

Interleukin-13 and tumour necrosis factor- α synergistically induce eotaxin production in human nasal fibroblasts

N. TERADA*, N. HAMANO*, T. NOMURA*, T. NUMATA*, K. HIRAI†, T. NAKAJIMA†, H. YAMADA‡, O. YOSHIE§, T. IKEDA-ITO¶ and A. KONNO*

*Department of Otorhinolaryngology, Chiba University School of Medicine, Chiba, †Department of Allergy and Rheumatology, University of Tokyo Graduate School of Medicine, Tokyo, ‡Department of Bioregulatory Function, University of Tokyo Graduate School of Medicine, Tokyo, §Department of Microbiology, Kinki University School of Medicine, Osaka Sayama and ¶Research Institute, Nissui Pharmaceutical Co. Ltd, Yuki, Japan

Summary

Background There is increasing evidence that eotaxin is a key mediator in the development of tissue eosinophilia. However, the mechanism involved in the production of eotaxin has yet to be clarified. Most recently, it has been shown that interleukin (IL) -4 induces eotaxin in dermal fibroblasts. A novel cytokine termed IL-13, which binds to the α -chain of the IL-4 receptor, shares many biological activities with IL-4. It is known that fibroblasts express the IL-4 receptor and produce collagen type I upon stimulation with IL-4.

Objective We investigated whether IL-13, as well as IL-4, are able to induce eotaxin production in human nasal mucosal fibroblasts (HNMFs). Furthermore, we investigated the effect of costimulation of IL-13 and TNF α on eotaxin production.

Methods HNMFs, isolated from inferior nasal mucosa samples, were stimulated by various kind of cytokines for 1–36 h at 37 °C in 5% CO₂. The change in the expression of eotaxin mRNA was then evaluated by reverse transcriptase-polymerase chain reaction and the Southern blot analysis. The amount of eotaxin in the culture media was measured by ELISA.

Results IL-13 as well as IL-4 dose-dependently induced eotaxin expression in HNMFs. Furthermore, IL-13 and TNF α synergistically induced eotaxin expression in HNMFs, while they hardly induced eotaxin expression in endothelial cells, epithelial cells or eosinophils. The synergy was observed when pre-incubation of HNMFs with IL-13 was followed by a stimulation with TNF α , or HNMFs were simultaneously stimulated with IL-13 and TNF α .

Conclusion These results strongly indicate that IL-13, as well as IL-4, may be important in eotaxin-mediated eosinophilic inflammation in nasal mucosa. In addition, in nasal mucosa, fibroblasts are the major cell source for eotaxin.

Keywords: chemokines, eosinophils, eotaxin, fibroblasts, interleukin-13, interleukin-4, nasal allergy

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Introduction

Eosinophils play a central role in the development of allergic diseases, including asthma, nasal allergy and atopic dermatitis. Eosinophil migration *in vivo* is regulated

by many cytokines and chemokines. The chemokines behave as chemoattractants for leucocytes and are classified into four subfamilies, C-X-C, C-C, C, and C-X3-C, based on the sequence of conserved cysteine residues in their primary structures [1]. Eotaxin is a C-C chemokine implicated in the recruitment of eosinophils in a variety of inflammatory disorders. Unlike other eosinophil chemoattractants, eotaxin is eosinophil specific, because its specific receptor, CCR-3,

Correspondence: N. Terada, Department of Otorhinolaryngology, Chiba University School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba City, Chiba 260-0856, Japan (e-mail: terada@med.m.chiba-u.ac.jp).

is expressed only on eosinophils [2–8]. In addition, eotaxin may help eosinophil accumulation to the nasal mucosa through its effect on the expression of adhesion molecules on microvascular endothelial cells [9]. Furthermore, recent reports have demonstrated that eotaxin stimulates the production and differentiation of myeloid haematopoietic progenitors in the bone marrow and that eotaxin induces a rapid release of eosinophils and their progenitors from the bone marrow [10,11]. Thus eotaxin has multifaceted effects on eosinophils and is a key mediator of tissue eosinophilia. However, the mechanism of production of eotaxin has yet to be clarified.

