Histochem Cell Biol (1998) 110:449–455

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ORIGINAL PAPER

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Fibrinolytic components in nasal mucosa and nasal secretion

Accepted: 25 May 1998

Abstract We evaluated a possible role for fibrinolytic components in nasal secretion by tissue localization with immunohistochemical techniques and by measuring their antigen concentrations in nasal discharge by means of ELISA and fibrin autography. Nasal mucosa was obtained surgically from the inferior turbinate. Urokinase-type plasminogen activator (u-PA) specific staining was observed in pseudostratified ciliated epithelium and was predominant in mucous cells of the seromucinous gland, while serous cells were almost devoid of stain. The pattern of staining of plasminogen activator inhibitor-2 was similar to that of u-PA. In contrast, plasminogen activator inhibitor-1(PAI-1) immunoreactive material was localized exclusively in serous cells of seromucinous glands. Positive staining for tissue-type plasminogen activator (t-PA) was observed in endothelial cells and basal cells, which differentiate into either ciliated or goblet cells. Nasal secretions were partially fractionated by immunospecific antibody-immobilized Sepharose. Subsequent fibrin autography patterns indicated the presence of u-PA, PAI-1, and t-PA. After methacholine provocation, the level of t-PA increased transiently but decreased rapidly with subsequent challenges. These differential stainings of fibrinolytic components and the existence of PAs and PAI-1 in the nasal discharge suggest that the fibrinolytic system may play a role in the movement and fluidity of nasal secretion.

Introduction

The fibrinolytic system plays an important role not only in degradation of fibrin clots but also in a variety of biological processes occurring in various human tissues, such as neovascularization, wound healing, ovulation, tumor invasion, and inflammation (Danf et al. 1985; Loskutoff et al. 1989; Carmeliet and Collen 1995). Whereas tissue-type plasminogen activator (t-PA) is involved primarily in clot dissolution, urokinase-type plasminogen activator (u-PA) has been implicated in pericellular proteolysis (Bugge et al. 1996a). u-PA is involved in tissue remodeling not only by converting plasminogen to plasmin, which directly or indirectly degrades tissue proteins via metalloprotease activation, but also directly cleaves tissue proteins such as fibronectin (Glod et al. 1992). Their action is controlled by plasminogen activator inhibitors (PAIs), of which PAI-1 appears to be the predominant physiological inhibitor of t-PA and u-PA. PAI-2, unable to inhibit t-PA as efficiently as PAI-1, is thought to be primarily a u-PA inhibitor (Kawano et al. 1968; Sprengers and Kluft 1987).

u-PA has been extracted from nasal polyps (Yamashiro et al.1992) and the maxillary mucosa (Hamaguchi et al. 1985) of patients with purulent rhinosinusitis. Åkerlund et al. (1993) demonstrated markedly increased fibrinogen levels in nasal lavage liquid from coronavirus-induced common cold patients. These studies, however, showed fibrinolytic factors in the tissue extract of the nasal mucosa or conditioned medium of the cultured tissue. The current study focuses on the trigger level of fibrinolysis by analysis of the tissue localization of u-PA, t-PA, PAI-1, and PAI-2 in nasal mucosa. In addition, to evaluate the relationship between fibrinolytic components in the nasal gland and in nasal secretion, we measured antigen concentrations in the secretions by ELISA.

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Materials and methods

Sample preparation of nasal mucosa

Specimens of nasal mucosa were obtained from 39 patients who underwent surgical resection or biopsy of the inferior turbinate (19 women and 20 men) and from 10 normal volunteers (3 women and 7 men). The diagnoses of these patients were as follows: 14 puru-

lent rhinosinusitis, 8 allergic rhinitis, 10 hypertrophic rhinitis, and 7 paranasal tumor. All samples were obtained with informed consent. Specimens were washed briefly with Hank's solution and fixed for 30 min in an ice-cold mixture of 2% paraformaldehyde, 0.2% glutaraldehyde, and 0.2% picric acid in 0.1 mol/l sodium phosphate buffer, pH 7.4, followed by postfixation for 10 h at 4°C in a second fixative consisting of 2% paraformaldehyde and 0.2% picric acid in sodium phosphate buffer. The fixed tissues were washed with sodium phosphate buffer containing 15% sucrose. They were placed in Tissue Tek OCT compound (Miles Laboratories, Naperville, Ill., USA) and immediately snap-frozen in n-hexane precooled with dry ice-acetone and then sectioned at 6 mm with a freezing microtome.

