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LABORATORY INVESTIGATION

The proteolytic effect of mast cell tryptase to eotaxin-1/CCL11·eotaxin-2/CCL24 and eotaxin-3/CCL26 produced by conjunctival fibroblasts

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Abstract

Purpose To investigate the proteolytic effect of mast cell tryptase on eotaxin-1/CCL11, eotaxin-2/CCL24 and eotaxin-3/CCL26 produced by conjunctival fibroblasts.

Study design Experimental.

Methods The production of eotaxin-1, -2 and -3 by conjunctival fibroblasts stimulated both with and without IL-4/IL-13 or/and TGF-β_1 was assessed by ELISA. The proteolytic activity of tryptase on eotaxins derived from conjunctival fibroblasts and recombinant eotaxins was also estimated by enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR).

Results Conjunctival fibroblasts produced eotaxin-1 and -3, but not eotaxin-2. Stimulation with IL-4/IL-13 and TGF-β_1 synergistically increased eotaxin-1 and -3 production. Tryptase reduced the immunoreactivity of eotaxin-1 and -3 but not of eotaxin-2, due to the proteolysis of these eotaxins but not the inhibition of their m-RNA expression.

Conclusion Mast cell tryptase may exercise proteolytic activity on eotaxin-1 and -3 produced by conjunctival fibroblasts, resulting in partial suppression of the ability of eotaxin-1 and -3 to accumulate eosinophils in the conjunctiva. Eotaxin-2 in the tears may be a suitable biomarker of severity of allergic conjunctival disease.

Keywords Vernal Keratoconjunctivitis · Tryptase · Eotaxins · Biomarker

Introduction

Humans possess two distinct mast cell (MC) subtypes distinguished on the basis of their granule neutral protease contents. The T subtype (MC_T) contains only a single neutral protease, tryptase, whereas the TC subtype (MC_{TC}) has both tryptase and chymase. In healthy subjects, conjunctival MCs consist predominantly of the MC_{TC} subtype distributed mainly in the substantia propria. In severe allergic conjunctival diseases such as vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC), both subtypes increase in the epithelium and substantia propria. The numbers and activity of MC_{TC} and MC_T subtypes may reflect the degree of severity of VKC. The determination of MC granule protease in tears of VKC patients also reflects the degree of disease severity. Several reports show that the amount of tear tryptase in VKC patients is higher than in healthy controls [1–3]. Previously we showed high tryptase and chymase activity in the tears of VKC patients [4]. The activity of tryptase was higher than that of chymase. However, whereas chymase activity correlated with severity of VKC, tryptase activity did not [4]. Therefore, we previously investigated the effects of chymase on conjunctival and corneal epithelial cells in vitro [5, 6]. Human chymase at the activity levels detected in the tear fluid of severe VKC patients caused the proteolysis of tight junction related proteins instigating effects such as occluding, resulting in a decreased barrier

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function of the corneal epithelium [5]. Similar levels of human chymase also caused the proteolysis of fibronectin, resulting in the inhibition of the migration of corneal epithelial cells and of the adhesion of conjunctival epithelial cells [6]. These results, together with the correlation between the activity of chymase in the tears of VKC patients and the severity of the disease, has led to the hypothesis that mast cell chymase plays a crucial role in the ongoing process of corneal and conjunctival tissue injury in VKC patients. Furthermore, human chymase cleaves cytokines and chemokines such as IL-3, IL-13, IL-6, IL-33, IL-18 and eotaxin-3 [7, 8]. However, in spite of the increased amount of tryptase in the tears of VKC patients there is still not enough of it to investigate the role it plays.

