Clara Cell 10-kD Protein Suppresses Chitinase 3-Like 1 Expression Associated with Eosinophilic Chronic Rhinosinusitis

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Rationale: Clara cell 10-kD (CC10) protein, an antiinflammatory molecule, is involved in inflammatory upper airway diseases, but its regulatory role is unclear, particularly in the process of chronic rhinosinusitis (CRS).

Objectives: To investigate the regulatory mechanisms of CC10 in eosinophilic CRS (ECRS) using an allergic mouse model.

Methods: Homozygous CC10-knockout mice were used to establish an allergic ECRS model. Phenotypic changes were examined by histology, cytokine ELISA, and gene microarray analysis. Differential expression of chitinase 3-like 1 (CHI3L1) was verified by quantitative reverse transcriptase-polymerase chain reaction and immunohistochemistry. The functional role of CHI3L1 *in vivo* was assessed by the use of anti-CHI3L1 antibody in ECRS mice. CHI3L1 gene expression regulated by inflammatory cytokines and CC10 protein was performed using BEAS-2B cell line.

Measurements and Main Results: Compared with wild-type mice, a significantly greater extent of inflammatory cell infiltration and tissue remodeling was found in CC10-knockout ECRS mice, which was associated with significantly higher levels of various cytokines and eotaxin-1. CHI3L1 was up-regulated in ECRS mice with a significant further increase in CC10-knockout mice. Anti-CHI3L1 treatment markedly ameliorated eosinophilic inflammation. Furthermore, nasal mucosal CC10 gene transfer in CC10-knockout mice attenuated eosinophilic inflammation and suppressed the levels of CHI3L1. Moreover, significantly up-regulated expression of CHI3L1 was noted in human ECRS. IL-1 β , tumor necrosis factor- α , and IL-13 were found to up-regulate CHI3L1 expression in BEAS-2B cells, whereas CC10 inhibited such up-regulation.

Conclusions: These results suggest that CHI3L1 is a novel molecule involved in ECRS and that CC10 plays a regulatory role in ECRS, presumably by attenuating CHI3L1 expression.

Keywords: inflammation; tissue remodeling; mouse

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Clara cell 10-kD protein (CC10) possesses antiinflammatory and immunomodulatory effects. Down-regulated expression of CC10 is associated with inflammatory upper airway diseases, but a causal role for CC10 in upper airway diseases has not been determined.

What This Study Adds to the Field

Using an allergic murine model, we demonstrated that CC10 plays a regulatory role in eosinophilic chronic rhinosinusitis by attenuating expression of chitinase 3-like 1 protein, a molecule with proinflammatory effects in eosinophilic chronic rhinosinusitis.

Chronic rhinosinusitis (CRS) represents a major public health problem. It is a complex and heterogeneous syndrome in which the sinonasal mucosa is abnormally and persistently inflamed. According to its pathological characteristics, CRS is divided into two major subtypes: eosinophilic CRS (ECRS) and non-eosinophilic CRS. ECRS is often complicated with nasal polyps (NPs) (1, 2). Variable evidence has suggested that ostial patency impairment, mucociliary dysfunction, allergy, microorganism infection, and genetic factors are associated with CRS, but the etiology of CRS still remains largely unknown (1, 2). Recently, based on previously published work (3, 4), we developed a murine model of allergic ECRS (5), which mimics human ECRS well at the immunopathologic level and may allow us to study the regulatory mechanisms underlying the prolonged and exaggerated inflammatory processes in CRS (5, 6).

Clara cell 10-kD protein (CC10) is the founding member of the newly recognized secretoglobin superfamily. It is constitutively expressed by the epithelial lining of lung and nose (7, 8). CC10 possesses antiinflammatory and immunomodulatory effects. It can antagonize the activity of secretory phospholipase A2, diminish inflammatory cell chemotaxis, down-regulate Th2 cell differentiation, and block prostaglandin D2 receptor-mediated nuclear factor- κ B activation (9–12). Our previous studies have shown that CC10-knockout mice represent exaggerated Th2-dominated eosinophilic inflammation in lung in response to allergen, and that CC10 can directly suppress Th2 cytokine production (9, 10). In humans, reduced levels of CC10 have been correlated with lung diseases (13, 14). Recently, studies from us and others indicated that CC10 expression in sinonasal mucosa is also down-regulated in subjects with upper airway diseases, such as allergic rhinitis and CRS (7, 8, 15). However, the precise role of CC10 in upper airway diseases has

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not yet been investigated. Moreover, despite significant progress in characterizing the pathophysiological roles of CC10, the regulatory mechanisms remain ill defined.

