INTRODUCTION

Because the pathophysiology of interstitial pneumonia (IP), including idiopathic interstitial pneumonia (IIP) and IP associated with collagen-vascular diseases (CVD-IP), is still unknown, a strategy for treating IP has yet to be established [1]. In medical practice, oral corticosteroid is often used with a variable response rate; still, some cases are spontaneously progressive or resistant to corticosteroid therapy as is usually the case with idiopathic pulmonary fibrosis (IPF) [2-5].

The turnover of fibrin matrix in the alveolar space has been recently reported to be critical in the development of pulmonary fibrosis in IP[6]. The balance of fibrinolytic and antifibrinolytic activities in the lung determines whether fibrin is deposited and organized or resolved, and the accumulation of fibrin is likely to be accompanied by the development of fibrosis [7]. Fibrinolytic activity is associated with the plasminogen activator (PA) and its inhibitor, plasminogen activator inhibitor 1 (PAI-1). While tissue-type PA mainly acts in vessels, it is well known that urokinase-type PA (u-PA) promotes plasmin generation in extra-vascular areas such as the alveolar space [8]. On the other hand, alveolar epithelial cells produce u-PA and PAI-1. These two molecules can greatly control the rate at which plasmin is generated in the alveolar epithelium and fibrin is cleared from the alveolar space [9].

Reduced fibrinolytic activity has been found in bronchoalveolar lavage fluids (BALF) from patients with IPF [10], suggesting that it is due to an imbalance between u-PA and PAI-1. However, there is little evidence of differences in fibrinolytic activity between the various forms of IP. The importance of this functional uPA/PAI-1 system in the prevention of pulmonary fibrosis is also supported by experiments performed on animals with bleomycin-induced pulmonary fibrosis. Bleomycin-treated mice deficient in plasminogen or u-PA showed enhanced pulmonary fibrosis when compared with controls [11], whereas the adenovirus-mediated transfer of u-PA to a bleomycin-treated lung resulted in a reduction in fibrosis [12], indicating a protective role for u-PA in the development of pulmonary fibrosis. Over-expression of the naturally occurring inhibitor of u-PA, PAI-1, in bleomycin-treated mice resulted in enhanced pulmonary fibrosis [13], indicating a profibrotic role for PAI-1. PAI-1 is strongly induced to express itself by the transforming growth factor-ß (TGF-ß) and may be partly responsible for TGF-ß-mediated fibrosis [7].

There have been few studies focusing on the relationship between clinically based fibrosis findings and the fibrinolytic balance or the origin of the fibrinolytic activities in IP. To clarify this relationship, the u-PA/PAI-1 system in the alveolar space should be evaluated. Various cells, including alveolar macrophages (AM) and alveolar epithelial cells, can regulate the fibrinolytic activities in the alveolar space. Although the regulation by alveolar epithelial cells, especially via TGF-ß and interleukin-1 ß (IL-1ß) - which were detected in BALF and considered important elements in IP [14, 15], has been elucidated [8, 9], the mechanism of regulation by AM is still to be determined.

In this study, levels of u-PA and PAI-1 antigen were measured in BALF obtained from patients with IP, and the data were compared between two groups, fibrosing and non-fibrosing, as determined by the findings of pulmonary high-resolution computed tomography (HRCT) and lung histology. In addition, the cell surface plasmin generation of various alveolar macrophages and its correlation with the u-PA/PAI-1 levels in each of the two groups were studied.

MATERIALS AND METHODS

Materials

Tissue culture plasticware was obtained from Coster (Cambridge, MA, USA), fetal calf serum from Life Technologies (Grand Island, NY, USA) H-D-Val-Leu-Lys-p-nitroanilide (S-2251), plasminogen and plasmin from CHROMOGENIX (Molndal, Sweden), IL-1ß and TGF-ß from Promega (Madison, WI, USA), and u-PA ELISA and PAI-1 ELISA kits, from American Diagnostica Inc. (Greenwich, CT, UK). All other reagents not specifically mentioned were of the highest grade available from Sigma Chemical Co. (St. Louis, MO, USA).

Store of BALF from patients with IP for u-PA and PAI-1 analysis

The BALF used in this study were collected according to the Ethical Principles for Medical Research Involving Human Subjects, Declaration of Helsinki. We routinely performed bronchoalveolar lavage (BAL) on patients admitted to Niigata University Medical and Dental Hospital from 1998 to 2000 in whom a diagnosis of IP, including IIP and CVD-IP, was suspected. BAL was performed with a fiberoptic bronchoscope (Model BF IT-300, Olympus Corporation, Tokyo) wedged into a segmental or subsegmental bronchus of the middle lobe and lavaged with 4 × 50 ml of sterile 0.9% saline. BALF were centrifuged at 1,000 rpm for 10 min at 4 °C, and BAL cells and cell-free fluids were obtained.