There are three groups of known human tryptase: α-, β-, and γ-tryptase or transmembrane tryptase. β-tryptase is the main type released during mast cell degranulation and detected in the tears of VKC patients [1–4]. Tryptase has been shown to cleave certain extracellular substrates, including vasoactive intestinal peptides, calcitonin gene-related peptides, fibronectin, and prostromelysin. Tryptase also cleaves several kinds of cytokines such as IL-33, RANTES and eotaxin-1 [9–11]. Eotaxin is a CC chemokine with a strong direct chemoattractive effect on eosinophils. Eotaxin can be produced by resident cells, including fibroblasts and smooth muscle cells, and resident cell production is believed to contribute directly to tissue eosinophilia. The eotaxin subfamily of CC chemokines consists of eotaxin-1/CCL11, eotaxin-2/CCL24 and eotaxin-3/CCL26. All eotaxins induce the trafficking of eosinophils to the sites of inflammation via CC chemokine receptor 3 (CCR3), which is also expressed by several different cell types, including basophils, dendritic cells, smooth muscle cells, epithelial cells and fibroblasts. Several reports reveal that eotaxins were found significantly increased in the tears of VKC patients compared with those of normal patients [12–14]. Eotaxins may play a crucial role in tissue eosinophilia in VKC [15]. In this study, therefore, we investigated the proteolytic effect of tryptase on eotaxin-1/CCL11, eotaxin-2/CCL24 and eotaxin-3/CCL26 produced by conjunctival fibroblasts.

Methods

Cell culture

Human conjunctival fibroblasts were purchased from ScienCell Research laboratories. Conjunctival fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS.

The use of cultured human cells and materials was approved by the Ethical Committee of Juntendo Graduate School of Medicine. Declaration of Helsinki protocols were followed.

Reagents

Human recombinant TGF-β_1, IL-4, IL-13, eotaxin-1, eotaxin-2 and eotaxin-3 were purchased from Peprotech. Human mast cell β-tryptase was obtained from Sigma. Tryptase was dissolved in phosphate buffered saline (PBS) and added to cultures at various concentrations (15ng/ml, 150ng/ml, 300ng/ml, 600ng/ml). Neither cell viability nor the appearance of conjunctival fibroblasts differed among the various concentrations of tryptase.

Enzyme-linked immunosorbent assay

To detect eotaxin-1, -2 and -3 in the conditioned medium of conjunctival fibroblasts, we used enzyme-linked immunosorbent assay (ELISA) kits (Quantikine; R&D Systems) according to the manufacturer's instructions. Conjunctival fibroblasts were grown to subconfluence in DMEM with 10% FCS, washed twice with PBS, and then incubated in serum-free DMEM for 24 hours with exposure to IL-4 or IL-13 (100ng/ml) and/or TGF-β_1 (30ng/ml) and/or tryptase (various concentrations).

Reverse transcriptase polymerase chain reaction for eotaxin-1

Total RNA from cells, treated with cytokines and tryptase for 24 hours, was harvested using an RNeasy Mini kit (Qiagen). Expression levels of m-RNA of eotaxin-1 were examined by reverse transcriptase polymerase chain reaction (RT-PCR). The primer used in the RT-PCR analysis is listed in Table 1, and one-step RT-PCR kit (Qiagen) according to the manufactures' instructions.

The PCR cycle was as follows: preincubation for 15 minutes at 94°C, incubation at 94°C for 30 seconds; a 30 seconds incubation period at the annealing temperature (62°C); a 30-s incubation period at 72°C; and a 10-minutes incubation period at 72°C for final elongation. Cycle numbers in the thermal cycler (GeneAmp® PCR System 9700, Applied Biosystems) was determined empirically as 40 cycles. For analysis of the RT-PCR products, electrophoresis was performed using 1.5% agarose gel, and the resultant products were visualized using ethidium bromide to reveal the DNA. Product size was 227bp for eotaxin-1.

Statistical analysis

Results are expressed as the mean ±SE. Differences were evaluated by Student's t-test (Figs. 1, 2, 3, 4, 5, 6) using analytical software (Excel; Microsoft).

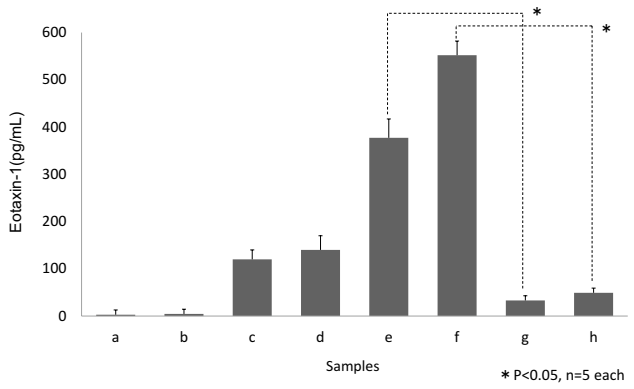

Fig. 1 Tryptase reduced the immunoreactivity of eotaxin-1/CCL11 derived from conjunctival fibroblasts. Both IL-4 and IL-13 increased the production of eotaxin-1/CCL11. The combination of TGF-β_1 and IL-4/IL-13 induced a marked increase in eotaxin-1/CCL11 production. Tryptase inhibited the immunoreactivities of these eotaxin-1/CCL11 productions. a control, b TGF-β_1 (30ng/mL), c IL-13 (100ng/mL), d IL-4 (100ng/mL), e IL-13+ TGF-β_1, f IL-4+TGF-β_1, g IL-13+TGF-β_1+Tryptase (150ng/mL), h IL-4+TGF-β_1+Tryptase (150ng/mL)

