Epithelium-derived cystatin SN enhances eosinophil activation and infiltration through IL-5 in patients with chronic rhinosinusitis with nasal polyps

Bing Yan, PhD, a, b Hongfei Lou, MD, PhD, a Yang Wang, BS, a, b Ying Li, BS, a, b Yifan Meng, MD, PhD, a Sihan Qi, MD, a, b Ming Wang, PhD, a, b Lei Xiao, MD, PhD, * c Chengshuo Wang, MD, PhD, a, b, d, s and Luo Zhang, MD, PhD a, b, d, s Beijing, China, and Chicago, Ill

Background: The interaction between epithelial cells and immune cells plays an important role in the pathogenesis of chronic rhinosinusitis with nasal polyps (CRSwNP); however, the mechanism or mechanisms underlying TH-biased inflammation in this process are largely unknown. Profiling protein expression in patients with CRSwNP by using shotgun proteomics suggested that cystatin SN (CST1), a type 2 cysteine protease inhibitor, might play a role because this was expressed with the greatest difference in patients with eosinophilic chronic rhinosinusitis with nasal polyps (ECRSwNP) and those with noneosinophilic chronic rhinosinusitis with nasal polyps (nonECRSwNP).

Objectives: We sought to investigate the expression and role of CST1 in modulating eosinophilic inflammation in patients with CRSwNP.

Methods: Sinonasal tissues were collected from 192 patients with ECRSwNP, 52 patients with nonECRSwNP, and 40 control patients with CRSwNP.

Results: CST1 was mainly expressed by epithelial cells and significantly increased in patients with ECRSwNP but decreased in patients with nonECRSwNP compared with that in control subjects. CST1 expression was further increased in patients with ECRSwNP and comorbid asthma and correlated with eosinophil percentages in tissue samples. CST1 was induced by IL-4 and IL-13 in tissue from both patients with ECRSwNP and those with nonECRSwNP and repressed by IL-17A in patients with nonECRSwNP in the presence of neutrophils. CST1 enhanced eosinophil activation and recruitment through induction of IL-5.

Conclusion: Epithelium-derived CST1 modulates eosinophil activation and recruitment, expression of which could be regulated by TH2 and TH17 cytokines. (Allergy Clin Immunol 2019;144:455-69.)

Key words: Chronic rhinosinusitis with nasal polyps, cystatin SN, eosinophil activation, eosinophil recruitment, epithelial cells, IL-5

Chronic rhinosinusitis (CRS) is an inflammatory disease with high prevalence worldwide.1 CRS can be divided into CRS without nasal polyps and chronic rhinosinusitis with nasal polyps (CRSwNP), with the latter type accounting for approximately 20% to 25% of patients with CRS, and is associated with greater recurrence after surgical or pharmaceutical treatment.2 CRSwNP can be further classified into 2 distinct immunohistologic subtypes, eosinophilic chronic rhinosinusitis with nasal polyps (ECRSwNP) and noneosinophilic chronic rhinosinusitis with nasal polyps (nonECRSwNP), based on observed eosinophil infiltration.3 The 2 subtypes are also characterized by differences in TH type inflammation.4,4-7 ECRSwNP is pronounced as TH2 skewed and with relatively high recurrence and asthma comorbidity rate, whereas nonECRSwNP is characterized by a TH1 type inflammation.8,4-7 CRSwNP is pronounced as TH2 skewed and with relatively high recurrence and asthma comorbidity rate, whereas nonECRSwNP is characterized by a TH1 type inflammation.8,4-7
Some studies have suggested that epithelial cells can also play a role in the pathogenesis of CRSwNP. Although different Th1-biased inflammatory pathways have been well established in patients with CRSwNP, relatively little is known about the influence of different Th1 status on expression of distinct epithelial pathways or mediator mediators responsible for the crosstalk between the epithelium and immune cells in patients with the ECRSwNP and nonECRSwNP inflammatory subtypes. In this regard preliminary studies involving assessment of samples from patients receiving a diagnosis of ECRSwNP or non-ECRSwNP and healthy control subjects subjected to shotgun proteomics, followed by selection of specific proteins using the COMPARTMENTS and UniProt databases and analysis of mRNA levels of each protein, indicated that the difference in expression of cystatin SN (CST1) was the most significant between patients with ECRSwNP and those with nonECRSwNP.

