Expression of Chloride Channel Protein CLC-3 in Patients With Allergic Rhinitis

Effect of Topical Corticosteroid Treatment

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Objectives: To determine whether the chloride channel protein CLC-3 is upregulated in patients with allergic rhinitis (AR) and whether topical corticosteroid treatment decreases the expression of CLC-3.

Design: Histologic study.

Setting: Academic research.

Patients: Eighteen patients with AR were included in the study.

Main Outcome Measures: Expression of CLC-3 was detected by immunohistochemistry and by reverse transcription–polymerase chain reaction before and at 4 weeks after treatment with the topical corticosteroid mometasone furoate.

Results: Strong CLC-3 expression was detected in epithelium and in submucosal glands. An increased presence of CLC-3 was demonstrated in nasal mucosa compared with that in normal nasal tissue. A statistically significant difference in CLC-3 gene expression level was found in nasal tissues before vs at 4 weeks after treatment with mometasone.

Conclusions: CLC-3 may have a role in modulating the pathogenesis of AR. Decreased expression of CLC-3 may be the mechanism of action of corticosteroid treatment in controlling AR.


Allergic rhinitis (AR) represents a global health problem. It is a common disease worldwide, affecting 10% to 25% of the population. Allergic rhinitis arises as a result of inflammation induced by an IgE-mediated immune response. It involves the release of inflammatory mediators and the activation and recruitment of cells to nasal mucosa. However, the mechanisms underlying this disease are not fully understood.

There is increasing evidence to suggest that ion channels play an important role in the development of AR. The proper homeostasis of liquid that lines the surface of the respiratory tract is vital to mucociliary clearance, which has an important role in host defense. Regulation of sodium absorption and chloride secretion may contribute to the development of AR and nasal polyps.

It has been reported that the chloride channel proteins CLC-2 and CLC-3 are expressed in rat lung by Northern blot analysis. CLC-3 was recently detected in submucosal gland cells of nasal tissue, but this has not been fully investigated, to our knowledge. The objectives of the present study were to detect the upregulation of CLC-3 in patients with AR and to evaluate the effect of topical corticosteroid treatment on CLC-3 expression. These findings may provide insight into the pathogenesis of AR and the effects of intranasal corticosteroid use on AR.

Methods

This study was approved by the local ethics committee, and informed consent was obtained from all subjects. Eighteen patients (10 women and 8 men, with a mean age of 31.4 years [age range, 24-45 years]) participated in this study. A full ear, nose, and throat examination was performed, and a skin prick test (SPT) was administered using a panel of common aeroallergens. Allergic rhinitis was identified based on findings of the patient history, physical examination, and SPT. All patients included in this study had a typical history of AR (sneezing, rhinorrhea, nasal obstruction, and itching), and a positive SPT result against house dust mites. Patients with marked septum deviation, prior nasal surgery, nasal polyps, or symptoms of infection were excluded.

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All medications (corticosteroids, antihistamines, and antibiotics) were withheld for a minimum of 6 weeks before the study. After this run-in period, initial nasal mucosal biopsy specimens were obtained from the inferior turbinate just beyond the anterior tip under local anesthesia. Subjects were instructed to use 50 µg of mometasone furoate aqueous nasal spray (Nasonex; Schering-Plough, Brussels, Belgium) per nostril once daily for 4 weeks, and biopsy samples were then obtained again. Nasal mucosal biopsy specimens obtained from the inferior turbinate of 9 healthy control subjects served as the normal control tissues for analysis. The controls were symptom free, had no history of AR or any other atopic disease, and had negative SPT results against common allergens.

Each specimen was cut into 2 portions. One portion was immediately frozen in liquid nitrogen and stored at −80°C for reverse transcription–polymerase chain reaction (RT-PCR). The other portion was fixed with 4% paraformaldehyde and then embedded in paraffin for immunohistochemical staining.

**IMMUNOHISTOCHEMICAL STAINING**

For immunohistochemical staining, blocks of formalin-fixed, paraffin-embedded tissues were sectionally cut into 4-µm-thick slices, collected on gelatin-coated slides, and dried for 15 minutes at 37°C. Immunostaining was performed using the avidin-biotin-peroxidase method. Briefly, sections were deparaffinized by xylene treatment. After blocking the endogenous peroxidase in 3% hydrogen peroxide and with 1% bovine serum albumin, the sections were incubated overnight at 4°C in the presence of a monoclonal rabbit antihuman CLC-3 antibody (Alomone Laboratories, Jerusalem, Israel) at a dilution of 1:200 according to the manufacturer’s instructions. Each of these sections was incubated with a secondary antibody (biotinylated goat antirabbit IgG) and then with horseradish peroxidase–labeled streptavidin complex (Zhongshan, Beijing, China). Distribution of peroxidase was revealed by incubating the sections in a solution containing 3% 3,3-diaminobenzidine tetrahydrochloride before being counterstained with hematoxylin-eosin and coverslipped. Negative control studies were performed by omitting the incubation step with the primary antibody.