Proteins and characterization of monoclonal antibodies

Human t-PA and PAI-1 were purified as described previously (Sakata et al. 1988). u-PA was a kind gift from Mochida Pharmaceutical (Tokyo, Japan). Bovine serum albumin (essentially fatty acid-free, globulin-free) was purchased from Sigma (St. Louis, Mo., USA). Monospecific antisera against t-PA, PAI-1, u-PA and PAI-2 were prepared in rabbits as described (Sakata et al. 1988) and affinity purified with each antigen immobilized to Sepharose 4B. Murine monoclonal antibodies against t-PA, PAI-1, and u-PA were prepared and characterized with standard methods as described (Wakabayashi et al. 1986; Sakata et al. 1988, Sakata et al.1991). A monoclonal antibody against PAI-2 (MAI-21) was purchased from Biopool (Umea, Sweden).

Immunohistochemical staining

Sections of the nasal mucosa were treated sequentially with 1% hydrogen peroxide in 0.05 M phosphate, 0.1 M NaCl, pH 7.4 (PBS) for 15 min to inhibit endogenous peroxidase activity in blood cells, and with 3% bovine serum albumin (BSA) in PBS containing 0.05% Triton X-100 for another 30 min at room temperature. The treated sections were incubated with the appropriate primary antibody at the desired dilutions (1:250 to 1:1000) in PBS containing Triton X-100 with 1.5% BSA for 24 h at 4°C. After washing extensively with PBS containing Triton X-100, they were processed further with an avidin-biotin-peroxidase system (Vectastain ABC kit, Vector, Burlingame, Calif., USA). Immunoreactive sites were visualized with hydrogen peroxide and diaminobenzidine, and counterstained with hematoxylin. Control sections were incubated with 5 μg/ml of non-immune mouse immunoglobulin G (Pierce, Rockford, Ill., USA) instead of the primary antibodies.

Collection of nasal secretion

Nasal secretion was obtained from ten normal volunteers without any finding of rhinitis or sinusitis, and from five patients with allergic rhinitis and five with purulent rhinosinusitis. The volunteers' mean age was 23.5 years (18–33); three were women and seven were men. The patients' mean age was 28 years (20–37); four were women and six were men. Nasal secretion from patients with allergic rhinitis or purulent rhinosinusitis was collected by carefully aspirating the secretion pooled in the nasal cavity through the anterior nostril without mechanical stimulation.

Nasal secretion from normal volunteers was collected after provocation with methacholine (acetyl-beta-methylcholine chloride) by careful aspiration while avoiding mechanical stimulation. The stimulation was necessary because the usual nasal secretion volume from normal volunteers was too small for sampling and analysis. Methacholine powder (Sigma) stored at –20°C, was dissolved in distilled water (20 mg/ml) immediately before use. Nasal provocation with methacholine was performed 5 times at 10 min intervals. At each time point, 0.5 ml of methacholine was administered by spray through the anterior nostril. Immediately after the spray, secretions were collected until just before the next stimulation. During this period, the volunteers sat with the head bent forward and were instructed to breath through the mouth.

Each nasal secretion collection was immediately mixed with an equal volume of 0.05 M TRIS, 0.1 M NaCl, pH 7.4, containing 10 mM EDTA, 500 KIU/ml aprotinin, and 50 μg/ml neutrophil elastase specific inhibitor, Ono-546 ([*N*-[2-[4-(2,2 dimethylpropionyloxy) phenylsulfonylamino]benzoyl] amino acetic acid; Ono Pharmaceutical, Osaka, Japan) in order to prevent proteolytic degradation of fibrinolytic components. Samples were centrifuged (2000×*g*, 4°C, 15 min) to remove cells and debris, and the supernatant was immediately frozen and stored at –80°C until use.

