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ORIGINAL ARTICLE

Proteome Analysis of Bronchoalveolar Lavage Fluid in Chronic Hypersensitivity Pneumonitis

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ABSTRACT

Background: Hypersensitivity pneumonitis (HP) is an immune-mediated lung disease induced by inhalation of numerous antigens. Pathologically, chronic HP tends to show usual interstitial pneumonia (UIP) and fibrotic nonspecific interstitial pneumonia (fNSIP) patterns. Patients with UIP pattern present insidious onset and a risk for acute exacerbations.

Methods: To evaluate the proteomic differences of bronchoalveolar lavage fluid (BALF) between UIP and fNSIP patterns, BALF from seven patients with UIP pattern and four patients with fNSIP pattern was examined using two-dimensional gel electrophoresis and mass spectrometry.

Results: By individually comparing each BALF sample, we found that the protein levels of surfactant protein A (SP-A), immunoglobulin heavy chain α, α-2 heat shock glycoprotein, haptoglobin β, and immunoglobulin J chain were significantly higher in the patients with UIP pattern than those in the patients with fNSIP pattern. In contrast, the protein levels of glutathione s-transferase, vitamin D-binding protein, and β-actin were significantly higher in the patients with fNSIP pattern than those in the patients with UIP pattern. To confirm the results of SP-A in the BALF proteome, we performed enzyme-linked immunosorbent assay in a larger group. The concentrations of SP-A in BALF from the patients with UIP pattern were significantly higher than those from the patients with fNSIP pattern (2.331 ± 1.656 μg/ml vs. 1.319 ± 1.916 μg/ml, $p = 0.034$).

Conclusions: We identified several proteins that may play roles in the development of pathological differences between UIP and fNSIP patterns of chronic HP.

KEY WORDS

bronchoalveolar lavage fluid, chronic hypersensitivity pneumonitis, mass spectrometry, proteomics, surfactant protein A

INTRODUCTION

Hypersensitivity pneumonitis (HP) is an immune-mediated lung disease induced by inhalation of antigens present in a wide variety of organic dusts. HP is classified into acute and chronic forms based on its clinical presentation.[1,2] Moreover, chronic HP is subgrouped into recurrent and insidious types.[3] The recurrent type is the subgroup of chronic HP patients presenting recurrent acute episodes, including low-grade fever. The insidious type is the subgroup of chronic HP patients with a slowly progressive course without acute episodes. Chronic HP is induced by persistent exposure to a small quantity of antigen. Patients with chronic HP often develop pulmonary fibrosis with the destruction of lung parenchyma, and the prognosis is poor. This clinical course is similar to that of idiopathic pulmonary fibrosis (IPF). However, the pathogenesis of fibrogenesis in chronic HP remains unclear.

In chronic HP, surgical lung specimens exhibit various histological patterns, including UIP pattern, fNSIP pattern, cellular nonspecific interstitial pneumonia (cNSIP) pattern, and organizing pneumonia (OP) pattern,[4] as observed in IIPs.[5] We have showed five clinical differences between UIP and fNSIP patterns in the previous reports.[3,4] First of all, all cases with UIP pattern were insidious type of chronic HP

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and half cases with fNSIP pattern were recurrent type.[6] Second, the percentage of lymphocytes in BALF with UIP pattern tended to be lower than that with fNSIP pattern (p = 0.052).[4] Third, in histology, giant cells or granulomas were observed in lung specimens from all of the fNSIP cases, whereas no granuloma was shown in the UIP cases. Fourth, the prognosis of fNSIP pattern tended to be better than that of UIP pattern (p = 0.065, median survival; 29 months vs. 63 months). Finally, the patients with UIP pattern at the time of diagnosis may be at higher risk for acute exacerbations (AE) than those with fNSIP or cNSIP patterns.[7] These lines of evidence suggest that comparative BALF analysis might provide some information of the pathogenesis in UIP and fNSIP patterns.

Proteomic analysis of human body fluids has become an important approach for the discovery of biomarkers. The analysis of the BALF proteome can potentially provide important information of differences or changes in protein expression and secretion during the course of pulmonary disorders. Previous studies have shown that the protein composition of BALF is altered in sarcoidosis,[8] IPF,[9] pulmonary alveolar proteinosis,[10,11] cystic fibrosis,[12] chronic obstructive pulmonary disease,[13] systemic sclerosis with pulmonary fibrosis,[14] and HP.[8] BALF has been widely used to collect cells and other soluble components from epithelial lining fluids that cover the airway and the alveoli.[15] BALF contains proteins secreted from various cell types, including epithelial and inflammatory cells as well as a wide variety of proteins from the bloodstream. Therefore, the analysis of BALF may reveal important biological activities related to inflammation, oxidation-reduction, tissue matrix turnover, and immunity.

