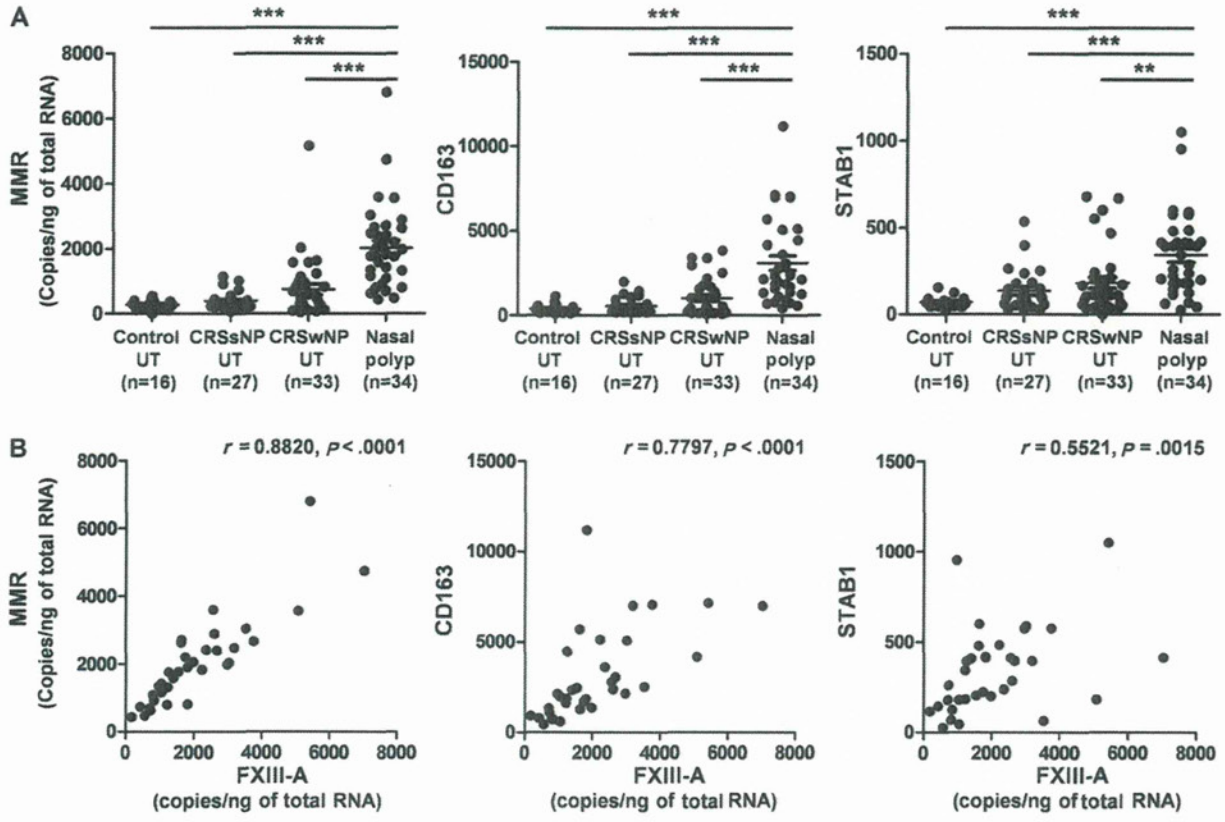

FIG 4. Correlation of FXIII-A with markers of M2 macrophages in NP tissue. **A,** Total RNA was extracted from UT from control subjects (n = 16), patients with CRSsNP (n = 27), and patients with CRSwNP (n = 33) and NP tissue (n = 34). The expression of FXIII-A and M2 macrophage markers MMR, CD163, and STAB1 was analyzed by using real-time PCR. **B,** The correlation in NP tissue was assessed by using a Spearman rank correlation test. $^{**}P < .01$ and $^{***}P < .001$.

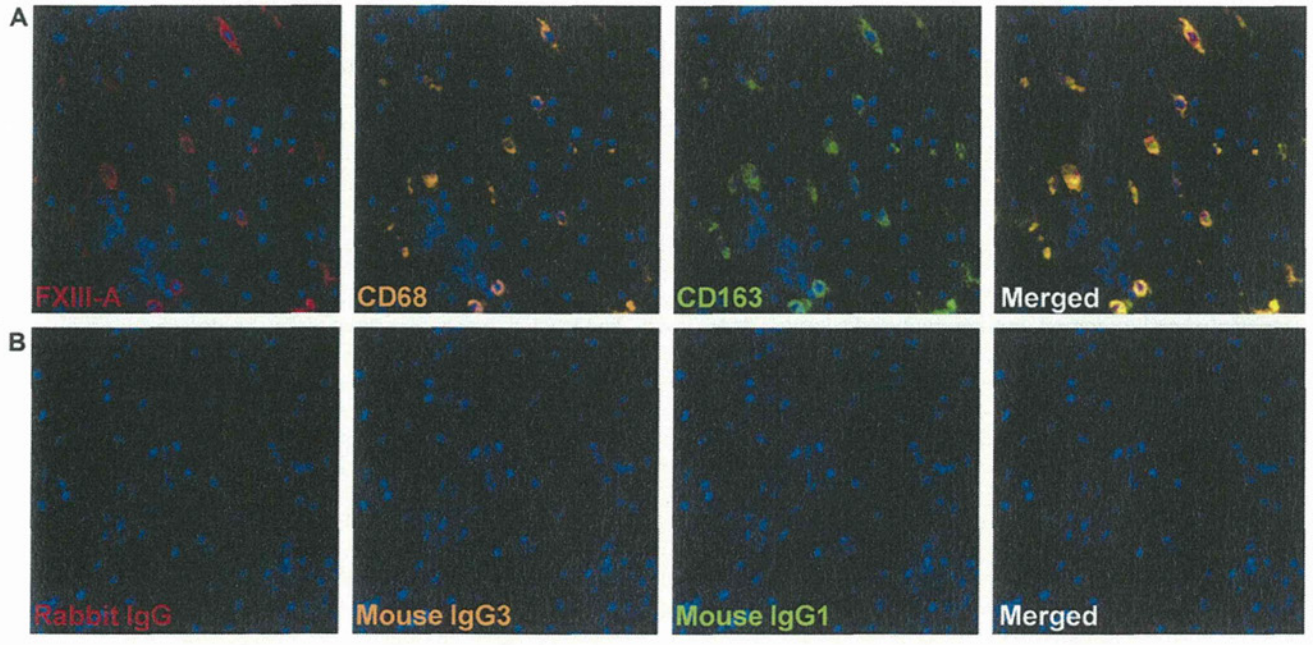

FIG 5. Detection of FXIII-A in M2 macrophages in NP tissue. Immunofluorescence assay was performed with anti-FXIII-A (*red* fluorescence), anti-CD68 mAb (*orange* fluorescence) for macrophages, and anti-CD163 mAb (*green* fluorescence) for M2 macrophage **(A)**, and control IgG **(B)**. Nuclei were counterstained with DAPI (*blue* fluorescence). The results are representative of 4 separate subjects. *DAPI*, 4′,6-diamidino-2-phenylindole.