The major cellular sources of eotaxin are thought to be the epithelial cells, endothelial cells and activated infiltrating leucocytes such as eosinophils [6–8]. Leucocytes and epithelial cells are stained for eotaxin in nasal polyps by immunohistochemistry [6–8]. Most recently, it has been shown that TH2 cytokine interleukin (IL) -4 induces eotaxin in dermal fibroblasts [12].

A novel cytokine termed interleukin 13 (IL-13), which binds to the α -chain of the IL-4 receptor (IL-4R), shares many biological activities on B lymphocytes and monocytes with IL-4 [13–18]. It is known that dermal fibroblasts express IL-4R and produce collagen type I upon stimulation with IL-4 [19]. Therefore, we speculate that, in addition to IL-4, IL-13 may induce eotaxin in human nasal mucosal fibroblasts (HNMFs).

In the present study we investigated whether IL-13 as well as IL-4 is able to induce eotaxin production in HNMFs. Furthermore, we investigated the effect of costimulation of IL-13 and TNF α on eotaxin production.

Materials and methods

Reagents

Recombinant human IL-13, IL-4 and TNF α were purchased from R & D systems (Minneapolis, MN, USA).

Antibodies

A monoclonal mouse antihuman IL-13 neutralizing antibody (clone, 32116.11, mouse IgG1) was purchased from R & D systems. Polyclonal rabbit antihuman TNF α neutralizing antibody was purchased from Genzyme (Cambridge, MA, USA). CD16 microbeads were purchased from Miltenyi Biotec Inc. (Sunnyvale, CA, USA).

Preparation of human eotaxin cDNA

Human eotaxin cDNA was prepared as previously described [2]. Briefly, a guinea pig eotaxin cDNA was used to screen a human lymphocyte genomic library (Stratagene, La Jolla,

CA, USA) under low stringency conditions. Hybridizing restriction fragments were sub-cloned into pBluescript II KS (Toyobo, Tokyo, Japan) and sequenced. A 217-bp genomic fragment corresponding to exon 3 of a novel human chemokine gene was then used to screen a human small intestine cDNA library (Clontech, Palo Alto, CA, USA). A positive phage clone designated 141 was converted to plasmid following the manufacturer's instructions. The cDNA insert was excised by *Xho*I and *Xba*I digestion, and sub-cloned into pBluescript II KS.

Collection of inferior nasal mucosa

Turbinal mucosa samples were collected from 10 perennial allergy patients sensitized to house dust and/or mites (six males and four females, mean age 25.8 ± 6.8 years). These patients had more than moderate nasal obstruction. Inferior nasal mucosa samples were obtained at the time of surgery for submucosal resection.

Isolation and propagation of epithelial cells, endothelial cells, and fibroblasts from human nasal mucosa

Human nasal epithelial cells (HNECs) and human mucosal microvascular endothelial cells (HMMECs) were prepared as described previously, with some modifications [20]. Surgically removed mucosa specimens were washed three times with a Ca²⁺- and Mg²⁺-free phosphate-buffered saline solution (PBS) containing 500 U/mL penicillin, 500 μ g/mL streptomycin and 6 μ g/mL Fungizon. The mucosal tissue specimens were cut into 4-mm square sections and then incubated separately for 1 h at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 0.25% collagenase (Wako Pure Chemicals, Osaka, Japan). Following incubation in collagenase, these sections were transferred to 10 mL of DMEM containing 50 μ g/mL gentamicin. HNECs and HMMECs were released into medium by applying pressure on the tissue across the surface with a pair of curve forceps 10 times. The cell suspension was transferred to a 15-mL centrifuge tube, and centrifuged for 5 min at 400 g. To separate HNECs from HMMECs, the cell pellets were resuspended in DMEM supplemented with 20% fetal calf serum (FCS) (purchased from ICN, Costa Mesa, CA, USA), and placed onto a 60-mm diameter plastic culture dish coated with type I-rich collagen (Sigma, St Louis, MO, USA). The dish was incubated for 1 h at 37 °C under a humidified atmosphere of 5% CO₂ to allow HMMECs to bound to the collagen matrix. The culture was then washed twice with plating medium to remove unattached cells and tissue fragments, and refed with 15 mL of MCDB 104 (Nissui, Tokyo, Japan) containing 10% FCS, 75 μ g/mL endothelial cell growth supplement (Collaborative Research Inc., Bedford, MA, USA), antibiotics. The floating HNECs

were collected, centrifuged and plated in MCDB153 (Nissui) medium containing 10 ng/mL epidermal growth factor and 1% bovine pituitary extract. The cultures of HMMECs and HNECs were fed twice weekly and subcultured before 70–80% confluence.

