

ORIGINAL**Human airway trypsin-like protease (HAT) is released into saliva**Susumu Yasuoka¹, Mari Miki², Rie Tsutsumi¹, Masami Yoshioka³, Takashi Bando⁴, and Fumiko Michishige⁵

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Abstract : We first discovered human airway trypsin-like protease (HAT) in human mucoid sputum. Precursor HAT (47 kDa), a cell surface type II transmembrane serine protease, is proteolyzed to mature HAT (27 kDa). Hitherto, HAT has not been detected in other biological fluids except for human sputum. We aimed to clarify whether human saliva contains mature HAT. Trypsin-like protease was isolated from saliva of healthy volunteers by a method adopted for isolation of HAT from sputum using Boc-Phe-Ser-Arg-MCA as the substrate. Biochemical properties of purified protease were similar to those of recombinant HAT (rHAT). HAT concentration in saliva was measured by ELISA, and immunoreactive HAT : total protein ratio (ng/mg) in saliva samples from healthy subjects was similar to that in mucoid sputum. RT-PCR showed that HAT mRNA was expressed in human gingival epithelial cells but not in gingival fibroblasts. Both indirect immunofluorescence and western blotting using monoclonal antibody for α -smooth muscle actin (α -SMA ; a myofibroblast marker) showed that HAT enhanced α -SMA fiber expression in gingival fibroblasts. These results indicate that both mucoid sputum and saliva from healthy subjects have similar concentrations of mature HAT, and HAT is related to certain physiological functions and pathological states of myofibroblasts in the oral cavity. *J. Med. Invest.* 65 : 258-267, August, 2018

Keywords : Human trypsin-like protease (HAT), saliva, gingival fibroblast, gingival epithelial cells, α -smooth muscle actin

INTRODUCTION

We have previously isolated a novel trypsin-like protease from human mucoid sputum and named it as human airway trypsin-like protease (HAT) (1). Analysis of the nucleotide sequence of cloned HAT cDNA suggested that HAT is originally translated as a precursor with a molecular size of 47 kDa (composed of 418 amino acid residues), whose N-terminal proximal region contains a hydrophobic putative transmembrane domain (2). Since the discovery of HAT, several serine proteases with properties similar to those of HAT have also been discovered, and recently, HAT has been classified as a member of the type II transmembrane serine proteases, and named TMPRSS11D (3, 4).

Precursor HAT is converted to an active enzyme by limited proteolysis between the Arg¹⁸⁶-Ile¹⁸⁷ peptide bond and is processed into the soluble form, mature HAT, containing 232 amino acid residues (187-418) in the extracellular space (2). This mature HAT may be involved in some important physiological processes.

Previous investigators have showed by immunohistochemistry that HAT protein is strongly expressed in the lower respiratory tract, e.g., bronchial epithelium (5-7), and in oral tissues, such as tonsils and buccal epithelium (7). Analysis of HAT mRNA expression in various human tissues showed that significant expression is observed in the trachea and in the upper gastrointestinal tract (particularly in the esophagus and tongue) as well as in other organs such as the bladder, testis, and spinal cord (4, 8, 9). These studies strongly indicated that HAT is distributed in various tissues, par-

ticularly in the lower and upper respiratory and upper gastrointestinal tracts, including oral tissues.

Mature HAT was first discovered in the sputum (1, 10-12). Hypertonic saline-induced sputum sample (12) and bronchial washings from healthy volunteers also contained mature HAT (unpublished data). These results indicated that the lower respiratory fluid, so-called bronchial secretions or sputum, contains mature HAT in both healthy and pathologic states. However, it is still unknown whether mature HAT is present in human biological fluids apart from airway secretions.

As described above, expression of HAT mRNA in oral tissues has been previously reported (8-9). In a preliminary study, we have shown by ELISA that human saliva contains HAT (13), and Sun *et al.* reported the presence of HAT by activity-based mass spectrophotometric characterization (14). However, till date no investigators have isolated mature HAT from saliva.

Saliva is involved in the defense mechanism of the oral mucous membrane via various processes (15). We hypothesized that mature HAT may be released into the saliva and may be related to some biological functions of the oral mucous membrane.

In the present study, we aimed to isolate mature HAT from saliva and demonstrated that the saliva of healthy subjects contained mature HAT at concentration similar to that in the sputum, and it was shown by RT-PCR analysis that HAT was released from oral epithelial cells. Moreover, recombinant HAT (rHAT) enhanced α -smooth muscle actin fiber (α -SMA ; a marker for myofibroblast) expression in gingival fibroblasts, indicating that HAT may promote fibroblast-to-myofibroblast differentiation in the oral cavity.

Received for publication January 17, 2018 ; accepted July 3, 2018.

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MATERIALS AND METHODS

Reagents and Antibodies

rHAT, which corresponds to mature HAT (2), and two different rabbit polyclonal IgG antibodies against rHAT were supplied by Teijin Institute for Bio-Medical Research, Tokyo, Japan. The biochemical properties of rHAT were almost similar to those of mature, native HAT purified from the mucoid sputum (2).

Recombinant human TGF- β 1 was obtained from R&D Systems (Minneapolis, MN).

Streptavidin-horse radish peroxidase conjugate was purchased from Biosource (Camarillo, CA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Nacalai Tesque, Inc (Kyoto, Japan) and Invitrogen Corp (Carlsbad, CA, USA), respectively. Keratinocyte-serum free medium (SFM) supplemented with bovine pituitary extract (BPE) and epidermal growth factor (EGF) was obtained from Invitrogen. Bovine serum albumin (BSA) and Micro BCA protein assay reagent kit were obtained from Sigma (St Louis, MO, USA) and Pierce (Rockford, IL, USA), respectively. Boc-Phe-Ser-Arg-4-methyl-coumaryl-7-amide (MCA) and other MCA substrates were obtained from the Peptide Institute (Osaka, Japan). Triton X-100 and sodium dodecyl sulfate (SDS) were obtained from Sigma-Aldrich (Tokyo, Japan). SephadexTM G-100 and Benzamidine-SepharoseTM-6B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Butyl-Toyopearl 650C and SP-Toyopearl 650 M were obtained from TOSOH (Tokyo, Japan).

Collection of whole saliva

The protocol was conducted in compliance with the guidelines of Declaration of Helsinki. Saliva samples were obtained from 40 non-smoker female volunteers aged 20–22-years-old and 62 non-smoker female volunteers aged 35–55-years-old. Before collecting the saliva, written informed consent was obtained from all subjects after a full explanation of the procedures involved. The experimental procedure was approved by the Ethics Committee of the Tokushima University Hospital (Approval No 286, 117).

Saliva, unless otherwise stated, was collected by suction using a low-pressure continuous aspirator (Sea Star Corporation, Kyoto) via a bronchial catheter (Type P, 10Fr, Thermo fisher scientific, CA), the tip of which was inserted into the floor of the mouth, by gentle continuous suction for 5–30 min.

