Evaluation of Chemokine mRNA Expression to Assess Allergic Inflammation of the Ocular Surface in Chronic Allergic Conjunctival Diseases

Hiroshi Aso, MD, Jun Shoji, MD, Yukiko Shiraki, MD, Noriko Inada, MD, and Satoru Yamagami, MD, PhD

Purpose: We validated the use of chemokine messenger RNA (mRNA) expression analysis for the assessment of ocular surface allergic inflammation in chronic allergic conjunctival diseases (ACDs) with proliferative lesions, including giant papillae and gelatinous infiltration of the limbus.

Methods: This prospective sectional study included 19 patients with chronic ACDs and 10 healthy volunteers as controls. Patients with chronic ACDs were divided into 2 subgroups according to the severity of the clinical score: active stage ACD subgroup (n = 9) and stable stage ACD subgroup (n = 10). Impression cytology using a filter paper for each upper tarsal conjunctiva of the patients with chronic ACDs and control subjects was performed, and the expression levels of IL1A, CXCL8, IL16, and CCL24 mRNAs encoding interleukin (IL)-1α, CXCL8/IL-8, IL-16, and CCL24/eotaxin-2, respectively, were determined by quantitative real-time polymerase chain reaction using impression cytology specimens.

Results: CCL24 and IL16 mRNA levels in the active ACD subgroup were significantly higher than those in the control group (P = 0.003 and 0.004, respectively). IL1A and CXCL8 expression levels in the active ACD subgroup were significantly higher than those in the stable ACD (P = 0.008 and 0.029, respectively) and control (P = 0.008 and 0.014, respectively) subgroups. Furthermore, significant correlations were detected between IL16 and CCL24 mRNA levels (r = 0.76, P = 0.0001) and between IL1A and CXCL8 (r = 0.67, P = 0.0004).

Conclusions: At least 2 kinds of inflammatory reactions, IL-1α- and CXCL8-associated inflammation and CCL24- and IL-16-associated inflammation, may be involved in the exacerbation of chronic ACDs.

Key Words: chronic allergic conjunctival diseases, interleukin-1α, interleukin-8, interleukin-16, eotaxin-2

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Allergic conjunctival diseases (ACDs), including allergic conjunctivitis, vernal keratoconjunctivitis, and atopic keratoconjunctivitis, are inflammatory diseases of the conjunctiva caused by the predominantly immunoglobulin E (IgE)-mediated immediate hypersensitivity response and accompanied by conjunctival eosinophilic inflammation. Furthermore, in refractory ACD with severe eosinophilic inflammation, other mechanisms, including neutrophilic inflammation, interleukin (IL)-17 producing helper T cell (Th17) response, IL-1α producing macrophage response, and innate immunity, have also been implicated.1–5 This etiological variety implies a very complex immunological basis of ACD.

ACDs are associated with various clinical findings, such as conjunctival hyperemia, conjunctival edema, limbal swelling, and papillary or giant papillary formation due to direct exposure of the conjunctiva to the allergens.1,6,7 ACDs comprise 5 different clinical forms: seasonal allergic conjunctivitis, perennial allergic conjunctivitis, atopic keratoconjunctivitis (AKC), vernal keratoconjunctivitis (VKC), and giant papillary conjunctivitis.8,9 Chronic ACDs, such as AKC and VKC, are particularly severe and refractory forms; their characteristic clinical findings are giant papillae, limbal gelatinous infiltration, and shield ulcer, developing because of severe allergic inflammation of the ocular surface. The allergic inflammation of the conjunctiva plays a crucial role in major pathological conditions of ACDs, but each clinical form has different clinical characteristics. Therefore, in addition to the immediate hypersensitivity reaction, several pathological conditions, including neutrophilic inflammation and exaggerated innate immune response, are believed to be associated with conjunctival allergic inflammation in patients with ACDs.5

In the clinical tests for ACDs, allergy-associated factors in tear samples and impression cytology specimens are usually investigated in the laboratory to assess the extent of allergic inflammation in the conjunctiva.10 The assay of the total IgE and antigen-specific IgE antibody levels in tears is believed to be a useful tool for diagnosing ACDs and detecting...
ACD-associated allergens.11,12 Because the invasion of eosinophils and type 2 helper T (Th2) cells is a major pathological condition of allergic inflammation in the ocular surface, the levels of the eosinophil cationic protein (ECP), eosinophil-associated and Th2-associated cytokines and chemokines, in tears are reportedly useful markers of the clinical severity of ACDs.2,13,14 Moreover, simultaneous measurements of various cytokines and chemokines in tears are helpful in the differential diagnosis of ocular surface inflammation that develops because of allergy, autoimmunity, or infection.