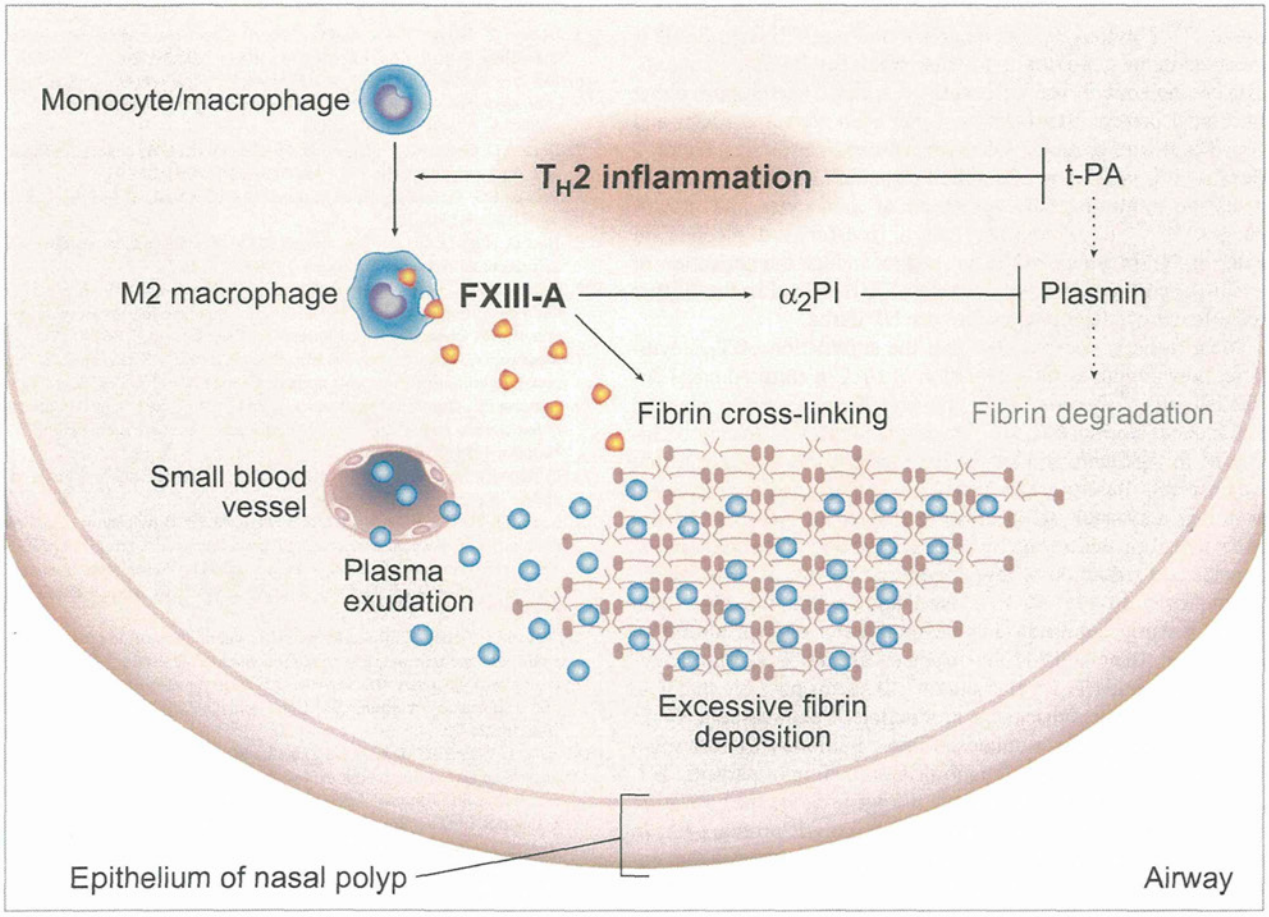

FIG 6. Hypothetical model in which the T_H2 milieu facilitates excessive fibrin deposition in NP tissue. T_H2 inflammation leads to the recruitment of M2 macrophages and the subsequent production of FXIII-A, which induces excessive fibrin deposition by cross-linking of fibrin and via the antifibrinolytic effect through binding α_2PI to fibrin. In the presence of T_H2 cytokines, t-PA levels are reduced, causing impaired plasmin generation, which, in turn, decreases fibrinolysis. *α_2PI*, α2-Plasmin inhibitor.

that FXIII-A is released by an alternative secretory pathway in human macrophages.[35,49,51] In fact, extravascular fibrin deposition is frequently observed within and around tumor matrix, and tumor-associated macrophages containing profound levels of FXIII-A have been reported.[52,53] It is reasonable to speculate that infiltrating M2 macrophages might contribute to excessive fibrin deposition by secreting FXIII-A in NP tissue, but this will require further experiments to test. In spite of presenting prominent inflammation, NP tissue shows low levels of fibrosis.[4,10] A previous report suggested that the downregulation of TGF-β may partially explain the low levels of collagen detected in NP tissue.[4] It has also been reported that M2 macrophages are involved in the suppression of tissue fibrosis by the production of IL-10, resistin-like molecule alpha, and arginase-1.[28] However, M1 macrophages, which have been identified as key regulators in demyelinating diseases of the central nervous system, produce significant amounts of TGF-β.[54] Thus, predominant infiltration of M2 macrophages might prevent or diminish fibrosis in NP tissue. Phagocytosis is one of the most important functions of macrophages, in which the rearrangement of cell cytoskeleton is deeply involved, and FXIII-A is implicated in phagocytic activities by catalyzing alterations in certain cytoskeletal components, including actin, myosin, vinculin, small heat shock protein HSP27, and thymosin β_4.[24,50] Macrophages from FXIII-A- deficient patients showed an impaired capacity of Fcγ, complement, and lectin-like receptor-mediated phagocytosis.[55] FXIII-A plays a critical intracellular role in receptor-mediated phagocytosis of macrophages. However, a recent study suggested that the phagocytic capacity of M2 macrophages is impaired in NP tissue and facilitated the increased presence of *Staphylococcus aureus* in CRSwNP.[44] Further studies are required to determine how and whether FXIII-A participates in the alteration of phagocytosis in M2 macrophages associated with nasal polyposis.

We found that M2 macrophages were major FXIII-A-expressing cells in NP. However, the regulation of macrophage recruitment in NP is poorly understood. We recently demonstrated that CCL23, known as a chemokine for macrophage, is elevated in NP tissue and CCL23 might play a critical role in macrophage infiltration.[56] It is possible that FXIII-A may play some role. Activated FXIII enhances the proliferation of peripheral blood monocytes, accelerates their migration, and inhibits monocyte apoptosis through the downregulation of thrombospondin-1 and due to the upregulation of c-Jun and

Egr-1.[25,43] Previous reports demonstrated that activated FXIII is involved in the generation of the complement C5-derived monocyte chemotactic factor.[57] Recently, it has also been reported that ribosomal protein S19 forms a dimer with activated FXIII and converts it into a monocyte-selective chemoattractive factor.[58] Because it is well known that the formation of a fibrin clot facilitates the infiltration and activation of monocytes and macrophages,[16,59,60] it is possible that a feed-forward mechanism exists in NP in which M2 macrophages induce the activation of FXIII-A and the actions of activated FXIII-A lead to the further recruitment of macrophages into the NP tissue.

