Comprehensive analysis of cytokines in bronchoalveolar lavage fluid of patients with idiopathic pulmonary fibrosis : a cross-sectional study

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a specific form of chronic, progressive fibrosing interstitial pneumonia of unknown cause, with a poor prognosis. The underlying molecular pathogenic mechanisms are not fully understood. The aim of this study was to investigate the relationship between concentrations of various proteins in bronchoalveolar lavage (BAL) fluid (BALF) and clinical parameters in consecutive patients with IPF, including those with preserved lung function. Concentrations of various proteins (SDF-1α, IP-10, IL-1β, IL-4, IL-6, IL-8, IL-13, IFN-γ, TNF-α, EGF, FGF, PDGF, Galectin-3, MMP-1, MMP-7, MMP-8, and TGF-β) in the BALF collected from ten patients with IPF were determined. As a result, BALF concentrations of TNF-α, MMP-1, -7, and -8 showed trends toward positive correlations with forced vital capacity (FVC) % predicted (r= 0.605, 0.527, 0.406, and 0.624, respectively), although statistical significance was not reached. These results suggested that these molecules are elevated more in the BALF of patients with earlier IPF. These proteins may be involved in the initial phase of pathogenesis and may possibly be new therapeutic targets in early IPF.

Key words : bronchoalveolar lavage, IPF, TNF-α, MMP, pulmonary function

Introduction

Idiopathic pulmonary fibrosis (IPF) is a specific form of chronic, progressive fibrosing interstitial pneumonia of unknown cause, with a poor prognosis. The underlying molecular mechanisms through which excessive collagen is deposited in fibrotic lesions are not fully understood, although it is thought that some mediators, such as tumor necrosis factor (TNF)-α, platelet-derived growth factor (PDGF), and transforming growth factor (TGF)-β have important roles in the pathological process.[1-3]

Two antifibrotic agents were developed and became available for the treatment of patients with IPF.[4-6] Nintedanib is an intracellular tyrosine kinase inhibitor that targets multiple tyrosine kinases, including the vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and PDGF receptors. Pirfenidone is an antifibrotic agent whose antifibrotic mechanism is unclear, but is suspected to be a modulator of the effect of TGF-β. These 2 drugs reduce disease progression.[5,6] Initiation of treatment in the early phase of IPF such as with forced vital capacity (FVC), % predicted of $\geqq$80% and/or diffusing capacity for carbon monoxide (DLco), % predicted of $\geqq$70% is important to maintain lung function.

Few studies have been conducted to reveal the mechanisms of disease progression in the early phase of IPF, probably because patients with mild IPF do not have severe symptoms. However, understanding the pathogenesis of the disease in the early phase is now considered very important to identify new drug targets. Therefore, disease-related molecules that were already evaluated in moderate to severe disease should be reevaluated in mild disease.

For this purpose, we measured the concentrations of various proteins in bronchoalveolar lavage (BAL) fluid (BALF), which seemed to directly reflect pathogenesis of lung fibrosis, and evaluated the correlations with clinical parameters in consecutive patients with IPF, including those with preserved lung function. This is a preliminary study to identify disease-related molecules that may have a role in pathogenesis of early-stage IPF.

Methods

Patients

This study was a cross-sectional study. Ten patients with a diagnosis of IPF were prospectively recruited from outpatient settings in our university hospital (Kindai University Hospital, Osakasayama, Osaka, Japan). The diagnosis of IPF was made according to the criteria of the American Thoracic Society (ATS), European Respiratory Society, Japanese Respiratory Society, and Latin American Thoracic Association.[7] Patients with a probable usual interstitial pneumonia (UIP) pattern and traction bronchiectasis were also eligible.[4] Patients were excluded if an infection or acute worsening of IPF had occurred within 3 months or if they had unstable comorbidities. Patients who could not undertake a pulmonary function test, were on any anti-inflammatory or antifibrotic therapy, and were receiving long-term oxygen therapy were also excluded.

Written informed consent was obtained from all patients who participated. The study protocol was approved by the ethics committee of Kindai University, Faculty of Medicine on March 22, 2016 (No. 27-153) and Meiji Seika Pharma Co., Ltd (No. 127-4).

