Rhinology, 45, 235-241, 2007

IL-4 and TNF-α increased the secretion of eotaxin from cultured fibroblasts of nasal polyps with eosinophil infiltration*

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SUMMARY

Background: *Nasal polyposis is considered a subgroup of chronic rhinosinusitis (CRS). Eosinophils are the most common inflammatory cells in nasal polyp and the degree of the tissue eosinophilia is correlated with the probability of the recurrence of nasal polyps. However, the mechanism by which eosinophils are selectively recruited in nasal polyp remains to be clarified. In the present study, fibroblasts were isolated from nasal polyps of patients with eosinophil-rich nasal polyps (Enp) and those with non-eosinophilic nasal polyps (NEnp) and the secreted levels of eotaxin, regulated upon activation normal T expressed and presumably secreted (RANTES), and vascular cell adhesion molecule-1 (VCAM-1) from the cultured fibroblasts were determined. The levels were compared between Enp and Nenp. The role of those chemokines and adhesion molecules in the pathogenesis of nasal polyp is discussed.*

Methods: *Fibroblasts isolated from nasal polyps of five patients with CRS with Enp and four patients with CRS with NEnp were cultured and stimulated with 10 ng/ml of tumor necrosis factor-α (TNF-α) and interleukin-4 (IL-4) for 24 hours. After stimulation, culture supernatants were collected and concentrations of eotaxin, RANTES, and VCAM-1 were quantified by Enzyme linked immunosorbent assay (ELISA).*

Results: *TNF-α enhanced the secretion of VCAM-1 and RANTES by fibroblasts derived from both NEnp and Enp, but did not affect the release of eotaxin. IL-4 increased the secretion of VCAM-1 and eotaxin but not that of RANTES. Furthermore, TNF-α and IL-4, when added together, induced a synergistic effect on the secretion of VCAM-1 and eotaxin. The effect of IL-4 and IL-4 plus TNF-α on eotaxin release was more marked for Enp fibroblasts compared with NEnp fibroblasts.*

Conclusions: *The results suggest that eotaxin plays an important role in the selective recruitment of eosinophils in Enp. Nasal fibroblasts in Enp are more sensitive than those in NEnp regarding eotaxin release induced by the stimulation with IL-4 and co-stimulation with TNF-α and IL-4. This difference might be associated with the pathogenesis of nasal polyposis having marked accumulation of eosinophils.*

Key words: eosinophil, eotaxin, fibroblast, nasal polyp

INTRODUCTION

Nasal polyposis is considered a subgroup of chronic rhinosinusitis (CRS) and eosinophils are the most common inflammatory cells in nasal polyps [(1)]. Nasal polyposis with marked accumulation of activated eosinophils is quite intractable and often complicated by non-atopic asthma and aspirin-induced asthma (AIA) [(2)]. Recently, Dhong et al. [(3)] compared the sinus mucosal histopathologies of CRS between asthmatic patients and non-asthmatic patients. They found that eosinophil infiltrations were more prominent in asthmatic patients compared to non-asthmatic patients. CRS in asthmatic patients showed worse outcomes than in nonasthmatic patients after endoscopic sinus surgery [(4)], indicating that eosinophil infiltration into nasal polyps and sinus mucosa is associated with the presence of asthma and the intractable pathology of CRS. Moreover, oral steroid effectively reduces eosinophilia and shrinks nasal polyps [(5)]. Surgical resection of nasal polyps reduces both eosinophilia and urinary concentration of cysteinyl leukotrienes in patients with AIA [(6)]. These findings suggest that eosinophilic accumulation in nasal polyps is not only a

*Received for publication: September 21, 2006; accepted: February 8, 2007

consequence but also a cause of systemic events in non-atopic asthma. However, the mechanism by which eosinophils are selectively accumulated in nasal polyps remains unclear.

