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ORIGINAL ARTICLE

Proteome Analysis of Bronchoalveolar Lavage Fluid in Lung Fibrosis Associated with Systemic Sclerosis

Ryutaro Shirahama[1], Yasunari Miyazaki[2], Tsukasa Okamoto[1], Naohiko Inase[1] and Yasuyuki Yoshizawa[1]

ABSTRACT

Background: Interstitial lung disease (ILD) is the major cause of mortality in collagen vascular diseases. However, its pathogenesis still needs to be elucidated.

Methods: To evaluate the alteration of certain proteins in bronchoalveolar lavage fluid (BALF) and clarify the causative role in the processes of ILD in systemic sclerosis (SSc), we compared a BALF protein profile between 5 patients with systemic sclerosis with pulmonary fibrosis (SSc-fib+) and 4 patients with systemic sclerosis without pulmonary fibrosis (SSc-fib-) using two-dimensional gel electrophoresis (2-DE), and matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS).

Results: We analyzed spots over the range of 10.1 kDa to 207.4 kDa. SSc-fib+ patients showed increased 3 proteins compared to SSc-fib- including α2-macroglobulin, α1-antitrypsin, and pulmonary surfactant protein A and decreased 2 proteins including α2 heat shock protein (HSP) and glutathione S-transferase (GST) compared to SSc-fib- patients.

Conclusions: In conclusion, we identified several interesting proteins that might have roles in ILD of SSc patients. Further studies are warranted to clarify the role of these proteins in the processes of pulmonary fibrosis in SSc.

KEY WORDS

bronchoalveolar lavage, inflammation, pulmonary fibrosis, scleroderma, Toll-like receptors

INTRODUCTION

Systemic sclerosis (SSc) is a disease of unknown origin characterized by excessive deposition of collagen and other extracellular matrix protein in skin and multiple internal organs. About 70% of SSc patients have abnormalities in pulmonary functions, and interstitial lung disease (ILD) is the main cause of mortality in this population because no reliable ameliorating treatment is available for such involvement.[1] However, the mechanisms involved in the severe and progressive pulmonary fibrosis remain unknown. As shown by the previous studies, nonspecific interstitial pneumonia (NSIP) pattern is the most frequent histopathologic pattern in ILD of SSc (SSc-ILD). NSIP pattern was diagnosed in 78% with SSc-ILD, while usual interstitial pneumonia (UIP) pattern was diagnosed in 7.5%.[2]

Major component of proteins in bronchoalveolar lavage fluid (BALF) are proteins derived from plasma. These proteins include α1-antitrypsin, α2-macroglobulin, apolipoprotein A1, α2-microglobulin, ceruloplasmin, complement factor 3 and 4, immunoglobulins and so on.[3] Many species of proteins were detected by differential proteomics analysis of BALF in ILD. These include immuno-inflammatory mediators, oxidant/antioxidant balance, protease/antiprotease balance, cytoskeletal, heat shock, coagulation system, calcium binding, lipid binding, and unknown function.[4] Proteomics analysis of BALF of interstitial lung disease was started from BALF of sarcoidosis, and hypersensitivity pneumonitis.[5,6] BALF is now the common way of sampling the components of the epithelial lining fluid and the most faithful reflect of the

[1]Department of Integrated Pulmonology and [2]Department of Sleep Modulatory Medicine, Tokyo Medical and Dental University, Tokyo, Japan.
Correspondence: Yasunari Miyazaki, MD, PhD, Associate Professor, Department of Sleep Modulatory Medicine, Tokyo Medical and Dental University, 1–5–45 Yushima, Bunkyo-ku, Tokyo 113–8519, Japan.
Email: miyazaki.pilm@tmd.ac.jp
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protein composition of the pulmonary airway. Few analyses were reported about BALF of interstitial lung disease with collagen vascular diseases. Eleven proteins (from 16.3 kDa to 69.8 kDa) were shown to be significantly different between SSc with pulmonary fibrosis (SSc-fib+) and SSc without pulmonary fibrosis (SSc-fib-) in the previous study.[7]

Aim of this study is to evaluate the alterations of the BALF proteins in comparison with the detected proteins in the previous studies and clarify the role of the proteins in the processes of pulmonary fibrosis in SSc.

