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Role of the coagulation system in allergic inflammation in the upper airways

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Abstract Thrombin has been detected and demonstrated to play a role in the airways of patients with bronchial asthma, but its role in the upper airways including during allergic rhinitis is unknown. This study was conducted to explore whether thrombin is presence in the upper airways and, if so, whether it affects mucin secretion. Fifteen patients with allergic rhinitis were enrolled in the clinical study; primary nasal septum epithelial cells and normal bronchial epithelial cells were used for *in vitro* evaluation, and rats as animal models. Significant concentrations of thrombin were found in nasal secretion after allergic provocation in allergic patients, and thrombin and its agonistic receptor peptide induced significant secretion of mucin in primary nasal cells and normal bronchial epithelial cells as compared to non-stimulated cells. Increased mucosubstance secretion in septum epithelial cells was also induced after nasal instillation of thrombin in rats. Further, the anticoagulant, activated protein C, significantly inhibited thrombin-induced mucin secretion from septum epithelial cells in rats. The results of this study suggest that activation of the coagulation system occurs during the allergic response and that thrombin plays a crucial role in the regulation of mucin production in the upper airways. © 2008 Elsevier Inc. All rights reserved.

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Introduction

Activation of the coagulation system occurs in patients with allergic diseases [1,2]. Studies from our laboratory have demonstrated the presence of coagulation proteases including thrombin in the bronchial secretion from patients with chronic bronchial asthma [3–6]. Thrombin is the effector enzyme of the coagulation system with important biological functions not only in thrombosis and hemostasis but also in inflammation [7]. The precise role of thrombin in allergy remains unknown but indirect evidence suggests that it may play a fundamental role. For example, thrombin may contribute to allergic inflammation by activating platelets and subsequent release of serotonin, thromboxane A₂, platelet factor 4, chemokines, IgE, platelet-derived growth factors and procoagulant factors [7]. Thrombin may also participate in the inflammatory process by increasing vascular permeability, by causing vasodilatation or vasoconstriction and by promoting the migration of granulocytes [7]. Tissue remodeling is also a frequent complication of chronic allergic diseases [8]. Thrombin may contribute to the pathogenesis of vascular or bronchial wall remodeling by stimulating the proliferation of fibroblasts or smooth muscle cells and the secretion of extracellular matrix proteins [7].

Mucus hypersecretion is a pathognomonic sign of airway inflammation and a common clinical complaint in patients with chronic bronchitis, bronchial asthma, cystic fibrosis, bronchiectasis, rhinosinusitis and nasal polyposis [9]. Increased mucus production is currently an area of much interest because no specific drug is currently available for its treatment. The constituents of airway mucus are plasma-derived proteins, mucin glycoproteins and cellular debris [10]. The molecular structure of mucin consists of a peptide backbone and several oligosaccharide chains that are products of MUC genes and glycosyltransferase genes, respectively [10]. To date more than 20 varieties of mucin have been identified but MUC5AC, MUC5B and MUC2 appear to be predominant in airway inflammatory diseases. Epidermal growth factor (EGF) and its receptor (EGFR) play a critical role in the production of mucin. In addition to EGF, EGFR may be activated by other ligands including transforming growth factor- α , bacterial products, cigarette smoke, neutrophil elastase and leukotrienes [9]. Although previous studies have shown that thrombin may transactivate the EGFR cascade, suggesting that it might be involved in mucin production [11] experiments on the role of thrombin in mucin production have not been reported. The present study was undertaken to evaluate the hypothesis that activation of the coagulation system increases the production of mucin by epithelial cells in the upper airway.