The aim of our study was to investigate the differences in the BALF proteome of patients with UIP and fNSIP patterns of chronic HP.

METHODS

PATIENT SELECTION

BALF samples were obtained from patients with chronic HP who underwent surgical lung biopsies in our hospital between 1998 and 2008. The diagnosis of chronic HP was based on clinical, radiological, and histological criteria, as described previously.[4] Out of 11 chronic HP patients, seven patients with UIP pattern and four patients with fNSIP pattern were enrolled in this study. Patients with UIP and fNSIP patterns were compared with smoking habits, laboratory test, including serum levels of C-reactive protein (CRP), LDH, Krebs von den Lungen 6 (KL-6), and surfactant protein D (SP-D), and pulmonary function test including predicted vital capacity percentage (%VC), percentage of forced expiratory volume in one second (%$FEV_{1.0}$), percentage of diffusing capacity for carbon monoxide (%DLco), and percentage of diffusing capacity for carbon monoxide corrected for alveolar volume (%DLco/VA). Patients were provided informed consent for bronchoscopy, and none of them were treated with prednisolone or other immunosuppressive agents at the time of enrollment. The study conformed to the Declaration of Helsinki and was approved by the internal review boards of our institution.

BRONCHOALVEOLAR LAVAGE (BAL)

We performed BAL using three 50-ml aliquots of sterile 0.9% saline, as previously described.[16] The cellular composition of the BALF was determined by counting 200 cells in a cytospun smear with Wright's stain. The analysis of lymphocyte phenotypes was performed by flow cytometry, using monoclonal antibodies for CD4 and CD8.

PATHOLOGICAL ASSESSMENT

Histological patterns were evaluated by two pulmonary pathology specialists (T.T. and T.A.) without the knowledge of the patient's clinical course. The patients were subgrouped into UIP, fNSIP, and cNSIP/OP patterns according to the international classification of idiopathic interstitial pneumonias proposed by the joint ATS/ERS statement in 2002.[4] When the opinions differed between the two pathologists, the final decision was reached by consensus.

TWO-DIMENSIONAL GEL ELECTROPHORESIS

Two-dimensional gel electrophoresis (2-DE) was performed as previously described.[17] Briefly, 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, Uppsala, Sweden) concentrated by acetone and swollen in 8 M urea, 4% CHAPS, 65 mM dithioerythritol, 0.1 M acetic acid, pharmalyte (pH 3 to 10 for isoelectrofocusing; GE healthcare), and a trace of bromophenol blue. Rehydration was performed in the strip holder of the IPG-IEF CoolPhoreStar system (Anatech, Tokyo, Japan) at 20℃ and isoelectrofocusing was terminated at 47 kV. The IPG strips were equilibrated first in the urea/SDS/Tris buffer for 30 minutes 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 gels. The gels were stained with SYPRO Ruby Protein Gel Stain (Molecular Probes, Carlsbad, CA, USA).

EVALUATION AND IDENTIFICATION OF PROTEINS

The gels were scanned using FluoroPhoreStar 3000 (Anatech), analyzed using Progenesis PG220 Soft-

Table 1 Patients characteristics

	UIP	fNSIP	*p* value
Number	7	4	-
Gender (M/F)	7/0	3/1	-
Age (yr)	58.9 ± 7.5	57.5 ± 11.3	0.925
Smoking history (pack-year)	40.2 ± 24.7	45.1 ± 37.6	0.927
Serum CRP (mg/dl)	0.2 ± 0.2	0.7 ± 0.8	0.774
Serum LDH (IU/l)	265.1 ± 69.1	262.0 ± 70.7	0.527
Serum KL-6 (U/ml)	2249.6 ± 1147.4	1062.0 ± 508.6	0.109
Serum SP-D (ng/ml)	293.3 ± 249.9	192.8 ± 171.7	0.527
%VC (%)	85.2 ± 22.8	86.8 ± 12.0	0.927
$\%FEV_{1.0}$ (%)	77.3 ± 17.5	78.0 ± 8.9	0.649
%DLco (%)	57.7 ± 12.5	54.8 ± 7.1	0.788
%DLco/VA (%)	77.5 ± 18.4	65.6 ± 28.7	0.412

Results were shown as number or mean ± SD.

