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METHODS

Patients and biopsies

Patients with CRS were recruited from the Allergy-Immunology Clinic and Otolaryngology Clinic of the Northwestern Medical Faculty Foundation, the group practice for physician faculty members of Northwestern University, and the Northwestern Sinus Center. Sinonasal and NP tissues were obtained from routine functional endoscopic sinus surgery in patients with CRS. All subjects met the criteria for CRS as defined by the American Academy of Otolaryngology-Head and Neck Surgery Chronic Rhinosinusitis Task Force. The presence of sinusitis or bilateral NP was confirmed by means of office endoscopy and computed tomographic imaging. All patients scheduled for surgery had previously failed to respond to adequate trials of conservative medical therapy (prolonged antibiotic regimens, nasal steroid sprays, oral steroids, saline irrigations, and decongestants) for control of symptoms. Patients with an established immunodeficiency, pregnancy, coagulation disorder, diagnosis of classic allergic fungal sinusitis, Samter's triad, Churg-Strauss syndrome, or cystic fibrosis did not participate in the study. Details of subjects' characteristics are included in Table I. Sinus tissues from disease-free control subjects were obtained during endoscopic skull-base tumor excisions, as well as intranasal procedures for obstructive sleep apnea and facial fracture repairs on patients with a history of CRS or asthma recruited from the otolaryngology clinic at the Northwestern Medical Faculty Foundation.

Subjects underwent skin tests to pollens, dust mites, pets, molds, and cockroaches by using Hollister-Stier (Spokane, Wash) extracts. A positive skin test response was defined as a wheal greater in size than that produced by the saline control by 3 mm or more. Histamine was used as a positive control. Atopic status was assessed in all subjects unless subjects declined or if the history did not suggest atopy.

All subjects signed informed consent, and the protocol and consent forms governing procedures for the study were approved by the Institutional Review Board of Northwestern University Feinberg School of Medicine.

Real-time PCR

Total RNA from sinus tissue was extracted with QIAzol (Qiagen) and was cleaned and treated with DNase I by using NucleoSpin RNA II (MACHEREY-NAGEL, Bethlehem, Pa) according to the manufacturer's instructions. The quality of total RNA from sinus tissue was assessed with a 2100 Bioanalyzer (Agilent Technologies, Carlsbad, Calif) by using an RNA 6000 Nano LabChip (Agilent Technologies). Single-strand cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen) and random primers. Semiquantitative real-time RT-PCR was performed with a TaqMan method by using an Applied Biosystems 7500 Sequence Detection System (Applied Biosystems, Foster City, Calif) in 15-μL reactions (7.5 μL of 2× TaqMan Master mix [Applied Biosystems], 400 nmol/L of each primer, and 200 nmol/L of TaqMan probe plus cDNA). Primer and probe sets for β-glucuronidase (human β-Glucuronidase endogenous control, PN; 4326320E), FXIII-A (Hs00173388_m1), MMR (Hs00267207_m1), CD163 (Hs00174705_m1), and STAB1 (Hs01109068_m1) were purchased from Applied Biosystems. A primer and probe set for β-Glucuronidase was chosen as the reference housekeeping gene in sinus tissue because previous studies have demonstrated no difference in the expression of this gene between patients and controls. To determine the exact copy number of the target genes, quantified aliquots of purified PCR fragments of the target genes were serially diluted and used as standards in each experiment. Aliquots of cDNA equivalent to 10 ng of total RNA were used for real-time PCR. The mRNA expression levels were normalized to the median expression of the housekeeping gene β-Glucuronidase.

Measurement of FXIII-A and albumin in tissue homogenates

Freshly obtained tissue specimens were weighed, and 1 mL of PBS supplemented with 0.05% Tween 20 (Sigma-Aldrich, St Louis, Mo) and 1% protease inhibitor cocktail (Sigma-Aldrich) was added for every 100 mg of tissue. The tissue was then homogenized with a Bullet Blender Blue (Next Advance, Averill Park, NY) at setting 7 for 8 minutes at 4°C. After homogenization, the suspension was centrifuged at 4000 rpm for 20 minutes at 4°C, and supernatants were stored at −80°C until analyzed.

The concentrations of FXIII-A (HYPHEN BioMed) and albumin (BETHYL, Montgomery, Tex) in cell-free supernatants were determined by using a specific ELISA kit. The color intensity was measured with a Bio-Rad Spectrophotometer Model 680 Microplate Reader (Bio-Rad, Hercules, Calif). Concentrations of FXIII-A in the tissue homogenate and cell lysate were normalized to the tissue weight.

Immunohistochemistry

Nasal tissue was dehydrated, infiltrated, and embedded in paraffin, and tissue was sectioned at 3 μm by using a Leica RM2245 Cryostat (Leica Microsystems, Inc, Bannockburn, Ill). Sections were rehydrated, and endogenous peroxidase activity was blocked with 3% H_2O_2/methanol. Tissue sections were then boiled in a citrate buffer (Dako, Carpinteria, Calif) for 15 minutes to induce antigen retrieval. After rinsing, nonspecific binding was blocked with 3% goat serum/0.3% Tween-20/PBS. Tissue sections were then incubated with 6.4 ng/mL of rabbit anti-human FXIII-A mAb (EP3372; CELL MARQUE) in blocking buffer overnight at 4°C. In control experiments, sections were incubated with the same concentrations of control rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, Pa). Sections were rinsed and then incubated in biotinylated secondary goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories) at a 1:500 dilution for 1 hour at room temperature. After another rinse, sections were incubated in ABC reagent (avidin–biotin–horseradish peroxidase complex; Vector Laboratories) for 1 hour at room temperature. Sections were rinsed again and incubated in diaminobenzidine reagent (Invitrogen) for 10 minutes at room temperature. They were then rinsed in deionized H_2O, counterstained with hematoxylin, dehydrated, cleared, mounted, and coverslipped by using Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, Mich) in preparation for microscopic analysis. Microscopic analysis was performed with an Olympus IX71 inverted research microscope by using ×40 objective lens, and images were collected with SlideBook software (Olympus, Center Valley, Pa). For the quantification of FXIII-A^+ cells, slides were blinded, and then 10 pictures were randomly taken from each slide. The number of FXIII-A^+ cells in nasal mucosa was counted by a blinded observer. For the immunofluorescence assay, rehydrated sections were blocked with 3% goat serum/0.3% Tween-20/PBS and then were incubated with 6.4 ng/mL of rabbit anti-human FXIII-A mAb (EP3372; CELL MARQUE), 24 ng/mL of mouse anti-human CD68 mAb (clone PG-M1, IgG_3, Thermo Fisher Scientific, Fremont, Calif), and 2.9 ng/mL of mouse anti-human CD163 mAb (clone 10D6, IgG_1, Thermo Fisher Scientific) in blocking buffer overnight at 4°C. The same concentrations of isotype control IgG were used in control experiments. After washing, sections were incubated with 4 μg/mL of Alexa Fluor 647-conjugated goat anti-rabbit IgG (Invitrogen), 4 μg/mL of Alexa Fluor 568-conjugated goat anti-mouse IgG_3 (Invitrogen), and 4 μg/mL of Alexa Fluor 488-conjugated goat anti-mouse IgG_1 (Invitrogen) for 1 hour at room temperature in the dark. Images from immunofluorescence slides were obtained with an Olympus IX71 inverted research microscope using ×40 objective lens, and images were collected with SlideBook software (Olympus).

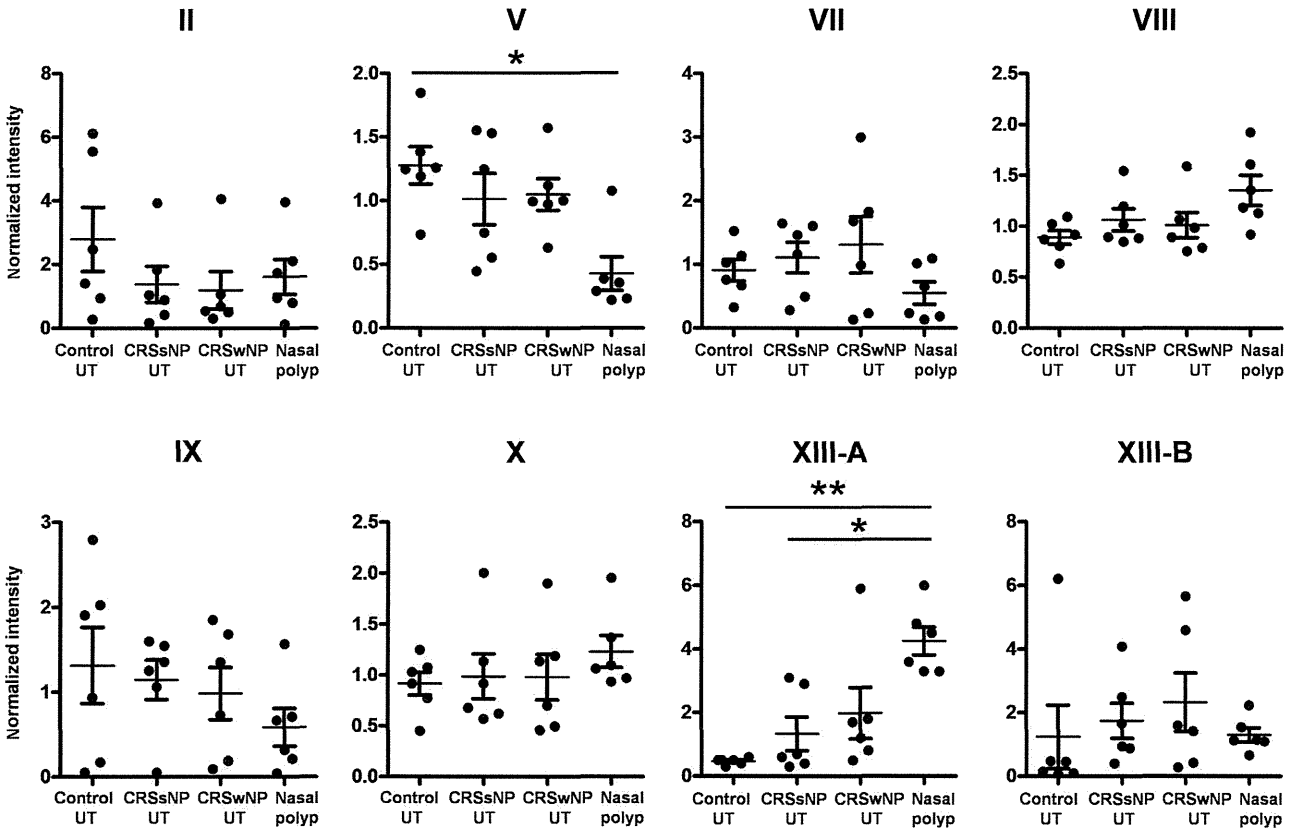

FIG E1. Microarray analysis of coagulation factors in sinonasal tissue. Microarray was used to assess the expression of coagulation factors in UT from control subjects (n = 6), patients with CRSsNP (n = 6), and patients with CRSwNP (n = 6) and in NP from patients with CRSwNP (n = 6). *$P < .05$ and **$P < .01$. All microarray data have been deposited to gene expression omnibus: GSE36830.

