Fibrinogenolytic activity of a novel trypsin-like enzyme found in human airway

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Abstract : Previously we isolated a new trypsin-like enzyme designated human airway trypsin-like protease (HAT) from human sputum. In this study, we examined in vitro whether HAT was related to the prevention of fibrin deposition in the airway lumen by cleaving fibrinogen. In mucoid sputum samples from patients with chronic airway diseases, the concentration of fibrinogen, as measured by ELISA, was in the range of 2-20 μg/mℓ, and trypsin-like activity, as measured by spectrofluorometry was in the range of 10-50 milliunits (mU)/mℓ. We showed by gel filtration that the trypsin-like activity of mucoid sputum was mainly due to HAT. We examined the effects of HAT on human fibrinogen at pH 7.4 and 8.6. Fibrinogen was used at concentrations of 4-2,000 μg/mℓ and HAT purified from sputum at concentrations of 0.6-10 mU/mℓ. As shown by SDS-polyacrylamide gel electrophoresis, HAT cleaved fibrinogen, especially its α-chain, regardless of the concentration of fibrinogen. Pretreatment of fibrinogen with HAT resulted in a decrease or complete loss of its thrombin-induced clotting capacity, depending on the duration of pretreatment with HAT and the concentration of HAT.

From these results we postulated that HAT may participate in the anticoagulation process within the airway, especially at the level of the mucous membrane, by cleaving fibrinogen transported from the blood stream. J. Med. Invest. 45 : 77-86, 1998

Key words **:** *trypsin-like enzyme, airway, fibrinogenolysis, sputum, chronic airway disease*

INTRODUCTION

As previously reported, we have isolated a new monomeric trypsin-like enzyme with a molecular weight of 27kDa from mucoid sputum samples from patients with chronic airway diseases, and have arbitrarily named this enzyme human airway trypsin-like protease (abbreviated as HAT for convenience) (1). Recently, Yamaoka et al. have cloned the cDNA for HAT, and have deduced the amino acid sequence of HAT from the nucleotide sequence of its cDNA (2). Their results indicated that HAT has a precursor with a molecular weight of 47 kDa, and that HAT isolated from the mucoid sputum corresponds to its mature form. Moreover, Northen blot analysis showed that the content of the messenger RNA for HAT in the trachea was the largest among 12 human tissues analyzed (2). From these results, it was considered that after the HAT precursor is synthesized in the airway walls, and is changed into its active and mature form (HAT) by limited proteolysis, HAT is secreted or released into the mucous membrane of the airway where it plays some physiological roles such as cleaving of exogenous proteins or endogenous proteins secreted or released into the mucous membrane.

In a preliminary experiment, we examined the hydrolyzing activities of mature HAT against major human serum proteins, and found that among various proteins, fibrinogen is the most prominently cleaved by HAT.

In this study, we measured the content of fibrinogen in sputum samples from patients with chronic airway diseases, and analyzed biochemically the fibrinogenolytic activity of HAT, to clarify whether

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HAT can prevent fibrin deposition on the airway mucous membrane by cleaving fibrinogen.

MATERIALS AND METHODS

Collection of sputum and purification of HAT

Mucoid and mucopurulent sputum samples were collected from patients with chronic bronchitis (CB) or bronchial asthma (BA) in a plastic container kept in an ice bath, and stored at -20℃ until use. HAT (approximately 7μg) was purified as previously reported (1) from about 900mℓ of the pooled sputum samples. The purified material showed a single band on SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

To measure fibrinogen in sputum, mucoid sputum samples were collected from 22 patients with chronic bronchitis (CB) and 26 patients with bronchial asthma (BA), and purulent sputum samples were collected from 27 patients with bronchiectasis. The diagnoses of CB and BA were based on the criteria of the Medical Research Council of the American Thoracic Society (3). The BA group consisted of patients with BA of a moderate degree.

Materials

Fibrinogen and thrombin, which were purified from human plasma, and bovine serum albumin (BSA), were purchased from Sigma (St. Louis, MO). Fibrinogen was further purified by affinity chromatography using lysine-Sepharose accord-ing to the method of Masuda *et al*. (4), after purification according to the method of Laki (5) with some modifications. All the fluorogenic synthetic substrates which had methylcoumarinamide (MCA) at their COOH-termini, were purchased from the Peptide Institute (Osaka, Japan). Sephadex G-200 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Human mast cell tryptase was purified as described by Smith *et al*. (6) with minor modifications.

Methods of biochemical analysis

1) Assay of activities of HAT and elastase

Activities of these serine proteases were measured by spectrofluorometry using fluorogenic MCA-substrates, which were synthesized by previous investigators for assaying each protease. HAT (trypsin-like) and elastase-like activities were measured by spectrofluorometry using Boc-Phe-Ser-Arg-MCA (7) and Suc-Ala-Pro-Ala-MCA (8) as substrates, respectively (1, 9). For assaying each protease, an assay mixture (1.5mℓ) containing 50 mU Tris-HCl-pH 8.6, 100μM the synthetic MCA-substrate, 100μg/mℓ BSA, and 50-200 μℓ of the test sample, was incubated at 37℃ for 10-60 min, and then the reaction was stopped by addition of 1mℓ of 30% acetic acid. The mixture was centrifuged at 2,500×g for 5 min, and the fluorescence intensity of the released aminomethylcoumarin (AMC) was measured. One unit of activity was defined as the amount that generated 1 μmole of AMC per min.

