Increased CXCL10 Expression in Nasal Fibroblasts from Patients with Refractory Chronic Rhinosinusitis and Asthma

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ABSTRACT

Background: Chronic rhinosinusitis (CRS) is characterized by local inflammation of the sinonasal tissues. CRS patients with nasal polyps and asthma often develop acute exacerbation of sinonasal symptoms after upper respiratory tract infections. However, the influence of concomitant asthma on the nasal immune response to viral infection remains unclear.

Methods: Specimens of nasal polyp and mucosal tissues were obtained from 3 groups of CRS patients (n = 14 per group): 1) patients without asthma (CRS group), 2) patients with aspirin-tolerant asthma (ATA group), and 3) patients with aspirin-intolerant asthma (AIA group). Nasal fibroblasts isolated from the specimens were stimulated with poly I:C. CXCL10 expression was analyzed by the quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assay. Biopsy specimens from CRS patients without asthma were subjected to immunohistochemistry for detection of T-bet and GATA-3 expression in CD3+ T cells by double-labeling.

Results: Nasal fibroblasts from the ATA and AIA groups showed significantly enhanced expression of CXCL10 mRNA and protein after poly I:C stimulation compared with cells from the CRS group and the control group (normal nasal mucosa). In addition to T helper (Th)2 cells, there was more abundant infiltration of Th1 cells into tissues from the AIA and ATA groups.

Conclusions: Our findings suggest that CRS associated with asthma may become intractable through the over-production of CXCL10 in response to viral infection.

KEY WORDS

asthma, CXCL10 chemokine, fibroblast, nasal polyps, rhinosinusitis

INTRODUCTION

Chronic rhinosinusitis (CRS) is a common chronic disease worldwide that is treated with medication (e.g., corticosteroids or macrolides) and by endoscopic sinus surgery. Although there has been a recent increase in the cure of CRS following the introduction of these treatments, a substantial number of patients still have a poor outcome. In CRS patients, nasal polyps arise from the paranasal sinus mucosa and prolapse into the nasal cavity, causing persistent nasal obstruction. We have often found that CRS patients with nasal polyps and asthma show acute exacerbation of nasal symptoms after upper respiratory tract infection. It has been reported that nasal polyps and asthma are linked by shared inflammation of the entire airway mucosa, and particularly by increased infiltration of eosinophils. In addition, mucosal eosinophilia is frequently associated with more severe disease and with recurrence of polyps after sur-
gery. Furthermore, CRS patients with aspirin-intolerant asthma often have particularly severe asthma that is associated with rhinorrhea and recurrent nasal polyps. These findings suggest that concomitant asthma may contribute to the pathophysiology of CRS.

Rhinoviruses are the most common cause of viral upper respiratory tract infections, being responsible for up to 50% of all episodes of the common cold. Viral infections have been shown to cause obstruction of the osteomeatal complex, which is thought to be a critical step in the development of CRS. Viral dsRNA and its synthetic analogue (poly I:C) are recognized by multiple pathways involving toll-like receptor 3 (TLR3), the protein kinase receptor, and two recently described genes: cytosolic RNA helicases retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5). Infection of airway epithelial cells leads to release of various proinflammatory mediators, including interleukin (IL)-1, IL-6, IL-8, interferon-inducible protein of 10 kDa (IP-10), regulated on activation normal T-cell expressed (RANTES), granulocyte macrophage-colony stimulating factor, and eotaxin. These cytokines and chemokines recruit inflammatory cells, such as neutrophils, lymphocytes, and eosinophils, to sites of inflammation, and contribute to the exacerbation of acute and chronic inflammation.

Histologically, fibroblasts are abundant in the stroma of nasal polyps and are thought to be involved in the pathogenesis of CRS associated with polyps. Although airway epithelial cells are the main target of viral infection, several studies have detected rhinovirus in the subepithelial layer by in situ hybridization and have shown that human airway fibroblasts are susceptible to rhinovirus infection. In addition, it has been reported that fibroblasts produce type I interferons (IFNs) and CXCL10 after stimulation with dsRNA. CXCL10 targets CXC chemokine receptor 3, leading to activation of natural killer cells and activated T cells, especially Th1 cells. CXCL10 plays an important role in various Th1-dominant diseases, such as viral and bacterial infections, autoimmune diseases, and transplant rejection. Thus, fibroblasts may also contribute to the pathogenesis of such diseases through an influence on the inflammatory response. However, little is known about the response of fibroblasts to viral infection in CRS patients with nasal polyps and concomitant asthma.