METHODS

PATIENTS SELECTION

BALF samples were obtained from SSc patients between April 2004 and March 2007. The preliminary criteria for disease classification American College of Rheumatology were used to define SSc.[8] Out of nine SSc patients the 5 patients with pulmonary fibrosis (SSc-fib+) and the 4 patients without pulmonary fibrosis (SSc-fib-), were examined in this study. SSc-fib- patients included 2 male and 2 female patients and SSc-fib+ patients included 2 male and 3 female. The diagnosis of SSc-associated pulmonary fibrosis was based on clinical, functional and radiographic signs. In all cases, carbon monoxide transfer, forced vital capacity, chest radiography were evaluated. Patients were given informed consent for bronchoscopy, and none were treated with prednisolone nor other immunosuppressive agents at the time of enrollment. The study conformed to the declaration of Helsinki and was approved by the internal review board of our institution. Written informed consent was obtained for each subject.

BROCHOALVEOLAR LAVAGE

Bronchoalveolar lavage (BAL) was performed as previously described using three 50 ml aliquots of sterile 0.9% saline.[9] The cellular composition of the BAL fluid (BALF) was determined using a cytospun smear with Wright stain by counting 200 cells. Lymphocyte phenotypes were analysed by flow cytometry with monoclonal antibodies for CD4 and CD8.

TWO DIMENSIONAL GEL ELECTROPHORESIS

Two-dimensional (2-D) gel electrophoresis was performed as previously described.[7] Shortly, BALF samples were freeze-dried and concentrated by acetone precipitation. In each analytical experiment, the same amount of samples (54 μl) were loaded on immobilized pH gradient (IPG) strips (pH 3 to 10,18 cm; GE healthcare, Sweden) which were concentrated by acetone and swollen in 8M urea, 4% CHAPS, 65mM dithioerythritol, 0.1M acetic acid and pharmalyte (pH 3 to 10 for IEF; GE healthcare) and a trace of bromophenol blue. Rehydration was performed in the strip holder of the IPG-IEF CoolPhoreStar system (Anatech, Japan) at 20℃ and isoelectrofocusing was terminated at 47 kV. The IPG strips were equilibrated first for 30 minutes in the urea/SDS/Tris buffer, and then in the same buffer containing 2.5% iodoacetamide. The second-dimensional run was performed on 10% polyacrylamide linear gradient gels with a constant current of 20 to 30 mA/gel at 20℃, until the dye front reached the bottom of the gel. Gels were stained with SYPRO Ruby Protein Gel Stain (Molecular Probes, USA).

EVALUATION AND IDENTIFICATION OF PROTEINS

The gels are scanned using FluoroPhoreStar 3000 (Anatech) and analyzed using Progenesis Same Spots (Nonlinear Dynamics Ltd, UK) to create a reference map for each patient group and to detect differentially expressed protein spot in SSc-fib+ patients compared with SSc-fib- patients. The software established the reference map by taking into account only the spots, which were consistently expressed in each patient of the same group. The differences between two groups were confirmed by analyzing the spot quantities of pooled samples of SSc-fib+ or SSc-fib-. Non-matching spots or the spots differed 1.5 folds higher or lower in quantity were regarded as differentially expressed in SSc-fib+ patients compared with SSc-fib- patients. Then we analyze BALF samples of individual between two groups to verify these results.

Proteins were identified by comparing the BALF maps, such as the SWISS-2D PAGE human plasma map and published BALF maps, or matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS) as follows. The selected spots, picked up from Coomassie-stained gels, were analyzed in Ettan MALDI-TOF Pro mass spectrometer (Amersham Biosciences, USA) at the Cellular and Proteome Research Laboratory of Tokyo Medical and Dental University, equipped with a reflectron analyzer and used in delayed extraction mode. Mass spectra was used for database searching with MASCOT peptide fingerprinting search program (Matrix Science, USA).