2) Gel filtration of HAT, mast cell tryptase and mucoid sputum extract through Sephadex G-200

The mucoid sputum samples from patients with CB or BA were mixed with 9 volumes of 0.05 M Tris-HCl buffer (pH8.6)-0.15 NaCl, homogenized with a Polytron homogenizer (Kinematica, PT/35, Littau, Switzerland) in an ice-bath for 30 sec, and then centrifuged at 10,000×g for 10 min. Two mℓ of the supernatant was applied to a column (2.2×65 cm) of Sephadex G-200 equilibrated with the same buffer. Elution was carried out at a flow rate of 15 mℓ/hr at 4℃and the eluate was collected in fractions of 4mℓ. About 80 mU of purified HAT or mast cell tryptase was subjected to gel filtration in the same way as described above, as control. Protease activity was measured using 50-200 μℓ of the eluate.

3) Analysis of HAT-induced cleavage of fibrinogen by SDS-PAGE

HAT-induced cleavage of fibrinogen was analyzed by SDS-PAGE, using HAT at concentrations of 0.62-10 mU/mℓ and fibrinogen at concentrations of 2,000μg/mℓ and 4-16μg/mℓ.

Cleavage of 2,000μg/mℓ fibrinogen by HAT :

The reaction mixture containing 2,000μg/mℓ fibrinogen, different concentrations of purified HAT (0.62-10 mU/mℓ),and 50 mU Tris-HCl (pH 8.6 or 7.4) in a total volume of 500 μℓ, was incubated at 37℃ for10min-16hrs, and then promptly cooled in an ice-bath. Ten μℓ of each of the mixtures was subjected to SDS-PAGE on a 4-20% gradient gel containing 0.1%SDS (Daiichi Pure Chemicals, Tokyo) under a denaturing and reducing condition by the method of Laemmli (10). The gels were stained with coomassie brilliant blue. The gels were calibrated with high range standards (Promega, Madison, WI).

Cleavage of 4-16μg/mℓ fibrinogen by HAT :

The reaction mixture contained HAT and Tris-HCl buffer at the same concentrations described above, but fibrinogen at concentration of 4-16μg/mℓ in a total volume of 5mℓ. It was incubated at 37℃ for 4hrs, concentrated using a vaporized centrifuge (CVE-100D,

Tokyo Rika Kikai, Tokyo) to 0.5-1mℓ, and then dialyzed against 1 ℓ of saline. Twenty μ ℓ of each sample was subjected to SDS-PAGE under a denaturing and reducing condition as described above. SDS-PAGE low range standards (APRO Science, Naruto-City, Japan), were used as molecular weight markers. The gels were silver-stained using a kit obtained from Daiichi Pure Chemicals.

4) Analysis of HAT-induced cleavage of fibrinogen by measuring released fibrinopeptide A (FPA)

Reaction mixtures which contained 2,000μg/mℓ fibrino-gen, 5mU/mℓ HAT, and 50mU Tris-HCl (pH 7.4-9.4) or Hepes (pH 6.8-7.6) in a total volume of 0.2mℓ, were incubated at 37℃ for 4hrs. Then released FPA (or FPA-reactive peptide) was measured using Asserachrom® (Diagnostica Stago, Stago, France), an EIA kit for FPA, according to the method of Amiral et al (11).

5) Assay of clotting capacity of HAT-treated fibrinogen

The reaction mixtures containing 2000μg/mℓ fibrinogen, 0.3-10 mU/mℓ HAT, and 50 mU Tris-HCl (pH 7.4) in a total volume of 0.25mℓ, were incubated at 37℃ for 10 min-16 hrs, and then cooled in an ice-bath to stop the reaction. The HAT-treated fibrinogen solution and a solution containing 2.5 unit/mℓ thrombin in 50 mU Tris-HCl buffer (pH 7.4) were kept at room temperature separately. Then the thrombin-induced clotting time of the HAT-treated fibrinogen was measured by incubating a mixture of 100 μ ℓ each of HAT-treated fibrinogen and thrombin solution in a plastic tube at 37℃. Clotting time was measured in triplicate and the mean value was recorded. When samples did not show formation of fibrin clot within 2 min, it was considered that fibrinogen had completely lost its clotting capacity.

6) Measurement of albumin and fibrinogen concentrations

Albumin and fibrinogen in the sputum samples were measured by sandwich enzyme-linked immunosorbent assay (ELISA) (12). The albumin concentration was measured according to the method of Tatenuma et al. (13).

For ELISA of fibrinogen, we used sheep IgG antibody against human fibrinogen (The Binding Site, Birmingham, England) as the first antibody, and goat IgG antibody against human fibrinogen (Organon Teknika N.V. Cappel Products Aurora, OH) as the second antibody. Protein standard serum Nor-Partigen (Behringwerke AG, Marburg, Germany) was used as the standard. The sputum samples were mixed with 9 volumes of saline, and homogenized using a Polytron homogenizer in an ice-bath for1min. The test samples were finally diluted 1,000- to10,000-fold with 10-fold diluted Block Ace (Snow Brand Milk Products, Tokyo).