Materials and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), L-glutamine, vitamin solution, sodium pyruvate, non-essential amino acids, transferrin, bovine pituitary extract, Trizol reagent and Superscript preamplification system™ were purchased from GIBCO (Grand Island, NY). Fetal bovine serum (FBS) was

from Bio Whittaker (Walkersville, MD) and penicillin, streptomycin and dexamethasone were from Nacalai Tesque (Kyoto, Japan). Bovine serum albumin (BSA), Ham's F12 medium, epinephrine, gentamycin, amphotericin B, insulin, triiodothyronine, cholera toxin, endothelial growth supplement, L-leucine, L-lysine, L-methionine, HEPES and non-specific protease from *Streptomyces griseus* were from Sigma (St. Louis, MO). Human thrombin was from Midori Juji Pharmaceuticals (Osaka, Japan) and activated protein C from Chemo-Sero-Therapeutic Research Institute, (Kumamoto, Japan); endotoxin was undetectable in the thrombin preparation. Human EGF was from Higeta Shoyu (Tokyo, Japan). Hydrocortisone and retinoic acid were from Wako (Osaka, Japan). All other chemicals and reagents used were of the highest quality commercially available.

Thrombin concentration in nasal secretion

A total of 15 patients with nasal allergy to house dust mite, positive in all allergy tests (anamnesis, rhinoscopy, nasal eosinophilia, skin test, and serum level of specific IgE antibody) were enrolled in this study. The range age of the patients was 15–83 years old and there were 9 males and 6 females. Informed consent was obtained from all subjects before sampling. The clinical protocol was approved by the Mie University Hospital Institutional Review Board for Clinical Investigation. Nasal secretion was collected from the nasal cavity by suction before and after the allergen provocation test. Stimulation was induced on the anterior and middle parts of the inferior turbinate by placing one round 3-mm diameter allergen paper disc containing 250 μ g of house-dust extract per disc. Sampling was carried out after 5 min of allergen challenge. The total volume of nasal secretion was measured, mixed with 5 volumes of phosphate buffered saline by shaking for 3 h at 4 °C, and then centrifuged at 500 $\times$ g for 30 min. The supernatants were preserved at –80 °C until use. Thrombin was measured spectrophotometrically using the synthetic substrate D-Phe-pipeconyl-Arg-p-nitroanilide (S-2238; Chromogenic, Molndal, Sweden) as follows: 100 μ l nasal secretion was incubated in the presence or absence of 250 antithrombin units of hirudin (50 μ l), a specific inhibitor of thrombin, for 30 min at 37 °C, and then 20 nM thrombin substrate S2388 was added. The difference between the apparent concentrations of thrombin in untreated samples and that of hirudin-treated samples was taken as the actual concentration of thrombin in nasal secretion. The concentration of thrombin was extrapolated from a curve drawn using standard concentrations of thrombin.

Cell culture

Normal human bronchial epithelial (NHBE) cells were purchased from Clonetics (Walkersville, MD). The human bronchial epithelial cell line BEAS-2B was obtained from the American Type Culture Collection (Rockville, MD). Both BEAS-2B and NHBE cells were assayed using conventional culture systems. NHBE cells were cultured in CCMD161 medium (Clonetics) supplemented with 30 μ g/ml bovine pituitary extract, 0.5 μ g/ml BSA, 0.5 μ g/ml epinephrine, 50 μ g/ml gentamycin, 50 ng/ml amphotericin B, 0.5 ng/ml human EGF, 0.5 μ g/ml hydrocortisone, 5 μ g/ml insulin, 7 ng/ml