Abbreviations: UIP, usual interstitial pneumonia; fNSIP, fibrotic nonspecific interstitial pneumonia; KL-6, Krebs von den Lungen 6; 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; %DLco/VA, percentage of diffusing capacity for carbon monoxide corrected for alveolar volume.

ware (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK), and automatically analyzed using the spot detection feature of the software, with automatic warping and matching. Spot volumes were corrected for background using the "mode of non-spot" method and were normalized. A normalization spot volume was an area and a density achieved using spot volumes multiplied by total area, when the total spot volume was defined as 100. The normalized spot volumes were used for comparison among different clinical groups. Proteins were identified by comparing the BALF maps, such as the SWISS-2D PAGE human plasma map and published BALF maps,[14,18] or by liquid chromatography nano electron spray ionization tandem mass spectrometry (LC-nESI-MS/MS) (QTRAP 5500 LC/MS/MS System, AB SCIEX, Concord, ON, Canada). The protein spots of interest picked up from the gels were analyzed by LC-nESI-MS/MS in the Laboratory of Cytometry and Proteome Research, Tokyo Medical and Dental University. LC separation was performed on a HiQ sil C18W-3P column (0.1 mmΦ × 100 mm, KYA TECH Corporation, Tokyo, Japan). Elution was performed at a flow rate of 300 nL/min, using water containing 0.1% (v/v) formic acid as the eluent A and acetonitrile containing 0.1% (v/v) formic acid as the eluent B with a linear gradient from 5% B to 45% B in 70 minutes. Data analysis was performed using ProteinPilot™ software (Version 3, Applied Biosystems, Warrington, UK).

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Since surfactant protein A (SP-A) and glutathione s-transferase pi (GST-pi) could be evaluated by ELISA, we measured serum and BALF SP-A and GST-pi in a larger group to confirm the result of SP-A in the BALF proteome. Using an ELISA kit (BioVendor-Laboratorni medicina a.s., Brno, Czech Republic), we measured the SP-A concentration of serum in 42 patients, including 25 patients with UIP pattern and 17 patients with fNSIP pattern, and that of BALF (diluted to 1 : 200) in 24 patients, including 15 patients with UIP pattern and 9 patients with fNSIP pattern. The sensitivity of this assay was to detect SP-A from 1 to 50 ng/ml. We measured the GST-pi concentration of serum and BALF in the same population using an ELISA kit (Immundiagnostik AG, Bensheim, Germany). The sensitivity of this assay was to detect GST-pi from 9.4 to 600 ng/ml.

STATISTICAL METHODS

Data were analyzed using Prism 5 (GraphPad Software, Inc., San Diego, CA, USA) and were described as the mean ± standard deviation (SD). The two groups were compared using the Mann-Whitney U test. Correlation coefficient was obtained using the Spearman's correlation coefficient test. All statistical comparisons were two-sided, and p values < 0.05 were considered statistically significant.

RESULTS

Proteome analysis of individual BALF samples was performed on 11 patients with chronic HP, including seven with UIP pattern and four with fNSIP pattern. Patient characteristics and BALF findings are shown in Table 1, 2. All of the patients with UIP pattern were males, and the patients with fNSIP pattern included three males and one female. All of the patients were ex-smokers. The levels of KL-6 and SP-D were ele-

Table 2 Bronchoalveolar lavage fluid analysis

	UIP	fNSIP	*p* value
Number	7	4	-
Recovery rate (%)	61.2 ± 16.8	50.4 ± 10.8	0.394
Total cell count (×10^5/ml)	3.53 ± 1.99	3.37 ± 1.89	1.000
Macrophage (%)	80.0 ± 10.3	71.0 ± 19.7	0.649
Neutrophils (%)	4.2 ± 6.9	4.5 ± 4.6	0.788
Eosinophils (%)	0.7 ± 0.5	5.2 ± 4.1	0.010
Lymphocytes (%)	15.2 ± 9.1	19.4 ± 18.8	0.924
$CD4^+/CD8^+$ ratio	3.4 ± 2.7	1.9 ± 0.8	0.256
Protein concentration (μg/ml)	261.7 ± 70.0	249.8 ± 59.8	0.927

Results were shown as mean ± SD.

Abbreviations: UIP, usual interstitial pneumonia; fNSIP, fibrotic nonspecific interstitial pneumonia.