Statistical analysis :

The protease activities and protein concentrations in the sputum samples from each patient group were expressed as the mean±SD. The significance of differences between values for each group was tested by the Mann-Whitney U-test.

RESULTS

Fibrinogen concentration in sputum samples from patients with chronic airway diseases

Figure 1 shows the fibrinogen concentration in mucoid sputum samples from CB and BA patients, and in purulent sputum samples from BE patients. The concentration of fibrinogen was significantly higher in mucoid sputum samples from BA patients

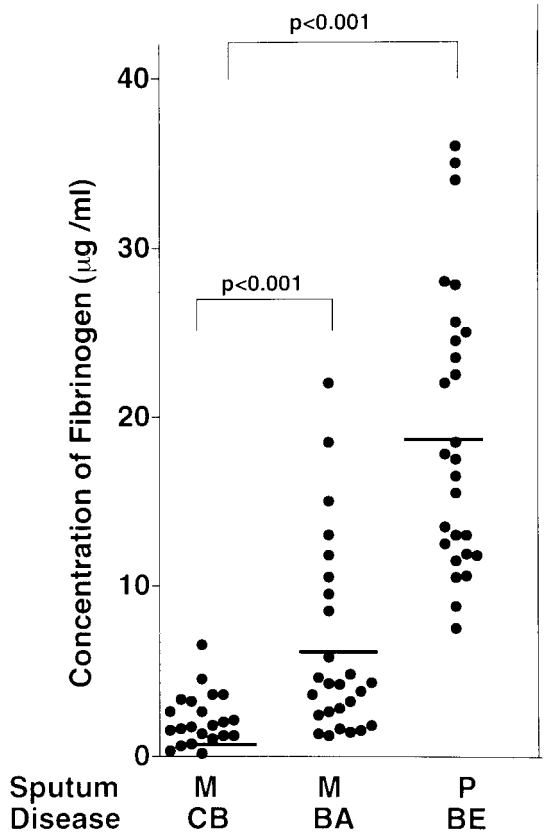

Fig.1. Concentration of fibrinogen in sputum samples from patients with chronic bronchitis, bronchial asthma and bronchiectasis Horizontal lines show mean values. Abbreviations M : mucoid ; P : purulent ; CB : chronic bronchitis ; BA : bronchial asthma ; BE : bronchiectasis $p<0.001$:Mann-Whitney U-test for difference from the mucoid sputum from CB patients

Table 1. Trypsin-like activity, elastase-like activity and albumin concentration in sputum samples of patients with chronic bronchitis, bronchial asthma and bronchiectasis

		Chronic bronchitis mucoid (n=22)	Bronchial asthma mucoid (n=26)	p※	Bronchiectasis purulent (n=27)	p※
Trypsin-like activity	milliunit/ml	23.46±18.03	46.90±43.96	p<0.05	14.92±8.23	p<0.001
Elastase-like activity	microunit/ml	15±18	6±14		16,975±8,545	p<0.001
Albumin	μg/ml	331±201	457±289	ns	1,206±679	p<0.001

Values are means±SD.
※Mann-Whitney U-test for difference from mucoid sputum samples of patients with chronic bronchitis.
ns : not significant

(6.3±5.5) than in those from CB patients (1.9± 1.1), and was significantly higher in the purulent sputum samples from BE patients (18.8±8.8) than in the mucoid sputa from both CB and BA patients.

Table 1 shows trypsin-like activity, elastase-like activity and albumin concentration in sputa, from patients with different diseases affecting the airway. Elastase activity was measured to estimate the extent of sputum purulence because neutrophil polymorphonuclear leukocytes contain much elastase, and actively release it extracellularly (14). As shown in this Table, elastase activity was about 1000-3000-fold higher in the purulent sputum samples from BE patients than in the mucoid sputum samples from both CB and BA patients. This result indicated that our judgment of purulence of sputum based on macroscopic appearance was roughly correct. The elastase activity in the mucoid sputum samples from CB patients was not compared with that in those from BA patients, because its activity was very low in both groups.

Trypsin-like activity was slightly higher in the mucoid sputum samples from BA patients than in those from CB patients. This activity was significantly lower in purulent sputum samples from BE patients, as compared with both types of mucoid sputum samples.

Albumin concentration was significantly (about 3-fold) higher in the purulent sputum samples from BE patients than in mucoid sputum samples from patients with CB or BA, and it had a tendency to be slightly higher in the mucoid sputum samples from patients with BA than in those from CB patients.

Gel filtration of extracts of mucoid sputum samples from patients with chronic bronchitis (CB)

Figure 2 shows the elution patterns of purified HAT and mast cell tryptase in gel filtration through Sephadex G-200. The peak concentrations of HAT (molecular weight, 27 kDa) (1) and that of lung mast cell tryptase (molecular weight, about 130 kDA) (6) were located in tube 50 and tube 34, respectively.

As shown in Fig.3, in gel filtration of mucoid sputum extracts from 2 patients with CB, prominant peak of trypsin-like activity was detectable only at tube 50. Thus trypsin-like activity was detectable at a fraction corresponding to HAT, and not at a fraction corresponding to mast cell tryptase. The elution patterns of mucoid sputum extracts from other patients with CB were similar to those shown in Fig.2.