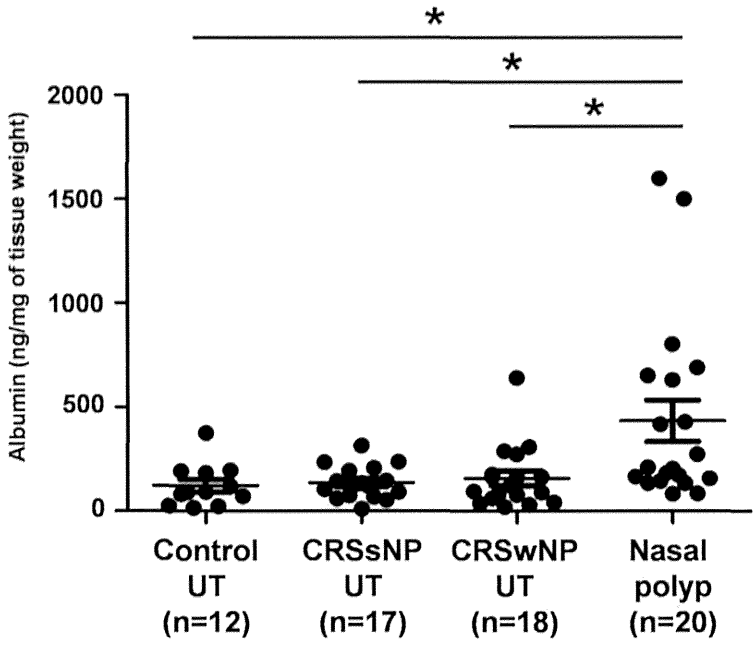

FIG E2. Increased levels of albumin in NP. Measurement of levels of albumin by ELISA in tissue homogenates of UT from control subjects, from patients with CRSsNP and CRSwNP, and in NP. $*P < .05$.

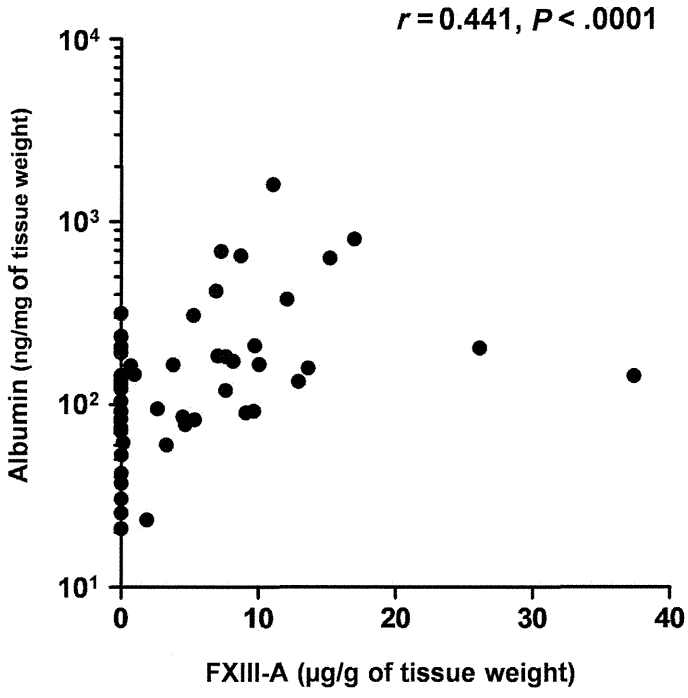

FIG E3. The relationship of FXIII-A and albumin in UT and NP was evaluated by using ELISA. The correlation was assessed by using the Spearman rank correlation test.

Excessive Fibrin Deposition in Nasal Polyps Caused by Fibrinolytic Impairment through Reduction of Tissue Plasminogen Activator Expression

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Rationale: Nasal polyps (NPs) are characterized by intense edema or formation of pseudocysts filled with plasma proteins, mainly albumin. However, the mechanisms underlying NP retention of plasma proteins in their submucosa remain unclear.

Objectives: We hypothesized that formation of a fibrin mesh retains plasma proteins in NPs. We assessed the fibrin deposition and expression of the components of the fibrinolytic system in patients with chronic rhinosinusitis (CRS).

Methods: We assessed fibrin deposition in nasal tissue from patients with CRS and control subjects by means of immunofluorescence. Fibrinolytic components, d-dimer, and plasminogen activators were measured using ELISA, real-time PCR, and immunohistochemistry. We also performed gene expression and protein quantification analysis in cultured airway epithelial cells.

Measurements and Main Results: Immunofluorescence data showed profound fibrin deposition in NP compared with uncinate tissue (UT) from patients with CRS and control subjects. Levels of the cross-linked fibrin cleavage product protein, d-dimer, were significantly decreased in NP compared with UT from patients with CRS and control subjects, suggesting reduced fibrinolysis ($P < 0.05$). Expression levels of tissue plasminogen activator (t-PA) mRNA and protein were significantly decreased in NP compared with UT from patients with CRS and control subjects ($P < 0.01$). Immunohistochemistry demonstrated clear reduction of t-PA in NP, primarily in the epithelium and glands. Th2 cytokine–stimulated cultured airway epithelial cells showed down-regulation of t-PA, suggesting a potential Th2 mechanism in NP.

Conclusions: A Th2-mediated reduction of t-PA might lead to excessive fibrin deposition in the submucosa of NP, which might contribute to the tissue remodeling and pathogenesis of CRS with nasal polyps.

Keywords: chronic rhinosinusitis; nasal polyps; tissue plasminogen activator; fibrin; fibrinolysis

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Management of patients with chronic rhinosinusitis with nasal polyps (CRSwNP) is unsatisfactory, and frequent recurrences occur despite medical treatment and surgical interventions. It is well known that intense edema and pseudocyst formation are major histopathological characteristics of nasal polyps (NPs), which are infiltrated with plasma proteins. However, the mechanisms by which NPs retain plasma proteins in their stroma remain unclear.

What This Study Adds to the Field

We demonstrate an impairment of fibrin degradation caused by reduction of tissue plasminogen activator and consequent abnormal fibrin deposition in NPs. Abnormal fibrin deposition might be involved in the formation of intense edema or pseudocysts in NPs. Excessive fibrin deposition resulting from reduced fibrinolysis may reflect Th2 inflammatory responses and may have a pathogenic role in CRSwNP. Stimulation of degradation of fibrin might have value as a therapeutic strategy for treating CRSwNP.

Chronic rhinosinusitis (CRS) is characterized by persistent symptomatic inflammation of nasal mucosa and is one of the most common chronic diseases in adults in the United States (1–3). The etiology and pathogenesis of CRS remain controversial; however, allergies, bacterial and fungal infections, and structural abnormalities have all been theorized to play a role (4). CRS is typically classified into CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). Sinonasal tissue from patients with CRSsNP displays a predominant infiltration of neutrophils, whereas CRSwNP tissue is characterized by more intense eosinophilic infiltration and a Th2-based cytokine profile (5). Management of patients with CRSwNP is still unsatisfactory, and symptoms can persist despite medical treatment and surgical intervention (3).

Nasal polyps (NPs) usually present as edematous masses originating in and around the middle nasal meatus or paranasal sinuses. The major histopathological characteristics of NPs are an infiltration by inflammatory cells, intense edematous stroma, and the formation of pseudocysts. It has been reported that the storage of albumin within the edema of pseudocysts determines the growth and size of NPs (6). However, plasma exudation may not readily induce edema but may rather pass through the airway epithelial layer (7). The mechanisms by which NPs retain plasma proteins in their stroma remain unclear.

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Fibrin is the major protein constituent of blood clots as a consequence of activation of the coagulation cascade. In inflamed tissue, vessel permeability is increased, resulting in the leakage of plasma proteins into the extravascular compartment. Much of the extravagated fibrinogen can be rapidly converted to fibrin. Activation of coagulation and fibrin deposition as a consequence of tissue inflammation are fundamental for host defense to confine infections and for repair processes (8). However, the proinflammatory effects of fibrin or the failure to degrade deposited fibrin may play an etiologic role in many diseases, including rheumatoid arthritis, multiple sclerosis, status asthmaticus, adult respiratory distress syndrome, and ligneous conjunctivitis (8–12).

The serine protease plasmin is responsible for the degradation of crosslinked fibrin (i.e., fibrinolysis). Plasmin is generated through cleavage of the proenzyme plasminogen by two physiological plasminogen activators, urokinase plasminogen activator (u-PA) and tissue plasminogen activator (t-PA). The activity of u-PA and t-PA is inhibited by plasminogen activator inhibitor-1 (PAI-1) (13).

We hypothesized that fibrin deposition as a consequence of inflammation retains exuded plasma proteins such as albumin, facilitating formation of intense edema and pseudocysts in NPs. To test this hypothesis, we investigated fibrin deposition and the expression of fibrinolytic components in sinonasal tissue from subjects with CRS. The results provide important new evidence suggesting that excessive fibrin deposition resulting from reduced fibrinolysis occurs in NP tissue. We have also discovered important differences in the fibrinolytic cascade between uncinate tissue (UT) and inferior turbinate tissue (IT).

METHODS

Patients

Patients with CRS were recruited from the Allergy immunology and Otolaryngology Clinics of the Northwestern Medical Faculty Foundation (NMFF) and the Northwestern Sinus Center at NMFF. Sinonasal and NP tissues were obtained from routine functional endoscopic sinus surgery in patients with CRS. All subjects met the criteria for CRS as defined by American Academy of Otolaryngology-Head and Neck Surgery Chronic Rhinosinusitis Task Force (1, 14). Details of the subjects' characteristics are included in Table 1. All subjects gave informed consent, and the protocol and consent forms governing procedures for study have been approved by the Institutional Review Board of Northwestern University Feinberg School of Medicine. Further details are provided in the online supplement.

Immunohistochemistry

Immunohistochemistry was performed as described previously (15). Briefly, blocked sections were incubated with antihuman fibrin antibody (Sekisui Diagnostics, Stamford, CT) or antihuman t-PA antibody (Sigma, St. Louis, MO) at 4°C overnight. Details of the methods for immunofluorescence and immunohistochemistry are provided in the online supplement.