Histochemical analyses were performed by 2 blinded investigators. The staining intensities of 5 randomly selected areas (×400 magnification) on each sample were summed and averaged. The staining intensity was semiquantitatively scored as 0 (no staining), 1 (weak staining), 2 (moderate staining), or 3 (strong staining).

**RESULTS**

**IMMUNOHISTOCHEMICAL STAINING OF CLC-3**

Immunohistochemical staining of the allergic nasal mucosa revealed strong staining of CLC-3 in all subjects with AR. By contrast, CLC-3 was weakly expressed in normal mucosa. CLC-3 was localized mainly in the cytoplasm of epithelial and submucosal nasal glandular cells. Furthermore, it partially localized in vessel endothelial cells and inflammatory cells (Figure 1A and B). In nasal mucosa obtained after 4 weeks of treatment with the topical corticosteroid mometasone, immunohistochemistry showed a continued but weaker presence of CLC-3 in epithelium and stroma (Figure 1C).

A statistically significant difference in staining intensity was seen between untreated AR (2.37 [0.41]) and control (0.79 [0.22]) samples (P < .05). A statistically significant difference was also seen between untreated (2.37 [0.41]) and corticosteroid-treated (1.54 [0.56]) samples (P < .05) (Figure 2).

**REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION**

Total RNA was extracted from 100 mg of frozen nasal mucosa using Trizol reagent (Invitrogen, Carlsbad, California) according to the manufacturer’s instructions. Reverse transcription was performed using 2 µg of total RNA from each sample in a first-strand complementary DNA (cDNA) synthesis reaction with reverse transcriptase (M-MLV; Promega, Madison, Wisconsin), and PCR was performed by adding 10 µL of the reverse transcription product to 50 µL of the total volume reaction containing 5 µL of 10× buffer, 2 µL of a solution of 5-mol/L magnesium chloride, 2 µL of deoxyribonucleotide triphosphate, 20 pmol of each oligonucleotide primer, and 0.4 U of Taq polymerase (Promega). Oligonucleotide primer synthesis was synthesized on the basis of the entire coding region of the human CLC-3 gene (GenBank X78520) and the inner control G3PDH gene (GenBank NM_002046). The sequences of the primer were as follows: G3PDH forward 5′-GGA GGT GAA GGT CGG AGT-C-3′ and reverse 5′-GAT GGT GTG GGG ATT TC-3′; CLC-3 forward 5′-GGG CAT TGG CCG CAT TAA TAG ACA-3′ and reverse 5′-GTG CAC CAA AAG CTA CAG AAG CCC-3′. The expected size was 225 base pair (bp) for G3PDH and 567 bp for CLC-3.

The PCR (38 cycles) was performed in a thermal cycler (Applied Biosystems, Foster City, California). Each cycle consisted of incubations at 94°C (1 minute) for denaturation, at 60°C (2 minutes) for annealing, and at 72°C (1 minute) for extension. The final extension was 12 minutes at 72°C. Amplified PCR products (10 µL) were detected by means of DNA gel electrophoresis in 2% agarose visualized by ethidium bromide staining and quantified by videodensitometry using a UV transilluminator. The level of CLC-3 gene expression was quantified by calculating the ratio of densitometric readings of the band intensities for CLC-3 and G3PDH from the same cDNA sample.

**STATISTICAL ANALYSIS**

Data are expressed as mean (SEM). Statistical significance of differences was analyzed using the Mann-Whitney test. P < .05 was considered statistically significant.
The present study was designed to evaluate the expression of CLC-3 mRNA in AR and normal nasal mucosa. To our knowledge, this is the first published study about the role of CLC-3 in the pathogenesis of AR. Our results demonstrate that CLC-3 was localized mainly in the cytoplasm of epithelial and submucosal nasal glandular cells. Statistically significant upregulation of CLC-3 was detected in all of the subjects with AR.