STATISTICAL METHODS

All data were described as mean ± standard error of the mean (SEM). The Mann-Whitney method was used for each statistic analysis. $P < 0.05$ were considered as statistical significance. Analyses were performed with Stat View 5.0 (Abacus Concept, USA).

RESULTS

Proteome analysis of the individual BALF was performed on 9 SSc patients, including 5 SSc-fib+ and 4 SSc-fib-. Patients' characteristics were shown in Table 1. No microbiological or clinical evidence of bacterial or fungal infections were found in all patients. SSc-fib+ patients showed a significant increase in KL-6

Table 1 Clinical, immunological and functional characteristics

Parameter	SSc fib+	SSc fib-	*p* value
number of patients	5	4	-
age	63.4 ± 2.0	63.5 ± 4.5	0.981
male/female	2/3	2/2	NS
smoking (pack year)	12.2 ± 7.6	9.0 ± 9.0	0.792
CRP (mg/dl)	0.6 ± 0.3	0.2 ± 0.1	0.323
LDH (IU/l)	228.2 ± 24.9	208.2 ± 22.0	0.570
ANA	304.0 ± 96.0	546.7 ± 375.5	0.453
Scl-70	15.8 ± 12.1	1.3 ± 1.3	0.405
KL-6 (U/ml)	1459.4 ± 197.4	561.3 ± 257.5	0.028
SP-D (ng/ml)	215.7 ± 62.9	135.0 ± 85.4	0.220
SP-A (ng/ml)	93.9 ± 36.7	58.2 ± 21.6	0.512
%VC (%)	73.1 ± 4.7	96.2 ± 13.1	0.111
$\%FEV_{1.0}$ (%)	81.2 ± 1.3	82.7 ± 1.1	0.448
%DLco (%)	56.9 ± 8.4	67.8 ± 12.0	0.473

Results were shown as mean ± SEM. SSc-fib+, systemic sclerosis patients with pulmonary fibrosis; SSc-fib-, systemic sclerosis patients without pulmonary fibrosis; ANA, anti nuclear antibody; Scl-70, anti scleroderma antibody; KL-6, sialylated carbohydrate antigen; SP-A, pulmonary surfactant protein-A; SP-D, pulmonary surfactant protein-D; %VC, percentage of volume capacity; $\%FEV_{1.0}$, percentage of forced expiratory volume in one second; %DLco, percentage of diffusing capacity for carbon monoxide.

and tended to be decreased in vital capacity and diffusing capacity for carbon monoxide compared to SSc-fib- patients. This tendency of a decrease was associated with abnormal chest radiograph and the presence of significant fibrosis on the HRCT. SSc-fib- patients revealed no evidence of lung involvement neither on the lung function tests nor by radiological findings. The mean recovery rates of BALF in SSc-fib+ and SSc-fib- patients were 62.7% (range; 59.4% to 64.2%) and 68.5% (range; 63.3% to 70.9%), respectively. In BALF profiles, no significant difference was observed in total BAL cell counts, the percentage of macrophages, neutrophils, eosinophils and lymphocytes between two groups were shown in Table 2. The ratio of $CD4^+$ to $CD8^+$ ratio was significantly higher in SSc-fib- patients than SSc-fib+ patients.

We analyzed the whole protein profiles of pooled BALF from SSc-fib+ and SSc-fib- by two-dimensional gel electrophoresis to evaluate the difference of proteins between two groups. Total numbers of spots on gels of pooled BALF from SSc-fib+ and SSc-fib- was 526 spots and 427 spots, respectively. Then we identified 41 species of proteins using MALDI-TOF MS and/or gel matching (GM) with 2-D gel electrophoresis database from Swiss-PROT (Fig. 1a, Table 3). In pooled BALF samples of SSc-fib+, we found 5 proteins including α2 macroglobulin, α2 antiplasmin, α1 antitrypsin, immunoglobulin heavy chain γ, and pulmonary surfactant protein A that were one and a half times as much as that of the corresponding proteins in SSc-fib- (Table 4). In comparison of each BALF sample of the individual patients, α1 antitrypsin, pulmonary surfactant protein A, and α2 macroglobulin were significantly increased in SSc-fib+ (Table 5).