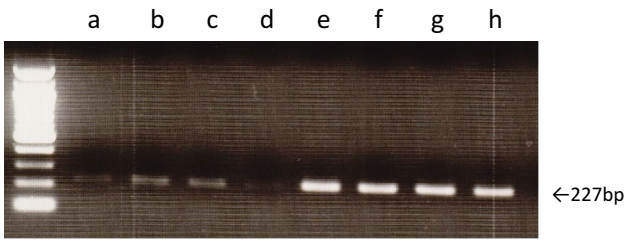

Fig. 2 mRNAs Expression of eotaxin-1 in conjunctival fibroblasts. Eotaxin-1/CCL11 mRNA was constitutively expressed in conjunctival fibroblasts, and was slightly enhanced by the stimulation of IL-4 or IL-13, was strongly enhanced by the stimulation of TGF-β_1 and IL-4/IL-13. However, tryptase had no effect on eotaxin-1/CCL11 mRNA expression. a control, b IL-4, c IL-13, d TGF-β_1, e IL-4+TGF-β_1, f IL-13+ TGF-β_1, g IL-4+TGF-β_1+Tryptase, h IL-13+TGF-β_1+Tryptase (The concentrations of IL-4, IL-13, TGF-β_1 and tryptase is same as Fig. 1)

Results

Tryptase reduced the immunoreactivity of eotaxin-1/CCL11 derived from conjunctival fibroblasts without affecting its mRNA expression (Fig. 1, 2, 3)

To examine the effect of tryptase on eotaxin-1 derived from conjunctival fibroblasts, we measured the immunoreactivity of the supernatant of cultured conjunctival fibroblasts both with and without tryptase for 24 hours.

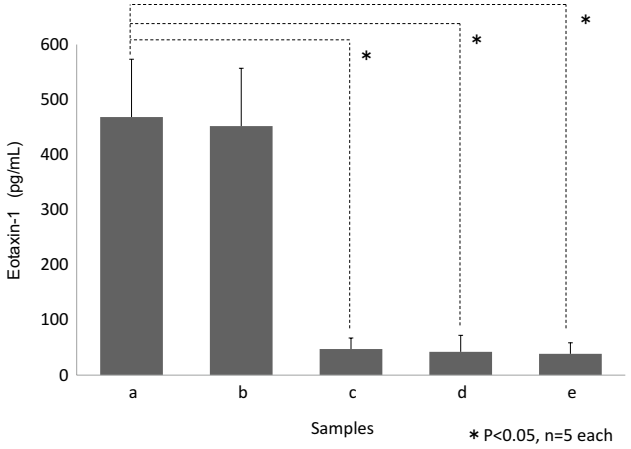

Fig. 3 Tryptase reduced immunoreactivities of recombinant eotaxin-1 in concentration dependent manner. Tryptase: a 0ng/ml, b 15ng/ml, c 150ng/ml, d 300ng/ml, e 600ng/ml