Pulmonary function tests

All patients included in the study underwent pulmonary function tests (CHESTAC-55V ; Chest, Tokyo, Japan), according to the method described in the ATS guidelines.[8] Single-breath DLco was also measured.[9] The values for FVC and DLco were related to the percent predicted values of Japanese.[10,11]

BAL procedure and analysis

BAL was performed via a bronchoscope (BF-1TQ290 or BF-260, Olympus, Tokyo, Japan). Briefly, after a total volume of 200 ml of sterile isotonic saline (50 ml x 4 times) was instilled into the targeted bronchi, BALF were recovered with a low aspiration after each aliquot. Then it was filtered through 2-layer sterile gauze and centrifuged at 500 X g for 10 minutes at room temperature. The supernatant was collected and stored at $-80°C$ for ELISA or Luminex. Cell pellets were resuspended in 10 mL RPMI-1640 for differential cell counts. 0.1 mL of cell suspension was sedimented by cytocentrifugation (Cytospin2, Shandon Instruments, Sewickley, PA, USA) onto glass slides at 20 X g for 3 min. The slides were dried and fixed, and then 500 cells were stained with Diff-Quick for morphology. Then, the number of each type of white blood cell was counted. The concentrations of stromal cell-derived factor (SDF)-1α/CXCL12, IFN-γ inducible protein (IP)-10/CXCL10, IL-1β, IL-4, IL-6, IL-8, IL-13, IFN-γ, TNF-α, epidermal growth factor (EGF), FGF, PDGF, Galectin-3, matrix metalloprotease (MMP)-1, MMP-7, and MMP-8 in BALF were quantified with multiplex analysis using Luminex xMAP technology (Luminex Corporation) in a 96-well microplate format according to the manufacturer's protocols (Invitrogen). The concentration of TGF-β in BALF was quantified with a sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according to the manufacturer's protocols.

Clinical data

Clinical data including Krebs von den Lungen-6 (KL-6) and arterial blood gas analysis were also obtained.

Statistical Analysis

The correlation between the concentrations of the proteins and clinical parameters was analyzed by the Spearman rank test. Spearman's correlation coefficients (r) were determined to examine the degree of the correlations. Analyses were performed with Microsoft Excel 2010. p values of less than 0.05 were considered to be statistically significant.

Results

Ten consecutive patients with IPF were recruited, and BALF samples were collected from all patients. The patient characteristics are shown in Table 1. Of the 17 proteins that were measured, the concentrations of only 9 (SDF-1α, IP-10, IL-8, TNF-α, EGF, Galectin-3, MMP-1, MMP-7, and MMP-8) were detected in the BALF. Other proteins (IL-1β, IL-4, IL-6, IL-13, IFN-γ, FGF, PDGF and TGF-β) were excluded from the analysis, because the levels did not reach detectable values in the majority of patients. The levels of the 9 proteins included in the analysis are shown in Table 2.

The relationships between the concentrations of these 9 proteins in the BALF and physiological variables are shown in Table 3. When considering a $r \geqq 0.4$ or $\leqq -0.4$ as a moderate correlation, the FVC % predicted showed a moderate correlation with TNF-α, MMP-1, MMP-7, and MMP-8 (r=0.605, 0.527, 0.406, and 0.624, respectively) (Figure 1). The PaO$_2$ showed a moderate negative correlation with IL-10 (r=–0.418), and KL-6 showed a moderate negative correlation with IL-10, IL-8, and TNF-α (r=–0.479, –0.406, and –0.587, respectively). The DLco % predicted showed no moderate correlations with any of the 9 proteins. There were no strong correlations of $r \geqq 0.7$ or $\leqq -0.7$ among them.

The relationships between the concentrations of 9 of the proteins in the BALF and the number of each type of white blood cell in the BAL are shown in Table 4. The percentage of lymphocytes was not correlated significantly with the concentration of any of the 9 proteins. However, the percentage of neutrophils was correlated significantly with the concentration of SDF-1α and MMP-1, and the percentage of eosinophils was correlated significantly with the concentration of SDF-1α. Several correlations in which $r \geqq 0.4$ or $\leqq -0.4$ were found.

Discussion

The novel finding of this study is that the concentration of TNF-α, MMP-1, -7, and -8 in the BALF of patients with IPF showed a trend toward a positive relationship with FVC % predicted. TNF-α, a cytokine associated with inflammation and fibrosis, is expressed not only in the lungs of animal models of pulmonary fibrosis,[12-14] but also in the lungs of patients with IPF.[15,16] Recently, it has been reported that an atypical fibrogenic monocyte, SatM, identified in animal models of pulmonary fibrosis, produces large amounts of TNF-α and activates fibroblasts via the production of TNF-α.[17] In addition, a TNF-α antagonist inhibited pulmonary inflammation and fibrosis in animal models of pulmonary fibrosis.[18] Therefore, it is considered that TNF-α is involved in

