups of patients following different clinical and functional features. Patients at the early stage of disease (≤ 4 yrs) had a higher PFP index (n = 7; median 1.24, range 0.98-1.28) compared to patients with SSc who had longer disease duration (> 4 yrs; n = 30; median 1.01, range 0.88-1.13; p = 0.03). Neither the form of SSc (limited or diffuse), nor the impairment of PFT measurements (such as FVC or DLCO), nor the presence of ILD significantly affected the ability of serum to induce lung fibroblast proliferation (data not shown).

Active alveolitis has been known to precede pulmonary fibrosis; we focused on the comparison of the serum ability to induce PFP between patients with SSc with $C_ANO > 4.3$ ppb and those with $C_ANO \leq 4.3$ ppb in patients with SSc who did not have ILD. The serum from the former group exhibited a stronger ability to induce PFP (n = 12, median 1.19, range 1-1.25) than serum from patients without ILD and lower C_ANO levels (n = 9, median 0.93, range 0.79-1.13; p = 0.02). However, among patients with established

Table 1. Demographic, clinical, and functional characteristics of patients with SSc and healthy controls.

Characteristics	SSc, n = 37	Controls, n = 10	p
Age, yrs, mean (range)	55 (47–63.5)	53.5 (42.5–57.0)	0.33
Males, n (%)	4 (10.8)	2 (20)	0.59
Duration of disease*, yrs, mean (range)	10.4 (4.9–18.7)		
Patients with dSSc, n (%)	9 (24)		
Patients with ILD, n (%)	16 (43)		
TLC (% predicted)	101 (81–113)		
TLC < 80% of predicted, n (%)	9 (24)		
FVC (% predicted)	96 (77–117)		
FVC < 80% of predicted, n (%)	10 (27)		
DLCO (% predicted)	70 (53–78)		
DLCO/AV (% predicted)	77 (66–88)		
Systolic PAP, mmHg	30 (27–37)		
F_ENO, 50 ml/s, ppb	12.4 (8.2–19.3)	13.7 (12.2–19.1)	0.38
C_ANO, ppb	5.77 (3.85–9.46)	2.38 (2.14–3.15)	< 0.001
PFP index (optical density)	1.04 (0.93–1.19)	0.82 (0.72–1.00)	0.007

Results are given as median and range (first and third quartiles). dSSc: diffuse SSc; ILD: interstitial lung disease; TLC: total lung capacity; FVC: forced vital capacity; DLCO: diffusing capacity of carbon monoxide; AV: alveolar volume; systolic PAP: systolic pulmonary artery pressure estimated by echocardiogram; F_ENO, 50 ml/s: fractional exhaled NO concentration at 50 ml/s constant flow rate, exhaled fraction of nitric oxide; C_ANO: alveolar concentration of exhaled nitric oxide; PFP: pulmonary fibroblast proliferation. * Calculated from first non-Raynaud's phenomenon clinical symptom of SSc to the date of enrollment into the study.

Table 2. Characteristics of patients with SSc with high levels of alveolar concentration of exhaled nitric oxide (C_ANO > 4.3 ppb) compared to those with C_ANO ≤ 4.3 ppb. Results are given as median and range (first and third quartiles).

Characteristics	C_ANO > 4.3 ppb, n = 25	C_ANO ≤ 4.3 ppb, n = 12	p
Age, yrs, mean (range)	54 (44–65.5)	57.5 (52.5–61.5)	0.47
Duration of disease*, yrs, mean (range)	10.8 (3.5–19.4)	9.8 (6.1–13.6)	0.82
Patients with dSSc, n (%)	7 (28)	2 (17)	0.46
Patients with ILD, n (%)	13 (52)	3 (25)	0.13
Systolic PAP, mmHg	30 (27–37)	33 (26.5–35.25)	0.76
TLC (% predicted)	101 (80–113)	99 (80–114)	0.96
TLC < 80% of predicted, n (%)	6 (24)	3 (25)	0.73
FVC (% predicted)	95 (75–113)	102 (90–120)	0.39
FVC < 80% of predicted, n (%)	8 (32)	2 (16.7)	0.55
DLCO (% predicted)	65 (43–77)	76 (62–86)	0.08
F_ENO, 50 ml/s, ppb	12.68 (9.97–21)	8.97 (5.59–15.62)	0.03
C_ANO, ppb	8.09 (5.71–11.07)	3.28 (2.2–3.88)	< 0.001
JawNO, ppb	11.71 (5.09–38.52)	16.76 (5.69–38.07)	0.85
R^2	0.98 (0.96–0.99)	0.98 (0.9–0.99)	0.88
PFP index (optical density)	1.1 (0.98–1.23)	0.93 (0.82–1.08)	0.01