In impression cytology (membrane biopsy), a method of cytological diagnosis in the ocular surface, goblet cell density, and keratinization of conjunctival epithelial cells are histologically evaluated.3 The real-time reverse transcriptase polymerase chain reaction (RT-PCR) method has recently improved, and mRNA expression levels can be evaluated on the ocular surface by using impression cytology specimens. Previously, we reported that impression cytology using a filter paper was a useful clinical test with low invasiveness for the patients. It enabled evaluation of mRNA expression levels of allergy-associated factors contained in the ocular surface lining fluid filled with epithelial cells, mucin, tear, and inflammatory cells.15

In allergic disorders such as bronchial asthma, allergic rhinitis, and atopic dermatitis, cytokines and chemokines secreted by epithelial cells and invading inflammatory cells are associated with the immune response and inflammatory reaction in cutaneous and mucosal tissues. IL-1α produced by the epithelial cells of the bronchus induces an innate immune response in human lung fibroblasts.16 Furthermore, in asthma, the CXCL8/IL-8 concentration in airway tissues increases with neutrophilia in sputum and is recognized as an aggravation factor in patients with a severe form of the disease.17 The increased chemotactic activity of CD4+ T cells and eosinophils in patients with asthma is mainly attributable to IL-16,18,19 Activation of the CCL24/eotaxin-2-dependent pathway reportedly exacerbates eosinophilic airway inflammation.20 In ACDs, we reported that CCL24 and IL-16 protein amounts increase in patients’ tears,21 and CCL24 mRNA expression is upregulated in conjunctival epithelial cells in patients with VKC.24 Therefore, simultaneous measurements of protein and mRNA levels of different functional cytokines and chemokines, for example, IL-1α, CXCL8, IL-16, and CCL24, on the ocular surface may be a valuable clinical laboratory test for evaluating the activity and severity of chronic ACDs in patients with various inflammatory reactions that accompany the allergic hypersensitivity reaction.

To verify this assumption, in this study, we investigated mRNA expression levels of inflammatory chemokines in the fluid lining the ocular surface of patients with chronic ACDs with severe and stable allergic inflammation.

**MATERIALS AND METHODS**

The study protocol, approved by the Institutional Review Board of the Nihon University School of Medicine (approval number: RK-120511-11), adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants.

**Subjects**

In total, 19 consecutive patients diagnosed with AKC or VKC at the Department of Ophthalmology of the Nihon University Itabashi Hospital (Tokyo, Japan) between July 2015 and November 2016 were included in this study as the chronic ACD group. This group was further divided into 2 subgroups according to the clinical severity of AKC and VKC: active ACD subgroup and stable ACD subgroup. Demographic data for the subjects are shown in Table 1. Only patients who had not received treatment or were treated with anti-allergic ophthalmic solutions, including mast cell stabilizers, histamine H1 receptor antagonists, corticosteroids, or immunosuppressive agents such as cyclosporine and tacrolimus, were included in the study (Table 1). Patients who used oral medicines or received injections for the treatment of allergic disease or immunotherapy were excluded. Patients with ocular surface disorders other than ACDs—including lagophthalmos, blepharospasm, conjunctival chalasis, dry eye, infectious conjunctivitis, infectious keratitis, Stevens–Johnson syndrome, or ocular pemphigoid—and those who could not provide a sufficient amount of tear sample were excluded. AKC and VKC were diagnosed according to the Japanese ACD guidelines.20 Healthy volunteers without any allergic diathesis or history of wearing contact lenses were recruited as controls (n = 10).

