Up-regulation of Chemokine Ligand 20 in Chronic Rhinosinusitis

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Objectives: To investigate the up-regulation of chemokine ligand 20 (CCL20) in chronic rhinosinusitis mucosa and to localize the distribution of CCL20 in the human paranasal sinus mucosa.

Design: Prospective study.

Setting: Tertiary academic institution.

Patients: Ten patients who underwent functional endoscopic sinus surgery for chronic rhinosinusitis without nasal polyps and 10 normal control subjects.

Interventions: Messenger RNA was extracted from the sinus mucosa, and semiquantitative reverse transcriptase–polymerase chain reaction was performed. Immunohistochemical staining was used to localize the CCL20 protein.

Results: The expression levels of CCL20 messenger RNA level in chronic rhinosinusitis without nasal polyps were significantly increased compared with those in normal sinus mucosa. The expression of CCL20 protein was greater in chronic rhinosinusitis without nasal polyps mucosa and was localized to the epithelial and submucosal glandular cells.

Conclusion: CCL20 is an inducible product of human paranasal sinus epithelium that may play a role in modulating mucosal immunity of the sinus mucosa.

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C HEMOKINES ARE CRUCIAL for the multistage process that involves recruitment of immature dendritic cells into the airway mucosa and for the ensuing maturation and migration of these dendritic cells to regional lymph nodes.1 Chemokine ligand 20 (CCL20) acts as a unique functional ligand for the chemokine receptor CCR6, which is selectively expressed on immature dendritic cells at mucosal surfaces.2 Thus, it is possible that CCL20 functions as an important cytokine for the recruitment of CCR6-expressing immature dendritic cells for subsequent antigen presentation.3

The expression of CCL20 has been shown to be up-regulated in response to inflammatory stimuli such as exposure to proinflammatory cytokines (eg, tumor necrosis factor α and interleukin 1β), ambient particulate matter, colonic inflammation, and lipopolysaccharides.3-5 Functions of this peptide have been described as an immune modulator participating in both the innate and adaptive immune systems.6 CCL20 has been demonstrated in human tonsillar crypts, inflamed intestinal epithelial cells, keratinocytes, lungs, appendix, and liver.7-9 However, to the best of our knowledge, there have been no published reports concerning the expression of CCL20 in the human sinus mucosa.

The aims of this study were to investigate the expression of CCL20 messenger RNA (mRNA) in human chronic rhinosinusitis mucosa using reverse transcriptase–polymerase chain reaction (RT-PCR) and to localize the CCL20 protein through immunohistochemistry.

METHODS

SUBJECTS

Ten patients (5 men and 5 women; age range, 28–40 years) who underwent surgery for chronic rhinosinusitis and 10 normal control subjects (5 men and 5 women; age range, 35–50 years) were recruited for this study. Clinical diagnosis of chronic rhinosinusitis followed the guidelines proposed by the American Academy of Otolaryngology–Head and Neck Surgery.10 In the chronic rhinosinusitis group, pa-
tients experienced typical symptoms, such as headache, nasal obstruction, and discolored nasal drainage arising from the nasal passages, that persisted for 12 weeks or longer and showed edema or erythema of the middle meatus, as identified by nasal endoscopy. Inflamed maxillary sinus mucosal tissues were removed during functional endoscopic sinus surgery through extended middle meatal antrostomy. For the controls, normal maxillary sinus mucosa was obtained from 10 patients with blowout fractures who underwent endoscopic endonasal reconstruction of the medial orbital walls. The patients in the control group had no sinonasal symptoms or signs and had no evidence of inflammation of paranasal sinus mucosa on preoperative computed tomography of the paranasal sinuses. The patients and controls had no personal or family history of allergy and had negative results on skin prick tests to 20 common airborne allergens and on multiple simultaneous allergen tests. Patients with nasal polyposis or immunodeficiency and individuals who had undergone prior sinus surgery were excluded from the study. No patient had been receiving any medication, including antibiotics, topical or oral corticosteroids, antihistamines, and mucokinetic agents, for at least 4 weeks before the study. Informed consent was obtained from all patients and control subjects, and the study protocols were approved by the Committee of Ethics, College of Medicine, Korea University, Seoul, South Korea.

PREPARATION OF SINUS MUCOSA

Tissues were cut into 2 portions. One portion was immediately flash frozen in liquid nitrogen and stored at −70°C for subsequent RNA studies. Another portion was fixed with 4% paraformaldehyde in 0.1M phosphate-buffered saline (pH, 7.4) overnight at 4°C and then embedded in paraffin for immunohistochemical staining.