Biochemical analysis of nasal secretion

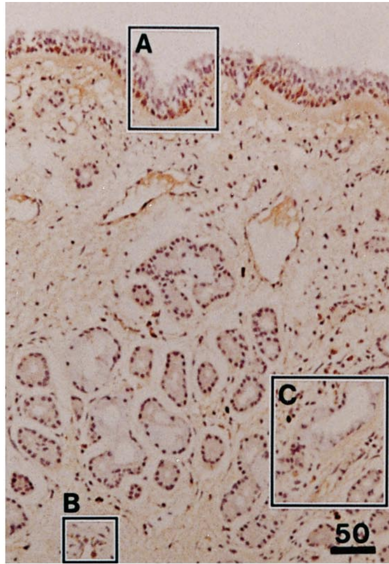
The fibrinolytic components of nasal secretion were analyzed by ELISA and by fibrin autography after SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE on slab gels was carried out using 9% resolving gels and 4% stacking gels according to Laemmli (1970). Fibrin agar indicator gels were prepared as previously described (Sakata et al. 1985). After electrophoresis, the gels were soaked in 2.5% Triton X-100 for 1.5 h at room temperature, patted dry with paper towels, and applied to the surface of fibrin agar indicator gels. For fibrin autography, the dark areas of the indicator film indicate the presence of PAs in the acrylamide gel. To determine the type of PA, the supernatant of the nasal secretion was mixed with an equal volume of 0.05 M TRIS, 0.1 M NaCl, pH 7.4, containing 0.5% Triton X-100 and applied to an anti-u-PA, anti-t-PA, or anti-PAI-1 immobilized Sepharose 4B column. The pass-through and the eluate obtained with SDS-PAGE sample buffer were collected and analyzed by fibrin autography.

Results

Fibrinolytic components in the epithelium

t-PA immunoreactive material was localized predominantly in the basal cells, while intermediate cells were almost devoid of stain (Fig. 1A). Strong staining for u-PA was seen predominantly in the cytoplasm of intermediate cells, but the basal cells were almost devoid of stain (Fig. 1D). The staining pattern of both activators was granular in the cytoplasm and devoid in the nucleus. Since PA activity is regulated by PAIs, the tissue distribution of the inhibitors was of interest. There was no significant staining of PAI-1 in the epithelium (Fig. 2A). We observed a similar distribution of PAI-2 and u-PA in the epithelium (Figs. 1D, 2D). There was no apparent difference in staining patterns between tissues from normal volunteers (one representative case is shown in Figs. 1, 2) and those from the patients with clinical disease.

Fig. 1A–F Immunolocalization of tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) in nasal mucosa. Nasal mucosa was immunostained with anti-t-PA (**A–C**) or anti-u-PA monoclonal antibody (**D–F**). *Upper panel* shows the sites corresponding to **A–F**. **A, D** *insets* Different magnification of the same object to show the basal cells and intermediate cells in more detail. *Arrows* Intermediate cells, *arrowheads* basal cells. *Bars* 10 μm and 50 μm



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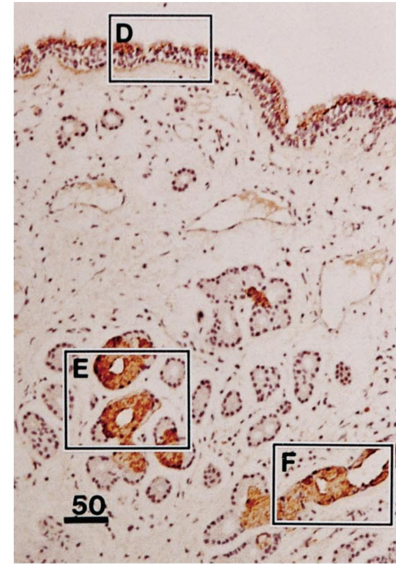

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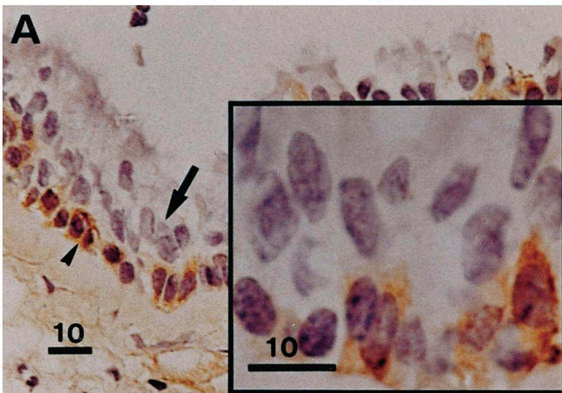