Morphological observations using a phase-contrast microscope showed that the HNECs consisted primarily of epithelial cells. More than 95% of the HNECs showed positive reactions with antihuman cytokeratin antibodies (Dakopatts, Glostrup, Denmark). Based on the morphology and positive reactions with antifactor VIII antibody (Dakopatts) in immunofluorescence staining tests, more than 95% of the HMMECs were shown to be endothelial cells.

HNMFs were isolated from the specimens by explant cultures following the removal of endothelial and epithelial cells. The specimens were cut into 1–2-mm square sections, and excess moisture was removed with sterile cotton plugs. Each of the explants was transferred to a well of a 12-well plate (Iwaki Grass Co. Ltd, Chiba, Japan) and centrifuged for 5 min at 110g using a swinging plastic plate holder. The attached explants were cultured with DMEM containing 2% FCS, 10 ng/mL recombinant basic fibroblast growth factor and antibiotics. The explant cultures were fed twice weekly and cells were subcultured in a T-25 tissue culture flask when the cells reached 50–80% confluence.

Based on the morphology and positive reactions with monoclonal antihuman fibroblasts (Dakopatts) in immunofluorescence staining tests, more than 97% of the HNMFs were shown to be fibroblasts.

Eosinophil purification

eosinophils were isolated from peripheral blood obtained from patients with allergic rhinitis. The diagnosis of allergic rhinitis was based on a typical history of perennial symptoms of allergic rhinitis, eosinophilia in nasal smears and a positive radioallergosorbent test to house dust and/or mites.

The leucocyte fraction was obtained by dextran sedimentation. Eosinophils were then isolated by negative immunomagnetic selection using CD16 microbeads and the MACS System (Miltenyi, Biotec, Germany) [21]. The resulting eosinophil purity was 98.7 ± 3.8 as determined by microscopic examination with Hinkelman staining. For this study we used eosinophils in which the viability (based on the trypan blue test) was >98%.

Cell culture

HNMFs, HMMECs and HNECs were cultured directly onto collagen type I-coated, 24-well culture plates (Sumitomo Bakelite Co. Ltd, Tokyo, Japan). They were checked by phase-contrast microscopy. At confluence, they were washed with PBS containing 10 µg/mL gentamicin

(Sigma) three times and were stimulated by various kind of cytokines for 1–36 h at 37 °C in 5% CO₂. Eosinophils in RPMI 1640 containing 10% FCS were also stimulated by various kind of cytokines for 36 h at 37 °C in 5% CO₂. In some experiments, eosinophils were stimulated with 1 µg/mL of calcium ionophore and 10^{−6} mol/L of PMA for 3 h. The supernatants were collected, centrifuged and stored frozen at −80 °C until use. Cells were harvested by trypsinization and immediately frozen in liquid nitrogen until use.

In some experiments, we cultured HNMFs alone, with IL-13, or with TNFα or in combination. After 24 h of culture, the cells were washed with PBS and cultured for further 24 h with or without cytokines.

Preparation of mouse anti-human eotaxin and ELISA for eotaxin

Preparation of mouse monoclonal antihuman eotaxin, antibody isotyping and purification were described elsewhere [22]. A double-sandwich ELISA for measurement of human eotaxin was also described previously [22].

The assay had a sensitivity of 3 pg/mL. Eotaxin production was expressed in picograms as 5×10^4 cells recovered at the end of the culture period.

Extraction of total RNA and RT-PCR

RNA extraction, cDNA synthesis, and PCR reaction were carried out essentially according to the methods described elsewhere [23,24].