CST1 is a member of the type 2 cystatin proteins superfamily. Recent studies have proposed that the members of the cystatin superfamily can be involved in a number of immunologic processes. However, the function of CST1 in the immunologic process is not fully understood. CST1 could inhibit the catalytic release. However, to our knowledge, very few studies have explored the function of CST1 in patients with CRSwNP. Therefore the aim of the present study was to investigate CST1 expression and its role in patients with CRSwNP.

**METHODS**

**Subjects and specimens**

A total of 284 subjects, including 192 patients with ECRSwNP, 52 patients with nonECRSwNP, and 40 healthy control subjects, were enrolled in the study. The diagnosis of CRSwNP was based on standard criteria issued in the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 guidelines. The definition of ECRSwNP and nonECRSwNP was in accordance with the previously reported criteria. Patients with immunodeficiency, coagulation disorder, fungal sinusitis, or cystic fibrosis and pregnant women did not participate in the study. None of the enrolled subjects had a history of aspirin sensitivity. Subjects undergoing septoplasty because of anatomic variations and without other sinonasal diseases were enrolled as control subjects.

Polyp tissues from patients with CRSwNP and inferior turbinate or uncinate mucosal tissues from control subjects were collected as biopsy specimens or during surgery. Detailed information on the subjects’ characteristics is provided in the Methods section, Fig E1, and Table E1 in this article’s Online Repository at www.jacionline.org.

This study was approved by the Ethics Committee of Beijing TongRen Hospital, Capital Medical University, and written informed consent was obtained from each patient before enrollment.

**Clinical assessment**

The diagnosis of asthma, allergic rhinitis, or atopy; measurements of routine hematologic parameters; and measurements of lower airway fraction of exhaled nitric oxide (FENO), a biomarker of eosinophilic inflammation, were made, as previously described. Detailed information on these procedures is provided in the Methods section in this article’s Online Repository.

**Hematoxylin and eosin staining and immunohistochemistry**

Hematoxylin and eosin and immunohistochemistry staining were performed, as previously described, to determine the inflammation type and the location of CST1 in the tissue. Detailed information is provided in the Methods section and Table E2 in this article’s Online Repository at www.jacionline.org.

**Assessment of protein profiles of tissues from patients with ECRSwNP, patients with nonECRSwNP, and control subjects by using shotgun proteomics and secreted protein selection**

Total proteins from 10 pooled samples each from patients with ECRSwNP, patients with nonECRSwNP, and control subjects were isolated and processed further for quality control. A minimum of 2 unique and nonredundant peptides per protein was used as the criterion for positive protein identification. Detailed information of these procedures is provided in the Methods section in this article’s Online Repository.

Differential proteins were subjected to the COMPARTMENTS (https://compartments.jensenlab.org) and UniProt (www.uniprot.org) databases for subcellular localization and secreted protein acquisition. Detailed information on these procedures is provided in the Methods section in this article’s Online Repository.

**RNA isolation, reverse transcription, and real-time PCR**

Total RNA from tissue samples and cultured cells were extracted with an RNA extraction kit (TaKaRa Biotechnology, Dalian, China). The quality of total RNA was assessed with the Nanodrop-2000 (Thermo Fisher Scientific, Waltham, Mass), and single-stranded cDNA was synthesized with the Prime-Script RT Master Mix (TaKaRa Biotechnology). Real-time PCR was then performed by using a SYBR Green method (TaKaRa Biotechnology) to assess mRNA levels of CST1 or mRNA coding eosinophilic granule proteins. Detailed information of the protocols and primer sequence are provided in the Methods section and Table E3 in this article’s Online Repository at www.jacionline.org.

**Assessment of CST1 and eosinophilic proteins by using ELISA**

The concentration of CST1 in tissue samples or epithelial cells was measured by using a commercially available ELISA kit (Sigma-Aldrich, St Louis, Mo). Similarly, eosinophil cationic protein (ECP) and eosinophil peroxidase (EPX) levels released from eosinophils in supernatant were determined by using specific ELISA kits obtained from Cusabio Life Sciences (Wuhan, China). Detailed information on these procedures is provided in the Methods section and Table E4 in this article’s Online Repository at www.jacionline.org.
Human nasal epithelial cell culture

Human nasal epithelial cells (HNECs) were prepared from nasal polyps or healthy mucosa after enzymatic digestion of tissue, and purified HNECs were cultured by using an air-liquid interface method. Cell cultures were treated with several mediators, including cathepsins B and C, IL-3, IL-4, IL-5, CXCL8, IL-13, IL-10, IL-17A, GM-CSF, and IFN-γ, for 24 hours and then harvested for assessment of CST1 mRNA by using quantitative RT-PCR, as described above. Detailed information on these procedures is provided in the Methods section in this article’s Online Repository.