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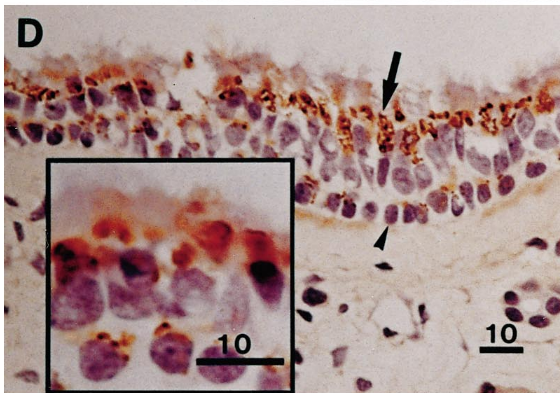

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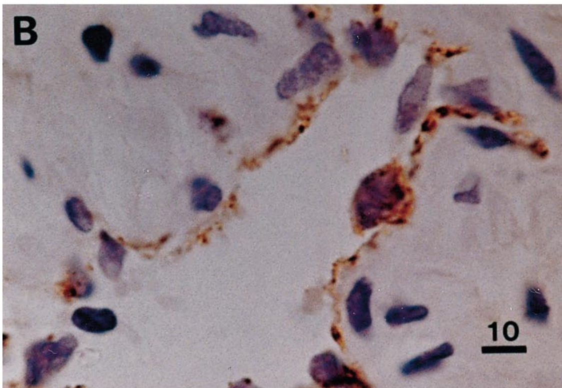

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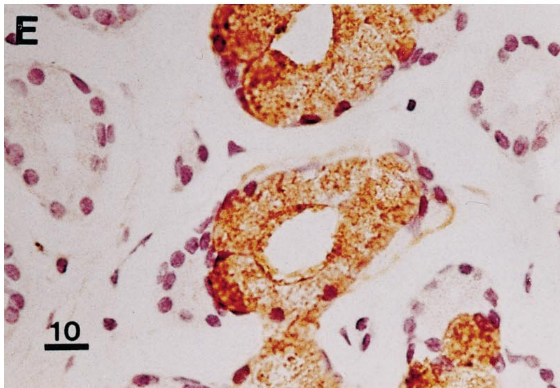

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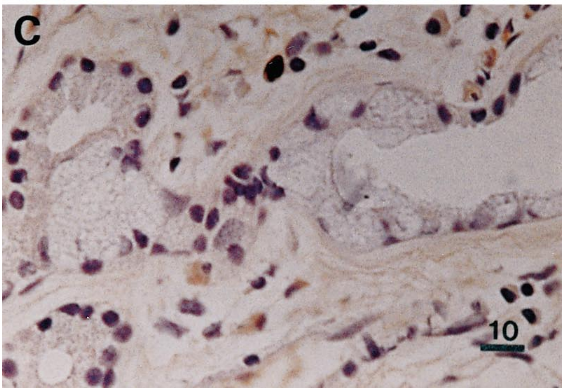

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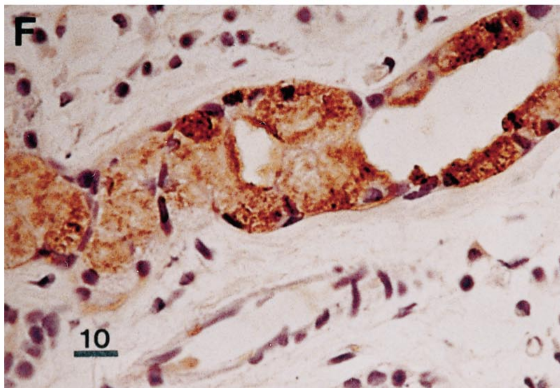

