ORIGINAL

The significance of cathepsins, thrombin and aminopeptidase in diffuse interstitial lung diseases

Makoto Kimura, Kenji Tani, Junya Miyata, Keiko Sato, Akihito Hayashi, Shinsaku Otsuka, Tomoyuki Urata, and Saburo Sone

Department of Internal Medicine and Molecular Therapeutics, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan

Abstract : To determine the significance of proteases in interstitial lung diseases, we examined the activity of cathepsins, thrombin, and aminopeptidase in bronchoalveolar lavage (BAL) fluid from patients with these disorders. Significantly increased activities of cathepsin H and aminopeptidase were detected in BAL fluid from patients with idiopathic pulmonary fibrosis (IPF), cryptogenic organizing pneumonia (COP), chronic eosinophilic pneumonia (CEP) and hypersensitivity pneumonitis (HP). Significantly higher activity of cathepsin B was found in BAL fluid from patients with CEP. The activity of thrombin was significantly higher in patients with IPF and CEP. In patients with IPF, there were significant correlations between neutrophil number and the activity of cathepsin B, cathepsin H or aminopeptidase. In patients with COP and HP, the activity of the proteases was significantly higher in patients with higher number of lymphocytes than in those with lower number of lymphocytes. The present study suggests that the activity of the proteases is a useful marker in activity of the interstitial lung diseases, and may have a role in the pathogenesis of these disorders.
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Keywords **:** *interstitial lung diseases, cathepsin H, cathepsin B, cathepsin G, thrombin, aminopeptidase*

INTRODUCTION

Interstitial lung diseases comprise a diverse group of lung infiltrations with inflammatory/fibrotic disorders that affect the interstitial and alveolar compartment of the lung (1). Idiopathic pulmonary fibrosis (IPF) is one of the more commonly occurring interstitial lung diseases of unknown etiology (2, 3). Cryptogenic organizing pneumonia (COP) is a distinct clinicopathological entity, proposed by Epler *et al*. (4), and is manifest radiologically by peribronchial ground glass opacities and subpleural consolidation with good response to corticosteroid therapy (5). Chronic eosinophilic pneumonia (CEP), a rare eosinophilic lung disease of unknown etiology, is characterized by peripheral blood eosinophilia, chest radiograph infiltrates, and prompt response to corticosteroid therapy (6). Hypersensitivity pneumonitis (HP) represents a group of immunologically mediated lung disorders provoked by recurrent exposure to various environmental agents (7).

Bronchoalveolar lavage (BAL) is a technique for sampling contents of the lower respiratory tract, and has been shown to be a clinically useful tool for various lung diseases including interstitial lung diseases (8, 9). For example, it is widely known that the cellular findings of BAL fluid in IPF and CEP are characterized by neutrophils and eosinophils, respectively, and those of COP and HP show increased number of lymphocytes (2, 5-7). In this study, to examine the significance

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Address correspondence and reprint requests to Saburo Sone, M.D., Department of Internal Medicine and Molecular Therapeutics, Institute of Health Biosciences, The University of Tokushima Graduate School, Kuramoto-cho, Tokushima 770-8503, Japan and Fax : +81-886-33-2134.

Table 1. Patient profiles and findings of bronchoalveolar lavage

Group	Number (male/female)	Age (yr)	Broncoalveolar lavage				
			Total cells ($\times 10^5$)	AM ($\times 10^5$)	Ly ($\times 10^5$)	Neut ($\times 10^5$)	Eo ($\times 10^5$)
NV	14/1	22.9±0.8	186.3±24.9	164.8±23.5	20.0±3.4	1.1±0.3	0.4±0.2
CP	6/4	59.0±4.4	173.3±32.8	157.6±31.3	14.2±3.8	1.1±0.6	0.3±0.2
IPF	20/6	65.7±1.6	252.0±48.7	169.4±25.3	44.1±17.4	19.9±8.2	19.0±11.3
COP	11/7	59.1±3.3	297.2±57.7	160.9±23.2	113.8±29.7[1), 2)]	9.9±5.3	12.6±6.1
CEP	9/6	40.3±3.6	1037.9±226.7[1), 2)]	252.4±52.4	139.3±45.4[2)]	23.3±13.5	623.3±206.0[1), 2)]
HP	6/4	54.8±3.0	552.0±134.8[1), 2)]	185.3±51.5	342.6±110.2[1), 2)]	8.4±3.9	16.3±9.9