It has been widely acknowledged that chemokines, such as eotaxin and regulated upon activation normal T expressed and presumably secreted (RANTES), and adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), are important in selectively recruiting eosinophils into the respiratory mucosa [7-9]. These factors are produced from epithelial cells, fibroblasts, and inflammatory cells and this production is up-regulated by stimulation from several inflammatory factors. In our previous study, VCAM-1 production from fibroblasts isolated from nasal polyps was enhanced by the stimulation with TNF-α [10]. Nasal fibroblasts also produce eotaxin and RANTES in response to TNF-α, IL-4, IL-13, and endotoxin [11-15]. These findings suggest that fibroblasts play an important role in the recruitment of eosinophils in nasal polyps.
Furthermore, it can be speculated that the production of eosinophil-specific chemokines and adhesion molecules from nasal fibroblasts is higher in eosinophil-rich nasal polyp (Enp) than non-eosinophilic nasal polyp (NEnp), and which might be associated with the selective recruitment of eosinophils in Enp. However, the released levels of eosinophil-specific chemokines and adhesion molecules from nasal fibroblasts and the differences between Enp and NEnp have not been investigated.

In the present study, nasal fibroblasts were isolated from Enp and NEnp and the released levels of eotaxin, RANTES, and VCAM-1 from those fibroblasts were determined. By comparing the released levels of these chemokines and adhesion molecules, their role in the pathogenesis of nasal polyposis is discussed.

MATERIALS AND METHODS

Classification of Enp and NEnp

Tissue from nasal polyps obtained by surgery from twelve patients with CRS was fixed in formalin and stained with hematoxylin and eosin, and the number of eosinophils was counted at x 200 magnification under light-microscopy. Five fields were examined for each section and the average was considered the number of eosinophils infiltrating the sample [16,17]. Among twelve nasal polyps, five samples having more than 100 eosinophils and four samples having 10 or fewer eosinophils were extracted and tentatively classified as Enp and NEnp, respectively. Three samples having eosinophils between 10 and 100 were excluded from the examination. The study was approved by the Institutional Review Board of Kagoshima University Hospital.

Clinical background of patients

The background of the nine patients involved in this study is shown in Table 1. In the NEnp group, all patients were male, while in the Enp group, three were male and two were female.

Table1. Clinical background of patients enrolled in the study.

	Nenp	**Enp**
No. of patients	4	5
(Male	4	3)
Female	0	2)
Age (y.o)	15-72	48-61
(mean	40.1	52.2)
Nasal allergy	0% (0/4)	0% (0/5)
Asthma	0% (0/4)	60% (3/5)
Eosinophils (No/foeld)	0-10	116-556
(Mean	3.0	349.2)

The NEnp and Enp groups did not differ significantly in mean age. Nasal allergy was not found in any subject. Asthma was a concomitant disease in three of five patients with Enp. Any medicines such as leukotriene antagonist, anti-histamine, and antibiotics had not been administered to the patients at least 2 weeks prior to surgery. None of the subjects had taken oral, nasal, or inhaled steroids for more than a year before the surgery.

Reagents

Human recombinant TNF-α and IL-4 were both purchased from CHEMICON International Inc. (Temecula, CA, USA).

Preparation of nasal fibroblasts

Nasal fibroblasts were isolated and cultured from Enp and NEnp as described previously [18]. In brief, nasal polyps were cut into small fragments and agitated in RPMI-1640 medium containing a mixture of 10 UI/ml DNAse, 500 UI/ml collagenase type IV, and 30 UI/ml hyaluronidase (all enzymes were purchased from Sigma, St Louis, MO) on a magnetic stirrer for 2 h at 37°C. The cells were then cultured at 37°C in 5% CO_2 until they reached confluence in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS). Fibroblasts were identified by phase-contrast microscopy and absence of contamination with epithelial cells and leukocytes was confirmed. The cells were then characterized by flow cytometry using an anti-human Thy-1 antibody (Dianova, Hamburg, Germany) in order to examine the purity; fibroblast purity was more than 99%. The cells were used throughout the study after two passages.