Most recently, we reported that the stimulation of T_H2 cytokines downregulates the expression of t-PA in cultured nasal epithelial cells.[10] Because t-PA converts plasminogen to plasmin and induces fibrinolysis, the downregulation of t-PA may be involved in the formation of the excessive fibrin deposition that we observed. Based on the current observation and our recent report, it is reasonable to speculate that the T_H2 milieu facilitates excessive fibrin deposition by both acceleration of the coagulation cascade and reduction of the process of fibrinolysis in NP tissue (summarized in Fig 6). It is well known that infection with many parasitic helminths induces activation of T_H2 immunity in mucosal tissue and M2 macrophages are mobilized in the development of this T_H2 polarization.[61] It seems possible that T_H2 immunity, and the subsequent production of extravascular fibrin deposition, takes part in mucosal innate immunity by forming a fibrin mesh and impeding the motility or feeding of parasitic helminths. This process may promote fibrogenesis via the activation of FXIII-A and the suppression of the fibrinolytic protein t-PA. In this scenario, the excessive activation of type 2 cytokines that occurs in CRSwNP and may lead to the formation of NP can be viewed as a localized sterile antiparasite response.

We report here that tissue levels of FXIII-A were profoundly increased in NP tissue and that M2 macrophages are the sole or major FXIII-A-producing cell in NP. Overproduction of FXIII-A may lead to the acceleration of the coagulation cascade, resulting in excessive fibrin deposition, which, in turn, retains exuded plasma proteins and participates in tissue remodeling, intense edema, or pseudocyst formation in the submucosa of NP tissue. Our results imply that targeting the local production of FXIII-A from M2 macrophage might therefore be of therapeutic value for treating patients with CRSwNP.

Clinical implications: Overexpression of FXIII-A may have a pathogenic role in CRSwNP and strategies to reduce the activity of the coagulation cascade might have therapeutic value in the treatment of CRSwNP.

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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).

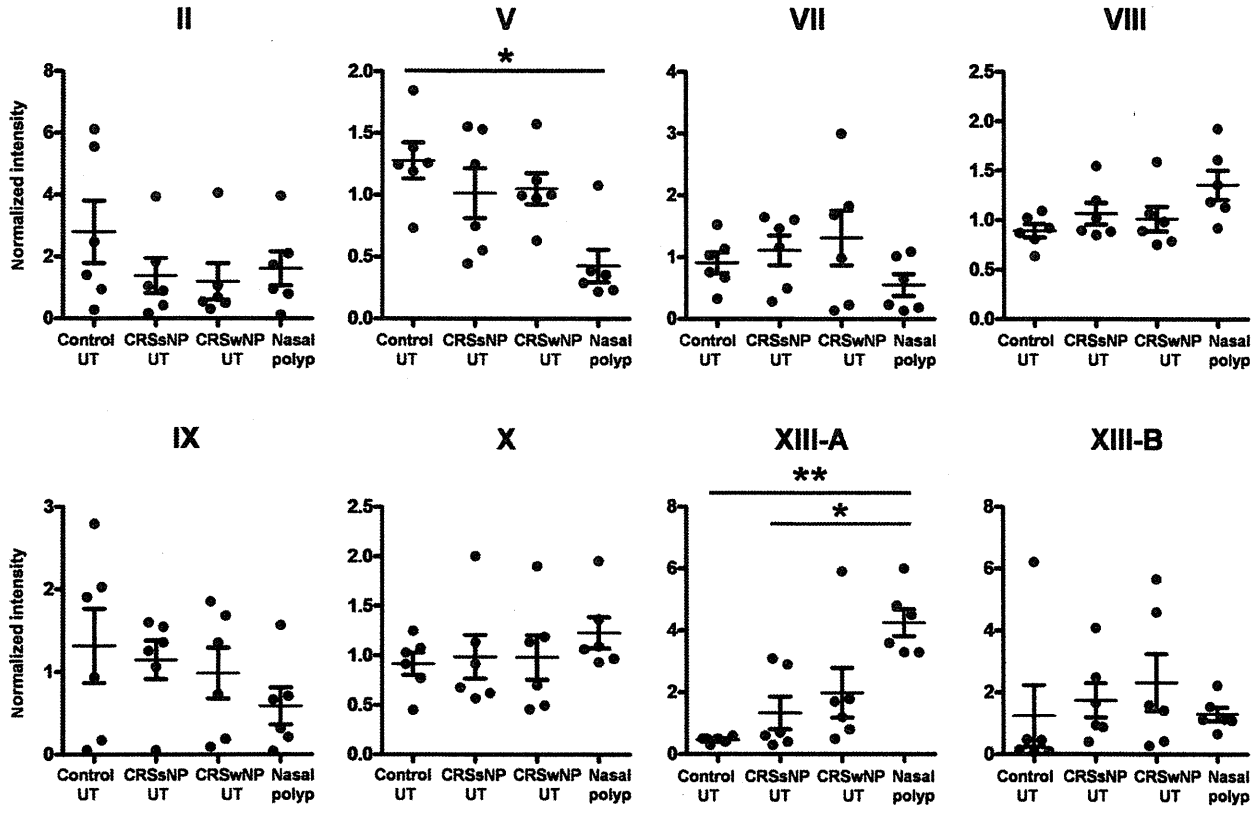

FIG E1. Microarray analysis of coagulation factors in sinonasal tissue. Microarray was used to assess the expression of coagulation factors in UT from control subjects (n = 6), patients with CRSsNP (n = 6), and patients with CRSwNP (n = 6) and in NP from patients with CRSwNP (n = 6). *$P < .05$ and **$P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

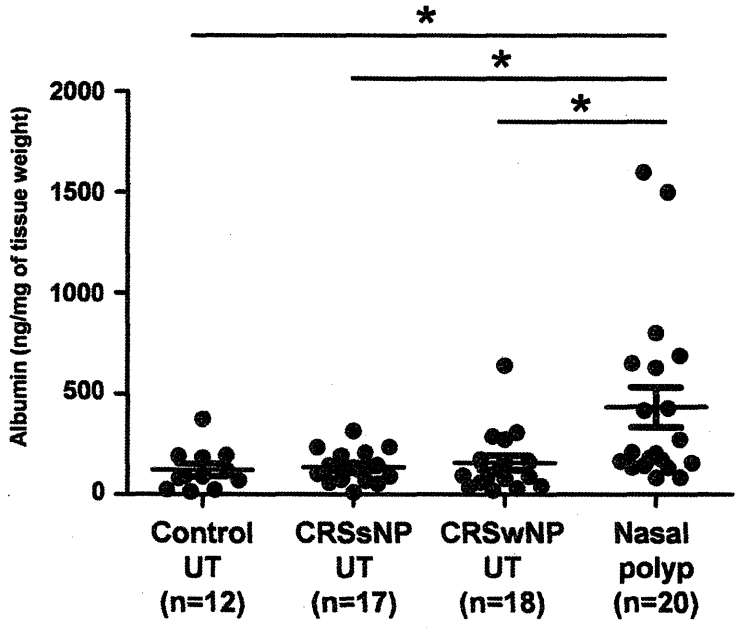

FIG E2. Increased levels of albumin in NP. Measurement of levels of albumin by ELISA in tissue homogenates of UT from control subjects, from patients with CRSsNP and CRSwNP, and in NP. *$P < .05$.