Table 1. Characteristics of the 10 patients

Characteristic	mean (SD)	range
Age, y.o.	74.2 (5.2)	61 - 80
Gender, male/female	7/3	
Pulmonary function		
FVC, L	2.33 (0.579)	1.51 - 3.44
FVC, % predicted	79.2 (11.4)	64.5 - 100
DLco, mL/min/mmHg*	10.9 (1.78)	7.64 - 13.5
DLco, % predicted*	72.3 (17.3)	43.3 - 94.2
Blood gas analysis		
pH	7.42 (0.02)	7.39 - 7.45
PaCO$_2$, mmHg	37.9 (2.9)	33.6 - 43.7
PaO$_2$, mmHg	80.3 (8.9)	63.8 - 92.3
KL-6, U/mL	1457 (1123)	665 - 4337
BAL findings		
Recovery ratio, %	57 (9.6)	47 - 69.5
Total cell count, $\times 10^5$ cells/mL	1.61 (1.05)	0.4 - 3.4
Macrophages, %	78.4 (17.7)	40 - 94
Lymphocytes, %	12.7 (8.9)	4.4 - 32.6
Neutrophils, %	6.94 (9.8)	0.6 - 33.2
Eosinophils, %	1.92 (2.0)	0.2 - 5.8

*n=9

BAL bronchoalveolar lavage, *DLco* diffusing capacity for carbon monoxide, *FEV₁* forced expiratory volume in 1s, *FVC* forced vital capacity, *KL-6* Krebs von den Lungen-6, *PaCO₂* arterial carbon dioxide tension, *PaO₂* arterial oxygen tension

Table 2. Protein levels in the BALF of the patients

Protein	mean (SD)	range
SDF-1α, pg/mL	296 (148)	87 - 582
IP-10, pg/mL	163 (62)	74 - 268
IL-8, pg/mL	145 (93)	54 - 352
Galectin-3, pg/mL	246517 (263491)	42450 - 974405
TNF-α, pg/mL	21 (7)	14 - 35
EGF, pg/mL	17 (10)	8 - 40
MMP-1, pg/mL	181 (199)	25 - 468
MMP-7, pg/mL	5428 (1865)	1699 - 7599
MMP-8, pg/mL	1685 (1188)	277 - 4145

EGF Epidermal Growth Factor, *IL-8* Interleukin-8, *IP-10* Interferon γ-induced protein 10, *MMP* matrix metalloproteinase, *SDF-1α* Stromal cell-derived factor-1α, *TNF-α* Tumor necrosis factor-α

Table 3. Correlations between BALF protein levels and physiological variables

Protein		%FVC	%DLco	PaO$_2$	KL-6
SDF-1α	r	0.236	0.133	0.261	−0.127
	p	0.513	0.734	0.470	0.725
IP-10	r	0.273	0.233	−0.418	−0.479
	p	0.450	0.551	0.243	0.179
IL-8	r	0.212	−0.150	0.067	−0.406
	p	0.557	0.702	0.854	0.257
Galectin-3	r	0.030	0.133	−0.200	−0.248
	p	0.933	0.734	0.580	0.491
TNF-α	r	0.605	−0.176	−0.167	−0.587
	p	0.084	0.654	0.645	0.095
EGF	r	−0.091	0.283	0.248	0.042
	p	0.802	0.469	0.491	0.907
MMP-1	r	0.527	0.100	0.224	−0.236
	p	0.137	0.798	0.535	0.513
MMP-7	r	0.406	0.267	0.345	−0.188
	p	0.257	0.496	0.337	0.604
MMP-8	r	0.624	0.300	0.297	−0.345
	p	0.074	0.443	0.410	0.337

EGF Epidermal Growth Factor, *IL-8* Interleukin-8, *IP-10* Interferon γ-induced protein 10, *MMP* matrix metalloproteinase, *SDF-1α* Stromal cell-derived factor-1α, *TNF-α* Tumor necrosis factor-α

Table 4. Correlations between BALF protein levels and the BAL findings

Protein		Total cell counts	Macrophages (%)	Lymphocytes (%)	Neutrophils (%)	Eosinophils (%)
SDF-1α	r	0.358	−0.733	0.515	0.863	0.729
	p	0.320	0.031	0.147	0.006	0.032
IP-10	r	0.418	−0.030	0.273	−0.292	−0.298
	p	0.243	0.933	0.450	0.418	0.409
IL-8	r	0.661	−0.358	0.115	0.419	0.401
	p	0.056	0.320	0.751	0.241	0.263
Galectin-3	r	0.018	−0.164	0.224	0.073	0.261
	p	0.960	0.651	0.535	0.840	0.469
TNF-α	r	0.654	−0.278	0.049	0.344	0.279
	p	0.059	0.441	0.892	0.339	0.440
EGF	r	−0.370	−0.236	0.248	0.097	0.170
	p	0.303	0.513	0.491	0.788	0.638
MMP-1	r	0.418	−0.406	0.103	0.717	0.468
	p	0.243	0.257	0.776	0.035	0.189
MMP-7	r	0.345	−0.309	0.127	0.432	0.359
	p	0.337	0.391	0.725	0.228	0.318
MMP-8	r	0.515	−0.394	0.200	0.584	0.353
	p	0.147	0.272	0.580	0.097	0.327