Assessment of cytokines using the Luminex system

Concentrations of cytokines, including IL-5, IL-17A, IFN-γ, GM-CSF, CXCL8, and TNF-α in tissue samples and IL-4, IL-5, IL-10, IL-13, IL-17A, IL-1β, IFN-γ, CCL5, CCL11, CCL24, CCL26, and GM-CSF in cultured cells, were determined by using the Luminex assay (Luminex, Austin, Tex). Specific details of the cytokines analyzed are provided in the Methods section and Table E2 in this article’s Online Repository.

Assessment of the CST1 mRNA transcriptional site by using in situ hybridization

The CST1 mRNA transcriptional site in tissue sections of samples from patients with ECRSwNP was analyzed by using in situ hybridization. Detailed information on the procedure is provided in the Methods section in this article’s Online Repository.

Eosinophil isolation and culture

Peripheral blood eosinophils were purified by using an eosinophil isolation kit (Miltenyi Biotec, San Diego, Calif). Eosinophil purity was assayed by using flow cytometry. Isolated eosinophils were cultured in RPMI 1640 medium (Thermo Fisher Scientific), supplemented with 10% FBS (Thermo Fisher Scientific), and stimulated with recombinant CST1, IL-5, GM-CSF, IL-1β, IFN-γ, CCL5, CCL11, CCL24, CCL26, and GM-CSF in cultured cells, were determined by using the Luminex assay (Luminex, Austin, Tex). Specific details of the cytokines analyzed are provided in the Methods section and Table E4 in this article’s Online Repository.

Eosinophil migration assay

Eosinophils isolated from peripheral blood of subjects were added into the upper compartments of transwells at a 5 × 10^5 eosinophils in 200 µL of RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% FBS (Thermo Fisher Scientific). The lower chambers were added with the same medium containing 100 ng/mL recombinant CST1 or 10 µmol/L E64d. The cells were incubated at 37°C and 5% CO2 for 1 to 4 hours. At the end of incubation, the medium in the lower chambers was collected and assessed for the number of eosinophils present. Detailed information on the assay is provided in the Methods section in this article’s Online Repository.

Eosinophil recruitment

Polyp tissues were rinsed in PBS containing 1% penicillin, 1% streptomycin, and 1% amphotericin and then weighed and cut into smaller sections of about 0.1 g in weight. Ten microliters of 10 µg/mL recombinant CST1, 10 µg/mL recombinant IL-5, 10 µg/mL recombinant GM-CSF, 10 µg/mL recombinant CCL24, 1 µmol/L E64d, or PBS were injected separately or together with 10 µL of 10 µg/mL IL-5 blocking antibody into each section and cocultured with WBCs (10^6 cells in each well) for 96 hours for assessment of eosinophils infiltrating the tissue. Detailed information on the assay is provided in the Methods section in this article’s Online Repository at www.jacionline.org.

Statistical analysis

All statistical analyses were performed with SPSS software (version 23.0; IBM, Armonk, NY). All data are presented as means ± SEMs, unless otherwise noted. Differences between groups were analyzed by using Kruskal-Wallis ANOVA. The Pearson χ^2 or Fisher exact test was applied to sets of categorical data. Correlations were assessed by using Spearman correlation and defined as strong when the R value was greater than 0.7, moderate when the R value was in the range of 0.5 to 0.69, or weak when the R value was in the range of 0.3 to 0.49. A paired t test was used to test the significance of cell-culture data. Samples before and after glucocorticoid treatment were compared by using a paired t test. Cells treated with recombinant CST1, E64d, and/or IL-5 neutralizing antibody were analyzed by using 2-way ANOVA. A P value of less than .05 was considered statistically significant. All significance levels presented are 2-tailed.

RESULTS

Protein profiles and screening of secreted proteins in nasal polyp tissue

Polyp tissues from patients with ECRSwNP and patients with nonECRSwNP and mucosa from healthy control subjects subjected to shotgun proteomics demonstrated a total of 1022, 872, and 944 proteins, respectively. To characterize inflammatory types present among these groups, IL-5, IL-17A, IFN-γ, GM-CSF, TNF-α, and CXCL8 (IL-8) were assessed in appreciable quantities. Levels of IL-5, GM-CSF, and IFN-γ were significantly greater in tissue from patients with ECRSwNP compared with control tissue, whereas levels of CXCL8, IL-17A, and IFN-γ were significantly greater in tissue from patients with nonECRSwNP compared with tissue from control subjects (see Fig E1).