**Clinical Staging of Chronic Allergic Conjunctival Diseases**

Clinical severity of chronic ACDs was scored using the 5-5-5 exacerbation grading scale for ACDs.7 In this method, 100 points are assigned for each of the 5 severe clinical findings (active giant papillae, gelatinous infiltrates of the limbus, exfoliative epithelial keratopathy, shield ulcer, and papillary proliferation at lower palpebral conjunctiva); 10 points are assigned for each of the 5 moderate clinical findings (blepharitis, papillary proliferation with velvety

| TABLE 1. Demographic Data of Study Subjects |
|--------------------------------------------|
| No. of patients (cases) | Control | ACD (Stable Stage) | ACD (Active Stage) | P |
| Age [mean ± SD (yrs)] | 24.7 ± 3.47 | 21.8 ± 12.3 | 29.1 ± 14.9 | 0.371 |
| Gender (male:female) | 5:5 | 8:2 | 9:0 | 0.024 |
| VKC/AKC ratio | — | 6:4 | 4:5 | 0.656 |
| Clinical score* [median (points)] | 12 | 114 | 0.0002 |
| Topical treatment | Anti-allergy drugs | 9 | 8 | 1.000 |
| Corticosteroids | — | 1 | 3 | 0.303 |
| Immunosuppressive agents (C:T ratio3) | — | 9 (1:8) | 9 (0-9) | 1.000 |

N/A, not applicable

*Score on the 5-5-5 exacerbation grading scale for allergic conjunctivitis.

1C:T ratio: cyclosporine ophthalmic solution 0.1%: tacrolimus ophthalmic suspension 0.1% ratio.
appearance, Horner–Trantas spots, edema of bulbar conjunctiva, and superficial punctate keratopathy); and 1 point is given for each of the 5 mild clinical findings (papillae at the upper palpebral conjunctiva, follicular lesions at the lower palpebral conjunctiva, hyperemia of palpebral conjunctiva, hyperemia of bulbar conjunctiva, and lacrimal effusion). The sum of total points in each grade determined the severity score on the 5–5–5 exacerbation grading scale.

According to the results of the 5–5–5 exacerbation grading scale for ACDs, the active stage subgroup had 9 patients with a clinical score of ≥100 points, whereas the stable stage subgroup comprised 10 patients with a clinical score of <100 points.

Sample Collection from the Ocular Surface

The membrane biopsy of the ocular surface was performed by a method identical to that of impression cytology but using the 5-mm tip of Schirmer test papers (Schirmer Tear Production Measuring Strips; Ayumi Pharmaceutical Corporation, Tokyo, Japan) instead of the nitrocellulose membrane. The Schirmer test paper was applied to the upper tarsal conjunctiva without local anesthesia or washing the eye, pressed gently using a glass rod, then removed, and preserved in RNAlater RNA stabilization reagent (Qiagen, Hilden, Germany) until RT-PCR analysis.

Measurements of mRNA Levels of IL1A, CXCL8, IL16, and CCL24 on the Ocular Surface

Expression levels of IL1A, CXCL8, IL16, and CCL24 mRNAs encoding IL-1α, CXCL8, IL-16, and CCL24, respectively, on the ocular surface of control subjects and patients with chronic ACDs were evaluated using membrane biopsy samples obtained from the affected eye in unilateral cases, from the more severely affected eye in bilateral cases of chronic ACDs, and from the right eye of control subjects.

To detect IL1A, CXCL8, IL16, and CCL24 expression by real-time RT-PCR, total RNA from each sample was extracted with a RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions and used to synthesize cDNA with a High-Capacity cDNA Reverse Transcription Kit (Life Technologies Japan, Tokyo, Japan). Real-time RT-PCR was performed using the TaqMan gene expression assay (Life Technologies Japan, Tokyo, Japan) on a Step One Plus system (Life Technologies Japan, Tokyo, Japan). Target Ct values were normalized to those of GAPDH (Hs99999905_m1) from the same sample. Expression levels were determined by the comparative threshold cycle (ΔΔCT) method.