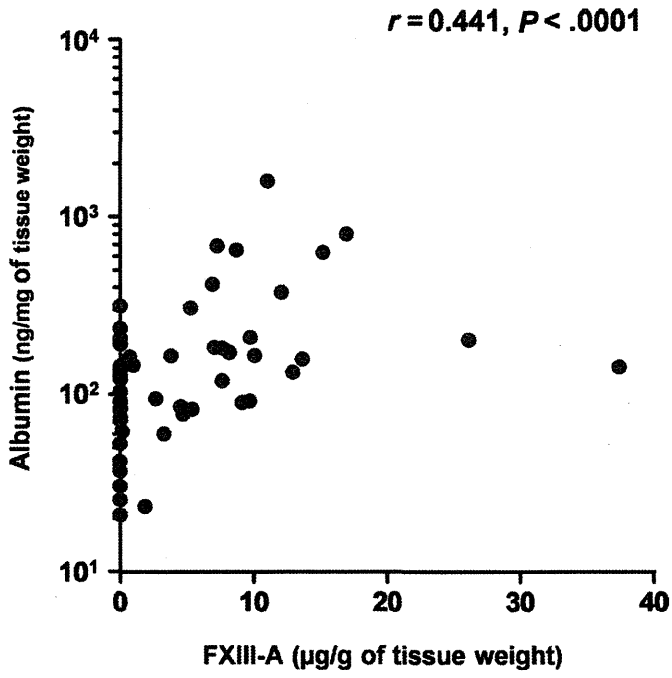

FIG E3. *The relationship of FXIII-A and albumin in UT and NP was evaluated by using ELISA. The correlation was assessed by using the Spearman rank correlation test.*

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.

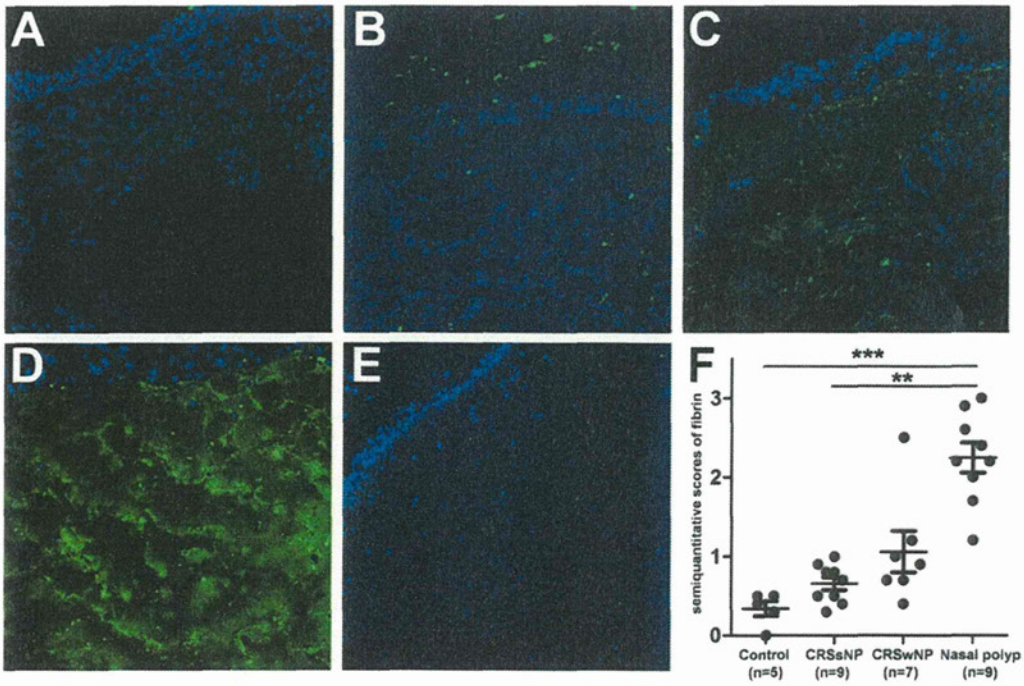

Figure 1. Immunofluorescence of fibrin in nasal tissues. Immunofluorescence was performed with antifibrin (*green fluorescence*). (*A–D*) Representative immunostaining for fibrin in uncinate tissue (UT) from a control subject (*A*), a patient with chronic rhinosinusitis without nasal polyps (CRSsNP) (*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. $^{**}P < 0.01$; $^{***}P < 0.001$.

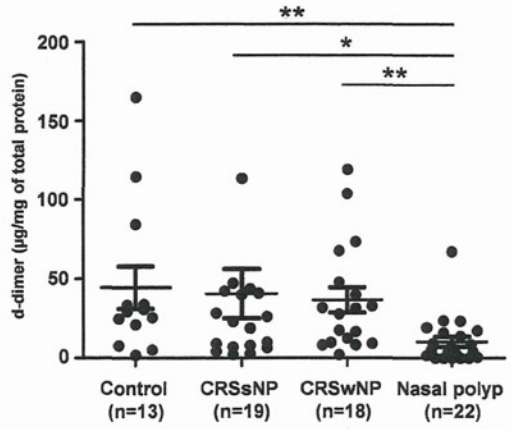

Figure 2. d-Dimer levels were decreased in nasal polyp tissue. Measurement of d-dimer in tissue homogenates of uncinate tissue from control subjects, from patients with chronic rhinosinusitis without nasal polyps (CRSsNP), from patients with chronic rhinosinusitis without nasal polyps (CRSwNP), and in 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

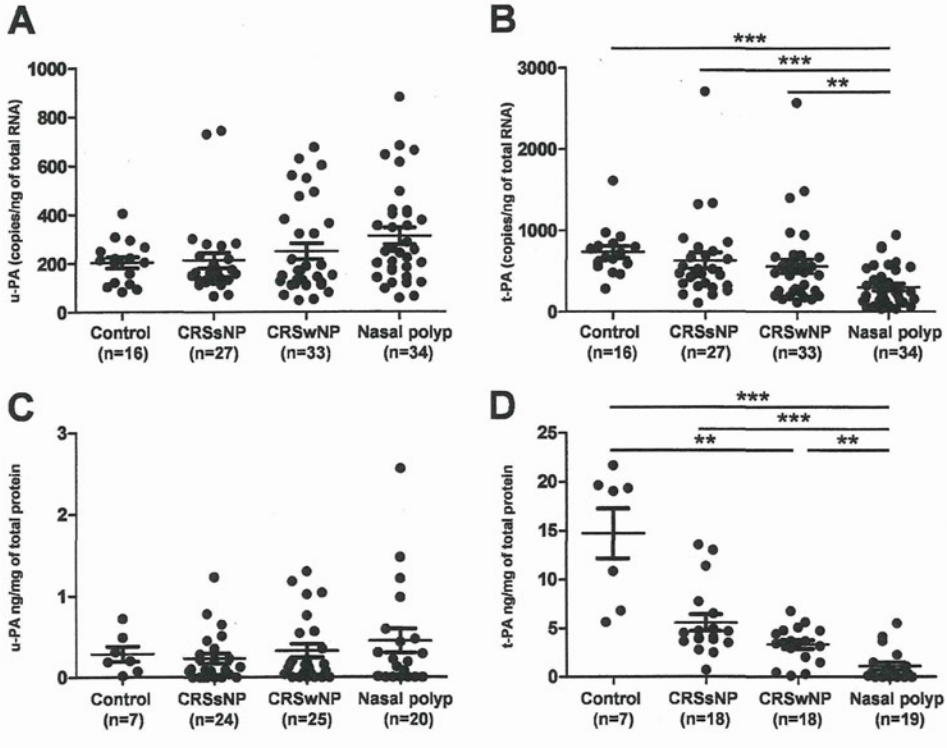

Figure 3. Expression of plasminogen activators in nasal tissues. Total RNA was extracted from uncinate tissue and nasal polyps, and expression of urokinase plasminogen activator (u-PA) (*A*) and t-PA (*B*) was analyzed using real-time PCR. Expression of u-PA (C) and t-PA (*D*) protein in tissue homogenates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