triiodothyronine, 10 $\mu\text{g/ml}$ transferrin and 0.1 ng/ml retinoic acid. BEAS-2B cells were cultured in serum free Ham's F-12 medium supplemented with 5 $\mu\text{g/ml}$ insulin, 5 $\mu\text{g/ml}$ transferrin, 20 ng/ml human EGF, 0.1 μM dexamethasone, 20 ng/ml cholera toxin, 30 $\mu\text{g/ml}$ bovine pituitary extract and 1 μM retinoic acid. Nasal septum epithelial cells were isolated from nasal polyps of patients with chronic sinusitis. Briefly, stroma from polypoid lesions was removed and the epithelial cells were dissociated by incubating with 0.1% non-specific protease from *Streptomyces griseus* in Ham's F12 medium at 4 °C for 20 h. The cell suspension was then filtered through a 60 μm Nitex mesh, centrifuged at 500 $\times g$ for 10 min, washed and resuspended in culture medium (DMEM/Ham's F12 medium containing 0.5 mM L-leucine, 0.5 mM L-lysine, 0.1 mM L-methionine, 0.3 mM MgCl_2 , 0.4 mM MgSO_4 , 1 mM CaCl_2 , 9 mg/l phenol red, 14 mM NaHCO_3 , 7 mM L-glutamine, 50 $\mu\text{g/ml}$ penicillin, 50 $\mu\text{g/ml}$ streptomycin, 10 $\mu\text{g/ml}$ insulin, 0.1 $\mu\text{g/ml}$ hydrocortisone, 0.1 $\mu\text{g/ml}$ cholera toxin, 5 $\mu\text{g/ml}$ transferrin, 25 ng/ml human EGF, 8 $\mu\text{g/ml}$ endothelial growth supplement, 50 $\mu\text{g/ml}$ bovine pituitary extract and 30 mM HEPES). Dissociated cells were plated onto Transwell tissue culture inserts (24.5 mm diameter with 0.45 μm pore size, Costar, Cambridge, MA) pre-coated with 0.4 ml type-I collagen gel (Cellmatrix, Nitta gelatin, Osaka, Japan), at a density of 5×10^4 cells/well in 0.5 ml of culture medium. The bottom compartment was filled with 2 ml of culture medium containing 10% FBS for the first 24 h of culture only and then the medium in both compartments was

changed every other day. When the cells become confluent at day 10, an air-liquid interface was created by removing the apical medium and filling only the bottom compartment with 2 ml of culture medium supplemented with 5×10^{-8} retinoic acid [12].

Effect of thrombin, PAR-1 and APC on mucin secretion

Nasal epithelial cells (1×10^6 cells) were cultured for 24 h and NHBE cells (1×10^6 cells) for 48 h in the presence or absence of thrombin and then the level of MUC5AC was measured in the cell supernatants. The participation of the thrombin receptor, PAR-1, was assessed by incubating BEAS-2B cells for 24 h in the presence of several concentrations of PAR-1 agonist (Sigma, St. Louis, MO,) and then the level of MUC5AC was measured in the cell supernatants. To evaluate the inhibitory effect of APC on MUC5AC secretion, BEAS-2B bronchial epithelial cells were cultured in the presence of APC (30 $\mu\text{g/ml}$) and epithelial growth factor (EGF, 100 ng/ml), tumor necrosis factor- α (TNF- α , 100 U/ml) or thrombin (250 U/ml) and cell supernatants were collected after 48 h and stored at -80 °C until use.

Level of MUC5AC in cell supernatant

The level of MUC5AC protein was measured by ELISA [13] in which 50 μl of cell supernatant was incubated with bicarbonate buffer (50 μl) at 40 °C in a 96-well plate

