
CLINICAL INVESTIGATION

Concentration of Soluble Interleukin-6 Receptors in Tears of Allergic Conjunctival Disease Patients

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Abstract

Purpose: To evaluate the significance of soluble interleukin-6 receptor (sIL-6R) in tears as a clinical indicator of disease exacerbation in patients with allergic conjunctivitis disease (ACD).

Methods: The study groups comprised 13 patients (13 eyes) with vernal keratoconjunctivitis (VKC group), 13 patients (13 eyes) with atopic keratoconjunctivitis (AKC group), 11 patients (11 eyes) with giant papillary conjunctivitis (GPC group), and 10 healthy volunteers (10 eyes) as a control. Tear samples were collected by the Schirmer I method using filter paper. Tear samples were eluted and the concentration of sIL-6R in tear samples was determined by enzyme-linked immunosorbent assay. Impression cytology using a nitrocellulose membrane was applied to the upper tarsal conjunctiva, and the mRNA of the signal-transducing molecule glycoprotein 130 (gp130) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was extracted from the membrane and analyzed by semiquantitative reverse transcription-polymerase chain reaction. In the VKC group, the clinical score was graded on the basis of allergic inflammation of the ocular surface, and the correlation between the concentration of sIL-6R and the clinical score was studied.

Results: The concentration of sIL-6R in tears in the VKC, AKC, GPC, and control groups was 5.1 ± 4.7 , 1.2 ± 1.7 , 2.6 ± 2.8 , and 0.5 ± 4.3 (mean $\pm$ SD) ng/ml, respectively. The concentrations of sIL-6R in tears in the VKC and GPC groups were significantly higher than those in the control group ($P < 0.001$ and 0.05 , respectively). The AKC group showed no significant change in the concentration of sIL-6R in tears in comparison with the control group. The gp130/GAPDH mRNA ratio was significantly higher in the patients with ACD than in control individuals ($P < 0.05$). The concentration of sIL-6R in tears and the clinical score of allergic inflammation of the ocular surface were significantly correlated in the VKC group ($r = 0.53$, $P < 0.01$).

Conclusions: We concluded that IL-6/sIL-6R complex trans-signaling via gp130 is an exacerbating factor of ACD, and that the concentration of sIL-6R in tears is a useful clinical biomarker in patients with ACD. **Jpn J Ophthalmol** 2007;51:332–337 © Japanese Ophthalmological Society 2007

Key Words: enzyme-linked immunosorbent assay, giant papillary conjunctivitis, soluble interleukin-6 receptors, vernal keratoconjunctivitis

Introduction

Allergic conjunctivitis disease (ACD) is an inflammatory disorder caused by immediate hypersensitivity of the ocular surface.¹ ACD can be nonproliferative, such as in allergic

conjunctivitis, or proliferative, such as in vernal keratoconjunctivitis (VKC). Proliferative lesions of the conjunctiva, giant papillary proliferation and limbal gelatinous infiltration are important clinical features of severe ACD caused by infiltration of eosinophils, CD4-positive T cells, and CD45RO-positive T cells,^{2–4} and the inflammatory cytokines produced by infiltrated inflammatory cells in the conjunctiva play a key role in maintaining allergic inflammation. In our previous study of cytokine profiles in tears of patients with VKC and giant papillary conjunctivitis (GPC), we reported that soluble interleukin-6 receptor (sIL-6R)

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Table 1. Subject characteristics

	VKC group	AKC group	GPC group	Control
No. of subjects	13	13	11	10
Sex (Male:female)	12:1	8:5	5:6	2:8
Age (years) (Mean $\pm$ SD)	21.6 $\pm$ 9.3	32.6 $\pm$ 12.9	27.9 $\pm$ 10.6	39.8 $\pm$ 13.5
No. of patients undergoing each examination				
ELISA	13	13	11	10
RT-PCR	4	4	NT	6

ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription-polymerase chain reaction; NT, not tested; VKC, vernal keratoconjunctivitis; AKC, atopic keratoconjunctivitis; GPC, giant papillary conjunctivitis.

increased significantly in both VKC and GPC patients compared with in normal individuals, on the basis of an antibody array analysis.⁵ Therefore, sIL-6R may be an important factor in the pathophysiology of severe ACD.

