Synergistic Induction of Eotaxin Expression in Human Keratocytes by TNF-α and IL-4 or IL-13

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PURPOSE. To investigate the effects of tumor necrosis factor (TNF)-α, interleukin (IL)-4, and IL-13 on expression of the chemokine eotaxin by cultured human keratocytes.

METHODS. Cultured human keratocytes were incubated with various combinations and concentrations of TNF-α, IL-4, and IL-13. The concentration of eotaxin in the culture supernatant was subsequently measured by enzyme-linked immunosorbent assay, and the amount of eotaxin mRNA in cell lysates was determined by reverse transcription–polymerase chain reaction analysis.

RESULTS. Keratocytes incubated in the absence of cytokines did not release detectable amounts of eotaxin into the culture medium. Whereas incubation of keratocytes with TNF-α, IL-4, or IL-13 alone or with the combination of IL-4 and IL-13 had only a small effect on eotaxin release, exposure of the cells to TNF-α in combination with either IL-4 or IL-13 resulted in a marked increase in eotaxin production that was both time and dose dependent. The abundance of eotaxin mRNA in keratocytes was also increased in a synergistic manner by incubation of cells with TNF-α together with either IL-4 or IL-13.

CONCLUSIONS. Stimulation of human keratocytes with the combination of TNF-α and either IL-4 or IL-13 resulted in synergistic increases in both the abundance of eotaxin mRNA and the release of eotaxin protein. This cytokine-induced increase in eotaxin production by keratocytes may contribute to eosinophil infiltration in inflammatory ocular diseases such as vernal keratoconjunctivitis.

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Vernal keratoconjunctivitis (VKC) is characterized by chronic allergic inflammation of the conjunctiva that is accompanied by ocular itching and conjunctival hyperemia. Corneal epithelial lesions often develop secondary to the conjunctival allergic reaction, with eosinophils infiltrating the conjunctiva and releasing various types of eosinophilic granular proteins that are toxic to corneal epithelial cells. Extravasation and local accumulation of eosinophils during allergic inflammation are regulated by specific chemokines such as RANTES (regulated on activation, normal T expressed and secreted) and eotaxin, the latter of which is a potent and specific eosinophil chemoattractant. Furthermore, the activation of accumulated eosinophils is modulated by stromal cells. Thus, coculture of eosinophils with lung fibroblasts increases the extent of eosinophil degranulation. The interaction between eosinophils and fibroblasts is an important regulatory step in the pathogenesis of allergic diseases.

Various types of cytokines are produced by inflammatory cells during allergic reactions. The proinflammatory cytokines and T helper cell-2 (Th-2)-type cytokines play important roles in the pathogenesis of allergic diseases. The proinflammatory cytokine tumor necrosis factor (TNF)-α is expressed by mucosal mast cells and is released during acute allergic reactions.

The Th-2 type cytokine interleukin (IL)-4 induces immunoglobulin isotype switching in B cells and maintains the production of IgE. Increased concentrations of IL-4 and TNF-α have been detected in the tear fluid of individuals with VKC. IL-13 is another Th-2–type cytokine that also induces IgE production and modifies IgE-mediated allergic responses. Although the presence of IL-13 in tear fluid has not been demonstrated, the concentration of IL-13 in bronchoalveolar lavage fluid is increased in allergen-challenged individuals with asthma. It is thus possible that cytokines, especially TNF-α, IL-4, and IL-13, modify the functions of inflammatory and structural cells in the cornea of individuals with VKC.

Previous studies have suggested that corneal keratocytes may release chemokines and thereby contribute to the local accumulation of eosinophils. Understanding the pathogenesis of corneal epithelial disorders associated with VKC necessitates characterization of the possible effects of proinflammatory cytokines and Th-2 cytokines on the release of chemokines from corneal stromal keratocytes. We therefore examined the effects of TNF-α, IL-4, and IL-13 on eotaxin expression, at both the protein and mRNA levels, in cultured human keratocytes.