[1)]Significantly different from NV [2)]Significantly different from CP
Abbreviations : AM ; alveolar macrophages, Ly ; lymphocytes, Neut ; neutrophils, Eo ; eosinophils, NV : normal volunteers, CP : control patients, IPF ; idiopathic pulmonary fibrosis, COP ; cryptogenic organizing pneumonia, CEP ; chronic eosinophilic pneumonia, HP ; hypersensitivity pneumonitis.

of proteases in determining the diagnosis and activity of interstitial lung diseases, we examined the activities of cathepsins, thrombin and aminopeptidase in BAL fluid, and compared them with clinical parameters.

MATERIALS AND METHODS

Study population

Clinical data of patients used in this study are shown in Table 1.

Patients with interstitial lung diseases : The subjects studied consisted of 69 patients with interstitial lung diseases ; 26 IPF, 18 COP, 15 CEP and 10 HP. The diagnosis was based on clinicopathological evaluation. Control subjects : The control population consisted of 15 normal volunteers (NV) and 10 control patients (CP). None of the NV showed any abnormalities on physical examination, chest radiography or in lung function tests. All the CP were free of interstitial lung disease ; 8 had localized lung cancer and 2 had no detectable lesion in the lungs although they complained of hemosputum.

Bronchoalveolar lavage

BAL was performed for patients with interstitial lung diseases and control subjects as described previously (9). Briefly, a flexible fiberoptic bronchoscope (Model 1T 20 ; Olympus Co., Tokyo, Japan) was wedged into a segmental or subsegmental bronchus of the middle lobe or lingula, and lavage was performed with a total volume of 150 ml of sterile 0.9% saline in three 50-ml portions. The lavage fluid was gently aspirated by syringe after deep inspiration. The fluid recovered was passed through a sterile gauze, and centrifuged at 250xg for 10 min at 4°C to precipitate cells, and the supernatant (BAL fluid) was stored at -70°C until examination.

Analysis of bronchoalveolar cells

The precipitated cells were analyzed. The total number of cells, suspended in an appropriate volume of saline, was counted in a hemocytometer. Differential counts on 500 cells were carried out on smears of sedimented cells stained with May-Giemsa stains, and the number of alveolar macrophages, lymphocytes, neutrophils and eosinophils were calculated.

Determination of cathepsin H, cathepsin B, thrombin and aminopeptidase

Protease activities were assayed fluorometrically using the specific substrate as described previously (9, 10). Arg-4-methyl-coumaryl-7-amide (MCA), Z-Arg-Arg-MCA, Boc-Val-Pro-Arg-MCA, and Leu-MCA (Peptide Institute, Osaka, Japan) were used to measure the activities of cathepsin H, cathepsin B, thrombin, and aminopeptidase, respectively. In brief, 80 μl of 0.1M Tris-HCl buffer, 100 μl of 100 μM substrate diluted with the buffer and 20 μl of samples were added into the wells of 96-multiwell plates (C8 White Maxisorp, Nunc, Denmark). The standard contained various amounts of MCA. The plates were incubated at 37°C for 1 h. The fluorescence intensity was measured in MTP-32 (Corona Electric Co., Ibaragi, Japan) with 365 nm for excitation and 450 nm for emission wavelengths. The protease activity is expressed in nanomoles of substrate cleaved per hour.

Determination of cathepsin G activity

Leukocye and mast cell proteases are classified as tryptic (tryptase and granzyme A), elastolytic (neutrophil elastase and proteinase-3) and chymotryptic