Assessment of secreted proteins in nasal polyp tissues by using the COMPARTMENTS and UniProt databases followed by RT-PCR to assess the genes encoding secreted proteins present exclusively in one group or with over 5-folds difference in between-group comparison demonstrated that CST1 was expressed, with the greatest difference between patients with ECRSwNP and those with nonECRSwNP.
CST1 expression in patients with CRSwNP

Assessment of CST1 expression relative to that of the housekeeping gene glyceraldehyde–3–phosphate dehydrogenase (GAPDH), indicated that CST1 mRNA levels were significantly increased in tissues from patients with ECRSwNP compared with those from patients with nonECRSwNP (P < .001; Fig 1, A). Furthermore, CST1 mRNA levels were significantly decreased in tissue from patients with nonECRSwNP compared with those in control subjects (P < .05; Fig 1, A). We further subclassified patients with ECRSwNP into asthmatic and nonasthmatic groups and found that the asthmatic patients demonstrated even greater levels of CST1 mRNA compared with the nonasthmatic subjects (P < .01; Fig 1, B). Measurement of the concentration of CST1 protein by using ELISA demonstrated that CST1 levels were significantly increased in patients with ECRSwNP (P < .001) and decreased in patients with nonECRSwNP (P < .05) compared with control subjects (Fig 1, C). Similarly, CST1 levels were significantly increased in asthmatic patients with ECRSwNP compared with nonasthmatic patients with ECRSwNP (P < .01; Fig 1, D), thus confirming the findings for increased expression of CST1 mRNA in patients with ECRSwNP.

CST1 is mainly expressed in nasal epithelial cells

Immunohistochemical staining of tissues from the 3 groups of subjects indicated that CST1 was localized mainly to the epithelium (Fig 2, A). Furthermore, semiquantitative analysis of
the staining indicated that consistent with the results obtained by using ELISA, CST1 levels were significantly increased in patients with ECRSwNP ($P < .001$) and decreased in patients with non-ECRSwNP ($P < .05$) compared with those in control subjects (Fig 2, B). In situ hybridization for determination of the CST1 mRNA transcriptional site further confirmed epithelial cells to be the major site in this regard (Fig 2, C).

Flow cytometric assessment to detect potential expression of CST1 in hematopoietic cells indicated that very low levels of CST1 were expressed in these cells, both at baseline and after stimulation with IL-4 (see Fig E2 in this article’s Online Repository at www.jacionline.org).

**Correlation of clinical parameters with CST1 mRNA and protein levels**

For patients with ECRSwNP, both tissue CST1 mRNA and protein levels were significantly but weakly correlated positively with percentages of eosinophils infiltrating the tissues ($P = .003$, $R = 0.443$ for mRNA and $P = .026$, $R = 0.335$ for protein) and FENO values ($P = .013$, $R = 0.411$ for mRNA and $P = .005$, $R = .461$ for protein; Fig 3). In contrast, no significant correlation was observed between CST1 mRNA or protein levels and percentages of peripheral blood eosinophils ($P = .382$ for mRNA and $P = .068$ for protein).

**Regulation of CST1 in patients with CRSwNP**

We explored factors to regulate the production of CST1. Because cathepsin family members act as the substrate for CST1, we assessed expression of these proteins by using data mining of microarrays, RNA sequencing (RNA-seq), and our proteomic data and found that 2 members of the family, cathepsins B and C, were upregulated in patients with ECRSwNP (see Table E5 in this article’s Online Repository at www.jacionline.org). Thus we further assessed the function of cathepsins B and C in CST1 expression. In this regard the recombinant cathepsins B and C were not able to induce CST1 expression in epithelial cells (see Fig E3 in this article’s Online Repository at www.jacionline.org).

Next, typical $T_h$ cytokines, which affect CST1 expression, were assessed, and in accordance with previous reports, we found that IL-4 induced CST1 expression (Fig 4). IL-13 also induced CST1 production, although not to the same level as IL-4. In contrast, IL-17A significantly decreased CST1 expression in polyps infiltrated with neutrophils but not in polyps from patients with ECRSwNP (Fig 4). However, IL-3, IL-5, IL-8, IL-
10, GM-CSF, and IFN-γ had little effect on CST1 expression (see Fig E3).