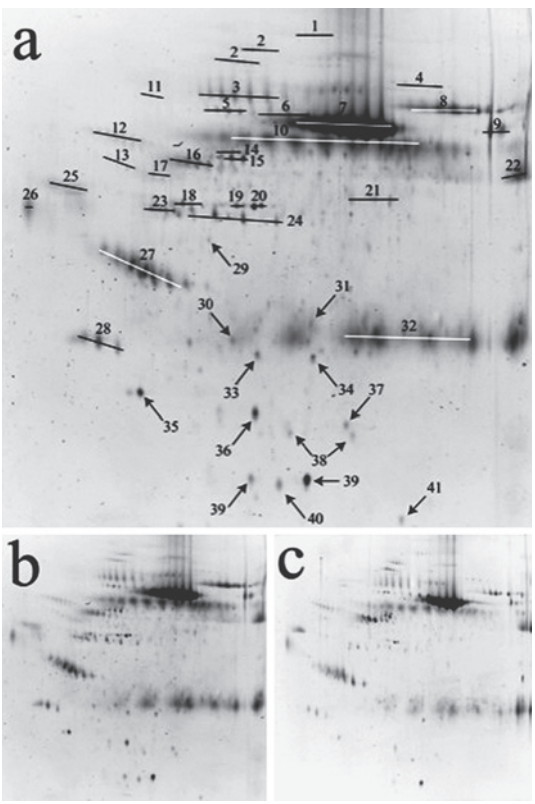

Fig. 1 Two-Dimensional Electrophoresis (2-DE) of BALF from a chronic HP patient with UIP pattern (**a**), performed using a nonlinear immunobiline gradient 4-7, IPG strip followed by 10% SDS PAGE. Proteins were detected by SYPRO-Ruby. Numbers indicate proteins and are listed in Table 3. Representative 2-DE patterns of BALF from UIP pattern (**b**) and fNSIP pattern (**c**).

vated, and the diffusing capacity of the patients, shown by the pulmonary function test, was reduced in both groups. The KL-6 level in the patients with UIP pattern tended to be higher than that in those with fNSIP pattern. In their BALF profiles, the percentage of eosinophils was significantly higher in the patients with fNSIP pattern than that in those with UIP pattern. The percentages of lymphocytes were elevated in both groups, and no difference was observed between the two groups. Other clinical characteristics were similar between the two groups. No microbiological or clinical evidence of bacterial or fungal infection was found in any patient.

We analyzed the BALF proteome in the patients with UIP and fNSIP patterns by 2-DE to evaluate the difference in protein expression between the two groups. The 2-DE patterns of BALF are shown in Figure 1. The total number of spots was significantly higher in BALF from the patients with UIP pattern than that from those with fNSIP pattern (550 ± 109.3 spots vs. 410 ± 69 spots, $p = 0.042$; Fig. 1b, c). We identified 41 species of proteins using LC-nESI-MS/MS and/or gel matching (GM) with the 2-DE database from Swiss-PROT (Fig. 1b, c; Table 3). By individually comparing the BALF samples, we found that the levels of SP-A, immunoglobulin heavy chain α, 60-kDa α-2 heat shock glycoprotein (HSP60), haptoglobin β, and immunoglobulin J chain were significantly higher in the patients with UIP pattern than those in the patients with fNSIP pattern (Table 4). In contrast, the levels of GST-pi, vitamin D-binding protein (VDBP), and β-actin were significantly higher in the patients with fNSIP pattern than those in the patients with UIP pattern (Table 5). SP-A was shown as nine spots, representing different isoforms, along a downward-sloping line (Fig. 2). These isoforms were likely to be categorized into two types of spots (number 1, 2, 4, 6, 8, and 9 were vertically long, whereas number 3, 5, and 7 were obliquely long). The quantity of number 5 spot in UIP pattern was significantly higher than that in fNSIP pattern, although the other eight spots showed no difference between UIP and fNSIP patterns.

The concentrations of SP-A in serum and BALF were determined by ELISA (Fig. 3). The level of SP-A in BALF from the patients with UIP pattern was significantly higher than that in those with fNSIP pattern (2.331 ± 1.656 μg/ml vs. 1.319 ± 1.916 μg/ml, p =

