Table 2 The number of intraocular and intranasal pollen grains in the first part of the study.

Subject No.#	1	2	3 *	4	5	6	7	8	9	10	Mean ± SD
Intraocular pollen	2	13	3	6	15	20	12	6	49	10	13.6 ± 13.6 (grains)
Intranasal pollen	175	175	303	160	198	522	90	260	281	328	249.2 ± 120.9 (grains)

One subject (*) exited the chamber 10 min earlier since she was about to sneeze.
The remaining subjects were able to stay for an hour without developing symptoms.
As to the subject numbers, see Table 1

Table 3 The number of subjects who showed nose or eye symptoms during 2-hour allergen exposure.

	15	30	60	90	120	(min)
Rhinorrhea	1	3	3	6	8	
Sneezing	0	1	1	4	4	
Nasal obstruction	1	4	6	6	7	
Nasal itching	2	4	4	7	8	
Eye itching	2	5	6	7	6	
Watery eyes	0	2	3	3	3	
Nasal secretion (g/hr)	←	0.23 ± 0.39	→ ←	0.73 ± 0.83	→	

There were increases in the number of subjects who developed nasal and ocular symptoms in a time dependent manner. Six subjects developed nasal obstruction after 60 minutes of exposure, of which 3 subjects marked their symptoms as moderate while 1 subject marked them as severe. Although 8 subjects developed rhinorrhea and nasal itching after 2 hours of exposure to the allergen, only 4 subjects developed sneezing and their symptoms were mild. We measured the amount of nasal secretion from 3 subjects in the first half of the study and from 7 in the latter half of the study. Ocular symptoms also developed as time went by. During the study period, 1 subject showed neither nasal nor ocular symptoms.

tomation Inc, Tokyo, Japan) to nasal lavage fluid in order to reduce its viscosity. After shaking the nasal or ocular lavage fluid for 5 minutes at room temperature, it was put into a suction filtration device using 0.5 μm filter papers (Advantee Cellulose Nitrate, Toyo Roshi Inc, Tokyo, Japan). Hereafter, the leftover solution was washed once more with saline and the solution was put into a suction filtration device to keep any single grain from being left behind. In this way, all the filter papers were examined under a light microscope (×100) and the pollen count was recorded.

RESULTS

The subjects consisted of 5 men and 5 women with mild symptoms. Their ages ranged from 32 to 48 years (the average age was 38.5 ± 5.4). Background factors of the subjects are shown in Table 1.

In the first part of this study, the temperature, the humidity, and the concentration of pollen grains in the room were kept within the targeted values. That is, the average number of pollen grains was 2572.8 ± 264.5 grains /m^3, the average temperature 22.1 ± 0.14℃, and the average humidity 44.7 ± 0.48% (Fig. 4). Intranasal pollen counts in the subjects ranged from 90 to 522 and intraocular grains ranged from 2 to 49. No relation was seen between the positions of the subjects within the chamber and the numbers of pollen detected in the nose and eyes. Even though 1 subject had to leave the chamber 10 minutes earlier than the scheduled time because of symptom development, the rest of the subjects were able to stay in the room for an hour without developing symptoms. The average number of intranasal pollen grains and intraocular grains was 249.2 ± 120.9 and 13.6 ± 13.6, respectively (Table 2).

In the second part of this study, the temperature, the humidity, and the concentration of pollen grains inside the chamber were within targeted values: the average number of pollen grains was 4367 ± 207 grains/m^3, the temperature 22.2 ± 0.35℃, and the humidity 44.8 ± 0.65% (Fig. 5). As time went by, more and more subjects started developing nasal symptoms such as nasal discharge, sneezing, nasal obstruction, and itchy nose. Only 3 subjects showed nasal discharge 60 minutes after the start of exposure, but 6 subjects after 90 minutes and 8 subjects after 120 minutes. Likewise, the number of subjects who developed nasal obstruction increased after 60 minutes of exposure, of which 3 subjects marked their symptoms as moderate while 1 subject marked severe. Although 8 subjects showed nasal itching after 120 minutes, only 4 subjects developed sneezing during exposure and their symptoms were mild. Three subjects blew their nose within 1 hour of the first half of the study, and 7 in the latter half. The amount of nasal secretion in the first half and the latter half of the study was 0.23 ± 0.39 g/hour and 0.73 ± 0.83 g/