Saliva was collected from some subjects by the spitting method according to the method of Nagler and Hershkovich (16). Saliva samples were stored in a deep freezer at -80°C until use.

Assay of protease activity

Trypsin-like activity was measured according to the method of Yasuoka *et al.* (1). Briefly, the assay mixture (1.5 ml), containing 50 mM Tris-HCl (pH 8.6), 100 μM Boc-Phe-Ser-Arg-MCA, 100 $\mu\text{g}/\text{ml}$ BSA, and 100 μl of the test sample, was incubated at 37°C for 60 min, and the reaction was stopped by adding 1 ml of 30% acetic acid. The fluorescence intensity of the released aminomethyl coumarin (AMC) was measured using a fluorescence spectrophotometer (F-3010 Hitachi Co, Japan) at 440 nm with excitation at 380 nm. The amount of AMC released was calculated from a standard curve. One unit of enzyme was defined as the amount that produced 1 μmole of AMC per min.

Thirty nanomolar HAT exhibited an activity of 100 mU/ml.

ELISA for determining HAT concentration

In brief, 96-well plates (Micron, Greiner Labortechnik) were coated with 50 μl of the primary antibody, a rabbit polyclonal anti-rHAT antibody (10 $\mu\text{g}/\text{ml}$) in PBS, at 4°C overnight. The plates were blocked with 1% BSA in PBS at room temperature for 3 h. Plates containing 50 μl each of the test sample or rHAT solution and

secondary biotinylated rabbit polyclonal anti-rHAT antibody solution (10 $\mu\text{g}/\text{ml}$) per well were incubated at 4°C overnight. Then, the plate containing 100 μl of a 6×10^5 -fold diluted streptavidin-horse radish peroxidase conjugate solution per well was incubated at room temperature for 1 h. Finally, the plate containing 100 μl of 0.023% tetramethylbenzidine dihydrochloride dihydrate (TMBZ-HCl)/2.5 mM H_2O_2 was incubated at room temperature for 30 min. The reaction was terminated using 100 μl of 1 N H_2SO_4 per well, and absorption at 450 nm was measured using a microplate reader (Model 550, Bio-Rad). The concentration range of HAT as measured by ELISA was 0.03–10 ng/ml .

Purification of trypsin-like protease obtained from saliva

When the activities of HAT purified from the sputum with various MCA-substrates were previously tested, Boc-Phe-Ser-Arg-MCA (a trypsin substrate) was the most preferentially hydrolyzed substrate by HAT (1). Therefore, a Boc-Phe-Ser-Arg-MCA-hydrolyzing protease was purified from the pooled saliva by a minor modification in the method previously adopted for purification of HAT from the mucoid sputum (1). Briefly, equal volumes (approximately 650 ml) of pooled saliva and 0.10 M Tris-HCl (pH 7.5)/0.30 M NaCl, were mixed and homogenized using a Polytron homogenizer and centrifuged. The supernatant was subjected to hydrophobic gel column chromatography using Butyl-Toyopearl 650C column (column size, 4×12 cm) as described previously (1). The active fraction eluted from the gel was precipitated with 65% ammonium sulfate, dissolved in 30 ml of 0.05 M sodium acetate (pH 4.0)/10% glycerol, dialyzed in 3 l of the same buffer, and then centrifuged. The supernatant was successively subjected to purification procedures using a cation exchange gel column (SP-Toyopearl 650M) by both the bathwise method and column chromatography (column size, 1.2×18 cm). The active fraction eluted with a linear 0–0.2 M NaCl gradient from the SP-Toyopearl 650M column was finally subjected to affinity column chromatography using Benzamidine-Sepharose 6B column equilibrated with 0.05 Tris-HCl buffer (pH 9.2)/0.5 M NaCl/10% glycerol (column size, 2×5 cm). After the column was washed with the equilibration buffer, most of the trypsin-like protease was eluted with 0.05 M acetate buffer (pH 4.0)/0.5 M NaCl/10% glycerol. The purification procedure was performed four times. Each active fraction from Benzamidine-Sepharose 6B column chromatography was combined, concentrated by ultrafiltration, and dissolved in 5 ml of 0.05 M Tris-HCl buffer (pH 7.5)/0.1 M NaCl. Approximately 2 μg of the purified enzyme (8% yield) was obtained after purification, with activity about 330,000-fold that of the starting material.

Analyses of trypsin-like activity and HAT concentration in whole saliva

For determining trypsin-like activity and HAT concentration in the saliva samples, collection of whole saliva samples was performed under resting conditions in a quiet room during the afternoon between 14:00 and 15:00 h, at least 1 h after eating. The saliva samples were mixed with nine parts of physiological saline and homogenized using an IKA homogenizer (IKA-Werke, Ultra-Turrax T8) for 1 min in an ice bath. Trypsin-like activities were measured using 100 μl of the whole homogenate diluted to 10-fold with 0.05 M Tris-HCl buffer (pH 8.6)/BSA (100 $\mu\text{g}/\text{ml}$). Thus, the whole homogenate was finally diluted to 1500-fold in an assay mixture of 1.5 ml.

HAT concentrations were measured by ELISA as described above using 50 μl of the whole homogenate finally diluted to 100-fold with PBS/2% Triton X-100/0.6% SDS.

Measurement of total protein

Unless otherwise stated, total protein was measured by the method of Lowry *et al.* (17) using BSA as the standard. For purifica-

tion of trypsin-like enzyme, total protein was measured using a Micro BCA assay kit (Pierce, Rockford, IL).

Culture of oral cells

Human gingival fibroblasts (HGF) and gingival epithelial cells (HGE) were cultured from human gingival tissues obtained during surgery for cancer and tooth extraction. Before the surgery, written informed consent was obtained from the patients after a full explanation of the procedures involved. The experimental procedure was approved by the Ethics Committee of the Kawashima Hospital at Tokushima City (Approval No 0011).

In a previous study, we cultured human bronchial fibroblasts (HBF) in DMEM supplemented with 10% FBS (11). HGF were cultured in almost the same manner as previously reported using gingival tissues. Experiments were performed using cells between passages 2 and 5.

HGE (human gingival epithelial cells) were cultured as previously reported (18), using keratinocyte-SFM/supplemented with BPE and EGF. All experiments were performed with cell passages 1 (P1) or 2 (P2).

RT-PCR for HAT and GAPDH mRNA extracted from human oral cells

Oral cells (both HGF and HGE) were seeded into 60-mm cell culture dishes and incubated at 37°C until 100% confluence. Total cellular RNA was extracted from the cells using Isogen (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. RT-PCR procedures for HAT were performed as described previously (19). Sense and antisense primers for RT-PCR were synthesized, and the sequences were as follows: sense primer, 5'-CATTG TCGTC GCAGG GGTAG-3'; antisense primer, 5'-TCAGC CTCAG TGCCT CCAAG-3'. The length of the expected PCR product was 519 bp. GAPDH was used as an internal control. The PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide solution and visualized by UV transillumination.