Analysis of HAT fibrinogenolytic activity by SDS-PAGE

1) Effect of HAT on 2,000 μg/mℓ fibrinogen

Figure 4 shows the time courses (between 15 min and 16 hrs) of HAT fibrinogenolytic activity at a concentration of 10mU/mℓ, at pH8.6. Lane8 shows electrophoretic mobility of control fibrinogen incubated in buffer alone for 4 hrs. In this lane, there were three major bands, whose molecular weights were 68,000, 58,000 and 48,000, respectively. This result was roughly in accordance with the reports of previous investigators who showed that fibrinogen consisted of two of three subunits, α-chain (MW68,000), β-chain (MW54,000) and γ-chain (MW 48,000) (15). Mills and Karpatkin (16) reported that SDS-PAGE of reduced fibrinogen revealed two α-chain species which differed in molecular weight by 3000, because the α-chain band consisted of two bands, a major and a minor one. In lane 8 of Fig.4,

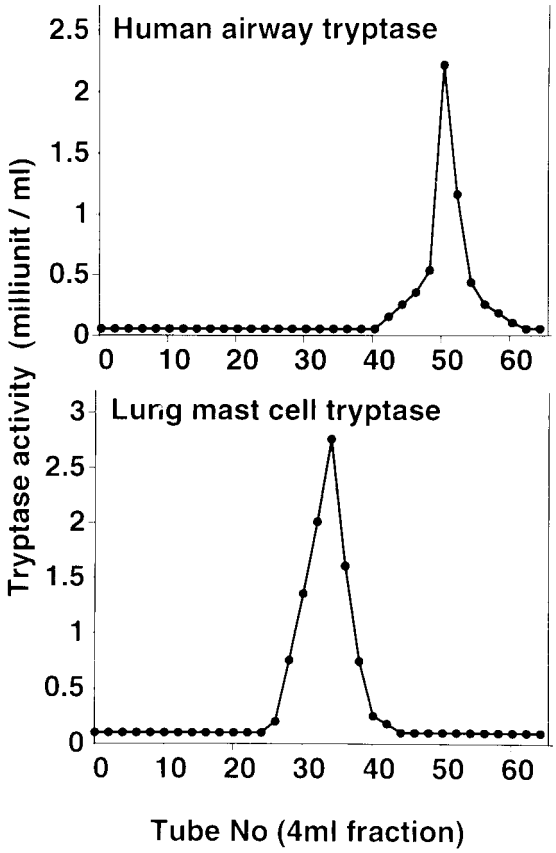

Fig.2. Gel filtration of purified HAT and lung mast cell tryptase
About 80 mU of purified HAT or mast cell tryptase dissolved in 0.05M Tris-HCl buffer-0.15M NaCl was applied to a column (2.2×65 cm) of Sephadex G-200 equilibrated with the same buffer. Elution was carried out at a flow rate of 15mℓ/hr at 4℃, and the eluate was collected in fractions of 4mℓ. HAT activity was measured with Boc-Phe-Ser-Arg-MCA, and mast cell tryptase activity with Boc-Gln-Ala-Arg-MCA, as the substrate.

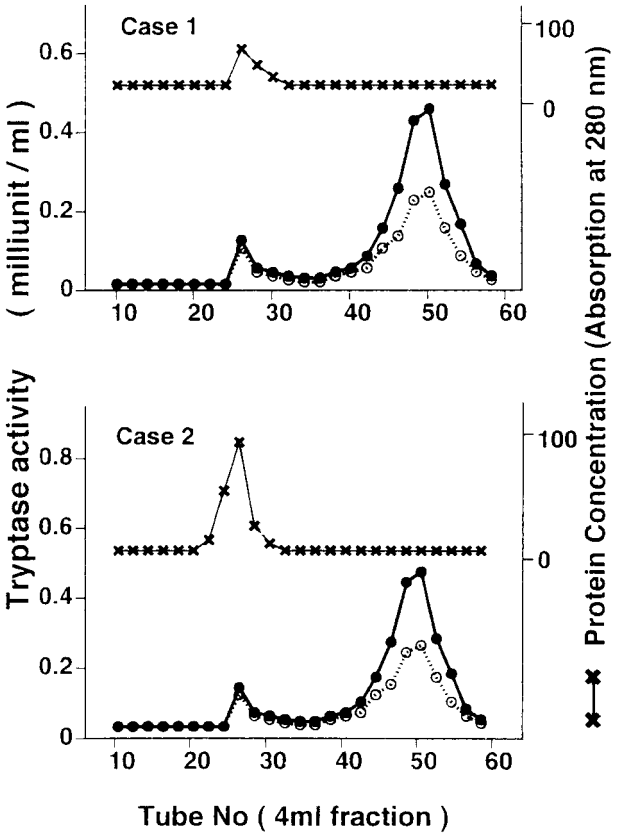

Fig.3. Gel filtration of extract of mucoid sputum from patients with chronic bronchitis on Sephadex G-200
Two mℓ of mucoid sputum extract prepared as described in Materials and Methods, was subjected to gel filtration through Sephadex G-200 in the same way as described for Fig.2.
●——● measured with Boc-Phe-Ser-Arg-MCA as the substrate
○·····○ measured with Boc-Gln-Ala-Arg-MCA as the substrate

the α-chain band was wide but stained less intense compared with the β-and γ-chain bands. This is thought to be due to the fact that the chain consists of two species.