The purposes of the present study were: (1) to examine whether CC10 has a regulatory role in the development of ECRS using an established allergic mouse model (5), and (2) to explore potential novel genes involved in CC10-mediated regulation of allergic upper airway inflammation. Our results demonstrate that CC10 can inhibit the inflammation associated with ECRS. Most importantly, by using gene microarray, it was discovered that chitinase 3-like 1 (CHI3L1) is up-regulated in ECRS, and such up-regulation is significantly more prominent in CC10-knockout mice. Neutralization of CHI3L1 can markedly ameliorate inflammation in ECRS. Using cultured BEAS-2B cell line, we further show that proinflammatory and Th2 cytokines can stimulate the expression of CHI3L1 and CC10 can suppress such induction. Moreover, increased expression of CHI3L1 is also noted in sinonasal samples from patients with ECRS. Therefore, we propose that CC10 can attenuate the development of ECRS, in part, by inhibiting the expression of CHI3L1.

METHODS

Animals and Patients

Wild-type C57BL/6 mice were purchased from Shanghai Experimental Animal Center (Shanghai, China). Homozygous CC10-knockout mice on C57BL/6 background were obtained from an intercross of heterozygous CC10-knockout mice (16) (kindly provided by Dr. A. B. Mukherjee, National Institutes of Health, Bethesda, MD), and germ-line transmission of the mutant CC10 allele was identified by PCR as described (9). All mice were used following protocols approved by the Animal Care and Use Committee of Tongji Medical College of Huazhong University of Science and Technology.

Eighteen patients with CRS without NPs and 30 patients with CRS with NPs (16 ECRS with NPs/14 noneosinophilic CRS with NPs) who had bilateral CRS and underwent endoscopic sinus surgery were recruited. Eighteen patients undergoing septoplasty because of anatomic variations and not having any sinus disease were enrolled as control subjects. Surgical samples were collected during surgery and processed for histology, ELISA, and reverse transcriptase-polymerase chain reaction (RT-PCR) study. More information is provided in the online supplement. All studies involving human subjects were approved by the Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology and conducted with written informed consent from patients.

ECRS Model, Nasal Lavage, Histology, and RNA Extraction

An allergic ECRS mouse model was established as previously described (5). Twenty-four hours after the last challenge (Day 103), nasal lavage fluid (NLF) was collected and the levels of interleukin IL-1 β , tumor necrosis factor (TNF)- α , interferon (INF)- γ , IL-5, IL-13, and eotaxin-1 in NLF were determined by ELISA as previously described (5). Sinonasal cavity structure was dissected, decalcified, embedded in paraffin, and sectioned as mentioned elsewhere (4, 5). Histological examination was performed according to the methods previously reported (4, 5). In some experiments, respiratory sinonasal mucosa was dissected under a microscope and total RNA was extracted by using an RNeasy kit (Qiagen, Valencia, Calif). Equal quantities of total RNA from five mice in the same study group were pooled together and subjected to microarray and quantitative RT-PCR analysis, and three independent experiments were done. In experiments to study the kinetics of CC10 expression after ovalbumin (OVA) challenge, five time points were studied, including Days 20, 34, 48, 76, and 104 (5). More information is provided in the online supplement.

Microarray Hybridization and Analysis

Briefly, 300 ng total RNA was amplified with Illumina RNA TotalPrep Amplification kit (Ambion, Austin, TX). During *in vitro* transcription, reaction cRNA was biotinylated. A hybridization mixture containing 1.5 mg biotinylated cRNA was hybridized to Sentrix mouse WG-6

BeadChips (Illumina, San Diego, CA). Hybridization was detected with 1 mg/ml cyanine3-streptavidin (Amersham Biosciences, Little Chalfont, UK) and the chips were scanned with Illumina BeadArray Reader (Illumina). Data analysis and absent-present call were performed on raw fluorescence intensity values with BeadStudio software (Illumina). Raw data were normalized and genes with detection precision value greater than 0.95 were filtered out. In comparison, genes with greater than twofold change and statistically significant difference between two groups were designated as genes differentially expressed.

In Vivo CHI3L1 Neutralization Experiment

Following the methods previously described (17), anti-CHI3L1 neutralizing antibody was raised through immunizing rabbit with keyhole limpet hemocyanin-conjugated mouse CHI3L1 peptide 325–339 amino acid residue (n-WVGYYDDQESVKS/NKVQ-c) using standard protocols and affinity purified (Aviva Antibody Corp., Beijing, China). The specificity and reactivity of anti-CHI3L1 was confirmed by Western blotting and ELISA. To define the role of CHI3L1, 1 mg of anti-CHI3L1 antibody or preimmune IgG control from same rabbits (Aviva Antibody Corp.) was administered intraperitoneally to wild-type ECRS mouse model twice a week starting the day before the first OVA challenge according to a previous report (17). More information is provided in the online supplement.