Immunofluorescence and western blotting

When fibroblasts differentiate into myofibroblasts, α -SMA fiber expression is observed in the fibroblasts (20–22). Therefore, expression of SMA fiber was used as a marker for fibroblast-to-myoblast differentiation in this study. Effect of HAT on α -SMA fiber expression in HGF was examined by both indirect immunofluorescence and western blotting.

HGF were obtained from the gingival tissues of a total of 10 subjects, seeded on cover slips (18 mm \times 18 mm) in 6-well plates (Greiner), and cultured in DMEM supplemented with 10% FBS for 2–3 days in a 5% CO₂ incubator until 50–60% confluence. After incubation for 24 h in serum-free DMEM supplemented with 0.01% BSA, the cells were stimulated with HAT at 7.5–60 nM for 24–48 h. The cells were gently washed with PBS, fixed with 4% paraformaldehyde at room temperature for 20 min, and then treated with 0.1% Triton X-100 in PBS for 3–4 min at 4°C. After blocking the fixed cells with PBS/1% BSA for 20 min at room temperature, cells were incubated with 1 : 200 dilution of monoclonal anti- α -smooth actin antibody (Sigma) at 4°C overnight, followed by incubation with Alexa 488-anti-mouse IgG antibody for 1–2 h at room temperature. Fluorescent images of cell sections excited at 488 and 560 nm were captured using a confocal laser scanning microscope (Leica TCS NT, Heidelberg, Germany) equipped with an argon-krypton laser source. As a control, the immunostaining procedure was performed with normal mouse IgG instead of anti-actin antibody.

Western blotting

HGF (15 \times 10⁴ cells) were cultured in a 6-well culture plate

(Greiner) in DMEM supplemented with 10% FBS in a 5% CO₂ incubator for 24 h at 37°C. The culture medium was changed to serum-free DMEM, and the cells were cultured for an additional 24 h. Fibroblasts were stimulated with HAT (7.5–60 nM) in the same medium for 12–72 h. The cells were washed with PBS, scraped off the wells in 0.4 ml cell lysis buffer (Cell Signaling, Denvers, MA) containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), sonicated, and centrifuged at 14,000 \times g for 10 min at 4°C.

The protein concentration of the resultant supernatant was measured using the Micro BCA protein assay reagent kit.

The supernatants were diluted to 2–4-fold with SDS sample buffer and boiled for 5 min. The same amount of cell lysate protein (0.2 μ g) was separated by SDS-PAGE on 10% gel (Greiner) under reducing condition. Pre-stained broad-range protein standards (Apro Science, Naruto city, Japan) were used to estimate the molecular mass. The proteins were transferred onto a polyvinylidene difluoride membrane (Bio-craft Co., Tokyo, Japan). The amount of α -SMA was estimated by western blotting using a monoclonal anti- α -smooth muscle actin antibody as the primary antibody and a peroxidase-conjugated monoclonal anti-mouse IgG antibody (GE Healthcare) as the secondary antibody. GAPDH, which was used as the loading protein control, was also detected as described above using a monoclonal anti-GAPDH antibody (Sigma) as the primary antibody.

Positive reactivity was detected using the ECL Western Blotting detection kit (GE Healthcare). Signals on a film (GE Healthcare) were detected by an X-ray film automatic processor using a TC-DF 202 processing system developer replenisher fixer (Konica Minolta Me).

Effect of HAT on HGF proliferation

HGF were seeded at 4000 cells/well in a 96-well plate containing 100 μ l of DMEM supplemented with 10% FBS until 70–80% confluence. The cells were then cultured in serum-free DMEM supplemented with 0.01% BSA for 24 h, stimulated with HAT for 48 h in the same medium, harvested from the plate by trypsinization, and counted using a hemocytometer, as described previously by Matsushima *et al.* (11).

Statistical analysis

Data are represented as mean \pm SD. Significant differences between samples were tested by the nonparametric Wilcoxon–Mann–Whitney test. The significance of correlation between trypsin-like activity and HAT concentration in the saliva samples was tested using Pearson's product–moment correlation coefficient. $p < 0.05$ was considered as statistically significant.

RESULTS

I Biochemical properties of trypsin-like protease purified from saliva

The distribution of trypsin-like activity in the purified trypsin-like protease from pooled saliva was very similar to that of mucoid sputum (1) in each purification step, including hydrophobic gel using Butyl-Toyopearl C column chromatography, anion-exchange column chromatography using SP-Toyopearl 650M, and affinity chromatography using Benzamidine-Sepharose 6B. Trypsin-like protease was absorbed on the Butyl-Toyopearl C column equilibrated with 40% ammonium sulfate (pH 7.5) and eluted with 5% ammonium sulfate (pH 7.5), absorbed on SP-Toyopearl 650M column equilibrated with 0.05 M sodium acetate (pH 4.0) and eluted with a linear 0–0.20 M NaCl gradient, and absorbed on a Benzamidine-Sepharose 6B column equilibrated with 0.05 M Tris-HCl buffer/0.5 M NaCl (pH 9.2) and eluted with 0.05 M sodium

acetate/0.5 M NaCl (pH 4.0).

Finally, approximately 2 µg of the purified enzyme (8% yield) was obtained after purification, with activity about 330,000-fold that of the starting material.

The substrate specificity of this material for synthetic model substrates was examined as previously reported (1). For this, various kinds of MCA substrates developed for assaying several proteases were used, with rHAT as the control. The activity profiles of rHAT on these substrates were almost similar to those of native HAT purified from mucoid sputum (1). As shown in Table 1, the activity profiles of the trypsin-like protease purified from saliva on these substrates were almost similar to those of rHAT. Of the MCA substrates tested, Boc-Phe-Ser-Arg-MCA (trypsin substrate) was most preferentially hydrolyzed by both the purified enzyme and rHAT.

Table 1 Activities of trypsin-like protease purified from saliva on synthetic model substrates

Substrate	Relative activity (%)**	
	Purified protease	rHAT
Boc-Phe-Ser-Arg-MCA*	100	100
Boc-Val-Pro-Arg-MCA	88	88
Boc-Gln-Ala-Arg-MCA	51	48
Boc-Gln-Arg-Arg-MCA	32	34
Boc-Leu-Thr-Arg-MCA	80	76
Boc-Ile-Glu-Gly-Arg-MCA	7	6
Glt-Gly-Arg-MCA	12	11
Boc-Gln-Gly-Arg-MCA	68	70
Pro-Phe-Arg-MCA	13	12
Boc-Val-Leu-Lys-MCA	4	3
Suc-Ala-Ala-Pro-Phe-MCA	0	0
Suc-Ala-Pro-Ala-MCA	0	0

* MCA : Methyl-Coumaryl-7-Amide

** Activity as a percentage of that with Boc-Phe-Ser-Arg-MCA

Effects of protease inhibitors on the purified protease were also examined as previously reported for native HAT (1) (Table 2). Inhibitors, other than EDTA, were added at a concentration of 10 µM. The activity of purified protease was completely inhibited by 10 µM diisopropyl fluorophosphate (DFP) and was almost completely inhibited by trypsin inhibitors such as leupeptin, antipain, soybean trypsin inhibitor, aprotinin, and urinary trypsin inhibitor ; however, the activity was not inhibited by amastatin, phosphoramidon (a metalloprotease inhibitor), or 1 mM EDTA. As shown in Table 2, the effects of these protease inhibitors on the purified protease were almost similar to those on rHAT.