The total cell number was counted by hemocytometer, and differential cell counting was carried out using a Gimsa-stained cytocentrifuge preparation. After the regular BAL analysis, the left BALF were stored at -80°C for additional analyses. A total of 26 stored samples were prospectively analyzed and measured for u-PA and PAI-1 antigen using ELISA kits.

Classification of cases in which BALF were analyzed

The patients with IP in whom BALF were analyzed were grouped into either a fibrosing group or a non-fibrosing group, based on the findings of HRCT and, when necessary and available, lung histology. We assigned a patient to the fibrosing group if a honeycomb formation was detected. When no such formation was detected, a patient was placed in the non-fibrosing group; however, if the lung biopsy showed usual interstitial pneumonia (UIP) or fibrotic nonspecific interstitial pneumonia (NSIP), that patient was classified in the fibrosing group even in the absence of a honeycomb formation. The actual course of each patient was evaluated based mainly on changes in HRCT findings more than six months after the first HRCT. All lung biopsies were done by video-assisted thoracoscopy. Six patients with stage I sarcoidosis (no obvious interstitial shadows detected in the lungs with HRCT) were employed as the controls. The absence of any interstitial lung shadows during the long follow-up period (mean±SD:61.5±3.0 months) confirmed their suitability as the controls.

Preparation of human and rat macrophages

Human alveolar macrophages were obtained by BAL and prepared as described above from healthy volunteers. Rat alveolar macrophages were obtained by BAL, which was performed with a 10 ml syringe wedged into the trachea of a sacrificed rat under anesthesia and lavaged with 4 × 10 ml of sterile 0.9% saline. Both human and rat alveolar macrophages were suspended in a RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 mg/ml). The media were changed to serum-free 12 h before the application of the cells.

Cell surface generated plasmin assay

Cells (3×10^6) in 15 ml conical-tubes were treated with either IL-1ß (1, 10 and 100 U/ml), TGF-ß (0.15, 1.5 and 15 ng/ml), or left untreated for 24 h. After centrifugation at 1,000 rpm for 10 min, the medium was discarded, and the cells were then washed in PBS and incubated in a serum free-medium, RPMI1640-containing plasminogen (20 µg/ml), for 3 h. After centrifugation at 1,000 rpm for 3 min, the cell lysate was collected and washed again in PBS. Plasmin was then dissociated from the cell membrane by incubation of the cells in 250 µl of 1 mM tranexamic acid in PBS, pH 7.4 for 15 min [14]. Aliquots of the samples (50 µl) were assayed in duplicate in a total volume of 125 µl of 0.1 M Tris, pH 8.0, containing a final concentration of 1.0 mM of the chromogenic substrate, S-2251. The amount of p-nitroaniline released after 2 h at 37°C was measured at 410 nm with a Molecular Devices UV MAX plate reader and referenced to a plasmin standard (Sigma, Catalog #P4895) run in parallel with the samples. One unit of plasmin activity was defined as a change in absorbance of 2.5 per minute under the stated assay conditions. Data were expressed as mU (10-3 units) per tube.

Statistical analysis

The statistical significance of differences between the patients with IP and the patients with sarcoidosis, or the fibrosing and non-fibrosing groups, was analyzed using Mann-Whitney and chi-square tests. The data were analyzed by a Macintosh computer with StatView software, version J-5.0. P values of less than 0.05 were considered to indicate a statistical significance.

RESULTS

Clinical characteristics, respiratory function and blood gas analyses, and the background of patients with IP or sarcoidosis

The mean age of patients in the fibrosing and non-fibrosing groups was 64.6±8.7 years (mean±SD) and 59.0+11.9 years old, respectively. The mean age of the fibrosing group was significantly higher ($p<0.05$) than that of patients with sarcoidosis (40.3±18.9 years old) as the control (N=6). There was no significant difference in gender among the three groups. The clinical characteristics of the patients in the fibrosing and non-fibrosing groups are summarized in Table 1. In the fibrosing group, there were six patients with IPF and seven with CVD-IP. In the non-fibrosing group, there were 11 patients with CVD-IP and four with non-IPF IIP. Six lung biopsies were performed in the fibrosing group to reveal UIP in three cases and fibrotic NSIP in another three, while one biopsy was done in the non-fibrosing group and showed cellular NSIP. Six patients in the fibrosing group were treated with oral