Real-Time PCR

Total RNA was extracted using NucleoSpin RNA II (Macherey-Nagel, Bethlehem, PA) and was treated with DNase I. Single-strand cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time RT-PCR was performed with a TaqMan method as described previously (16). Further details are provided in the online supplement.

ELISA

The plasminogen activators u-PA and t-PA (Assaypro, St. Charles, MO), eosinophilic cationic protein (ECP) (MBL, Woburn, MA), and d-dimer (Diagnostica Stago, Asnieres-Sur-Seine, France) were assayed with specific ELISA kits as detailed in the online supplement.

Cell Culture

The methods for culture of primary normal human bronchial epithelial (NHBE) cells are detailed in the online supplement.

Statistical Analysis

All data are reported as mean ± SEM unless otherwise noted. Differences between groups were analyzed with the Kruskal-Wallis ANOVA with Dunnett *post hoc* testing and Mann-Whitney U test. Correlations were assessed by using the Spearman rank correlation. A P value of less than 0.05 was considered statistically significant.

RESULTS

Fibrin Deposition in Patients with CRS

Sinonasal and polyp tissues were collected from 126 subjects with CRSsNP, 156 subjects with CRSwNP, and 73 control subjects to

TABLE 1. SUBJECT CHARACTERISTICS

	Control (*n* = 73)	CRSsNP (*n* = 126)	CRSwNP (*n* = 156)	CRSwNP Polyp
Male/female	36/37	50/76	92/64	—
Age, yr, median (range)	43 (16–78)	36 (18–73)	45 (22–74)	—
Atopy				
Yes	4	51	73	—
No	49	55	52	—
Unknown	20	20	31	—
Asthma				
Yes	0	16	66	—
No	67	101	84	—
Unknown	6	9	6	—
Methodology used				
Tissue RNA, n (M/F)	16 (7/9)	27 (8/19)	33 (21/12)	33 (22/12)
Age, yr, median (range)	45 (16–62)	35 (20–59)	38 (23–67)	39 (23–67)
Tissue extract, n (M/F)	31 (16/15)	64 (21/43)	61 (39/22)	55 (34/21)
Age, yr, median (range)	45 (19–72)	36 (18–73)	44 (26–73)	45 (26–73)
Immunohistochemistry, n (M/F)	14 (5/9)	18 (8/10)	16 (9/7)	17 (11/6)
Age, yr, median (range)	43 (19–64)	43 (24–70)	52 (33–64)	50 (27–74)
Nasal lavage, n (M/F)	36 (20/16)	49 (22/27)	48 (35/13)	—
Age, yr, median (range)	42 (18–78)	36 (18–73)	45 (29–72)	—

Definition of abbreviations: CRSsNP = chronic rhinosinusitis without nasal polyps; CRSwNP = chronic rhinosinusitis with nasal polyps; F = female; M = male.

determine the fibrin deposition and the expression of fibrinolytic components in patients with CRS. Subjects' characteristics are shown in Table 1.

To evaluate the fibrin deposition in nasal mucosa, we performed immunofluorescence of surgical samples from control subjects and patients with CRS. Only a small amount of fibrin was seen in UT from control subjects or patients with CRSsNP, and a moderate level of fibrin staining was seen in UT from patients with CRSwNP (Figures 1A–1C); intense staining of fibrin was found in submucosa of NP from patients with CRSwNP (Figure 1D). Cellular staining was graded by blinded observers for intensity, as described in the online supplement. This semiquantitative analysis showed significantly more intense fibrin staining in NP from patients with CRSwNP compared with staining seen in control subjects or in UT from patients with CRSsNP ($P < 0.01$) (Figure 1F). We observed similar results using Masson's Trichrome stain, which highlights fibrin as a pink color (*see* Figure E1 in the online supplement). In addition, NP had much less collagen (blue color), which confirms a previous report (Figure E1) (17).

Extravascular fibrin is ordinarily degraded to fibrin degradation products (FDPs) by plasmin to prevent excessive fibrin deposition (18). To assess the levels of FDPs in nasal tissue, we measured the levels of d-dimer, which is an important FDP. d-Dimer protein levels were significantly decreased in NP from patients with CRSwNP ($P < 0.05$) in comparison with levels in UT from patients with CRS or control subjects (Figure 2). Taken together, these findings suggest the presence of excessive fibrin deposition associated with reduced fibrin degradation in NP.

The Expression of Plasminogen Activators in Patients with CRS

Fibrin is cleaved by plasmin, which is generated from plasminogen by two plasminogen activators, u-PA and t-PA. We therefore assessed the expression of u-PA and t-PA in UT from patients with CRSsNP or CRSwNP and from control subjects as well as in NP from patients with CRSwNP. Although the expression of mRNA for u-PA was not different among the four groups (Figure 3A), t-PA mRNA levels were significantly decreased in NP tissues from patients with CRSwNP ($P < 0.01$) in comparison with UT from patients with CRS or control subjects (Figure 3B). To confirm this observation at the protein level, we made detergent extracts from homogenates of UT and NP tissues and measured the concentration of u-PA and t-PA by ELISA. In agreement with the mRNA data, although u-PA protein levels were not different among the four groups (Figure 3C), t-PA protein levels were significantly decreased in NP from patients with CRSwNP ($P < 0.01$) in comparison with UT from patients with CRS or control subjects (Figure 3D). Tissue plasminogen activator activity was also significantly decreased in NP ($P < 0.01$) (Figure E2). Together, these results show clear reduction of t-PA mRNA, protein, and activity and suggest that the fibrinolytic pathway is severely compromised in NP tissue.

Immunohistochemical Analysis of Plasminogen Activators in Sinonasal Tissue

To further characterize the expression of plasminogen activator proteins in patients with CRS, we performed immunohistochemical analysis of surgical samples from control subjects and patients with CRS to determine whether t-PA expression could be detected. We detected t-PA staining in glands and in mucosal epithelium and endothelium in tissues (Figure 4). Consistent with ELISA data, t-PA staining in glandular and mucosal epithelium of control tissue (Figures 4C and 4D) was more intense when compared with that seen in NP (Figures 4I and 4J and *see* Table E1 in the online supplement) in patients with CRSwNP.

Comparison of Plasminogen Activator Expression between UT and IT

NPs are known to arise from nasal and paranasal sinus mucosa that are mainly situated in the middle nasal meatus but rarely arise from the inferior turbinate (6). We therefore examined the expression level of plasminogen activators between UT and IT from control subjects and patients with CRS using ELISA.

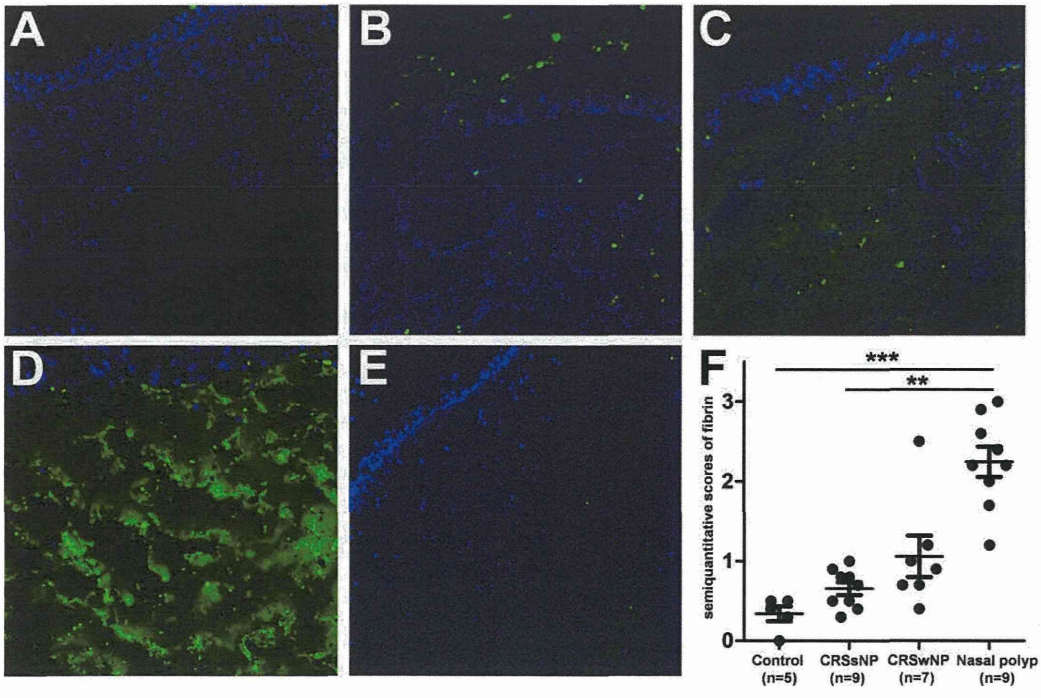

Figure 1. Immunofluorescence of fibrin in nasal tissues. Immunofluorescence was performed with antifibrin (*green fluorescence*). (*A–D*) Representative immunostaining for fibrin in uncinate tissue (UT) from a control subject (*A*), a patient with chronic rhinosinusitis without nasal polyps (CRSsNP) (*B*), a patient with chronic rhinosinusitis with nasal polyps (CRSwNP) (*C*), and nasal polyp (NP) tissue (*D*). (*E*) Negative control antibody staining in NPs from a patient with CRSwNP. (*F*) Semiquantitative analysis of fibrin in UT from control subjects (n = 5), patients with CRSsNP (n = 9), and patients with CRSwNP (n = 7) and NPs (n = 9) was performed. Magnification: ×400. $^{**}P < 0.01$; $^{***}P < 0.001$.

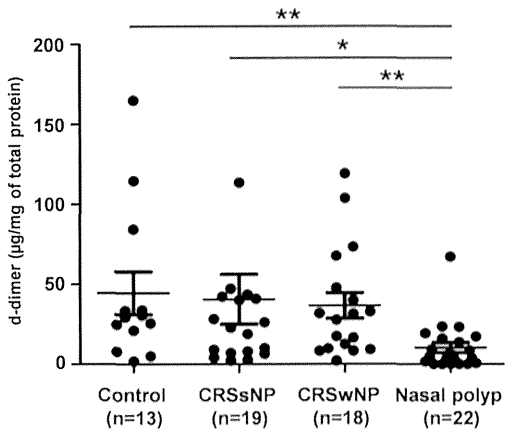

Figure 2. d-Dimer levels were decreased in nasal polyp tissue. Measurement of d-dimer in tissue homogenates of uncinate tissue from control subjects, from patients with chronic rhinosinusitis without nasal polyps (CRSsNP), from patients with chronic rhinosinusitis without nasal polyps (CRSwNP), and in nasal polyps using ELISA. d-Dimer concentration was normalized to the concentration of total protein. $*P < 0.05$; $**P < 0.01$.

u-PA protein levels were significantly lower in UT in comparison with those in IT from control subjects ($P < 0.05$), patients with CRSsNP ($P < 0.001$), or patients with CRSwNP ($P < 0.05$) (Figure 5A). t-PA protein levels were also significantly lower in UT in comparison with those seen in IT from patients with CRSsNP ($P < 0.001$) or patients with CRSwNP ($P < 0.01$) (Figure 5B). Although not statistically significant, t-PA protein levels were also lower in UT from control subjects ($P = 0.068$) compared with IT from control subjects (Figure 5B). These results suggest that the overall fibrinolytic capacity is higher in the inferior turbinate than in the uncinate, and we speculate that low expression of both plasminogen activators in UT might confer susceptibility to fibrin deposition and polyp formation in this region due to reduced capacity for fibrin degradation.