(cathepsin G and mast cell chymase). In this study, cathepsin G enzyme was measured using a thiobenzylester substrate, Succ-Phe-Leu-Phe-S-Bzl (Sigma Chemical Co., U.S.A.) which was shown to be the most sensitive substrate for cathepsin G (11, 12). To monitor enzyme activities, assays were performed at room temperature by using 0.5 mM 5, 5*-ditiobis-(2-nitrobenzoic acid) (DTNB ; Sigma Chemical Co., U.S.A.) to detect the thiobenzyl leaving group. The activity was determined using a microtiter assay activity. In brief, 50 μl of sample was added to 100 μl of 1 mM DTNB made up in 10 mM Hepes, 1mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.2. The reaction was initiated by the addition of 50 μl of Succ-Phe-Leu-Phe-S-Bzl to give a final concentration of 62.5 μM. The duration of the assay depended on color development, the rate of which was measured on a microplate reader at 410 nm. Medium control, DTNB alone, and DTNB and substrate were always run in parallel. Cathepsin G activity was expressed as μg/ml by determining a standard curve using cathepsin G.

Statistical analysis

All results are expressed as mean ± SEM. Statistical analysis was performed using the Student's two-tailed unpaired t test for comparisons between two groups. Correlations between two parameters were evaluated using Pearson's test. Differences were considered significant if p values were 0.05 or less. Data were analyzed on a computer using Statview software.

RESULTS

BAL cell analysis

The total and differential counts of cells in BAL fluid were shown in Table1. The number of total cells in BAL fluid from patients with CEP and HP was significantly higher than that from NV and CP. Differential cell analysis of BAL cells showed significantly increased number of lymphocytes in patients with COP, CEP and HP when compared with NV and CP. Increased number of neutrophils was detected in patients with IPF, COP, CEP and HP though there was no significant defference in the results when compared with NV or CP. Significantly higher number of eosinophils was detected in BAL fluid from patients with CEP than in that from NV and CP.

Activities of cathepsin H, cathepsin B and cathepsin G

Low activities of cathepsin H, cathepsin B and cathepsin G were detected in BAL fluid from NV and CP. Significantly higher activity in CP than in NV was observed only in data of cathepsin H (Fig. 1). Significantly increased activity of cathepsin H was detected in BAL fluid from patients with IPF, COP, CEP and HP when compared with the value of NV. BAL fluid from patients with IPF, COP and CEP contained increased activity of cathepsin B, but significantly higher activity than the value of NV was found only in patients with CEP. Increased activity of cathepsin G was detected in some of patients with interstitial lung diseases, but there was no significant difference when compared with the activity of NV. Patients with higher protease activity are defined as those whose protease activities exceeded the mean±2SD of NV (normal

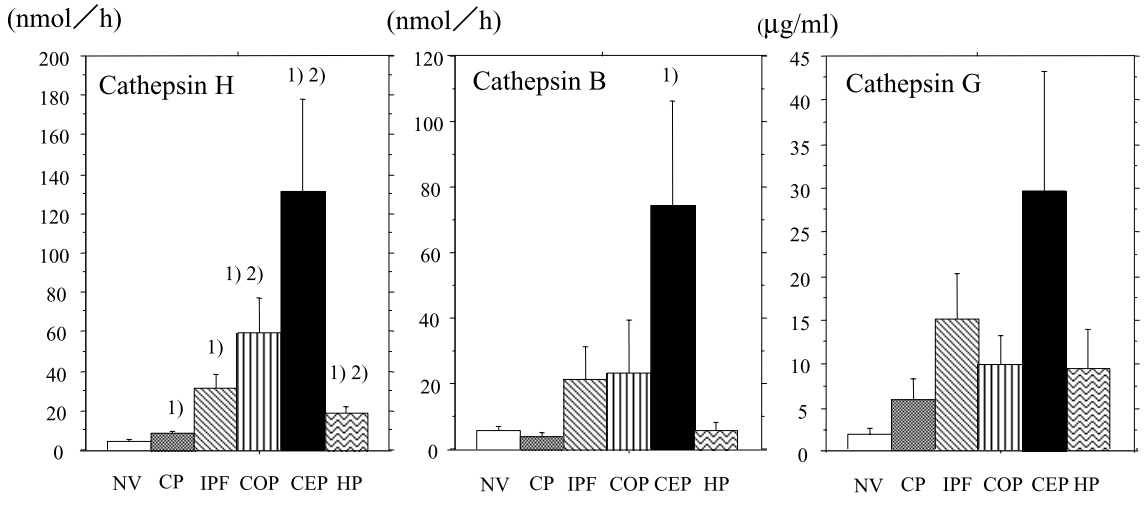

Figure. 1. The activity of cathepsins in the BAL fluid from normal volunteers (NV), control patients (CP), and patients with interstitial lung diseases. The activity was assayed as shown in Materials and Methods.[1)]Significantly different from the value of NV ($p<0.05$).[2)]Significantly different from the value of CP ($p<0.05$).