METHODS

Materials

Eagle’s minimum essential medium (MEM) was obtained from the Research Foundation for Microbial Diseases of Osaka University (Osaka, Japan), fetal bovine serum (FBS) was from Dainippon Pharmaceutical (Osaka, Japan), OPTI-MEM and trypsin (0.25%)-EDTA (0.02%) were from Gibco (Grand Island,
were endotoxin minimized. Bioproducts (Rockland, ME). All reagents used for cell culture (Toyama, Japan), and agarose (NuSieve 3:1) was from FMC molecular size standards (Marker 11) were from Nippongene 2400-R; Basel, Switzerland), ethidium bromide and DNA mo-
cation system was from Roche Diagnostic Systems (GeneAmp AMV) was from Takara Shuzo (Shiga, Japan), the PCR amplifi-
Qiagen (Hilden, Germany), the PCR kit (TaKaRa One-Step RNA; from Tosoh (Tokyo, Japan). The RNeasy mini kit was from
llonsorbent assay (ELISA) kit for eotaxin was from R&D Systems from Peprotech (Rocky Hill, NJ). The enzyme-linked immu-
Genzyme (Cambridge, MA), and human recombinant IL-13 was

were endotoxin minimized.

Isolation, Culture, and Stimulation of Human Keratocytes

Four human corneas were obtained from Mid-America Transplant Service (St. Louis, MO). The donors were white men and women ranging in age from 4 to 65 years. After the center of each donor cornea was punched out for corneal transplantation surgery (performed by TN), the remaining rim of the cornea was used for the present experiments. The human tissue was used in strict accordance with the basic principles of the Declaration of Helsinki. Each cornea was digested separately with collagenase to provide a suspension of keratocytes.14 The cells from each cornea were cultured separately in MEM supplemented with 10% FBS, and they were used for the present studies after four to six passages. The purity of the keratocyte cultures was judged on the basis of both cell morphology and reactivities with antibodies to vimentin and to cytokeratin12 (Fig. 1). All the cells were positive for vimentin and negative for cytokeratin, suggesting the absence of contamination of the cultures by epithelial cells. No changes in cell morphology or immunoreactivity were apparent after culture for up to four to six passages.

Cells (5 × 10^5) were seeded in 60-mm dishes and grown until they became subconfluent, after which the culture medium was replaced with OPTI-MEM (without FBS), and the cells were incubated for 3 days. The medium was then replaced with OPTI-MEM supplemented with various concentrations of TNF-α, IL-4, or IL-13. After incubation for the indicated periods of time, the medium was collected from each culture dish and centrifuged at 120g for 5 minutes. The resultant supernatants were frozen at −80°C for subsequent assay of eotaxin. The keratocytes remaining in the dish were exposed to trypsin-EDTA, and their number was determined with a hemocytometer.

Determination of Eotaxin Concentration by ELISA

The concentration of eotaxin in culture supernatants was quantified with an ELISA kit, with absorbance at 450 nm being determined with a microplate reader. The limit of detection was 5 pg/ml. The morphology and number of cells were not affected by incubation with cytokines for 24 hours. The concentration of eotaxin in the culture medium was therefore normalized by expression as nanograms of eotaxin per 10^6 cells.

RT-PCR Analysis of Eotaxin mRNA

Keratocytes were cultured and stimulated as described, after which the cells were washed with phosphate-buffered saline, and total RNA was extracted with the commercial kit. The abundance of eotaxin mRNA was determined by reverse transcription–polymerase chain reaction (RT-PCR) analysis with the PCR kit. Transcripts of the constitutively expressed gene for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) served as an internal control to confirm that equal amounts of RNA were analyzed. The sequences of the PCR primers were as follows: eotaxin forward, 5'-AAGCTTAGCCAAAAGCTCA-CACCT-3'; eotaxin reverse, 5'-GAATCCCTGGCTTGGAGTTGGGATGG-3'; G3PDH forward, 5'-GCCAAAAGGGTCATCATCTC-3'; and G3PDH reverse, 5'-ACCACCTGGTGCTCAGTGTA-3'.