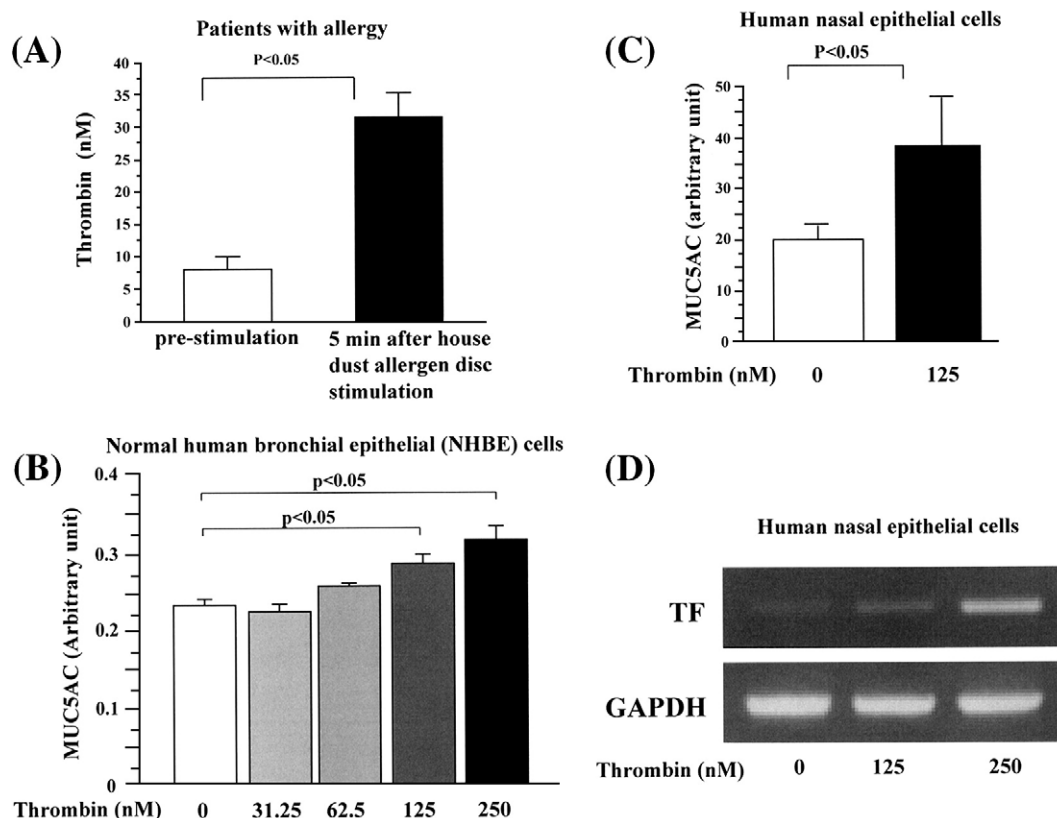


Figure 1 Thrombin in nasal secretion and its effect on mucin and tissue factor expression. Increased concentration of thrombin was found in nasal secretion after house dust mite challenge in patients with allergic rhinitis (A), and thrombin significantly increased the secretion of MUC5AC from normal bronchial epithelial cells (B) and from nasal epithelial cells (C). Thrombin also increased the expression of tissue factor (TF) as evaluated by RT-PCR (D). Data are the mean $\pm$ SE.

(Nunc) until dry. Plates were washed three times with PBS and then blocked with 5% BSA for 1 h at room temperature. Plates were again washed three times with PBS and then incubated with 50 μ l of mouse monoclonal anti-MUC5AC antibody (1:100 dilution) (Thermo, Waltham, MA) with PBS containing 0.05% Tween 20 and applied to each well. After 1 h, plates were washed three times with PBS, and 100 μ l of horseradish peroxidase-goat anti-mouse IgG conjugate (1:10000) was added to each well, incubated for 1 h before washing three times with PBS. Color reaction was developed using TMB peroxidase solution and stopped with 1 M H_2SO_4 . Absorbance was read at 450 nm.

Immunohistochemistry

Staining of PAR-1 in nasal epithelial cells was performed as described previously using specific antibodies (Santa Cruz Biotech, CA) [14].

Reverse-transcriptase polymerase chain reaction

Total RNA was extracted from cultured cells and tissues by the guanidine isothiocyanate procedure using Trizol Reagent (GIBCO, Grand Island, N.Y.). Two μ g of total RNA were reverse transcribed using oligo-dT primers and then the cDNA was amplified by PCR using the Superscript

Preamplification system kit (GIBCO Life Technologies) following the manufacturer's instructions. Samples without reverse transcriptase were included in these assays to check for DNA contamination. Sense and antisense primers used for amplification of PAR-1, epithelial growth factor receptor (EGFR), tissue factor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were reported in previous studies [15–17]. PCR products were then electrophoresed on a 2% agarose gel and stained with 0.5 μ g/ml ethidium bromide.