Interleukin-6 (IL-6) is one of the proinflammatory cytokines, the biological effect of which is thought to be host defense against infection and tissue injury. IL-6 exerts its effect through the IL-6 receptor complex, which is composed of membrane-bound IL-6 receptor (mIL-6R) and the signal-transducing molecule glycoprotein 130 (gp130).⁶ A 55-kDa sIL-6R has also been described as having both the capacity to bind to IL-6 and agonistic activity with gp130 on cells not expressing IL-6 receptor.⁷ Therefore, sIL-6R augments the biologic activities of IL-6 through IL-6/sIL-6R complexes that can bind to gp130.

However, the possible relationship between sIL-6R in tears and allergic inflammation in patients with ACD has not been thoroughly investigated. In this study, to elucidate the significance of sIL-6R in the pathophysiology of ACD with proliferative lesions of the conjunctiva, we examined sIL-6R levels in tears of patients with ACD.

Subjects and Methods

Subjects

This study was approved by the Ethics Committee for Clinical Study of the Nihon University School of Medicine. The subjects comprised 47 patients (47 eyes) with ACD treated at the Department of Ophthalmology of the Nihon University Itabashi Hospital during January and December 2005. Subject data are shown in Table 1. The subjects of this evaluation were limited to nontreated patients or patients treated only with eye drops. Patients who used oral or injected medicines for allergic treatment and those who received immunotherapy were excluded. The treatment regimen with eye drops used by the subjects is shown in Table 2.

The patients with ACD were divided into a vernal keratoconjunctivitis (VKC) group (13 patients, 13 eyes), an atopic keratoconjunctivitis (AKC) group (13 patients, 13 eyes), and a giant papillary conjunctivitis (GPC) group (11 patients, 11 eyes). ACD was diagnosed on the basis of the guidelines of the Japanese Ophthalmological Society.⁸ Each patient was diagnosed as having AKC, VKC, or GPC on the

Table 2. Treatment modalities used in the allergic conjunctivitis disease group

Clinical signs	VKC	AKC	GPC
Mast cell stabilizer only	4	0	0
Steroid only	0	0	0
Mast cell stabilizer and steroid	8	0	0
No treatment	1	13	11

basis of a clinical examination using a slit-lamp microscope and currently applied clinical guidelines.⁸ Patients with AKC who had proliferative conjunctival lesions were excluded. The enrolled patients with ACD had at least one positive allergen-specific IgE antibody to house dust mites or Japanese cedar pollen detected by AlaSTAT (Mitsubishi Kagaku Iatron, Tokyo, Japan). Informed consent for participation in this study was obtained from all subjects before enrollment. The tears were sampled from either the affected eye in the unilateral cases or the more severely affected eye in the bilateral cases. The right eyes of ten healthy normal volunteers who had neither allergic diathesis nor a history of contact lens wear were used as a control group.

Tear Sampling Methods and Enzyme-Linked Immunosorbent Assay

We used the previously reported method for sampling tears.⁹ Tears were sampled by the Schirmer I method using filter paper (Schirmer Tear Production Measuring Strips, Showa Yakuin Kako, Tokyo, Japan). After sampling, the Schirmer strip was stored at -20°C until use. Each Schirmer strip with a sample was thawed and eluted with 0.5 M NaCl and 0.5% Tween 20 containing 0.05 M phosphate-buffered solution (PBS, pH 7.2) at room temperature overnight. At that time, the amount of tears was calculated by converting 1 mm on a wet Schirmer strip to 1 μl of tear volume, and the end concentration of the elution solution contained a 40-fold-diluted tear sample.

Concentrations of sIL-6R in tear samples were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Human IL-6sR immunoassay, R&D Systems, Minneapolis, MN, USA) according to the

manufacturer's instructions. The lower detection limit given by the manufacturer was 6.5 pg/ml for sIL-6R.

Impression Cytology and Reverse Transcription-Polymerase Chain Reaction for gp130 Messenger RNA

Impression cytology was performed after instillation of topical 0.4% oxybuprocaine (Benoxil, Santen, Osaka, Japan). A strip of nitrocellulose membrane (Millipore, Bedford, MA, USA) was applied to the upper tarsal conjunctiva, pressed gently by a glass rod, and then removed. The membrane was preserved in RNAlater RNA stabilization reagent (QIAGEN, Hilden, Germany).