Table 2 Bronchoalveolar lavage fluid profiles

	SSc-fib+	SSc-fib-	*p* value
number of patients	5	4	-
total cell counts ($\times 10^6$)	26.0 ± 6.0	28.0 ± 14.0	0.906
macrophages (%)	63.7 ± 4.1	67.7 ± 7.8	0.688
neutrophils (%)	13.1 ± 5.1	3.3 ± 1.4	0.075
eosinophils (%)	5.0 ± 1.0	1.4 ± 0.4	0.057
lymphocytes (%)	18.1 ± 7.4	27.6 ± 8.1	0.415
$CD4^+/CD8^+$ ratio	1.4 ± 0.4	4.6 ± 1.5	0.046

Results were shown as mean ± SEM. SSc-fib+, systemic sclerosis patients with pulmonary fibrosis; SSc-fib-, systemic sclerosis patients without pulmonary fibrosis.

In pooled BALF samples of SSc-fib+, we found 9 proteins including immunoglobulin heavy chain α, liver type fatty acid protein (L-FABP), immunoglobulin J chain α1 antichymotrypsin, haptoglobulin heavy chain α, α2 heat shock protein (HSP), hemopexin, and glutathione S-transferase (GST) that were lower by two-thirds than that of the corresponding proteins in SSc-fib- (Table 6). In comparison of each BALF sample of the individual patients α2 HS glycoprotein and glutathione S-transferase were significantly increased in SSc-fib- (Table 7).

DISCUSSION

We identified 5 proteins whose quantity was different between SSc-fib- and SSc-fib+. In the recent study, Fietta *et al*. reported that α1-acid glycoprotein, albumin, cytohesin-2, mitochondrial DNA topoisomerase 1, Cu-Zn superoxide dismutase, calgranulin B, haptoglobin-α, GST and cystein SN were significantly different between SSc-fib- and SSc-fib+.[7] Those proteins were picked up surveying the protein from 16.3 kDa to 69.8 kDa of low molecular weight. By contrast, we identified 5 species of proteins surveying the wider range of 10.4 kDa to 207.4 kDa that were significantly different between SSc-fib- and SSc-fib+ by MALDI-TOF MS and GM, with a reference of database from Swiss-PROT. Comparison of BALF protein expression in both the Fietta's and the present study was demonstrated in Table 8.

In patients' characteristics, SSc-fib+ patients showed an increase of the level of KL-6 and tended to be decreased in vital capacity and diffusing capacity, which may relate to the lung fibrosis. In BALF profiles, there was no significant difference in total BAL cell number, the numbers of macrophages, and lymphocytes between two groups. The number of neutrophils and eosinophils were tended to be higher in