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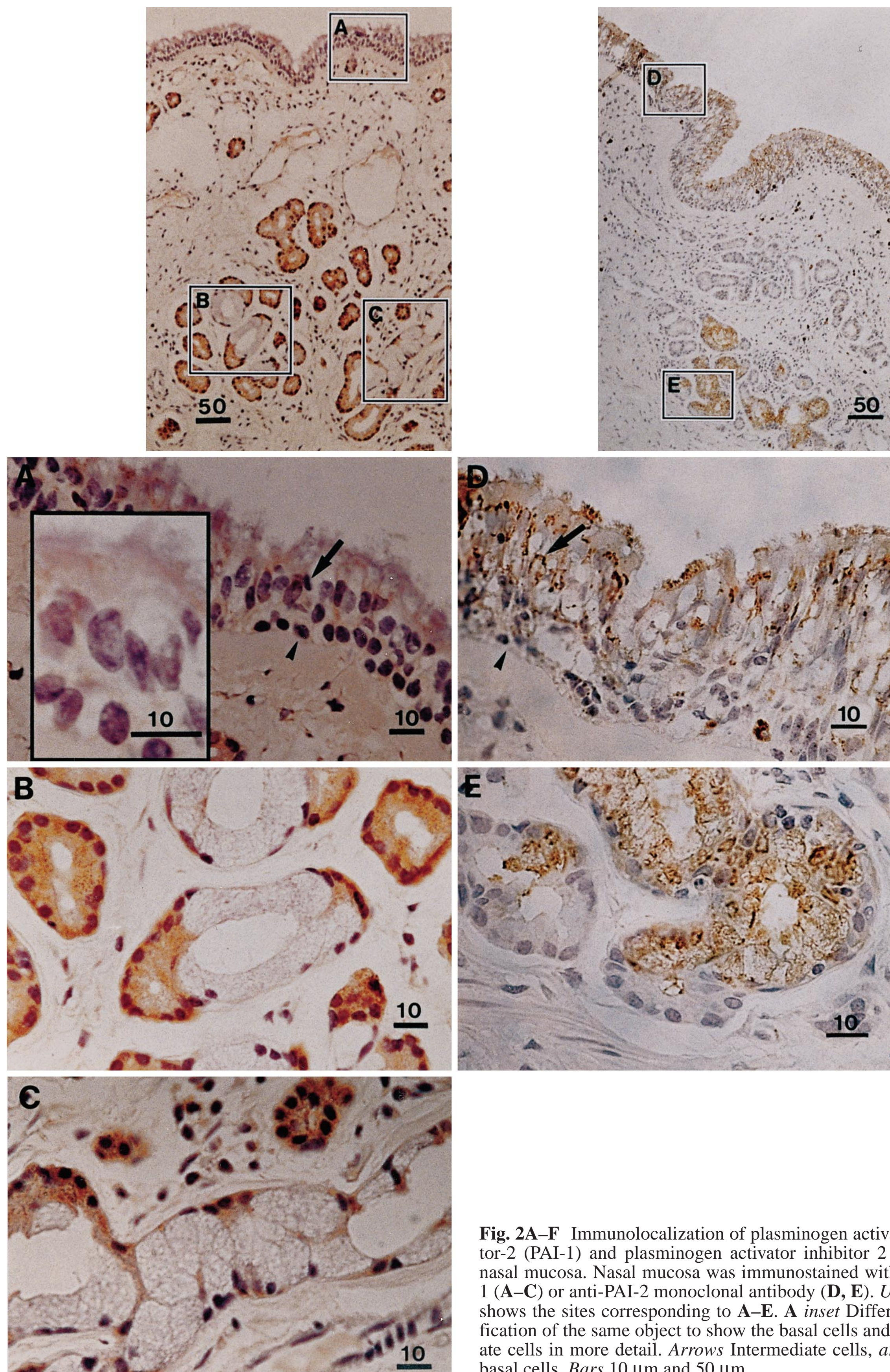

Fig. 2A–F Immunolocalization of plasminogen activator inhibitor-2 (PAI-1) and plasminogen activator inhibitor 2 (PAI-2) in nasal mucosa. Nasal mucosa was immunostained with anti-PAI-1 (**A–C**) or anti-PAI-2 monoclonal antibody (**D, E**). *Upper panel* shows the sites corresponding to **A–E**. **A** *inset* Different magnification of the same object to show the basal cells and intermediate cells in more detail. *Arrows* Intermediate cells, *arrow heads* basal cells. *Bars* 10 μm and 50 μm