Statistical Analyses

Statistical analyses were performed using MAC Toukei–Kaiseki v.2 software (Esumi, Tokyo, Japan). Differences between active and stable stage subgroups of the chronic ACD group and the control group were evaluated with the χ² test and Kruskal–Wallis H test. IL1A, CXCL8, IL16, and CCL24 mRNA expression levels on the ocular surface of control subjects are shown in Table 2. The cutoff value for the respective cytokine/chemokine in the control group, so that if the sample’s expression level was equal to or larger than the cutoff value, the expression was considered to

RESULTS

IL1A, CXCL8, IL-16, and CCL24 mRNA Expression Levels on the Ocular Surface Increase Depending on the Clinical Severity of Chronic Conjunctival Diseases

The median, 95th percentile, and 5th percentile of IL1A, CXCL8, IL16, and CCL24 mRNA expression levels on the ocular surface of control subjects are shown in Table 2. The cutoff value of ocular surface mRNA expression for each cytokine/chemokine was set up based on the 95th percentile value for the respective cytokine/chemokine in the control group, so that if the sample’s expression level was equal to or larger than the cutoff value, the expression was considered to

| TABLE 2. Relative mRNA Levels of CCL24, CXCL8, IL16, and IL1A in the Control Group |
|-----------------------------------------------|
| CCL24 | CXCL8 | IL16 | IL1A |
| Median (ΔΔCT) | 1.7 | 0.88 | 0.48 | 0.0098 |
| 95th percentile (ΔΔCT) | 7.4 | 2.8 | 2.0 | 4.0 |
| Fifth percentile (ΔΔCT) | 0.0017 | 0.021 | 0.038 | 0.001 |

| TABLE 3. Two-Factor Analysis of CXCL8 and CCL24 mRNA Levels in Control Group |
|-----------------------------------------------|
| Control (n = 10) | High (>7.4) | Low (<7.4) |
| CXCL8 |
| | |
| High (>2.8) | 0 | 1 |
| Low (<2.8) | 1 | 8 |

| TABLE 4. Two-Factor Analysis of CXCL8 and CCL24 mRNA Levels in ACD Group |
|-----------------------------------------------|
| ACD Group (n = 19) | High (>7.4) | Low (<7.4) |
| CXCL8 |
| | |
| High (>2.8) | 10 | 1 |
| Low (<2.8) | 4 | 4 |

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TABLE 5. Characteristics of CCL24\textsuperscript{high}/CXCL8\textsuperscript{high} and CCL24\textsuperscript{high}/CXCL8\textsuperscript{low} Subgroups

| Subgroup                  | No. of patients | Gender (%male) | Age (median) | VKC/AKC ratio | Clinical score (median) (range) |
|---------------------------|-----------------|----------------|--------------|----------------|--------------------------------|
| CCL24\textsuperscript{high}/IL-8\textsuperscript{high} | 10              | 90%            | 14           | 3/1            | 62.5 (3–223)                  |
| CCL24\textsuperscript{high}/IL-8\textsuperscript{low} | 4               |                | 20           | 75%            | 63 (3–213)                    |

\( P > 0.05 \), \( P > 0.05 \)

*The recurrence was calculated by the number of recurrences for the past 1 year.

Correlation Between IL1A and CXCL8 and Between IL16 and CCL24 mRNA Expression Levels on the Ocular Surface

Partial correlation analysis revealed significant correlations between mRNA levels of IL16 and CCL24 \((P = 0.004)\), and of IL1A and CXCL8 \((P = 5.4 \times 10^{-11})\) in all subjects (combined ACD and control groups; Table 6). Significant correlations between CCL24 and IL16 mRNA levels \((P = 0.002)\), Spearman correlation coefficient; Fig. 2A) and between IL1A and CXCL8 mRNA levels \((P = 0.029\) and control \((P = 0.008\) and 0.029, respectively) groups (Figs. 1C, D, respectively).
TABLE 6. Values of Partial Correlation Coefficient r Indicating the Extent of Correlation Between Expression Levels of Different Cytokines

|        | CCL24 | CXCL8 | IL16 | IL1A |
|--------|-------|-------|------|------|
| CCL24  | 1     |       |      |      |
| CXCL8  | 0.002 | 1     |      |      |
| IL16   | 0.564*| 0.206 | 1    |      |
| IL1A   | 0.007 | 0.909**| −0.076 | 1 |

*P < 0.005; **P < 0.0001.