Table 1. Summary of patients

Group	Gender	Age	Clinical diagnosis	Histological diagnosis	Management	Changes in HRCT
Fibrosing	Male	48	CVD-IP	Fibrotic NSIP	OCS+ImSx	No change
Fibrosing	Male	68	IPF	-	Observation	Progress
Fibrosing	Female	50	CVD-IP	Fibrotic NSIP	OCS+ImSx	No change
Fibrosing	Male	75	CVD-IP	-	OCS	No change
Fibrosing	Female	64	CVD-IP	-	Observation	No change
Fibrosing	Male	72	CVD-IP	Fibrotic NSIP	OCS+ImSx	No change
Fibrosing	Male	72	IPF	-	Observation	No change
Fibrosing	Male	71	IPF	-	Observation	Progress
Fibrosing	Male	68	IPF	UIP	OCS	No change
Fibrosing	Male	57	IPF	UIP	Observation	No change
Fibrosing	Female	59	CVD-IP	-	OCS+ImSx	Progress
Fibrosing	Male	65	IPF	UIP	Observation	No change
Fibrosing	Female	71	CVD-IP	-	Observation	No change
Non-fibrosing	Male	63	Non IPF IIP	-	Observation	No change
Non-fibrosing	Female	71	Non IPF IIP	-	OCS	Improved
Non-fibrosing	Female	66	Non IPF IIP	Cellular NSIP	OCS	Improved
Non-fibrosing	Male	63	CVD-IP	-	Observation	No change
Non-fibrosing	Female	60	CVD-IP	-	Observation	No change
Non-fibrosing	Female	60	CVD-IP	-	OCS	Improved
Non-fibrosing	Male	29	CVD-IP	-	OCS	Improved
Non-fibrosing	Female	66	Non IPF IIP	Cellular NSIP	Observation	No change
Non-fibrosing	Male	46	CVD-IP	-	OCS	Improved
Non-fibrosing	Female	49	CVD-IP	-	OCS	Improved
Non-fibrosing	Female	51	CVD-IP	-	OCS	No change
Non-fibrosing	Female	79	CVD-IP	-	OCS	Improved
Non-fibrosing	Female	58	CVD-IP	-	OCS	Improved
Non-fibrosing	Female	57	CVD-IP	-	OCS	Improved
Non-fibrosing	Female	67	CVD-IP	-	Observation	No change

CVD-IP, interstitial pneumonia associated collagen vascular diseases; IP, interstitial pneumonia; IIP, idiopathic interstitial pneumonia; IPF, idiopathic pulmonary fibrosis; NSIP, non-specific interstitial pneumonia; UIP, usual interstitial pneumonia; OCS, oral corticosteroid; ImSx, immunosuppressive agent; HRCT, high resolution computer tomography.

Table 2. Summary of respiratory function and blood-gas analysis

	Sarcoidosis	Non-fibrosing group	Fibrosing group
%VC(mean+/-SD)	113.5+/-6.0	89.9+/-17.3	90.4+/-18.3
FEV1%(mean+/-SD)	83.1+/-11.4	81.6+/-7.2	86.8+/-17.8
%DLco(mean+/-SD)	ND	62.7+/-15.8	48.3+/-11.0##
PO2(torr mean+/-SD)	ND	86.0+/-8.4	85.2+/-5.2
PCO2(torr mean+/-SD)	ND	42.8+/-2.4	42.6+/-3.3
AaDO2(torr mean+/-SD)	ND	11.8+/-9.5	12.8+/-6.0

##, P<0.01 vs non-fibrosing group.

corticosteroid or oral corticosteroid plus immunosuppressive agents for a minimum of six months; among them, one patient showed disease progression on HRCT after the treatment while no changes were seen in the other five patients. The other seven patients in the fibrosing group were observed without treatment and again one showed disease progression on HRCT. On the other hand, 11 patients in the non-fibrosing group treated with oral corticosteroid all showed improvement. The other four patients in the non-fibrosing group were observed without treatment and showed no changes.

Results of respiratory function and blood gas analyses are summarized in Table 2. There were no significant differences in percent vital capacity (%VC) and FEV1% among the three groups. The %DLco was significantly lower in the fibrosing group than in the non-fibrosing group ($p<0.05$). There were no significant differences in PaO2, PaCO2, or AaDO2 between the fibrosing and the non-fibrosing group.