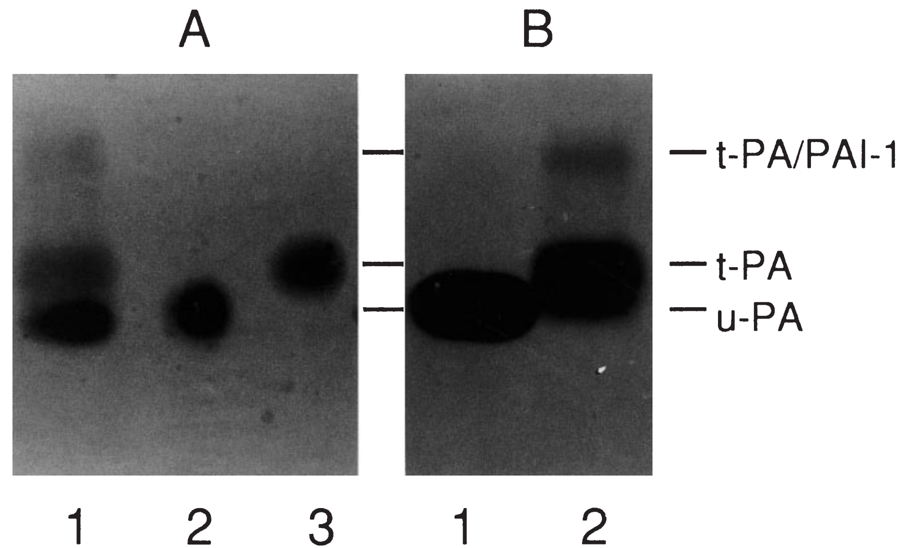

Fig. 3A, B Electrophoretic and fibrin autography analysis of fibrinolytic factors in nasal secretion. Samples were fractionated by SDS-PAGE under non-reducing conditions and subjected to fibrin autography. **A** Nasal secretion, 100 µl obtained from normal volunteers by single methacholine provocation (*lane 1*), pass-through of the nasal secretion from anti-t-PA monoclonal antibody-immobilized column (*lane 2*) and pass-through from anti-u-PA monoclonal antibody-immobilized column (*lane 3*). **B** Eluate, 50 µl, from anti-u-PA monoclonal antibody-immobilized column (*lane 1*) and from anti-t-PA monoclonal antibody-immobilized column (*lane 2*)

Table 1 Immunohistochemical localization of fibrinolytic factors in human nasal mucosa. Immunohistochemical staining of 49 samples of nasal mucosa was performed. + Strong or moderate immunoreactivity in almost all specimens, – no immunoreactivity in almost all specimens (*t-PA* Tissue-type plasminogen activator, *u-PA* urolcinase-type plasminogen activator, *PAI-1* plasminogen activator inhibitor-1, *PAI-2* plasminogen activator inhibitro-2)

	t-PA	u-PA	PAI-1	PAI-2
Surface epithelium				
Intermediate	–	+	–	+
Basal	+	–	–	–
Seromucous glands				
Mucous cells	–	+	–	+
Serous cells	–	–	+	–
Endothelium	+	–	–	–

Immunohistochemical staining of 49 nasal mucosa was performed as described in Materials and Methods. +, strong or moderate immunoreactivity in almost all specimens; -, no immunoreactivity in almost all specimens.

Immunolocalization of fibrinolytic components in the lamina propria mucosa

Interestingly, there was a distinct difference of staining for u-PA, PAI-2, and PAI-1 between mucinous and serous cells of the nasal gland. The data shown is from normal volunteers and, again, no significant differences were observed between normal tissues and those obtained from patients with clinical disease. u-PA (Fig. 1E, F) and PAI-2 (Fig. 2E) were clearly localized in the mucinous cells, while almost no definitive localization was seen in the serous cells. In contrast, PAI-1 immunoreactive material was localized in the serous cells, whereas the mucinous cells were almost devoid of stain (Fig. 2B, C). t-PA immunoreactivity was not found in either mucinous or serous cells (Fig. 1C), but positive staining was observed only in the vessel wall (Fig. 1B). The immunohistochemistry results are summarized in Table 1.

Analysis of fibrinolytic components in nasal secretion

Nasal secretion was collected and applied to anti-u-PA, anti-t-PA, or anti-PAI-1 antibody-immobilized Sepharose. Each pass-through fraction (Fig. 3A, lanes 2, 3) and eluate (Fig. 3B, lanes 1, 2) was analyzed by fibrin autography after SDS-PAGE. As shown in Fig. 3A, both u-PA and t-PA activity was found in nasal secretion. In addition, almost all u-PA existed as the single-chain form because no fibrinolytic activity corresponding to a u-PA/PAI-1 complex was observed in the fibrin autography (Fig. 3B, lane 1). The fibrinolytic activity corresponding to a t-PA/PAI-1 complex (Fig. 3B, lane 2) was confirmed by removing this activity from the secretion with anti-PAI-1 Sepharose (data not shown). Although the mean PAI-1 level of purulent rhinosinusitis (15.1±6.3 ng/ml, n=5) was higher than that of normal volunteers (3.1±1.2 ng/ml, n=10), the absolute levels may vary considerably between individuals, because each secretion volume was different and the secretion from the normal volunteers was obtained after a methacholine provocation. However, although the size of the lytic zone was different, there was no apparent difference in the fibrin autography pattern between normal and patient secretions (Fig. 4).

