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Cysteine proteinases and cystatin C in bronchoalveolar lavage fluid from subjects with subclinical emphysema

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ABSTRACT: This study examined the role of cysteine proteinases and their inhibitor in the development of emphysema in comparison with neutrophil elastase (NE) complexed with α_1-protease inhibitor (NE-α_1-PI), which was previously demonstrated to be increased in bronchoalveolar lavage (BAL) fluid from subjects with subclinical emphysema.

Eight nonsmokers and 31 current smokers with (n=17) and without (n=14) emphysema, as evidenced by lung computed tomographic scans, were studied.

The concentrations of immunologically detected cathepsin L and cystatin C, but not cathepsin B, were significantly increased in BAL fluid from the smokers with emphysema compared with those without emphysema, although the activity of cathepsin L, measured using a synthetic substrate and cathepsin L released from cultured alveolar macrophages at 24 h, did not show any significant difference between the two groups. When comparison was made only for the subjects aged <60 yrs, the difference between the two groups disappeared for cathepsin L, but remained for NE-α_1-PI. There was no significant correlation between the level of cathepsin L and that of NE-α_1-PI in BAL fluid from the subjects with emphysema.

In conclusion, increased levels of cathepsin L and cystatin C were demonstrated in bronchoalveolar lavage fluid from subjects with subclinical emphysema. However, the roles of cathepsin L and neutrophil elastase in the development of emphysema may vary between subjects and between the young and the old.

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Protease–antiprotease imbalance theory has been a central theme for the last 30 years in the study of the pathogenesis of pulmonary emphysema [1, 2]. Although smoking is a major exogenous cause of pulmonary emphysema, only a small percentage of smokers develop clinically apparent pulmonary emphysema. Numerous studies have examined the effect of cigarette smoking on the protease–antiprotease balance in body fluids, but few have focused on individual differences in the susceptibility to pulmonary emphysema. Most studies have simply compared smokers and nonsmokers [3, 4] or healthy subjects and patients with chronic obstructive pulmonary disease (COPD), mostly clinically diagnosed through pulmonary function tests [5]. However, the data from subjects with established COPD could be influenced by possible concomitant infections of the respiratory tract.

It was previously demonstrated that neutrophil elastase (NE) complexed with α_1-protease inhibitor (α_1-PI; NE-α_1-PI complex) in bronchoalveolar lavage (BAL) fluid was significantly increased in community-based older volunteers who had subclinical emphysema, as evidenced by lung computed tomographic (CT) scans, compared with those who had a comparable smoking history but no evidence of emphysema [6]. In addition, the releasability of NE from alveolar macrophages (AM) was shown to be high in those subjects with emphysema [7]. FINLAY *et al.* [8] recently reported that the levels of collagenase and gelatinase B in BAL fluid from subjects with clinically apparent emphysema were significantly higher than in healthy subjects.

Another important class of proteases which is a candidate for causing chronic pulmonary destruction consists of the cysteine proteases, one of which, cathepsin L, has potent elastolytic activity at acidic pH [9, 10] and is capable of inactivating α_1-PI catalytically [11]. The expression of cathepsin L messenger ribonucleic acid (mRNA), as well as the activity of cathepsin L, was reported to be higher in BAL cells from smokers than in nonsmokers [12]. It is also possible that chronic smoking may decrease the level of cystatin C, an inhibitor of cathepsin L in the lungs, so that emphysematous changes are more likely to occur [13]. However, to our knowledge, there have been no reports on the levels of cathepsin L and cystatin C in BAL fluid from subjects with subclinical emphysema.

In this study, the activities and immunological quantities of cathepsin L and cystatin C in BAL fluid from asymptomatic community-based older volunteers were evaluated and the releasability of cathepsin L from AM was examined *in vitro*. Cathepsin B was also measured in BAL fluid, which has little elastolytic activity. In particular, the

study investigated whether the data on cathepsin L and cystatin C in BAL fluid and AM were similar to those found for NE in previous studies [6, 7].