Th2 Cytokines Down-Regulate the t-PA Expression in NHBE Cells

NP from patients with CRSwNP have long been known to be characterized by Th2-dominant eosinophilic inflammation (19). We examined whether levels of plasminogen activators correlated with eosinophilic inflammation in nasal tissues. We assayed the levels of ECP as a marker for the presence of eosinophils in nasal tissue. The concentration of t-PA in UT and NP was significantly negatively correlated with the concentration of ECP ($r = -0.5395$; $P < 0.0001$) (Figure 6A); however, the concentration of u-PA in nasal tissue did not correlate with the concentration of ECP (data not shown). Immunohistochemistry data demonstrated that t-PA staining was mainly observed in glandular and mucosal epithelium in nasal tissue (Figure 4). Therefore, to assess the t-PA mRNA level in epithelium, we used nasal scraping-derived epithelial cells. Although not statistically significant, as shown in immunohistochemistry, t-PA mRNA levels were decreased in epithelial scraping cells from NP ($P = 0.063$) compared with levels in UT from control subjects (Figure 6B). Given that expression of t-PA was reduced in nasal tissue and negatively correlated with ECP, we hypothesized that Th2 cytokines might regulate t-PA expression in airway epithelial cells. To study the regulation of plasminogen activators in airway epithelial cells, primary NHBE cells were stimulated with Th2 cytokines, IL-4, or IL-13 for 24 hours. Although the levels of u-PA mRNA were not altered by Th2 cytokine stimulation (Figure 6C), the levels of t-PA mRNA were significantly down-regulated by both Th2 cytokines in a dose-dependent manner (Figure 6D). To confirm this observation at the protein level, we made cell lysate of NHBE cells and measured the concentration of plasminogen activators using ELISA. Although the levels of u-PA protein were not altered by Th2 cytokine stimulation (Figure 6E), the levels of t-PA protein were significantly down-regulated by both Th2 cytokines (Figure 6F). We also observed that stimulation with Th2 cytokines down-regulated t-PA expression in primary nasal epithelial cells (Figure E4). This result suggests that Th2 cytokines

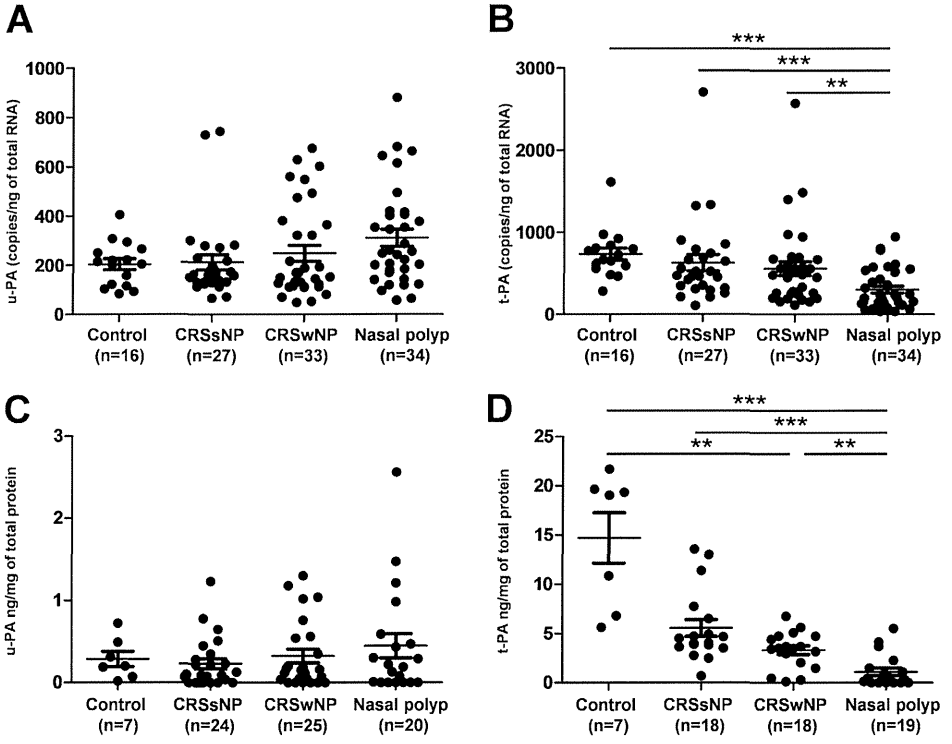

Figure 3. Expression of plasminogen activators in nasal tissues. Total RNA was extracted from uncinate tissue and nasal polyps, and expression of urokinase plasminogen activator (u-PA) (*A*) and t-PA (*B*) was analyzed using real-time PCR. Expression of u-PA (*C*) and t-PA (*D*) protein in tissue homogenates of uncinate tissue and nasal polyps was measured using ELISA. The concentration of plasminogen activators was normalized to the concentration of total protein. $**P < 0.01$; $***P < 0.001$.

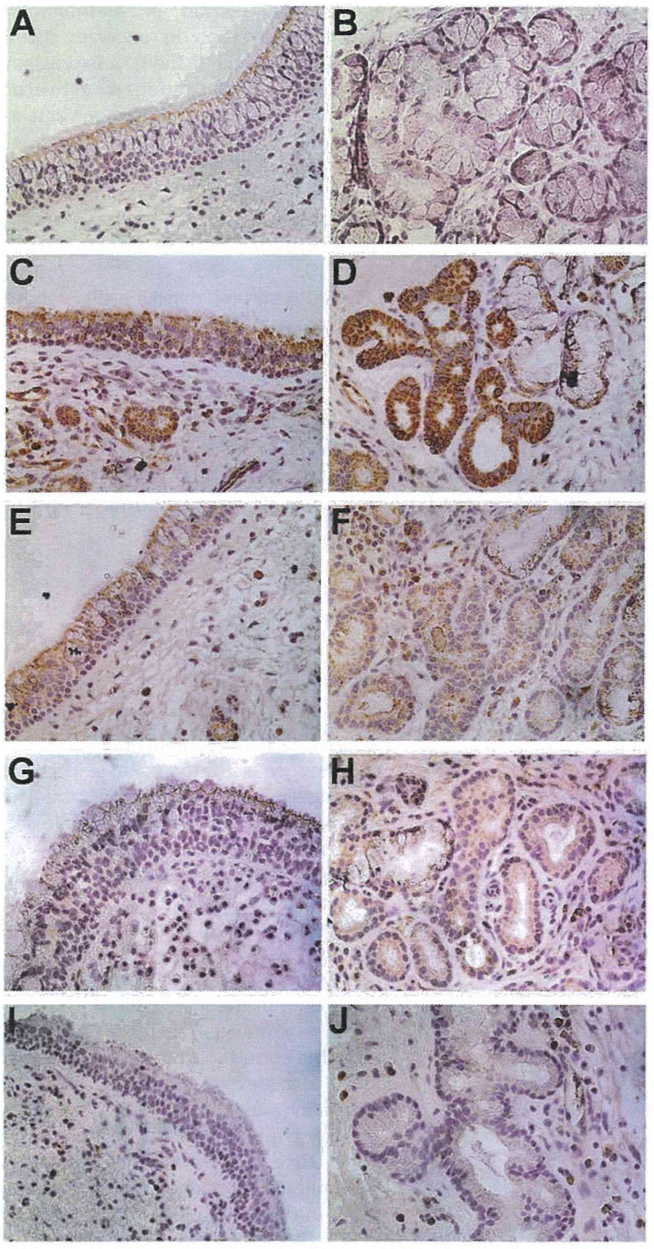

Figure 4. Immunohistochemical staining for tissue plasminogen activator (t-PA) in representative tissue samples from uncinate tissue (UT) and nasal polyps (NPs). (*A, B*) Negative control of UT from a control subject did not stain. (*C–H*) t-PA staining of UT from control subject (*C, D*) showed intense staining in epithelial and glandular tissue, whereas light-to-moderate staining of t-PA was seen in UT from a patient with chronic rhinosinusitis without NPs (*E, F*) and a patient with chronic rhinosinusitis with NPs (*G, H*). (*I, J*) Less staining was seen in NP tissue. Magnification: ×400.

down-regulate expression of t-PA but not u-PA in airway epithelial cells.

DISCUSSION

It is well known that intense edema and pseudocyst formation are major histopathological characteristics of NP tissues, which are infiltrated with plasma proteins, mainly albumin (6). In spite of the presence of considerable albumin in the stroma of NP, the levels of albumin were not increased in nasal lavage from patients with CRSwNP compared with albumin levels in control subjects or patients with CRSsNP (Figure E3). The mechanism by which NP tissue retains plasma proteins in the stroma has not been explored. The current study demonstrates for the first time that fibrin deposition is profoundly increased in NP from patients with CRSwNP in comparison with that seen in UT from patients with CRS or control subjects (Figure 1). We also found that although there is a great deal of fibrin deposition, d-dimer, a major fibrin degradation product, was significantly decreased in NP compared with UT in the three groups of subjects (Figure 2). These results indicate that excessive fibrin deposition in NP might be caused by a disorder of fibrin degradation. Because fibrin degradation is facilitated by plasmin, which is generated through cleavage of plasminogen by u-PA and t-PA, we examined the levels of these two plasminogen activators. The levels of t-PA, but not u-PA, were significantly decreased in patients with CRSwNP, especially in NP tissue (Figures 3B and 3D). t-PA promotes fibrinolysis by virtue of the presence of t-PA binding sites on fibrin strands, where plasminogen is also localized. It is therefore generally believed that t-PA acts as a central plasminogen activator for fibrinolysis (8). These results suggest that decreased levels of t-PA in NP tissue lead to a deceleration of the rate of conversion of plasminogen to plasmin, reducing fibrinolytic tone. In the face of plasma exudation, reduced degradation of fibrin would in turn facilitate excessive deposition of fibrin in NP. Fibrin deposition might also be involved in retention of albumin in NP stroma. An outline of this hypothetical model is given in Figure 7.