In this study, we classified CRS patients with nasal polyps based on the presence or absence of asthma and investigated CXCL10 expression by nasal fibroblasts in response to stimulation by factors associated with viral infection. In addition, biopsy specimens from these patients were subjected to immunohistochemistry with double labeling to detect the expression of T-bet (the master regulator of Th1 responses) and its antagonist GATA-3 in CD3+ T cells.

**METHODS**

**SUBJECTS**

A specimen of nasal polyp mucosa was obtained during surgery from 49 patients who had been referred to Jikei University Hospital for endoscopic sinus surgery (Table 1). Specimens were obtained from 3 different groups of CRS patients: 1) 14 CRS patients who had nasal polyps without asthma (CRS group), 2) 14 patients who had polyps and aspirin-tolerant asthma (ATA) (ATA group), and 3) 14 patients with polyps and aspirin-intolerant asthma (ATA group). We excluded CRS patients without polyps because our aim was to investigate the features of polyp tissues in this disease. Specimens from 7 controls were also examined (middle turbinate mucosa was obtained from 5 patients with non-allergic rhinitis and middle meatus mucosa was harvested from 2 patients with blowout fractures). Written informed consent was obtained from all of the patients prior to enrolment, and this study was approved by the Ethics Committee of Jikei University Hospital (reference number: 22-130 6307). The clinical data of the subjects are summarized in Table 1. Before endoscopic sinus surgery, each patient’s serum immunoglobulin E level and peripheral eosinophil count were measured, and sinus CT scanning was performed.

**CLINICAL ASSESSMENT**

The diagnosis of CRS was based on typical symptoms (nasal congestion, dysosmia, etc.) documented in the medical history, the presence of endoscopically visible nasal polyps arising from the middle meatus, and

| Table 1 Clinical characteristics of the four groups |
|---|---|---|---|---|
| | Control | CRS | ATA | AIA |
| No. | 7 | 14 | 14 | 14 |
| Sex (F/M) | 1/6 | 2/12 | 4/10 | 7/7 |
| Age (yr), mean | 26.75 ± 9.29 | 44.14 ± 16.08 | 43.75 ± 14.42 | 47.17 ± 20.21 |
| Age (yr), range | 19-40 | 20-71 | 18-65 | 26-71 |
| Allergic rhinitis | 0 (0%) | 5 (35.7%) | 5 (35.7%) | 2 (14.3%) |
| Total IgE (IU/ml) | 96.5 ± 58.48 | 100.25 ± 142.65 | 257.91 ± 258.73 | 232.33 ± 373.03 |
| Peripheral blood eosinophils (%) | 3.03 ± 2.49 | 2.57 ± 1.26 | 8.33 ± 4.44 | 13.5 ± 10.82 |
involvement of the ethmoidal and maxillary sinuses on CT scans of the paranasal sinuses. The diagnosis of asthma was made by physicians at the Department of Pulmonology of Jikei University Hospital, based on a history of typical symptoms, while AIA was diagnosed from a history of exacerbation of asthma, nasal congestion, and/or rhinorrhea after intake of aspirin or other nonsteroidal anti-inflammatory drugs.

**CELL CULTURE**

Nasal tissues obtained during endoscopic sinus surgery were chopped into small pieces and fibroblasts from each specimen were cultured at 37°C in Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12; Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Life Technologies, Grand Island, NY, USA) under an atmosphere of 5% CO₂ and humidified air. Nasal fibroblasts were used after 3 or 4 passages.

**STIMULATION OF NASAL FIBROBLASTS**

Nasal fibroblasts were seeded in 6-well and 24-well culture plates (Becton-Dickinson, Franklin Lakes, NJ, USA). When the cells had reached 80% confluence, the culture medium was replaced with DMEM/F12 (Life Technologies). Then cells were stimulated with 10 μg/ml of poly I:C (Life Technologies), 10³ units of IFN-β (PBL InterferonSource, Piscataway, NJ, USA), 10 ng/ml of IFN-γ (R&D Systems, Minneapolis, MN, USA), or 10 ng/ml of TNFα (R&D Systems) for 3 hours before harvest of mRNA and for 24 or 48 hours before harvesting the culture supernatant.

**QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (qRT-PCR) FOR CXCL10 mRNA EXPRESSION IN NASAL FIBROBLASTS**

Total RNA was isolated by using an RNaseasy Mini Kit that included DNase (Qiagen, Hilden, Germany) and was transcribed to obtain cDNA by using superscript II (Life Technologies). Then the quantitative polymerase chain reaction (qRT-PCR) was performed with an ABI PRISM 7700 Sequence Detector System (Applied Biosystems, Foster City, CA, USA) and SYBR green PCR master mix (Life Technologies). The primers were as follows: CXCL10: 5’-TGTCACTGACTGCTGACTCACCCT-3’ (forward) and 5’-CTGTGTATCACAAGACACGATCA-3’ (reverse), GAPDH: 5’-GAGGGTGAAGGTTGAGTC-3’ (forward) and 5’-GAAGATGTGATGGATTCTTTC-3’ (reverse). The level of CXCL10 mRNA expression was normalized by the average expression of GAPDH.

**ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR CXCL10 PROTEIN**

Briefly, 96-well plates (Nunc, Roskilde, Denmark) were coated with 2.0 μg/ml of anti-human CXCL10 monoclonal antibody (R&D Systems) overnight at 4°C. Then the plates were washed and blocked with blocking solution (Boehringer, Mannheim, Germany). After adding a supernatant sample or standard to the wells, the plates were incubated overnight at 4°C. After washing with PBS containing 0.05% Tween 20, 100 ng/ml of biotinylated polyclonal CXCL10 antibody was added, and the plates were incubated for 2 hours at room temperature. Further washing was done and 100 μl of streptavidin-horseradish peroxidase (Life Technologies) was added, after which the plates were let stand for 20 min at room temperature. Next, the wells were washed, and TMB (KPL, Gaithersburg, MD, USA) was added. The reaction was stopped with 2N H₂SO₄, and absorbance was measured at 450 nm with a microtiter ELISA reader (Model 550; Bio-Rad Laboratories, Hercules, CA, USA). Data were analyzed with Microplate Manager Software version 5.2.1 (Bio-Rad Laboratories), and the CXCL10 concentration in each culture supernatant was calculated from standard titration curves by linear regression analysis.

**IMMUNOHISTOCHEMISTRY**

Immunohistochemistry was performed with deparaaffinized 4 μm sections cut from paraffin-embedded nasal tissue blocks. The paraffin-embedded sections and slides were blocked by incubation with 5% diluted normal donkey serum (Chemicon International, Temecula, CA, USA) for 30 minutes at room temperature. Then sections were incubated with primary mouse anti-T-bet polyclonal antibody (R&D Systems), mouse anti-GATA-3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-CD3 polyclonal antibody (Abcam, Cambridge, UK) overnight at 4°C. The secondary antibodies were Alexa 555-conjugated donkey anti-mouse IgG (Life Technologies) and Alexa 488-conjugated donkey anti-rabbit IgG (Life Technologies), and incubation was done for 30 minutes. After washing with PBS, sections were incubated for 5 min with 4’,6-diamidino-2-phenylindole dihydrochloride (Dojindo, Kumamoto, Japan) and mounted with Permafluor (Beckman Coulter, Miami, FL, USA). Then the sections were viewed using a Biorevo BZ-9000 microscope (Keyence, Osaka, Japan).

**STATISTICAL ANALYSIS**

Analyses were performed using a statistical package (SPSS version 19; IBM, Armonk, NY, USA). All analyses were nonparametric. Between-group comparisons were performed by the nonparametric Mann-Whitney’s U-test with Bonferroni correction, and differences were considered significant at P < 0.05.
Yoshikawa M et al.

RESULTS

INCREASED CXCL10 EXPRESSION IN NASAL FIBROBLASTS FROM THE AIA AND ATA GROUPS

We measured CXCL10 mRNA expression in nasal fibroblasts from 4 groups of subjects (CRS, ATA, AIA, and control) by qRT-PCR and the results are shown in Figure 1a. In the absence of stimulation, no CXCL10 mRNA expression was found in any of the four groups. In response to stimulation with poly I:C (10 μg/ml), CXCL10 mRNA expression was increased in nasal fibroblasts from all four groups. In particular, CXCL10 mRNA expression was significantly higher in cells from the ATA group and the AIA group than in cells from the control group or the CRS group, while no significant difference was noted between the ATA and AIA groups.

We also detected CXCL10 protein in the culture supernatant of nasal fibroblasts (Fig. 1b). The concentration of CXCL10 culture supernatant from the ATA group and the AIA group was significantly higher than in supernatant from the control group or the CRS group, and the increase was time-dependent. These findings were similar to those for CXCL10 mRNA expression.