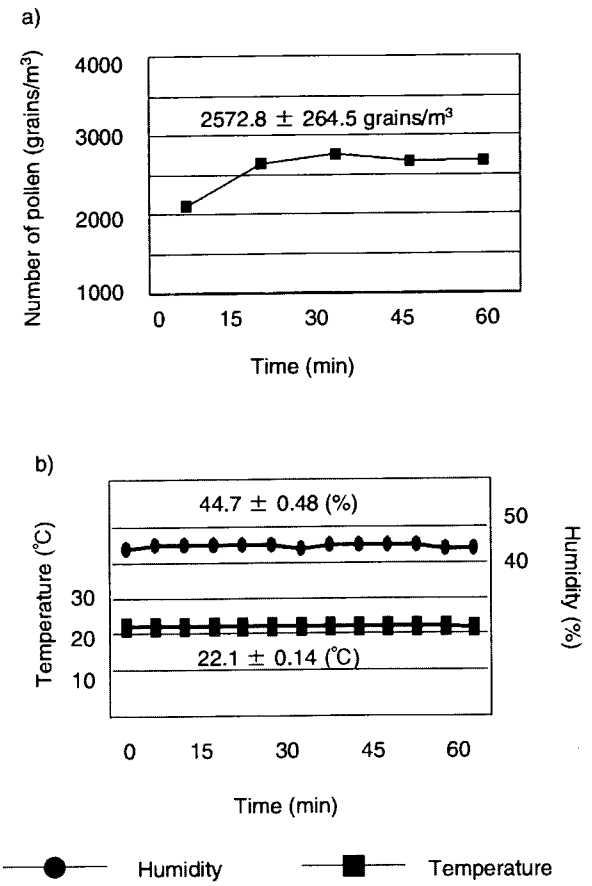

Fig. 4 The pollen count (**a**) and temperature and humidity (**b**) in the chamber during the first part of the study. The pollen count at each point shows the average value during 15 minutes. The average number of pollen grains was 2572.8 ± 264.5 grains/m^3, the average temperature 22.1 ± 0.14℃, and the average humidity 44.7 ± 0.48%.

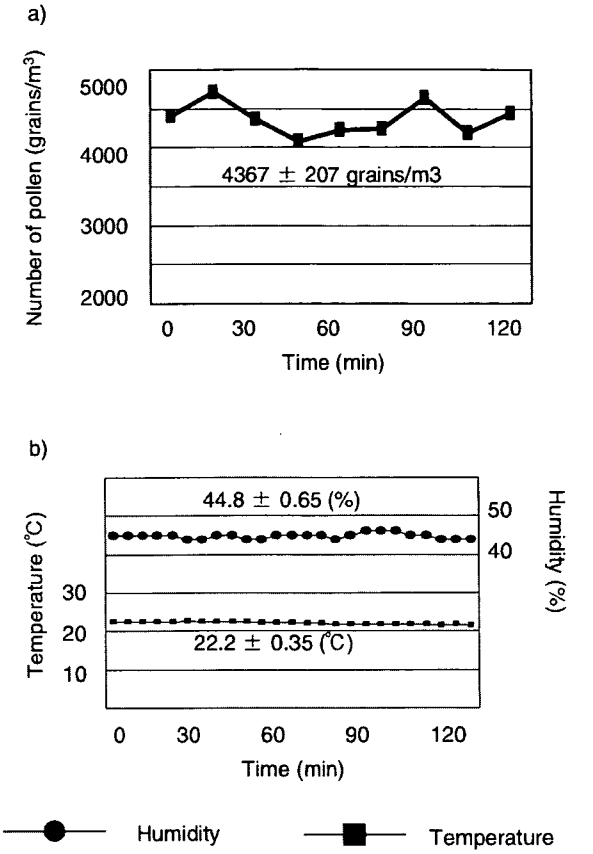

Fig. 5 The pollen count (**a**) and temperature and humidity (**b**) in the chamber during the second part of the study. The average number of pollen grains was 4367 ± 207 grains/m^3, the average temperature 22.2 ± 0.35℃, and the average humidity 44.8 ± 0.65%.

hour respectively (Table 3).

With regard to ocular symptoms, half of the subjects developed itchy eyes within 30 minutes after entering the chamber, increasing to 7 subjects as time went by. Their symptoms, however, were mild. In addition, only 3 subjects developed epiphora while other 3 subjects experienced neither itchy eyes nor epiphora in the chamber. During the 2-hour exposure to pollen, 1 subject showed no nasal or ocular symptoms. No adverse event was observed in these studies. None of the subjects showed late nasal reaction after returning home.