Fibrin, as the final product of the coagulation cascade, plays a major role in blood clotting. In addition, because components of the coagulation cascade reside in, or are transported to, tissues and can stimulate extravascular fibrin formation (20), fibrin deposition in response to inflammation can be integral to normal repair and restoration of tissues. This is believed to play a role in the confinement of microbial or toxic agents to a limited area and in the formation of provisional matrix for the influx of monocytes, fibroblasts, and endothelial cells (21, 22). However, disorder of fibrin turnover facilitates abnormal fibrin deposition and can be deleterious because of its proinflammatory properties (8, 23). Fibrin can directly stimulate expression of IL-1β and TNF-α in mononuclear cells and can induce production of the chemokines CXCL8 and CCL2 by endothelial cells and fibroblasts, promoting the migration of leukocytes and macrophages (8, 24). Indeed, some evidence suggests that removal of fibrin can diminish disease development and symptoms (8, 25–28).

t-PA converts plasminogen into proteolytically active plasmin, which in turn degrades fibrin and other extracellular matrix proteins (8). In addition, t-PA facilitates the posttranslational activation of several growth factors, such as hepatocyte growth factor or transforming growth factor (TGF)-β via proteolysis, and TGF-β can induce endogenous t-PA expression in an autocrine manner (29, 30). We observed reduced collagen in NP (Figure E1D); other studies have reported that reduced collagen is seen in NP compared with control subjects as a consequence of decreased TGF-β (17). Taken together, the presence of low levels of t-PA and TGF-β provides a milieu for low collagen production in NP (Figure 7). Growing evidence suggests t-PA can act as a cytokine and binds to the cell membrane receptor low-density-lipoprotein receptor–related protein-1 (LRP-1). Independent of its proteolytic capacity, binding by t-PA to LRP-1 induces receptor tyrosine phosphorylation, triggers intracellular signal transduction, and induces collagen production by fibroblasts (30–33). We detected LRP-1 expression in nasal tissue by real-time PCR, and there was no significant difference between UT and NPs from control subjects and patients with CRS(data not shown). In normal wound healing

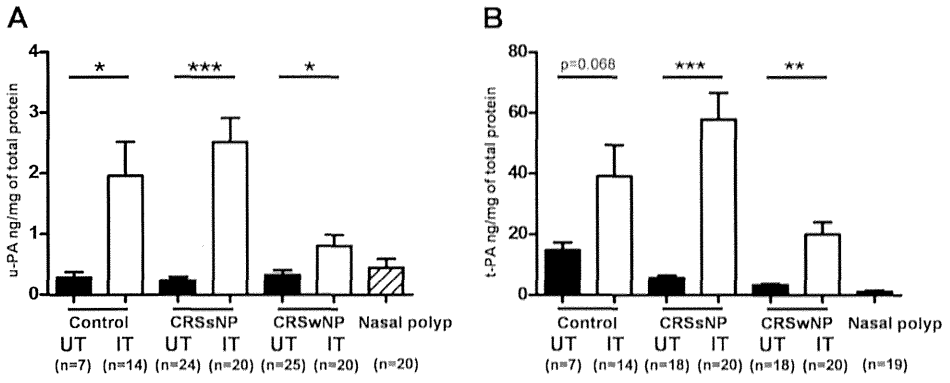

Figure 5. Comparison of plasminogen activator expression in uncinate tissue (UT) and turbinate tissue (IT). Expression of urokinase plasminogen activator (u-PA) (*A*) and tissue plasminogen activator (t-PA) (*B*) protein in tissue homogenates of UT, IT, and nasal polyps was measured using ELISA. The concentration of plasminogen activators was normalized to the concentration of total protein. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. CRSsNP = chronic rhinosinusitis without nasal polyps; CRSwNP = chronic rhinosinusitis with nasal polyps.

processes, the early deposition of fibrin matrix is replaced with collagen produced by fibroblasts, and inadequate removal of fibrin impedes this process (22). In this regard, low levels of t-PA/LRP-1 signaling might hinder fibrin removal and prolong inflammation in NP. In addition, recent studies suggest that t-PA/LRP-1 pathways induce nitric oxide (NO) production in the central nervous system (34). Because it has been reported that the levels of NO were decreased in NP tissue (35), low levels of t-PA might be involved in down-regulation of NO in NP tissue (Figure 7).

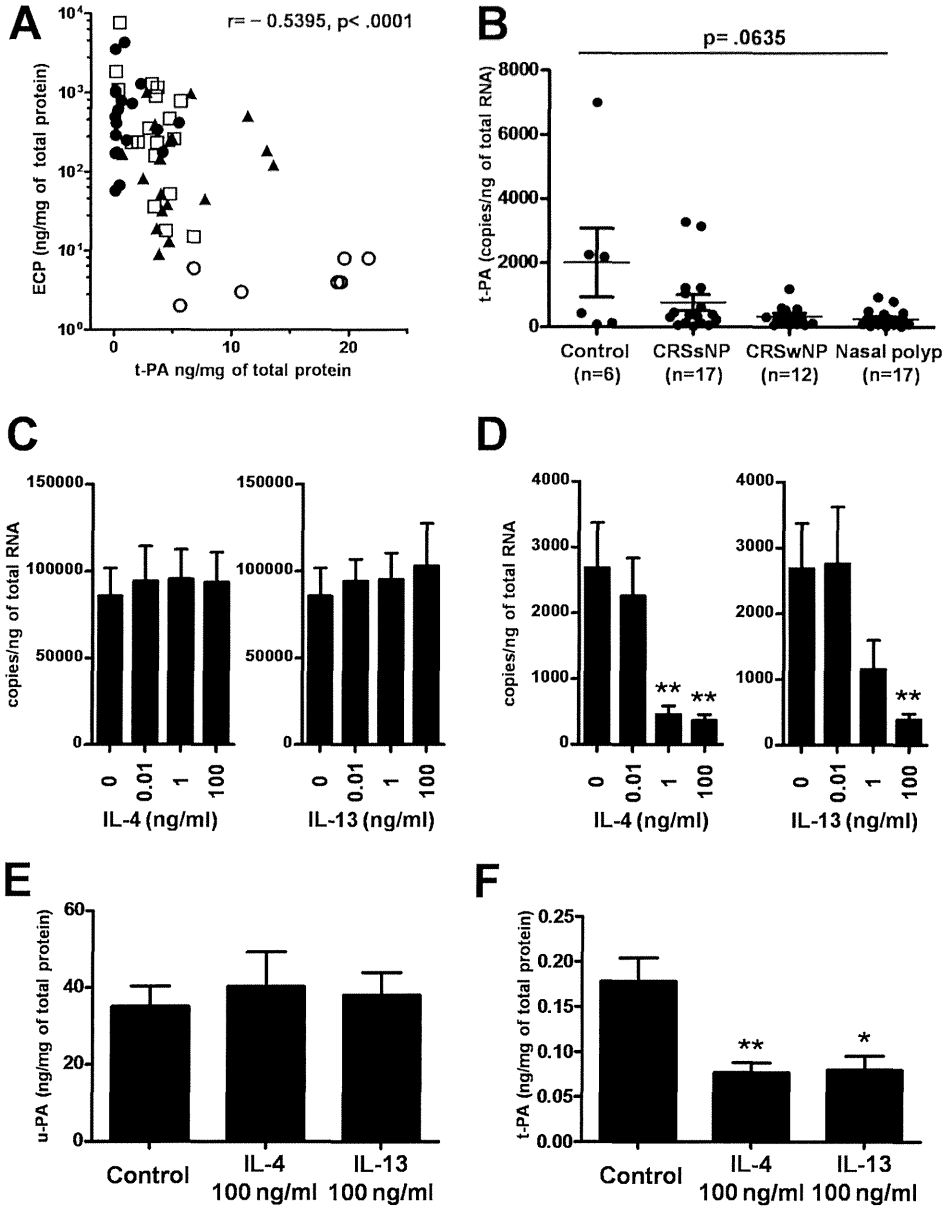

Figure 6. Potential regulation of tissue plasminogen activator (t-PA) expression in epithelial cells by Th2 cytokines. The relationship of t-PA and eosinophilic cationic protein (ECP) in nasal tissue was evaluated using ELISA (*open circles*, control uncinate tissue [UT]; *triangles*, chronic rhinosinusitis without nasal polyps [NPs] UT; *open squares*, chronic rhinosinusitis with NPs UT; *closed circles*, NP). None of the individual groups produced a correlation between ECP and t-PA. The correlation shown was assessed using all values with the Spearman rank correlation test (*A*). Total RNA was extracted from epithelial scraping cells from UT and NPs, and expression of t-PA mRNA was analyzed with real-time PCR. The levels of t-PA were decreased in NPs ($P = 0.063$) compared with levels in UT from control subjects (*B*). Normal human bronchial epithelial cells were stimulated with 0.01 to 100 ng/ml IL-4 or IL-13 for 24 hours. The levels of urokinase plasminogen activator (u-PA) (*C*) and t-PA (*D*) mRNA were determined by real-time PCR. Concentrations of u-PA (*E*) and t-PA (*F*) protein in cell lysates from normal human bronchial epithelial cells were measured by ELISA. The concentration of plasminogen activators was normalized to the concentration of total protein. Results shown are mean ± SEM of six independent experiments (*C–F*). $^{*}P < 0.05$; $^{**}P < 0.01$.