Materials and methods

Subjects

A total of 39 asymptomatic community-based older volunteers were enrolled in the study, which examined concentration and/or activity of cathepsins L and B, and cystatin C in the supernatant of BAL fluid. Informed consent was obtained from all subjects. The study was approved by the Ethics Committee of Hokkaido University School of Medicine. The subjects ages ranged 32–78 yrs, with a mean±SD of 60±11. The nonsmokers consisted of five nonsmokers and three exsmokers who had stopped smoking >5 yrs previously and whose mean value for pack-yrs of smoking was only 4. Smokers were classified into those with (n=17) or without (n=14) emphysema according to the lung CT scans. None of the subjects were receiving regular medication or had a history of asthma or other respiratory disorders. They had had no episodes of respiratory tract infection during at least the preceding 3 months. They showed no abnormalities on physical examination and chest radiography and had no signs of inflammation in the blood tests described below.

Five of the 39 subjects in the above study and 10 newly-enrolled community-based older volunteers served as subjects for the study examining the releasability of cathepsin L from AM. They were classified into two groups: subjects with emphysema (n=8) and those without emphysema (n=7). All of them gave informed consent. Their mean age was 60±11 yrs.

Pulmonary function tests

Pulmonary function tests were performed within 1 month before the BAL procedure. Forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1) were measured with a spirometer (CHESTAC-55V; Chest Co., Tokyo, Japan). The diffusing capacity of the lung for carbon monoxide ($D_{L,CO}$) was measured using the single-breath method.

Lung computed tomography scans

All subjects underwent lung high-resolution CT scans at a window level of -700 Hounsfield units (HU) and a width of 1,500 HU *via* a Yokokawa CT 9000 unit (Yokokawa Co., Tokyo, Japan). Serial horizontal slices with a slice width of 2 mm were obtained at 10 mm intervals from the apex to below the diaphragm. Breath was held at the midinspiratory state during scanning. Three pulmonary physicians, who were blinded to any information about individual smoking history, pulmonary function tests and BAL fluid data, independently evaluated the presence of low-attenuation areas in all slices. The subject was defined as having emphysema if all three doctors agreed that low-attenuation areas were present in any slice, regardless of size and severity. The severity of emphysema for the "emphysema" group was visually quantified, by scoring the overall areas involving emphysematous changes, for all slices, into five grades as described previously [14]: low-attenuation area with involvement of 1, <5%; 2, 5–25%; 3, 25–50%; 4, 50–75%; 5, 75–100%.

Blood analysis

Venous blood was taken 30 min before the BAL procedure. Routine tests for blood cell count, blood chemistry and C-reactive protein were conducted to ascertain that the subject had a normal nutritional state and liver function and no signs of inflammation. The plasma concentration of cotinine, which is the main metabolite of nicotine and biochemically represents the cumulative dose of exposure to cigarette smoke for the previous few days [15], was measured using gas chromatographic techniques. Measurement of the cotinine concentration in plasma, therefore, helped to identify and reconfirm noncurrent *versus* current smokers who had been classified by history-taking.

Bronchoalveolar lavage procedure

BAL was carried out in one of the subsegmental bronchi of the right middle lobe, using a modification of the technique introduced by RENNARD *et al*. [16], which has been described previously [6]. All subjects had refrained from smoking for at least 12 h before the procedure to eliminate the acute effects of smoking. Four separate 50 mL aliquots of sterile 0.9% saline were infused through a wedged flexible fibreoptic bronchoscope (Olympus BF- B3R; Olympus, Tokyo, Japan). The fluid returned from the first 50 mL aliquot was not used for this study because it is known to contain cells and material more from large airways than from peripheral regions. The remaining lavage fluid was used as the alveolar fluid fraction. The sample did not contain visible blood contamination.