Animal model

Male F344 rats (specific pathogen free; Japan SLC, Inc., Shizuoka, Japan) 8–10 weeks of age were used. Rats were anesthetized with ether, and then 0.05 ml of saline, thrombin (20 μ M) or activated protein C (APC; 750 μ g/mL) was instilled into both nasal cavities each day for 3 days. 24 h after the last instillation, rats were killed by an intraperitoneal overdose of sodium pentobarbital. The head of each rat was removed and fixed in 10% neutral buffered formalin for 24 h, then decalcified in 5% trichloroacetic acid for 5 days. The nasal cavity was transversely sectioned at the incisive papillar level of the hard palate, and the tissue block was embedded in paraffin. 4- μ m-thick tissue sections were cut and stained with Alcian blue (pH, 2.6)-periodic acid-

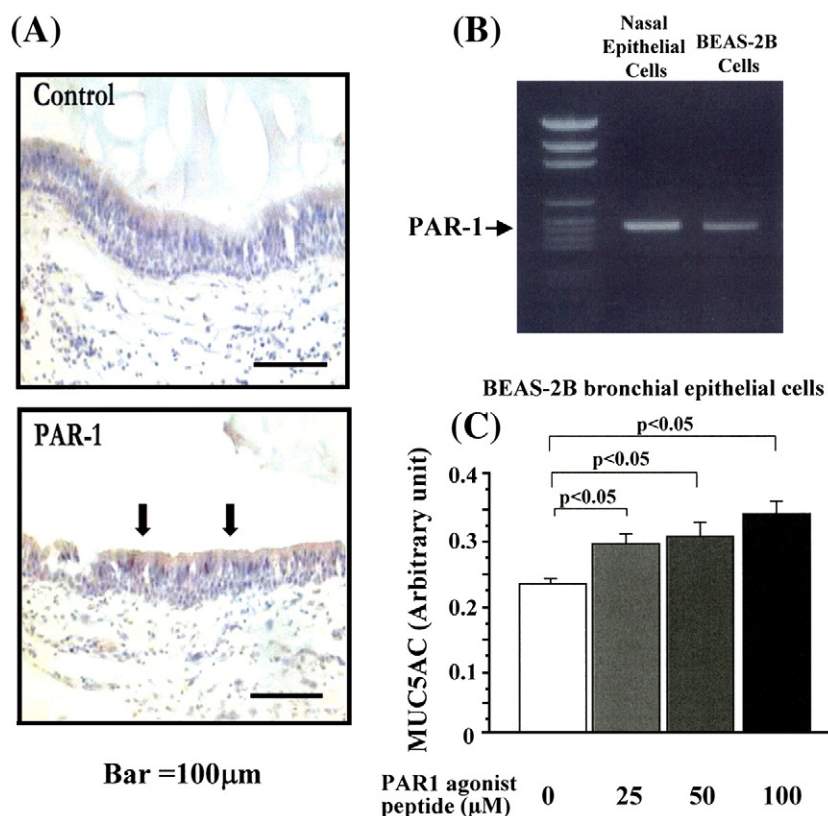


Figure 2 PAR-1 mediates the action of thrombin. Immunohistochemical analysis showed that nasal epithelial cells express PAR-1 (A). PCR analysis also confirmed mRNA expression of PAR-1 by primary nasal epithelial cells and BEAS-2B bronchial epithelial cells (B). PAR-1 agonist significantly stimulated the secretion of MUC5AC from normal human epithelial cells (C). Data are the mean $\pm$ SE. Arrows indicate sites of expression.

Schiff and hematoxylin (AB-PAS-H). The percent area of AB-PAS-H-stained mucosubstance on the surface epithelium was determined using an image analyzer (SP500; Olympus, Tokyo, Japan). The experimental protocol was approved by the Committee for Animal Care and Ethics of Mie University and it was carried out following the guidelines of the National Institute of Health.

Results

Thrombin in nasal secretions of allergic rhinitis patients

The total concentration of thrombin was measured in nasal secretions from patients with allergic rhinitis to house dust mite before and after provocation. The concentration of thrombin after provocation with disc of house dust mite was significantly increased compared to that before allergic provocation (Fig. 1A) in patients with allergic rhinitis.