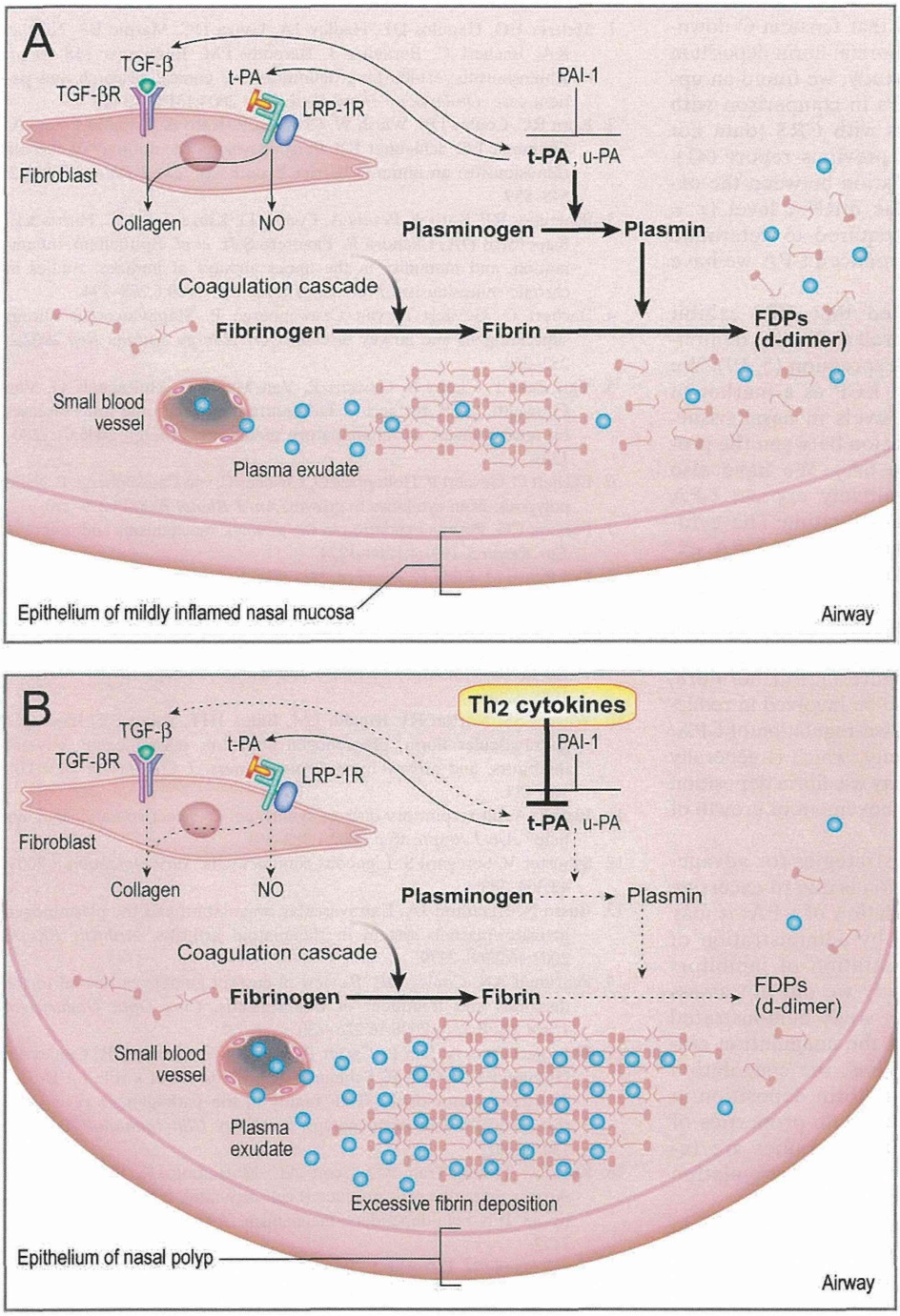

Figure 7. Hypothetical model to explain the role of tissue plasminogen activator (t-PA) in excessive fibrin deposition and reduced collagen in nasal polyps. As a protease, t-PA converts plasminogen to plasmin, which promotes fibrin degradation. As a cytokine, t-PA binds to its receptor lipoprotein receptor–related protein-1 (LRP-1), leading to collagen production and nitric oxide (NO) synthesis by fibroblast (*A*). In the presence of Th2 cytokines, t-PA levels are reduced, promoting fibrinogenesis. Reduced tissue levels of t-PA facilitate abnormal fibrin deposition and diminish collagen expression in nasal polyps (*B*). FDP = fibrin degradation product.

In the study of CRS, one of the most intriguing questions is "Why do NPs arise only from mucous membranes in and around the middle nasal meatus?" In the current study, we found that protein levels of u-PA and t-PA were lower in UT in comparison with those seen in IT in diseased samples and controls (Figure 5). This suggests that low levels of plasminogen activators might confer an increased susceptibility to excess fibrin deposition in UT and may provide an explanation of why NP arise from mucous membranes in and around the middle nasal meatus but not in the IT. In previous studies, we have found that IT and UT differ dramatically in levels of host defense molecules, so such a regional difference is not unprecedented (36, 37).

It is known that the activation of t-PA is tightly controlled by PAI-1, which directly binds t-PA and inactivates it. We observed that the levels of t-PA protein and the activity of t-PA were decreased in NP in comparison with UT from control subjects and patients with CRS (Figures 3 and E2). However, the levels of PAI-1 protein in NP were not elevated in comparison with control subjects and CRS samples (data not shown), suggesting that PAI-1 is not responsible for inactivation or reduction of t-PA in NP. The regulation of t-PA gene expression is not well described. t-PA is produced by a number of airway cells, including mast cells, macrophages, fibroblasts, endothelial cells, glandular cells, and epithelial cells (38, 39). Our immunohistochemistry data demonstrated that t-PA staining was most prominently

observed in epithelial and glandular cells in UT from control subjects. Recently, it has been reported that tenascin-C down-regulates t-PA expression resulting in abnormal fibrin deposition in a mouse model (40). In the present study, we found an up-regulation of tenascin-C mRNA in NPs in comparison with UT from control subjects and patients with CRS (data not shown), which is consistent with this previous report (41). However, we could not find any correlation between the expression of tenascin-C and t-PA at the mRNA level ($r = 0.182$; P = NS). Further studies are required to determine whether tenascin-C plays a role in the reduced t-PA we have observed in NPs.

Previous studies have demonstrated that NPs exhibit a high degree of tissue eosinophilia as well as T cells, demonstrating skewing toward Th2 cytokine expression (5, 19). We therefore examined the correlation of ECP as a marker of Th2 inflammation with t-PA protein levels in nasal tissue. We found a significant negative correlation between the protein levels of ECP and t-PA (Figure 6A). We have also shown here that NHBE cells constitutively express t-PA and that stimulation with the STAT6-activating Th2 cytokines IL-4 or IL-13 significantly down-regulated t-PA expression while leaving u-PA expression unaltered (Figures 6C–6F). These findings suggest that Th2-related inflammation in NPs might down-regulate the expression of t-PA and play a role in the induction of excessive fibrin deposition through suppression of fibrinolysis (Figure 7). Furthermore, the reduction in levels of t-PA might also be involved in reduction of collagen production in NPs by down-regulation of t-PA/LRP-1 signaling (Figure 7). Th2 immunity, which is generally associated with antiparasite responses, may use fibrin deposition in the pathways designed to impede the movement or growth of parasite worms in tissues.

Our findings suggest potential new strategies for advancing the treatment of NPs. If NP formation is due to excessive fibrin deposition caused by down-regulation of t-PA, it may be feasible to diminish NP formation by administration of t-PA or activators of t-PA or administration of inhibitors of fibrinogenesis. Although in this study we did not assess the coagulation status in NPs, a recent study demonstrated that thrombin, a central component of the coagulation cascade, was up-regulated in NPs (42). Thus, the coagulation cascade might be involved in excessive fibrin deposition in NPs, interacting with the reduced fibrinolytic properties of the tissue that we describe herein. Future studies are required to determine the relationship between coagulation and NP development.

In summary, we report here that excessive fibrin deposition and low levels of d-dimer are observed in NP tissue from patients with CRSwNP. Tissue levels of t-PA were profoundly decreased in NPs, suggesting that down-regulation of t-PA may lead to insufficient fibrin degradation resulting in fibrin deposition. Furthermore, the constitutive levels of protein for both plasminogen activators were very low in UT in comparison with IT, suggesting that low levels of fibrinolysis in UT may lead to a particular susceptibility for fibrin deposition in the ethmoid sinus. This difference of fibrinolytic capacity might be one reason that NPs almost exclusively arise in the proximity of the middle nasal meatus. Our findings indicate that profound fibrin deposition might be involved in the retention of plasma proteins and the formation of the apparent tissue remodeling, intense edema, or pseudocysts in NP tissue and provide potential new targets for novel therapeutic approaches to CRSwNP.

Author disclosures are available with the text of this article at www.atsjournals.org.

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Risk factors associated with severity of eosinophilic otitis media

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ABSTRACT

Objective: Eosinophilic otitis media (EOM) is an intractable otitis media associated with bronchial asthma. Clinical characteristics of EOM are apparent, but severe EOM, which is unresponsive to ongoing treatments, can occur. The present study aimed to investigate potential risk factors associated with the severity of EOM.

Methods: We scored the EOM severity of 26 patients according to quantity of middle ear effusion (MEE), thickness of the middle ear mucosa, use of topical and oral corticosteroids, and use of antibiotics, all measured over a 3-month period. The scores for four 3-month periods (1 year) were averaged. We analyzed the prevalence of clinical variables by partial regression: sex, age, body mass index (BMI), duration of bronchial asthma, association of aspirin-intolerant asthma, Lund–Mackay score for sinusitis, mastoid pneumatization, width of the bony Eustachian tube at the tympanic orifice, percentage of eosinophils and immunoglobulin E in peripheral blood, and association of allergic rhinitis. Duration of bronchial asthma was defined as the period from onset of bronchial asthma to the age of first consultation at our hospital. Samples of MEE were taken for bacterial culture.

Results: The average severity score was 6.6 (out of 16). The severity score in the pathogen-positive MEE group was significantly higher than that in the pathogen-negative MEE group ($p < 0.05$). The score was not significantly different between the seasons. Linear multiple regression analysis showed that BMI and the duration of bronchial asthma significantly affected the EOM severity score ($p < 0.05$). The presence of aspirin intolerant asthma tended to be correlated with the severity score. The Lund–Mackay score tended to be negatively correlated with it.

Conclusions: There is a significant association between the severity of EOM and obesity, as well as with the duration of bronchial asthma.

1. Introduction

Eosinophilic otitis media (EOM) is an intractable otitis media characterized by a highly viscous effusion containing eosinophils, and is associated with bronchial asthma (BA). The clinical characteristics of EOM were clarified [1,2] and diagnostic criteria were proposed in 2011 [3]. The number of patients diagnosed as having EOM has increased in Japan and other countries [4]. EOM is unresponsive to conventional treatments, such as myringotomy or insertion of a tympanostomy tube. Intratympanic instillation of triamcinolone acetonide has been used to treat middle ear effusion (MEE) and otorrhea [5]. The condition of most patients with EOM remains stable by systemic, topical, or oral administration of corticosteroids. However, some patients experience regular recurrences, despite ongoing treatment. They often have MEE or purulent otorrhea with bacterial infection, and need systemic or topical administration of antibiotics. The present study was carried out to investigate potential risk factor(s) for severe EOM and to investigate new treatment strategies for controlling this intractable disease.

2. Materials and methods

2.1. Subjects

Between October 2011 and September 2012, we studied 26 patients with EOM who had been followed up at Jichi Medical University Saitama Medical Center for more than 1 year. The

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Table 1
Scoring system used to evaluate the severity of eosinophilic otitis media (Iino et al. [6]).