Total RNA was extracted by using the Acid Guanidium Phenol Chloroform method, and synthesis of the complementary DNA was performed using a cDNA synthesis kit (Boehringer Mannheim, Mannheim, Germany). A cDNA equivalent of 500 ng total RNA was amplified in a 50-µL reaction volume containing 200 mmol/L (each) of dNTPs, 3 units of Amplitaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT, USA), 25 pmol of 5' and 3' primer in 10×PCR reaction buffer (1.5 mmol/L MgCl₂, 50 mmol/L KCl, 10 mmol/L TRIS-HCl, 100 mg of gelatin, pH 8.3). The PCR was performed in a DNA thermal cycler (Quick Thermo Systems, Nippon Genetics, Tokyo, Japan). The PCR was performed for 30 cycles under the following conditions: (1) predenaturation at 94 °C for 5 min, (2) denaturation at 94 °C for 2 min, (3) annealing at 55 °C for 2 min, and (4) extension at 72 °C for 2 min.

After repeating steps (2) through (4) for 30 cycles, re-extension was performed at 72 °C for 5 min.

The sequences of the 5' sense primers and the 3' antisense primers used in this study are as follows:

eotaxin, 5'-CCTCTCAGCCAAAGCTCACA-3' and 5'-TAGGCAACACTCAGGCTCTGG-3' (product size 375 bp); β-actin, 5'-CTTCTACAATGAGCTGCGTG-3'

and 5'-TCATGAGGTAGTCAGTCAGG-3' (product size 305 bp).

Southern blot analysis

The specificity of the PCR product for eotaxin was determined by Southern blot analysis [25]. A portion of PCR products was subjected to electrophoresis on a 3% agarose gel. After denaturation in alkaline solution, this sample was transferred to a nylon membrane filter for Southern blot analysis. Subsequent to transfer, DNA was fixed by UV irradiation and was hybridized using a ^{32} P-labelled human eotaxin cDNA probe prepared by random-primed DNA labelling. The conditions for the hybridization were: first, prehybridization at 42 °C for 2 h; and second, hybridization at 42 °C for 1 day. The washing conditions were: washing twice with 2 $\times$ saline sodium citrate (SSC) (1 $\times$ SSC = 150 mmol/L of NaCl and 15 mmol/L of trisodium citrate, pH = 7.0) and 0.1% sodium dodecyl sulphate (SDS) at room temperature for 20 min and then three times with 0.2 $\times$ SSC and 0.1% SDS at 52 °C for 15 min. An X-ray film was exposed for 1 h.

The ratio of eotaxin mRNA to β -actin mRNA was determined with a densitometer.

Inhibition assay

To confirm that the induction of eotaxin in HNMFs by IL-13 and TNF α are both cytokine specific, we performed an inhibition assay. Before stimulation with IL-13 and TNF α , HNMFs were preincubated for 30 min at 37 °C in the presence or absence of saturating concentrations of a monoclonal mouse antihuman IL-13-neutralizing antibody and polyclonal rabbit antihuman TNF α -neutralizing antibody. The viability of cells as determined by the trypan blue exclusion test after the pre-incubation was more than 98%.

Statistical analysis

The results of the study were expressed as mean value $\pm$ SEM. Statistical significance was determined by using paired Wilcoxon test, defining $P < 0.05$ as statistically significant.

Results

Unstimulated HNMFs expressed eotaxin mRNA at low levels. After stimulation with IL-4 or IL-13 without or with TNF α , eotaxin mRNA in HNMFs reached peak levels within the first 6 h of stimulation and gradually decreased in the following 36 h (Fig. 1). By stimulation with IL-13, HNMFs showed significant enhancement of eotaxin mRNA levels compared with unstimulated

HNMFs ($P < 0.05$). Stimulation with IL-4 showed similar results. In addition, the combined stimulation of IL-13 and TNF α as well as that of IL-4 and TNF α showed strong enhancement of eotaxin mRNA levels (Fig. 1).

To evaluate whether the production of eotaxin protein accompanied the expression of eotaxin mRNA, we determined the amount of eotaxin in the supernatants of HNMFs. Eotaxin production is expressed as picograms per 5×10^4 cells. Unstimulated HNMFs produce a small amount of eotaxin. In time-course experiments, eotaxin production continued to increase for at least 36 h by IL-13 stimulation as well as IL-4 stimulation (Fig. 2a). By combined stimulation with IL-13 and TNF α , the cells produced a dramatic amount of eotaxin. The amount of eotaxin in the culture medium of unstimulated HNMFs after the 36-h incubation period is 2.3 ± 0.5 pg/mL. It increased to 175.8 ± 50.8 pg/mL after the combined stimulation with IL-13 and TNF α .