When the purified protease was subjected to gel filtration on a Sephadex G-100 column, the elution pattern of its trypsin-like activity was very similar to that of rHAT, with peaks of both trypsin-like activity of purified protease and rHAT (mature HAT) located in fraction 38 (Fig 1).

Thus, the results showing that biochemical properties of the purified protease were almost similar to those of rHAT indicate that purified protease was primarily composed of mature HAT. Therefore, mature HAT is assumed to be released into the saliva.

Table 2 Effects of protease inhibitors on the trypsin-like protease purified from saliva

Addition	Final concentration	Relative activity (%)	
		Purified protease	rHAT
None		100	100
DFP	10µM	0	0
Leupeptin	10µM	2.8	2.5
Antipain	10µM	3.3	1.9
Soybean trypsin inhibitor	10µM	9.7	5.2
Aprotinin	10µM	0.5	0.2
UTI	10µM	0.2	0.2
Benzamidine	10µM	0.9	0
Amastatin	10µM	100	100
α-1-protease inhibitor	10µM	46.7	53.5
Phosphoramidon	10µM	100	100
EDTA	1mM	100	100

DFP : diisopropyl fluorophosphate

UTI : urinary trypsin inhibitor

EDTA : ethylenediamine tetraacetic acid

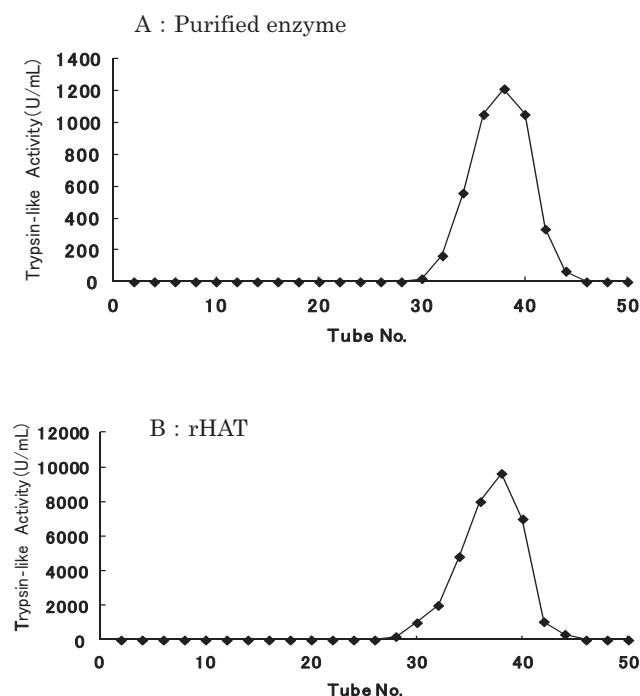


Fig 1 Gel filtration of a trypsin-like protease purified from the saliva on a Sephadex G-100 column

A : Purified protease ; 28.8 mU of the trypsin-like protease purified from the saliva was dissolved in 2 ml of 0.05 M Tris-HCl buffer (pH 7.5)/0.30M NaCl and applied to a Sephadex G-100 column (2.2 × 65 cm) equilibrated with the same buffer. Elution was performed at a flow rate of 15 ml/h at 4°C, and the elute was collected in fraction of 4 ml. Trypsin-like activity was measured with Boc-Phe-Ser-Arg-MCA as the substrate. B : rHAT ; 0.2 µg of rHAT was subjected to gel filtration in the same way as described above.

II Measurement of trypsin-like activity and HAT concentration in the saliva samples

Saliva contains insoluble substances that precipitate on centrifugation. We assumed that a considerable portion of proteins,

including proteases, in the saliva were co-precipitated with insoluble substances by centrifugation. When saliva samples were mixed with equal volumes of 0.1 M Tris-HCl buffer (pH 7.5)/0.30 M NaCl, homogenized, and centrifuged at 8,000 rpm for 10 min, approximately 40% of trypsin-like protease activity in the original saliva was precipitated with other macromolecules. Therefore, in the following experiments to measure the trypsin-like activity and HAT concentration in the saliva samples, we analyzed whole saliva homogenate at the following dilutions: 1500-fold dilution with 0.05 M Tris-HCl buffer (pH 8.6)/BSA (100 µg/ml) for assaying trypsin-like activity and 100-fold dilution with PBS/2% Triton X-100/0.6% SDS for assaying HAT concentration with ELISA for preventing the loss of trypsin-like activity and HAT due to centrifugation.

The trypsin-like activities in the saliva samples from 60 middle-aged healthy subjects were measured. In all the saliva samples diluted to 1500-fold, trypsin-like activity was detectable in a range of 5,350–58,880 µU/ml, with the mean value and standard deviation being 23.9 ± 16.2 mU/ml. Moreover, immunoreactivity against HAT in the present ELISA was also detectable in all the saliva samples diluted to 100-fold.

A saliva sample (5 ml) was diluted with five volumes (25 mL) of buffer, concentrated by ammonium sulfate fractionation (20–80%), and subjected to gel filtration on a Sephadex G-100 column. The elution pattern of immunoreactivity against ELISA for HAT was very similar to that of trypsin-like activity. Both trypsin-like activity and immunoreactivity to anti-HAT antibody in ELISA yielded only a single peak, and peaks of both trypsin-like activity and immunoreactivity were located in fraction 38, where the rHAT peak was located. Fig 2 shows the result of gel filtration of the saliva sample. In gel filtration, almost similar results were obtained in the seven saliva samples tested.

Correlation between trypsin-like activity and HAT concentration was tested in healthy subjects ($N = 60$). As shown in Fig 3, trypsin-like activity was significantly correlated with HAT concentration, as measured by present ELISA ($r = +0.922$; $p < 0.01$).

These results support the idea that saliva samples from healthy subjects contain mature HAT, indicating that the Boc-Phe-Ser-Arg-MCA-hydrolyzing activity of the saliva was primarily due to mature HAT.

III Comparison of trypsin-like activity level and HAT concentration in the saliva with those in the mucoid sputum

In a previous experiment (13), the mean value of the total protein concentration, trypsin-like activity, and HAT concentration were not significantly different between the saliva samples collected by the suction and spitting methods (data not shown). Therefore, in the following experiment, saliva samples collected by the suction method were analyzed.

In the saliva sample from healthy subjects ($N = 60$), the mean values of total protein concentration (µg/ml), trypsin-like activity (mU/ml), and HAT concentration (ng/ml) were 3340 ± 1638 , 23.9 ± 16.7 , and 153 ± 112 , respectively.