Table 3 BALF proteins identified in UIP and fNSIP patients

Protein No.	Protein	AC	Identification method
1	α-2 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	α-1 B-glycoprotein	P-04217	GM, MS
6	Hemopexin	P-02790	GM, MS
7	Albumin	P-02768	GM, MS
8	Transferin	P-02787	GM, MS
9	Complement C3β	P-01024	GM
10	Immunoglobulin heavy chain α	P-99002	GM, MS
11	α-2 antiplasmin	P-08697	GM, MS
12	α-1 antichymotrypsin	P-01011	GM, MS
13	α-2-HS-glycoprotein	P-02765	GM, MS
14	Antithrombin III	P-01008	GM, MS
15	Vitamin D-binding protein	P-02774	GM, MS
16	α-1 antitrypsin	P-01009	GM, MS
17	Zinc finger protein GLIS1	P-08151	MS
18	Apolipoprotein A-IV	P-06727	GM
19	β-actin	P-60709	GM, MS
20	Fibrinogen γ, α chain	P-02679	GM
21	Immunoglobulin heavy chain μ	P-99009	GM, MS
22	Immunoglobulin heavy chain γ	P-99006	GM
23	Zinc α-2-glycoprotein	P-25311	GM
24	Haptoglobin β	P-00737	GM, MS
25	Toll-like receptor 1	P-38593	MS
26	Orosomucoid 1	P-02763	GM
27	Pulmonary surfactant protein A	P-07714	GM, MS
28	Immunoglobulin J chain	P-01591	GM, MS
29	Complement factor I	P-05156	GM
30	C-reactive protein	P-02741	GM
31	Proapolipoprotein AI	P-39133	GM
32	Immunoglobulin light chain κ, λ	P-99007	GM
33	Apolipoprotein A-1	P-02647	GM
34	Glutathione S-transferase pi	P-09211	GM, MS
35	Translationally controlled tumor protein	P-13693	GM
36	Serum retinol binding protein	P-02753	GM
37	Haptoglobin α	P-00738	GM
38	Superoxide dismutase (Cu-Zn)	P-00441	GM
39	Transthyretin	P-02766	GM, MS
40	Immunoglobulin binding factor	P-15923	GM
41	Calgranulin B	P-31725	GM

Protein No.: refer to the annotations in Fig. 1.

Abbreviations: AC, accession number from the SWISS-PROT database; MS, LC-nESI-MS/MS; GM, Gel matching with two dimensional gel electrophoresis database from SWISS-PROT.

0.034; Fig. 3b); however, the serum level of SP-A showed no difference between the two groups (94 ± 38 ng/ml vs. 101 ± 53 ng/ml, $p = 0.959$; Fig. 3a). The concentrations of GST-pi in serum and BALF showed no difference between two groups (serum; 37.0 ± 34.2 ng/ml vs. 53.1 ± 61.6 ng/ml, $p = 0.710$, BALF; 17.8 ± 15.4 ng/ml vs. 12.2 ± 9.2 ng/ml, $p = 0.551$; Fig. 4). There was no correlation between the serum level and the BALF level of SP-A ($p = 0.583$, r = -0.124; Fig. 5a). In contrast, there was a significant negative correlation between the SP-A level and the percentage of eosinophils in BALF ($p = 0.0009$, r = -0.631; Fig. 5b), whereas there was no correlation between the SP-A level in BALF and other clinical parameters, includ-

Table 4 Proteins of higher normalization volume in UIP pattern than fNSIP pattern

Protein	MW (kDa)/pI	Normalization volume of UIP ($\times 10^{-3}$)	Normalization volume of fNSIP ($\times 10^{-3}$)	*p* value
60 kDa α2 heat shock glycoprotein	61.1/5.7	79.4 ± 60.1	17.8 ± 14.8	0.029
Haptoglobin β	45.2/6.1	438.6 ± 392.4	90.5 ± 97.2	0.012
Immunoglobulin heavy chain α	37.7/6.1	946.7 ± 502.8	126.5 ± 99.1	0.024
Surfactant protein A (isoforms number 5)	26.2/5.1	1153.3 ± 641.5	339.6 ± 285.5	0.024
Immunoglobulin J chain	18.1/5.1	243.6 ± 197.9	27.1 ± 53.9	0.029

Results were shown as mean ± SD.

Abbreviations: UIP, usual interstitial pneumonia; fNSIP, fibrotic nonspecific interstitial pneumonia.

Table 5 Proteins of higher normalization volume in fNSIP pattern than UIP pattern

Protein	MW (kDa)/pI	Normalization volume of UIP ($\times 10^{-3}$)	Normalization volume of fNSIP ($\times 10^{-3}$)	*p* value
Vitamin D-binding protein	53.0/5.4	146.9 ± 81.8	375.3 ± 172.0	0.029
β-actin	42.0/5.9	36.0 ± 29.5	315.0 ± 194.3	0.024
Glutathione s-transferase pi	23.4/5.4	65.3 ± 29.1	189.6 ± 73.1	0.012

Results were shown as mean ± SD.

Abbreviations: UIP, usual interstitial pneumonia; fNSIP, fibrotic nonspecific interstitial pneumonia.