Processing of bronchoalveolar lavage fluid

Recovered BAL fluid was filtered through several layers of gauze to remove excess mucus and debris and then centrifuged at 400×*g* for 5 min at 4°C to separate the supernatant from cells. The supernatant was then frozen at -70°C for later analysis. The cell pellet was washed twice and resuspended in 5 mL phosphate-buffered saline (PBS). Cell viability was estimated by exclusion of trypan blue, which was >90% in all cases. Differential cell counts were made from cytocentrifuge preparations using Diff-Quik (International Reagents, Kobe, Japan) staining. In the second study, BAL cells were resuspended in RPMI- 1640 medium (Gibco, Grand Island, NY, USA) containing 100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin at 1×10^6 $cells \cdot mL^{-1}$. Cell suspension, 1 mL, was layered on to 30 mm poly-L-lysine-coated culture dishes (Corning Glass Works, Corning, NY, USA) and incubated for 1 h at 37°C in 5% CO_2, 95% air to allow the AM to adhere. The dishes were washed vigorously with PBS to remove all nonadherent cells. No neutrophils were seen in any of these preparations of cells in culture. Adherent AM were then

incubated under the same ambient conditions for an additional 1, 6 or 24 h. At the end of each culture period, the medium was collected, frozen and stored at -70°C for later analysis.

Immunological quantitation of cathepsins L and B

Cathepsins L and B were measured, using an enzyme-linked immunosorbent assay (ELISA) kit purchased from RD Laboratory (Munich, Germany), in unconcentrated BAL fluid, and cathepsin L in culture medium at 1×10^6 cells·mL^{-1} at 1, 6 and 24 h. This ELISA procedure employed a goat anti-cathepsin L or B antibody coated on a plastic plate and a rabbit anti-human cathepsin L or B antibody conjugated with horseradish peroxidase. Albumin was measured (mg·L^{-1}) in the same unconcentrated BAL fluid supernatant using an immunoradiometric assay and all of the data from unconcentrated BAL fluid were expressed relative to the concentration of albumin.

Because cell viability under these culture conditions could affect the releasability of cathepsin L, lactate dehydrogenase (LDH) was measured simultaneously in the culture medium as an index of cell viability [17].

Measurement of cathepsin L activity

To determine the activity of cathepsin L, the cleavage of the methylcoumaride substrate, carbobenzoxy-phenylalanine-L-arginine 4-methyl-coumaryl-7-amide (Z-Phe-Arg-MCA), specific for cathepsins L and B [18], was measured fluorometrically in the presence of a selective cathepsin B inhibitor, a synthetic epoxysuccinyl peptide, *N*-(L-3-trans-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline (CA-074; Peptide Institute, Osaka, Japan) [19]. The incubation buffer was 0.2 M sodium acetate, adjusted to pH 5.5, containing 2 mM ethylenediaminetetraacetic acid (EDTA) and with 8 mM L-cysteine added just before use. Activity was defined as the amount of enzyme required to cleave 1 mmol substrate·h^{-1}. Specific activity in BAL fluid was expressed in mmol methylcoumarylamide produced·h^{-1}·mg albumin^{-1}.

Immunological quantitation of cystatin C

This ELISA procedure employed a rabbit anti-human cystatin C antibody (Dako, Glostrup, Denmark) coated on a plastic plate, followed by a mouse monoclonal anti-human cystatin C antibody conjugated with horseradish peroxidase (Mitsui Pharmaceutical Company, Tokyo, Japan). After the addition of stop solution, the resulting colour was measured spectrophotometrically at 450 nm. Results from samples were extrapolated on the standard curve. The detection limit of this system was 16.9 ng·mL^{-1}.

Immunological quantitation of neutrophil elastase-α_1-protease inhibitor complex in bronchoalveolar lavage fluid

The immunoreactive NE-α_1-PI complex was measured using an ELISA kit purchased from Merck (Darmstadt, Germany) in unconcentrated BAL fluid for all subjects. Details of measurements have been described previously [6].