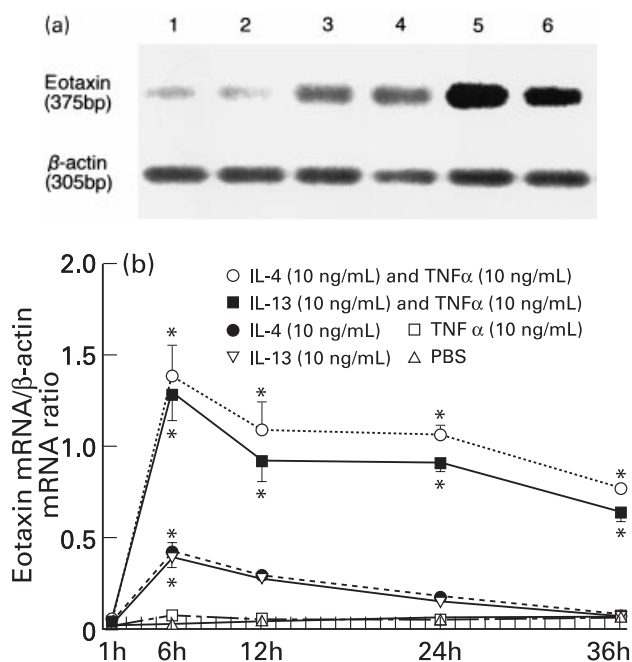


Fig. 1. (a) The representative result of HNMFs which had 6 h incubation with IL-4 (10 ng/mL), IL-13 (10 ng/mL) or TNF α (10 ng/mL) alone or in combination. Lane 1, unstimulated HNMFs. Lanes 2–4, HNMFs stimulated with TNF α (10 ng/mL), IL-4 (10 ng/mL) and IL-13 (10 ng/mL), respectively. Lane 5, HNMFs incubated with the combined stimulation of IL-4 and TNF α . Lane 6, HNMFs incubated with the combined stimulation of IL-13 and TNF α . Total RNA was isolated from HNMFs. PCR-assisted Southern blot analysis was performed as described in the text. (b) Kinetics of eotaxin mRNA expression in HNMFs. The ratio of eotaxin mRNA to β -actin mRNA was determined with a densitometer. All values are mean $\pm$ SEM of five experiments. * $P < 0.05$ compared with unstimulated HNMFs