RNA EXTRACTION AND RT-PCR

Total RNA was extracted from maxillary sinus mucosa specimens using a commercially available reagent (TRIzol; Gibco BRL, Grand Island, NY) according to the manufacturer’s recommendations. Total RNA from each sample was reverse transcribed in 20 µL of reaction mixture containing 2.5 U of Moloney murine leukemia virus RT (Gibco BRL) and 50pM random hexanucleotide at 42°C for 60 minutes. Based on the published sequences, oligonucleotide primers for PCR were commercially synthesized (Bioneer Co, Daejon, South Korea). Oligonucleotide primers were designed according to the following published sequences for CCL20: 5’-CTG TAC CAA GAG TTT GCT CC-3’ and 5’-ACG CAC ATT ATA TTT CAC CC-3’.

Amplification of the complementary DNA was carried out using 35 cycles at 94°C for 45 seconds, at 55°C for 30 seconds, and at 72°C for 1 minute, followed by a final extension cycle at 72°C for 7 minutes. Specificity of the 156–base pair (bp) PCR product was verified by its predicted size, restrictive digestion, and DNA sequencing. The primers 5’- GTG GAT ATT GTT GCC ATC AAT GAC C-3’ and 5’-GCC CCA GCC TTC ATG GTG GT-3’ for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) were run simultaneously as an internal control (the expected size of this PCR product was 248 bp). Each PCR cycle for GAPDH consisted of denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute, and elongation at 72°C for 1 minute, for a total of 20 cycles. To semiquantitatively analyze the RT-PCR result, we scanned the gel images and measured the intensity of the PCR product using a commercially available image software package (National Institutes of Health, Bethesda, Md). We determined the relative intensity of individual bands on a gel image as the ratio of the intensity of CCL20 to the intensity of GAPDH. The PCR products obtained from the sinus mucosa were cut from the agarose gel, subconed with a TA cloning kit (Invitrogen, Carlsbad, Calif), which is used to clone Taq polymerase-generated PCR products, and sequenced. To establish the specificity of responses, we used negative controls in which input RNA was omitted or in which RNA was used but RT was omitted. Messenger RNA extracted from lung tissues known to express CCL20 was used as the positive control.

PARAFFIN EMBEDDING AND TISSUE PREPARATION

After the maxillary sinus mucosa samples were fixed in 4% paraformaldehyde, they were prepared for paraffin embedding with graded ethanol. The specimens were then soaked in 100% xylen solution for 2 hours. After this period, the tissues were embedded in paraffin for 2 more hours. The paraffin blocks were cooled down to solidify them. The specimens were cut into 4-µm sections.

IMMUNOHISTOCHEMICAL STAINING

Before immunohistochemical staining, the sections were deparaffinized and rehydrated in xylene and a graduated dilution of ethanol. Immunohistochemical staining was performed by the avidin-biotin-peroxidase method (Vectastain ABC-Elite kit; Vector Laboratories, Burlingame, Calif). For CCL20 immunostaining, monoclonal anti–mouse CCL20 antibody (R&D Systems, Inc, Minneapolis, Minn) was used. Distribution of peroxidase was revealed by incubating the sections in a solution containing 3,3’diaminobezidine tetrahydrochloride (Sigma-Aldrich, St Louis, Mo). The antibody concentrations, incubation time, and other conditions were determined according to the instructions of the supplier and the results of preliminary experiments carried out by the authors. For negative control, non-immune serum IgG or phosphate-buffered saline, instead of the primary antibody, was used.

STATISTICAL ANALYSIS

The Mann-Whitney U test was used to test for differences in each of CCL20/GAPDH ratios between the 2 groups. The difference in CCL20/GAPDH ratio between the 2 groups was considered statistically significant at P<.05.

RESULTS

CCL20 mRNA was expressed in the paranasal sinus mucosa from both normal controls and patients with chronic rhinosinusitis. The PCR products extracted from the chronic rhinosinusitis mucosa had the size (156 bp) that was expected from the selected primers. There were no significant differences in the degree of CCL20 mRNA expression among the 10 chronic rhinosinusitis mucosa samples examined (P>.05). The same-size product was expressed in the positive control. For negative control, RT-PCR was performed without the CCL20 primer or RT (Figure 1). As a control for RNA integrity and RT-PCR procedure, the same RNA samples were also analyzed for GAPDH gene expression. The GAPDH primer produced...
a PCR product (248 bp) from each of the tissue samples. All samples were standardized to their corresponding GAPDH control. The expression of CCL20 mRNA in chronic rhinosinusitis was increased significantly compared with that in normal sinus mucosa (P < .05) (Figure 2). The PCR products obtained from lung and sinus mucosa were isolated and sequenced. In both tissues, the sequences obtained were identical to the corresponding sequences published for human CCL20, confirming that the correct transcript was being amplified in the PCR. These data indicate that CCL20 mRNA expression in the human sinus mucosa is up-regulated in the presence of inflammation.