Table 2. Percentages of patients with higher protease activity

Proteases	Percentages of patients with higher protease activity			
	IPF	COP	CEP	HP
Cathepsin H	53.8	72.2	86.7	60.0
Cathepsin B	26.9	17.6	64.3	10.0
Cathepsin G	40.0	47.1	60.0	30.0
Thrombin	40.0	52.9	85.7	0.0
Aminopeptidase	65.4	72.2	100.0	50.0

Patients with higher protease activity are defined as those whose protease activities exceeded the mean±2SD of NV (normal volunteers).

Abbreviations : IPF ; idiopathic pulmonary fibrosis, COP ; cryptogenic organizing pneumonia, CEP ; chronic eosinophilic pneumonia, HP ; hypersensitivity pneumonitis.

volunteers), and their percentages are shown in Table 2. Higher activity of cathepsin H was detected in most of patients with COP (72.2%) and CEP (86.7%), whereas percentages of patients with higher activity of cathepsin B were high only in patients with CEP (64.3%). Patients with higher activity of cathepsin G were found in 60.0% of patients with CEP, in 47.1% of those with COP, in 40.0% of those with IPF, and in 30.0% of those with COP.

Activities of thrombin and aminopeptidase

Low activities of thrombin and aminopeptidase were detected in BAL fluid from NV and CP (Fig. 2). There was no significant difference in the activities between NV and CP. The activity of thrombin was significantly increased in BAL fluid from patients with IPF and CEP when compared with the value of NV. Significantly higher activity of aminopeptidase was detected in patients with IPF, COP, CEP and HP than the value of NV. As shown in Table 2, percentages of patients with higher activity of thrombin were 85.7% in CEP, 52.9% in COP, 40.0% in IPF and 0% in HP. Higher activity of aminopeptidase was detected in BAL fluid from most of patients with interstitial lung diseases.

Comparison of protease activities in the BAL fluid with the number of BAL cells

Neutrophils have been shown to play a critical role in the pathogenesis of IPF (2, 3). On the other hand, COP and HP are diseases characterized by lymphocytes which are responsible for pathogenesis of these disorders (5, 7). Therefore, to determine the role of the proteases in the pathogenesis of interstitial lung