BAL data for patients with IP or sarcoidosis

The BAL data are summarized in Table 3. Apart from CD4/8, there were no significant differences in the BAL data between patients with sarcoidosis and those with IP or between patients with sarcoidosis and the non-fibrosing group. There were no significant differences in the recovery rates of BAL among the three groups either. On the other hand, the alveolar macrophage counts (MCC) were significantly larger in the fibrosing group than in the patients with sarcoidosis ($p<0.01$). Moreover, MCC and the proportion of alveolar macrophages were significantly higher in the fibrosing group than non-fibrosing group ($p<0.01$ and $p<0.05$, respectively), while the proportion of lymphocytes was lower ($p<0.05$). These results suggest that numbers of AM increased not in the non-fibrosing but rather in the fibrosing group, compared with the sarcoidosis patients as the controls.

u-PA and PAI-1 levels in IP or sarcoidosis

The levels of u-PA and PAI-1 in BALF are summarized in Table 4. There was a significant decrease in the level of u-PA ($p<0.05$) in patients with IP compared with the patients with sarcoidosis. When IP patients were divided into fibrosing and non-fibrosing groups, no significant difference in the level of u-PA was found between the two groups although the fibrosing group had significantly lower values of u-PA ($p<0.05$) than the sarcoidosis cases. Levels of PAI-1 did not differ between the patients with sarcoidosis and the patients with IP, or the fibrosing and the non-fibrosing groups. These results indicate that the reduced fibrinolytic activity in BALF from patients with IP is probably not dependent on the presence of IP but on the fibrosis of IP.

Effects of IL-1ß and TGF-ß on plasmin generation in the macrophages

We already have adequate information about levels of u-PA/PAI-1 and plasmin generation in the pulmonary

Table 3. Summary of BAL data

	Sarcoidosis	Interstitial pneumonia	Non-fibrosing group	Fibrosing group
%recovery(mean+/-SD)	57.0+/-14.5	52.0+/-14.3	56.1+/-15.1	47.3+/-12.1
TCC($\times 10^5$/ml mean+/-SD)	2.22+/-1.04	2.52+/-1.76	2.71+/-2.78	3.17+/-1.16
%MC(mean+/-SD)	58.7+/-34.2	69.5+/-19.2	62.6+/-21.9	77.6+/-11.7#
MCC($\times 10^5$/ml mean+/-SD)	1.06+/-0.53	1.92+/-1.16	1.44+/-0.97	2.49+/-1.14**,##
%lymphocyte(mean+/-SD)	41.0+/-34.2	26.6+/-18.8	34.1+/-21.2	18.0+/-10.8#
LC($\times 10^5$/ml mean+/-SD)	1.15+/-1.11	0.89+/-1.06	1.18+/-1.36	0.55+/-0.38
CD4/8 ratio(mean+/-SD)	6.14+/-5.46	1.14+/-0.81***	0.91+/-0.87**	1.40+/-0.63**,#

**, P<0.01; ***, P<0.001 vs Sarcoidosis; #, P<0.05; ##, P<0.01 vs non-fbrosing group; TCC, total cell count; MC, macrophage; MCC, macrophage count; LC, lymphocyte count.

Table 4. Summary of u-PA and PAI-1 level in BALF

	Sarcoidosis	Interstitial pneumonia	Non-fibrosing group	Fibrosing group
u-PA(ng/ml mean+/-SD)	1.54+/-0.86	0.71+/-0.55*	0.75+/-0.61	0.66+/-0.50*
PAI-1(ng/ml mean+/-SD)	0.02+/-0.04	0.04+/-0.10	0.06+/-0.12	0.02+/-0.05

*, P<0.05 vs sarcoidosis.

Table 5a. Effects of IL-1ß on plasmin generation (mU/tube)

	Control	IL-1ß 1 U/ml	IL-1ß 10 U/ml	IL-1ß 100 U/ml
Human macrophage	12.8+/-0.5	14.0+/-1.3	14.7+/-0.7	14.4+/-2.0
Rat macrophage	33.9+/-2.1	35.9+/-2.3	35.5+/-1.5	35.9+/-1.0

No significant difference vs CTL; CTL, Control.

Table 5b. Effects of TGF-ß on plasmin generation (mU/tube)

	Control	TGFß 0.15 ng/ml	TGFß 1.5 ng/ml	TGFß 15 ng/ml
Human macrophage	12.8+/-0.5	15.0+/-1.1	15.2+/-0.6	13.6+/-1.0
Rat macrophage	33.9+/-2.1	33.5+/-2.0	32.4+/-0.5	33.0+/-5.2

No significant difference vs Control.

epithelial cells [8, 9]. Disclosing the mechanism of plasmin generation in alveolar macrophages was necessary to interpret the data (Table 4). We therefore investigated the effects of IL-1ß and TGF-ß on plasmin generation by AM. IL-1ß, which is an important inducible cytokine on the plasmin generation and u-PA induction in pulmonary epithelial cells, did not have any effect on plasmin generation by human AM and rat AM (Table 5a). TGF-ß, which is a major suppressor of plasmin generation and PAI-1 induction in pulmonary epithelial cells, did not influence plasmin generation in these cells either (Table 5b). These results indicate that plasmin generation by the AM in IP is probably not regulatory but constant.