Gene Construct and Transfer

CC10 gene was constructed as previously described (10) (*see* online supplement). Mucosal gene transfer was given 3 days before the first OVA challenge and repeated every 7 days. The groups of mice received intranasal injection of 50 μ l pcDNA + lipofectamine (mock; 10 μ g plasmid/25 μ l phosphate-buffered saline [PBS] + 25 μ l lipofectamine) or pCC10 + lipofectamine (pCC10; 10 μ g plasmid/25 μ l PBS + 25 μ l of lipofectamine). The expression of transduced genes was confirmed as previously reported (10).

Cell Culture and Cytokine Stimulation

The BEAS-2B cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle medium/F-12 supplemented with 5% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37°C with 5% CO₂ in humidified air. When the cells reached 80 to 90% confluence, medium was changed to Dulbecco's modified Eagle medium/F-12 without serum, and the cells were stimulated with one or combination of the following: TNF- α , IL-1 β , INF- γ , IL-4, and IL-13 at 10 ng/ml (Peprotech, Placentia, CA). Before the stimulation, the cells were pretreated with or without 30 ng/ml human recombinant CC10 (R&D Systems, Minneapolis, MN) for 2 hours as previously described (10). Five hours after cytokine stimulation, cells were harvested. Total RNA was extracted with Trizol (Invitrogen, San Diego, CA) and then subjected to quantitative RT-PCR. Seven to 13 independent experiments were repeated.

Immunohistochemistry

Immunohistochemical staining was conducted using the streptavidin-peroxidase complex method as previously described (18). Rabbit antimouse CC10 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit antihuman and mouse CHI3L1 (1:200; Beijing Biosynthesis Biotechnology, Beijing, China) were used as primary antibodies. Color development was achieved with 3', 3'-diaminobenzidine, which rendered positive cells brown. Control isotype rabbit IgG was used as a negative control. The number of CC10 positive cells per millimeter of epithelium and the number of CHI3L1 positive cells per square millimeter of epithelium and lamina propria were counted as previously described (5, 7, 8).

CC10 ELISA

CC10 levels in tissue homogenates were determined by ELISA as previously described (8). Briefly, the human sinonasal tissues were homogenized and the supernatants were used to detect CC10 levels with a commercial kit according to the manufacturer's protocol (Bio Vendor Laboratory Medicine, Inc., Brno, Czech Republic) (*see* online supplement).

RT-PCR

CHI3L1 mRNA expression was detected by means of quantitative RT-PCR. cDNA was reverse transcribed as stated elsewhere (7, 19). By using the specific primer pairs described in Table E2 in the online supplement and SYBR Premix Ex Taq kit (TaKaRa Biotechnology, Dalian, China), cDNA equivalent to 40 ng total RNA was used to perform quantitative PCR as mentioned elsewhere (19). Relative gene expression was calculated by using the comparative CT method (7, 8, 19). Glyceraldehyde-3-phosphate dehydrogenase (for human and BEAS-2B cell line samples) and β -actin (for mouse samples) were used as housekeeping genes for normalization and a "no template" sample was used as a negative control.

The CC10 mRNA expression in nose was confirmed with conventional RT-PCR analysis. The total RNA (1 μ g) was reverse transcribed, and PCR was performed with 5 μ l of reverse transcription product using mouse CC10 and glyceraldehyde-3-phosphate dehydrogenase-specific primers (Table E2) as previously described (12).

Statistical Analysis

For continuous variables, results are expressed as mean $\pm$ SE. The Student *t* test was used in microarray and tissue culture data analysis. The Mann-Whitney *U* test was used for other paired sets of data. Differences in proportions between groups were tested by chi-square test. Statistical analysis was performed using SPSS statistical software for windows (SPSS Inc, Chicago, IL), and *P* < 0.05 was considered statistically significant.

RESULTS

CC10 Expression Is Down-Regulated during the Development of Mouse ECRS

With lung tissues as positive controls, we demonstrated CC10 mRNA and protein expression in mouse sinonasal mucosa (Figures 1A and 1B). Immunohistochemical staining showed that CC10 protein expression was located in epithelial cells

(Figure 1A). Our previous study showed that a mouse model with allergic ECRS developed a marked Th2 skewed chronic eosinophilic inflammation (5). In the present study, a significant time-dependent down-regulation of CC10 expression was found during the development of ECRS, as compared with control mice (Figures 1C and 1D).