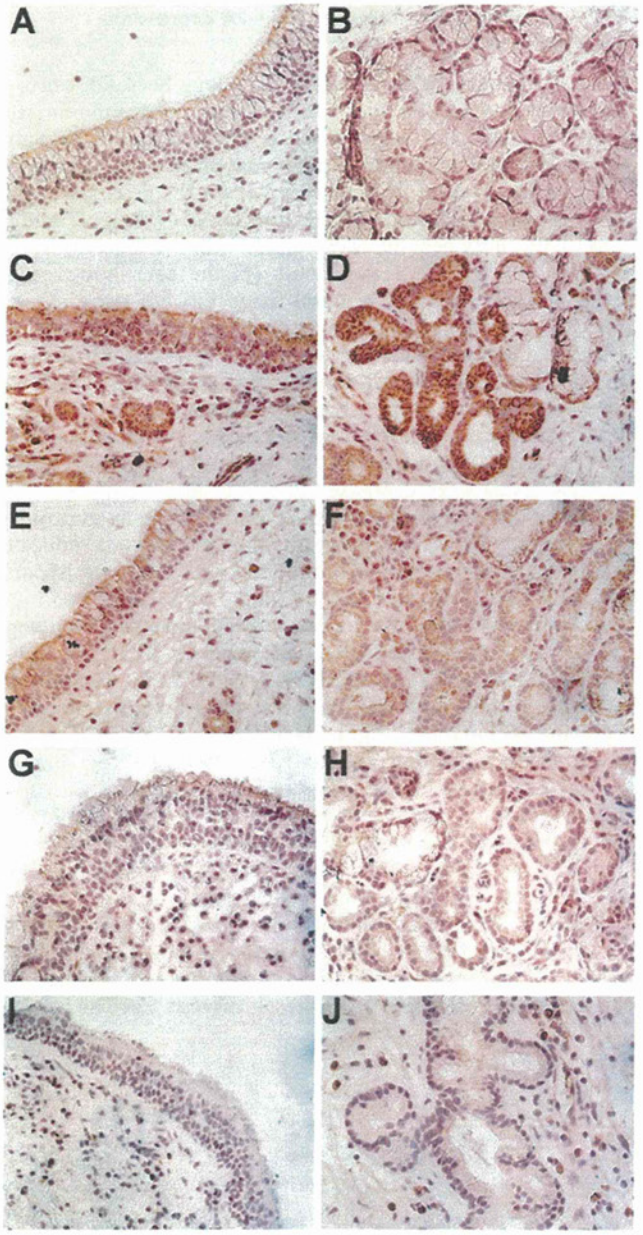

Figure 4. Immunohistochemical staining for tissue plasminogen activator (t-PA) in representative tissue samples from uncinate tissue (UT) and nasal polyps (NPs). (*A, B*) Negative control of UT from a control subject did not stain. (*C–H*) t-PA staining of UT from control subject (*C, D*) showed intense 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

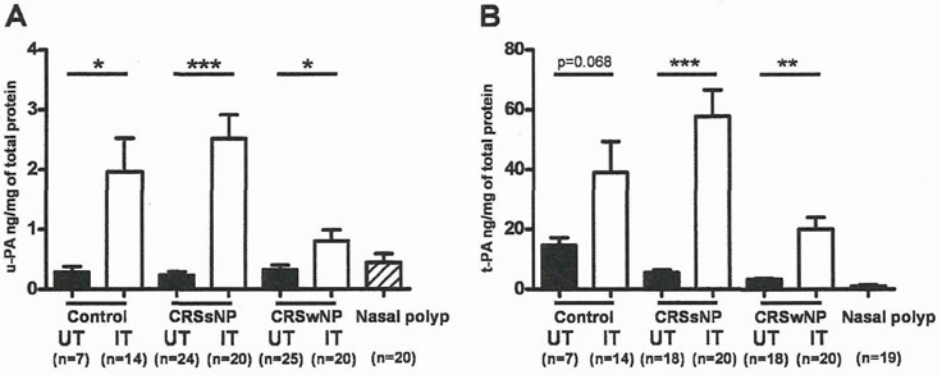

Figure 5. Comparison of plasminogen activator expression in uncinate tissue (UT) and turbinate tissue (IT). Expression of urokinase plasminogen activator (u-PA) (*A*) and tissue plasminogen activator (t-PA) (*B*) protein in tissue homogenates of UT, IT, and nasal polyps was measured using ELISA. The 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).