ELISA

After reaching confluence, the medium was removed and the fibroblasts were exposed to serum-free medium with human recombinant TNF-α and IL-4. The negative control consisted of cells cultured without stimulation. The cultured supernatants were collected and the concentrations of eotaxin, RANTES, and VCAM-1 in the culture supernatants were measured with sandwich ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

RT-PCR

After stimulation with TNF-α or IL-4, total mRNA was isolated from cultured fibroblasts for RT-PCR using a High Pure RNA Isolation kit (Roche, Mannheim, Germany) and reverse-transcribed using 1st Strand cDNA Synthesis kit (Roche). Real time PCR was then performed with Light Cycler Fast Start DNA Master SYBR Green (Roche) using specific primer sets for IL-4 receptor (IL-4R) and ,-actin as the internal control. The primer combinations were: 5'-CATAGCACAACAGGCAGACG-3' and 5'-GACCTGGAGCAACCCGTATC-3' for IL-4R (predicted 335-bp fragment) [19] and 5'GTGGGGCGCCCCAGGCACCA3' and 5'CTCCTTAATGTCACGCACGATTTC-3' for β-actin (predicted 540-bp fragment).

Statistical analysis

The concentrations of eotaxin, RANTES and VCAM-1 measured by ELISA are expressed as means ± standard error (SE). Data were statistically analyzed using two-way analysis of variance (ANOVA). Values of $p < 0.05$ were considered significant.

RESULTS

Time-dependent eotaxin secretion from fibroblasts in response to TNF-α and IL-4

In order to determine the optimum culture time for nasal fibroblasts to produce eotaxin, fibroblasts isolated from NEnp were stimulated with either 10 ng/ml of TNF-α or IL-4 and the concentrations of eotaxin in culture supernatants were examined at 12, 24, and 48 hours after incubation. The secretion of eotaxin increased in a time-dependent manner until 24 hours in the cells stimulated with TNF-α or IL-4 and in unstimulated control cells (Figure 1). Stimulation with IL-4 significantly increased eotaxin secretion compared to the control, while that with TNF-α did not. In all groups, eotaxin secretion was not enhanced by 48 hours incubation compared to 24 hours incubation. Based on the results, cells were cultured for 24 hours in all experiments. Furthermore, a previous study [11] and preliminary experiments showed that the secretion of VCAM-1 and RANTES from nasal fibroblasts plateaued at 24 hours after the incubation with 10 ng/ml of TNF-α or IL-4 (data not shown).

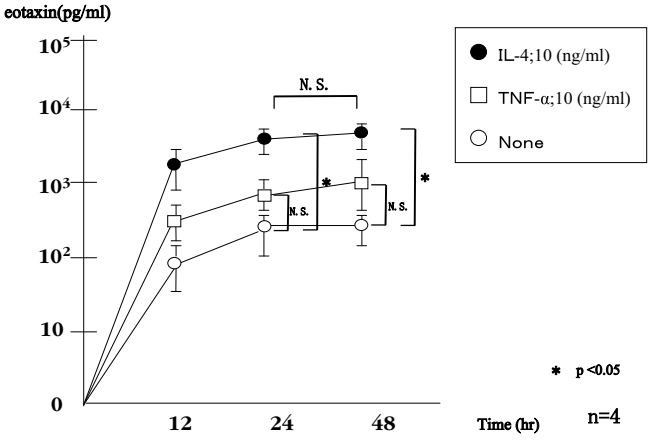

Figure 1. Time-dependent eotaxin secretion from fibroblasts in response to TNF-α and IL-4.

Fibroblasts isolated from NEnp were stimulated with either 10 ng/ml of TNF-α or 10 ng/ml of IL-4 and the concentrations of eotaxin in culture supernatants were examined. Secreted levels of eotaxin increased time-dependently and plateaued at 24 hours. Data are expressed as mean ± SEM. *, $p < 0.05$ versus control group. N.S.: not significant.