Development of ECRS Is Augmented in CC10-Knockout Mice

In line with our previous findings about CC10's effect on lung inflammation after OVA challenge (9), after establishment of allergic ECRS, CC10-knockout mice displayed exaggerated chronic eosinophilic inflammation compared with wild-type mice. There was a markedly increased number of eosinophils (55.88% increase), mononuclear cells (54.31% increase), and total infiltrating cells (24.86% increase) in CC10-knockout mice in comparison with wild-type mice (Figure 2A). Most interestingly, we further found that CC10-knockout mice demonstrated significantly greater extent of goblet cell hyperplasia (34.98% increase), subepithelial fibrosis (53.09% increase), and epithelial thickening (59.74% increase) compared with wild-type mice (Figure 2A). In addition, there was a significantly higher level of TNF- α (42.47% increase), IL-1 β (64.43% increase), IL-5 (25.92% increase), IL-13 (39.13% increase), and eotaxin-1 (42.32% increase) in NLF in CC10-knockout ECRS mice than in wild-type mice; however, the level of INF- γ did not show significant difference between CC10-knockout and wild-type mice (Figure 2B).

Changes in Gene Expression in Sinonasal Mucosa from ECRS Mice

Differences in gene expression profiles in sinonasal mucosa between different study groups were compared by gene microarray analysis. The false discovery rate of microarray assay was

Figure 1. The expression of Clara cell 10-kD protein (CC10) in eosinophilic chronic rhinosinusitis (ECRS) mice. (A) Representative photomicrographs of CC10 immunohistochemical staining of normal murine nose and lung tissue sections. Original magnification $\times 400$. (B) CC10 mRNA expression in the normal murine nose and lung tissues by semiquantitative reverse transcriptase-polymerase chain reaction. (C) Representative photomicrographs of CC10 immunohistochemical staining of sinonasal tissue sections from ECRS mice at various time points. Original magnification $\times 400$. (D) The number of CC10-positive cells decreased gradually during the development of ECRS. **P* < 0.05 and ***P* < 0.01 for control mice (solid dots) challenged with saline compared with corresponding ECRS mice (open dots); *n* = 3 to 5 mice per group. GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

Figure 2. An exaggerated inflammation in Clara cell 10-kD protein (CC10)-knockout mice compared with wild-type mice after establishment of eosinophilic chronic rhinosinusitis (ECRS). (A) The analysis of the number of eosinophils, mononuclear cells, total inflammatory cells, and goblet cells, and the extent of fibrosis and epithelial thickening in sinonasal mucosa. (B) Cytokine and chemokine levels in nasal lavage fluid assessed by ELISA. $^{\#}P < 0.05$ and $^{##}P < 0.01$ for ECRS mice compared with corresponding control mice challenged with saline. $^{\$}P < 0.05$ and $^{$$$}P < 0.01$ for CC10-knockout ECRS mice compared with wild-type ECRS mice; $n = 3$ to 5 mice per group. Open columns = control mice; solid columns = ECRS mice. INF = interferon; TNF = tumor necrosis factor.

less than 5%. First, we compared the difference between OVA-challenged wild-type and saline-challenged wild-type mice, which allowed us to find the novel genes involved in the pathophysiological processes of ECRS. We identified 259 up-regulated and 51 down-regulated genes in ECRS mice (those with fold change greater than or equal to four are listed in Table E3). Second, for the purpose of identification of genes involved in the CC10-modulated chronic eosinophilic sinonasal inflammation, we compared the difference between wild-type and CC10-knockout mice after establishment of ECRS and identified 152 up-regulated and 27 down-regulated genes in CC10-knockout mice (those with fold change greater than or equal to four are listed in Table E4). Finally, we picked out genes identified in both comparisons, which were believed to be important not only in the development of ECRS but also in CC10-modulated responses in ECRS. Using this method, we identified 133 up-regulated and 25 down-regulated genes (those with fold change greater than or equal to four for both comparisons are listed in Table E5). Some of these genes are involved in immune system processes, whereas others are involved in various metabolic pathways, enzyme regulation, signal transduction, biological adhesion, and other functions.

CHI3L1 Gene Expression Is Augmented in CC10-Knockout ECRS Mice

Gene array analysis identified CHI3L1 as one of the top four up-regulated genes involved both in the pathological processes of ECRS (wild-type ECRS vs. wild-type control mice: 4.87-fold increase) and CC10-modulated pathway (CC10-knockout ECRS vs. wild-type ECRS mice: 5.98-fold increase). Recently, very limited but interesting studies have underscored a novel and important role for chitinases and chitinase-like proteins in

airway diseases (20–27). Nevertheless, the mechanistic function of CHI3L1 in airway diseases remains largely unexplored. Therefore, we selected this gene for further study. Quantitative RT-PCR and immunohistochemistry analysis confirmed that mRNA and protein expression levels of CHI3L1 were significantly increased in ECRS mice, with an even greater increase in CC10-knockout ECRS mice (Figure 3). Slightly different from the results obtained from gene array assay, quantitative RT-PCR showed that CHI3L1 mRNA expression was increased by 7.82-fold in wild-type ECRS mice as compared with wild-type control mice, and by 1.84-fold in CC10-knockout ECRS mice as compared with wild-type ECRS mice. Immunohistochemical staining showed the CHI3L1 protein was mainly expressed by epithelial cells and infiltrating inflammatory cells (Figure 3C).