Total RNA from each impression cytology specimen was isolated with an RNeasy Mini kit (QIAGEN). In eight patients with ACD and six control individuals (Table 1), the expression of messenger RNA (mRNA) of gp130 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control were examined by reverse transcription-polymerase chain reaction (RT-PCR). The primers used for the RT-PCR analysis are listed in Fig. 1, and one-step RT-PCR was performed with the Qiagen one-step RT-PCR kit (QIAGEN) according to the manufacturer's instructions. In brief, the 20- μ l RT-PCR amplification solution contained the following final components: a 2- μ l aliquot of total RNA, 4 μ l of 5 $\times$ buffer, 0.8 μ l of deoxyribonucleotide triphosphate, 0.5 μ M each of sense and antisense primers, and 0.8 μ l of enzyme mix. For gp130, reverse transcription was performed at 50°C for 30 min. Initial PCR activation was performed at 94°C for 15 min, and PCR for 30 cycles in a thermal cycler (Geneamp PCR system 9700, Applied Biosystems Japan, Tokyo, Japan). Each PCR cycle contained a denaturation step at 95°C for 1 min, an annealing step at

55°C for 1 min, and an elongation step at 72°C for 1 min. For analysis of RT-PCR products, electrophoresis was performed on a 1.5% agarose gel, and the resultant products were visualized using ethidium bromide to reveal the DNA. Product sizes were 550 bp for gp130 and 432 bp for GAPDH.

Semiquantification of the results was carried out by a densitometric analysis. The optical density (OD) ratio of gp130/GAPDH was calculated using the Gel-Pro analyzer software (Media Cybernetics, Silver Spring, MD, USA).

Clinical Score

In the VKC group, the correlation between the concentration of sIL-6R in tears and clinical score of allergic inflammation of the ocular surface was evaluated in both eyes of each patient. The severity of allergic inflammation of the ocular surface was scored at the time of tear sampling on a scale from 0 to 6, on the basis of the presence (score, 1) or absence (0) of six major clinical signs (Table 3).

Table 3. Clinical signs for scoring allergic inflammation of the ocular surface

Clinical Signs
1. Giant papillary proliferation surrounded by mucus strands
2. Limbal gelatinous infiltrates
3. Horner-Trantas dots
4. Superficial punctate keratopathy with multiple discrete gray spots
5. Shield ulcer
6. Blepharitis
Each sign is scored as 1 if present or as 0 if absent; total score (0–6 points) = sum of the scores for the six clinical signs.

A

	Forward	Reverse
gp130	5'-TCTCCTTGAGCAAACCTTTGGGG-3'	5'-ACATGCTTTGGGTGGAATGG-3'
GAPDH	5'-ACCACAGTCCATGCCATCAC-3'	5'-TCCACCACCCTGTTGCTGTA-3'

B

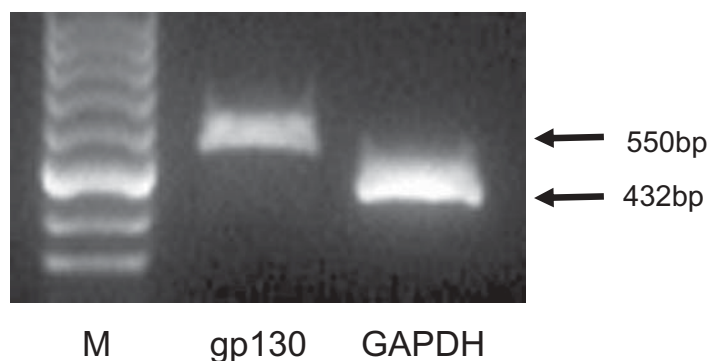


Figure 1A, B. Reverse transcription-polymerase chain reaction (RT-PCR) of signal-transducing molecule glycoprotein 130 (gp130) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was carried out. **A** PCR primers. **B** Results of the RT-PCR analysis of gp130 and GAPDH in a representative patient with vernal keratoconjunctivitis. Lane M, marker lane.

