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ONO-5046 is a potent inhibitor of neutrophil elastase in human pleural effusion after lobectomy

Tsutomu Sakuma [a,*], Keiji Takahashi [a], Nobuo Ohya [a], Katsuo Usuda [b], Masashi Handa [b], Tatsuya Abe [c]

[a] *Department of Respiratory Medicine, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan*
[b] *Department of Surgery, Sendai Kosei Hospital, Sendai, Japan*
[c] *Department of Respiratory Oncology and Molecular Medicine, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan*

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Abstract

The imbalance of neutrophil elastase and α_1-antitrypsin in pleural effusion after lobectomy and the effects of the neutrophil elastase inhibitors, sodium *N*-[2-[4-(2,2-Dimethylpropionyloxy)phenyl-sulfonylamino]benzoyl]aminoacetic acid (ONO-5046) and purified α_1-antitrypsin, on neutrophil elastase activity were determined. The amount of neutrophil elastase complexed to α_1-antitrypsin, measured by an enzyme-linked immunosorbent assay, was 170 times higher in pleural effusion than in blood 3 h after lobectomy. The α_1-antitrypsin levels measured by laser nephelometry did not increase in either blood or pleural effusion. Although neutrophil elastase activity, measured by the hydrolysis of succinyl-$(Ala)_3$-p-nitroanilide, was not detected in blood, it was increased in pleural effusion 3 h and 24 h after lobectomy. ONO-5046, but not α_1-antitrypsin, reduced the neutrophil elastase activity in pleural effusion. There is an imbalance of neutrophil elastase and α_1-antitrypsin in pleural effusion after lobectomy. ONO-5046 is a potent inhibitor of neutrophil elastase activity in human pleural effusion.

Keywords: Neutrophil; Proteinase–antiproteinase imbalance; Proteinase inhibitor; Pleural space; Thoracotomy

1. Introduction

Previously, we reported the pathway through which neutrophils moved into the pleural spaces in patients who underwent thoracotomy and in rabbits (Sakuma et al., 1992). Neutrophils release proteinases that play a role at sites of inflammation (Ohlsson and Olsson, 1974). However, little is known about whether proteinase activity is present in pleural effusion in patients after lobectomy.

Neutrophil elastase is a potent lysosome which hydrolyses tissue (Weiss, 1989; Weissmann and Korchak, 1984). Since neutrophil elastase is inactivated by α_1-antitrypsin, the destruction of tissue occurs only when there is an imbalance between neutrophil elastase and α_1-antitrypsin (Birrer et al., 1994; Lee et al., 1981; Ossanna et al., 1986). Therefore, purified α_1-antitrypsin has been administered as augmentation therapy for α_1-antitrypsin deficiency associated with emphysema (Wewers et al., 1987).

ONO-5046, sodium *N*-[2-[4-(2,2-Dimethylpropionyloxy)phenyl-sulfonylamino]benzoyl]aminoacetic acid, is a newly synthesized inhibitor of human neutrophil elastase (Kawabata et al., 1991). It is highly specific for neutrophil elastase and does not inhibit trypsin, thrombin, plasmin, plasma kallikrein, pancreas kallikrein, chymotrypsin or cathepsin G (Kawabata et al., 1991). The effects of ONO-5046 have been examined in lung injury (Kaneko et al., 1997; Kubo et al., 1994; Uchiba et al., 1996; Yoshimura et al., 1994), gastric ulcer (Harada et al., 1997; Murakami et al., 1997), ischemia–reperfusion liver injury (Kushimoto et al., 1996; Yamaguchi et al., 1997), rheumatoid arthritis (Kawabata et al., 1996), and endothelial cell injury (Furuno et al., 1997), but little information is available about human pleural effusion.

Therefore, we measured the time-course of changes in neutrophil counts, the amount of neutrophil elastase complexed to α_1-antitrypsin, the α_1-antitrypsin concentration, and neutrophil elastase activity in pleural effusion and in blood, and determined whether an imbalance between neutrophil elastase and α_1-antitrypsin is present in pleural

* Corresponding author. Tel.: +81-76-286-2211; Fax: +81-76-286-0980.