As shown in Table 3, mean value of the total protein concentration, trypsin-like activity, and HAT concentration in the mucoid sputum ($N = 60$) were 1.9-, 2.62-, and 2.69-fold higher, respectively, in the mucoid sputum than those in the saliva samples; the difference in each parameter between the saliva samples and mucoid sputum was significant. As shown in Fig 4, the ratio of HAT concentration to total protein concentration (HAT:TP ratio; ng/mg) was almost similar in the saliva and mucoid sputum samples.

IV Expression of HAT mRNA in HGE and HGF

In this study, as a method to clarify the origin of HAT in saliva, RT-PCR for HAT mRNA was performed using HGF and HGE as target cells.

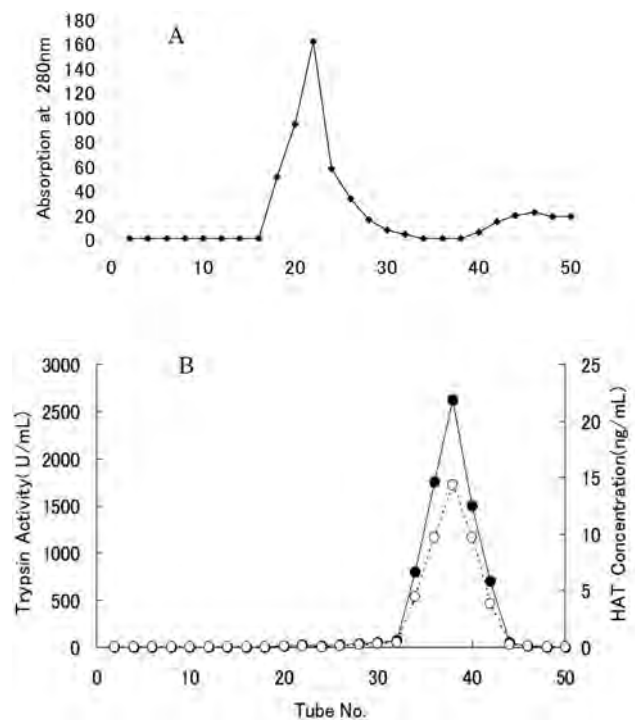


Fig 2 Gel filtration of saliva from a healthy subject on a Sephadex G-100 column

Five milliliter of saliva was mixed with equal volume of 0.100 M Tris-HCl buffer (pH 7.5)/0.30 M NaCl and centrifuged at 8,000 rpm at 4°C. The resultant supernatant was subjected to ammonium sulfate fractionation, and 20–80% ammonium sulfate fraction was dissolved in 4 ml of 0.05 M Tris-HCl buffer/0.30 M NaCl. Two milliliter of this solution was subjected to gel filtration, and trypsin-like activity was measured as described in Fig 1. HAT concentration was measured by ELISA as described in the Materials and Methods.

A: Absorption at 280 nm. B: Trypsin-like activity (closed), HAT concentration (opened).

PCR products were electrophoresed on 1.5% agarose gels. As shown in Fig 5, the expression of HAT mRNA was clearly detected in HGE but not in HGF, whereas expression of GAPDH mRNA was almost the same in these cells.

V Analyzing the effect of HAT on α -SMA fiber expression in HGF by indirect immunofluorescence and western blotting

Expression of intracellular SMA fiber was used as the marker for observing fibroblast-to-myoblast differentiation in this study. Ten HGF samples were obtained from various gingival tissues. When the HGF was not stimulated with HAT, the intracellular immunofluorescence intensity of α -SMA varied considerably among the samples. Of the total unstimulated HGF, α -SMA fiber was either not detectable or very weak in six samples (group A), as shown in Fig 6A, whereas in another four samples (group B), it was clearly and prominently visible even in unstimulated condition (Fig 7A). This result was thought to be a consequence of the fact that HGF were obtained from the gingival tissues with various kinds of inflammation in the present study.

In group A HGF, HAT clearly induced α -SMA fiber expression and also increased the cell size (Fig 6C). The cell sizes of HAT-treated HGF were 2–4-fold larger than those of control cells (Fig 6A). TGF- β 1, a positive control that differentiates fibroblasts into myofibroblasts, also induced α -SMA expression in HGF (Fig 6 B). Furthermore, even in HGF, in which α -SMA fiber formation

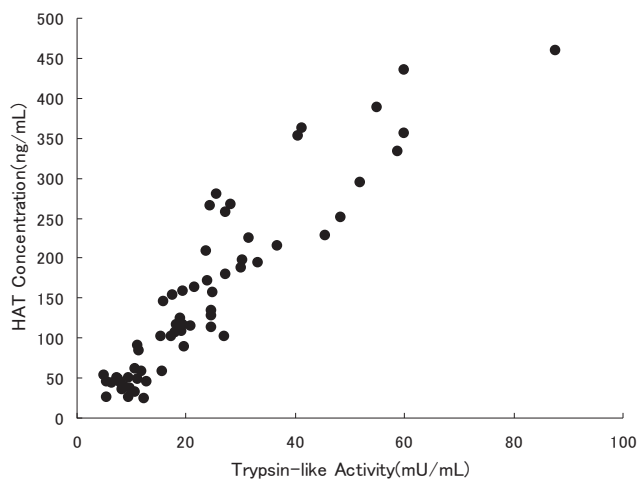


Fig 3 Relationship between trypsin-like activity and HAT concentration in the saliva samples from healthy subjects. Each saliva sample was mixed with nine parts of physiological saline and homogenized using a IKA homogenizer (IKA-Werke, Ultra-Turrax T8) for 1 min in ice-bath.

For assaying trypsin-like activity, the saliva whole homogenates were finally diluted to 100-fold with 0.05 M Tris-HCl buffer (pH 8.6)/BSA (100 µg/ml), and the trypsin-like activities were measured using 100 µl of the diluted whole saliva homogenate. For assaying HAT concentration, the saliva whole homogenates were finally diluted to 100-fold with PBS/2% Triton X-100/0.6% SDS. HAT concentration was measured by ELISA using 50 µl of the whole homogenate.

had already occurred, HAT significantly enhanced α -SMA fiber expression compared to that in the control (Fig 7A and B).

VI Western blotting

Western blot analysis was performed in HGF obtained from group A donors. When HGF were stimulated with HAT, HAT clearly induced α -SMA expression in the range of 7.5–60 nM in a dose-dependent fashion after 48 h. Extent of α -SMA expression varied among the fibroblasts obtained from different donor patients and was at the maximum level at a HAT concentration of approximately 30–60 nM. Fig 8 is a typical example of the effect of HAT concentration on α -SMA expression. HAT-induced α -SMA expression was at the maximum level at 48–72 h (data not shown). Increase in SMA expression with 30 nM HAT was similar to that with 100 pg/ml of TGF- β 1 (Fig 9).