dSSc: diffuse SSc; ILD: interstitial lung disease; TLC: total lung capacity; FVC: forced vital capacity; DLCO: diffusing capacity of carbon monoxide; PAP: systolic pulmonary artery pressure estimated by echocardiogram; F_ENO, 50 ml/s: fractional exhaled NO concentration at 50 ml/s constant flow rate, exhaled fraction of nitric oxide; C_ANO: alveolar concentration of exhaled nitric oxide; JawNO: maximal bronchial flow rate of nitric oxide. * Calculated from first non-Raynaud's phenomenon clinical symptom of SSc to the date of enrollment into the study.

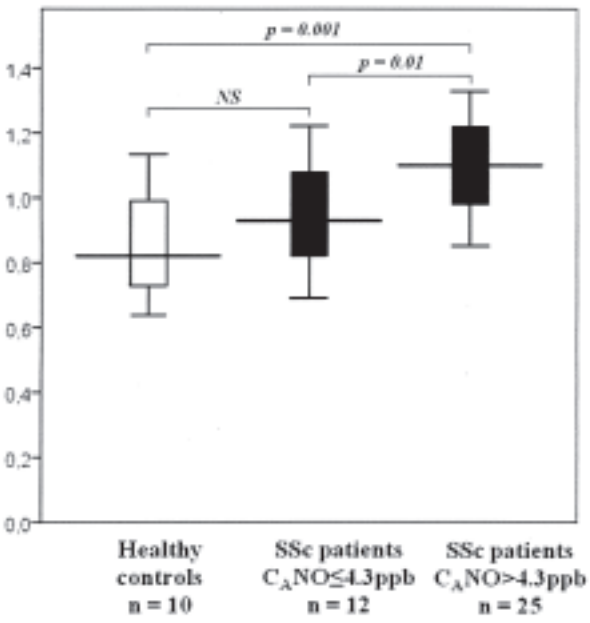

Figure 1. Comparison of pulmonary fibroblast proliferation index of healthy controls, patients with SSc with C_ANO ≤ 4.3 ppb, and those with C_ANO > 4.3 ppb. C_ANO: alveolar concentration of exhaled nitric oxide; NS: not significant.

ILD, the serum proliferative capacity on lung fibroblasts was not significantly different (p > 0.05) between patients with high levels of C_ANO (> 4.3 ppb; n = 13, median 1.03, range 0.97-1.13) and those with C_ANO ≤ 4.3 ppb (n = 3, median 0.93, range 0.87-0.96).

Lung fibroblast conversion into myofibroblasts induced by serum from patients with SSc and controls. PFP induced by serum from patients with SSc came with a conversion to myofibroblasts, characterized by the presence of α-SMA in the cytoplasm. Immunofluorescent staining showed that the expression of α-SMA was significantly higher in fibroblasts stimulated by serum from patients with SSc compared with that in fibroblasts stimulated by controls (p = 0.012). Similar to PFP index results, α-SMA levels were higher in fibroblasts cultured with serum from patients with C_ANO > 4.3 ppb compared to those stimulated by serum from patients with C_ANO ≤ 4.3 ppb (p < 0.001; Figure 2). The results of lung fibroblast transformation were also confirmed by Western blot analysis (Figure 3).

Relationship between the PFP index and C_ANO levels and skin score. Importantly, the serum proliferative capacity on lung fibroblasts was related to the lung inflammation reflected by levels of C_ANO (p = 0.48; p = 0.002) but not with the extent of skin fibrosis assessed by mRSS (p = 0.092, p = 0.6).

Followup. During the followup period, the PFT measurements of 10 patients worsened, defined as a decrease of > 10% in FVC or TLC (median followup 27 mo, range 15.3-36.8; Table 3). Twenty-seven patients had stable lung disease (median followup 24 mo, range 20-34.5). Seven out of 25 patients with C_ANO > 4.3 ppb, and 3 out of 12 patients with C_ANO ≤ 4.3 ppb, had worse PFT measurements. However, the PFP index of patients with worse subsequent lung function (median 1.11, range 0.93-1.24) was not sig-