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effusion after lobectomy. Then, we determined the effects of the neutrophil elastase inhibitors, ONO-5046 and purified α_1-antitrypsin, on neutrophil elastase activity in ex vivo human pleural effusion.

2. Materials and methods

2.1. Materials

Purified human neutrophil elastase (Elastic Products, MO, USA), α_1-antitrypsin and catalase (Sigma, St Louis, MO, USA), and rabbit anti-human α_1-antitrypsin antibody (Dako, Glostrup, Denmark) were purchased. ONO-5046 was generously donated to this study (Ono Pharmaceutical, Osaka, Japan).

2.2. Methods

2.2.1. Neutrophil elastase and α_1-antitrypsin imbalance

To determine the neutrophil elastase and α_1-antitrypsin imbalance in pleural effusion of patients who had undergone lobectomy, we measured the neutrophil count, amount of neutrophil elastase complexed with α_1-antitrypsin, α_1-antitrypsin concentration, and neutrophil elastase activity sequentially in pleural effusion and in peripheral blood collected as previously reported (Sakuma et al., 1992). Briefly, lobectomies with dissection of the mediastinal lymph nodes were carried out in 10 patients with bronchogenic carcinoma. At the end of the thoracotomy, the pleural cavity was rinsed with 3000–5000 ml of warmed saline solution to remove blood and neutrophils. Then, two chest tubes (28F, Sherwood, St. Louis, MO, USA) were placed in the pleural cavity. Pleural effusion was drained through the tubes at a pressure of -10 cmH_2O. We collected samples of pleural effusion 3 h and 24 h postoperatively and samples of peripheral blood preoperatively, 3 h, 24 h, and 1 w postoperatively. The samples were collected in glass tubes with EDTA-2Na for measuring the neutrophil counts, the levels of neutrophil elastase, and the activity of neutrophil elastase and in glass tubes without EDTA-2Na for measuring the level of α_1-antitrypsin. To remove cells from the samples, the tubes were centrifuged at 3000 rpm for 10 min at 4°C. Supernatants of pleural effusion, plasma and serum were collected and stored at -80°C until the day of measurement. This study was approved by the Human Research Committee in Sendai Kosei Hospital. Informed consent was obtained from each patient before surgery.

2.2.2. Effects of neutrophil elastase inhibitors on neutrophil elastase activity

To determine whether the neutrophil elastase inhibitors affected neutrophil elastase activity in ex vivo pleural effusion, we investigated the effect of purified human α_1-antitrypsin or ONO-5046 on neutrophil elastase activity. We collected pleural effusion 3 h after lobectomy from patients who had undergone lobectomy for bronchogenic carcinoma ($n = 5$). Samples of pleural effusion were centrifuged at 3000 rpm and 4°C for 10 min. The supernatants were collected and incubated with different concentrations of purified human α_1-antitrypsin or ONO-5046 at 37°C for 1 h. Then, the neutrophil elastase activity in pleural effusion was measured.

Since purified α_1-antitrypsin did not inhibit the neutrophil elastase activity in the ex vivo pleural effusion, the following ex vivo experiments were performed. First, the effect of purified human α_1-antitrypsin on the activity of purified neutrophil elastase was measured in saline solution. Second, the production of neutrophil elastase-α_1-antitrypsin complex and the cleavage of α_1-antitrypsin were determined in pleural effusion, using Western blot analysis.

2.3. Measurements

2.3.1. Neutrophil counts

Neutrophils in pleural effusion were counted using a Neubauer hemocytometer. White blood cells in the peripheral blood were counted using an automatic counting machine (CC-800, Toua Iyoudenshi, Tokyo, Japan). Cell differentiation was performed by counting samples stained with May-Giemsa dye.

2.3.2. Complex of neutrophil elastase and α_1-antitrypsin

The levels of neutrophil elastase complexed with α_1-antitrypsin were determined by an enzyme-linked immunosorbent assay (Neumann et al., 1984).

2.3.3. α_1-antitrypsin

The levels of α_1-antitrypsin in the samples that were mixed with anti-α_1-antitrypsin serum from rabbits were determined by laser nephelometry (Shulman, 1979).