Fibrinolytic components in nasal secretion after successive stimulations with methacholine

Each methacholine provocation produced similar measurable amounts of secretion that varied between individuals from 0.7 to 2.2 ml. Each sample was analyzed by ELISA and fibrin autography (Fig. 5). After the second provocation, t-PA antigen decreased relative to that observed from the first provocation. The u-PA antigen level as well as the PAI-1 antigen level decreased gradually with each successive provocation. The fibrin autography pattern (Fig. 5, inset) corresponded to the data obtained

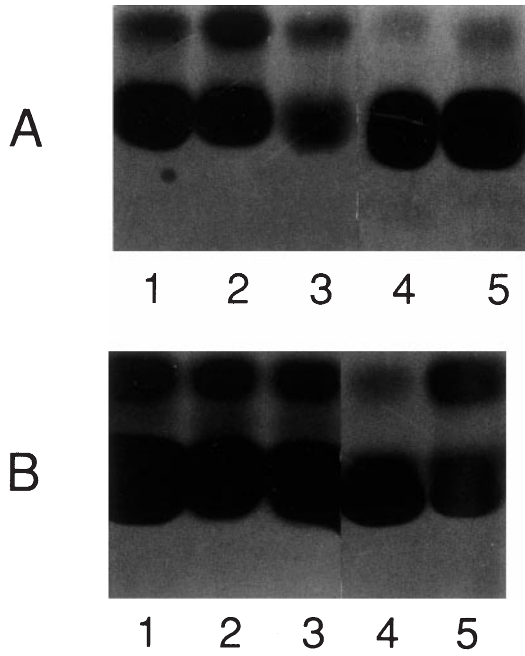

Fig. 4A, B Fibrin autography analysis of fibrinolytic components in nasal secretion from patients. Samples were analyzed by fibrin autography. **A** Nasal secretion 100 µl, obtained from allergic rhinitis patients (*lanes 1, 2–3*) and that from normal volunteers by single methacholine provocation (*lanes 4, 5*). **B** Nasal secretion, 100 µl, obtained from purulent rhinosinusitis patients (*lanes 1, 2, 3*) and that from allergic rhinitis patients (*lanes 4, 5*)

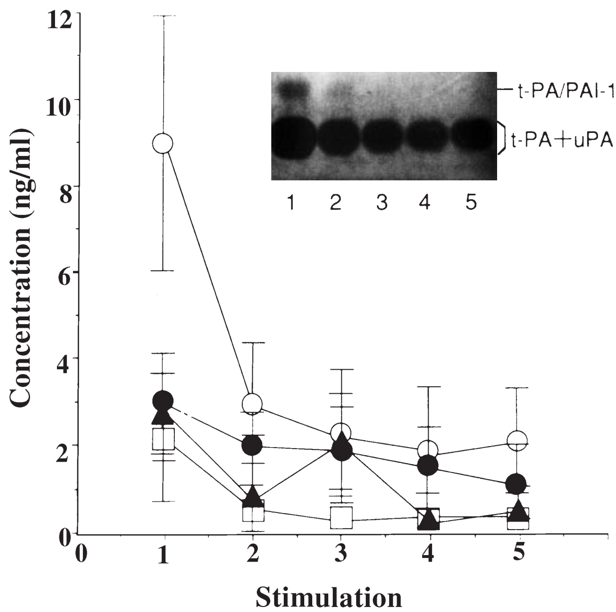

Fig. 5 Level of fibrinolytic factors with successive methacholine provocations in nasal secretion. Level of fibrinolytic factors in nasal secretion from normal volunteers obtained by successive methacholine stimulation were measured by ELISA for t-PA (○), u-PA (□), PAI-1 (▲), and t-PA/PAI-1 complex (●). Results are expressed as mean±SD, n=6. *Inset* shows the fibrin autography of a representative sample

by ELISA. Although the antigen levels of fibrinolytic components in the nasal secretion decreased with successive methacholine provocations, the pattern and immunoreactivity of PAs and PAIs in the nasal mucosa from normal volunteers were similar before and after provocation.