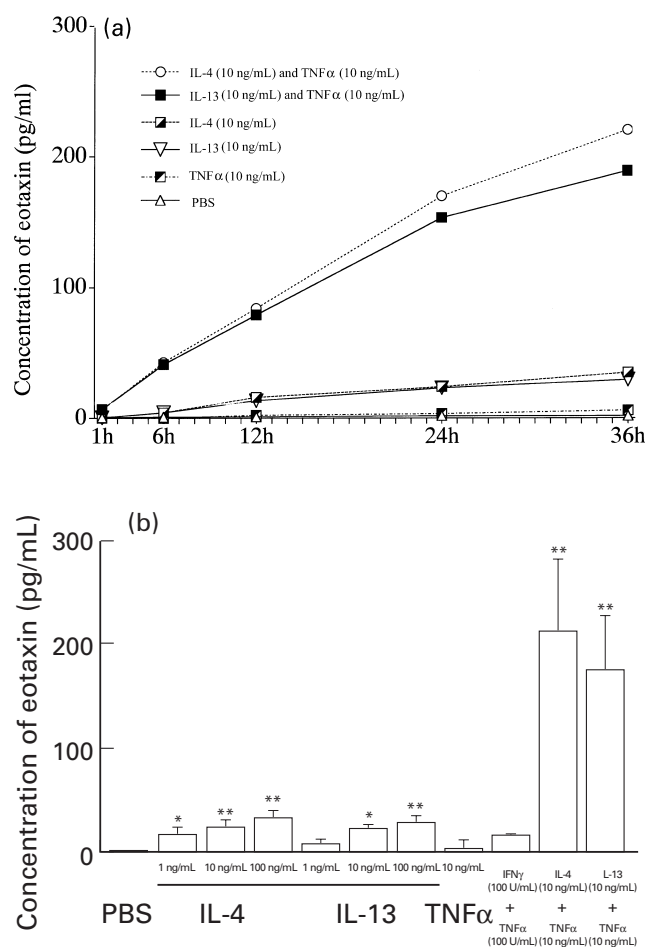


Fig. 2. (a) Kinetics of eotaxin production in HNMFs. HNMFs were cultured with IL-4 (10 ng/mL), IL-13 (10 ng/mL) or TNFα (10 ng/mL) alone or in combination. Results shown are from one of three experiments. (b) Synergistic effect of IL-13 and TNFα on eotaxin production. HNMFs were cultured for 36 h with the indicated concentrations of IL-4, IL-13 or TNFα alone or in combination. All values are mean $\pm$ SEM of six experiments. * P < 0.05 compared with unstimulated HNMFs. ** P < 0.01 compared with unstimulated HNMFs

(Fig. 2b). The differences were statistically highly significant (P < 0.01). To confirm that the induction of eotaxin in HNMFs by IL-13 and TNFα was specific for both cytokines, we performed an inhibition assay. A monoclonal-antihuman IL-13 neutralizing antibody and polyclonal rabbit antihuman TNFα neutralizing antibody completely blocked the eotaxin production induced by IL-13 and TNFα (2.5 ± 0.6 pg/mL).

On the other hand, neither the combined stimulation with IL-13 and TNFα nor the combined stimulation with IL-4 and TNFα induced significant levels of eotaxin production by HMMECs and HNECs (Table 1). Although the costimulation of TNFα and IFNγ induced eotaxin mRNA in HMMECs, the amount of eotaxin in the culture medium was much lower than that in HNMFs induced by costimulation with IL-4 and TNFα, or that with IL-13 and TNFα (Fig. 3 and Table 1). Eosinophils expressed eotaxin mRNA and produced a small amount of eotaxin when they were stimulated with calcium ionophore and PMA, but not when they were stimulated with combination of IL-4 and TNFα.

To investigate the mechanism of synergy between IL-13 and TNFα on eotaxin production, cells were incubated for two consecutive 24-h periods with IL-13 and TNFα. As shown in Fig. 4, there was no synergy when the pre-incubation of HNMFs with TNFα was followed by a stimulation with IL-13. By contrast, pre-incubation of HNMFs with IL-13 strongly sensitized these cells to the stimulating effect of TNFα. The simultaneous stimulation with IL-13 and TNFα was most effective for eotaxin production.

Discussion

Eotaxin has a chemoattractant effect for eosinophils and also increases the expression of adhesion molecules on microvascular endothelial cells [9]. Besides local eosinophil accumulation, eotaxin can exert eosinophil differentiation and induce a rapid release of eosinophils and their progenitors from the bone marrow [10,11].

Additions	HNMFs	HMMECs	HNECs	Eosinophils
None	2.3 ± 0.47	1.4 ± 0.13	1.7 ± 0.23	1.7 ± 0.21
IL-4 (10 ng/mL)	$24.9 \pm 5.6^{**}$	1.2 ± 0.11	2.3 ± 0.22	1.8 ± 0.22
IL-13 (10 ng/mL)	$22.8 \pm 4.7^{*}$	1.4 ± 0.12	2.2 ± 0.43	1.9 ± 0.22
TNFα (10 ng/mL)	3.3 ± 0.88	1.9 ± 0.13	2.4 ± 0.46	1.7 ± 0.32
TNFα + IFNγ	24.3 ± 1.7	$6.9 \pm 0.14^{*}$	2.9 ± 0.33	2.2 ± 0.20
IL-4 + TNFα	$211.8 \pm 69.3^{**}$	2.3 ± 0.12	3.3 ± 0.17	2.9 ± 0.34
IL-13 + TNFα	$175.8 \pm 50.8^{**}$	2.5 ± 0.11	4.0 ± 0.24	2.7 ± 0.27
Ca ionophore + PMA				4.4 ± 0.37