Dose-dependent eotaxin secretion from fibroblasts in response to IL-4 and TNF-α

The optimum doses of TNF-α and IL-4 to stimulate nasal fibroblasts were determined by the application of various concentrations of TNF-α and IL-4 to cultured fibroblasts derived from NEnp for 24 hours. The concentration of eotaxin in culture supernatants was increased in a dose-dependent manner by stimulation with TNF-α together with IL-4 (Figure 2). Increased secretion of eotaxin was not observed when cells were stimulated at any dose of TNF-α alone. Stimulation with 10 and 100 ng/ml of IL-4 alone slightly but significantly increased the secretion of eotaxin compared to the control. The secretion of eotaxin plateaued at 10 ng/ml of IL-4 and at 10ng/ml TNF-α. Cells were therefore cultured with 10 ng/ml of TNF-α or IL-4 in all experiments. A previous study (11) and preliminary experiments confirmed that 10 ng/ml of TNF-α or IL-4 optimally induced the secretion of VCAM-1 and RANTES (data not shown).

VCAM-1 secretion from nasal fibroblasts by stimulation with TNF-α and IL-4

In both NEnp and Enp, VCAM-1 secretion from fibroblasts

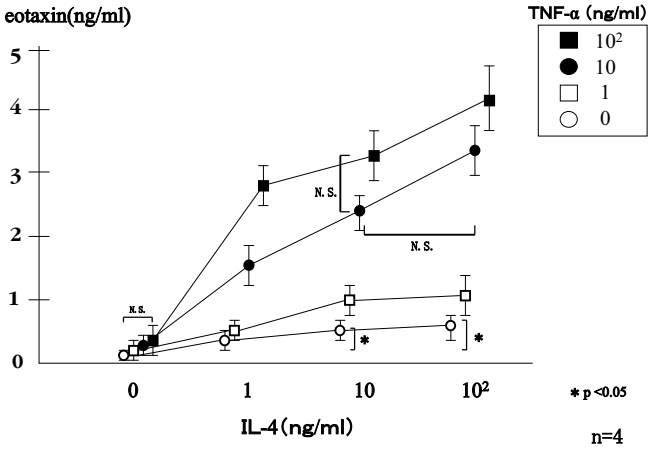

Figure 2. Dose-dependent eotaxin secretion from fibroblasts in response to IL-4 and TNF-α.

The optimum doses of TNF-α and IL-4 required to stimulate nasal fibroblasts were determined by the application of various concentrations of TNF-α and IL-4 for 24-hours to cultured fibroblasts derived from NEnp. The concentrations of eotaxin in culture supernatants were increased by co-stimulation with TNF-α and IL-4 in a dose-dependent manner. Data are expressed as mean ± SEM. *, $p < 0.05$ versus control group. N.S.: not significant.