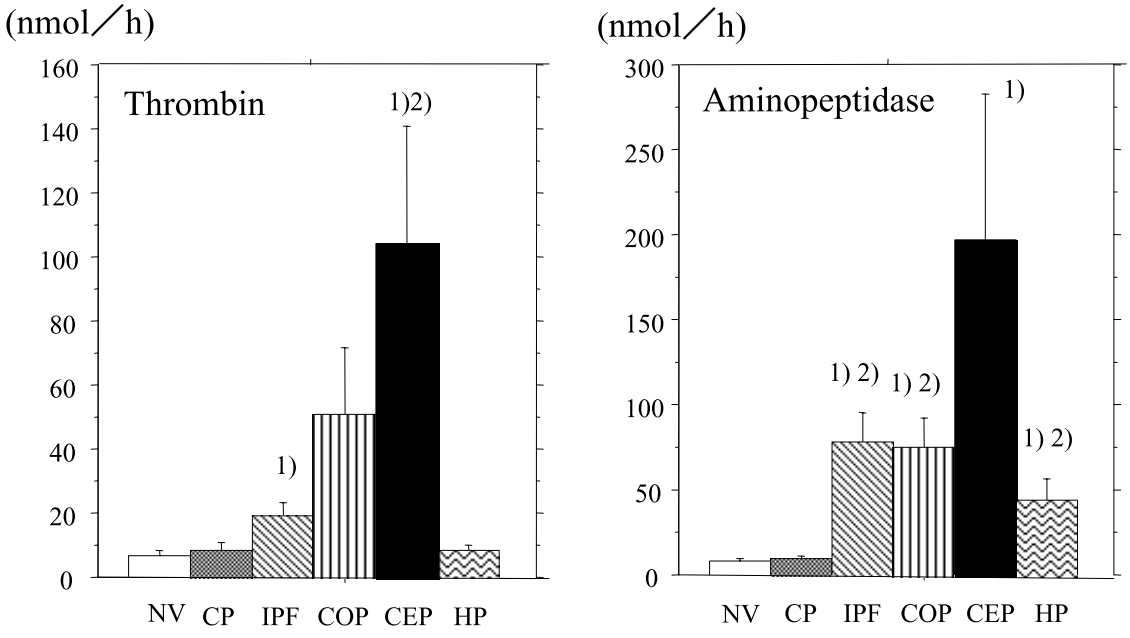

Figure. 2. The activity of thrombin and aminopeptidase in the BAL fluid from normal volunteers (NV), control patients (CP), and patients with interstitial lung diseases. The activity was assayed as shown in Materials and Methods. [1)]Significantly different from the values of NV ($p<0.05$).[2)]Significantly different from the value of CP ($p<0.05$).