2.3.4. Neutrophil elastase activity

Neutrophil elastase activity was determined by the hydrolysis of succinyl-$(Ala)_3$-*p*-nitroanilide (Katagiri et al., 1979). Briefly, samples (0.1 ml) were incubated with 0.5 M succinyl-$(Ala)_3$-*P*-nitroanilide in 2 ml of 0.05 M Tris–HCl buffer (pH 7.5) at 37°C for 2 h. The reaction was terminated by adding 2 ml of 10% trichloroacetic acid and the mixture was centrifuged at 3000 rpm for 10 min to remove the precipitate. The supernatant (3 ml) was diazotized by adding 0.4 ml of 0.1% $NaNO_2$ in 6.5% HCl. The excess $NaNO_2$ was neutralized by 0.5 ml of 0.5% ammonium sulfamate, and 0.4 ml of 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 10% ethanol was added to this solution for color development. The pink to scarlet diazotized compound was measured at 550 nm. One unit (U) of elastase activity was defined as the amount of enzyme that converted 1 μmol of succinyl-$(Ala)_3$-*p*-nitroanilide in 1 min at 37°C.

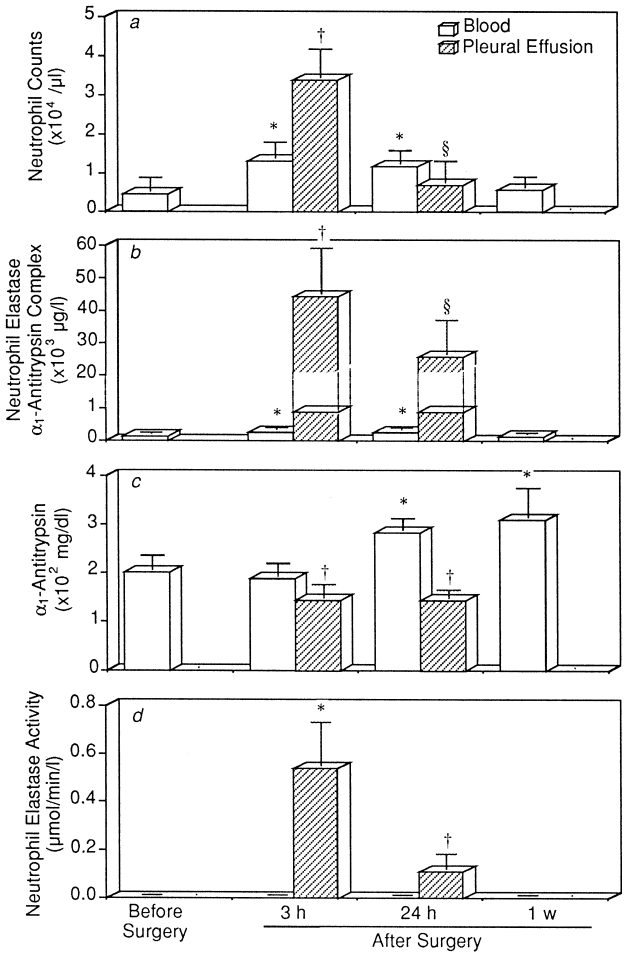

Fig. 1. Time course of changes in neutrophil counts (a), amount of neutrophil elastase complexed with α1-antitrypsin (b), α_1-antitrypsin (c), and neutrophil elastase activity (d) in pleural effusion and blood. The activity was under the limit of detection in the samples of blood taken before and after surgery. $* P < 0.05$ vs. the corresponding values before surgery. $\dagger P < 0.05$ vs. the corresponding values in blood. $\S P < 0.05$ vs. the corresponding values in pleural effusion 3 h after surgery.

2.3.5. Western blot analysis

Western blot analysis was performed to determine the activity of purified α_1-antitrypsin. The samples (100 μl) were incubated for 30 min at 37°C and then heated at 100°C for 5 min in 2.5% SDS, 0.1 M Dithiothreitol, 0.001% bromophenol blue, and 62.5 mM Tris–HCl (pH 6.8). The analysis was carried out by electrophoresis using 10% polyacrylamide gel. After electrophoresis, the protein was transferred to an acetate cellulose membrane and reacted with rabbit anti-human α_1-antitrypsin antibody and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins.