Considering both the increased MMC in BALF from the fibrosing group compared with the non-fibrosing group and lack of difference in fibrinolytic activity between the fibrosing and non-fibrosing groups, we speculate that the reduced fibrinolytic activity is not associated with AM because plasmin generation by AM is constant.

DISCUSSION

Previous studies have confirmed the key role that altered fibrinolytic activity in the alveolar space plays in the pathogenesis of IP [6, 7, 10-13, 16]. The current study was undertaken in line with this hypothesis. Both human and experimental studies revealed that reduced fibrinolytic activity in BALF was likely to be related to the development of pulmonary fibrosis in IP. However, the origin of this activity and its relationship to the fibrosis in IP is still unclear.

Our data clearly showed that the fibrinolytic activity in the BALF was associated with IP. The reduced fibrinolytic activity in IP compared with sarcoidosis was likely to be directly related to the development of pulmonary fibrosis due to the impaired absorption of fibrin in the alveolar space as previously reported. However, we now propose another function for the fibrinolytic activity. It was reported that metalloprotease-1 (MMP-1) derived from skin fibroblasts was activated by plasmin [17, 18], and we also confirmed that pulmonary epithelial cells activate MMP-1 by producting plasmin on the cell surface [19]. These findings indicate that reduced fibrinolytic activity might have resulted in the direct accumulation of collagen via its reduced absorption. Although it is unclear which pathway is dominant, we believe that fibrinolytic activity plays an important role in the development of fibrosis in IP.

Our study has provided new insight into the relationship between reduced fibrinolytic activity in the alveolar space and the fibrosis in IP. Table 4 shows that there was less fibrinolytic activity in the alveolar space in IP compared with the sarcoidosis group, but there was no difference between the fibrosing and non-fibrosing groups. Table 3 indicates that MCC was increased significantly in the fibrosing group compared with the non-fibrosing group, and there was no difference between the sarcoidosis group and the non-fibrosing group. On the other hand, Tables 5a and b show that the ability of AM to generate plasmin was not regulatory but constant. Considering these three results, it can be concluded that the reduced fibrinolytic activity in the fibrosing IP might have resulted from cells other than AM, which can be regulated by cytokines including IL-1ß and TGF-ß. Although plasmin generation by lymphocytes has been previously reported [20], it is generally considered that lymphocytes play a minor role in plasmin generation. Therefore, we speculate that other cells in the alveoli, probably the epithelial cells, were responsible for the reduced fibrinolytic activity in the fibrosing IP.

In our study, patients were classified as fibrosing or non-fibrosing based first on the findings of HRCT and second on histological findings, when necessary and available. Eleven patients in the non-fibrosing group were treated with oral corticosteroid; HRCT showed evident improvement at six months after the treatment, whereas none of the patients in the fibrosing group had

improved HRCT findings. The relationship between the clinical course of the disease (Table 1) and the profile of lymphocytes and macrophages in the BALF (Table 3) corresponded with that reported previously [21, 22]. Therefore, the fibrinolytic activity might be related not only to the fibrosis in IP but also to the clinical course and response to therapy in IP. Furthermore, we could not explain why it is not the AM but probably the alveolar epithelial cells -- which influenced fibrinolytic activity in the alveolar space -- that contributed to the clinical course in our study. Further study with a histological approach including histochemical staining of different cell types should help resolve this issue.

In summary, we analyzed the fibrinolytic activity in BALF from patients with IP. The u-PA level was significantly lower in IP patients than sarcoidosis patients. The IP patients were divided into two groups, fibrosing and non-fibrosing, based on the HRCT. Although u-PA and PAI-1 levels in BALF did not differ between the two groups, the MMC and the percentage of AM in BALF were significantly greater in the fibrosing than non-fibrosing group. The major regulatory cytokines, IL-1ß and TGF-ß, however, did not affect cell surface plasmin generation by AM, indicating that the fibrinolytic activity in AM was constant. These results indicate that the reduced fibrinolytic activity in BALF from the fibrosing group was not related to AM. Other cells such as alveolar epithelial cells might contribute to the fibrosis although further clinical studies will be required.

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