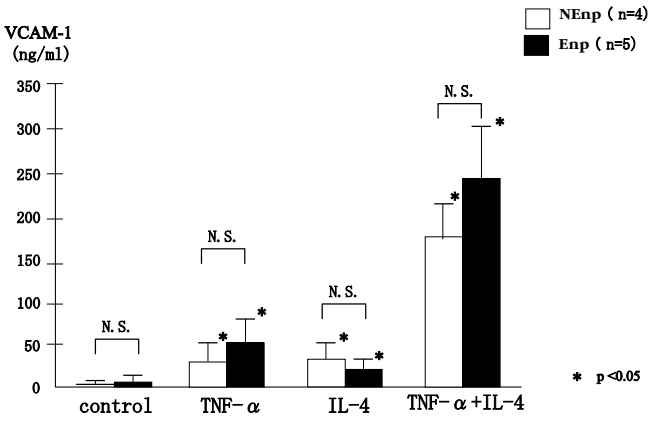

Figure 3. VCAM-1 secretion from nasal fibroblasts by stimulation with TNF-α and IL-4.
VCAM-1 secreted from fibroblasts was induced by TNF-α and by IL-4; the effects were enhanced when co-stimulation was performed. VCAM-1 secretion induced by co-stimulation with TNF-α and IL-4 was significantlyhigher than that by TNF-α or IL-4 alone ($p < 0.05$, respectively), suggesting that the effect was synergistic. However, there was no significant difference in the levels of VCAM-1 between Enp and NEnp. Data are expressed as mean ± SEM. *, $p < 0.05$ versus control group. N.S.: not significant.

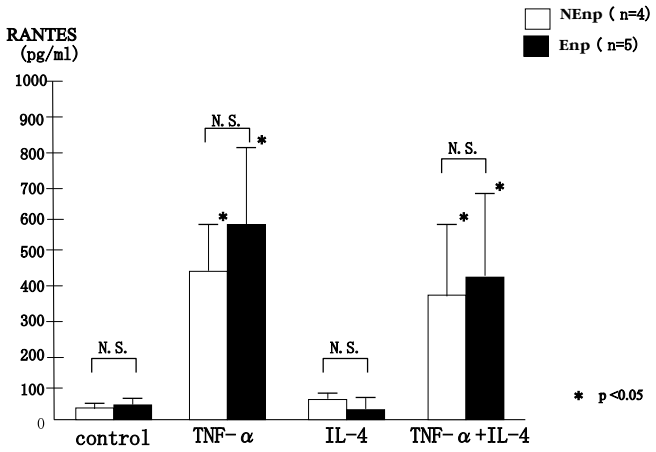

Figure 4. RANTES secretion from nasal fibroblasts by stimulation with TNF-α and IL-4.
RANTES secretion from fibroblasts was significantly enhanced by TNF-α, but not by IL-4. Co-stimulation with TNF-α and IL-4 did not enhance the secretion of RANTES. The level of RANTES did not differ significantly between Enp and NEnp. Data are expressed as mean ± SEM. *, $p < 0.05$ versus control group. N.S.: not significant.

was slightly but significantly increased by the stimulation with TNF-α or IL-4 alone when compared to the control (Figure 3). In contrast, VCAM-1 secretion was remarkably increased by co-stimulation with TNF-α and IL-4 in both NEnp and Enp. Further, VCAM-1 secretion induced by co-stimulation with TNF-α and IL-4 was significantly higher than that by TNF-α or IL-4 alone, suggesting that the effect was synergistic. However, the levels of VCAM-1 did not differ significantly between Enp and NEnp.

RANTES secretion from nasal fibroblasts by stimulation with TNF-α and IL-4

RANTES secretion from fibroblasts was significantly enhanced by stimulation with TNF-α in both NEnp and Enp, but not by stimulation with IL-4 (Figure 4). Co-stimulation with TNF-αand IL-4 enhanced the secretion of RANTES above control levels, but did not elevate it to levels seen with TNF-α alone. Furthermore, the levels of RANTES did not differ significantly between Enp and NEnp.

Eotaxin secretion from nasal fibroblasts by stimulation with TNF-α and IL-4

Eotaxin secretion from fibroblasts was significantly increased by stimulation with IL-4 in both NEnp and Enp, but was not increased by stimulation with TNF-α (Figure 5). The level of eotaxin induced by IL-4 was significantly greater in Enp than in NEnp and co-stimulation with TNF-α and IL-4 also significantly enhanced eotaxin release; this occurred to a significantly greater degree in Enp than in NEnp. Further, eotaxin secretion induced by co-stimulation with TNF-α and IL-4 was significantly higher than that by TNF-α or IL-4 alone, suggesting that the effect was synergistic.