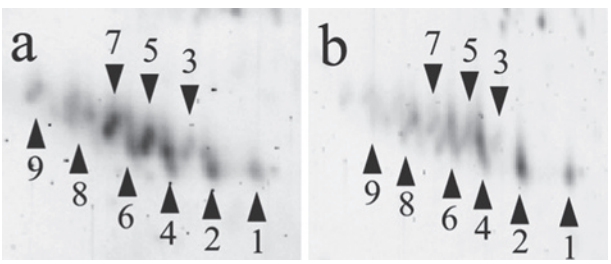

Fig. 2 The SP-A isoforms of UIP (**a**) and fNSIP (**b**) patterns. The normalization volume of number 5 in UIP pattern was significantly higher than that of fNSIP pattern.

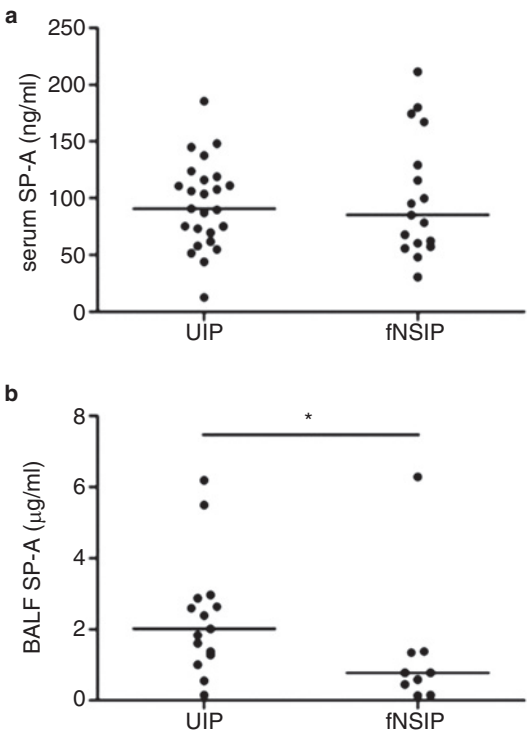

Fig. 3 The concentrations of SP-A in serum (**a**) and BALF (**b**) in comparison between UIP and fNSIP patterns. Bars showed the median values. *$p = 0.034$.

ing serum KL-6, SP-D, LDH, pulmonary function, the percentages of lymphocytes and neutrophils, and the ratio of CD4 to CD8.

DISCUSSION

This study is the first report of the protein expression in BALF from patients with chronic HP, in which different histological patterns were analyzed using a proteomic approach. We identified eight proteins whose expression levels were significantly different between the histological patterns of UIP and fNSIP. Additionally, we found a number of SP-A isoforms differing in molecular weight and pH. Those proteins and isoforms might be associated with the pathogenesis of histological patterns of UIP and fNSIP.

In patient characteristics, there was no difference between UIP and fNSIP patterns. In BALF profiles, the percentage of eosinophils in fNSIP pattern was significantly higher than that in UIP pattern. This result is consistent with our previous study, showing that the group having an episode of AE in chronic HP presented a lower percentage of eosinophils than the group without an episode of AE, and that the pathological type of almost all except one with AE was UIP

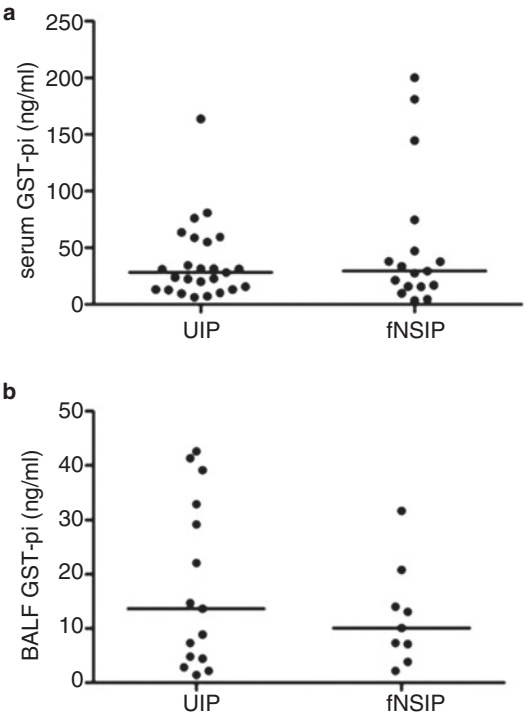

Fig. 4 The concentrations of GST-pi in serum (**a**) and BALF (**b**) in comparison between UIP and fNSIP patterns. Bars showed the median values.