Figure 1 shows very low eotaxin levels in the culture medium of cells incubated in the absence of cytokines. Addition of IL-4 or IL-13 increased the production of eotaxin-1, while incubation of cells with the combination of TGF-β_1 and IL-4 or IL-13 induced a marked increase in eotaxin-1production. However, tryptase inhibited the immunoreactivity of eotaxin-1 in the culture medium of conjunctival fibroblasts stimulated with the combination TGF-β_1 and IL-4/IL-13. To explore whether the effect of tryptase on the immunoreactivity of eotaxin-1 derived from conjunctival fibroblasts was due to its inhibition of eotaxin-1 mRNA expression, we investigated the expression of mRNA of eotaxin-1 in cultured conjunctival fibroblasts treated both with and without cytokines. Eotaxin-1 mRNA was constitutively expressed in conjunctival fibroblasts, and was slightly enhanced by the stimulation of IL-4 or IL-13; it was strongly enhanced by the stimulation of TGF-β_1 and IL-4 or IL-13 after 2 hours' treatment. However, tryptase had no effect on IL-4/IL-13 and TGF-β_1 induced eotaxin-1 mRNA expression (Fig. 2). Next, we estimated the effect of tryptase on the immunoreactivity of recombinant eotaxin-1. Recombinant eotaxin-1 (500pg/ml) was incubated with various concentrations of tryptase at 37°C for 30 minutes. As shown in Figure 3, a small loss in the immunoreactivity of eotaxin-1 was observed after incubation in the absence of tryptase, whereas tryptase reduced the immunoreactivity of eotaxin-1 in a concentration dependent manner. (A marked reduction was observed at a concentration as low as 150ng/ml). These results suggest that tryptase-induced loss of immunoreactivity of eotaxin-1 derived from conjunctival fibroblasts is likely to be post-transcriptional, involving its proteolytic activity.

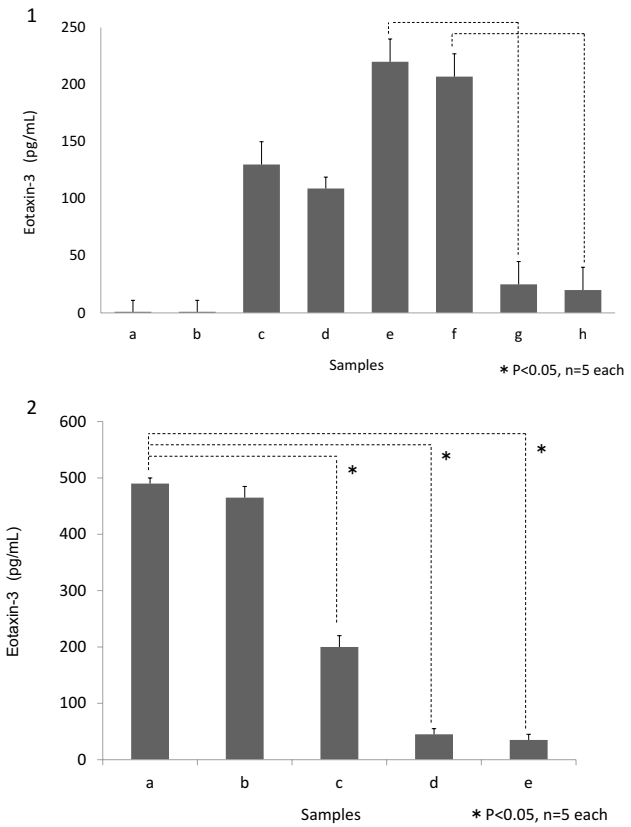

Fig. 4 Tryptase reduced the immunoreactivities of eotaxin-3/CCL26 derived from conjunctival fibroblasts and recombinant eotaxin-3/CCL26. 4-1. Both IL-4 and IL-13 increased the production of eotaxin-3/CCL26. The combination of TGF-β_1 and IL-4/IL-13 induced a marked increase in eotaxin-3/CCL26 production. Tryptase inhibited the immunoreactivity of eotaxin-3/CCL26 production. a control, b TGF-β_1, c IL-13, d IL-4, e IL-13+ TGF-β_1, f IL-4+TGF-β_1, g IL-13+TGF-β_1+Tryptase, h IL-4+TGF-β_1+Tryptase (The concentrations of TGF-β_1, IL-4, IL-13 and tryptase is same as in Fig. 1). 4.2 Tryptase reduced immunoreactivy of recombinant eotaxin-3/CCL26 in concentration dependent manner. Tryptase: a 0ng/ml, b 15ng/ml, c 150ng/ml, d 300ng/ml, e 600ng/ml