DISCUSSION

In an attempt to expand the range of available methods to assess allergic inflammation of the ocular surface in chronic ACDs, we determined the mRNA levels of cytokines/chemokines in impression cytology specimens. We found that mRNA expression levels of the cytokines IL-1α, CXCL8, IL-16, and CCL24 increased in patients with chronic ACDs in the active stage compared with their levels in patients with chronic ACDs in the stable stage and in control subjects. Furthermore, alterations in the expression pattern of cytokines concerned 2 systems: the CXCL8/IL-1α axis and the IL-16/CCL24 axis. These results mean that there are likely 2 different types of pathophysiological events associated with the exacerbation of allergic inflammation in the conjunctiva of patients with chronic ACDs.

ACDs are believed to be conjunctival inflammatory disorders that exhibit the immediate hypersensitivity response as a basic pathological condition. However, some pathological conjunctival manifestations of AKC and VKC, such as giant papillae and limbal gelatinous infiltration, cannot be explained only by the immediate hypersensitivity response. Thus, the details of the pathological conditions that cause conjunctival proliferative alterations are not fully understood. Because severe infiltration of eosinophils, basophils, and CD4+ cells, including Th2 cells, in conjunctival tissues was reported in a histopathological study of giant papillae, it was suggested that AKC and VKC pathological conditions are strongly associated with the late-phase reaction of the immediate hypersensitivity response. In addition, depending on the assay of the allergy-associated factors in tears, increases in antigen-specific IgE antibodies, ECP, Th2 cytokine, CCL11/eotaxin-1, CCL24, soluble IL-6 receptor, CCL20, and CXCL8 were reported and it has been shown that VKC-associated conditions involve complex immune responses. Therefore, we investigated the expression of IL1A, CXCL8, IL16, and CCL24 mRNAs on the ocular surface because cytokines encoded by these genes might be associated with inflammation of the conjunctiva of patients with chronic ACDs. Furthermore, the noninvasive membrane biopsy method with a filter paper was employed to obtain ocular surface samples, which could be useful for the quantitative follow-up of patients with chronic ACDs. We are convinced that a prognostic ocular surface marker that can predict the therapeutic effect would be a useful clinical tool in the treatment of chronic ACDs.

One of the major pathophysiological features of allergic inflammation in the conjunctiva is eosinophilic inflammation. Eosinophils express the C-C chemokine receptor CCR3 on their cell surface, and CCR3 ligands, including CCL11, CCL24, CCL26/eotaxin-3, MCP-4, and Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES), are crucial for the infiltration of eosinophils in allergically inflamed tissues. In addition, ECP levels in tears in VKC and AKC groups were significantly increased compared with those in a control group. In addition, ECP levels in tears significantly correlated with the severity of clinical findings. Furthermore, we reported that out of all mRNAs encoding the members of the eotaxin family, CCL24 mRNA was most abundantly expressed on the ocular surface and its level significantly correlated with tear ECP levels in the patients with ACDs. Therefore, CCL24 mRNA expression on the ocular surface may be a useful biomarker of eosinophilic inflammation of the conjunctiva. It has been reported that allergic inflammation is characterized mainly by the infiltration of eosinophils and CD4+ lymphocytes. Th2 cells that express CD4 on their cell surface are a major type of inflammatory cells mediating allergic inflammation in the conjunctiva. IL-16 is a chemokine associated with the migration of CD4+ cells and may be also implicated in the migration of eosinophils. Therefore, our data that were obtained from membrane biopsies and suggested that CCL24 mRNA level significantly correlated with that of IL16 on the ocular surface are quite expected in chronic ACD patients with allergic inflammation in the conjunctiva. Accordingly, these results showed that allergic inflammation caused by the immediate hypersensitivity response enhanced the cooperative expression of CCL24 and IL-16 on the ocular surface; therefore, the

FIGURE 2. Evaluation of the correlation between CCL24 and IL16 mRNA expression levels, and between IL1A and CXCL8 mRNA expression levels on the ocular surface in all subjects. To examine the correlation, data from the ACD group were merged with those from the control group. Spearman correlation coefficients showed significant correlation between CCL24 and IL16 mRNA expression levels (ρ = 0.757, P = 0.0001), and between IL1A and CXCL8 (ρ = 0.670, P = 0.0004).
combined levels of their mRNAs and/or proteins may be a suitable biomarker of allergic inflammation in the conjunctiva.