Thrombin-mediated expression of mucin

To evaluate the effect of thrombin on MUC5AC secretion, we cultured primary normal human bronchial epithelial cells and stimulated them with thrombin. Thrombin stimulated the secretion of MUC5AC in the supernatant from normal bronchial epithelial cells in a dose-dependent manner (Fig. 1B). Similar *in vitro* experiments were performed using primary cultured cells from nasal epithelium. Thrombin significantly increased the secretion of MUC5AC from primary nasal epithelial cells (Fig. 1C).

Tissue factor expression by nasal epithelial cells

Tissue factor is the main initiator of coagulation activation [7]. Therefore we explored whether thrombin stimulates the expression of tissue factor, and found that thrombin increases the expression of tissue factor from primary nasal epithelial cells in a dose-dependent manner (Fig. 1D).

Mediation of protease-activated receptor-1

The main mediator of thrombin action on cells is protease-activated receptor (PAR)-1 [7]. Immunohistochemical analysis showed that human nasal epithelial cells express PAR-1 (Fig. 2A). PAR-3 but not PAR-4 was also detected (data not shown). PCR analysis confirmed the expression of PAR-1 by human nasal epithelial cells and BEAS-2B bronchial epithelial cells (Fig. 2B). PAR-1 agonistic peptide stimulates the secretion of MUC5AC in a dose-dependent manner in normal BEAS-2B human bronchial epithelial cells (Fig. 2C).

Effect of thrombin and anticoagulant activated protein C (APC) on mucin secretion *in vivo*

To explore the effect of thrombin and anticoagulation on mucin secretion *in vivo*, rats were intranasally instilled with thrombin alone, APC alone or thrombin plus APC (instilled 1 h

after thrombin) and then the amount of mucosubstance was measured in nasal epithelium in histopathological samples. Rats instilled with saline alone were used as control animals. Thrombin induced hypertrophy of goblet cells in rat nasal epithelium, and APC inhibited this thrombin-induced goblet cell hyperplasia (Fig. 3A). The quantitative measurement revealed increased secretion of mucosubstance in nasal epithelium from rats treated with thrombin alone as compared to rats treated with saline. Rats instilled with both thrombin and APC had decreased secretion of mucosubstance in nasal epithelium compared to rats that were instilled with thrombin alone (Fig. 3B). Interestingly, the production of mucosubstance in rats treated with APC alone, or thrombin plus APC was significantly increased as compared to the saline group.

Inhibition of MUC5AC secretion from airway epithelial cells by APC

EGF and TNF- α are known stimulators of mucin secretion, while APC has been reported to have anti-inflammatory activity by inhibiting the secretion of inflammatory cytokines. In bronchial epithelial cells APC was found to inhibit the secretion of MUC5AC induced by EGF or TNF- α (Fig. 4A)