Data analysis

All values are expressed as mean±SEM. In the study examining the concentration and/or activity of cathepsins L and B and cystatin C in the supernatant of BAL fluid, the differences in the mean values in the three groups were analysed using the nonparametric Kruskal–Wallis test because the data were not normally distributed. The Mann–Whitney U-test was then applied for comparison between the two groups of three as a *post hoc* test, where appropriate, and p-values <0.05 were accepted as significant. In the study examining the releasability of cathepsin L from AM, the differences in the mean values were analysed using the Mann–Whitney U-test and p-values <0.05 were accepted as significant.

Results

Characteristics of subjects

According to smoking habits and CT findings, the subjects were classified into three groups: eight nonsmokers without emphysema, 14 smokers without emphysema and 17 smokers with emphysema (table 1). All of the nonsmokers, but none of the smokers, had a plasma cotinine concentration of <5.0 ng·mL^{-1}, which proved them to be biochemically free from smoke exposure. Neither the pack-yrs smoking nor the plasma cotinine concentration differed significantly between the current smokers with and without emphysema, although a few subjects with emphysema had a markedly higher number of pack-yrs smoking, giving a higher mean value (NS) in the subjects with emphysema than in the others. No subjects with α_1-PI-deficiency were found in this study and there was no significant difference in the plasma levels of α_1-PI between the three groups.

Pulmonary function tests revealed that FEV1/FVC was slightly, but significantly, lower in the smokers with emphysema, but the mean value was still within the normal range. The differences in $D_{L,CO}$ (% predicted) did not reach statistical significance between the three groups. Among subjects who had emphysematous changes on lung CT scans, the scores ranged 1–3, with a mean±SD of 1.7±0.8, which indicated that they had, at most, mild emphysema. The proportions of AM in the smokers were significantly higher than those in the nonsmokers. The recovery rate of fluid and total cell numbers, and the proportions of macrophages and neutrophils were not significantly different in the smokers with and without emphysema (table 2).

In the study examining the releasability of cathepsin L from AM, $\%D_{L,CO}$ was significantly lower and FEV1/FVC tended to be lower in subjects with emphysema than in those without emphysema (mean±SEM 85±6 *versus* 104±5%, p<0.05 for $D_{L,CO}$; 67±4 *versus* 74±2%, NS for FEV1/FVC). The two groups had similar mean ages (64±3 *versus* 62±5 yrs, NS).

Table 1. – Characteristics of subjects

	Nonsmokers	Smokers	
		Nonemphysematous	Emphysematous
Number of subjects	8	14	17
Age yrs	68±2	58±3	59±3
Smoking pack-yrs	4±1*	38±4	47±8
Cotinine ng·mL^{-1}	<5.0±0†	118±22	152±34
FEV1/FVC %	80±1	79±1	70±2‡
$D_{L,CO}$ % pred	113±8	107±2	95±5

Values are means±SEM. *: Smoking duration is shown for exsmokers (n=3) alone and does not include nonsmokers (n=5). FEV1: forced expiratory volume in one second; FVC: forced vital capacity; $D_{L,CO}$: diffusing capacity of the lung for carbon monoxide. †: p<0.01 *versus* smokers; ‡: p<0.01 *versus* nonsmokers and nonemphysematous smokers.

Cathepsins L and B in bronchoalveolar lavage fluid

Cathepsin L was detected in unconcentrated BAL fluid supernatant for all subjects. The concentration of cathepsin L·mg albumin^{-1} in BAL fluid was significantly increased in smokers with emphysema compared not only with nonsmokers, but also with smokers without emphysema (table 3). With regard to cathepsin B, although the smokers as a whole (n=19) had a significantly higher level than nonsmokers (n=5; 0.26±0.05 *versus* 0.08±0.02 μg·mg albumin^{-1}, p<0.05), there was no significant difference between the smokers with and without emphysema (table 3). The concentration of cathepsin L in the smokers did not correlate with either the plasma cotinine concentration or pack-yrs smoking. In addition, it did not correlate significantly with FEV1/FVC and %$D_{L,CO}$ in the subjects with emphysema.