IL-1α is known as the main inflammatory cytokine of innate immunity and one of the alarmins released from damaged epithelium. In addition, IL-1α secreted by epithelial cells reportedly upregulated expression levels of IL-6, CXCL8, monocyte chemotactic protein-1, and granulocyte macrophage colony-stimulating factor in cultured primary human lung fibroblasts. Moreover, IL-1α released from necrotic corneal epithelial cells was shown to increase the expression levels of IL-6 and CXCL8 in intact cultured human epithelial cells. In addition, the present study, we showed that CXCL8 mRNA expression on the ocular surface of the patients with chronic ACDs significantly correlated with the expression level of IL1A mRNA. Therefore, these results strongly suggest that the proinflammatory cytokine or danger signal associated with innate immunity and not allergen-specific IgE antibody-dependent immediate hypersensitivity may be involved in the pathological condition of the chronic ACD.

We established the cut-off levels of CCL24 and CXCL8 mRNAs on the basis of the 95th percentile levels of the control group and classified patients with chronic ACDs into 4 subgroups. As a result, in addition to the most numerous CCL24^high--CXCL8^high subgroup and CCL24^low--CXCL8^low subgroup, there were patients with the CCL24^low--CXCL8^high and CCL24^high--CXCL8^low expression profiles. These results indicate that it may be helpful to examine the background of the patients with such binary inflammation profiles. The common characteristics of the 4 cases with CCL24^high--CXCL8^low expression profile included refractory AKC or VKC; although tacrolimus eye drops were continuously administered to those patients, inflammatory manifestations in the conjunctiva sometimes recurred. Furthermore, the recurrence count in the CCL24^high--CXCL8^low subgroup was significantly lower than that in the CCL24^high--CXCL8^high subgroup. Therefore, with high CCL24 mRNA expression and low CXCL8 mRNA expression on the ocular surface may have persistent eosinophilic inflammation in the conjunctiva despite treatment with immunosuppressive drugs. Therefore, it is necessary to be careful about therapeutic drug selections in common practice for these patients to avoid chronic ACD recurrence.

This study had several limitations. First, this study is a cross-sectional study for outpatients with ACD, and, therefore, the impact of interpatient differences on the cytokine profiles in tears could not be prevented. Second, mRNA expression levels of the selected cytokines and chemokines in this study could not ascertain the difference between AKC and VKC samples. The clinical utility of the suggested ocular allergy test for evaluating disease progression and severity should be improved considering medical treatment and cytokine profiles in tears in each clinical form of ACD. Third, the male-to-female ratio in our control group was 1:1, although men predominated in stable ACD and active ACD groups in our study, which is consistent with numerous reports in the literature. Although we believe that this circumstance does not diminish the utility of the selected cytokines/chemokines as biomarkers, the impact of sex is an important factor that will be clarified in our future studies. Fourth, we did not evaluate whether our ocular allergy test that utilized an impression cytology method correlated with changes in inflammatory cells associated with allergic inflammation in the conjunctiva. However, our method can estimate the variability and severity of allergic inflammation simultaneously if the ocular allergy test using real-time RT-PCR substitutes for conjunctival cytology. Further studies of the correlation between cytokine/chemokine mRNA expression levels as biomarker for ACD and the type of cytological inflammation in nontreated patients with chronic ACD are needed.

In conclusion, we demonstrated correlated expression of 2 different sets of ocular surface inflammation markers, IL16/CCL24 and CXCL8/IL1A, in chronic ACDs. Therefore, simultaneous monitoring of these markers may be a useful clinical test for the evaluation of inflammation severity and disease prognosis in patients with chronic ACDs.

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