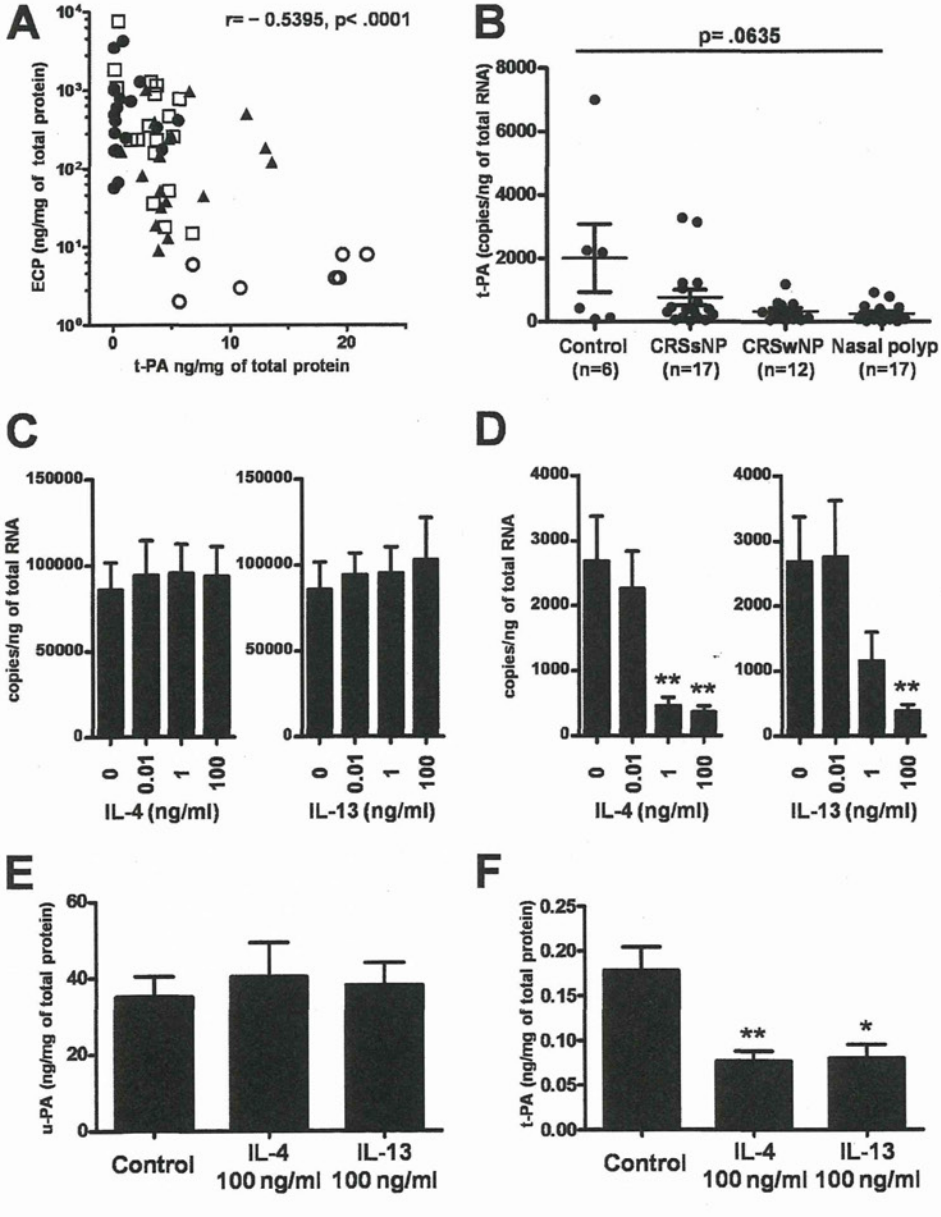

Figure 6. Potential regulation of tissue plasminogen activator (t-PA) expression in epithelial cells by Th2 cytokines. The relationship of t-PA and eosinophilic cationic protein (ECP) in nasal tissue was evaluated using ELISA (*open circles*, control uncinate tissue [UT]; *triangles*, chronic rhinosinusitis 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*). $^{*}P < 0.05$; $^{**}P < 0.01$.

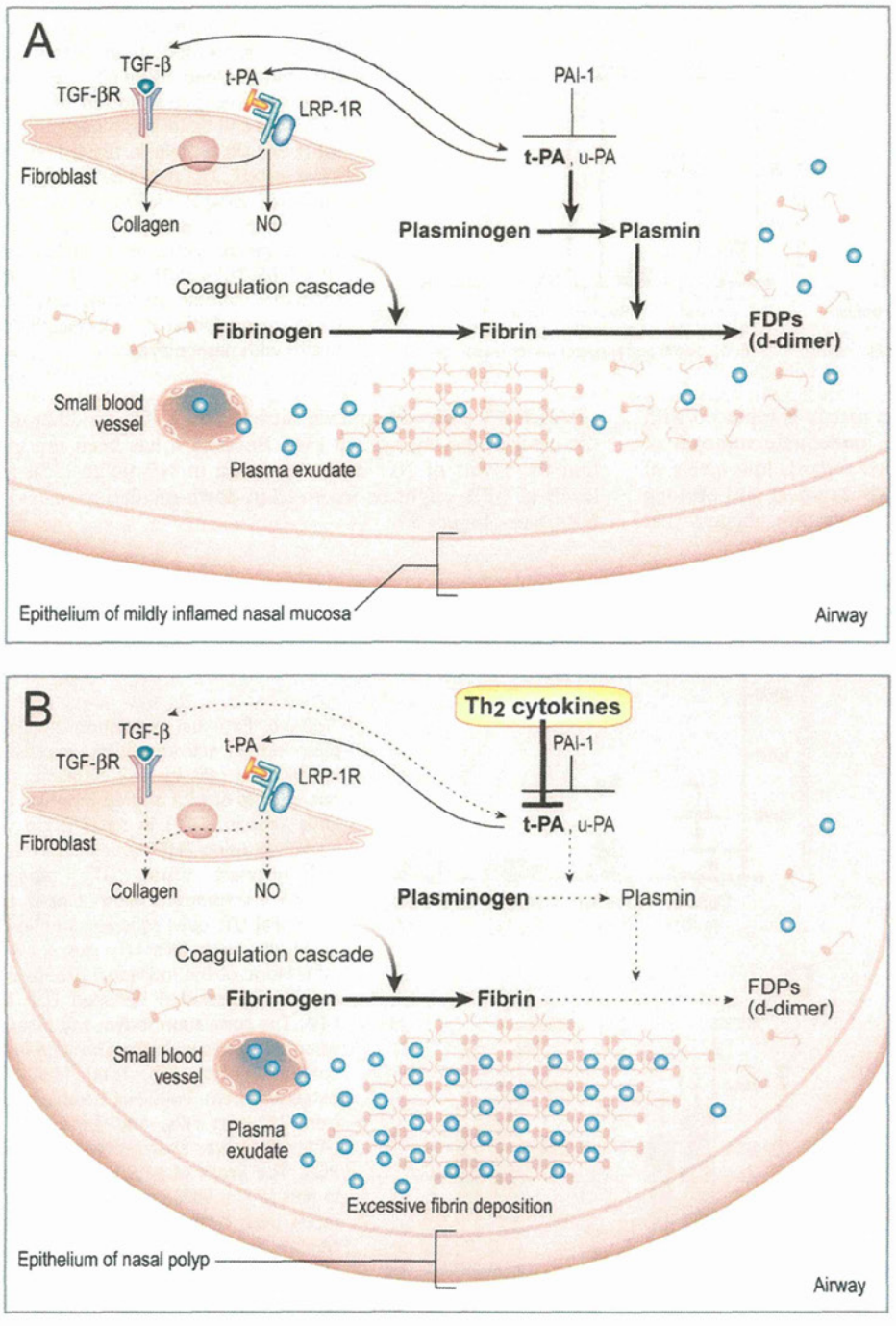

Figure 7. Hypothetical model to explain the role of tissue plasminogen activator (t-PA) in excessive fibrin deposition and reduced collagen in nasal polyps. As a protease, t-PA converts plasminogen to plasmin, which promotes fibrin degradation. As a cytokine, t-PA binds to its receptor lipoprotein receptor–related 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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Glandular mast cells with distinct phenotype are highly elevated in chronic rhinosinusitis with nasal polyps

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Background: Although chronic rhinosinusitis (CRS) with nasal polyps (CRSwNP) is characterized by T_H2 inflammation, the role of mast cells is poorly understood.
Objective: The objective of this study was to investigate the presence, localization, and phenotype of mast cells in patients with CRS.
Methods: We collected nasal tissue and nasal lavage fluid from patients with CRS and control subjects. We analyzed mRNA for the mast cell proteases tryptase, chymase, and carboxypeptidase A3 by using real-time PCR and measured mast cell protease proteins by using ELISA, immunohistochemistry, and immunofluorescence.
Results: Tryptase mRNA was significantly increased in nasal polyps (NPs) from patients with CRSwNP ($P < .001$) compared with uncinate tissue from patients with CRS or control subjects. Tryptase protein was also elevated in NPs and in nasal lavage fluids from patients with CRSwNP. Immnohistochemistry showed increased numbers of mast cells in epithelium and glands but not within the lamina propria in NPs. The mast cells detected in the epithelium in NPs were characterized by the expression of tryptase and carboxypeptidase A3 but not chymase. Mast cells expressing all the 3 proteases were abundant within the glandular epithelium of NPs but were not found in normal glandular structures.
Conclusions: Herein we demonstrated a unique localization of mast cells within the glandular epithelium of NPs and showed that mast cells in NPs have distinct phenotypes that vary by tissue location. Glandular mast cells and the diverse subsets of mast cells detected may contribute to the pathogenesis of CRSwNP. (J Allergy Clin Immunol 2012;130:410-20.)