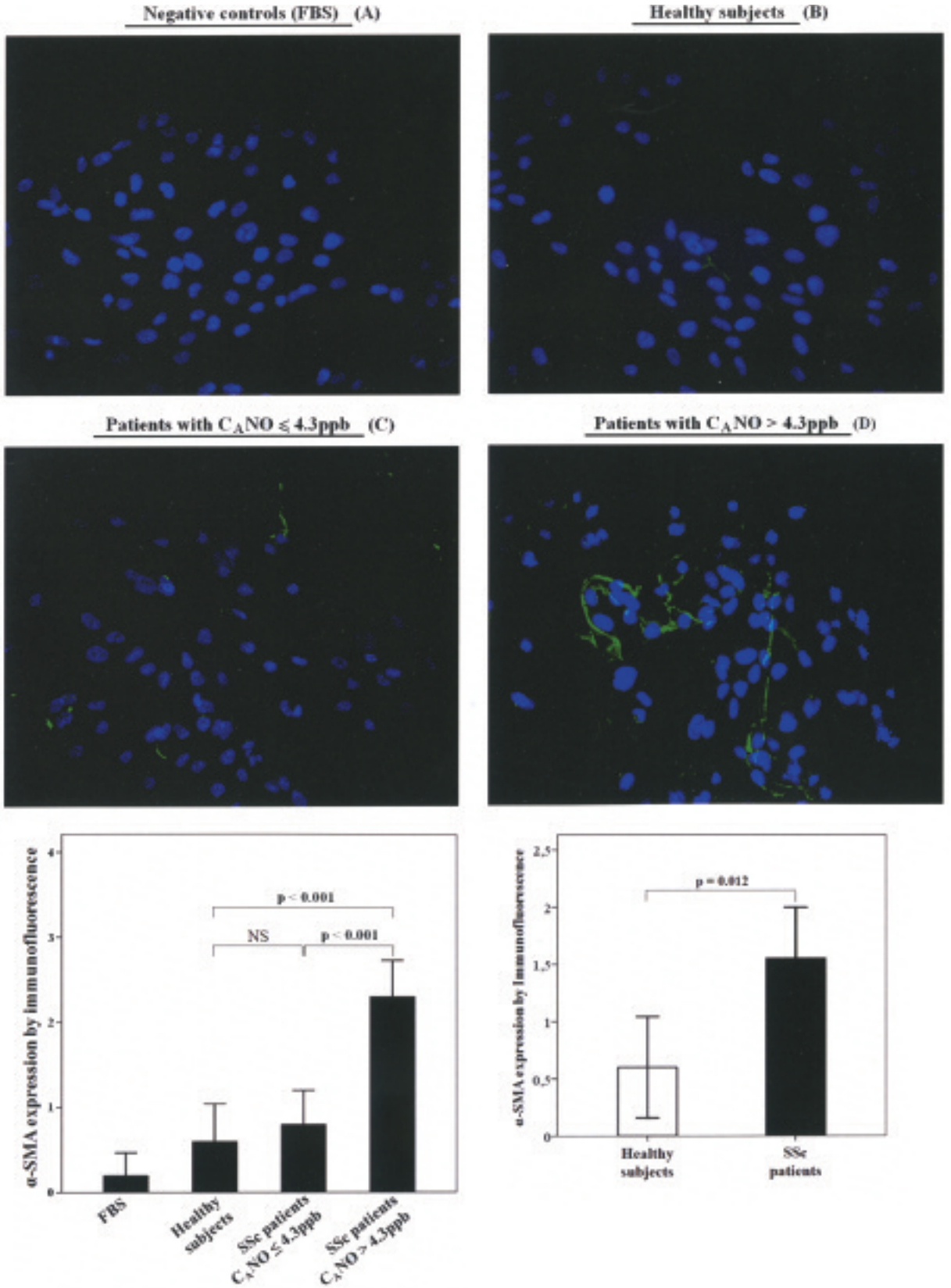

Figure 2. Immunofluorescence staining of α-smooth muscle actin (α-SMA) in lung fibroblasts stimulated by serum from patients with SSc or healthy controls (A-D). Semiquantitative results were given as mean ± SEM and compared by Student's 2-tailed test. Each group contained at least 5 samples in duplicate. NS: not significant; FBS: fetal bovine serum; SSc: systemic sclerosis; C_ANO: alveolar concentration of exhaled nitric oxide.