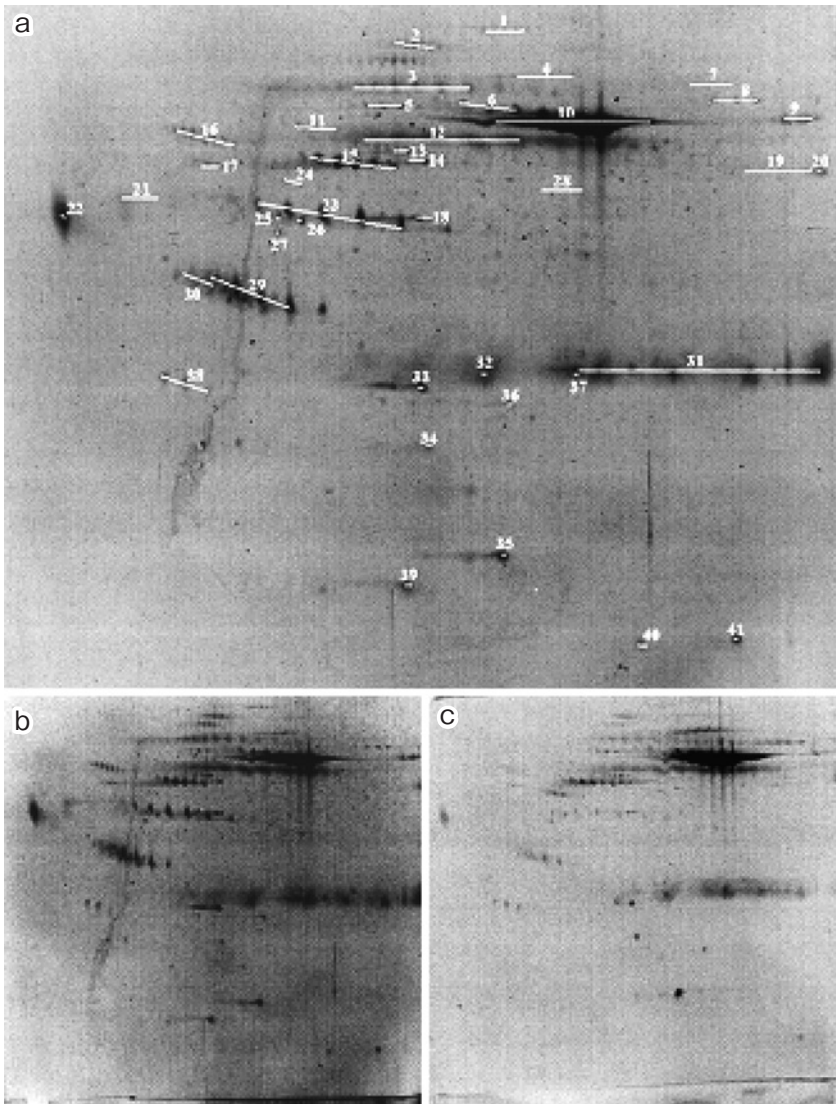

Fig. 1 2-DE of BALF from SSc-fib- patient (**a**), performed using a nonlinear immunobiline gradient 4-7, IPG strip followed by 9-16% SDS PAGE. Proteins were detected by SYPRO-Ruby. Numbers indicate proteins and are listed in Table 3. The 2-DE patterns of BALF from SSc-fib- patient (**b**) and SSc-fib+ (**c**).

SSc-fib+ patients almost in line with the Fietta's study. Whereas, the number of lymphocytes showed no difference between two groups although Fietta *et al*. found a difference.[7] The ratio of $CD4^+$ to $CD8^+$ in SSc-fib- was higher than that in SSc-fib+. It could be due to increased levels of $CD4^+$ or decreased levels of $CD8^+$ cells. Wallace *et al*. suggest an inhibitory effect of $CD4^+$ T cells on fibroblast function,[9] Whereas, Murayama *et al*. suggest a possible role of fibrogensis in the lungs by $CD4^+$ T cells.[10] These lines indicate that the role of the $CD4^+$ T cells might be controversial. For $CD8^+$ T cells, Rottoli *et al*. showed $CD8^+$ lymphocytes in SSc-fib+ could promote pulmonary fibrosis.[11]

In comparison of each BALF samples, we identified 3 species of proteins whose quantity of SSc-fib+ is higher by two-thirds than that of SSc-fib- including α1-antitrypsin, α2-macroglobulin, and pulmonary surfactant protein A (SP-A). Those proteins were thought to relate to the main mechanism driving alveolitis in SSc patients.[2] According to the previous report, an accumulation of many plasma proteins was observed in BALF from SSc-fib+. In this study, we found an increase in α1-antitrypsin and α2-macroglobulin in BALF from SSc-fib+. Concerning these proteins, the levels of α1-antitrypsin and α2-macroglobulin in BALF of sarcoidosis were higher than those of IPF patients.[5] And the level of α1-antitrypsin in BALF from SSc fib+ was higher than that of IPF patients.[5,12,13] The increase in these proteins in BALF may result from the activation of im-