Key words: Chronic rhinosinusitis, nasal polyps, mast cells, tryptase, chymase, carboxypeptidase A3 (CPA3)

Abbreviations used
CRS: Chronic rhinosinusitis
CPA3: Carboxypeptidase A3
CRSsNP: CRS without nasal polyps
CRSwNP: CRS with nasal polyps
ECP: Eosinophil cationic protein
GUSB: β-Glucuronidase
MC-T: Mast cell-tryptase
MC-TC: Mast cell-tryptase/chymase
NPs: Nasal polyps
SCF: Stem cell factor
SP: Substance P
UT: Uncinate tissue

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Chronic rhinosinusitis (CRS) is one of the most common chronic diseases in adults in the United States and affects up to 15% of the population.[1,2] The prevalence and medical costs of CRS are increasing and have become important social issues. This disease is typically classified into 2 types: CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). The etiology and pathogenesis of CRS remain controversial, but bacteria, viruses, and fungi have all been implicated in the establishment of an inflammatory process.[3-5] Particularly, CRS is often comorbid with asthma and is resistant to therapeutic interventions.[6] Surgical intervention is frequently necessary to clear the nasal and sinus passages and repeat endoscopic sinus surgery is often required. Recent studies suggest that CRSwNP is characterized by a T_H2-skewed eosinophilic inflammation characterized by significantly elevated levels of IL-5, IL-13, eotaxin, and eosinophil cationic protein (ECP).[7-9] Taken together, these results point to a critical role for eosinophils in the pathophysiology of CRSwNP and further suggest that factors triggering eosinophil degranulation may also be associated with the formation of nasal polyps (NPs).[10]

The potential importance of mast cells has been considered in CRS disease pathogenesis because they produce an abundance of cytokines that activate eosinophils, molecules that directly promote tissue remodeling and chemical mediators that can produce profound tissue edema.[11] Mast cells play key roles in host defense, homeostasis, tissue repair, and mechanisms of allergic inflammation.[12] In the case of human mast cells, 2 subtypes have been recognized by variable expression of granular neutral proteases: mast cell-tryptase (MC-T) and mast cell-tryptase/chymase

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TABLE 1. Subjects' characteristics

	Control			CRSsNP			CRSwNP			CRSwNP polyp
Total no. of subjects	n = 42 (17 M/25 F)			n = 70 (26 M/44 F)			n = 91 (56 M/35 F)			—
Age (y), median (range)	37 (16-63)			36 (18-69)			44 (22-75)			—
	Y	N	U	Y	N	U	Y	N	U	—
Atopy	2	32	8	35	27	8	45	24	22	—
Asthma	0	35	7	17	46	7	44	44	3	—
Methodology used										
Tissue RNA	n = 16 (7 M/9 F)			n = 26 (10 M/16 F)			n = 33 (21 M/12 F)			n = 34 (22 M/12 F)
Age (y), median (range)	45 (16-62)			35 (20-59)			38 (23-67)			39 (23-67)
Nasal scraping RNA	n = 7 (4 M/3 F)			n = 15 (8 M/7 F)			n = 10 (6 M/4 F)			n = 13 (7 M/6 F)
Age (y), median (range)	47 (29-62)			37 (18-61)			54 (27-73)			48 (22-73)
Tissue extract	n = 7 (5 M/7 F)			n = 19 (7 M/12 F)			n = 16 (9 M/7 F)			n = 19 (13 M/6 F)
Age (y), median (range)	49 (19-64)			36 (26-69)			46 (26-73)			45 (29-71)
Nasal lavage	n = 15 (5 M/10 F)			n = 14 (5 M/9 F)			n = 15 (9 M/6 F)			—
Age (y), median (range)	31 (21-63)			38 (24-64)			46 27-73)			—
Immunohistochemistry	n = 10 (3 M/7 F)			n = 10 (4 M/6 F)			n = 10 (6 M/4 F)			n = 10 (7 M/3 F)
Age (y), median (range)	28 (16-62)			31 (21-58)			45 (26-75)			50 (30-75)

F, Female; M, male; N, no; U, unknown; Y, yes.

(MC-TC). MC-T–type predominantly express high levels of tryptase but not chymase or carboxypeptidase A3 (CPA3), whereas MC-TC express all the 3 proteases.[13] MC-TC are essentially the exclusive type of mast cell found in normal skin and account for a small minority of mast cells in the lung. MC-T predominate in the alveolar wall and epithelium of the lung, whereas MC-TC favor the bronchial smooth muscle of patients with asthma and correlate with their bronchial hyperreactivity.[14] MC-T numbers in the respiratory epithelium increase during the pollen season in sensitive subjects.[15,16] These distinct tissue distributions and disease associations suggest a purposeful presence for each type of mast cell. Recent studies have demonstrated increased levels of mast cells in the presence of highly eosinophilic allergic inflammatory diseases such as asthma and eosinophilic esophagitis.[17,18] Moreover, mast cells in these studies have been shown to have a unique protease phenotype (tryptase and CPA3 high and chymase low).[19,20] The expression, phenotype, regulation, and protease expression patterns of mast cells in CRS have not been explored in detail.

In this study, we examined the distribution and phenotype of mast cells in uncinate tissue (UT) from control as well as from patients with CRS, including both CRSsNP and CRSwNP; we also evaluated NPs in patients with CRSwNP. We used quantitative RT-PCR, ELISA, and immunohistochemistry to determine the expression and distribution of mast cells as well as mast cell tryptase, chymase, and CPA3 in subjects with CRS. We detected a profound elevation of mast cells located within glands in NPs, an observation not previously described.

METHODS

Patients

Patients with CRS were recruited from the allergy and otolaryngology clinics at Northwestern University and the Northwestern Sinus Center. Sinonasal and polyp tissues were obtained from routine functional endoscopic sinus surgery in patients with CRS. All subjects met the criteria for CRS as defined by the Sinus and Allergy Health Partnership.[1]

Patients with an isolated antrochoanal polyp, cystic fibrosis, or unilateral NPs were excluded from the study. Details of subjects' characteristics are included in Table I and in the Methods section of this article's Online Repository at www.jacionline.org. 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 RT-PCR

Total RNA from sinus tissue was extracted by using QIAzol (Qiagen, Valencia, Calif), and the quality of total RNA from sinus tissue was assessed with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, Calif). Semiquantitative real-time RT-PCR was performed by using the TaqMan method. The mRNA expression levels were normalized to the median expression of the housekeeping gene β-glucuronidase (GUSB). Further details can be found in the Methods section of this article's Online Repository.

ELISA

Tryptase was assayed by using a commercially available assay kit (Uscn Life Science, Inc, Wuhan, China). The color intensity was measured with a Bio-Rad Spectrophotometer Model 680 Microplate Reader (Bio-Rad Laboratories, Hercules, Calif). Concentrations of tryptase protein were normalized to the concentration of total protein. Details can be found in the Methods section of this article's Online Repository.