In the HAT-treated fibrinogen, the α-chain was completely lost within 15 min. On the other hand, cleavage of the β-chain was not detectable up to 180 min, but the band almost completely disappeared after 16 hrs of incubation. The cleavage of γ-chain was not clear at any determination point.

When fibrinogen was incubated with HAT at pH 7.4, it was cleaved by HAT in almost the same manner as at pH 8.6 (data not shown).

Figure 5 shows the effect of HAT concentration on the cleavage of fibrinogen. The reaction mixtures containing HAT at concentrations varying from 0.6 to 10.0 mU/mℓ, and 50 mU Tris-HCl (pH 8.6 or pH 7.4), were incubated at 37℃ for 4hrs. As clearly shown in Fig.5, HAT cleaved fibrinogen in a dose-dependent manner. The cleavage of α-chain

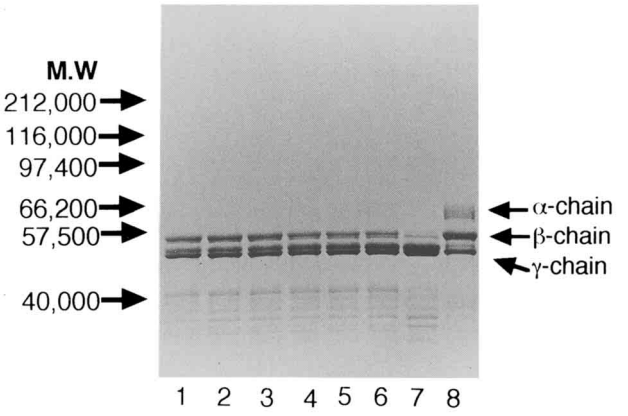

Fig.4. Fibrinogenolytic activity of HAT against human fibrinogen
— Time course —
Fibrinogen at a concentration of 2,000μg/mℓ was mixed with HAT at a concentration of 10 mU/mℓ, and 50 mU Tris-HCl (pH 8.6) in a total volume of 0.5mℓ, and then incubated at 37℃ for 15 min-16 hrs. The reaction was stopped by placing the mixture in an ice-bath. Ten μℓ of each of the reaction mixture was subjected to SDS-PAGE on a gradient gel (Multigel 10/20, Daiichi Pure Chemicals) under a denaturing and reducing condition by the method of Laemmli (11).The gel was calibrated with high range standards (Promega). The proteins trapped in the gel were stained with coomassie brilliant blue.
Incubation time : lane1, 15min ; lane2, 30min ; lane3, 60min ; lane4, 120 min ; lane 5, 180 min ; lane 6, 240 min ; lane 7, 16 hrs ; lane 8, control incubated without HAT for 240 min

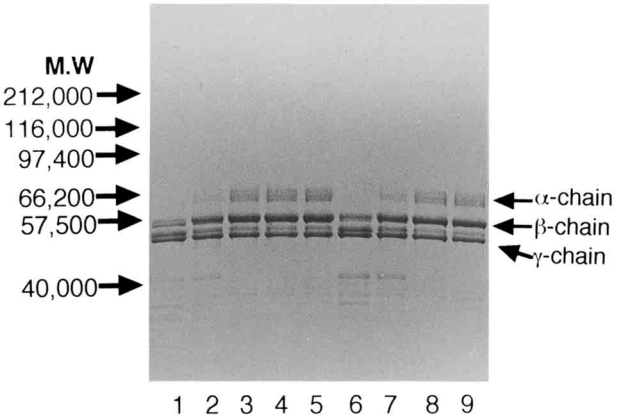

Fig.5. Fibrinogenolytic activity of HAT against human fibrinogen
--- Effect of the concentration of HAT ---
Experimental procedures, unless otherwise stated, were carried out in the same way as described for Fig.4. The reaction mixtures containing HAT at concentrations varying from 0.625 to 10.0 mU/mℓ, and 50mU Tris-HCl (pH8.6 or pH7.4), were incubated at 37℃ for 240 min. Lanes 1-5 correspond to reaction mixtures incubated at pH 8.6, and lanes 6-9 to those incubated at pH 7.4
Concentration of HAT (mU/mℓ) : lane 1, 10.0 ; lane 2, 5.0 ; lane 3, 2.5 ; lane4, 1.25 ; lane5, 0.625 ; lane6, 10.0 ; lane7, 5.0 ; lane8, 2.5 ; lane 9, 1.25