Table 1. Effects of cytokines on eotaxin production in HNMFs, HMMECs, HNECs and eosinophils. All values are mean $\pm$ SEM of six experiments. * P < 0.05 compared with unstimulated cells. ** P < 0.01 compared with unstimulated cells

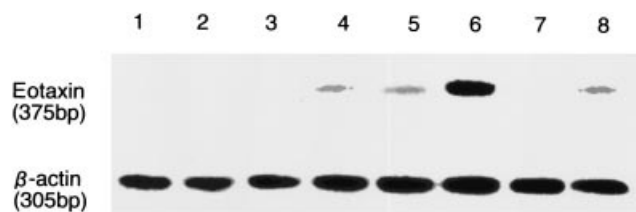


Fig. 3. The representative result of eotaxin mRNA in HNECs, HMMECs, HNMFs and eosinophils which had 6 h incubation with indicated cytokines. Lane 1, unstimulated HNECs. Lane 2, HNECs incubated with the combined stimulation of IL-13 and TNF α . Lane 3, unstimulated HMMECs. Lane 4, HMMECs stimulated with the combined stimulation of TNF α and IFN γ . Lane 5, unstimulated HNMFs. Lane 6, HNMFs incubated with the combined stimulation of IL-13 and TNF α . Lane 7, unstimulated eosinophils. Lane 8, eosinophils incubated with the combined stimulation of calcium ionophore and PMA. Total RNA was isolated from HNECs, HMMECs, HNMFs and eosinophils. PCR-assisted Southern blot analysis was performed as described in the text

We conducted a series of experiments to determine the role of IL-13 and TNF α in eotaxin generation in HNMFs. RT-PCR analysis showed that the eotaxin message was induced by IL-13 and began to increase at 1 h in HNMFs and was still present at 36 h (Fig. 1b). Following the message expression, eotaxin protein increased for at least 36 h after the IL-13 stimulation or the combined stimulation with IL-13 and TNF α (Fig. 2). The amount of eotaxin induced in HNMFs by IL-4 and IL-13 is almost identical. The combined stimulation of IL-4 and TNF α , as well as that

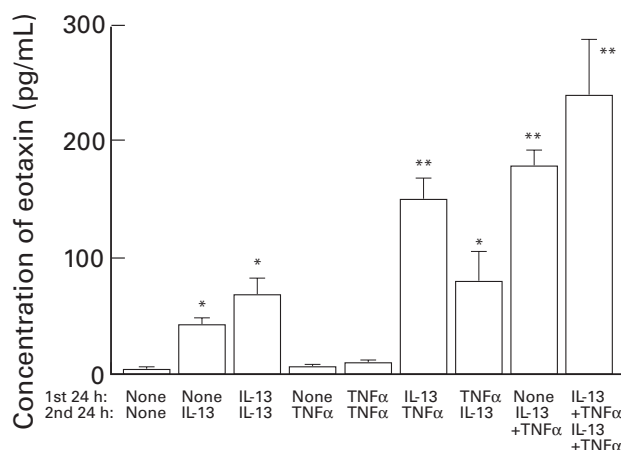


Fig. 4. Effect of sequential addition of IL-13 and TNF α on eotaxin production. HNMFs were cultured alone, with IL-13 (10 ng/mL) or with TNF α (10 ng/mL) or in combination. After 24 h culture, cells were washed with PBS and cultured for a further 24 h with or without cytokines. All values are mean $\pm$ SEM of six experiments. * P < 0.05 compared with unstimulated HNMFs. ** P < 0.01 compared with unstimulated HNMFs

of IL-13 and TNF α , dramatically induced eotaxin (Table 1). On the other hand these stimulations did not induce eotaxin in HMMECs and HNECs. When HNMFs were pretreated with mAb to TNF α , or IL-13 neutralizing antibody, the eotaxin induction by these cytokines was almost completely blocked.

The major sources of eotaxin are thought to be the epithelial cells, endothelial cells and activated infiltrating leucocytes such as eosinophils [3,6,26]. Leucocytes and epithelial cells are immunoreactive for eotaxin on nasal polyps by immunohistochemistry [3]. In our experiments, however, we were unable to detect any eotaxin immunoreactive signals in epithelial cells in the inferior turbinate (unpublished observation). It is highly unlikely that we would have failed to detect eotaxin immunoreactivity in epithelial cells of inferior turbinates, because it was easily detectable in those of polyps with the same methods. Eosinophils are significantly more numerous in the epithelium of nasal polyps than in the inferior turbinate mucosa [27]. This fact suggests that epithelial cells of nasal polyps might produce a higher amount of eosinophil chemoattractants, such as eotaxin or RANTES, than those of inferior turbinate mucosa.