Table 3 BALF protein porfiles identified in SSc-fib+ patients and SSc-fib- patients

Protein No.	Protein	AC	Identification method
1	α2 macroglobulin	P-01023	GM
2	ceruloplasmin	P-00450	GM, MS
3	immunoglobulin A S-chain	P-99003	GM, MS
4	complement factor B	P-00751	GM
5	α1 B-glycoprotein	P-04217	GM, MS
6	hemopexin	P-02790	GM, MS
7	complement factor B	P-00751	GM
8	transferin	P-02787	GM, MS
9	complement C3 β	P-01024	GM
10	albumin	P-02768	GM, MS
11	α2 antiplasmin	P-08697	GM, MS
12	immunoglobulin heavy chain α	P-99002	GM
13	antithrombin III	P-01008	GM, MS
14	vitamin D-binding protein	P-02774	GM, MS
15	α1 antitrypsin	P-01009	GM, MS
16	α1 antichymotrypsin	P-01011	GM, MS
17	α2 heat shock protein	P-02765	GM, MS
18	fibrinogen γ, α chain	P-02679	GM
19	immunoglobulin heavy chain γ	P-99006	GM
20	undetermined		
21	Toll-like receptor 1	P-38593	MS
22	orosomucoid 1	P-02763	GM
23	haptoglobin β	P-00737	GM
24	Zinc finger protein GLIS1		MS
25	Zinc α-2-glycoprotein		MS
26	transmembrane protein		MS
27	clusterin	P-10909	GM
28	immunoglobulin heavy chain μ	P-99009	MS
29	pulmonary surfactant protein A	P-07714	MS, GM
30	undetermined		
31	immunoglobulin heavy chain κ, γ	P-99007	GM
32	glutathione S-transferase	P-09211	MS
33	apolipoprotein A-1	P-02647	GM
34	serum retinol binding protein	P-02753	GM
35	transthyretin	P-02766	GM, MS
36	cytidylate kinase	P-30049	GM
37	albumin fragment	P-02768	GM, MS
38	immunoglobulin J chain	P-01591	GM
39	liver type fatty acid binding protein	P-07148	GM
40	hemoglobulin β chain	P-02023	GM
41	cystain SN	P-01037	GM

Protein No.: Refer to the annotations in Fig. 1. BALF, bronchoalveolar lavage fluid; SSc-fib+, systemic sclerosis patients with pulmonary fibrosis; SSc-fib-, systemic sclerosis patients without pulmonary fibrosis; AC, accession number from the SWISS-PROT database; MS, MALDI-TOF MS; GM, Gel matching with two dimensional gel electrophoresis database from Swiss-PROT.

Table 4 Comparison of pooled BALF samples between SSc-fib+ patients and SSc-fib- patients (SSc-fib+/SSc-fib->1.5)

Protein	MW (kDa)	pooled SSc-fib+/ pooled SSc-fib-
α2 macroglobulin	207.4	1.64
α2 antiplasmin	65.6	2.77
immnunoglobulin heavy chain γ	51.0	4.06
α1 antitrypsin	46.9	1.83
pulmonary surfactant protein A	26.7	2.03

SSc-fib+, systemic sclerosis patients with pulmonary fibrosis; SSc-fib-, systemic sclerosis patients without pulmonary fibrosis; pooled SSc-fib+, pooled samples of BALF from 5 SSc-fib+ patients; pooled SSc-fib-, pooled samples of BALF from 4 SSc-fib+ patients.