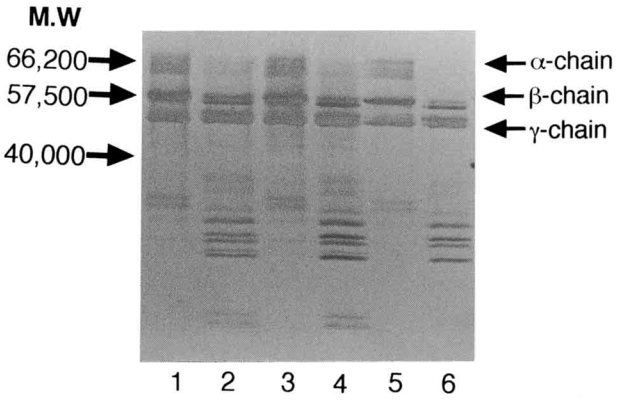

Fig.6. Fibrinogenolytic activity of HAT against a low concentration of human fibrinogen
Reaction mixtures containing fibrinogen at concentrations of 4, 8 or 16μg/mℓ, and 5 mU/mℓ HAT and 50 mU Tris-HCl (pH 8.6) in a total volume of 5mℓ, were incubated at 37℃ for 240 min, cooled in an ice-bath, concentrated using a vaporized centrifuge, and dissolved in0.25, 0.50 and 2.0mℓ, respectively, of 50mU Tris-HCl (pH 6.8). Ten μ ℓ of the mixtures was subjected to SDS-PAGE as described for Fig.4. The proteins trapped in the gel were silver-stained.
Concentration of fibrinogen :
lane 1, 4μg/mℓ without HAT ; lane 2, 4μg/mℓ with HAT ; lane 3, 8μg/mℓ without HAT ; line 4,8μg/mℓ with HAT ; lane 5,16μg/mℓ without HAT ; lane 6, 16μg/mℓ with HAT

was the most marked, that of β-chain was relatively clear, while that of γ-chain was not detectable. This figure also showed that the pattern of cleavage of fibrinogen by HAT incubated at pH7.4 was very similar to that at pH 8.6. This was more clear when fibrinogen was incubated with 5-10 mU/mℓ HAT.

The results of Figs 4 and 5 showed that HAT degradates first the α-chain of fibrinogen, and more slowly β-chain.

2) Effect of HAT on 4-16μg/mℓ fibrinogen

Fibrinogen in relatively low concentrations was incubated with 10 mU/mℓ HAT at pH 8.6 for 4 hrs. As shown in Fig.6, the α-chain completely disappeared at each fibrinogen concentration of 4, 8 and 16μg/mℓ, but cleavage of β-chain was not so prominent at any concentration of fibrinogen, compared with the respective control incubated without HAT. The cleavage of the γ-chain was almost not detectable when fibrinogen was used at concentrations of 4 and8μg/mℓ. The α-chain was completely cleaved by HAT at pH 7.4 and at pH 8.6.

Analysis of the optimum pH for HAT-induced cleavage of fibrinogen using a kit for measuring FPA

Thrombin cleaves the bond between arginine and glycine (arginyl-glycine bond) to release FPA (16 amino acids) from the NH_2-terminal segment of the fibrinogen α-chain(17). Previously, we showed that HAT also split the COOH-terminal side of

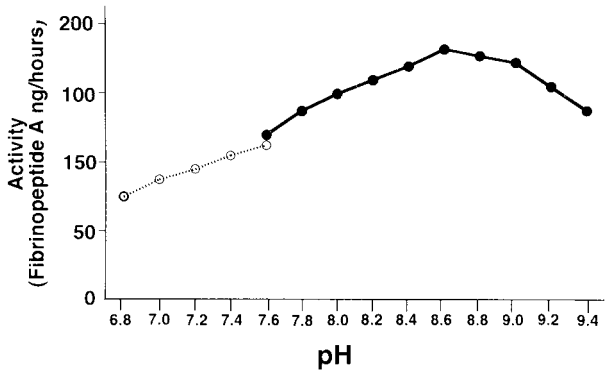

Fig.7. Effect of pH on the fibrinogenolytic activity of HAT
Reaction mixtures containing 2,000μg/mℓ fibrinogen, 5 mU/mℓ HAT and 50 mU Tris-HCl (pH 7.6-9.4) or Hepes (pH 6.8-7.6) in a total volume of 0.25mℓ, were incubated at 37℃ for 240 min. Then fibrinopeptideA (FPA) in the mixtures was measured by an EIA kit for FPA (Asserachrom®).

arginine in various kinds of model peptides like trypsin (18). The experiments of Figs.4, 5 and 6 showed that HAT can cleave human fibrinogen, especially the α-chain of fibrinogen. From these results, we thought that HAT may cleave the arginyl-glycine bond in the α-chain of fibrinogen like thrombin, and FPA or FPA reactive peptides may be released from fibrinogen incubated at 37℃with HAT. And we showed that when human fibrinogen was incubated with HAT under the same conditions described in the previous section, the amount of substances

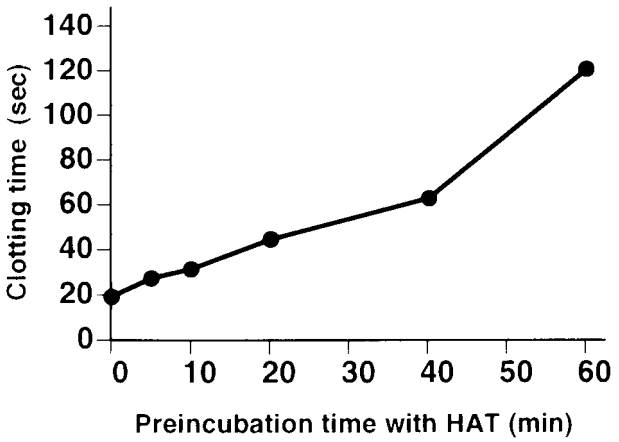

Fig.8. Effect of HAT treatment on thrombin-induced clotting time of the fibrinogen
— Effect of pretreatment time —
Reaction mixtures containing 2,000μg/mℓ fibrinogen, 10 mU/mℓ HAT and 50 mU Tris-HCl (pH 7.4), were incubated at 37℃ for 10-80min, and then cooled in an ice-bath. The reaction mixtures and a solution containing 2.5 unit/mℓ thrombin in 50 mU Tris-HCl buffer (pH 7.4) were kept at room temperature separately. Then the thrombin-induced clotting time of HAT-treated fibrinogen was measured by incubating a mixture of 100 μ ℓ each of the HAT-treated fibrinogen solution and thrombin solution in a plastic tube at 37℃.