EGF Epidermal Growth Factor, *IL-8* Interleukin-8, *IP-10* Interferon γ-induced protein 10, *MMP* matrix metalloproteinase, *SDF-1α* Stromal cell-derived factor-1α, *TNF-α* Tumor necrosis factor-α

pathological processes in lung fibrosis, and inhibition of TNF-α may be a new therapeutic approach in IPF. In the present study, the concentration of TNF-α in the BALF of patients with IPF showed a trend toward a positive relationship with FVC % predicted, although it is just a preliminary study. The concentration of TNF-α was higher in patients with FVC % predicted more than 80%. It is possible that a strong inflammatory or fibrotic response occurs even in patients without lung impairment as measured by pulmonary function tests.

On the other hand, MMPs can degrade all of the components of the extracellular matrix. Then, a wide range of growth factors, cytokines, chemokines and cell surface receptors can be released and activated. Adhesion, proliferation, and differentiation, as well as recruiting and transmigration, and apoptosis of them can be also affected.[19] MMPs may contribute to changes in the lung microenvironment, and dysregulated expression of those may have an important biopathological role in the development of IPF. MMP-1

is significantly overexpressed in lungs of patients with IPF,[20,21] although its role in pulmonary fibrosis has not been fully elucidated. MMP-7 is also one of the molecules highly expressed in IPF, which is localized primarily in activated epithelial cells of alveolars and bronchioles.[22,23] It has been considered that MMP7 has a profibrotic role in lung fibrosis.[24] As for MMP-8, which is also highly expressed in lung tissue and BALF,[25-27] it has been considered to facilitate fibrocyte migration.[28-30] It is important that the concentration of MMP-1, -7, and -8 in BALF showed a trend toward a positive correlation with FVC % predicted in patients with IPF. Similarly, TNF-α, MMP-1, -7, and -8 were elevated more in patients with more preserved lung function.

Two anti-fibrotic agents, nintedanib and pirfenidone, are now available as treatments for patients with IPF.[5,6] Nintedanib reduces lung function decline, even in patients with preserved lung function.[31] Conversely, lung fibrosis progresses without treatment, even in patients whose lung function is preserved. Early intervention in pa-
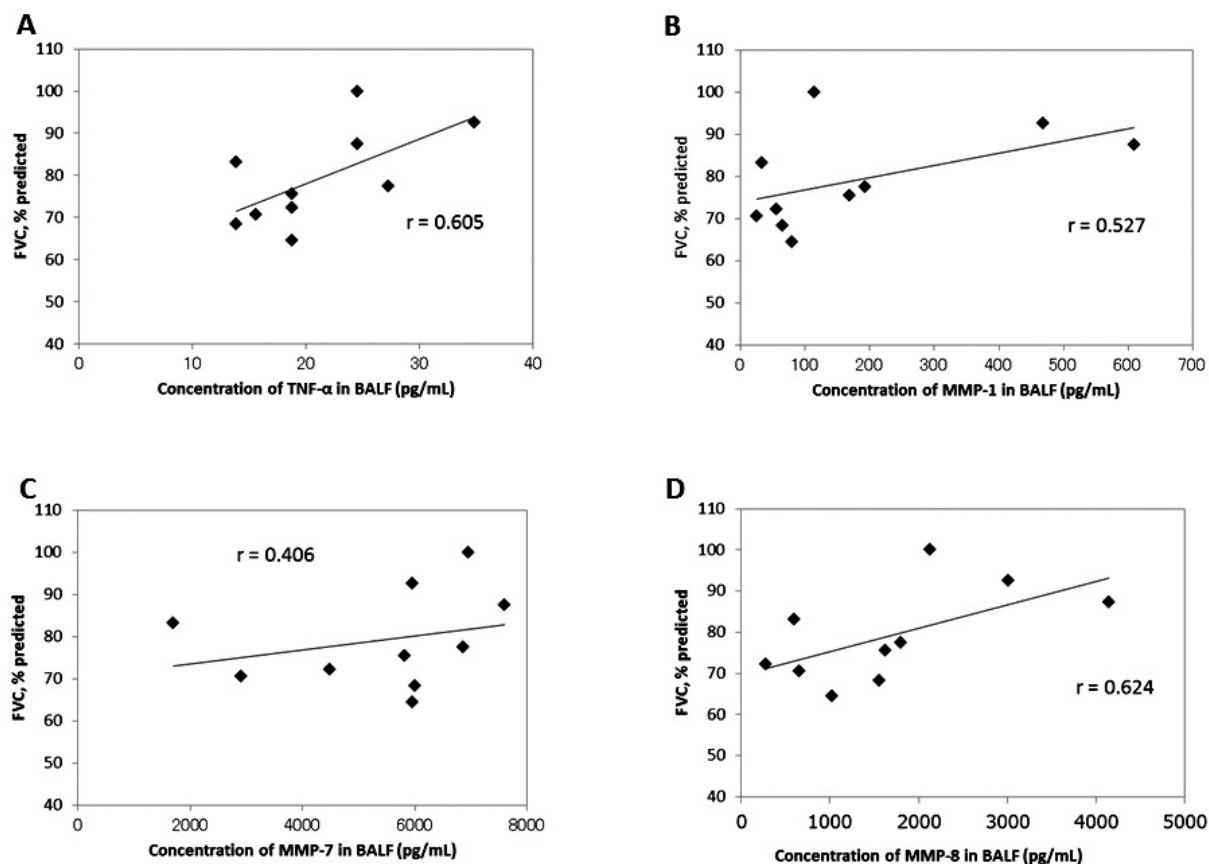