Anti-CHI3L1 Treatment Inhibits the Chronic Eosinophilic Inflammation in Sinonasal Mucosa

Compared with wild-type ECRS mice treated with preimmune IgG control, wild-type ECRS mice treated with anti-CHI3L1 antibody displayed fewer eosinophils (40.37% decrease) and mononuclear cells (41.53% decrease) and less overall inflammatory cell infiltration (23.30% decrease) (Figure 4A). Moreover, goblet cell hyperplasia (43.82% decrease), subepithelial fibrosis (30.25% decrease), and epithelial thickening (32.47% decrease) were also significantly inhibited in anti-CHI3L1-treated ECRS mice (Figure 4A). With respect to cytokine expression, anti-CHI3L1 treatment was found to markedly diminish the levels of TNF- α (36.44% decrease) and eotaxin-1 (42.98% decrease) in NLF (Figure 4B), whereas no significant measurable effect on the expression of IL-1 β , INF- γ , IL-5, and IL-13 was found (Figure 4B).

Figure 3. Increased chitinase 3-like 1 protein (CHI3L1) expression in eosinophilic chronic rhinosinusitis (ECRS) mice. (A) CHI3L1 mRNA expression levels in mice. (B) The number of CHI3L1-positive cells in mice. Open columns = control mice; solid columns = ECRS mice. (C) Representative photomicrographs of CHI3L1 immunohistochemical staining of sinonasal tissue sections from (a) wild-type mice challenged with saline, (b) wild-type ECRS mice, (c) Clara cell 10-kD protein (CC10)-knockout mice challenged with saline, and (d) CC10-knockout ECRS mice. Original magnification $\times 400$. Arrows, CHI3L1-positive infiltrating cells. $^{\#}P < 0.05$ and $^{##}P < 0.01$ for ECRS mice compared with corresponding control mice challenged with saline. $^{\$}P < 0.05$ and $^{$$}P < 0.01$ for CC10-knockout ECRS mice compared with wild-type ECRS mice. $n = 3$ to 5 mice per group.

CC10 Gene Mucosal Transfer Can Inhibit the Development of ECRS and Up-Regulation of CHI3L1 in CC10-Knockout Mice

Next, CC10's effect on the Th2-associated eosinophilic inflammation and on the CHI3L1 expression in ECRS was further studied by sinonasal mucosal gene transfer in CC10-knockout mice. The kinetic analysis of CC10 expression after CC10 gene reconstitution demonstrated detectable CC10 mRNA expression in sinonasal mucosa 1 day after gene transfer and robust expression 3 days after gene transfer, and the elevated expression continued for at least 4 days (Figure E2). Immunohistochemistry study showed significant expression of CC10 in the epithelium of pCC10-transduced mice 3 days after gene transfer and the expression continued for about 4 days; no detectable staining was found in mock-transduced mice (data not shown), which is consistent with our previous report (10). In line with our previous study (10), compared with mice treated with mock controls, mice receiving CC10 gene transfer demonstrated a significantly lower degree of inflammatory cell infiltration (30.02% decrease for eosinophil, 26.92% decrease for mononuclear cells, and 19.91% decrease for total infiltrating cells) and tissue remodeling (21.38% decrease for goblet cell hyperplasia, 22.37% decrease for subepithelial fibrosis, and 34.59% decrease for epithelium thickness), and lower levels of cytokines (37.08% decrease for IL-1 β , 30.69% decrease for TNF- α , 31.62% decrease for IL-5, 36.51% decrease for IL-13, and 46.66% decrease for eotaxin-1) in NLF, except for INF- γ (Figure 5). Moreover, the relative expression levels of CHI3L1 mRNA were significantly reduced in CC10 gene-transferred mice compared with mock plasmid-transferred mice (pCC10-transferred ECRS mice vs. mock-transferred mice: 1.77-fold decrease, $P < 0.01$).