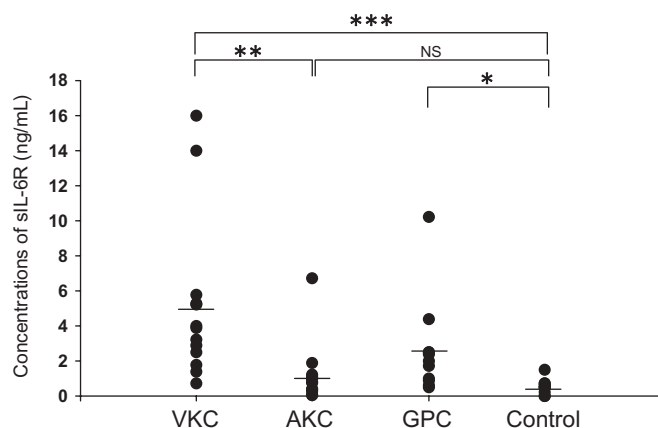


Figure 2. Comparison of the concentration of soluble interleukin-6 receptor (sIL-6R) in tears among the vernal keratoconjunctivitis (VKC), atopic keratoconjunctivitis (AKC), giant papillary conjunctivitis (GPC), and control groups. Concentrations of sIL-6R in tears in the VKC and GPC groups were significantly higher than those in the control group (** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; Kruskal-Wallis test). NS, not significant.

Statistical Analysis

The results of both the ELISA and semiquantitative RT-PCR are shown as means $\pm$ SD. Statistical significance was assessed by nonparametric analysis (Mann-Whitney U test, Kruskal-Wallis test). To analyze the relationship between the clinical score and the concentration of sIL-6R in tears, we used the Spearman rank correlation. For all tests, a P value < 0.05 was considered to be significant.

Results

Concentration of sIL-6R in Tears

The concentration of sIL-6R in tears in the VKC, AKC, GPC, and control groups was 5.1 ± 4.7 , 1.2 ± 1.7 , 2.6 ± 2.8 , 0.5 ± 4.3 (mean $\pm$ SD) ng/ml, respectively. The concentrations of sIL-6R in tears in the VKC and GPC groups were significantly higher than in the control group ($P < 0.001$ and 0.05 , respectively) (Fig. 2). The AKC group showed no significant difference in the concentration of sIL-6R in tears from that in the control group.

gp130 mRNA Expression on Ocular Surface

Expression of gp130 mRNA was identified in all patients with ACD and all control individuals (Fig. 1). As shown in Fig. 3, the OD ratio of gp130/GAPDH mRNA of the patients with ACD was significantly higher than that of control individuals ($P < 0.05$).

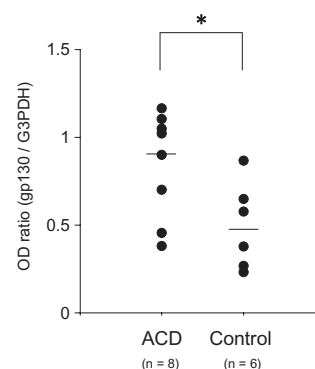


Figure 3. Results of the semiquantitative analysis of gp130 mRNA on the ocular surface by RT-PCR. The optical density (OD) ratio of gp130/GAPDH mRNA in the allergic conjunctivitis disease (ACD) group shows a significant increase in comparison with that in control individuals (* $P < 0.05$; Mann-Whitney U test).

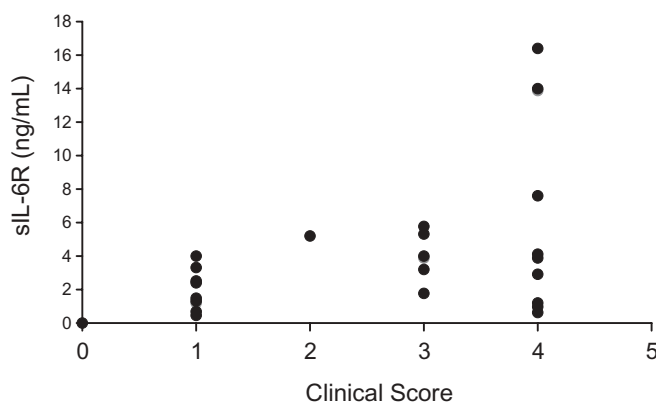


Figure 4. Relationship between the concentration of sIL-6R in tears and the clinical score of allergic inflammation of the ocular surface in patients with VKC. A positive correlation was found ($r = 0.53$; $P < 0.01$; Spearman's correlation).

Correlation Between Clinical Score and sIL-6R Concentration in Tears

In the VKC group, the clinical score of allergic inflammation of the ocular surface was 2.5 ± 1.4 (mean $\pm$ SD) points (range, 0 to 4 points), and the concentration of sIL-6R in tears was significantly correlated with the clinical score ($r = 0.53$, $P < 0.01$) (Fig. 4).