Table 5 Comparison of each BALF sample of the individuals between SSc-fib+ patients (n = 5) and SSc-fib- patients (n = 4)

Protein	MW (kDa)	Spot quantity values of SSc-fib+	Spot quantity values of SSc-fib-	p value
α2 macroglobulin	207.4	6.3 ± 1.6	1.2 ± 1.0	<0.05
α2 antiplasmin	65.6	17.5 ± 11.7	5.6 ± 1.9	0.29
immnunoglobulin heavy chain γ	51.0	16.7 ± 7.4	4.9 ± 2.4	0.13
α1 antitrypsin	46.9	18.2 ± 6.1	4.1 ± 1.8	<0.05
pulmonary surfactant protein A	26.7	25.5 ± 6.6	5.9 ± 3.1	<0.05

Results were shown as mean ± SEM. SSc-fib+, systemic sclerosis patients with pulmonary fibrosis; SSc-fib-, systemic sclerosis patients without pulmonary fibrosis.

mune and inflammatory cells within the lung. These cells may damage the pulmonary tissue, in particular the alveolar-capillary barrier, leading to an impairment of its barrier function followed by increased permeability to proteins.

In this study, we found an increase of SP-A in BALF of SSc-ILD+. The increase of SP-A in BALF may result from enhanced synthesis and/or release by secreting cells or from impaired clearance by alveolar macrophages, mucociliary transport, degradation, and absorption into the bloodstream.[1] SP-A is produced by several cell types, including the type II alveolar epithelial cells in the lung, and secreted into the lung alveolar space. SP-A plays a role in inflammation by regulating cytokines, such as tumor necrosis factor (TNF-α) and interleukin 8 (IL-8) which are produced by macrophages. Modification of surfactant proteins was recently analyzed by high-solution Fourier transform ion cyclotron resonance mass spectrometry (MALDI-FTICR) MS in BALF from patients with cystic fibrosis.[14,15] SP-A has important

Table 6 Comparison of pooled BALF samples between SSc-fib+ patients and SSc-fib- patients (SSc-fib+/SSc-fib-<0.67)

Protein	MW (kDa)	Pooled SSc-fib+/ pooled SSc-fib-
ceruroplasmin	123.0	0.31
immunoglobulin heavy chain α	62.0	0.23
α1 antichymotrypsin	56.7	0.23
hemopexin	52.4	0.30
haptoglobulin β	40.3	0.28
glutathione S-transferase	25.8	0.27
immunoglobulin J chain	23.9	0.26
α2 heat shock protein	13.6	0.00
liver type fatty acid binding protein	10.0	0.10

SSc-fib+, systemic sclerosis patients with pulmonary fibrosis; SSc-fib-, systemic sclerosis patients without pulmonary fibrosis; pooled SSc-fib+, pooled samples of BALF from 5 SSc-fib+ patients; pooled SSc-fib-, pooled samples of BALF from 4 SSc-fib+ patients.

Table 7 Comparison of each BALF sample of the individuals between SSc-fib+ patients (n = 5) and SSc-fib- patients (n = 4)

Protein	MW (kDa)	Spot quantity values of SSc-fib+	Spot quantity values of SSc-fib-	p value
ceruroplasmin	123.0	2.4 ± 2.0	7.7 ± 3.8	0.16
immunoglobulin heavy chain α	62.0	9.2 ± 2.8	26.0 ± 8.5	0.43
α1 antichymotrypsin	56.7	4.9 ± 2.0	10.2 ± 7.7	0.43
hemopexin	52.4	10.7 ± 6.7	26.2 ± 14.7	0.31
haptoglobulin β	40.3	18.7 ± 12.7	48.8 ± 23.2	0.25
glutathione S-transferase	25.8	6.0 ± 4.5	64.6 ± 22.3	<0.05
Immunoglobulin J chain	23.9	3.5 ± 2.9	9.8 ± 8.6	0.43
α2 heat shock protein	13.6	0.0 ± 0.0	6.2 ± 3.6	<0.05
liver type fatty acid protein	10.0	2.4 ± 2.0	7.7 ± 3.8	0.24

Results were shown as mean ± SEM. SSc-fib+, systemic sclerosis patients with pulmonary fibrosis; SSc-fib-, systemic sclerosis patients without pulmonary fibrosis.

functions for the immunological surveillance of the lung and has been shown to undergo several structural modifications of pathophysiological relevance in lung fibrosis.[16,17] In addition, SP-A was used as the disease marker of the lung fibrosis.