These primers yielded PCR products of the expected sizes of 322 bp for eotaxin mRNA15 and 500 bp for G3PDH mRNA.16,17 RT-PCR was performed with the commercial PCR system. The PCR protocol comprised 25 cycles of 1 minute at 94°C, 2 minutes at 60°C, and 3 minutes at 72°C, and the reaction was terminated by cooling to 4°C. The amplification products were subjected to electrophoresis on a 4% agarose gel, which was then stained with ethidium bromide (1 μg/ml). Densitometry was performed with a system (Nighthawk; pdi, Huntington Station, NY) comprising a charge-coupled device camera, an
ultraviolet transilluminator, and an analysis program (Quantity One). The abundance of eotaxin mRNA was normalized on the basis of the amount of G3PDH mRNA.

Statistical Analysis
Data are expressed as means ± SEM from four separate experiments unless indicated otherwise. Differences were analyzed by Student’s t-test or by analysis of variance and Fisher’s protected least significant difference (PLSD) test. \( P < 0.01 \) was considered statistically significant.

RESULTS
Synergistic Effects of TNF-α and Either IL-4 or IL-13 on Eotaxin Release by Human Keratocytes
We first examined the effects of TNF-α, IL-4, and IL-13 on the production of eotaxin by human keratocytes isolated from four different donors. The cells were cultured for 24 hours with each cytokine present at a concentration of 10 ng/ml, either alone or in various combinations.

Eotaxin was not detected in the culture medium of cells incubated in the absence of these agents. For all four keratocyte preparations examined, the addition of TNF-α, IL-4, IL-13, or the combination of IL-4 and IL-13 resulted in only a small increase in the amount of eotaxin in the culture medium (Fig. 2). However, incubation in the presence of TNF-α and either IL-4 or IL-13 induced a marked increase in the release of eotaxin by all four cell preparations. No correlation was apparent between the extent of eotaxin release and the age or sex of the corneal donors. Given that the responses of the cells from the four different donors were almost identical, we performed subsequent experiments with keratocytes from one donor.

We next investigated the time course of eotaxin release by keratocytes incubated for up to 24 hours with TNF-α (10 ng/ml) and either IL-4 (10 ng/ml) or IL-13 (10 ng/ml). Whereas incubation of cells with TNF-α, IL-4, or IL-13 alone, or with the combination of IL-4 and IL-13, had only a small effect on the amount of eotaxin in the culture medium at any of the time points examined (data not shown), exposure of keratocytes to the combination of TNF-α with either IL-4 or IL-13 resulted in an approximately linear increase in eotaxin release with incubation time (Fig. 3); the increase was statistically significant \( P < 0.01 \) at 12 and 24 hours for both combinations of cytokines, compared with either the zero time point or with the corresponding times for cells incubated in the absence of cytokines.

The dose dependence of the effect of the combination of TNF-α and IL-4 on the production of eotaxin by keratocytes was then examined. Keratocytes were cultured for 24 hours with various concentrations of TNF-α in the absence or presence of IL-4 (10 ng/ml; Fig. 4), or with various concentrations of IL-4 in the absence or presence of TNF-α (10 ng/ml; Fig. 5). In the absence of IL-4, TNF-α had only a small effect on eotaxin

FIGURE 2. Effects of cytokines on eotaxin production by human keratocytes derived from different donors. Keratocytes prepared from four different donors were incubated for 24 hours with TNF-α (10 ng/ml), IL-4 (10 ng/ml), IL-13 (10 ng/ml), or various combinations thereof, after which the concentration of eotaxin in the culture supernatant was measured by ELISA. Symbols represent the mean values obtained from four separate experiments with cells from a single donor; horizontal bars represent the overall mean values for cells from all four donors.