Under normal conditions, the nasal mucosa humidifies and cleans inspired air by means of water and mucin lining the airway surfaces, which may accomplish normal mucociliary clearance for host defense. In AR, these mechanisms go awry and contribute to the clinical signs and symptoms, including sneezing, rhinorrhea, and nasal congestion. Excessive secretion can occur in nasal mucosa during inflammation or after allergen challenge. Histologic studies of secretory liquid reveal that it is composed mainly of water, as well as mucin, lysozyme, lactoferrin, secretory IgA, and other elements. It is produced primarily by submucosal glands and epithelial goblet cells. Transepithelial transport of water depends on the osmotic gradients of 2 sides of the epithelial layers. Previous data revealed that ion transport by epithelium regulates the volume and composition of liquids. Until now, there has been little speculation about the role of liquid imbalance in the pathogenesis of AR.

Aquaporin plays a key role in water transport in the skin and respiratory tract. The role of cystic fibrosis transmembrane conductance regulator in bronchial epithelium has been described in the development of cystic fibrosis. Bernstein and Yankaskas suggest that mediators of inflammation in nasal polyps may have an
expression of CLC-3. Our results confirmed these hypotheses.

Whether the use of topical corticosteroids decreases the expression of nasal inflammation.

Transport by nasal epithelium causes the development of nasal polyps. Taken together, it can be deduced that ion transport by nasal epithelium causes the development of nasal inflammation.

In the present study, our objectives were to evaluate whether CLC-3 is upregulated in patients with AR and whether the use of topical corticosteroids decreases the expression of CLC-3. Our results confirmed these hypotheses. There are 9 different chloride channel proteins in mammals. CLC-1, CLC-2, and 2 CLC-K chloride channels reside in plasma membranes, whereas CLC-3, CLC-4, CLC-5, CLC-6, and CLC-7 are thought to reside predominantly in membranes of intracellular organelles. Oshima et al report that CLC-3 is expressed in submucosal nasal glands in chronic sinusitis, but this has not been fully investigated, to our knowledge.

A previous study demonstrated that CLC-3 is induced in nasal epithelial cells by interleukin 4 (IL-4), a major type 2 cytokine. Consistent with that, we showed herein that CLC-3 is increased in nasal mucosa with AR and that topical corticosteroid treatment decreases the expression of CLC-3 and mRNA based on immunohistochemistry and RT-PCR. These findings may enhance our understanding of the action of topical corticosteroids in allergic disorders. It has been known for some time that AR is amenable to treatment with intranasal corticosteroids. Studies demonstrated the efficacy of topical corticosteroids in the alleviation of clinical symptoms of AR, as well as the suppression of inflammatory mediators. Recently, topical corticosteroid treatment has been recommended as first-line therapy of AR in the Allergic Rhinitis and Its Impact on Asthma guidelines. Corticosteroid treatment has shown extensive anti-inflammatory action, including decreases in the local expression of IL-4 and IL-5, as well as tissue eosinophilia and tissue remodeling.

To our knowledge, the pathogenesis of AR and the mechanism of action of corticosteroids have not been elucidated until now. Corticosteroids may act on multiple levels in the reduction of CLC-3 expression. The reduction of CLC-3 levels after corticosteroid treatment may be a direct or an indirect mechanism. For example, intranasal corticosteroid administration may inhibit the production of IL-4 in inflammatory cells. Our finding that treatment with topical corticosteroids results in decreased CLC-3 expression sheds further light on the effects of corticosteroids in controlling AR. This improved understanding of the expression of CLC-3 in AR and of the decrease in CLC-3 by topical corticosteroids adds insight into the pathogenesis of this disease and into the effects of intranasal corticosteroid treatment on AR.

We demonstrated that CLC-3 expression was detected in nasal epithelium and in submucosal glands in patients with AR. The expression of CLC-3 and mRNA is up-regulated in AR and decreases at 4 weeks after treatment with mometasone. The finding that CLC-3 may have a role in modulating the pathogenesis of AR and in decreasing the expression of CLC-3 in patients with AR by topical corticosteroid administration may have potential therapeutic implications. Therefore, insight is gained into the pathogenesis of this disease and the effects of intranasal corticosteroid treatment on AR.

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Author Contributions: Drs Li and Wen contributed equally to this work, Dr Xu had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Li and Xu. Acquisition of data: Wen. Drafting of the manuscript: Li and Wen. Critical revision of the manuscript for important intellectual content: Xu. Statistical analysis: Li. Administrative, technical, and material support: Wen. Study supervision: Xu.

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REFERENCES