Activity of cathepsin L in bronchoalveolar lavage fluid

There were no significant differences in the activity of cathepsin L in BAL fluid between the three groups (fig. 1) and comparison of the two groups of current smokers revealed little difference for this parameter. However, >35% of all the smokers, regardless of the presence of emphysema on lung CT scans, had cathepsin L activity in BAL fluid >0.19 μmol·mg albumin^{-1}·h^{-1}), which was higher than the highest value for nonsmokers. Thus, the activity of cathepsin L in BAL fluid in the combined group of smokers tended to be higher than that in nonsmokers (0.16± 0.03 *versus* 0.07±0.02 μmol·mg albumin^{-1}·h^{-1}, respectively, p=0.09).

Cathepsin L released from alveolar macrophages

Cathepsin L was detected in the culture medium of AM at 1 h in all eight subjects examined and its concentration increased in a time-dependent manner (n=8), although a high degree of variation was noted between subjects (fig. 2). The concentration of cathepsin L released from AM at 24 h of culture was not significantly different between the subjects with and without emphysema (218±14 *versus* 209±16 ng·mL medium^{-1}, NS) (fig. 3). Since no significant difference in the level of LDH in culture medium was found between the subjects with and without emphysema, it was assumed that the viability of the AM was not different between the two groups.

Cystatin C in bronchoalveolar lavage fluid

Cystatin C was detected in unconcentrated BAL fluid from four of seven nonsmokers, three of 11 smokers without emphysema and 12 of 15 smokers with emphysema. If all of the values below the limit of detection were assumed to be 8.45 ng·mL^{-1} for statistical analysis, the concentration of cystatin C·mg albumin^{-1} in BAL fluid was significantly higher in smokers with emphysema than in those without emphysema (table 3).

Comparison of cathepsin L with neutrophil elastase-α_1-protease inhibitor complex in bronchoalveolar lavage fluid

The level of cathepsin L showed no significant correlation with that of NE-α_1-PI in BAL fluid from the smokers with emphysema (fig. 4). When only subjects aged <60 yrs were chosen for comparison, a significant difference in the level of NE-α_1-PI was seen between the smokers with emphysema and those without emphysema, but not in the level of cathepsin L (fig. 5). In the older subjects, aged >60 yrs, a significant difference between the two groups was found only for cathepsin L, although a similar trend was observed for NE-α_1-PI.

Discussion

In this study, the concentrations of cathepsin L and cystatin C, but not cathepsin B, were significantly increased

Table 2. – Bronchoalveolar lavage findings

	Nonsmokers*	Smokers	
		Nonemphysematous	Emphysematous
Recovery rate %	51±6	50±5	38±4
Total cell numbers cells×10^4·mL^{-1}	13±4	23±4	34±9
Macrophages %	82±5†	91±1	93±1
Neutrophils %	0.8±0.3	0.8±0.2	1.5±0.5

Values are means±SEM. *: nonsmokers, n=5; exsmokers, n=3. †: p<0.01 *versus* smokers.

Table 3. – Cathepsin L, cathepsin B and cystatin C in bronchoalveolar lavage fluid

	Nonsmokers	Smokers	
		Nonemphysematous	Emphysematous
Cathepsin L μg·mg albumin^{-1}	0.17±0.04 (n=8)	0.29±0.07 (n=13)	0.54±0.10† (n=17)
Cathepsin B μg·mg albumin^{-1}	0.08±0.02 (n=5)	0.29±0.09 (n=6)	0.24±0.06 (n=13)
Cystatin C μg·mg albumin^{-1}	0.38±0.05 (n=7)	0.31±0.04 (n=11)	0.54±0.07‡ (n=15)