Discussion

Recently, many investigators have demonstrated the contribution of the PA-plasmin system in ovulation (Liu et al. 1991), arteriosclerosis (Carmeliet and Collen 1995, Schneiderman et al. 1995), and keratinocyte movement (Bugge et al. 1996b), as well as in smooth muscle cell movement and angiogensis (Carmeliet et al. 1997). Fibrinolytic components in nasal tissues have been examined mainly by measuring antigen levels in tissue extracts (Kosugi et al. 1982; Hamaguchi et al. 1988). However, their distribution and physiological roles in nasal tissues are not yet understood. In this study, two novel observations arose from the immunohistochemistry data. Firstly, the type of PA in the epithelial basal cells was different from that in the intermediate cells (Fig. 1) and secondly, u-PA colocalized with PAI-2 in the mucinous cells in the lamina propria and in the epithelial cells (Figs. 1, 2). PAI-1 immunoreactive material was localized solely in the serous cells.

According to a hypothesis put forward many years ago (Breeze and Wheeldon 1977, Jafek 1983), intermediate cells are derived from the basal cells and represent intermediate stages of development into either ciliated or goblet cells. The current observation that u-PA and t-PA distributions differ between the basal and intermediate cells raises the possibility that specific receptor and/or signal transduction pathways for expression of the PAs change during differentiation. The observation that u-PA stained the pseudostratified ciliated epithelium suggests that u-PA activity may be help the ciliated cells to move the mucous blanket lining of the nasal epithelium by moderately reducing the viscosity of the fluid.

Like amylase (Tachibana et al. 1986), PAI-1 was produced predominantly by the serous cells of the nasal glands. Although PAI-2 is currently thought to be primarily a u-PA inhibitor, PAI-1 can still inhibit both t-PA and u-PA almost 10- to 100-fold more efficiently (Sprengers and Kluft 1987; Loskutoff et al. 1989). In addition, PAI-1 and single-chain u-PA make a complex, although it easily dissociates during SDS-PAGE (Manchanda and Schwartz 1995). u-PA is synthesized and secreted as a single-chain polypeptide and is involved mainly in the fibrinolytic process occurring in tissues (Carmeliet and Collen 1995). The different localizations of PAI-1 (serous cells) and u-PA (mucinous cells) may contribute to a delayed inhibition of u-PA activity after secretion. It is also possible that PAI-2 is a primary inhibitor of u-PA in these tissues because of colocalization. Although further study is necessary, this selective localization of fibrinolytic components suggests a role for these proteins in maintaining normal nasal gland function.

Although t-PA was found in nasal secretion (Fig. 3), t-PA immunoreactive material was not observed in the nasal gland (Fig. 1). Since methacholine enhances vascular permeability only slightly (Raphael et al. 1988), and the level of t-PA in the secretion with the second provocation decreased significantly (Fig. 5), it is unlikely that plasma t-PA was induced by methacholine. Furthermore, successive methacholine provocations did not induce either continuous secretion or depletion of fibrinolytic components from the gland or endothelial cells. Therefore, it is more likely that secretion of the fibrinolytic components was induced by some stimulus other than the methacholine, and they stayed in the duct as a constituent of the mucous blanket. Most probably, methacholine provocation simply increased the volume of the nasal discharge and washed out these fibrinolytic components from the duct and blanket.

These components may play an important role in maintaining the fluidity of the nasal discharge. The production of the fibrinolytic components are regulated by several cytokines (Sadwey and Loskutoff 1991; Jensen and Rodeck 1993). Inflammatory cytokines, such as interleukin-1, upregulate the production of PAI-1 as well as fibrinogen (Loskutoff et al.1989). This would decrease fibrinolytic activity in the nasal secretion and may contribute, at least in part, to the increased viscosity of the secretion in the case of infectious rhinosinusitis.

Acknowledgements This work is supported in part by Grant-in-aid for Scientific Research number 09470234 to Y.S. from the Ministry of Education, Science, and Culture of Japan.

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