CXCL10 mRNA EXPRESSION BY NASAL FIBROBLASTS SUBJECTED TO VARIOUS STIMULI

There are two proximal NF-κB recognition sites and the proximal interferon-stimulated response element (ISRE) within the promoter region of CXCL10.24 After poly I:C stimulation of nasal fibroblasts, TLR-3 and RIG-I signaling activates two cellular kinases known as TANK-binding kinase-1 and IκB kinase-s, leading to the activation of IFN regulatory factor-3 (IRF-3) and NF-κB.25-30 IRF-3 can also bind directly to several DNA-binding motifs, including the ISRE, leading to the direct induction of interferon-stimulated genes, such as those for IFN-α and IFN-β.24,31,32 Therefore, we assessed CXCL10 mRNA expression by nasal fibroblasts after stimulation with IFN-β, IFN-γ, or TNFα. As shown in Figure 2, CXCL10 mRNA expression was induced by all three cytokines. In the control group, CXCL10 mRNA expression was more...
CXCL10 in Fibroblasts of Refractory CRS

strongly induced by IFN-β, which activates the ISRE pathway, than by IFN-γ or TNF-α. In the ATA group, CXCL10 mRNA expression showed a significantly greater increase in response to TNF-α, which activates the NF-κB pathway, than in the control group or the CRS group. On the other hand, in the AIA group, CXCL10 mRNA expression was significantly higher than in the CRS group after stimulation with either TNF-α or IFN-β.

ANALYSIS OF CELLS EXPRESSING TRANSCRIPTION FACTORS IN NASAL POLYP TISSUES

T-bet and GATA-3 are known to be the principal transcription factors involved in the differentiation of type-1 (Th1) and type-2 (Th2) helper T cells, respectively. In order to determine whether Th1 or Th2 cells were increased in nasal polyp tissues, we detected T-bet or GATA-3 and CD3 by double immunofluorescence staining. CD3+ cells were restricted to the perivascular and subepithelial regions of nasal biopsy specimens. Representative double-stained images of T-bet+CD3+ cells (Th1 cells) and GATA-3+CD3+ cells (Th2 cells) in nasal biopsy specimens are shown in Figure 3a, b. CD3+ cells were detected in all specimens, but the number of CD3+ cells was significantly higher in the ATA and AIA groups than in the control and CRS groups (Fig. 3c). In addition, the number of T-bet+CD3+ cells and GATA-3+CD3+ cells was significantly higher in the AIA group than in the control or CRS groups (Fig. 3d, e). In the ATA group, the number of T-bet+CD3+ cells and GATA-3+CD3+ cells was slightly higher than in the control group or the CRS group (Fig. 3d, e), but there were no significant differences. Thus, Th1 and Th2 cells were significantly increased in nasal polyp tissues from the AIA group compared with the control, CRS, and ATA groups.

DISCUSSION

CRS is a heterogeneous and multifactorial disease of unknown etiology. In CRS patients, inflammation of the upper and lower airways is well documented, and epidemiological and pathophysiological links between CRS with or without nasal polyps, asthma, and/or eosinophilic inflammation have been established by recent investigations. It has been shown that viral infection increases the production of a variety of proinflammatory cytokines and chemokines by epithelial cells and fibroblasts, which probably contribute to the exacerbation of airway inflammation.33,34 In this study, we focused on the susceptibility to viral infection-related factors of fibroblasts from CRS patients with asthma (ATA and AIA). We found that the expression of CXCL10 mRNA by nasal fibroblasts from CRS patients with asthma (ATA and AIA) was significantly upregulated in response to poly I:C compared with fibroblasts from CRS patients without asthma or controls. We also observed that both Th1 and Th2 cells were abundant in the nasal polyp tissues from CRS patients with asthma (ATA and AIA).

A previous study revealed that poly I:C significantly increases the expression of mRNAs for CXCL10, CCL5, CXCL8, and IL-6 by airway epithelial cells through TLR3 signaling that is dependent on NF-κB and/or interferon regulatory factor (IRF)-3.35 Poly I:C has also been shown to dose-dependently induce the production of CXCL10 by human fibroblasts derived from diploid skin-muscle.36 However, this is the first report about upregulation of CXCL10 mRNA and protein expression in nasal fibroblasts by poly I:C stimulation, and we also showed that the response was significantly greater in the ATA and AIA groups. It has been reported that fibroblasts from different individuals display distinctive characteristics that are maintained even after prolonged culture, suggesting that fibroblasts may have a highly stable imprinted phenotype.37,38 Our results suggested that viral infection might initiate an intense inflammatory response with massive release of CXCL10 from nasal fibroblasts that are susceptible to viral infection-related factors, such as the fibroblasts isolated from the ATA and AIA groups.