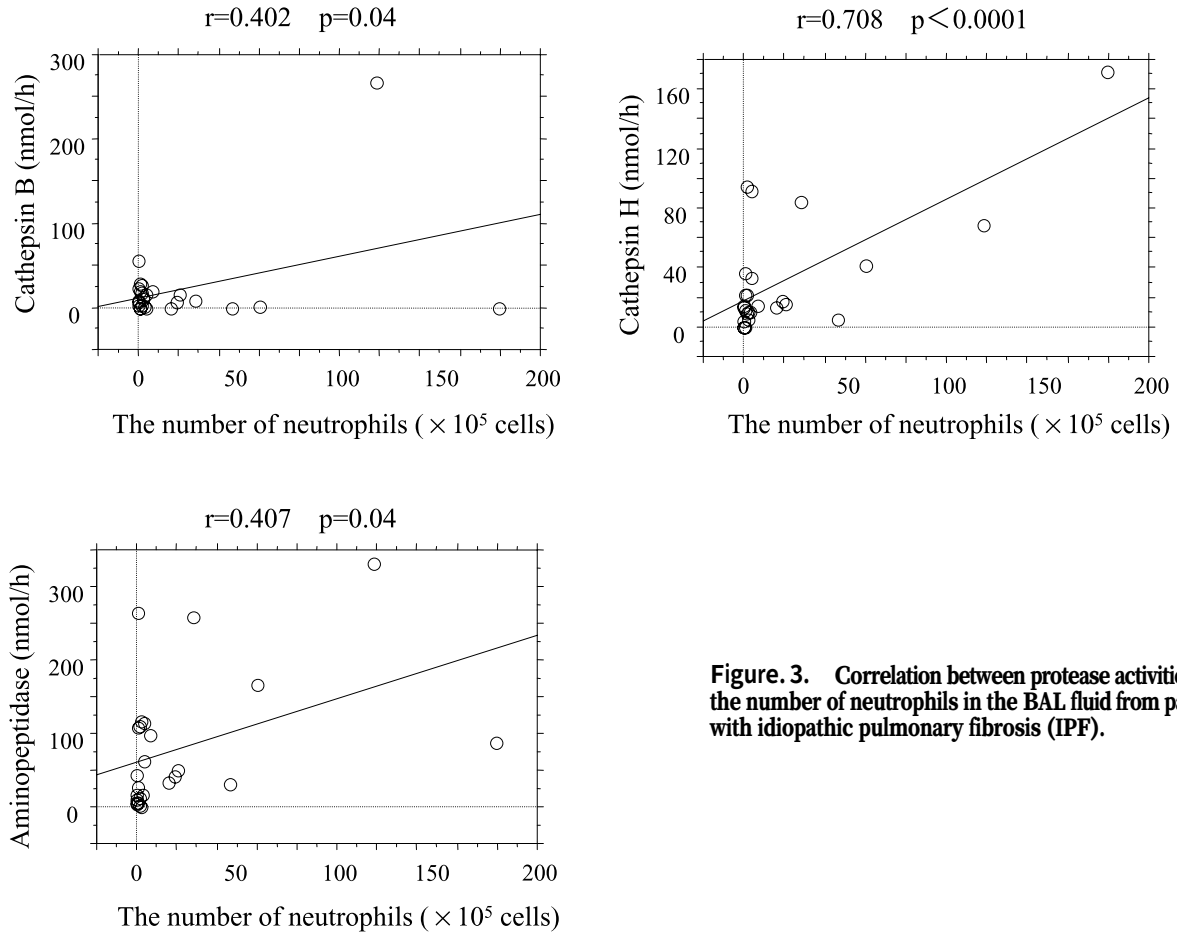

Figure. 3. **Correlation between protease activities and the number of neutrophils in the BAL fluid from patients with idiopathic pulmonary fibrosis (IPF).**

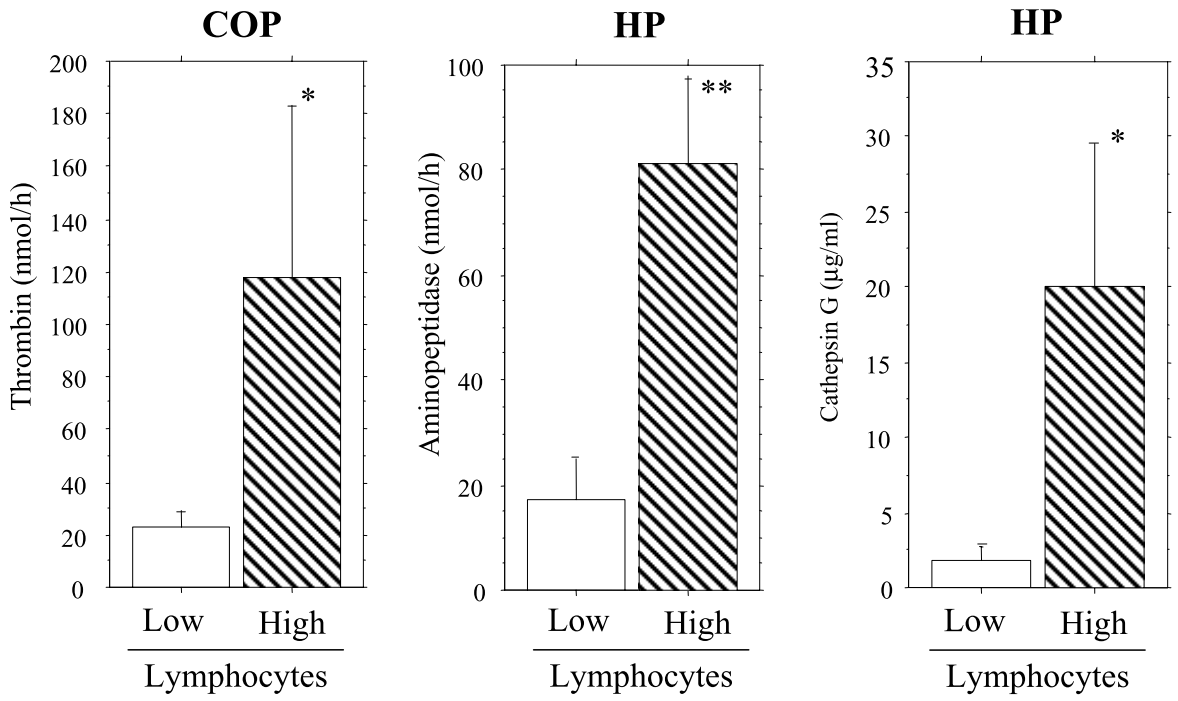

Figure. 4. **Comparison of protease activities in BAL fluid with higher number of lymphocytes and lower number of lymphocytes from patients with COP and HP. COP : High>120×10⁵ of lymphocytes, Low<120×10⁵of lymphocytes. HP : High>350×10⁵of lymphocytes, Low<350×10⁵of lymphocytes. * p<0.05,**p<0.01.**

diseases, the correlation between the protease activity and the number of BAL cells was examined. In patients with IPF, there were significant correlations between neutrophil number and the activity of cathepsin B, cathepsin H or aminopeptidase (Fig. 3). In patients with COP, the activity of thrombin was significantly higher in patients with higher lymphocyte number than in those with lower lymphocyte number (Fig. 4). In patients with HP, the activities of aminopeptidase and cathepsin G were significantly higher in patients with higher lymphocyte number than in those with lower lymphocyte number.