**IMMUNOHISTOCHEMICAL STAINING OF CCL20 PROTEIN**

To localize the distribution of the CCL20 protein, the paranasal sinus mucosal samples from the controls and the patients with chronic rhinosinusitis were immunostained for CCL20. Immunohistochemical staining of the chronic rhinosinusitis mucosa showed strong staining for the CCL20 protein in the epithelial and submucosal glandular cells (Figure 3). In contrast, the CCL20 protein was weakly expressed in the normal sinus mucosa. These results demonstrate that the CCL20 protein is localized in epithelial and submucosal glandular cells and is up-regulated in chronic rhinosinusitis.

**COMMENT**

The present study demonstrated expression of CCL20 mRNA in normal and chronically inflamed human paranasal sinus mucosa and showed its up-regulation in chronic rhinosinusitis using RT-PCR. Furthermore, we localized the distribution of the CCL20 protein in the human sinus mucosa to the epithelial cells and submucosal glandular cells and demonstrated its up-regulation in chronic rhinosinusitis mucosa through immunohistochemistry. Chemokines are small-molecular-weight proteins that regulate leukocyte migration via the activation of 7 transmembrane-spanning G protein–coupled receptors. CCL20 has been demonstrated in human tonsillar crypts, inflamed intestinal epithelial cells, keratinocytes, lungs, appendix, and liver. It is released upon stimulation of cells with various proinflammatory cytokines, such as interleukin 1, tumor necrosis factor alpha, interleukin 17, and interferon gamma, as well as by other stimuli, including lipopolysaccharides, 12-O-tetradecanoylphorbol-13-acetate, and viral infection.

CCL20 is a unique functional ligand for the chemokine receptor CCR6. The receptor is selectively expressed on immature dendritic cells, such as Langerhans cell precursors, a subpopulation of dendritic cells that reside at mucosal surfaces. It is also expressed on distinct subpopulation of antigen-activated CD4⁺ T lymphocytes and B cells. Therefore, it is possible that CCL20 functions as an important chemokine for the recruitment of a distinct population of CCR6-expressing immature dendritic cells to the airway for subsequent antigen presentation.

In this study, RT-PCR analyses of human sinus mucosal cells indicated that CCL20 mRNA is constitutively expressed in the normal paranasal sinus mucosa and is
up-regulated in chronic rhinosinusitis mucosa. Also, immunohistochemical staining demonstrated predominant localization of CCL20 protein in the epithelium and submucosal glands in chronic rhinosinusitis mucosa. The localization pattern of CCL20 protein suggests that the expression of CCL20 may be induced by external stimuli, because the sinus mucosa first contacts the external milieu in the epithelium and submucosal glands. The constitutive and up-regulated expression of CCL20 by the human sinus mucosa demonstrated in the present study is consistent with a role for epithelial cell–produced CCL20 in modulating mucosal adaptive immune responses.

The up-regulation of CCL20 mRNA and protein in chronic rhinosinusitis epithelium is in agreement with previous reports of its up-regulation in other organs in response to inflammatory stimuli. This up-regulation of CCL20 in chronic rhinosinusitis suggests that human paranasal sinus epithelial cells regulate the expression of CCL20, which is an important chemokine for the recruitment of immature dendritic cells that are essential for subsequent antigen presentation and the development of host adaptive immune response. Thus, in response to inflammatory stimuli, the paranasal sinus mucosal epithelial cells may develop the ability to alter the cytokine microenvironment to influence the recruitment of immature dendritic cells that reside in close proximity to epithelial cells. It is also possible that constitutive low levels of epithelial expression of CCL20 in normal human sinus mucosa may serve to keep immature dendritic cells and memory T cells close to the epithelial surface, the first site of contact with antigen.

In conclusion, the expression of CCL20 protein and mRNA is up-regulated in chronic rhinosinusitis mucosa, and the CCL20 protein is localized in the epithelial and submucosal glandular cells. These findings support the current view that CCL20 is an inducible product of human airway epithelium that plays a role in modulating mucosal immunity. Further studies regarding the exact signaling pathways that induce the regulation of the CCL20 gene and protein are warranted.

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