CST1 activates eosinophils

The positive correlation noted in polyp tissue for CST1 expression and eosinophil percentages suggested that CST1 can affect eosinophils. In this regard incubation of DNPCs for 24 hours with recombinant CST1 or E64d, a chemical cysteine protease inhibitor that can mimic the activity of cystatins, demonstrated that both these treatments significantly increased mRNA levels of EPX and ECP (see Fig E4 in this article’s Online Repository at www.jacionline.org). Although EPX and ECP proteins were also detected in medium, levels of these proteins were lower than the limit of detection in several samples, and thus these data were not available. However, incubation of eosinophils isolated from peripheral blood of patients with ECRSwNP with recombinant CST1 or E64d demonstrated that, consistent with the results from studies using DNPCs, levels of mRNAs for EPX and ECP were significantly increased after recombinant CST1 or E64d treatment for 24 hours in comparison with control medium (mock; Fig 5, A). Furthermore, levels of EPX and ECP proteins were also significantly increased after recombinant CST1 treatment for 24 hours (Fig 5, B). Similarly, assessment of expression of CD69, a protein with increased levels during eosinophil activation that is necessary for degranulation, on eosinophils demonstrated that both recombinant CST1 and E64d significantly enhanced the number of CD69⁺ eosinophils (Fig 5, C). The purity of the blood eosinophil preparation was assessed by using flow cytometry and found to be 93.4% (see Fig E5 in this article’s Online Repository at www.jacionline.org). Eosinophils isolated from several patients were also treated with recombinant IL-5 or GM-CSF as a positive control (see Fig E6 in this article’s Online Repository at www.jacionline.org).

Because activated eosinophils secrete granule contents through a mechanism termed piecemeal degranulation, which ensures that the cells do not die after degranulation and can produce more granules, we assessed the mechanism by which CST1 can promote the synthesis of eosinophils granule (ie, by enhancing the proliferation of eosinophils or by stimulating synthesis). Assessment of numbers of cultured eosinophils incubated with recombinant CST1 and E64d demonstrated that eosinophil numbers were not significantly greater after 48 hours compared with those in control subjects (Fig 5, D), suggesting that CST1 can enhance granule synthesis of “resident eosinophils.” Because PU.1, a transcriptional factor, has been reported to be crucial for the synthesis of eosinophil granule proteins, such as ECP and EPX, we also assessed the effect of recombinant CST1 and E64d on PU.1 expression and demonstrated that this was also significantly increased by CST1 and E64d compared with control medium (Fig 5, A).

CST1 promotes eosinophil recruitment

Assessment of the effect of CST1 on eosinophil recruitment using transwells with eosinophils treated with recombinant CST1 or E64d for up to 4 hours demonstrated no significant increase in
FIG 4. Regulation of CST1. A, Representative figures showing hematoxylin and eosin staining for each type of CRSwNP. Bars = 20 μm (×400 magnification). B and C, mRNA (Fig 4, B) and protein (Fig 4, C) levels of CST1 measured in epithelial cells cultured from nasal mucosa from control subjects or polyp tissues stimulated with IL-4, IL-13, and IL-17A. *P < .05, **P < .01, and ***P < .001.
FIG 5. Effect of CST1 on activation of eosinophils isolated from peripheral blood of patients with ECRSwNP. Isolated eosinophil cultures were treated with 100 ng/mL recombinant CST1 or 10 μmol/L E64d for 24 (A-C) or 48 (D) hours and then assessed for relative expression of ECP, EPX, and PU.1 mRNA in eosinophils (Fig 5, A), concentrations of ECP and EPX released in culture medium (Fig 5, B), CD69 expression in eosinophils (Fig 5, C), and eosinophil proliferation (Fig 5, D). For Fig 5, A and B, samples from the same subject are shown in the same color, and mean values for each group are shown as green squares. FITC, Fluorescein isothiocyanate.
numbers of migrating eosinophils compared with control values (see Fig E7 in this article’s Online Repository at www.jacionline.org). However, polyps injected with recombinant CST1 or E64d and cultured with WBCs of the same subject from whom the polyps were obtained demonstrated that after 96 hours, large numbers of eosinophils were present in tissue sections treated with recombinant CST1 and E64d compared with PBS (Fig 6 and see Figs E8 and E9 in this article’s Online Repository at www.jacionline.org). Injection of recombinant IL-5, GM-CSF, and CCL24 (eotaxin-2) as positive controls also demonstrated significantly increased numbers of eosinophils compared with PBS and thus confirmed the effects of CST1, as well as the validity of the model in assessing the efficacy of specific mediators in the recruitment of eosinophils (see Fig E8). This finding suggests that recruitment of eosinophils by CST1 is unlikely to be due to ligand or receptor binding on the eosinophils but is probably mediated through cytokines or molecules.