Discussion

IL-6 is a multifunctional cytokine that exerts its biological activity through binding to a specific receptor (IL-6R α) and a 130-kDa signal-transducing unit (gp130). sIL-6R has also been reported to be able to activate gp130 after binding to IL-6. Therefore, the sIL-6R/IL-6 complex acts on cells, such as endothelial cells and type 2 helper T cells (Th2), that do not primarily possess mL-6R through binding to gp130. This process of the IL-6/sIL-6R complex is termed trans-signaling.¹⁰

sIL-6R, which has been identified in various body fluids,^{10,11} is generated by two independent mechanisms, shedding¹² and splicing.¹³ Clinically, elevated plasma sIL-6R levels have been found in multiple myeloma,¹⁴ rheumatoid arthritis,¹⁵ and asthma.¹⁶ Therefore, it is assumed that it has various clinical manifestations. Furthermore, it is interesting to note that a high level of production of sIL-6R is associated with T-cell abnormalities, such as acquired immunodeficiency syndrome and adult T-cell leukemia.¹⁷

Regarding allergic disorders, there are several reports on the local detection of sIL-6R, but local levels of sIL-6R are still controversial. In the nasal fluid, levels of sIL-6R were reported to not differ significantly between patients with allergic rhinitis and healthy controls.¹⁸ However, in the bronchoalveolar lavage fluid (BALF), the concentration of sIL-6R in BALF of patients with asthma was reported to be significantly higher than that of control subjects.¹⁹ To evaluate variations of sIL-6R levels in tears, it is important to establish an index to determine the severity of ACD. Therefore, we aimed to evaluate the concentration of sIL-6R in tears and the expression of gp130 mRNA on the ocular surface, and to investigate their significance in clinical manifestations.

In this study, the patients with ACD were divided into three groups depending on whether giant papillae, the proliferative lesions of the conjunctiva, were present. In VKC group patients, the condition was atopic and giant papillae were present; in AKC group patients, the condition was atopic but no giant papillae were present; and in GPC group patients, giant papillae were present, but the condition was not atopic. The concentration of sIL-6R in tears was significantly higher in the VKC and GPC groups, but did not differ between the AKC and control groups. Therefore, the concentration of sIL-6R in tears was associated with the clinical activity of giant papillae, but it did not matter whether or not the condition was atopic. In particular, in the VKC group, the clinical score, an indicator of the severity of the allergic inflammation of the ocular surface, significantly correlated with the concentration of sIL-6R in tears. These results suggest that the concentration of sIL-6R in tears may be a useful biomarker for the pathophysiologic condition of giant papillary proliferation. The IL-6/sIL-6R complex has been reported to promote cell proliferation in both multiple myeloma cell lines²⁰ and retinal pigment epithelial cell lines.²¹ Therefore, the IL-6/sIL-6R complex plays an agonist role in cell proliferation. However, the mechanism of conjunctival proliferation caused by sIL-6R is unclear at present. Further studies are necessary to determine the proliferative effect of sIL-6R on the conjunctival tissues.

Regarding allergic inflammation, Th2 lymphocytes play a key role in the pathogenesis of allergic inflammatory diseases such as VKC, and these lymphocytes lack mIL-6R on the cell surface. Therefore, the IL-6/sIL-6R complex may affect Th2 lymphocytes via gp130, and regulate the production of Th2 cytokine. Moreover, a significant correlation between sIL-6R and IL-5 (a Th2 cytokine) concentrations has been observed in the BALF of patients

with asthma, and sIL-6R is reported to be a clinical index of lymphoid alteration in allergic inflammation.¹⁹ Pathologic findings of giant papillary proliferation in VKC are characterized by infiltration of Th2 lymphocytes and eosinophils.^{22,23} Therefore, an increased level of the IL-6/sIL-6R complex in tears may affect the Th2 lymphocyte-infiltrating giant papillae, and Th2 cytokine synthesis from Th2 lymphocytes may be enhanced. These processes are thought to be related to the exacerbation of the clinical activity of giant papillae.

On the other hand, gp130 mRNA expression on the ocular surface was significantly higher in the ACD group than in control individuals in our study. It is assumed that expression of gp130 mRNA is a constitutive cellular activity. However, an increase of gp130 expression has been observed in acutely inflamed tissues.²⁴ Therefore, our results indicate that the expression of gp130 mRNA on the ocular surface in ACD might be upregulated by allergic inflammation of conjunctival tissues.

We conclude that IL-6/sIL-6R complex trans-signaling via gp130 is an exacerbating factor in ACD, and the concentration of sIL-6R in tears is a potent biomarker in patients with a proliferative type of ACD.

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