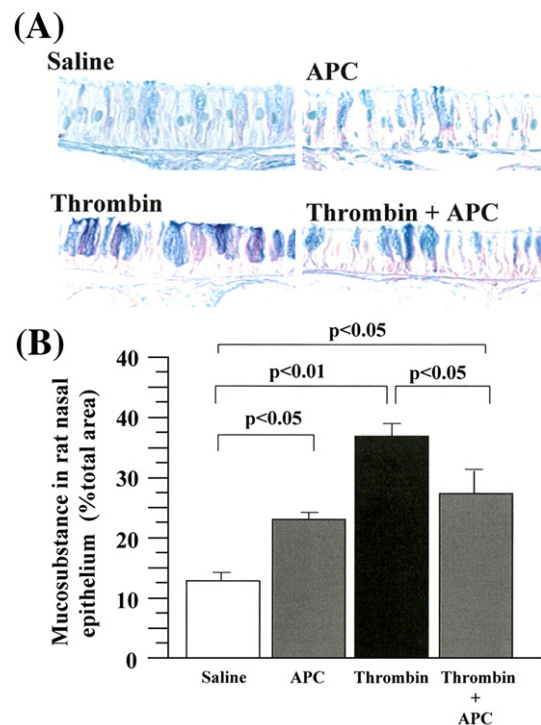


Figure 3 Effect of thrombin and the anticoagulant activated protein C (APC) on mucin secretion in rats. (A) Nasal tissue sections were stained with AB-PAS-H. Intranasal instillation of thrombin caused hypertrophic changes of goblet cells in rat nasal epithelium. APC inhibited thrombin-induced goblet cell hyperplasia. (B) Thrombin induced significant increase in epithelial mucosubstance compared to saline exposure. APC significantly suppressed thrombin-induced mucus production. Data are the mean $\pm$ SE of experiments performed in five mice in each group.

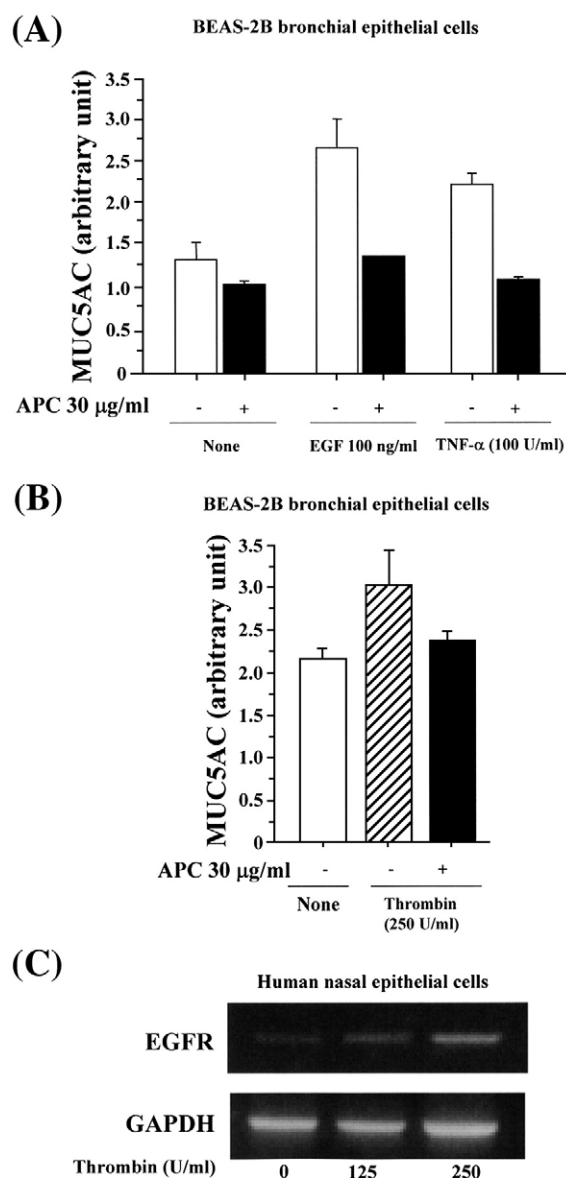


Figure 4 Effect of APC on MUC5AC secretion induced by EGF and TNF- α and effect of thrombin on EGFR expression. APC inhibited the secretion of MUC5AC from BEAS-2B cells induced by EGF, TNF- α and thrombin. Thrombin increased the expression of EGFR from nasal epithelial cells (C).

and thrombin (Fig. 4B). The receptor for EGF, EGFR, was stimulated by thrombin treatment of nasal epithelial cells (Fig. 4C).

Discussion

The results of this investigation showed that stimulation with the allergen enhances the production of thrombin in the nasal secretion of patients with allergic rhinitis, that thrombin is involved in the expression of mucin from nasal and bronchial epithelial cells and that this action is mediated by the PAR-1 receptor.