DISCUSSION

In this study, we showed the increased activities of cathepsins, thrombin and aminopeptidase in BAL fluid of patients with various interstitial lung diseases when compared with the value of NV and CP.

Significantly higher activity of cathepsin B was found only in patients with CEP. Higher cathepsin B activity was detected in 64.3% of patients with CEP. These results suggest that the activity of cathepsin B may be useful in the diagnosis of CEP. Cathepsin B belongs to lysosomal thiol proteinases (13), and is found in lysosomal fractions of a variety of cells throughout the body including monocytes/macrophages (14, 15). Cathepsin B is present in monocytes/macrophages and the activity is increased during differentiation of human monocytes into macrophages (15, 16). Human lung macrophages were shown to highly express cathepsin B (17). Although the role of cathepsin B in CEP is unclear, cathepsin B may have a role in connective tissue degradation in this disorder because this protease has been shown to be involved in lysosomal proteolysis, protein processing (18-20) and degradation of native collagen and elastin (17, 21-23).

This study showed the most remarkable increase in the activity of cathepsin H in BAL fluid from patients with interstitial lung diseases. The activity of cathepsin H was significantly increased in BAL fluid from patients with IPF, COP, CEP and HP. In patients with IPF, the activity of cathepsin H significantly correlated with the number of neutrophils. Cathepsin H belongs to lysosomal thiol proteinases (24) but is distinct from cathepsin B. Cathepsin H has strong aminopeptidase activity and little endoproteinase activity, whereas cathepsin B mainly exhibits endoproteinase activity (25). Cathepsin H is found at high levels in the lung, liver, kidney and spleen. On the other hand, cathepsin B is mainly detected in the kidney, spleen and adrenal gland. The lung contained higher levels of cathepsin H than cathepsin B (25), corresponding to this result that the activity of cathepsin H was higher than that of cathepsin B in BAL fluid from patients of interstitial lung diseases. Ishii *et al*. showed that cathepsin B is secreted from alveolar macrophages, but cathepsin H is secreted from both Type II pneumocytes and alveolar macrophages (26). In this study, most (72.2%) of patients with COP contained higher activity of cathepsin H in BALF but a few (17.6%) of them contained higher activity of cathepsin B. These results suggest that there are differences between cathepsin B and cathepsin H in their roles in the pathogenesis of interstitial lung diseases.

Significantly higher activity of aminopeptidase was detected in patients with IPF, COP, EP and HP. The activity of aminopeptidase was significantly increased in BAL fluid from 100% of patients with CEP, 72.2% of COP, 65.4% of IPF, and 50.0% of HP. The activity of aminopeptidase was significantly higher in HP patients with higher lymphocyte number than in those with lower lymphocyte number. Our previous reports showed that the activity of aminopeptidase in BAL fluid is mainly due to aminopeptidase N derived from macrophages which has chemotactic activity for T lymphocytes (9, 27). These results suggest that aminopeptidase has a role in migration of T lymphocytes into the disease site of interstitial lung diseases.

Increased activity of thrombin was detected in BAL fluid from patients with IPF, CEP and COP, but not with HP. The activity of thrombin was significantly higher in patients with higher lymphocyte number than in those with lower lymphocyte number in patients with COP. These results suggest that thrombin has a role in the pathogenesis of alveolitis of interstitial lung diseases. Thrombin is a multifunctional protease which is converted from circulating prothrombin synthesized in the liver at sites of tissue injury by factor Xa(28). Although the reason of low thrombin activity in HP patients is unclear, it is possible that there is a difference in the role of blood coagulation in the pathogenesis between HP and other interstitial lung diseases.

The data presented here indicate that the activity of cathepsins, thrombin and aminopeptidase in BAL fluid may serve as a marker of diagnosis and activity of interstitial lung diseases, and suggest that they may participate in the pathogenesis of tissue inflammation in these disorders. A greater understanding of the regulation of the production and action of these proteases may lead to new insights for the control and

treatment of interstitial lung diseases.

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