**CST1 enhances IL-5 expression**

Because previous studies have indicated that CST1 mainly plays a role in eosinophilic inflammation through some mediators, to illustrate the whole vision of the inflammation bias, we assessed the effect of CST1 on induction or inhibition of a variety of TH cytokines, including IL-4, IL-5, IL-10, IL-13, IL-17A, IL-1β, and IFN-γ. Incubation of DNPCs from tissues and eosinophils of patients with ECRSwNP isolated from patients with ECRSwNP with both recombinant CST1 and E64d for 24 hours significantly upregulated expression of IL-5 in both DNPCs and eosinophils (Fig 7). IL-17A and IFN-γ were not detectable in the eosinophils by using the Luminex system in the present study.

We further explored the effect of CST1 on concentrations of CCL5, CCL11, CCL24, CCL26, and GM-CSF, which are also known to be effective mediators in recruitment and activation of eosinophils. Our study demonstrated that incubation of eosinophils isolated from patients with ECRSwNP with recombinant
CST1 did not significantly alter the concentrations of CCL5, CCL11, CCL24, and CCL26 released from eosinophils, whereas the concentration of GM-CSF was less than the detection limit for the assay (see Fig E10 in this article’s Online Repository at www.jacionline.org).

IL-5 is involved in patients with CST1-induced eosinophilic inflammation

Eosinophils were pretreated with medium containing 10 μg/mL IL-5 blocking antibody (R&D Systems) or medium alone for 1 hour and then incubated for a further 24 hours with recombinant CST1.
FIG 8. Effect of IL-5 blocking antibody (10 μg/mL) on CST1- or E64d-induced relative mRNA expression of ECP, EPX, and PU.1 (A); ECP and EPX protein expression (B); and eosinophil recruitment (C and D). For Fig 8, A and B, the mean value for each group is shown as the green square. Fig 8, C, shows representative images for recruitment of eosinophils induced by recombinant CST1 or E64d. Ab, Antibody. Bars = 50 μm (×200 magnification). Fig 8, D, shows average numbers of eosinophils recruited in each group of 5 high-power fields (HPF) at ×400 magnification. N.S., Not significant.
CST1 or E64d to determine the role of IL-5 in the function of CST1. Both CST1 and E64d significantly increased EPX, ECP, and PU.1 mRNA expression (Fig 8, A) and release of EPX and ECP protein in cell-culture media (Fig 8, B) in the absence of IL-5 blocking antibody. However, these CST1- and E64d-induced increases were abolished by IL-5 blocking antibody (Fig 8, A and B, respectively).

Similarly, to determine whether IL-5 blocking antibody could decrease CST1- and E64d-induced infiltration of eosinophils, polyp tissues were injected with PBS, recombinant CST1, or E64d in the absence or presence of IL-5 blocking antibody. Similar to the findings for EPX, ECP, and PU.1, recombinant CST1 or E64d significantly increased eosinophil numbers infiltrating the polyp tissues compared with mock PBS, and this increase in eosinophil counts was significantly attenuated in the presence of IL-5 blocking antibody (Fig 8, C and D).

**DISCUSSION**

Research into CRSwNP over the last few decades has shown that this is a complex and heterogeneous disease. A recent study has indicated that several clusters of CRSwNP can be identified based on distinct clinical pathobiological features and differences in response to treatment. Immunohistologically, CRSwNP can be divided into ECRSwNP and nonECRSwNP, with ECRSwNP predominating in white patients, demonstrating T_{H}2-biased cytokine profiles, and nonECRSwNP predominating in mostly Asian patients, demonstrating T_{H}1/T_{H}17-biased patterns. Furthermore, ECRSwNP is associated with a greater likelihood of comorbid asthma and thus can be further divided into the subtypes according to asthma comorbidity or not. As a clustering axis extending from nonECRSwNP to ECRSwNP without asthma to ECRSwNP with comorbid asthma, peripheral blood eosinophil counts and eosinophil percentages are generally increased, suggesting that the probability of the disease to be a systemic disorder is increased.