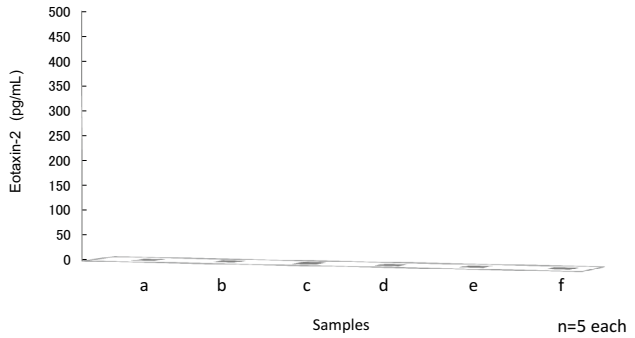

Fig. 5 IL-4/IL-13 and/or TGF-β_1 did not induce the production of eotaxin-2/CCL24 by conjunctival fibroblasts. Conjunctival fibroblasts did not produce eotaxin-2/CCL24. IL-4/IL-13 and/or TGF-β1 did not induce the production of eotaxin-2/CCL24 by conjunctival fibroblasts. a control, b TGF-β_1, c IL-13, d IL-4, e IL-13+ TGF-β_1, f IL-4+TGF-β_1 (The concentration of IL-4, IL-13, TGF-β1 tryptase is same as Figure 1)

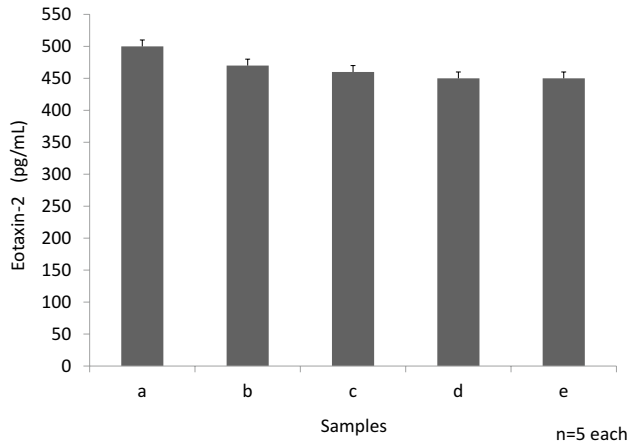

Fig. 6 Tryptase did not reduce the immunoreactivities of recombinant eotaxin-2/CCL24. Tryptase: a 0ng/ml, b 15ng/ml, c 150ng/ml, d 300ng/ml, e 600ng/ml

Tryptase reduced the immunoreactivity of eotaxin-3/CCL26 derived from conjunctival fibroblast and recombinant eotaxin-3/CCL26 (Fig. 4-1, 2)

To examine the effect of tryptase on eotaxin-3 derived from conjunctival fibroblasts, we also measured the immunoreactivity of the supernatant of cultured conjunctival fibroblasts treated with and without tryptase for 24 hours. Figure 4-1 shows that eotaxin-3 levels were very low in the supernatant of cells with no stimulation. Addition of Il-4 or IL-13 increased the production of eotaxin-3, while incubation of cells with the combination of TGF-β_1 and IL-4/IL-13 induced a marked increase in eotaxin-3 production. However, tryptase inhibited the immunoreactivity of eotaxin-3 in the supernatant of cells stimulated with the combination TGF-β_1 and IL-4/IL-13. Next, we estimated the effect of tryptase on the immunoreactivity of recombinant eotaxin-3. Recombinant eotaxin-3 (500pg/ml) was incubated with tryptase at 37°C for 30 minutes. As shown Figure 4-2, a small loss in immunoreactivity of eotaxin-3 was observed after incubation in the absence of tryptase, whereas tryptase reduced the immunoreactivity of eotaxin-3 in a concentration dependent manner. (A marked reduction was observed at a concentration as low as 150ng/ml). These results suggest that tryptase-induced loss of immunoreactivity of eotaxin-3 derived from conjunctival fibroblasts is likely to be post-transcriptional, involving its proteolytic activity.

IL-4/IL-13 and/or TGF-β_1 did not induce the expression of eotaxin-2/CCL24 by conjunctival fibroblasts (Fig. 5)

To examine whether conjunctival fibroblasts produce eotaxin-2, we estimated the immunoreactivity of the supernatant of cultured cells by ELISA. The production of eotaxin-2 by these cells was not noticeable even when cells were stimulated with IL-4/IL-13 or/and TGF-β_1.

Tryptase did not reduce the immunoreactivity of recombinant eotaxin-2/CCL24 (Fig. 6)

We estimated the effect of tryptase on the immunoreactivity of recombinant eotaxin-2. Recombinant eotaxin-2 (500pg/ml) was incubated with tryptase at 37°C for 30 minutes. As shown in Figure 6, a small loss in immunoreactivity of eotaxin-2 was observed after incubation in various concentrations of tryptase.