Expression of IL-4R mRNA

The expression of IL-4R mRNA in nasal fibroblasts was quantitatively analyzed by RT-PCR. IL-4R mRNA was expressed in control cells and this expression was not enhanced by stimulation with TNF-α (Figure 6). Although co-stimulation with TNF-α and IL-4 increased the expression of IL-4R mRNA, the increase was not significant. Expression of IL-4R mRNA did not differ significantly between NEnp and Enp.

DISCUSSION

Previous reports have already demonstrated that cultured fibroblasts isolated from nasal polyps can produce several adhesion molecules and chemokines [10-15]. VCAM-1 is an eosinophil-specific adhesion molecule whose production is enhanced by TNF-α [10]. In the present study, the secretion of VCAM-1 was induced by IL-4 as well as TNF-α. Furthermore, IL-4 and TNF-α acted synergistically in inducing VCAM-1 from nasal fibroblasts. Silvestri et al. [20] investigated VCAM-1 expression in nasal polyp fibroblasts by flow cytometry and reported that VCAM-1 was not up-regulated by TNF-α and IL-4 in combination. The characteristics of the fibroblasts and the methods used to examine VCAM-1 secretion might explain why the results of Silvestri's study differed from those of the present study.

The role of VCAM-1 secreted from nasal fibroblasts is not yet fully understood. Jahnsen et al. [21] demonstrated that both the

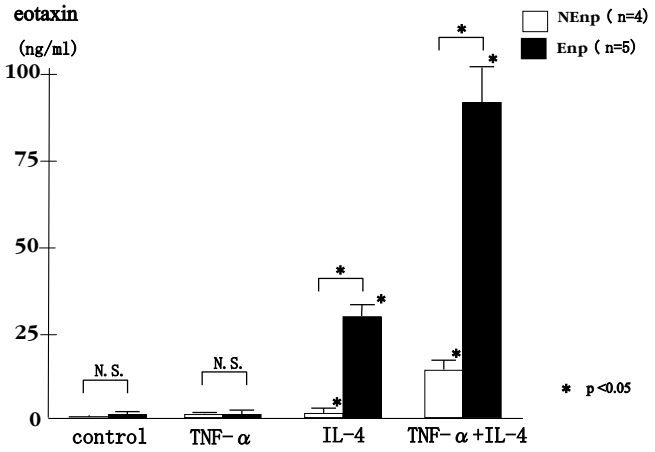

Figure 5. Eotaxin secretion from nasal fibroblasts by stimulation with TNF-α and IL-4.
Eotaxin secretion from fibroblasts was significantly increased by IL-4, but not by TNF-α. Secretion of eotaxin induced by co-stimulation with TNF-α and IL-4 was significantly higher than that by TNF-α or IL-4 alone ($p < 0.05$, respectively), suggesting that the effect was synergistic. NEnp and Enp differed significantly in terms of eotaxin levels induced by IL-4 and by simultaneous stimulation with TNF-α and IL-4. Data are expressed as mean ± SEM. *, $p < 0.05$ versus control group. N.S.: not significant.

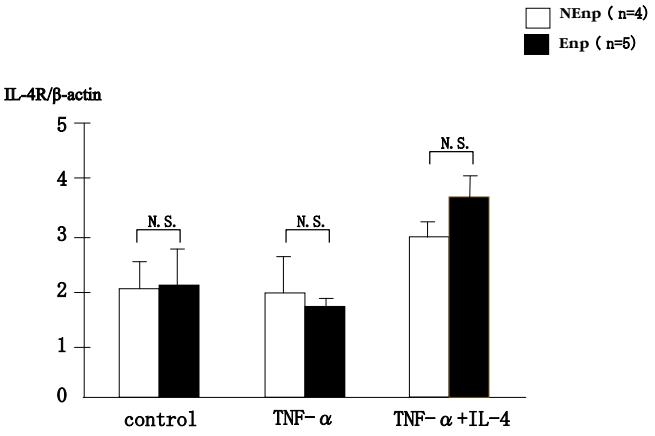

Figure 6. Expression of IL-4R mRNA.
The expression of IL-4R mRNA in nasal fibroblasts was quantitatively analyzed by real time RT-PCR. IL-4 mRNA expression was not enhanced by stimulation with TNF-α. The expression of IL-4R mRNA did not differ significantly between NEnp and Enp. N.S.: not significant.