Values are means±SEM. †: p<0.05 *versus* nonsmokers and smokers without emphysema; ‡: p<0.05 *versus* smokers without emphysema.

in BAL fluid from smokers who had emphysema as evidenced by lung CT scans, compared with those who did not, although the two groups of smokers had comparable smoking histories and similarly increased numbers of total cells and AM in BAL fluid. The functional activity of cathepsin L in BAL fluid, however, did not differ between the two groups. These data demonstrate that the increased levels of cathepsin L and cystatin C in BAL fluid are another characteristic of subclinical emphysema, as was shown previously for NE-α_1-PI [6].

These data, however, do not necessarily indicate that cathepsin L and NE exert a similar effect in the development of emphysema. Firstly, an increased level of one protease in BAL fluid is not necessarily associated with an increased level of another protease, as is shown in figure 4. Secondly, when only the subjects aged <60 yrs were chosen for comparison, a significant difference in the level of NE-α_1-PI was found between the smokers with emphysema and those without emphysema, but not in the level of cathepsin L (fig. 5). Thirdly, the releasability of cathepsin L from AM was not enhanced in the subjects with emphysema in this study compared with those without emphysema, in contrast to the previous finding that NE-α_1-PI released from AM was significantly elevated in the former subjects [7].

Numerous studies have shown the effects of smoking on a number of proteases in BAL fluid; therefore, those proteases are all considered to be candidates for involvement in chronic lung destruction. They include serine proteases such as neutrophil elastase [3, 20], cysteine proteases such as cathepsin L [12] and metalloproteases such as the 92 kDa gelatinase [8]. It is, however, not fully understood whether or not those proteases present in the lungs are equally affected by smoking or whether or not they contribute equally to the development of pulmonary emphysema.

Cathepsin L is a cysteine protease that is stored together with other cysteine proteases in the lysosomes of macrophages, fibroblasts and other cells in the lungs as an active lysosomal enzyme [9]. The reasons why we focused on cathepsin L in this study are as follows. Firstly, alveolar macrophages, one of the main sources of this enzyme, are increased in number not only in BAL fluid from smokers [21] but also in lung specimens from patients with emphysema, where destructive lung changes are most prominent [22, 23]. Secondly, it has been shown that AM are capable of releasing this enzyme in a precursor (43 kDa) form and of changing it to a mature and active (25 kDa) form by acidifying the interface between the cell surfaces where the cells adhere [24–26]. Thirdly, this enzyme has powerful elastolytic activity at its acidic pH optimum that is two or three times as strong as leukocyte elastase at its own neutral pH optimum [9, 27]. LESSER *et al.* [28] demonstrated that intratracheal instillation of cathepsin B, another type of cysteine proteinase, can induce emphysema in hamsters, but its elastolytic activity is not prominent. In contrast to cathepsin B, cathepsin L can degrade elastin approximately 100 times more efficiently at its pH optimum of 5.5 [10]. It is of note that cathepsin L, immunologically detected in BAL fluid, but not cathepsin B, was a factor that differentiated the smokers who were developing emphysema from those who were not. In addition, cathepsin L can inactivate α_1-PI, an inhibitor of NE, by cleavage in the reactive site region [11].

In this study, the activity of cathepsin L in BAL fluid did not show any significant difference between the group