1. Quantity of middle ear effusion (MEE)/otorrhea (without eardrum perforation)
Score 0No MEE
Score 1MEE with partial intratympanic aeration
Score 2Mesotympanum totally filled with MEE
Quantity of MEE/otorrhea (with eardrum perforation)
Score 0No otorrhea
Score 1Otorrhea limited to the mesotympanum
Score 2Otorrhea extrudes to the external auditory canal
2. Condition of the middle ear mucosa
Score 0Nearly normal or slightly edematous
Score 1Edematous or thickened
Score 2Highly thickened or granulated, and extending beyond the eardrum
3. Frequency of intratympanic administration of corticosteroids
Score 0None
Score 1Once in the previous 3 months
Score 2Two or more times in the previous 3 months
4. Frequency of systemic administration of corticosteroids
Score 0None
Score 1Seven days or less in the previous 3 months
Score 2More than 7 days in the previous 3 months
5. Frequency of systemic administration of antibiotics
Score 0None
Score 17 days or less in the previous 3 months
Score 2More than 7 days in the previous 3 months

patients were 13 male and 13 female patients aged 26–75 years (mean ± standard deviation, 49.8 ± 13.3 years). All the patients were diagnosed as having BA and EOM on the basis of previously reported diagnostic criteria [3]. They visited our hospital every 1–3 months. Their blood testing was obtained at the initial visit. Intratympanic instillation of triamcinolone acetonide was regularly used at the same time and systemic administration of antibiotics was performed if bacterial infection was present in the middle ear. If acute inflammation became worse, tympanostomy or topical saline irrigation was chosen. Patients also regularly visited the respiratory medicine department for management of BA. All participants used nasal spray for sinusitis and inhaled corticosteroids and five participants also used continuous systemic corticosteroids to control BA. Some of the patients received systemic corticosteroids at the time of exacerbation of BA or EOM. The duration of BA and EOM was defined as the onset of BA and EOM to the age of the first consultation to our hospital.

This study was approved by the Ethics Committee of Jichi Medical University Saitama Medical Center (study number: RIN 13-49).

2.2. Evaluation of severity of EOM

The degree of severity of EOM was evaluated according to five items: (1) quantity of MEE or otorrhea; (2) condition of the middle ear mucosa; (3) frequency of intratympanic injection of triamcinolone acetonide; (4) frequency of administration of systemic corticosteroids; and (5) frequency of administration of antibiotics. These items were scored on a scale from 0 to 2 (Table 1 [6]). Patients were evaluated every 3 months. The scores for four 3-month periods (1 year) were averaged to resolve seasonal differences in clinical symptoms. Three of the items (1, 2, and 3) were evaluated separately for each ear. Two of the items (3 and 5) in each participant were also evaluated, including medications prescribed by physicians other than the otolaryngologist. The 3-month assessment periods were assigned to correspond with Japanese seasons (from October to March: cold season; from April to September: warm season).

2.3. Bacterial and fungal cultures in MEE or otorrhea

MEE or otorrhea was obtained from each patient for bacterial and fungal cultures. Detection of bacteria and fungi was performed by routine laboratory analysis at the clinical microorganism division of our University Hospital.

2.4. Width of the bony portion of the Eustachian tube at the tympanic orifice

We measured the width of the bony portion of the Eustachian tube at the tympanic orifice from an anatomical perspective [7]. Measurements were obtained from 0.5-mm sliced axial and coronal sections of a computed tomography (CT) scan of the temporal bone (Fig. 1).

2.5. Lund–Mackay score

The extent of sinus disease identified by CT scan was evaluated by using the Lund–Mackay scoring system [8]. To evaluate sinus contents, the five major right and left sinuses (frontal, maxillary, anterior and posterior ethmoid, and sphenoid), and the ostiomeatal complex were scored on a 2-point scale as follows: 0, clear; 1, partial opacification; and 2, total opacification. This score applied to not only pre-operative condition but also post-operative condition after endoscopic sinus surgery (ESS).

2.6. Mastoid pneumatization

Mastoid pneumatization was evaluated according to the extent of pneumatization. Pneumatization was classified as follows: 1, poorly pneumatized (cells were not well developed); 2, moderately pneumatized (cells were developed within the sigmoid sinus); and 3, well pneumatized (cells were developed beyond the sigmoid sinus). We used the mean score of both sides.

2.7. Statistical analysis

Associations between variables for some characteristics and the severity score were assessed by the partial correlation coefficient. The variables were used as independent variables in multiple linear regression analysis, which was performed to identify risk

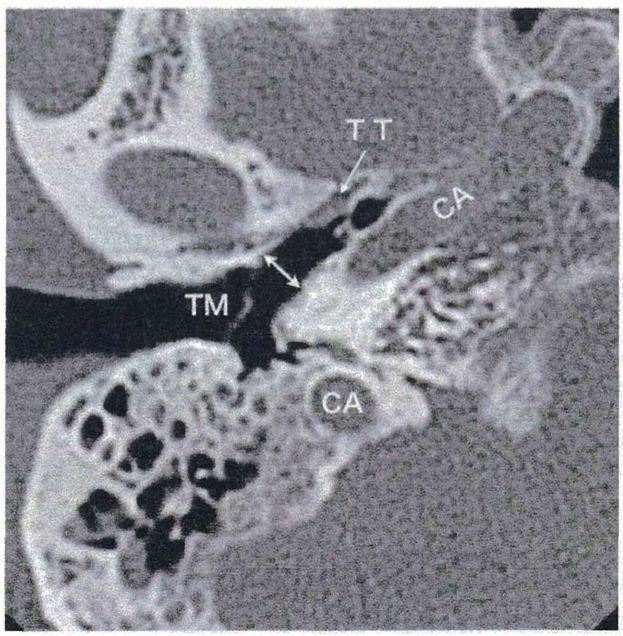

Fig. 1. Width of the bony portion of the Eustachian tube at the tympanic orifice (measured from 0.5-mm sliced axial and coronal sections of a temporal bone CT scan). TT, tensor tympani muscle; TM, tympanic membrane; CA, carotid artery.

Table 2
Clinical and demographic features of the enrolled patients.

No. of patients	26 (13 men and 13 women)
Age (y)	49.8 ± 13.3 (26–75)
Eosinophil count in PB (%) (<7%)*	11.4 ± 8.1 (0–36)
Total serum IgE levels (IU/ml) (<173 IU/ml)**	653.3 ± 913.6 (53.1–4160)
BMI (kg/m^2)	23.7 ± 4.1 (16.0–35.3)
Duration of BA, y	16.0 ± 12.9 (0–54)
Duration of EOM, y	6.2 ± 6.5
Width of tympanic orifice of ET formed by the bony septum (mm)	3.6 ± 0.6 (2.4–4.4)
Associated diseases	
Aspirin-intolerant asthma (+/−)	11/15
Allergic rhinitis evaluated by MAST (none/seasonal-pollen/perennial-mites, HD/both seasonal and perennial)	8/6/5/7
Baseline mean scores (range)	
Lund–Mackay score	16.1 ± 6.8 (1–24)
Mastoid pneumatization (average of both sides)	2.3 (1–3)
Severity of EOM	6.6 ± 3.8

Values are mean ± SD (range in parentheses) unless indicated otherwise.
PB, peripheral blood; IgE, immunoglobulin E; BMI, body mass index; BA, bronchial asthma; EOM, eosinophilic otitis media; ET, Eustachian tube; MAST, multiple-antigen simultaneous test.
* The normal value of eosinophil count in PB in our medical center.
** The normal value of total serum IgE levels in our medical center.

factors associated with severe EOM. We investigated as many potential factors as possible. The severity score in bacterial cultures in MEE or otorrhea was compared with the Student's t test. Seasonal changes of the severity score were analyzed by the Wilcoxon matched-pairs signed-rank test. All values of $p < 0.05$ were considered significant.

3. Results

3.1. Patients' characteristics

The baseline characteristics of enrolled patients are shown in Table 2. 8 of 26 patients were BMI ≧ 25 kg/m^2, including 2 patients who were ≧30 kg/m^2. All of the patients had adult onset of BA, which preceded that of EOM. The eosinophil counts in peripheral blood (%) and total serum immunoglobulin E (IgE) levels were higher than the normal range in most of the patients.

3.2. Bacterial and fungal culture in MEE or otorrhea

Table 3 shows the results of bacterial and fungal culture in MEE or otorrhea of EOM. A high prevalence (69.2%) of pathogens was found in samples from MEE or otorrhea. The most frequent bacterium was *Staphylococcus aureus*, including four patients with *Methicillin-resistant S. aureus* infection. A patient showed only non-pathogen positive (*coagulase-negative Staphylococcus*).

Table 3
Bacteria and fungi detected from MEE or otorrhea in patients with eosinophilic otitis media.

MEE (n=26)	
Pathogen positive	18 (69.2%)
Staphylococcus aureus (MRSA)	12 (4)
Pseudomonas aeruginosa	5
Streptococcus pneumonia	1
Hemophilus influenzae	0
Candida	0
Non-pathogen only	1 (3.8%)
Negative	7 (26.9%)

MEE: middle ear effusion; MRSA: methicillin-resistant *Staphylococcus aureus*.

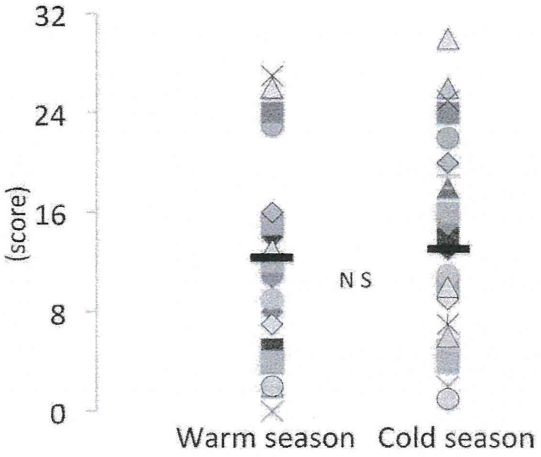

Fig. 2. Difference in the mean severity score between the cold and warm seasons.

3.3. Severity score in different seasons

Changes between the mean severity score in the cold and warm seasons are shown in Fig. 2. The paired score was not significantly different between the seasons.

3.4. Relationship between variables and the severity score

The relationship between the presence of pathogens in MEE or otorrhea and the severity score of EOM is shown in Fig. 3. The severity score in the pathogen-positive group was significantly higher than that in the pathogen-negative group ($p < 0.05$). Linear multiple regression analysis showed that BMI and the duration of BA significantly affected the severity score of EOM ($p < 0.05$, Table 4). The presence of aspirin-intolerant asthma tended to be correlated with the severity score ($p < 0.1$). The Lund–Mackay score tended to be negatively correlated with the severity score.