In our studies, cultured epithelial cells also failed to express eotaxin mRNA. We could detect neither eotaxin mRNA in epithelial cells nor eotaxin protein in the medium of epithelial cells stimulated with IL-4, IL-13, TNF α and combinations thereof. Although the costimulation of TNF α and IFN γ induced eotaxin mRNA in HMMECs, the amount of eotaxin in the culture medium was much lower than that in HNMFs induced by costimulation with IL-4 and TNF α , or that with IL-13 and TNF α (Fig. 3 and Table 1). In nasal mucosa, almost all eosinophils express the immunoreactivity of eotaxin (our unpublished observation). However, the amounts of eotaxin from eosinophils induced by calcium ionophore were much lower than those from HNMFs induced by the costimulation with IL-4 and TNF α or that with IL-13 and TNF α . These observations suggest that eosinophils and epithelial cells have comparatively minor roles as cellular sources for eotaxin in nasal mucosa.

The mechanisms whereby IL-13 and TNF α synergize to induce eotaxin production remain obscure. We wondered whether this synergy required both cytokines to be present simultaneously, or whether one cytokine could 'prime' for the other. To address this question, we incubated HNMFs for two consecutive 24-h periods with or without the indicated cytokines. As shown in Fig. 4, there was no synergy when the pre-incubation of HNMFs with TNF α was followed by stimulation with IL-13. Lugli *et al.* demonstrated that the stimulation with TNF α induced a two- to threefold increase in the IL-4R α expression [28]. TNF α stimulation led to increased activation of the IL-4-specific signal transducers and activators of the transcription protein

(STAT6) by IL-4 and IL-13. However, our data suggest that upregulation of IL-13 receptor expression by TNF α is not likely to be responsible for the synergy we observed. In contrast, pre-incubation of HNMFs with IL-13 strongly sensitized the HNMFs to the stimulating effect of TNF α . The simultaneous stimulation with IL-13 and TNF α was most effective for eotaxin production. It is therefore likely that the synergy between TNF α and IL-13 on eotaxin production involves both pre- and post-receptor events.

Mast cells and basophils appear to play key roles in regulating the production of eotaxin in HNMFs. In nasal mucosa, IL-4 is produced mainly by mast cells [29].

The cell source for IL-13 in nasal mucosa has been reported by Pawankar *et al.* [30]. They demonstrated that IL-13 at the protein level was localized to lymphocytes and mast cells. Both mast cells and basophils are known to produce TNF α [31]. Most recently, it has been revealed that eosinophils in nasal polyp also produce IL-13 (Nakano *et al.*, unpublished observation). In recent years, the production of cytokines by eosinophils has been substantiated; thereby, productions of many kinds of cytokines including TNF α , have been reported so far [32,33]. These cytokines are associated with autoregulations inherent in eosinophils, including differentiation, proliferation, migration, activation and degranulation, indicating that eosinophils exert regulatory effects on allergic inflammation, in addition to playing their own autoregulatory role through cytokine production and release. In co-ordination with mast cells and basophils, eosinophils induce an elevation of the tissue levels of both IL-13 and TNF α , and stimulation of the eotaxin production from HNMFs, thus resulting in an eventual fostering of eosinophil migration.

In conclusion, we have shown that IL-13 as well as IL-4 caused a dose-dependent induction of eotaxin in HNMFs. Furthermore, costimulation of IL-13 and TNF α caused a dramatic increase in eotaxin expression in HNMFs, whereas it did not induce a significant level of eotaxin in HMECs and HNECs. This synergy was observed when the pre-incubation of HNMFs with IL-13 was followed by stimulation with TNF α . The simultaneous stimulation with IL-13 and TNF α was most effective for eotaxin production. These results strongly indicate that IL-13, as well as IL-4 may be important in eotaxin-mediated eosinophilic inflammation in nasal mucosa.

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