For the purpose of treatment, confirming the disease subtype is possibly the first and most important thing because this helps in deciding the strategy for the route of administration, as either local or systemic treatment, in minimizing the systemic risks and reducing the drug load as well as the costs. Hence determining an effective biomarker for the clustering is likely to aid in the diagnosis and appropriate treatment for CRSwNP.

The present study has indicated that CST1 might be a novel useful biomarker for ECRSwNP because its expression was significantly increased in tissue from patients with ECRSwNP and decreased in tissue from patients with nonECRSwNP compared with inferior turbinate or uncinate tissue from control subjects. Furthermore, CST1 expression was significantly increased in patients with ECRSwNP and comorbid asthma compared with that seen in patients with ECRSwNP without asthma. Indeed, levels of both CST1 mRNA and protein were also found to be positively correlated with percentages of tissue eosinophils and with Fex0 levels in patients with ECRSwNP. Similar to our findings, Kato et al have recently demonstrated that the expression of CST1 was significantly increased in patients with ECRSwNP compared with that in patients with non-ECRSwNP. Collectively, these findings suggest that CST1 might serve as a reliable biomarker for ECRSwNP.

Activation and recruitment of eosinophils are major features of eosinophilic inflammation, and in this regard it is likely that CST1 plays a role in both processes. Activated eosinophils undergo degranulation, releasing proteins, such as EPX, ECP, and eosinophil-derived neurotoxin, that can be toxic to invading cells and also be responsible for tissue damage and remodeling. It has been reported that apoptosis of eosinophils is delayed in patients with CRSwNP and that infiltrating eosinophils release the granules without undergoing self-destruction. Although different mechanisms of eosinophil degranulation have been postulated, mechanisms underlying the regeneration/sustain-ability of granules/granule proteins in activated eosinophils are presently not fully understood. Findings from the present study suggest that epithelial cell–derived CST1 promotes eosinophil granule protein synthesis by inducing IL-5. Although several cells, including T_{H}2 and group 2 innate lymphoid cells, could significantly higher compared with that in healthy control subjects (Fig 9).

**Glucocorticoids partially suppress expression of epithelial CST1**

We investigated the effect of glucocorticoids on epithelial CST1 mRNA and protein expression. Samples were acquired before and after treatment by means of biopsy and after surgery. Patients underwent a 2-week oral corticosteroid therapy (24 mg of methylprednisolone once daily for 7 days, followed by a 4-mg reduction every other day during the other 7 days). This study demonstrated that glucocorticoids significantly suppressed both CST1 mRNA and protein expression compared with baseline values. However, regular doses of glucocorticoids could not attenuate CST1 expression in patients with ECRSwNP to the level of healthy control subjects, and even after treatment, CST1 expression on epithelial cells of patients with ECRSwNP was
be the source of IL-5, our study has further demonstrated that CST1 also enhances production of IL-5 in eosinophils. Thus it is possible that CST1-induced IL-5 synthesis in eosinophils can be involved in autocrine regulation of granule protein synthesis. Furthermore, the present study has demonstrated that CST1-mediated activation and recruitment of eosinophils through IL-5 can be reciprocated by IL-4 or IL-13 released from eosinophils.

Our study has further suggested that CST1 expressed in tissue from patients with CRSwNP also has the potential to enhance the recruitment and infiltration of eosinophils directly from the blood through involvement of IL-5 and activation of eosinophils involving degranulation. Eosinophil infiltration was significantly enhanced by treatment of recombinant CST1 or E64d for 96 hours, which reflects the promotion of eosinophilic inflammation of CST1 in vivo. Overall, these data indicate that CST1 is involved in activation of existing eosinophils in the tissue and is responsible for eosinophil recruitment under TH2 microenvironments.

Furthermore, the finding from the present study that recombinant CST1 did not recruit eosinophils effectively over a relatively short period of 4 hours but significantly increased eosinophil recruitment over a longer period of 96 hours suggests that CST1 is unlikely to affect eosinophils directly by binding to specific receptor or receptors on eosinophil surfaces. Indeed, although eosinophils are known to express numerous cell-surface receptors, including chemokine receptors, Fc receptors, pattern recognition receptors, lipid mediator receptors, cytokine receptors, complement receptors, and adhesion receptors, to our knowledge, there are no reports of cystatin receptors on eosinophils.