VII Effect of HAT on HGF proliferation

Effect of HAT on HGF proliferation was examined in other HGF

samples different from those subjected to western blot analysis. As shown in Table 4, direct counting of cell number using a hemocytometer showed that HAT at 15, 30, and 60 nM slightly but significantly increased the cell number by 15–40% above the control value. Although this stimulatory effect of HAT on HGF proliferation was slight, and varied among samples, it was reproducible in all the four separate samples tested.

DISCUSSION

Following the discovery of mature HAT for the first time in human mucoid sputum from patients with chronic airway diseases (1), this study was primarily undertaken to clarify whether mature HAT is released in human saliva. When purification of trypsin-like protease from pooled saliva was performed using a method similar to that adopted for purification of HAT from mucoid sputum, distribution of trypsin-like activity of saliva in each purification step was very similar to that of HAT in the mucoid sputum (1). In this study, the yield of trypsin-like activity in the purified product was approximately 10%, with 330,000-fold increase in activity compared to that starting material. This value was similar to the yield after purification of HAT from the mucoid sputum. Moreover, biochemical properties, such as substrate specificity for various synthetic model substrates, behavior to protease inhibitors, and molecular weight estimated by gel filtration using Sephadex G-100 column, of this purified protease were almost similar to those of rHAT. These results strongly indicated that human saliva contains mature HAT.

Preliminary experiments showed that when saliva samples were diluted 2-fold and centrifuged, approximately 40% of trypsin-like protease activity was precipitated along with other macromolecules. Therefore, in the following experiments, to measure the total trypsin-like activity and HAT concentration in saliva samples, we used whole saliva homogenate as the test material. The saliva homogenates were finally diluted to 1500-fold for assaying trypsin-like activity and to 100-fold in PBS/2% Triton X-100/0.6% SDS for assaying HAT by ELISA. Therefore, most of the HAT contained in the whole homogenates was probably soluble in the present assay mixture used for determining trypsin-like activity and HAT concentration.

Trypsin-like activity was detected in all the 1500-fold diluted saliva homogenates from all healthy subjects, and immunoreactivity to anti-HAT antibody in ELISA was also detected in all the 100-fold diluted saliva homogenates. As shown in Fig 3, trypsin-like activity was significantly and positively correlated with HAT immunoreactivity in ELISA ($r = +0.922, p < 0.01$) for whole saliva homogenate. Moreover, in gel filtration of the concentrated original saliva on a Sephadex G-100 column, peaks of both trypsin-like activity and immunoreactivity to anti-HAT were observed in the fraction where rHAT was eluted. These results indicated that

Table 3 The trypsin-like activity and HAT concentration in saliva samples from healthy subjects and mucoid sputum samples from patients with chronic airway diseases

Sample	n	Total protein µg/ml	p	Trypsin-like activity mU/ml	p	HAT ng/ml	p
Saliva	61	3,340±1,638	p<0.001	23.9±16.7	p<0.001	153±112	p<0.001
Sputum	61	6,567±6,221		62.8±68.7		413±511	

Values are means ± SD.

P: p value tested for difference between saliva and sputum samples by the Wilcoxon-Mann-Whitney test

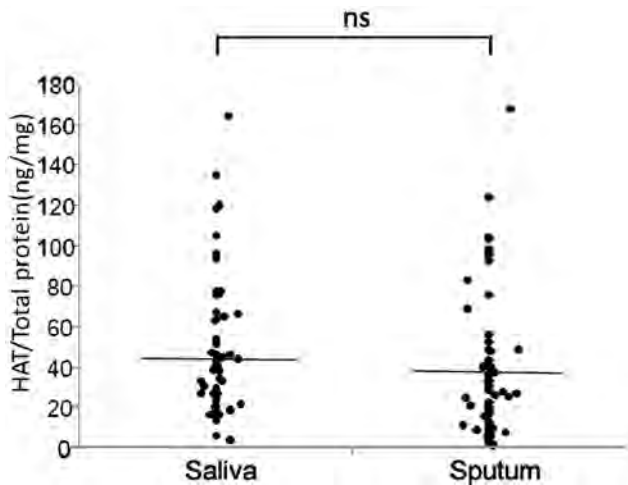


Fig 4 The ratio of HAT concentration to the total protein concentration in the saliva and mucoid sputum samples
Horizontal lines indicate mean values. * ns : not significant

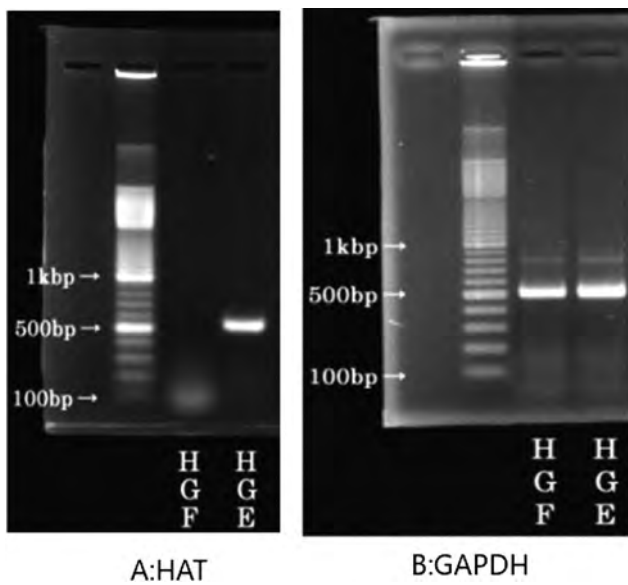


Fig 5 Analysis of the expression of HAT mRNA in primary human gingival epithelial cells (HGE) and human gingival fibroblast (HGF) by RT-PCR

Oral cells (both HGF and HGE) were cultured as described in the Materials and Methods. These cells were seeded into 60-mm cell culture dishes and proliferated in each growth medium until confluence.

Total cellular RNA was extracted from the cells using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RT-PCR procedures for HAT were performed as described previously (19). The length of the expected PCR product was 519 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as the control for HAT mRNA. The PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide.

A : HAT mRNA ; line 1, Molecular markers ; line 2, HGF ; line 3, HGE.
B : GAPDH mRNA.