FIGURE 3. Time course of eotaxin release from keratocytes stimulated with a combination of TNF-α and either IL-4 or IL-13. Human keratocytes were cultured for the indicated times in the presence of 10 ng/ml TNF-α and 10 ng/ml of either IL-4 (●) or IL-13 (○), after which culture supernatants were assayed for eotaxin by ELISA. Data are means ± SEM of values from four separate experiments. * \( P < 0.01 \) (Student’s t-test) versus the zero time point; * \( P < 0.01 \) versus the corresponding time point for cells incubated in the absence of cytokines.

FIGURE 4. Dose dependence of the effect of TNF-α in the absence or presence of IL-4 on eotaxin release by keratocytes. Human keratocytes were cultured for 24 hours with the indicated concentrations of TNF-α in the absence (○) or presence (●) of IL-4 (10 ng/ml). Culture supernatants were then assayed for eotaxin by ELISA. Data are means ± SEM of values from four separate experiments. * \( P < 0.01 \) (Fisher’s PLSD test) versus the corresponding value for cells incubated in the absence of IL-4.
release at any of the concentrations examined; however, in the
presence of IL-4, TNF-α induced a dose-dependent increase in
eotaxin release (Fig. 4). Similarly, in the absence of TNF-α, IL-4
had only a small effect on eotaxin release at any of the con-
centrations examined; however, in the presence of TNF-α, IL-4
induced a dose-dependent increase in eotaxin release (Fig. 5).

The dose dependence of the effect of TNF-α and IL-13 on
the production of eotaxin by keratocytes was similarly exam-
in. Whereas TNF-α had only a small effect on eotaxin produc-
tion at any of the concentrations examined in the absence
of IL-13, in the presence of IL-13 it induced a dose-dependent
increase in eotaxin release that was maximal at 1 ng/ml (Fig. 6).
Similarly, in the absence of TNF-α, IL-13 had only a small effect
on eotaxin production at any concentration examined; how-
ever, in the presence of TNF-α, eotaxin release was increased
by IL-13 in a dose-dependent manner that was maximal at an
IL-13 concentration of 10 ng/ml (Fig. 7).

**Synergistic Effects of TNF-α and Either IL-4 or IL-13 on the Abundance of Eotaxin mRNA in Keratocytes**

Finally, we investigated the effects of TNF-α, IL-4, and IL-13 on
the abundance of eotaxin mRNA in human keratocytes. Cells
were cultured for 24 hours in the absence or presence of
cytokines at concentrations of 10 ng/ml, after which the
amount of eotaxin mRNA in cell lysates was assayed by RT-PCR
(Fig. 8) and quantified by densitometry. Eotaxin mRNA was not
detected in lysates of unstimulated keratocytes, but was appar-
ent in keratocytes exposed to either TNF-α, IL-4, or IL-13. The
abundance of this transcript was increased further by simulta-

**Figure 5.** Dose dependence of the effect of IL-4 in the absence or presence of TNF-α on eotaxin release by keratocytes. Human keratocytes were cultured for 24 hours with the indicated concentrations of IL-4 in the absence (○) or presence (●) of TNF-α (10 ng/ml). Culture supernatants were then assayed for eotaxin by ELISA. Data are means ± SEM of values from four separate experiments. *P < 0.01 (Fisher’s PLSD test) versus the corresponding value for cells incubated in the absence of TNF-α.

**Figure 6.** Dose dependence of the effect of TNF-α in the absence or presence of IL-13 on eotaxin release by keratocytes. Human keratocytes were cultured for 24 hours with the indicated concentrations of TNF-α in the absence (○) or presence (●) of IL-13 (10 ng/ml). Culture supernatants were then assayed for eotaxin by ELISA. Data are means ± SEM of values from four separate experiments. *P < 0.01 (Fisher’s PLSD test) versus the corresponding value for cells incubated in the absence of IL-13.

**Figure 7.** Dose dependence of the effect of IL-13 in the absence or presence of TNF-α on eotaxin release by keratocytes. Human keratocytes were cultured for 24 hours with the indicated concentrations of IL-13 in the absence (○) or presence (●) of TNF-α (10 ng/ml). Culture supernatants were then assayed for eotaxin by ELISA. Data are means ± SEM of values from four separate experiments. *P < 0.01 (Fisher’s PLSD test) versus the corresponding value for cells incubated in the absence of TNF-α.