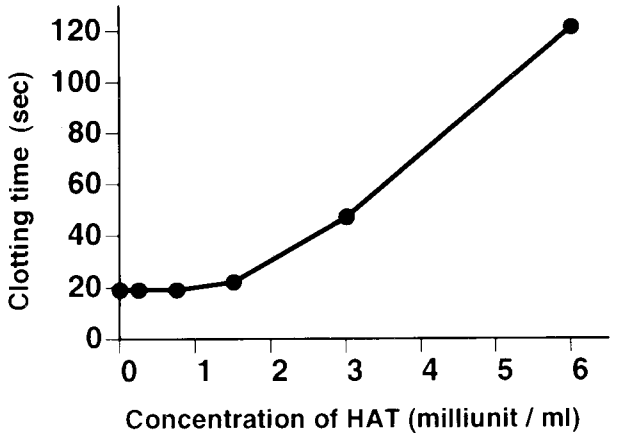

Fig.9. Effect of the concentration of HAT on the thrombin-induced clotting time of HAT-treated fibrinogen
2,000μg/mℓ fibrinogen was preincubated with various concentrations of HAT (0.3-6 mU/mℓ) at 37℃ for 240 min. The thrombin-induced clotting time of HAT-treated fibrinogen was measured as described for Fig.8.

measured by the assay kit for FPA increased in proportion to the incubation time and HAT concentration. Therefore the effect of pH on the fibrinogenolytic activity of HAT was examined using this kit for FPA. As shown in Fig.7, the fibrinogenolytic activity of HAT was maximal at pH 8.5-8.6.

Thrombin-induced clotting capacity of HAT-treated fibrinogen

After the reaction mixtures containing 2,000μg/mℓ fibrino-gen, 10 mU/mℓ HAT and 50 mU Tris-HCl (pH 7.4), were incubated at 37℃ for 10-80 min, the thrombin-induced clotting time of HAT-treated fibrinogen was measured. In the present assay system, the clotting time of intact fibrinogen was 20±5 sec (n=5). As shown in Fig.8, HAT prolonged the clotting time of treated fibrinogen in a time-dependent manner, from the initial value of 20 sec to about 60 sec after 40 min of incubation with HAT. We thought that the fibrinogen which did not clot at 120 sec after it had been incubated with thrombin, had almost completely lost its capacity to clot, because it finally did not clot after a longer time. Thus Fig.8 shows that fibrinogen treated with HAT for over 60 min almost completely lost its capacity to clot.

Fibrinogen was treated with HAT at various concentra-tions (0.36-6mU/mℓ) at 37℃ for 4hrs. As shown in Fig.9, HAT prolonged the clotting time of fibrinogen in a dose-dependent manner from the initial value (20sec) to 45sec at concentrations from 0 to 3mU/mℓ, and the fibrinogen treated with 6 mU/mℓ HAT almost completely lost its capacity to clot.

DISCUSSION

Previous reports on serine proteases of human airways have indicated that at least two kinds of trypsin-like proteases, mast cell tryptase (19-20) and HAT (1-2), are secreted or released into the human airway lumen from specific inflammatory cells or secretory cells localized in the airway walls. Mast cells are thought to release tryptase mainly into the airway walls, because in the airway, they are mainly localized in the submucosal layer (20), and they actively release tryptase which is already activated and translocated into secretory granules at their degranulation (20). On the other hand, HAT is thought to be released or secreted into the airway lumen, because much of it is contained in the airway mucus, such as sputum. Besides, we have postulated based on the analysis of the cloned cDNA for HAT that HAT is released or secreted from airway walls after its precursor (MW47kDa) is subjected to limited proteolysis and changed into its active and mature form, HAT (2). Gel filtration of extracts of mucoid sputum samples supported this idea, because the trypsin-like activity of these sputum samples was mainly due to HAT.

The elastase activities of purulent sputa from BE patients were much higher than those of mucoid sputa

from CB and BA patients. This result was in good accordance with the report of Stockley *et al*. (14) that purulent sputa from BE patients contained much neutrophil elastase. On the other hand, the trypsin-like activity was significantly lower in the purulent sputa from BE patients than in the mucoid sputa from CB and BA patients. The lower level of trypsin-like activity in BE patients is thought to be partly due to the fact that HAT released into airway lumen is hydrolyzed by proteases such as neutrophil elastase, because neutrophi elastase can hydrolyze various kinds of endogenous proteins (21).

Considering localization of HAT, we think that HAT plays some physiological role mainly in the airway lumen, on the airway mucous membranes, by causing limited cleavage of some endogenous proteins (peptides) or inhaled proteins (peptides).