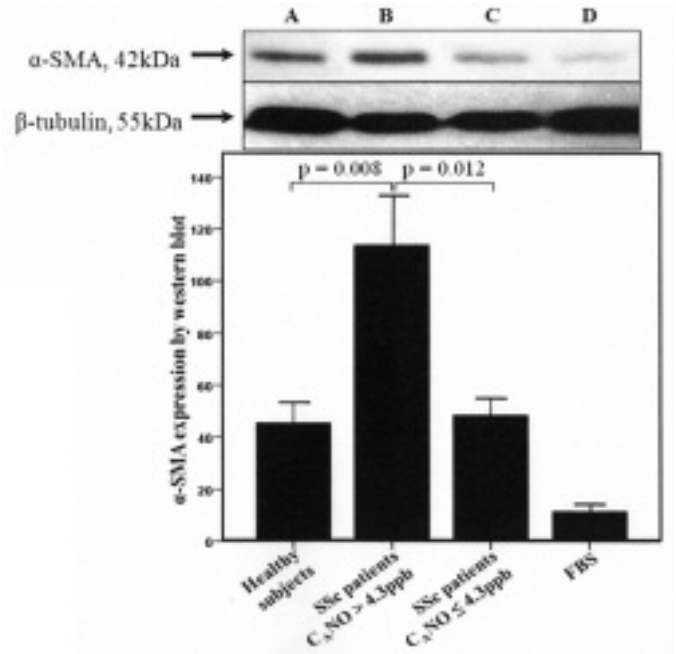

Figure 3. Western blot analysis of α-smooth muscle actin (α-SMA) levels in lung fibroblasts stimulated by serum from patients with systemic sclerosis (SSc) or healthy controls. Data were given as mean ± SEM, compared by Student's 2-tailed test. Each group contained 6 samples. C_ANO: concentration of exhaled nitric oxide; FBS: fetal bovine serum.

nificantly higher than that of patients with stable lung function (median 0.99, range 0.87-1.22; p = 0.45). Similarly, there were no significant differences (p > 0.05) in PFT measurements (FVC, TLC, and DLCO) and radiologic results between these 2 groups of patients.

DISCUSSION

The main result of our study showed that alveolar NO production was related to serum-induced PFP and myofibroblast conversion, linking active alveolitis process to cell proliferation and lung fibrosis in patients with SSc. It was also found that serum-induced lung fibroblast proliferation and its conversion to myofibroblast phenotype was significantly greater in patients with SSc than in healthy controls. Among patients with SSc, PFP index and its myofibroblast conversion were significantly increased in patients with SSc at an early stage of the disease (≤ 4 years). However, this proliferative activity of the serum could be predicted only by the levels of C_ANO, and not by the form of the disease (limited vs diffuse), modified Rodnan skin score, or lung function impairment, reflecting the extent of fibrosis independent of the levels of lung inflammation.

The lung fibroblasts taken from the fibrotic lungs of patients with SSc have a constitutively activated myofibroblast-like phenotype[4]. Fibroblast activation is a key event in the development of fibrosis. However, it is difficult to noninvasively assess the activity of the lung fibrosis process in clinical management of SSc lung disease. It has been reported that several soluble molecules including cytokines[26], chemokines[27], autoantibody[9], and low molecular weight peptides[28] are involved in various pathways leading to lung fibrosis. Indeed, studies have demonstrated the humoral mediated biological effect on mice fibroblasts or dermal fibroblast strains[9,16]. One of those studies showed that fibroblasts stimulated *in vitro* by cytokines such as interferon-γ, interleukin 1ß, and tumor necrosis factor-α increased proliferation rates, expressed highly inducible NOS, and therefore produced a great amount of NO[10]. Other reports using serum from patients with SSc to stimulate murine and human dermal fibroblasts implicated advanced oxidation protein products that triggered intracellular oxidative stress to increase fibroblast proliferation[16]. Recently, Baroni, *et al* discovered stimulatory antiplatelet-derived growth factor-receptor (PDGFR) antibody in serum from patients with SSc that could induce extracellular matrix production and cause phenotypic changes of normal fibroblasts[9]. Anti-PDGFR antibodies contained in the serum of some patients with SSc could induce fibroblast proliferation,

Table 3. Followup time, alveolar concentration of nitric oxide, fibroblast proliferation index, initial PFT, and radiologic results from patients with systemic sclerosis with decline in and stable lung function. Data at entry were given as median (first and third quartiles).

Clinical Data	SSc Patients with Decline in Lung Function, n = 10	SSc Patients with Stable Lung Function, n = 27	p
Followup, mo	27 (15.3–36.8)	24 (20–34.5)	0.54
C_ANO, ppb	5.4 (3.8–11.5)	6.6 (3.6–8.9)	0.67
PFP index (optical density)	1.11 (0.93–1.24)	0.99 (0.87–1.22)	0.45
TLC (% of predicted)*	106 (75–110)	95 (81–110)	0.77
FVC (% of predicted)*	113 (80–120)	94 (75–108)	0.09
DLCO (% of predicted)*	66 (42–80)	67 (53–77)	0.98
ILD, n	4	12	0.99

C_ANO: alveolar concentration of exhaled nitric oxide; PFP: pulmonary fibroblast proliferation; TLC: total lung capacity; FVC: forced vital capacity; DLCO: diffusing capacity of carbon monoxide; PFT: pulmonary function tests; ILD: interstitial lung disease. * Significant if p < 0.05.