Discussion

In this study, we revealed that the production of eotaxin-1 and -3 by cultured conjunctival fibroblasts worked constitutively. The stimulation with IL-4/IL-13 increased the production of these eotaxins and TGF-β_1 and IL-4/IL-13 synergistically increased the production of these eotaxins. However, the production of eotaxin-2 by conjunctival fibroblasts stimulated with or without IL-4/IL-13 and/or TGF-β_1 was not detected. It is known that high concentrations of IL-4/IL-13 and TGF-β_1 can be detected in the tears of eyes with severe allergic conjunctival diseases, such as VKC and AKC [16–19]. Therefore, it is possible that increased levels of IL-4/IL-13 and TGF-β_1 in the tears enhance the production of eotaxin-1 and -3 by conjunctival fibroblasts and induce tissue eosinophilia associated with severe allergic conjunctival diseases.

We also revealed that tryptase reduced the immunoreactivity of eotaxin-1 and -3 by conjunctival fibroblasts due to the proteolysis of these eotaxins. Tryptase also reduced the immunoreactivity of recombinant eotaxin-1 and -3, but not eotaxin-2. These findings support the view that tryptase derived from mast cells can modulate local concentrations of eotaxin-1 and -3 in the conjunctiva.

Therefore, eotaxin-1 and -3 derived from conjunctival fibroblasts were stimulated by tryptase. Tryptase selectively reduced the immunoreactivity of recombinant eotaxin-1 and -3, while having little effect on eotaxin-2. Eotaxin-2 is only 39% homologous to eotaxin-1 and is located on chromosome 7q 11.23 (whereas eotaxin-1 is located on chromosome 17q 21.1). The eotaxin-3 gene lies close to the eotaxin-2 gene on chromosome 7 but shares only 33% homology with eotaxin-2. As eotaxin-2 and other eotaxins differ structurally, only eotaxin-2 may not be susceptible to cleavage by tryptase.

In this study, eotaxin-1 and -3 were produced by conjunctival fibroblasts. Eotaxin-2 was not produced by conjunctival fibroblasts and epithelial cells (data not shown). Watanabe et al. reveal that under basal conditions or when stimulated with IL-4/IL-13, human dermal fibroblasts produced eotaxin-1 but did not generate eotaxin-2. However, monocytes produced eotaxin-2 constitutively and macrophages stimulated with IL-4 produced eotaxin-2 [20]. Leonardi et al. reveal that in severe allergic conjunctival diseases eotaxin-2 was produced primarily by monocytes and macrophages [11]. Therefore, in the tears of patients with VKC or AKC, eotaxin-1 and -3 may be mainly produced by conjunctival fibroblasts and eotaxin-2 by inflammatory cells in the conjunctiva.

Recently, Shoji J et al. used ELISA to examine the concentration of eotaxin-1 and 2 in tear samples obtained from 25 VKC patients [14, 21]. The expression ratio of eotaxin-1 and 2 in tears was significantly higher in the VKC group than in the control group. However, in the VKC group, the concentration of eotaxin-2 in tears was higher than that of eotaxin-1 (median values; eotaxin-1: 0.7pg/ml vs eotaxin-2: 1440.5pg/ml). There was a significant correlation between the concentration of eotaxin-2 and that of eosinophil cationic protein in tears in the VKC group. On the other hand, in the RT-PCR analysis, the positive ration of eotaxin-1 and 2 mRNA in conjunctival epithelial cells obtained from VKC patients using impression cytology was not different between eotaxin-1 and eotaxin-2. These results may depend on the differences in sensitivities to proteolysis by tryptase.

In conclusion, Eotaxin-1 and -3 in the tears of patients with severe VKC were mainly produced by conjunctival fibroblasts. Eotaxin-2 in the tears of these patients may have been mainly produced by infiltrating cells in the conjunctiva. Cleavage of eotaxin-1, 3 by tryptase may therefore provide a feedback regulation to prevent the excessive eosinophils infiltration into the conjunctiva of VKC patients. Therefore, eotaxin-2 in the tears may be a suitable biomarker of disease severity of VKC.

Conflicts of interest Y. Miyagawa, None; A. Murakami, None; N. Ebihara, None.

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