Mucins are high molecular weight glycoproteins that under physiological conditions, together with ciliated

epithelial cells, play an essential role in the maintenance of sterile and unobstructed airways [9]. However, under pathological conditions overproduction of mucins may be the cause of increased morbidity and mortality of acute and chronic airway diseases such as bronchial asthma and chronic obstructive pulmonary disease [9]. In this study we focused on MUC5AC as it mainly expressed in epithelial goblet cells and both its mRNA and protein are significantly increased in bronchial tissues from patients with asthma [18,19]. Several factors including inflammatory cytokines (IL-4, IL-13, TNF- α) and growth factors (EGF) have been found to increase the secretion of mucin from the upper airways [10]. In the present study, besides these stimuli we found that thrombin, the effector enzyme of the coagulation system, also enhances the production of mucin from airway epithelial cells in a dose-dependent manner via its PAR-1 receptor, suggesting that the level of thrombin may regulate mucin production in the airways. In addition to its crucial role in thrombosis and haemostasis, thrombin can also stimulate the inflammatory response by enhancing the secretion of inflammatory cytokines and growth factors from a variety of cells [7,15], and in our present study we found that thrombin can also stimulate the expression of both TF and EGF receptor from nasal epithelial cells. These observations indicate that thrombin can induce the secretion of mucin, not only directly, but also indirectly by increasing the expression of inflammatory cytokines and EGF receptor from airway epithelial cells. The exact intracellular mechanism by which thrombin stimulation of PAR-1 leads to an increase in mucin secretion is not clear but transactivation of EGF receptor is a potential mechanism [11].

In our present study, the increased generation of thrombin in nasal secretion induced by allergen stimulation in patients with allergic rhinitis, and the increased secretion of mucin after instillation of thrombin in rat septum epithelial cells suggest that activation of the coagulation system occurs at sites of allergic reaction and that its product, thrombin, can actively stimulate the secretion of mucin *in vivo*. In addition, we found that thrombin enhances the expression of tissue factor, the initiator of the coagulation system, suggesting that thrombin can induce a vicious circle causing continuous activation of the coagulation system and subsequent thrombin generation in the upper airways [7]. To confirm the role of the coagulation system activation in the upper airways we treated rats with intranasal instillation of APC together with thrombin. Intranasal instillation of the anti-coagulant APC significantly decreased the secretion of mucin in the septum epithelium induced by thrombin in rats, suggesting that activation of the coagulation system plays a fundamental role in the upper airways. APC has been also reported to exert anti-inflammatory activity by its ability to inhibit the secretion of several cytokines and growth factors [6,20]. Therefore, we also explored whether APC inhibits the secretion of mucin induced by EGF or TNF- α and found that the secretion of MUC5AC induced by these factors from airway epithelial cells is suppressed by APC. These observations suggest that APC may protect against mucin overproduction not only by inhibiting the formation of thrombin but also by inhibiting mucin production induced by several stimulators. Instillation of APC alone also increased significantly the secretion of mucosubstance in the septum epithelium compared to rats instilled with saline. Mucin is

a defense mechanism against allergen and microbes, thus enhancement of its secretion by APC may be important for removal of allergen and microbes from the upper airways under physiological conditions. It has been reported that different environmental factors may affect the composition, in particular, the degree of glycosylation and sulfation of mucin components, therefore, it is also conceivable that APC and thrombin differently affect the composition the composition of mucin [21,22].

One limitation of the present study was the use of bronchial epithelial cells instead of nasal epithelial cells in some of the *in vitro* experiments. However, the use of bronchial epithelial cells as surrogates of nasal epithelial cells was based on the following observations: (1) upper and lower airways are closely associated in terms of anatomy, physiology, pathology, pharmacology and epidemiology, [23], (2) there is increasing evidence that supports the concept of "united airway disease" [24] and (3) there is similarity between nasal and bronchial epithelial cells in the pattern expression of surface receptors and in the response to cytokine stimulation [25], (4) and it was found significant correlation between nasal and bronchial cells mediator release [25].

In summary, the results of this study showed that factors of the coagulation systems including thrombin and APC play a regulatory role in inflammatory and allergic responses in the upper airways.

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