2.4. Statistics

Data are expressed as means ± S.D. We used an analysis of variance and then Student's unpaired *t*-test to compare the data between the groups. We also used Student's paired *t*-test to compare the data in the same group. Significance was $P < 0.05$.

3. Results

3.1. Neutrophil elastase and α_1-antitrypsin imbalance

The number of neutrophils in pleural effusion increased more than the number in blood 3 h after lobectomy (Fig. 1a), but was lower than the number in blood after 24 h. The blood neutrophil counts returned to the preoperative counts 1 w after lobectomy. The amount of neutrophil elastase complexed with α_1-antitrypsin in the pleural effusion was 170 times higher than the amount in blood 3 h after lobectomy and was 100 times higher 24 h after lobectomy (Fig. 1b). The levels of α_1-antitrypsin in blood were not increased 3 h after lobectomy but were increased 24 h and 1 w after lobectomy (Fig. 1c). The levels of α_1-antitrypsin in pleural effusion were 70% of the blood levels 3 h after lobectomy and stayed at this level 24 h after lobectomy. The neutrophil elastase activity in all blood samples was too low to be measured (Fig. 1d). However, the neutrophil elastase activity in pleural effusion was 0.54 ± 0.18 and 0.11 ± 0.06 μmol min^{-1} l^{-1} at 3 and 24 h after lobectomy, respectively.

3.2. Effects of neutrophil elastase inhibitors on neutrophil elastase activity

Pleural effusion collected 3 h after lobectomy was used because it had the highest neutrophil elastase activity.

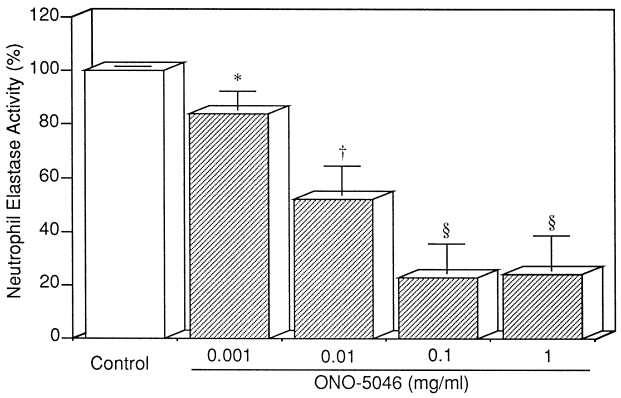

Fig. 2. Effects of ONO-5046 on the activity of neutrophil elastase in ex vivo pleural effusion. The activity of neutrophil elastase is expressed as a percentage of control activity in the absence of ONO-5046. Activity under the limit of detection (0.08 μmol min^{-1} l^{-1}) is regarded as zero. ONO-5046 reduced the activity of neutrophil elastase dose-dependently. $* P < 0.05$ vs. the activity in the control experiments. $\dagger P < 0.05$ vs. the activity in the experiments with 0.001 mg/ml of ONO-5046. $\S P < 0.05$ vs. the activity in the experiments with 0.01 mg/ml of ONO-5046.

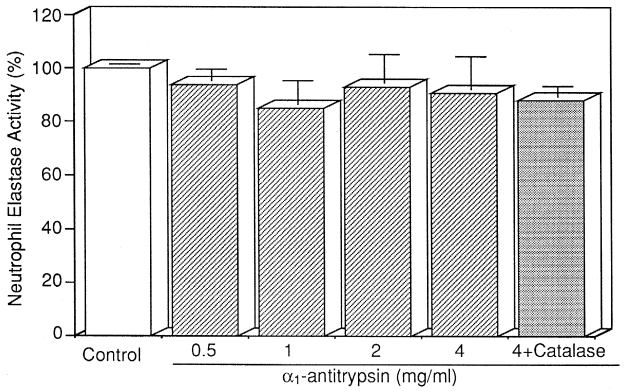

Fig. 3. Effect of purified human α_1-antitrypsin in the absence or presence of catalase on the activity of neutrophil elastase in pleural effusion 3 h after lobectomy.