Figure 1. The relationship between the protein level in the BALF (A. TNF-α, B. MMP-1, C. MMP-7, D. MMP-8) and the FVC % predicted.

tients with IPF may lead to continued preserved lung function and longer survival. The mechanism of nintedanib against lung fibrosis is anti-PDGF, anti-VEGF, and anti-FGF.[4,5] The mechanism of pirfenidone remains uncertain. Given the fact that TNF-α, MMP-1, -7, and -8 were active in patients with IPF and preserved lung function, our results may reveal new therapeutic targets other than those of the two existing anti-fibrotic drugs in early IPF.

A clinical trial of etanercept, a recombinant soluble human TNF receptor that binds to TNF and blocks its interaction with cell surface, was conducted in patients with moderate IPF (FVC % predicted, 64.7 ± 14.1%). However, its efficacy was not confirmed in that trial.[32] Given that the BALF concentration of TNF-α was elevated more in patients with IPF whose lung function is more preserved, patients who may benefit from the drug may be those with preserved lung function. If patients whose lung function is still normal are recruited, different results may be obtained.

As for MMPs, earlier efforts to block the activity of MMPs in patients with cancer did not achieve clinical success.[33] Trials of MMP inhibitors should be conducted in IPF as well. However, the selectivity of the MMP inhibitors should be taken into consideration. Further study is needed to elucidate which MMPs mainly have a major role in the pathogenesis in IPF. As with TNF-α, patients with preserved lung function would be good candidates for the trial.

Some limitations of the present study should be mentioned. First, the number of patients was too small to demonstrate statistically significant relationships between the proteins and lung function. However, the correlation coefficients were more than moderate (0.4-0.7). Because this study was a preliminary study for generating hypotheses, we did not recruit more patients to achieve statistical significance, but will proceed to the next assessment; for example, focusing on only IPF patients with normal lung function. Second, the pathogenetic mechanisms of TNF-α, MMP-1, -7, and -8 for fibrosis were not examined in early-phase IPF. The exact roles of these molecules in early-phase IPF should be examined in further studies. Third, the study was conducted at a single institute. Although physicians experienced in IPF diagnosed the patients, the way of diagnosing IPF might be different at other institutes. Recruiting patients from mul-

tiple institutes might be preferable. Fourth, the study was a cross-sectional study. The relationship between changes in TNF-α, MMP-1, -7, and -8 over time and lung function decline should be examined in further researches.

A trend toward a positive correlation between the BALF concentration in TNF-α, MMP-1, -7, and -8 and FVC % predicted was observed. These molecules are elevated more in the BALF of patients with earlier IPF. Given that these proteins are involved in the initial inflammation and fibrosis of IPF, they are possibly new therapeutic targets in early IPF to prevent disease progression.

Author contributions
Conception and experimental design (TM, ON, YC), Data analysis and interpretation (TM, ON, YT), Data acquisition (TM), Drafting the manuscript (TM, ON), Critical manuscript revision (ON, YC, HS, TI, YT). All authors read and approved the final manuscript.

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