CC10 Can Suppress Cytokine-Induced CHI3L1 Expression in Airway Epithelial Cells

Because epithelial cells are the major sources of CHI3L1 in sinonasal mucosa, we used BEAS-2B cell line, a human bron-

chial epithelial cell line, to study the CHI3L1 gene expression regulation by cytokines. IL-1 β , IL-13, and TNF- α were found to significantly induce CHI3L1 mRNA expression in BEAS-2B cells (Figure 6A). No significant effect was observed for IL-4 and INF- γ (Figure 6A). CC10 pretreatment can markedly inhibit IL-13- and TNF- α -induced CHI3L1 expression (Figure 6A). To investigate whether there is a synergistic effect between proinflammatory and Th1 and Th2 cytokines, BEAS-2B cells were stimulated with TNF- α combined with either IL-4, IL-13, or INF- γ . We found that CHI3L1 mRNA expression can be synergistically induced by TNF- α with IL-13 or IL-4, and such effect was more prominent for TNF- α combined with IL-13 (Figure 6B). The CHI3L1 expression induced by combination of cytokines can also be significantly inhibited by CC10 treatment (Figure 6B).

CHI3L1 Expression Is Up-Regulated in Human ECRS with NPs

Because positive CHI3L1 mRNA expression was only found in some subjects, the result of mRNA expression was expressed as proportion of subjects having positive expression. We found that the mRNA expression rate and protein expression levels of CHI3L1 in sinonasal mucosa were significantly increased in patients with CRS with NPs, but not in patients with CRS without NPs, who have predominant noneosinophilic inflammation and a Th1 milieu (1, 2, 28), compared with control subjects (Figure 7). Because our recently published data showed that in contrast to white patients, about 50% of Chinese patients with CRS with NPs display noneosinophilic inflammation (28), we further divided patients with CRS with NPs into eosinophilic and noneosinophilic subgroups and found that CHI3L1 expression was only significantly increased in ECRS with NPs with Th2-dominated inflammation, but not in patients with noneosinophilic CRS with NPs as compared with control subjects (Figure 7). Immunohistochemical staining further revealed that CHI3L1 was mainly expressed by epithelial cells and infiltrating cells in sinonasal mucosa (Figure 7C). In addition, in line with

Figure 4. Anti-chitinase 3-like 1 protein (CHI3L1) antibody treatment ameliorates the inflammation in sinonasal mucosa. (A) The analysis of the number of eosinophils, mononuclear cells, total inflammatory cells, and goblet cells, and the extent of fibrosis and epithelial thickening in sinonasal mucosa. (B) Cytokine and chemokine levels in nasal lavage fluid assessed by ELISA. [#] $P < 0.05$ and ^{##} $P < 0.01$ for wild-type eosinophilic chronic rhinosinusitis (ECRS) mice compared with corresponding control mice challenged with saline. ^{\$} $P < 0.05$ and ^{\$\$} $P < 0.01$ for wild-type ECRS mice treated with anti-CHI3L1 compared with wild-type ECRS mice treated with preimmune rabbit IgG. $n = 3$ to 5 mice per group. Open columns = control mice; solid columns = ECRS mice. INF = interferon; TNF = tumor necrosis factor.

our previous findings (8), CC10 expression was found to be down-regulated in patients with CRS with and without NPs compared with normal control subjects (Figure 7D). CC10 levels were much lower in patients with ECRS with NPs than in patients with CRS without NPs and patients with non-eosinophilic CRS with NPs (Figure 7D).

DISCUSSION

Patients with CRS who do not respond to antibiotic and surgery therapy often have an inflammatory infiltrate characterized by Th2-type lymphocytes and eosinophils, which has more resemblance to asthmatic inflammation (1, 2). In this study, to explore the role of CC10 in the inflammatory responses in ECRS, we used an allergic ECRS mouse model established through prolonged repetitive 12-week challenge with OVA (5). In this model, an apparent Th2-skewed eosinophilic inflammation with tissue remodeling was observed not only in nasal cavity mucosa but also in sinus mucosa (4, 5, 29), which closely approximates that of human ECRS (1, 2, 28), except that no NPs formation was found (4, 5). Therefore, this model provides us with a useful tool to study the cellular and immunological changes in ECRS on a functional basis. In line with our findings in humans (7, 8), this study demonstrated that CC10 was also expressed by murine upper airway epithelial cells in addition to lung epithelial cells. During the development of ECRS, gradually down-regulated CC10 expression was found, which confirms the involvement of CC10 in CRS. To examine whether CC10 has a regulatory role during the development of ECRS, we used CC10-knockout mice and CC10 gene transfer approach to examine the effect of CC10 on the development of ECRS. Consistent with our previous studies of lung inflammation (9, 10), a similar exaggerated eosinophilic Th2-skewed reaction was

found in sinonasal mucosa in CC10-knockout mice after establishment of ECRS, which could be suppressed by CC10 reconstitution. Besides its previously demonstrated effect on infiltration of inflammatory cells and on cytokine and chemokine expression, we further showed here that CC10 deficiency could lead to a much more profound tissue remodeling, which is an essential feature of chronic airway inflammation. These results indicate an inhibitory role of CC10 in the inflammatory reactions in ECRS.