ONO-5046 reduced neutrophil elastase activity dose-dependently up to 0.1 mg/ml of ONO-5046 (Fig. 2). However, purified human α_1-antitrypsin did not affect the neutrophil elastase activity (Fig. 3). Since α_1-antitrypsin has been reported to be inactivated by oxygen radicals (Carrell, 1986; Ossanna et al., 1986), we incubated pleural effusion with 1 mg/ml of catalase, an inhibitor of H_2O_2, for 30 min. Addition of 1 mg of human α_1-antitrypsin did not affect the neutrophil elastase activity (Fig. 3).

To determine whether the purified α_1-antitrypsin was active, it was added to purified neutrophil elastase in saline solution. At 1 mg/ml, α_1-antitrypsin added to 0.1 and 0.01 mg/ml of purified neutrophil elastase in saline solution decreased neutrophil elastase activity from 8.4 to 0.2 μmol min^{-1} l^{-1} and from 0.6 μmol min^{-1} l^{-1} to under detectable limits, respectively (Fig. 4).

Western blot analysis showed that purified α_1-antitrypsin (52 kDa) was active: the α_1-antitrypsin–neutrophil elastase complex (81 kDa) was formed when α_1-antitrypsin was added to purified neutrophil elastase in pleural

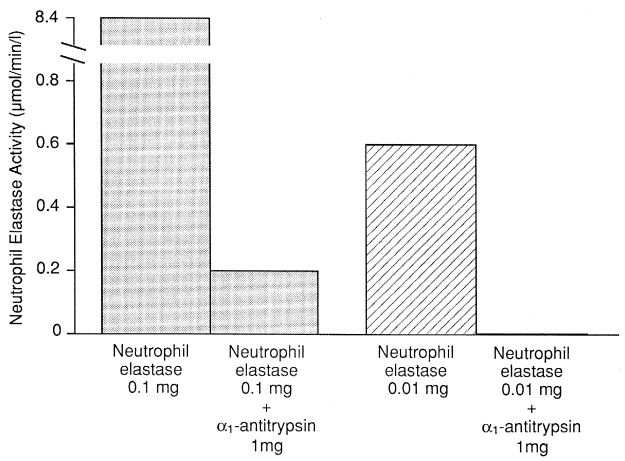

Fig. 4. Effect of purified human α_1-antitrypsin on the activity of purified neutrophil elastase. Purified human α_1-antitrypsin (1 mg/ml) inhibited the activity of neutrophil elastase (0.1 or 0.01 mg/ml).