number of eosinophils and the proportion of vessels positive for VCAM-1 were significantly increased in nasal polyps and that the relative number of eosinophils in nasal polyps was well correlated with the percentage of vessels positive for VCAM-1. However, in the present study, no significant difference was found between Enp and NEnp in the secreted levels of VCAM from nasal fibroblasts. Soluble VCAM-1 can increase the viability and promote the survival of eosinophil in a dose- and time-dependant manner by stimulating autocrine production of GM-CSF [22]. Corneal fibroblasts are also known to express VCAM-1 when activated with IL-4 and TNF-α. Then, eosinophils adhere to the activated corneal fibroblasts and induce subsequent fibroblast damage through these adhesion molecules [23]. Those findings suggest that VCAM-1 released by nasal fibroblasts might be associated with the survival of eosinophils rather than the chemotaxis and tissue remodeling of nasal polyps.

RANTES can cause chemotaxis of eosinophils, T cells, and monocytes, and is produced by mononuclear cells, epithelial cells, and fibroblasts after stimulation with IL-4, IL-13, TNF-α, or IFN-γ [24]. The present study demonstrated that TNF-α remarkably enhanced RANTES secretion from nasal fibroblasts. However, there was no significant difference between Enp and NEnp in this regard. IL-4 was not associated with RANTES release and had no synergistic effect with TNF-α. The findings suggest that increased secretion of RANTES might not be associated with eosinophilic infiltration in nasal polyps.

Shin et al. [14] investigated the role of RANTES in the recruitment of eosinophils into allergic and non-allergic nasal polyps and normal inferior turbinates by using quantitative RT-PCR for RANTES mRNA expression. They found that RANTES mRNA expression was similar among the three groups and not correlated with tissue eosinophilia. Pods et al. [15] found that expression of mRNA and protein synthesis of RANTES was similar in nasal polyps of patients suffering from chronic nasal polypous sinusitis, intrinsic asthma, aspirin-intolerance, and aspirin-triad. In contrast, Meyer et al. [25] reported that nasal polyps with a high tissue eosinophilia had a significant higher RANTES gene expression and protein production than nasal polyps without tissue eosinophilia. Association with AIA enhanced the amount of RANTES mRNA expressed in nasal polyps. The difference might be due to the use of different clinical definition for eosinophilic nasal polyps in each study.

Eotaxin is an eosinophil-specific chemokine that facilitates the infiltration of eosinophils in the nasal mucosa through its effect on the expression of adhesion molecules on microvascular endothelial cells. Recently, Schaefer et al. [26] analyzed the expression of eotaxin-2 (CCL24) mRNA in nasal turbinates and nasal polyps in order to localize the cellular source of eotaxin and found that nasal polyp endothelial and epithelial cells are the main source of CCL24. Fibroblasts and unstimulated cells did not express CCL24 mRNA. In contrast, Terada et al. [13] reported that fibroblasts are the major source of eotaxin in nasal mucosa, since stimulation with TNF-α or IL-4 caused minimal eotaxin expression by endothelial cells and epithelial cells in the human nasal mucosa. Furthermore, it has been reported that eotaxin is produced from nasal fibroblasts in a time- and dose-dependent manner after stimulation with TNF-α or IL-4 [11-15]. Since the definition of nasal polyposis

and CRS is different in each study, differences in the background of the subjects and in the experimental methods might have affected the findings. In the present study, IL-4 significantly increased the secretion of eotaxin from nasal fibroblasts and co-stimulation with TNF-α and IL-4 remarkably enhanced the response. The effects of IL-4 and the synergy of TNF-α and IL-4 on eotaxin release were greater in Enp than in NEnp. This indicates that the fibroblasts present in Enp are more sensitive than those in NEnp to stimulation with IL-4 and combined stimulation with TNF-α and IL-4.