DNA microarray analysis is one of the most powerful approaches for the potential identification of unexpected genes involved in pathogenic processes. By using this approach, CHI3L1 was found to be one of the most up-regulated genes potentially involved in the pathogenesis of ECRS and associated with CC10's regulatory pathway. CHI3L1 belongs to chitinase and chitinase-like protein family (20, 21). Recent studies in human subjects and rodents have identified several chitinases with true enzyme activity to chitin and chitinase-like proteins without enzyme activity but having strong binding affinity to chitin (20, 21). Although a number of studies have suggested a role of chitinases and chitinase-like proteins in inflammation and tissue remodeling (17, 22), only few recent studies demonstrated the involvement of chitinases and chitinase-like proteins in inflammatory lower airway diseases (23–27). CHI3L1 levels were found to be increased in serum and lungs in patients with asthma and were correlated with severity of asthma (23, 24). Smokers with chronic obstructive pulmonary disease were demonstrated to have elevated CHI3L1 levels in serum and bronchoalveolar lavage fluids (26). Despite these findings, the mechanistic function of CHI3L1 in airway inflammatory processes has remained largely unexplored. During the period of time when our article was under review, Lee and colleagues published their study exploring the role of CHI3L1

Figure 5. Clara cell 10-kD protein (CC10) gene reconstitution inhibits the inflammation in CC10-knockout eosinophilic chronic rhinosinusitis (ECRS) mice. (A) The analysis of the number of eosinophils, mononuclear cells, total inflammatory cells, and goblet cells, and the extent of fibrosis and epithelial thickening in sinonasal mucosa. (B) Cytokine and chemokine levels in nasal lavage fluid assessed by ELISA. [#] $P < 0.05$ and ^{##} $P < 0.01$ for ECRS mice compared with corresponding control mice challenged with saline. $P < 0.05$ and $$$$P < 0.01$ for CC10-knockout ECRS mice with mock plasmid transfer compared with CC10-knockout ECRS mice with pCC10 transfer. $n = 3$ to 5 mice per group. Open columns = control mice; solid columns = ECRS mice.

in allergic lower airway disease by using CHI3L1 knockout and transgenic mice (27). They found that CHI3L1 can promote Th2 responses possibly through inducing dendritic cell accumulation and alternative macrophage activation (27). In the present study, for the first time, we found that anti-CHI3L1 treatment can markedly inhibit the chronic eosinophilic inflammation and tissue remodeling in sinonasal mucosa, suggesting a proinflammatory effect of CHI3L1 in ECRS. Similar to acidic mammalian chitinase (25), anti-CHI3L1 did not alter the induction of Th2 cytokine, but did diminish the expression of eotaxin, suggesting that CHI3L1 does not play a role in Th2 cytokine production but rather mediates the effector responses of Th2 cytokines.

However, Lee and colleagues found that CHI3L1-knockout mice demonstrated elevated levels of Th2 cytokines in lung (27). The reasons contributing to the discrepancy between our results and those of Lee and colleagues are not clear, but may relate to the different models and techniques used. Moreover, we found that CHI3L1 can modulate the expression of proinflammatory cytokine TNF-α. The reduced levels of eotaxin after anti-CHI3L1 treatment may contribute to the decreased infiltration of eosinophils. In our study, besides eosinophils, a significant inhibition of mononuclear cell infiltration was also noted after treatment with anti-CHI3L1; therefore, whether there is a change of expression of chemokines for mononuclear

Figure 6. Clara cell 10-kD protein (CC10) protein inhibits cytokine-induced chitinase 3-like 1 protein (CHI3L1) mRNA expression in BEAS-2B cells. (A) CC10 inhibits CHI3L1 production induced by individual cytokine. ^{*} $P < 0.05$ compared with medium controls, [#] $P < 0.05$ compared with the corresponding condition

with CC10 treatment. Gray columns = with CC10; solid columns = without CC10. (B) CC10 inhibits CHI3L1 production induced by TNF-α alone or TNF-α combined with Th1 or Th2 cytokines. ^{*} $P < 0.05$ compared with the condition with TNF-α alone, [#] $P < 0.05$ compared with the corresponding condition with CC10 treatment. $n = 7$ to 13. Solid columns = medium; light gray columns = TNF-α; dark gray columns = TNF-α + CC10.