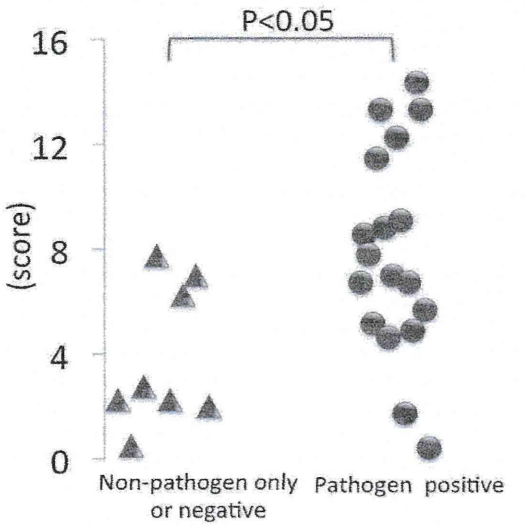

Fig. 3. Pathogens detected in cultures of the otorrhea and nasopharynx obtained from participants.

Table 4
Relationship between the severity score and variables tested by partial correlation analysis.

Variable	Partial correlation coefficient	*p* value
Body mass index	0.58	0.02
Duration of bronchial asthma	0.55	0.03
Lund–Mackay score	−0.49	0.05
Aspirin-intolerant asthma	0.46	0.07
Sex	0.40	0.12 (ns)
Allergic rhinitis	0.39	0.13 (ns)
Lateromedial width of the Eustachian tube	0.37	0.16 (ns)
Age	−0.33	0.22 (ns)
Total serum IgE level	0.18	0.50 (ns)
Percentage of eosinophils (peripheral)	−0.11	0.68 (ns)
Pneumatization of the mastoid	0.07	0.81 (ns)

ns, not significant.

4. Discussion

This study aimed to investigate which factors affected the control of EOM. We identified the risk factors of obesity and a long duration of BA.

Our definition for the severity of EOM was consistent with the severity of BA. Large scale clinical studies have investigated severe or difficult-to-treat asthma, including The Severe Asthma Research Program study (SARP) [9], The European Network For Understanding Mechanism of Severe Asthma (ENFUMOSA) [10], and The Epidemiology and Natural History of Asthma: Outcomes and Treatment Regimens (TENOR) [11], which used different definitions, but are similar to each other in terms of inclusion of current control status and exacerbations in the preceding year as an indication of asthma severity and treatment resistance. Our definition of the severity of EOM is related to the definitions. Our observation duration was chosen as 3 months because most of the patients visited our hospital with each change in symptoms every 1–3 months to check their state of the middle ear. In particular, patients with severe EOM required more frequent treatment. The scoring of our definition can be applied to a larger study because of its relatively simple way of evaluation.

We found that BMI was a risk factor for severe EOM. BMI is considered as a potential risk factor for severe asthma [12–15]. Recently, progressive research of the pathophysiology of severe asthma showed the heterogeneity of the phenotypic presentation [9]. The ethnic/genetic background, of Japanese in particular, may especially effect the association between obesity and asthma severity [16]. There is an ethnic difference in the risk of severe asthma. The SARP 2010 study has shown that one phenotype which consists mainly of older women (mean age, 50 years) with the highest BMI (58% with BMI > 30) and late-onset asthma (all older than 23 years of age) and non-atopy exists in 10% of the patients with severe asthma [17]. Fukutomi also found an association between obesity (according to the World Health Organization classification, $\geq$30.0 kg/m^2 [18]) and aspirin intolerance and difficult-to-treat asthma in Japanese non-atopic women [15]. In this study there were not gender differences in severity of EOM. The cause of the mechanism of the effect of obesity, respiratory inflammation, or stenosis is unclear [13,14]. The main symptom of EOM is thickness of the middle ear mucosa and a viscous otorrhea, which resembles eosinophilic chronic rhinosinusitis (ECRS) [19]. Obesity increases the number of adipose tissue-resident macrophages, which secrete a variety of inflammatory cytokines and adipokines (e.g., leptin and adiponectin), and these macrophages directly affect the airway [20,21]. The localization of these adipose tissue-resident macrophages in the middle ear is unknown.

We found that a long duration of BA affected the severity of EOM. The duration of BA in this study was defined as from the onset of BA to the age of the first consultation to our hospital. Our previous study showed that the onset of EOM is 10 years later than that of BA [22]. This result indicates that early diagnosis and adequate treatment are important. Physicians need to pay attention to the symptoms of EOM, such as an echo to an ambient sound, feeling of ear stuffiness, habitual sniffing, and autophony [23].

In our study, the Lund–Mackay score tended to be negatively correlated with the severity score of EOM. This result might be influenced by some factors such as a different operation method and/or systemic steroid application pre- and post-operatively. ECRS has been considered as one of the potential risk factors and strong relationship with severe asthma [12,19,23,24]. ESS for ECRS has been reported as an important first line treatment for removing polyps and also for opening the sinuses widely to enhance the effect of post-operative maintenance therapy, such as a nasal irrigation and topical and systemic corticosteroids [19]. Furthermore, systemic corticosteroid for recurrence of ECRS with asthma after ESS had a higher efficacy than those for non-ECRS [19]. In our series, 11 of 26 cases had the history of ESS with various surgical approaches at different hospitals. Therefore, postoperative sinus conditions and anatomical features varied in our series. Systemic steroids tend to apply for severe EOM for long period of time. As systemic steroids reduced the mucosal edema of ECRS more effectively than that of EOM, these differences of steroid effectiveness and steroid application for ECRS and EOM pre- and post-operatively seems to modify the relationship with Lund–Mackay score and severity of EOM.

In this study, we measured the width of the tympanic orifice of the Eustachian tube formed by the bony septum because the existence of a patulous or insufficient Eustachian tube in EOM is already known [22,25]. Antigenic material can easily invade the middle ear through the Eustachian tube and stimulate inflammatory cells, leading to accumulation of eosinophils, and induction of fibroblasts or endothelial cells to produce eosinophil chemoattractants in the middle ear [25]. In our study, the native width of the tympanic orifice of the Eustachian tube formed by the bony septum did not have a significant effect on the severity score compared with other factors.

High rates of *MRSA* and *Pseudomonas aeruginosa* infection in the middle ear were observed in our study. We eliminated bacterial cultures as independent variables in multiple linear regression analysis because the frequency of antibiotics was already included in the severity score of EOM. Most patients had received long-term treatment at other hospitals, resulting in drug-resistant microbes and infection hidden within the thickness of the middle ear mucosa. Recurrent infection implies insufficient bacterial residue. We do not know whether this infection, which might cause the middle ear mucosa to become thickened, is a cause or result. However, this complicated pathology in a small space makes choosing treatment difficult.

5. Conclusion

We studied a limited number of participants with EOM, and found a significant association between severe EOM and obesity, as well as with a long duration of BA. The participants in this study were a small group of patients from a single center, which has less external validity than a multicenter study. However, the patients come from far and near in east Japan because even otolaryngologists are unsure of the course of action if ongoing treatment is insufficient. To validate our results, a large-size randomized and controlled study is needed.

Conflicts of interest

None.

Acknowledgements

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Antigen-specific IgE in middle ear effusion of patients with eosinophilic otitis media

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ABSTRACT

Background: Eosinophilic otitis media (EOM) is an intractable otitis media characterized by a highly viscous effusion that contains eosinophils. We previously reported that high levels of IgE were detected in middle ear effusion (MEE) of patients with EOM.
Objective: To examine antigen-specific IgE in MEE of patients with EOM to clarify the pathogenesis of EOM.
Methods: Twenty-six patients with EOM associated with bronchial asthma were included in this study. Antigen-specific IgE against inhalant and bacterial antigens were measured in the serum and MEE of these patients.
Results: In patients with EOM, 1 or more antigen-specific IgEs were detected from the MEE of 16 of 26 patients (62%), whereas 1 of 9 control patients (11%) had antigen-specific IgE ($P < .01$). Total serum IgE concentrations were not different between the groups who tested positive (16 patients) and negative (10 patients) for antigen-specific IgE. None of the fungi-specific IgEs were detected in serum even though 11 patients tested positive for 1 or more fungal antigens detected in MEE. The severity score of EOM in the antigen-specific IgE–positive group was significantly higher than that in the antigen-specific IgE–negative group ($P < .05$).
Conclusion: Antigen-specific IgE against inhalant and bacterial antigens may be locally produced in the middle ear mucosa in patients with EOM. In particular, local sensitization against fungi together with *Staphylococcus aureus* could result in local IgE production in the middle ear and may be responsible for the severity of EOM.

Introduction

Eosinophilic otitis media (EOM) is a newly recognized middle ear disease first reported by Tomioka et al[1] in 1994. Since then, there have been several case reports, as well as studies, concerning the pathogenesis of EOM.[2–4] In 2011, the EOM study group established diagnostic criteria for EOM.[5] The major criterion of EOM is otitis media with effusion or chronic otitis media with eosinophil-dominant effusion. Minor criteria are as follows: (1) highly viscous middle ear effusion (MEE), (2) resistance to conventional treatment for otitis media, (3) association with bronchial asthma, and (4) association with nasal polyposis. Definitive cases are defined as positive for the major criterion plus 2 or more of the minor criteria. Strikingly, some patients have gradual or sudden hearing loss, and the incidence of deterioration of bone conduction and total deafness in patients with EOM is 59% and 6%, respectively.[5]

The cause of eosinophilic inflammation in the middle ear has not been determined. We previously reported that patients with EOM have a significantly longer eustachian tube opening duration compared with that of control patients.[6] This finding indicates that patients with EOM tend to have insufficient closure of the eustachian tube or a patulous eustachian tube.[6] Such a condition allows some antigenic material to enter the middle ear via the eustachian tube, causing eosinophilic inflammation with atopic predisposition, such as bronchial asthma. We also previously found that high levels of IgE were detected in the MEE of patients with EOM.[7] We speculate that IgE may be locally produced in the middle ear mucosa. In chronic rhinosinusitis, massive infiltration of eosinophils is also observed in the sinus mucosa or nasal polyps of some patients. This condition is called eosinophil-dominant nasal polyposis or eosinophilic chronic rhinosinusitis.

Bachert et al[8] found that *Staphylococcus aureus* enterotoxin is an important factor in causing eosinophilic inflammation in nasal polyps and is characterized by an increased level of IgE antibody against *S aureus* enterotoxin. Recently, certain fungi, such as *Aspergillus* and *Alternaria*, were also found to be responsible for

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