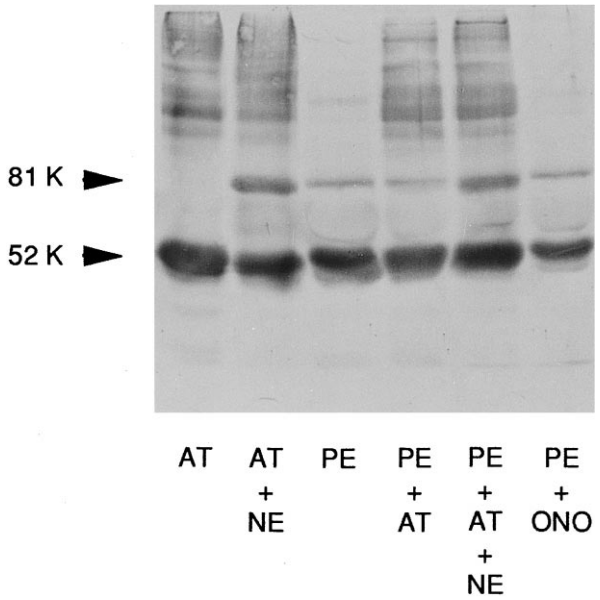

Fig. 5. Western blot analysis. Lane 1: Purified human α_1-antitrypsin (1 mg/ml) in saline solution. Lane 2: Purified α_1-antitrypsin (1 mg/ml) added to purified neutrophil elastase (0.1 mg/ml) in saline solution. Lane 3: Pleural effusion alone. Lane 4: Purified α_1-antitrypsin (3 mg/ml) added to pleural effusion. Lane 5: Purified neutrophil elastase (0.1 mg/ml) added to purified α_1-antitrypsin (1 mg/ml) in pleural effusion. Lane 6: ONO-5046 (1 mg/ml) added to pleural effusion. Molecular sizes of α_1-antitrypsin and the complex of neutrophil elastase and α_1-antitrypsin are 52 kDa and 81 kDa, respectively. AT: α_1-antitrypsin, NE: neutrophil elastase, PE: pleural effusion, ONO: ONO-5046.

effusion (Fig. 5, lane 5) as well as in saline solution (Fig. 5, lane 2). Purified α_1-antitrypsin or ONO-5046 did not affect the formation of the α_1-antitrypsin-neutrophil elastase complex in pleural effusion (Fig. 5, lanes 4 and 6).

4. Discussion

The first objective of this study was to determine whether there was an imbalance between neutrophil elastase and α_1-antitrypsin in pleural effusion after lobectomy. Two approaches were used. One approach was to compare the increase in neutrophil elastase and α_1-antitrypsin levels in pleural effusion as well as in blood. Although the amount of the neutrophil elastase–α_1-antitrypsin complex in pleural effusion increased, the amount of α_1-antitrypsin in pleural effusion did not increase 24 h after lobectomy. An imbalance of neutrophil elastase and α_1-antitrypsin has been proposed as playing an important role in neutrophil elastase-dependent tissue injury in emphysema (Damiano et al., 1986), rheumatoid arthritis (Weissmann and Korchak, 1984), adult respiratory distress syndrome (Cochrane et al., 1983; Lee et al., 1981), septic shock (Jochum et al., 1986), and cystic fibrosis (Birrer et al., 1994). However, because the enzyme-linked immunosorbent assay does not indicate whether the enzyme is active, our second ap-

proach was to measure neutrophil elastase activity using artificial substrates that are hydrolyzed by the enzyme (Bieth et al., 1974; Neumann et al., 1984). In the present study, elastase activity was measured by spectrophotometry with suc-$(Ala)_3$-*p*-nitroanilide used as substrate. Although neutrophil elastase activity was not detected in plasma, it was in pleural effusion (Fig. 1d). Since neutrophil elastase and α_1-antitrypsin form a 1:1 complex (Baugh and Travis, 1976), the large increase in neutrophil elastase levels and the absence of an increase in α_1-antitrypsin levels explain why neutrophil elastase activity was detected in pleural effusion. The data therefore indicate the presence of an imbalance between neutrophil elastase and α_1-antitrypsin in pleural effusion.

Increased levels of neutrophil elastase were detected 3 h after lobectomy. In contrast, the increase in α_1-antitrypsin levels was delayed in blood and the levels of α_1-antitrypsin were not increased in pleural effusion 24 h after lobectomy. More tissues than previously suspected express human α_1-antitrypsin mRNA, and the α_1-antitrypsin produced at the sites of inflammation is suggested to have an important role (Koopman et al., 1989). However, it is unlikely that large amounts of α_1-antitrypsin were produced in the pleural spaces after lobectomy because its levels did not increase in the pleural effusion. Since α_1-antitrypsin is synthesized mainly in the liver and is secreted into the blood (Alper et al., 1980), the delayed increase in the blood might result from delayed production in the liver.

Why was there no increase in α_1-antitrypsin levels in pleural effusion 24 h after lobectomy, although levels were increased in serum? This question seems important because the efficacy of α_1-antitrypsin replacement therapy is doubted (Hutchison and Hughes, 1997). In the present study, the ability of α_1-antitrypsin to permeate across the pleura was estimated by the ratio of the level of α_1-antitrypsin in pleural effusion to that in serum. The ratio at 24 h after lobectomy was lower than that at 3 h after lobectomy. Therefore, it is likely that α_1-antitrypsin did not increase in pleural effusion because the migration of α_1-antitrypsin decreased 24 h after lobectomy. The results are consistent with those for albumin (Sakuma et al., 1992), which has a similar Stokes radius and diffusion coefficient (Hastings et al., 1992; Salahuddin, 1991). Although it has been suggested that recombinant α_1-antitrypsin moves efficiently from the blood to alveolar epithelial lining fluid (Wewers et al., 1987) and from the airway into the lung interstitium (Hubbard et al., 1989), this study suggests that the efficacy also depends on vascular or epithelial permeability.