Figure 7. Increased chitinase 3-like 1 protein (CHI3L1) expression in human eosinophilic chronic rhinosinusitis (ECRS). (A) CHI3L1 mRNA expression rate in control subjects and patients with CRS. (B) The number of CHI3L1-positive cells in control subjects and patients with CRS. (C) Representative photomicrographs of CHI3L1 immunohistochemical staining of sinonasal tissue sections from (a) control, (b) CRS without NPs, (c) noneosinophilic CRS with NPs, and (d) ECRS with NPs. No obvious positive staining in a and b. Original magnification $\times 400$. Arrows, CHI3L1-positive infiltrating cells. (D) Clara cell 10-kD protein (CC10) expression levels in control subjects and patients with CRS. * $P < 0.05$ and ** $P < 0.01$.

cells is an area for further investigation. Moreover, although our present study suggests a role of CHI3L1 in upper airway tissue remodeling, the underlying mechanisms obviously remain to be determined.

In the present study, CC10-knockout ECRS mice displayed a higher degree of CHI3L1 expression up-regulation compared with wild-type ECRS mice, with reconstitution of CC10 expression in CC10-knockout mice being able to markedly suppress the increased CHI3L1 expression, indicating that CC10 may modulate CHI3L1 expression in its regulatory processes in airway inflammation. Because CC10 can affect the levels of proinflammatory and Th2 cytokines in ECRS and immunohistochemical staining demonstrated that epithelial cells are a main resource of CHI3L1 expression in sinonasal mucosa, we first studied the effect of proinflammatory, Th1, and Th2 cytokines on CHI3L1 expression in a human airway epithelial cell line. We found that IL-1 β , TNF- α , and IL-13 could up-regulate the CHI3L1 mRNA expression in airway epithelial cells and TNF- α could work synergistically with IL-13 or IL-4, suggesting that the elevated CHI3L1 expression in ECRS may result, at least in part, from increased expression of proinflammatory and Th2 cytokines and that CC10 can modulate CHI3L1 expression indirectly through regulating proinflammatory and Th2 cytokine expression. In our study, INF- γ had no significant effect on CHI3L1 expression in airway epithelial cells. Nevertheless, Lee and colleagues reported that CHI3L1 mRNA levels in lung can be induced after adoptive transfer of *in vitro*-polarized, antigen-specific Th1 cells (27), which may result from the modulation on alveolar macrophages, another important resource of CHI3L1 production in lung besides epithelium (26, 27). Despite the fact that IL-4 and IL-13 can usually signal through the common type 2 IL-4 receptor, our current study demonstrated distinct effects of IL-4 and IL-13 on CHI3L1 expression. Different type 2 IL-4 receptor affinities and the coexistence of other unique receptors for IL-4 or IL-13 may contribute to the difference between IL-4 and IL-13 (30). Furthermore, we found that CC10

was able to significantly suppress cytokine-induced CHI3L1 expression in airway epithelial cells, indicating a direct link between CC10 and CHI3L1. However, the precise mechanism awaits further investigation. Therefore, our study indicates that CC10 can inhibit CHI3L1 expression directly or indirectly in ECRS.

To investigate the involvement of CHI3L1 in human CRS, we detected CHI3L1 expression in human sinonasal mucosa. Consistent with observations in the murine model, we found that CHI3L1 expression was only markedly increased in human ECRS with NPs. Although our current and previous studies demonstrated CC10 down-regulation in both eosinophilic and noneosinophilic human CRS, only ECRS demonstrated increased CHI3L1 expression. Several reasons may explain this discrepancy. First, CC10 levels were down-regulated more greatly in ECRS with NPs. Second, exaggerated Th2 response was only found in ECRS with NPs (28). Third, other factors may also participate in the regulation of CHI3L1 expression, which is supported by the fact that CHI3L1 was only modestly induced in CC10-knockout mice. On the other hand, the absence of enhanced CHI3L1 expression in noneosinophilic CRS suggests that other factors may be involved in the CC10-mediated process in noneosinophilic CRS, which remains to be defined. We previously found that Th1 cytokines can promote, whereas Th2 cytokines can suppress, CC10 expression in sinonasal mucosa (8). Therefore, the feedback loop between CC10-, CHI3L1-, and Th2-dominated eosinophilic inflammation may contribute to the sustained inflammation in ECRS.

It should be noted that our ECRS mouse model has some inherent limitations. First, mice do not have true sinuses like human beings (4, 5). Second, the etiology of human ECRS might be heterogeneous and allergy might not be the only or important cause for human ECRS (1, 2, 6). Third, no polyp formation was found in our model (4, 5).

In summary, our current study indicates that CC10 and CHI3L1 may possess a regulatory function in the development

of ECRS. CHI3L1 may be a novel molecule in CC10-modulated eosinophilic airway responses.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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