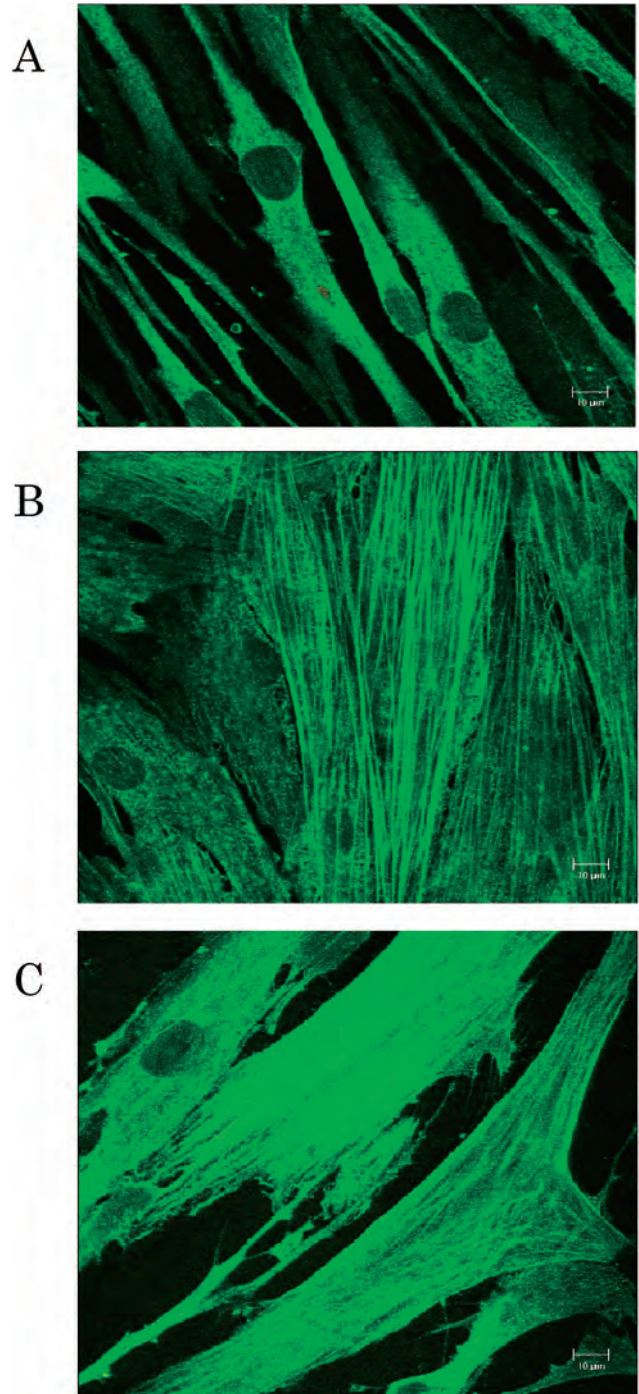


Fig 6 Analysis of the effect of HAT on the expression of α -smooth muscle actin in HGF by indirect immunofluorescence staining (1) Group A HGFs were seeded onto 18 \times 18 mm cover glasses in 6-well plates in DMEM supplemented with 10% FBS, and cultured to 60% confluence. After they were cultured in serum-free DMEM for 24 h, the cells were either stimulated with HAT (30 nM) or TGF- β 1 (100 pg/ml) for 48 h or were not stimulated. Indirect immunofluorescence staining of α -smooth muscle actin was performed as described in the Materials and Methods.

A : control cells. B : TGF- β 1 (100 pg/ml)-treated cells. C : HAT (30 nM)-treated cells.

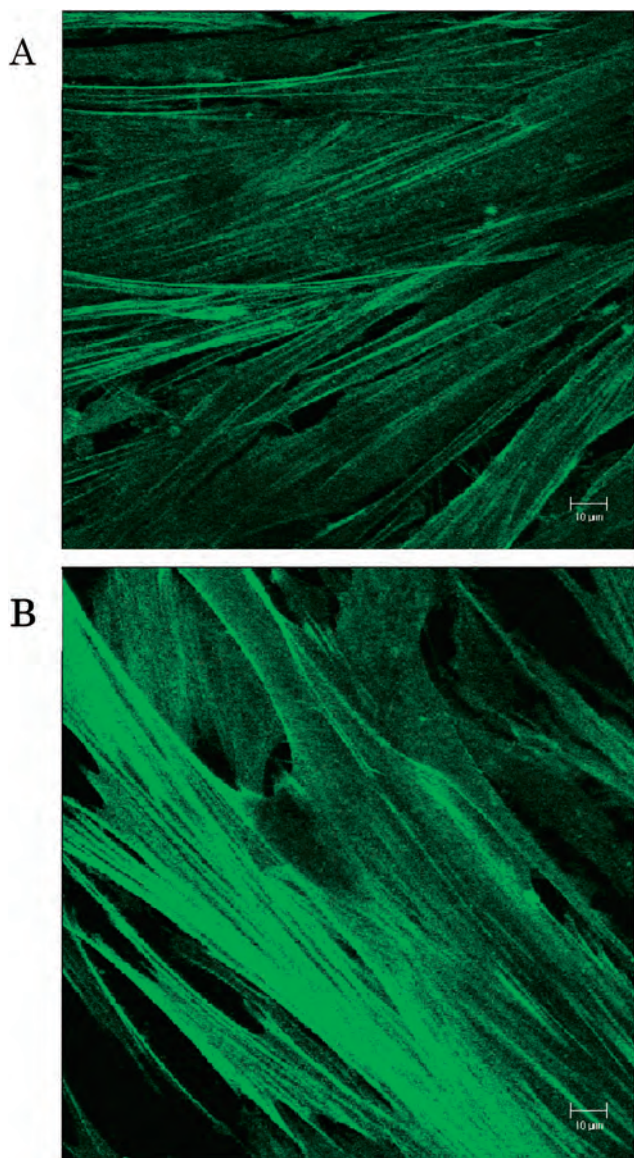


Fig 7 Analysis of the effect of HAT on α -smooth muscle actin expression in HGF by indirect immunofluorescence staining (2) Group B : Experimental procedures were performed as described in Fig 6. A : control cells. B : HAT (30 nM) -treated cells.

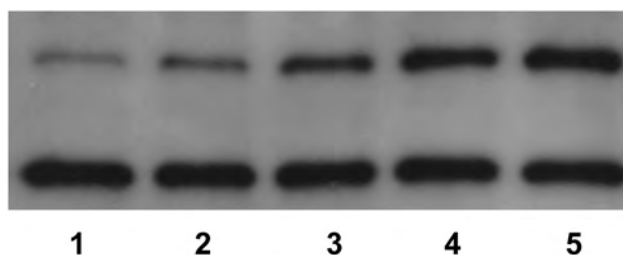


Fig 8 Analysis of the effect of HAT on α -smooth muscle actin expression in HGF by western blotting (1)
Dose response : HGFs were seeded into in 6-well plates (Greiner) in DMEM supplemented with 10% FBS and cultured to 60% confluence. After they were cultured in serum-free DMEM for 24 h, the cells were cultured without stimulus or with HAT (7.5-60 nM) for 48 h. HAT concentration : 1, control ; 2, 7.5 nM ; 3, 15 nM ; 4, 30 nM ; 5, 60 nM. Western blotting was performed as described in the Materials and Methods.