**Figure 8.** Effects of cytokines on the abundance of eotaxin mRNA in human keratocytes. Keratocytes were cultured for 24 hours in the absence (−) or presence of 10 ng/ml TNF-α, IL-4, or IL-13 or the combination of TNF-α with either IL-4 or IL-13, after which cell lysates were assayed for eotaxin mRNA by RT-PCR. Lane M: DNA molecular size standards. The positions and sizes of the amplification products derived from eotaxin and G3PDH mRNAs are indicated. Data are from a representative experiment that was repeated three times with similar results.
neous exposure of cells to TNF-α together with either IL-4 or IL-13.

**DISCUSSION**

We have shown that the combination of TNF-α with either IL-4 or IL-13 induces a synergistic increase in the expression of eotaxin at both the mRNA and protein levels in cultured human corneal keratocytes. Exposure of keratocytes to each of these cytokines individually had only a small effect on eotaxin production. Our results are thus consistent with the previous observation that eotaxin production by skin fibroblasts is increased by costimulation with TNF-α and IL-4. Local production of eotaxin by fibroblast-like cells may therefore be widespread throughout the body. In skin, eotaxin is released from fibroblasts, which are biologically similar to corneal keratocytes, but not from keratinocytes, which are similar to corneal epithelial cells. Similarly, we have also shown that simian virus-40-transformed human corneal epithelial cells do not produce detectable amounts of eotaxin when exposed to TNF-α and either IL-4 or IL-13 (unpublished observations, June, 1999). The reason for this difference in the ability to produce eotaxin between keratocytes and corneal epithelial cells requires further investigation.

Our results suggest that activation of keratocytes is an important step in the pathogenesis of eosinophilic infiltration in the ocular region and that keratocytes and local immune cells communicate through cytokines and chemokines released into tear fluid. Various cytokines participate in allergic inflammatory reactions. Whereas proinflammatory cytokines such as TNF-α play important roles in inflammatory reactions regardless of causative factor, such as injury, infection, immunologic response, or allergy, Th-2 cytokines such as IL-4 and IL-13 are the principal players in allergic reactions. The initial pathobiology of allergic reactions in the conjunctiva is almost identical in individuals with allergic conjunctivitis and in those with VKC. However, only individuals with VKC develop corneal ulceration, shield ulcer, or corneal plaque. Whereas an increased concentration of IL-4 in tear fluid has been detected in individuals with either seasonal allergic conjunctivitis or VKC, an increased concentration of TNF-α in tear fluid has been demonstrated in only a few patients with VKC. It is thus possible that the increased concentrations of both IL-4 and TNF-α in tear fluid of individuals with VKC induce the production of eotaxin by keratocytes. Given that eotaxin is a potent and specific chemoattractant for eosinophils, activated keratocytes may induce the infiltration of eosinophils into the corneal stroma. The release from the infiltrated eosinophils of degradative enzymes that target extracellular matrix proteins then is likely to result in the corneal ulceration associated with VKC.

Both IL-4 and IL-13 increased the production of eotaxin in keratocytes in the presence of TNF-α. The actions of IL-4 and IL-13 are similar, because the corresponding receptor complexes both share the IL-4 receptor α chain, although these two cytokines are produced by different T-cell subsets and dendritic cells. However, recent studies have provided evidence for important differences between these two cytokines, such as in their effects on airway mucus secretion and airway fibrosis. In the present study, the effect of IL-4 on eotaxin production was greater than that of IL-13 when keratocytes were stimulated with low concentrations of these cytokines in the presence of TNF-α. Differences in the roles of IL-4 and IL-13 in ocular allergic reactions require further investigation.

Although the main function of eotaxin in allergic reactions is to promote eosinophil accumulation, other actions of this chemokine, such as induction of the expression of adhesion molecules on vascular endothelial cells and on epithelial cells, as well as enhancement of eosinophil degranulation, may contribute to allergic eye diseases.

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**References**