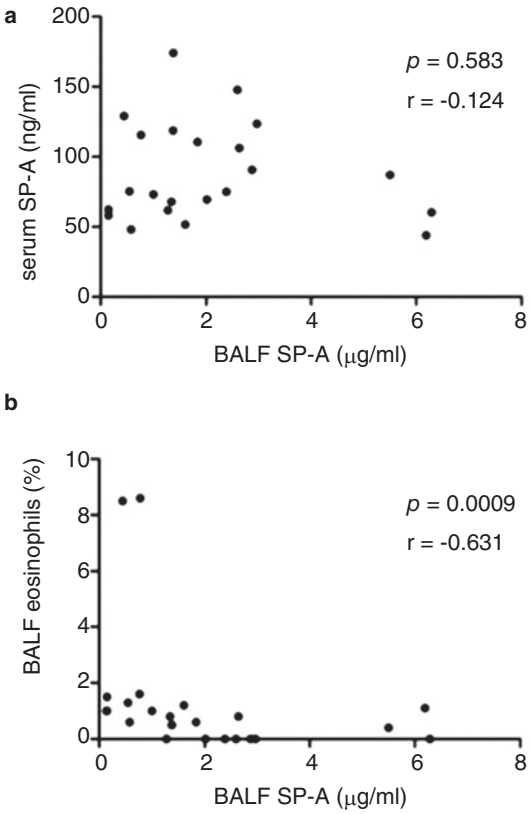

Fig. 5 Correlation relationship of the concentrations between serum SP-A and BALF SP-A (**a**), and correlation relationship between the percentage of eosinophils and the concentrations of SP-A in BALF (**b**).

pattern.[7] However, the relationship between eosinophils and AE was still unknown.

Moreover, we identified five proteins whose normalization volume of UIP pattern was higher than that of fNSIP pattern, including SP-A and HSP60. SP-A is a hydrophilic calcium-dependent lectin, known as collectin, which is primarily produced by the type II alveolar epithelial cells in the lung and is secreted into the lung alveolar space. Surfactant stabilizes the gas-exchanging surface of the alveoli and plays a role in pulmonary host defense by binding, aggregating and opsonizing various microorganisms. SP-A also affects the function of alveolar macrophages, other immune cells, and lung fibroblasts. Some studies suggested that SP-A is associated with the regulation of matrix metalloproteinases, extracellular matrix components, and potentially the repair and remodeling of damaged tissue.[19] However, controversial results on the expression of SP-A in BALF samples of IPF patients have also been reported.[20-22] By comparing the gene expression profile between relatively stable IPF and progressive IPF, a previous study reported that SP-A was upregulated in the progressive group.[23] Similar to their finding, in the present study, we observed elevated protein expression of SP-A in the patients with UIP pattern. Regarding HP, previous studies have shown that the level of SP-A in BALF from patients with HP is elevated, compared to that from healthy volunteers.[24] However, there has been no report regarding the relative concentrations of SP-A in BALF among a variety of histological patterns of HP. In this study, the difference of SP-A between the two groups may result from the synthesis and/or release of SP-A by secreting cells or from impaired clearance by alveolar macrophages, mucociliary transport, degradation, and absorption into the bloodstream. Therefore, we suggest that the synthesis and release of SP-A in UIP pattern may increase or that the clearance of SP-A in UIP pattern may be impaired, compared to that in fNSIP pattern. There was a significant negative correlation between the SP-A level and the percentage of eosinophils in BALF. Madan *et al*. showed that knockout of SP-A gene induced the increase of eosinophils in serum and BALF through IL-5 and TNF-α in murine model.[25] These data might support that SP-A have a role in regulating the eosinophils infiltration and modification in the lung in response to environmental stimuli.

In this study, 2-DE gel electrophoresis of BALF revealed nine spots of SP-A. When the SP-A precursor is processed into mature SP-A, it undergoes several posttranslational modifications, including N-linked

glycosylation, hydroxylation of proline residues, addition of sialic acid to the oligosaccharide, formation of disulfide bonds, and signal peptide cleavage. These posttranslational modifications may be important for some SP-A functions. The heterogeneity of the mass and charge of SP-A isoforms has been studied using 2-DE or mass spectrometry[12,13]; however, its molecular nature remains unclear. Posttranslational modifications of surfactant proteins were previously analyzed by matrix-assisted laser desorption/ionization high-solution Fourier transform ion cyclotron resonance mass spectrometry (MALDI-FT-ICR MS) in BALF from patients with cystic fibrosis.[12] Ohlmeier *et al*. identified nine isoforms of SP-A in lung tissue of chronic obstructive pulmonary disease (COPD).[13] In the present study, we observed nine spots representing different SP-A isoforms and that these spots were categorized into two types of shapes (number 1, 2, 4, 6, 8, and 9 were vertically long, whereas number 3, 5, and 7 were obliquely long; Fig. 2). We considered that the difference in shape may reflect the difference in posttranslational modifications. The quantity of number 5 spot in UIP pattern, shown in Figure 2 as a SP-A isoform, was significantly higher than that in fNSIP pattern, suggesting that the expression of number 5 spot might play an important role in the development of pathologic differences between UIP and fNSIP patterns.