Studies in cystatin F (CSTF) knockout mice have reported that eosinophils from these mice had reduced lifespans, reduced granularity, and disturbed granule morphology. Furthermore, cysteine protease inhibitors restored granularity, demonstrating that control of cysteine protease activity by CSTF was critical for normal eosinophil development. However, microarray, RNA-seq, and proteomics assessments have not detected the expression of either CSTF mRNA or protein in patients with CRSwNP, whereas CST1, cystatin SA (CST2), cystatin A (CSTA), cystatin B (CSTB), and cystatin C (CST3) have been shown to be present. RNA-seq assays indicated that CST1 was upregulated in patients with ECRSwNP compared with that in healthy control subjects, and the fold change of CST1 for patients with ECRSwNP versus control subjects is 1732.2, which also indicated that CSTA and CSTB were upregulated in both patients with ECRSwNP and patients with non-ECRSwNP compared with healthy control subjects, with no significant difference between the 2 subtypes: the fold change of CSTA for patients with ECRSwNP versus control subjects is 4.32, and that for patients with nonECRSwNP versus control subjects is 3.95; the fold change of CSTB for patients with ECRSwNP versus control subjects is 1.34, and that for patients with nonECRSwNP versus control subjects is 2.28. Proteomics demonstrated CSTB and CST3 to be present in the nonECRSwNP group, but they were not detectable in control subjects and patients with ECRSwNP (unpublished data). Similarly, microarray analysis demonstrated CST1, CSTA, and CST2 expression to be increased in patients with CRSwNP; however, expression of CSTA and CST2 is relatively low compared with that of CST1. The fold change of CST1, CSTA, and CST2 for patients with CRSwNP versus control subjects is 31.53, 2.1, and 8.34, respectively. Thus, unlike the internal signaling of CSTF for mice, the CST1-mediated external signaling might be the way to regulate eosinophils by cystatins in human subjects and reflects interaction between the epithelium and eosinophil counts.

**FIG 9.** Effect of glucocorticoids on epithelial CST1 expression. A and B, Representative CST1 staining in nasal tissue and quantitative analysis before and after glucocorticoid treatment (n = 5). Bars = 20 µm (×400 magnification). C, relative mRNA level and protein expression of CST1 before and after glucocorticoid treatment (n = 10 and 11, respectively). N.S., Not significant.
The present study shows that CST1 could be regulated by a variety of cytokines, including IL-4 and IL-13, which promoted the transcriptional level of CST1 in epithelial cells, whereas IL-17A decreased its expression. Interestingly, because this decrease could only be observed in neutrophil-infiltrated polyps, it might reflect that IL-17A does not directly regulate CST1 but acts through another molecule that is absent or undetectable in healthy subjects or patients with ECRSwNP. Nevertheless, these results further support the concept that CRSwNP is a heterogeneous condition, and each subtype presents with the distinct genetic background. However, because activated eosinophils could also release IL-4, IL-5, and IL-13, this suggests that there might be a CST1-mediated positive feedback loop that propagates eosinophil recruitment, activation, and synthesis of eosinophil granular proteins in patients with ECRSwNP (see Fig E11 in this article’s Online Repository at www.jacionline.org).

Thus CST1 might be an effective biomarker for rapid detection of CRSwNP subtypes for several reasons. First, the threshold window is wide enough that the mRNA relative expression ratio of typical ECRSwNP to nonECRSwNP is as high as hundreds-fold.

Second, the absolute expression of CST1 is high in patients with ECRSwNP, which is as high as or even greater than the expression of housekeeping genes, such as GAPDH. The wide threshold window and the large amount of CST1 in patients with ECRSwNP make it easy for clinical examination.

Third, our study provides positive correlation of CST1 expression and eosinophil percentages, which could be the basis for the biomarker development. Together, these data indicate that CST1 participates in eosinophilia and that the cysteine protease inhibitor ability is required, particularly because tissue levels of CST1 were profoundly increased in tissue from patients with ECRSwNP and decreased in tissue from patients with nonECRSwNP. This difference in expression results from the different T3h type of inflammation. Epithelial cells are the major CST1-producing cell in patients with ECRSwNP, and CST1 expression is positively correlated with tissue eosinophil percentages and FENO levels. Overproduction of CST1 might lead to eosinophil granule protein synthesis and recruitment of eosinophils by IL-5. Collectively, these findings suggest that CST1 is a valuable biomarker for systemic CRSwNP and that targeting CST1 might be of therapeutic value in treating patients with T3h2-dominated CRSwNP.

Clinical implications: CST1 might be a valuable biomarker and a potential therapeutic target for management of CRSwNP.

REFERENCES

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