Nonaka et al. [12] demonstrated the synergistic effects of IL-4 and lipopolysaccharide on the production of eotaxin from normal nasal fibroblasts and from nasal polyp fibroblasts in a similar manner. Terada et al. [13] investigated the eotaxin production in human nasal fibroblasts isolated from inferior turbinate nasal mucosa of patients with perennial nasal allergy by RT-PCR and Southern blot analysis. They clearly demonstrated that both IL-13 and IL-4 induced eotaxin expression and that the combined stimulation of IL-4 and TNF-α, as well as that of IL-13 and TNF-α, synergistically enhanced the production of eotaxin. The mechanisms whereby TNF-α and IL-4 synergize to induce the production of eotaxin are not clear [13]. Lugli et al. [27] demonstrated that stimulation with TNF-α induced a two- to three-fold increase of IL-4R expression. However, our experiments showed that IL-4R expression was not increased by stimulation with TNF-α or by combined stimulation with TNF-α and IL-4. Moreover, expression of IL-4R did not differ significantly between Enp and NEnp. Furthermore, pre-incubation of nasal fibroblasts with TNF-α did not enhance eotaxin release induced by IL-4, and vice versa. Simultaneous stimulation with TNF-α and IL-4 was most effective in inducing the production of eotaxin (data not shown). These findings suggest that the increased production of eotaxin by combined stimulation with TNF-α and IL-4 in nasal fibroblasts of Enp as well as NEnp might be regulated by post-receptor events [13]. In fact, TNF-α stimulation leads to increased activation of the IL-4-specific signal transducers and activators of transcription protein (Stat6) by IL-4 [24]. It is also interesting that a significant difference between Enp and NEnp was observed only in the secretion of eotaxin induced by IL-4 or by simultaneous stimulation with TNF-α and IL-4. Hence, fibroblasts in Enp might be selectively primed for the production of eotaxin in response to stimulation with IL-4 and TNF-α.

In addition to the synergistic effects with TNF-α and IL-4, immunological balance between T helper 1 (Th1) and Th2 cytokines might be associated with the secretion of RANTES and eotaxin, since TNF-α is a Th1 and IL-4 is a Th2 cytokine. Fujisawa et al. [24] found that TNF-–induced RANTES production from BEAS-2B cells was markedly enhanced by Th1 cytokine IFN-γ and was not affected by Th2 cytokine IL-4. Moreover, IFN-γ inhibited eotaxin production induced by co-stimulation with TNF-α and IL-4. On the other hand, Lezcano-Meza et al. [28] found that IL-4 was the major stimulus for eotaxin-2 production from nasal polyps followed by IL-13 and IFN-γ. Those findings suggested that Th1 as well as Th2 cytokines regulate the production of RANTES and eotaxin and the degree of eosinophil infiltration in nasal polyps.

In conclusion, the present study showed that eotaxin secretion from nasal fibroblasts was induced by stimulation with IL-4 and synergistically enhanced by simultaneous stimulation with TNF-α and IL-4. The secreted level of eotaxin from fibroblasts was significantly higher in Enp than in NEnp. In contrast, the levels of VCAM-1 and RANTES did not differ significantly between Enp and NEnp. These findings suggest that eotaxin plays an important role in selective recruitment of eosinophils in Enp. Furthermore, nasal fibroblasts in Enp appear more sensitive than those in NEnp regarding eotaxin secretion induced by co-stimulation with TNF-α and IL-4. This might be associated with the pathogenesis of nasal polyposis having marked eosinophil infiltration.

ACKNOWLEDGEMENT

Financial support: Grant-in-Aids from the Ministry of Education, Science and Culture, and Ministry of Health and Welfare of Japan (B 17390463)

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