Immunohistochemistry

Immunohistochemistry was performed as described previously.[21] Tissue sections were incubated with mouse antihuman tryptase mAb (Thermo Scientific, Fremont, Calif) or mouse antihuman chymase mAb (Thermo Scientific) or rabbit antihuman CPA3 polyclonal antibody (HPA008689, Sigma, St Louis, Mo) overnight at 4°C. Sections were rinsed and then incubated in biotinylated secondary horse antimouse or goat antirabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, Pa) at a 1:500 dilution for 1 hour at room temperature. After another rinse, sections were incubated in ABC reagent (Vector Laboratories, Burlingame, Calif) for 1 hour. Sections were rinsed again and incubated in DAB reagent (Invitrogen, Carlsbad, Calif) and then counterstained with hematoxylin. Slides were blinded, and 10 pictures were randomly taken from each slide. The number of positive cells in epithelium, glands, and submucosa was counted by 2 independent observers. Details of the methods for immunofluorescence and immunohistochemistry are described in the Methods section of this article's Online Repository.

Statistical analysis

All data are reported as the median. Differences between groups were analyzed by using the 1-way ANOVA Kruskal-Wallis test. Correlations were

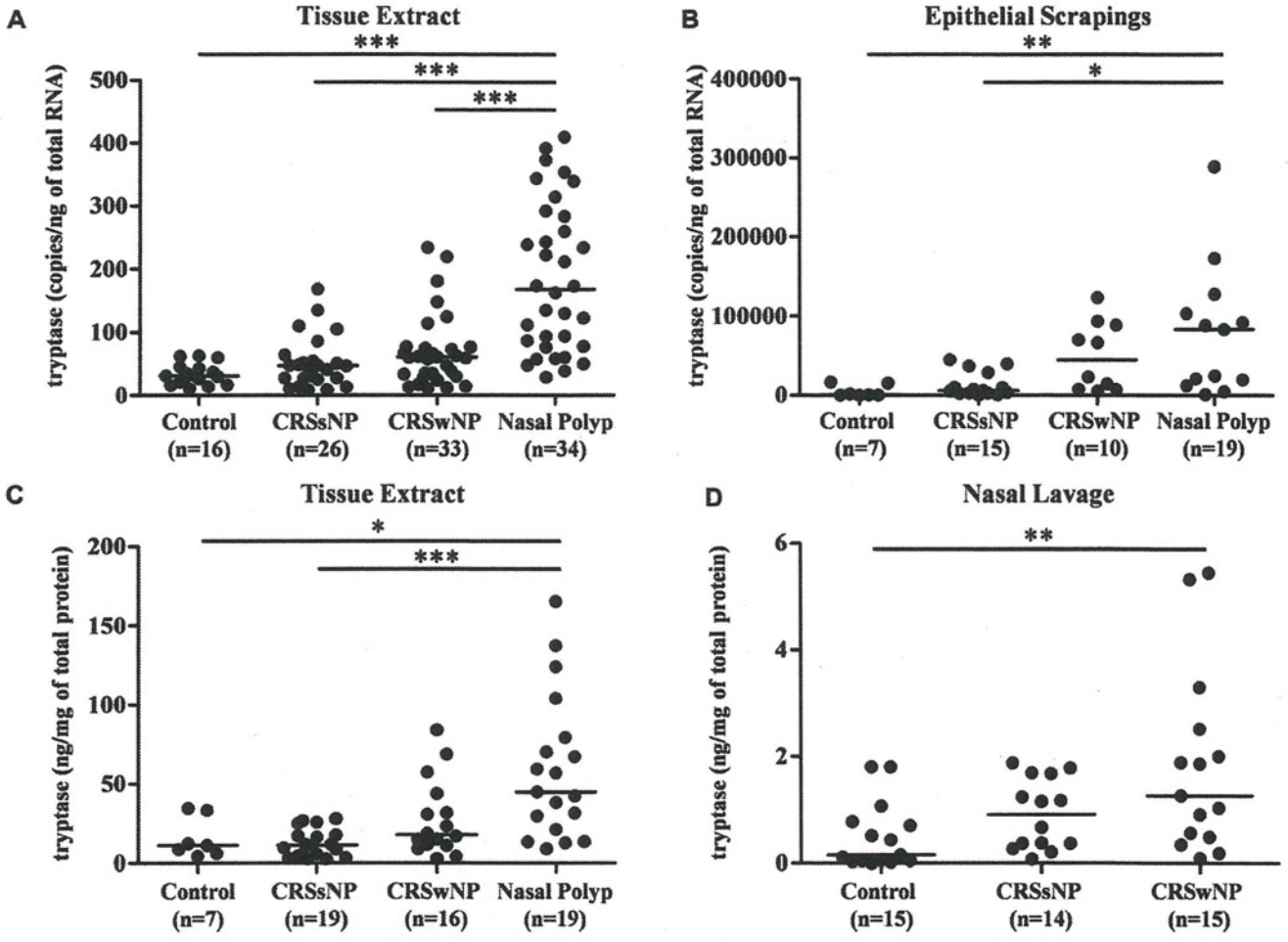

FIG 1. Increased expression of tryptase in NPs. The gene expression of tryptase in UT and NPs **(A)** and nasal scrapings **(B)** was measured by using real-time PCR. The concentration of tryptase in tissue homogenates of UT and NPs **(C)** and nasal lavage **(D)** was measured by using ELISA. Tryptase concentration was normalized to the concentration of total protein. $*P < .05$, $**P < .001$, $***P < .0001$.

assessed by using the Spearman rank correlation. A P value of less than .05 was considered statistically significant.

RESULTS

Mast cell expression and distribution in patients with CRS

Sinonasal and polyp tissues were collected from 70 subjects with CRSsNP, 91 subjects with CRSwNP, and 42 control subjects to determine the levels of expression of mast cells and their products in patients with CRS. Subject characteristics are shown in Table I.

To estimate the levels of mast cells in nasal mucosa, we first assessed the expression of mRNA for tryptase in UT from patients with CRSsNP, patients with CRSwNP, and controls, as well as in NPs from patients with CRSwNP. The expression of the housekeeping gene GUSB was not significantly different among the 4 groups (data not shown). Tryptase mRNA was significantly increased in NPs from patients with CRSwNP ($P < .0001$) in comparison with UT from either patients with CRS or control subjects (Fig 1, *A*). Likewise, the levels of tryptase mRNA were significantly elevated in NPs in 17 patients from which we had matched sets of both polyp and UT for RT-PCR evaluation ($P < .05$ data not shown). To assess the gene expression level in epithelium, we used nasal scraping–derived epithelial cells. Tryptase mRNA was significantly increased in scrapings from NPs in comparison with scrapings of the UT from either patients with CRSsNP or control subjects (Fig 1, *B*). To confirm this observation at the protein level, we made detergent extracts from homogenates of UT and NPs and then measured the concentration of tryptase by using ELISA. Tryptase protein was significantly increased in NPs ($P < .05$) compared with UT from either patients with CRSsNP or control subjects (Fig 1, *C*). We also determined tryptase protein expression in nasal lavage fluids collected from a separate group of subjects. Tryptase protein was significantly increased in lavage fluids from patients with CRSwNP ($P = .0079$) than in lavage fluids from control subjects (Fig 1, *D*).

To further characterize the mast cells in the nasal mucosa of patients with CRS, we performed immunohistochemistry by using surgical samples from control subjects and patients with CRS to detect tryptase-expressing cells. Consistent with the real-time PCR and ELISA data, increased numbers of