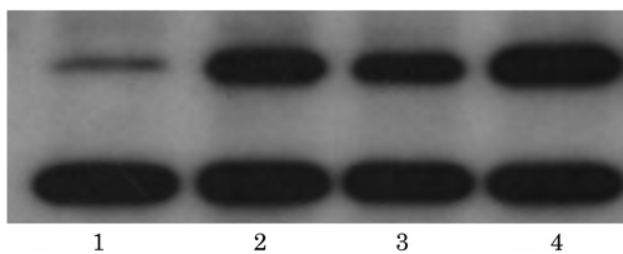


Fig 9 Analysis of the effects of HAT and TGF- β 1 on α -smooth muscle actin expression in HGF by western blotting (2)
Experimental procedures were performed as described in Fig8. Western blotting was performed as described in the Materials and Methods. 1, control ; 2, HAT (30 nM) ; 3, TGF- β 1 (50 pg/ml) ; 4, TGF- β 1 (100 pg/ml).

Table 4. Effect of HAT on proliferation of HGF

Protease	Concentration (nM)	Number of Cell/well($\times 10^4$)
Control		1.18 \pm 0.11
HAT	15	1.29 \pm 0.05*
HAT	30	1.39 \pm 0.07*
HAT	60	1.41 \pm 0.12*

Data are presented as means \pm SD from 8 well. *P< 0.05 compared with control.

the trypsin-like activity of saliva is primarily due to mature HAT, supporting the idea that saliva contains mature HAT.

It was considered that when saliva is collected by the suction method, precursor HAT of oral cells may be activated by negative pressure due to suction and processed into mature HAT. However, in the preliminary experiments, mature HAT was present in the saliva collected by the spitting method, wherein negative pressure was not inducted. Both trypsin-like activity and immunoreactive HAT concentration were almost similar in the saliva collected by the suction and spitting methods in healthy subjects. Therefore, mature HAT is thought to be released into the saliva, regardless of the method of saliva collection.

As shown in Fig 4, the HAT : TP ratio (ng/mg) was almost similar in the saliva and mucoid samples, suggesting that mature HAT is actively released in the saliva in the healthy state as it is released into the bronchial secretion in chronic airway disease.

As described previously (1), mature HAT was first isolated from the mucoid sputum. Because the mucoid sputum was expectorated by all patients, it was relatively contaminated with saliva. Therefore, it was considered that the mucoid sputum may contain both airway- and saliva-derived HAT. The ratio of airway-derived HAT to saliva-derived HAT in the expectorated mucoid sputum samples is considered to be different among the samples. Chronic airway diseases, such as chronic bronchitis, are accompanied with airway hypersecretion (23). In patients with hypersecretory chronic airway diseases, the proportion of airway-derived HAT to total HAT in the mucoid sputum samples is assumed to increase with the degree of airway hypersecretion.

Previous investigations have shown that HAT is expressed in various tissues, particularly in the epithelial cells of upper respiratory and gastrointestinal tracts, including oral tissues (5-9). Oral tissues are closely localized in both the upper respiratory and gastrointestinal tracts. These results indicate that HAT is expressed in the epithelial cells in healthy state in these organs. No investigators have analyzed HAT mRNA expression in oral cells cultured in vitro. The present study showed that HAT mRNA is prominently expressed in gingival epithelial cells, whereas it was not detected in gingival fibroblasts. These results further prove that HAT is expressed in epithelial cells in the oral cavity in healthy state. HAT mRNA expression has been observed in primary bronchial epithelial cells by RT-PCR in our laboratory (19).

We consider that HAT plays certain roles at least at two different locations in the airways and oral cavity; it functions on the epithelial cells where it is biosynthesized or in the neighboring cells and at locations distant from the epithelial cells after it is released. Mature HAT detected in the saliva and mucoid sputum is assumed to correspond to that released.

Previous investigators have reported that serine proteases, such as thrombin and mast cell tryptase, are involved in fibroblast proliferation (24-25) and in fibroblast-to-myofibroblast differentiation (20-22). In the present study, we examined whether HAT enhances the differentiation of HGF into myofibroblasts or not by assessing the expression of α -SMA fiber, a marker of myofibroblast (20-22, 26-27), by immunofluorescence and western blotting. Our results suggested that HAT enhances SMA fiber expression, irrespective of the existence of intracellular SMA fiber in HGF, because the SMA expression-enhancing activity of HAT was observed in HGF both in which intracellular SMA expression was not detected and in those in which SMA expression was detected.

The SMA expression-enhancing activity of HAT in HGF was also confirmed by western blotting. In this method, the extent of α -SMA expression induced by 100 mU/ml (30 nM) HAT was similar to that induced by 100 pg/ml TGF- β 1.

There were some differences between HAT and TGF- β 1 with respect to the dose response of SMA expression-enhancing activity. HAT activity reached a plateau at a concentration of 30–60 nM (100–200 mU/ml), whereas TGF- β 1 activity increased with concentration of 100–1000 pg/ml. This could be explained by the fact that the mechanism of the SMA expression-enhancing activity of HAT differs from that of TGF- β 1. TGF- β 1 induces fibroblast-to-myofibroblast differentiation via its cell surface receptor (28). However, HAT may enhance fibroblast-to-myofibroblast differentiation via cell surface protein receptors different from the TGF- β 1 receptor. Because HAT is a serine protease, it is assumed to hydrolyze several cell surface proteins that are related to various cellular functions.

Hitherto, it has been demonstrated that HAT attacked urinary type-plasminogen activator receptor (uPAR) in human bronchial epithelial cells (HBEC) (6), and protease-activated receptor-2 in HBEC, bronchial fibroblasts, and keratinocytes (11, 29-30). However, in fibroblast-to-myofibroblast differentiation, the target protein on the cell surface of HGF that HAT initially attacks is yet unknown. This problem is now under investigation in our laboratory.

We have previously reported in HBF that the extent of HBF growth-stimulating activity of HAT was slight, but this activity of HAT was reproducible by direct counting of cell number and the 3 H-thymidine uptake method (11). The present study showed that the fibroblast proliferation-stimulating activity of HAT was also low in HGF, similar to that in HBF. Furthermore, HAT enhanced the stimulation of fibroblast-to-myofibroblast differentiation and enlarged the cell size. These results strongly suggested that HAT had higher ability to stimulate fibroblast-to-myofibroblast differentiation than that to stimulate fibroblast proliferation in HGF.

Myofibroblast differentiation from fibroblasts contributes to the

healing process, tissue remodeling, and fibrotic disorders (20-22). Fibroblast-to-myofibroblast differentiation is regulated by various cytokines and proteases (20-21, 26-27). This study showed that HAT enhanced fibroblast-to-myofibroblast differentiation at a concentration of 10–60 nM (10 200 mU/ml) in vitro. Therefore, mature HAT may exist at an adequate concentration to induce myofibroblast transformation in the oral tissues.

These results indicate that HAT may be related to some physiological functions and pathological states, concerning myofibroblasts, in the oral cavity.

ACKNOWLEDGEMENTS

The authors thank Dr. Hiroshi Eguchi and his colleagues at Teijin Institute for Biomedical Research, Tokyo, Japan, for kind gifts of human airway trypsin-like protease, its polyclonal antibodies.

This study was supported in part by a Grand-in AID for Scientific Research (C) No. 09672401 from the Ministry of Education, Science, Sports and Culture of Japan

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