We also examined the response of CXCL10 mRNA expression to IFN-β, IFN-γ, and TNF-α, in order to find key differences from poly I:C signaling, since poly I:C was reported to activate both the IRF-3 and NF-κB pathways via TLR-3 or RIG-I.25-30 We showed that CXCL10 mRNA expression was enhanced by TNF-α in the ATA group, whereas it was significantly enhanced by both IFN-β and TNF-α in the AIA group. Activation of NF-κB in nasal polyps could be important because NF-κB induces the transcription of cytokines, chemokines, and adhesion molecules that play a major role in the inflammatory process.39 It was recently reported that IL-1β and TNF-α induce CXCL10 mRNA expression more rapidly in asthmatic than nonasthmatic airway smooth muscle cells, with this response being mediated by the activation of NF-κB and JNK.40 It has also been reported that TLR3 and MDA5 signaling through a common downstream intermediate (IRF3) is required for maximal sensing of rhinovirus dsRNA and is involved in CXCL10 expression.41 More studies are needed to elucidate the role of alterations in IRF signaling.

The present study showed that Th1 and Th2 cell infiltration of nasal polyp tissues was significantly increased in the AIA group compared with the control, CRS, and ATA groups. These findings suggested that migration of Th1 cells into nasal polyps might be induced when CXCL10 is produced by fibroblasts in response to viral infection, leading to an excessive Th1-type response in CRS patients with ATA and AIA. Although there was no significant difference of Th1 and Th2 cell infiltration between the ATA group and the CRS group, CXCL10 expression was significantly
**Fig. 3** Results of double immunostaining. (a) Representative double immunostaining of T-bet+CD3+ cells in nasal biopsy specimens. Cells were stained with DAPI (blue nuclei), CD3 (green), and T-bet (red). White arrows indicate T-bet+CD3+ cells in the merged image. Inset shows double-labeled T-bet+CD3+ cells marked by a square in the merged image. (b) Representative double immunostaining of GATA-3+CD3+ cells in nasal biopsy specimens. Cells were stained with DAPI (blue nuclei), CD3 (green), and GATA-3 (red). White arrows indicate GATA-3+CD3+ cells in the merged image. (c) CD3+ cells: The number of CD3+ cells was significantly higher in the ATA and AIA groups than in the control and CRS groups. (d) T-bet+CD3+ cells: The number of T-bet+CD3+ cells was significantly higher in the AIA group than in the control and CRS groups. In the ATA group, the number of T-bet+CD3+ cells was also higher than in the control and CRS groups, but the difference was not significant. (e) GATA-3+CD3+ cells: The number of GATA-3+CD3+ cells was significantly higher in the AIA group than in the control and CRS groups. In the ATA group, the number of GATA-3+CD3+ cells was also higher than in the control and CRS groups, but the difference was not significant.
higher in the former group, which is a finding that conflicts with our hypothesis. One possibility is that viral inflammation was not reflected by the changes in the ATA group. There might be other ways in which CXCL10 contributes to viral-induced exacerbation of CRS. For instance, CXCL10 upregulates eosinophil functions such as adhesion and O2 generation and also increases the release of eosinophil-derived neurotoxin when eosinophilic infiltration occurs during the exacerbation of asthma.\(^4\) CXCL10 also has a prominent role in the worsening of airflow obstruction and airway inflammation in patients with acute rhinovirus-induced asthma.\(^34\) Some recent studies have suggested that CXCR3 may be expressed by human CD25hi FOXP3+ CD4+ Tregs, a T cell subset with potent immunoregulatory properties,\(^43\) which suggests a paradoxical role for CXCL10. The exact functional implications of these findings can only be explained by further investigation.

The pathogenesis of ATA and AIA is different. AIA is attributable to inhibition of cyclooxygenase by aspirin-like drugs and does not arise from an allergic reaction. Biosynthesis of cysteinyl leukotrienes is also upregulated in patients with AIA.\(^44\) However, both AIA and ATA are associated with eosinophilic sinusitis and nasal polyposis. The clinical impact of ATA and AIA on CRS may be influenced by many factors, but the differences between ATA and AIA have not been well documented. Basement membrane hyperplasia, goblet cell proliferation, and eosinophil infiltration have been reported to be more prominent in the nasal polyps of asthma patients than in polyps from patients without asthma.\(^45\) Based on our findings in the present study, diseases of the lower airways such as ATA and AIA seem to influence gene expression in nasal polyp fibroblasts, suggesting that concomitant lower airway disease is a major reason why CRS may become refractory to treatment.

In conclusion, we found that CXCL10 expression was upregulated by Poly IC stimulation in nasal fibroblasts from CRS patients with asthma and this induced Th1 cell infiltration into nasal polyp tissues. Although the mechanism leading to differences of CXCL10 expression between CRS patients with or without asthma needs to be clarified, our findings suggest that CRS associated with asthma may become intractable due to the overproduction of CXCL10 in response to viral infection.

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502