Schwartz *et al*. (22) have reported that human lung mast cell tryptase cleaves fibrinogen. In a previous report (1), we showed that HAT also cleaved fibrinogen. Extravascular fibrin deposition accompanies tissue inflammation and serves as a provisional matrix for subsequent repair, and the fibrin gel participates in progression of tissue injury by modulating cell accumulation, activation and migration, angiogenesis, synthesis of granulation tissue, and collagen deposition (23). Moreover, fibrin gel on the mucous membranes of airways, is thought to disturb mucociliary movement, the most fundamental defense system of the airways, because of its rheological property. We thought that probably HAT may serve as an anticoagulant by cleaving the fibrinogen transported to the mucous membranes via the blood stream, because the capacity of HAT-degradated fibrinogen to clot in the presence of thrombin might decrease or be completely lost.

This study was undertaken to test in vitro the possibility of HAT being related to the prevention of fibrin deposition on the mucous membranes of airways due to its fibrinogenolytic action.

It is known that the concentration of fibrinogen in blood of healthy subjects is in the range of 2,000-4,000 $\mu g/m\ell$ or $6\text{-}12\times10^{-6}$ M (24). To roughly estimate the concentration of fibrinogen in the bronchial secretion (respiratory tract fluid) of patients with chronic airway diseases, we measured the concentration of fibrinogen in their sputum samples by ELISA. Regarding mucoid sputa, the concentration of fibrinogen was higher in those from BA patients than in those from CB patients, and it was higher in purulent sputa from BE patients than in the mucoid sputa from CB patients. The concentration of fibrinogen in the sputum samples was about 1/100-1/1,000 of that found in blood. We think that the concentration of fibrinogen in sputum sample may roughly reflect that on the mucous membrane of the airway. All the fibrinogen in the sputum or bronchial secretion derives from the blood stream. The higher concentration of fibrinogen found in the purulent sputum samples was thought to be due to increased transudation accompanied by infection, in the airways. The higher concentration of fibrinogen found in the mucoid sputum samples from BA patients compared with those from CB patients was also probably due to a more marked transudation in the airway in BA than in CB. Kawata et al. (25) reported using immunohistochemical techniques that fibrinogen is present in all mucoid sputum samples from patients with CB and BA, which they had examined, while fibrin was detectable only in a part of them.

As shown in Table 1, the concentration of HAT (trypsin-like enzyme) in the sputum samples was about 10-40mU/$m\ell$. Therefore, we examined the fibrinogenolytic activity of HAT using HAT at a concentration of 5-10 mU/$m\ell$. Our SDS-PAGE study clarified that when fibrinogen was incubated with HAT, the α-chain was preferentially cleaved, the β-chain was cleaved to a lesser degree, and the γ-chain was almost not cleaved by HAT, and that HAT can cleave fibrinogen not only when present at a concentration similar to that found in blood, but also when present at the concentration it is found in sputum samples. The Km value of the reaction between HAT and human fibrinogen is unknown, mainly because we did not analyze in detail the degradation products of HAT-treated fibrinogen. But the result of SDS-PAGE (Fig.6) strongly suggested that HAT can cleave fibrinogen at concentrations similar to that it is found in bronchial secretion. When thrombin attacks fibrinogen, it removes the NH_2-terminal fibrinopeptide moieties (fibrinopeptide A and fibrinopeptide B), amounting only to about 3% of the total weight of fibrinogen (15). Thus the resulting fibrin monomers represent 97% of the fibrinogen molecule. Therefore the SDS-PAGE study also showed that the degradation of fibrinogen induced by HAT is clearly different, because both the α-chain and the β-chain were completely lost after HAT treatment.

When fibrinogen was incubated with HAT in vitro, the production of FPA increased in proportion to the concentration of HAT and treatment time. As described above, we do not think this was due to the fact that HAT cleaves only the arginyl-glycine bond, which is attacked specifically by thrombin, in the α-chain of fibrinogen. However, the amount of FPA

reactive substances was thought to be proportional to the amount of fibrinogen cleaved by HAT. On the basis of this idea, the optimum pH for the fibrinogenolytic activity of HAT was examined using this kit, and was shown to be pH8.5-8.6 in vitro. However, HAT also cleaved fibrinogen at pH 7.4 in vitro.

The clotting activity of HAT-treated fibrinogen induced by thrombin decreased or completely disappeared in vitro. This result suggests that HAT may act as an anticoagulant in the airways, especially on the mucous membranes, if it cleaves fibrinogen before thrombin reacts with the latter.

It is postulated that two kinds of antibodies against human fibrinogen, which we used for the ELISA of fibrinogen, react with multiple antigenic sites of human fibrinogen and thus with considerable parts of fragments produced from human fibrinogen by HAT action, because they are polyclonal antibodies. When the concentration of human fibrinogen, which was treated at 37℃ by HAT at 10 mU/㎖ for 1 to 4 hr in vitro, was measutred by the ELISA, it was not decreased compared with non-treated control (our unpublished data). Therefore even if the fibrinogen contained in the sputum samples is already partially cleaved by HAT, probably we can measure roughly the fibrinogen concentration of the sputum samples by the present ELISA.

Further studies seem to be necessary to clarify whether HAT can cleave fibrinogen in vivo (in the airway lumens) as it cleaves the latter in vitro, and whether HAT participates in the anticoaglulation process in the airways.

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