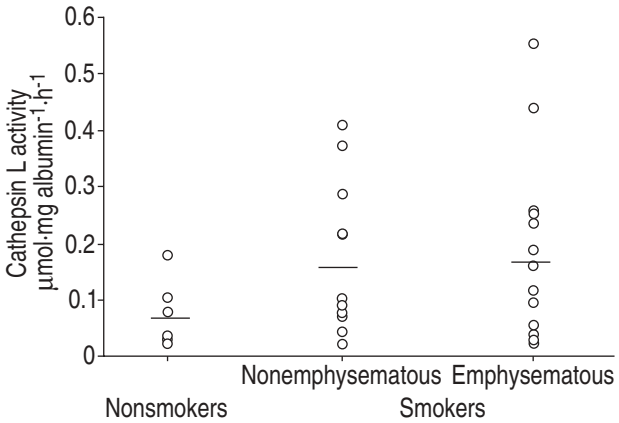

Fig. 1. – Activity of cathepsin L in bronchoalveolar lavage fluid from noncurrent smokers (n=8), smokers without emphysema (n=14) and smokers with emphysema (n=17). The term "emphysema" refers to emphysema diagnosed by computed tomographic scan. There were no significant differences between groups. Horizontal bars represent mean values.

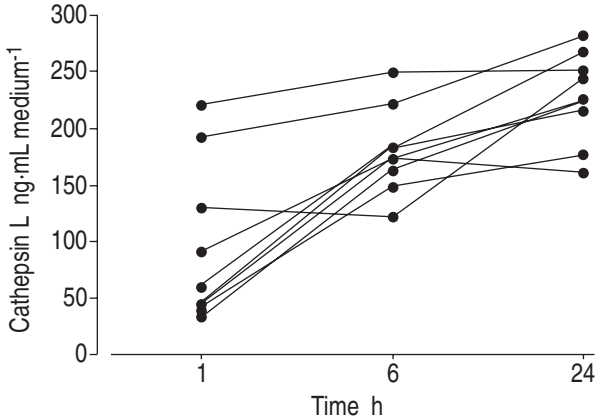

Fig. 2. – Concentrations of cathepsin L released from alveolar macrophages from current smokers (n=8) into culture medium at 1, 6 and 24 h.

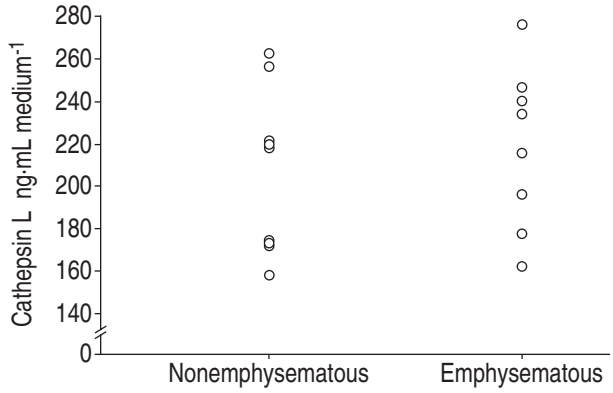

Fig. 3. – Concentrations of cathepsin L released from alveolar macrophages from subjects with emphysema (n=8) and those without emphysema (n=7) at 24 h of culture. There was no significant difference between the two groups.

of smokers who had emphysema and those who did not. This activity is reported to be elevated in BAL fluid as well as lysates of BAL cells from smokers [12], but other reports have suggested that the activity attributed to cysteine proteinases in BAL cell lysates may be due not only to cathepsin L but also to other cysteine proteinases [29]. As cathepsins S and K were recently discovered and reported to have an elastolytic activity, this may explain such undetermined activities in BAL cell lysates and/or in BAL fluid itself.

The level of cystatin C was significantly higher in BAL fluid in the smokers with emphysema than the smokers without emphysema. These data suggest that a low level of cystatin C does not account for the susceptibility to pulmonary emphysema. Cystatin C is a major inhibitor of cysteine proteinases and is found in a variety of human body fluids, such as spinal fluid, milk, seminal plasma and BAL fluid. There have been two conflicting reports on the level of cystatin C in the culture medium of alveolar macrophages from smokers and nonsmokers. CHAPMAN *et al*. [13] demonstrated that the level was lower in smokers than in nonsmokers, whereas WARFEL *et al*. [30] reported the opposite findings. However, there have been no reports on the level of cystatin C in BAL fluid from subjects with emphysema. This study is the first to demonstrate that the