Table 4. Relationship between fibroblast proliferation index and C_ANO adjusted for age and duration of disease.

	Fibroblast Proliferation Index (Optical Density) ß ± SE	p
C_ANO (by increased ppb)	0.428 ± 0.006	0.007
Age (by increased yr)	–0.255 ± 0.002	0.17
Duration of disease (by increased yr)	–0.022 ± 0.002	0.90

C_ANO: alveolar concentration of exhaled nitric oxide.

myofibroblast conversion, and an increased reactive oxygen species (ROS) output by the Ha-Ras-ERK1/2 transduction signal. These data indicate a plausible link between NO and ROS output from fibroblast and its ability to proliferate and to transform into myofibroblast. We showed that serum from patients with SSc, especially those with a high level of C_ANO (> 4.3 ppb), could convert normal human lung fibroblasts to a myofibroblast-like phenotype.

Several serum factors can be responsible for the observed capacity of serum from patients with SSc who have high levels of C_ANO to induce lung fibroblast proliferation. The factors causing proliferation of lung fibroblast and its conversion to myofibroblast have not yet been identified but the presence of these factors in the serum was likely related to increased NO synthesis in the lungs of patients with SSc.

Our 2007 report showed a relationship between PFT measurements and C_ANO[14]. However, this relationship, although reaching statistical significance, was loose, suggesting that C_ANO is more a marker of lung inflammation that is currently present rather than a reflection of impaired lung function that results from abnormal repair processes in response to inflammation. In our current study, we showed that high C_ANO reflected not only the alveolar NO output but also the increased level of mediators or cytokines involved in profibrotic pathways. The release of these substances into the bloodstream has probably rendered the serum from patients with SSc capable of inducing myofibroblast transition. Impairment of PFT could not be predicted by the PFP index. Patients with high C_ANO and in the early stage of disease had a stronger PFP index (Table 4). These results suggest that serum from patients with SSc who have high levels of C_ANO had high potential to induce fibrosis. Further, C_ANO, which assessed the biological pathways involved in inflammation, would provide additional information on PFT measurements in order to better characterize features of SSc lung disease.

However, our study was not initially designed to assess the predictive value of the PFP index. For that purpose, more patients and a longer disease followup period are required. Further studies with larger populations are needed to establish the evidence linking elevated values of C_ANO to disease progression and/or the presence of subclinical alveolitis, and to confirm the predictive value of the PFP index and C_ANO on the decline of lung function in SSc.

The levels of increased fibroblast proliferation induced by serum from patients with SSc compared to controls in our study were relatively lower than those reported elsewhere[15]. This discrepancy resulted essentially from different fibroblast cell lines and methods used to assess lung fibroblast proliferation as well as other cell culture measurements.

Our study was limited by the lack of direct evidence of alveolitis that could be documented by an invasive means such as BAL in patients with high levels of C_ANO. For ethical reasons, it was not possible to perform BAL in all patients with SSc, especially when the practical value of BAL fluid cell counts has been questioned, and its lack of predictive value for the response to treatment for patients with SSc[29] has been highlighted. Moreover, BAL could be normal in patients with well documented ILD, as shown by abnormal lung HRCT[30]. The meaning of increased C_ANO, which we assumed to reflect active alveolitis, should be investigated further. The negative relationship between DLCO and C_ANO could be linked to either increased thickness of alveolar membrane, impeding NO diffusion, or alveolar inflammation in SSc lung disease. No data are currently available showing concomitant measurement of C_ANO and lung diffusion of NO (DLNO). In SSc-associated ILD, the ratio of DLNO/DLCO recently reported by van der Lee, *et al*[31] was slightly higher than that from patients with chronic obstructive pulmonary disease, suggesting that reduced NO diffusion across the alveolar membrane was unlikely to account for increased NO concentration in the alveolar space. On the other hand, it has been reported that increased fractional concentration of exhaled NO was related to alveolitis documented by BAL cell counts[7]. These data are consistent with the hypothesis that alveolar inflammation is likely the main factor causing increased C_ANO in patients with SSc.

Our study demonstrated that alveolar production of NO, a surrogate cause of lung inflammation, was related to pulmonary fibroblast proliferation and myofibroblast conversion induced by serum from patients with SSc. The underlying mechanisms linking active alveolitis to lung fibroblast proliferation remain to be further investigated.

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