The second objective of this study was to determine the effects of purified human α_1-antitrypsin or ONO-5046 on the neutrophil elastase activity in ex vivo pleural effusion collected 3 h after lobectomy. Unexpectedly, purified human α_1-antitrypsin did not inhibit the neutrophil elastase activity in pleural effusion even when 4 mg/ml of α_1-antitrypsin was administered (Fig. 2). It is unlikely that purified α_1-antitrypsin was inactive because it inhibited the activity of purified neutrophil elastase in saline solution and the formation of neutrophil elastase–α_1-antitrypsin complex in pleural effusion (Fig. 5, lane 5). Also, it is unlikely that α_1-antitrypsin was inactivated by the cleavage of α_1-antitrypsin because no cleavage was detected by Western blot analysis (Fig. 5).

The effect of purified α_1-antitrypsin on the neutrophil elastase activity in pleural effusion was not consistent with the results of a previous study with patients with adult respiratory distress syndrome patients (McGuire et al., 1982). In that study, plasma from patients with α_1-antitrypsin deficiency failed to inhibit proteolytic activity and addition of α_1-antitrypsin to the deficient plasma resulted in inhibition of proteolytic activity. Since α_1-antitrypsin is very sensitive to oxidative inactivation by the myeloperoxidase–H_2O_2–halide system of human neutrophils (Clark et al., 1981; Matheson et al., 1979), pleural effusion was incubated with catalase, followed by the addition of human α_1-antitrypsin. However, the activity of neutrophil elastase was not inhibited. Therefore, it is unlikely that H_2O_2 played a role in the control of activity of neutrophil elastase in pleural effusion after pulmonary resection.

In contrast, ONO-5046 inhibited the neutrophil elastase activity dose-dependently (Fig. 2). The effects of ONO-5046 are consistent with those of the study in which ONO-5046 competitively inhibited human neutrophil elastase purified from purulent human sputum (Kawabata et al., 1991). One possible explanation for the difference between the effects of ONO-5046 and α_1-antitrypsin on the neutrophil elastase activity in pleural effusion might be the difference in their molecular size. It has been proposed that macro-molecular inhibitors are excluded from close contact with neutrophil elastase and substrates (Campbell et al., 1982). Since ONO-5046 is a low-molecular-weight inhibitor (mol.wt.: 434.47), it might be able to achieve a closer contact and so inhibit elastase dose-dependently as previously reported (Kawabata et al., 1991).

The activity of neutrophil elastase is also regulated by α_2-macroglobulin in body fluids. The nature of the interaction between α_2-macroglobulin and proteinases is unique, because the bound proteinases retain proteolytic activity against low-molecular-weight substances (Travis and Salvesen, 1983). Interestingly in synovial fluid from patients with rheumatoid arthritis, elastase damages cartilage even in the presence of α_1-antitrypsin and α_2-macroglobulin (Kawabata et al., 1996; Schalkwijk et al., 1987). Since α_2-macroglobulin is present in pleural effusion in patients after lobectomy, it is likely that α_2-macroglobulin forms a complex with neutrophil elastase in pleural effusion and also plays a role in degrading low-molecular-weight substrates. However, further studies are needed to understand the mechanisms by which neutrophil elastase remains active in the presence of high-molecular-mass proteinase inhibitors in pleural effusion after lobectomy.

5. Summary

An imbalance between neutrophil elastase and α_1-antitrypsin is present in human pleural effusion after lobectomy. The inflammation in the pleural space after lobectomy may be related to the imbalance between neutrophil elastase and α_1-antitrypsin. ONO-5046 is a potent inhibitor of neutrophil elastase activity in ex vivo human pleural effusion. However, further studies are needed to determine whether ONO-5046 plays a role in in vivo human pleural effusion.

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