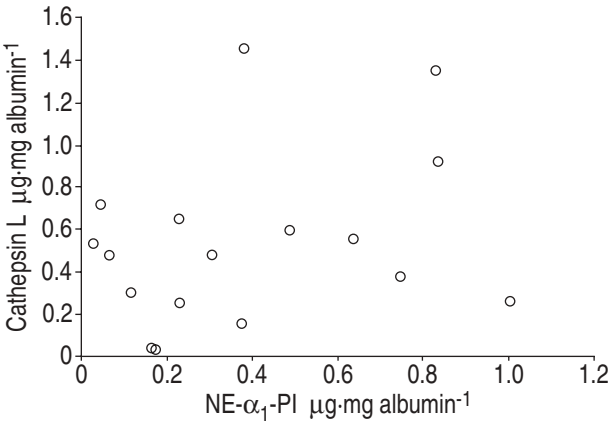

Fig. 4. – Relation between cathepsin L and neutrophil elastance complexed with α_1-protease inhibitor (NE-α_1-PI) levels in broncho-alveolar lavage fluid in the subjects who had emphysema. There was no significant correlation between cathepsin L and NE-α_1-PI levels.

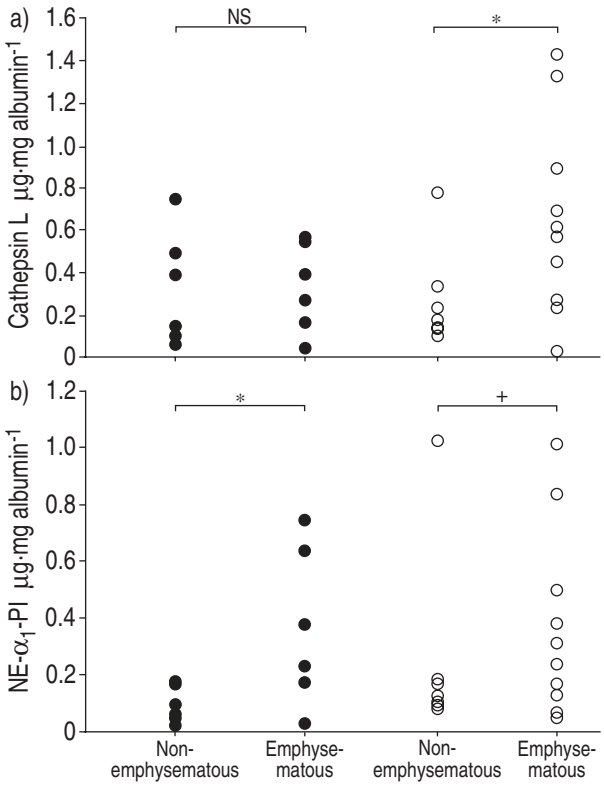

Fig. 5. – The concentrations of a) cathepsin L and b) neutrophil elastance complexed with α_1-protease inhibitor (NE-α_1-PI) in broncho-alveolar lavage fluid from 31 current smokers. Subjects were classified into four groups by age and presence or absence of emphysema; ●: subjects <60 yrs; ○: subjects Š60 yrs. +: p<0.1; *: p<0.05; NS: nonsignificant difference between groups.

level of cystatin C in BAL fluid is increased in subjects with subclinical emphysema.

Finally, FINLAY *et al*. [8] recently demonstrated that the levels of collagenase and gelatinase B in BAL fluid from subjects with emphysema were significantly higher than in those without emphysema. However, it remains to be elucidated whether this is true for subjects who are developing emphysema because the subjects in their study had more clinically apparent emphysema than those in the present study.

In conclusion, increased levels of cathepsin L and cystatin C were demonstrated in bronchoalveolar lavage fluid from subjects with subclinical emphysema. However, the roles of cathepsin L and neutrophil elastase in the development of emphysema may vary between subjects and between the young and the old.

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