HSP60 with a molecular mass of 60 kDa is mainly expressed in mitochondria. The cellular protective mechanisms of HSPs are related to their chaperone functions, which lead to the prevention of protein denaturation and the promotion of the refolding of damaged proteins caused by various stress, including oxidative stress.[26,27] HSP60 is also an antigen for the adaptive immune system. T cells response to HSP60 epitopes, regulating inflammatory diseases, such as rheumatoid arthritis, insulin-dependent diabetes mellitus, and atherosclerosis.[28,29] Although there have been no data regarding the induction of HSP60 in chronic HP, the high level of BALF HSP60 in UIP pattern suggested that HSP60 might be associated with a defense response against lung inflammation or the impairment of the immune system in chronic HP.

Additionally, we identified three proteins whose normalization volume of UIP pattern was higher than that of fNSIP pattern, including haptoglobin β, immunoglobulin heavy chain α, and immunoglobulin J chain. Haptoglobin β, a part of haptoglobin, is an acute phase protein in inflammation, tissue damage, infection, and cancers. The expression level of haptoglobin β is regulated by several cytokines, including IL-1, IL-6, TNF-α, and TGF-β.[30]

Meanwhile, we identified three proteins whose normalization volume of fNSIP pattern was higher than that of UIP pattern, including VDBP and GST-pi. VDBP belongs to the albumin superfamily of binding proteins that includes albumin, α-albumin, and α-fetoprotein. VDBP is expressed in many tissues, including liver, kidney, gonads, and fat. It is also expressed by neutrophils, contributing to macrophage activation,[31] augmenting monocyte and neutrophil chemotaxis to C5-derived peptides[32] and serving as a scavenger protein to clear extracellular G-actin released from necrotic cells.[33] The low level of VDBP in UIP pattern, which may reflect the impairment of its role as a scavenger protein or of protection against inflammation, might contribute to the high susceptibility to pulmonary fibrosis and progressive clinical course.

Oxidative stress is important for the pathogenesis of lung damage and for the development of lung fibrosis. Among various enzymatic and non-enzymatic mechanisms that protect cells and tissues from oxidants, GSTs and SODs play a key protective role, especially in the lung.[34] The lower level of GST-pi in UIP pattern might induce continuous oxidative stress to the lung. In the present study, we observed a lower level of GST-pi in the patients with UIP pattern, suggesting that the impairment of protection from oxidative stress by GST-pi might play a critical role in the pathogenesis of chronic HP and IPF. Recently, several studies have reported the efficacy of N-acetylcysteine as an antioxidant in the treatment of IPF.[35] Similarly, in chronic HP, GST could be a potential therapeutic target, and N-acetylcysteine might improve the oxidant-antioxidant imbalance in clinical settings. We could not find the difference of GST-pi expression in serum and BALF by ELISA. This was because ELISA and 2-DE might detect different forms of GST-pi differently.

We found that SSc with pulmonary fibrosis showed increased expression of three proteins, including α2-macroglobulin, α1-antitrypsin, and SP-A, and decreased expression of two proteins, including HSP60 and GST-pi.[17] We considered that the changes in SP-A and GST-pi are not disease-specific because the changes of these two proteins were very similar to the results in the present study. In contrast, the alteration of HSP60 showed the opposite tendency between these two studies. In the present study, the high level of HSP60 in BALF might be a consequence of the defense response against lung inflammation or of the impairment of immune system.

There were several limitations of this study. First, it was not clear whether the alternations in protein expression were systemic or local events in the lung. Second, we were not able to investigate hydrophobic proteins by the 2-DE technique. For example, surfactant protein C, a hydrophobic protein critically involved in surfactant homeostasis, was unable to be analyzed by 2-DE. Third, the power to detect statistically differences between UIP and fNSIP pattern is low because this study includes a low numbers of patients.

In conclusion, we identified several proteins that

might play roles in the development of pathologic differences between UIP and fNSIP patterns in chronic HP. Further studies are warranted to clarify the role of these proteins in the development of pulmonary fibrosis in chronic HP.

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