We identified 2 proteins whose quantity of SSc-fib+ is lower by two-thirds than that of SSc-fib- including α-2 HSP and glutathione S-transferase (GST). HSP is induced by the various stresses and is involved in the synthesis, conveyance and disassembly of the protein inside the cell as a molecular chaperon.[18] The cytokines, TNF-α, IL-6 and IL-8 are induced by HSP. In our study, the level of HSP was lower in BALF of SSc-fib+ implicating the impaired protection against the lung injury.

Table 8 Comparison of BALF proteomics between the Fietta's and present study

Protein	MW kDa	Fietta *et al.*	Present study
α1-acid glycoprotein	23.5	↑	N/A
albumin	69.4	↑	-
cytohesin-2	46.5	↑	N/A
mitochondrial DNA topoisomerase 1	69.8	↑	N/A
Cu-Zn superoxide dismutase	15.8	↓	N/A
calgranulin B	13.2	↓	N/A
haptoglobin-α	15.9	↓	-
cystein SN	16.3	↓	N/A
α2 macroglobulin	207.4	N/A	↑
α1 antitrypsin	46.9	N/A	↑
pulmonary surfactant protein A	26.7	N/A	↑
glutathione S-transferase	25.8	↓	↓
α2 heat shock protein	13.6	N/A	↓

↑, increased in BALF of SSc-fib+; ↓, decreased in BALF of SSc-fib+; -, unchanged between SSc+ and SSc-fib-; N/A, not available.

Recently, Fietta *et al*. reported that the levels of GST and S-O-D (SOD) in BALF of SSc-fib- patients are higher than those of SSc-fib+ patients.[6] GST is an anti-oxidation to detoxify toxic hydroxynonenal induced by the lipid peroxidizing reaction. GST is known to be protective against the injury. In our study, the lower level of GST of SSc-fib+ is in line with these findings suggesting that GST has the key protective role against oxidant-induced damage and fibroproliferation. Oxidative damage to proteins may be the direct result of reactive oxygen/nitrogen species or an indirect effect of secondary products of oxidative stress.[19] Formation of carbonyl groups is the most widely studied as oxidative stress-induced modification of proteins and regarded as a biomarker of oxidative stress and is used to quantify oxidative damage to polypeptide chains.[20,21] Magi *et al*. showed that carbonylated proteins, hemopexin, GST and ceruloplasmin were higher in BALF of patients with IPF than in BALF of SSc-ILD, sarcoidosis and healthy controls.[10] The oxidative stress may be important in the pathogenesis of lung damage and in the development of lung fibrosis.[22] GST and superoxide dismutase (SODs) may have the key protective role in the lung.

There are several limitations in this study. First, it is not clear whether the protein profiles in this study are specific for ILD in SSc or universal in most fibrotic lung disease. We should conduct BALF proteomics in other diseases such as idiopathic interstitial pneumonias, rheumatoid arthritis, and chronic

hypersensitivity pneumonitis in the next study. Second, it is not clear that the alternation of protein expressions is systematic or local event in the lungs. Since we have no comparative data between peripheral blood and BALF, this problem could not be concluded in this paper. Finally, minute fibrotic cases might be included in SSc-fib- group, because high resolution computed tomography (HRCT) was not applied in this study. In the diagnosis of SSc-fib+, we performed not only chest radiography but also pulmonary function tests including diffusing capacity. Therefore, most cases of lung fibrosis should be correctly evaluated.

In conclusion, we identified several interesting proteins that might have roles in ILD of SSc patients. Further studies are warranted to clarify the role of proteins in the processing of pulmonary fibrosis in SSc.

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