DISCUSSION

This is a preliminary study showing the amount of intranasal or intraocular pollen as well as allergy symptoms in mildly symptomatic subjects with JC pollinosis under well controlled conditions in an OHIO chamber.

There are several allergen exposure units in Europe and the US[6-8] and a lot of useful data are available in the treatment of allergic rhinitis.[10,11] However it is difficult to simply compare the figures for pollen dispersal between the results obtained from each exposure unit. Each chamber uses a different method to count the pollen grains. For instance, our OHIO chamber uses KC-20 (Rion Co, Tokyo, Japan), a laser particle counter which measures the number of particles between 10 and 100 m in diameter. On the other hand, the Japanese Environment Ministry, which has been measuring pollen counts at various stations and providing information during pollen seasons, uses a different kind of laser-counter (KH-3000: Yamato Seisakujo, Japan). We attempted measurements with both KC-20 and KH-3000 and discovered that there is a correlation between the outcomes of the two methods of measurement. We also discovered that KC-20 yields values 5.9 times larger than those obtained using KH-3000 (data not shown).

In the first part of the study, subjects were exposed

to 2500 grains/m^3 of JC pollen, which is equivalent to the amount of airborne pollen grains in the early stages of the pollen season. Although their allergic symptoms were reported to be mild, one of the subjects had to leave the room before the scheduled time since the subject was about to sneeze. The other 9 subjects were able to remain in the room for an hour without developing any allergic symptoms. We found that the average number of intranasal and intraocular pollen grains was 249.2 and 13.6, respectively. The subjects sat still during the study for up to 1 hour and did not move around, so their eyes simply received pollen grains, and their shedding tears and blinking cleared the pollen grains. On the other hand, subjects actively inhaled pollen grains through their noses, allowing more pollen to precipitate in the nose and they did neither sneeze nor blow their noses. These could be the possible reason why the number of intranasal pollen grains was much larger than that of the intraocular pollen grains. Gotoh *et al.*[12] conducted a study on the ratio of intranasal to intraocular pollen numbers, which were obtained from healthy volunteers walking in the open air at an ordinary speed for half an hour. Their study showed the almost same result with ours.

In the second part of the study, the concentration of pollen dispersed was increased to 4500 grains/m^3. This concentration is equivalent to the amount of airborne pollen grains during the midterm and late stages of the pollen season. Nasal and ocular symptoms gradually developed in a time dependent manner, but these symptoms were mild.

Okuda *et al.*[13] measured the number of intranasal grains of JC pollinosis patients during pollen seasons over several years. They showed that the average number of JC pollen found in a patient's nose was about 20, although the amount of pollen varies every year. They concluded that 90 to 150 pollen grains were considered to be sufficient to cause symptoms from the dynamic study of pollen in the nose.

In this study, we found that 90 to 500 pollen grains in the nose were not enough to develop nasal symptoms. The only exception was the subject who exited the room in 50 minutes due to sneezing, whose intranasal pollen counted 303. There is a difference in the number of pollen which develops nasal symptoms observed in our study and in the study conducted in a natural environment.[13] The following could be the reason for the difference; subjects in this study were mildly symptomatic patients with JC pollen; subjects had not received repetitive exposure to JC pollen, because the study was conducted 3 months ahead of the pollen season; and subjects were under psychological pressure since they had never experienced an environmental exposure study.

This is the first study to show the intranasal and intraocular pollen grains and allergic symptoms using the OHIO Chamber. As far as pollinosis is concerned, however, our data cannot be immediately generalized since the results depend on the amount of pollen, the priming effects of the nasal mucosa, and the severity of the patients' symptoms. We need to evaluate the results of our data carefully. Therefore, further investigations are required to decide an appropriate amount of pollen and exposure time to obtain reproducible results and to secure the safety of the subjects.

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TNF-α upregulates VCAM-1 and NF-κB in fibroblasts from nasal polyps

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Abstract

Objective: Lung and synovial fibroblasts produce VCAM-1 in response to TNF-α. However, the massive infiltration of eosinophils, the effects of the increased amount of TNF-α and the production of VCAM-1 in human nasal polyp fibroblasts are not yet fully understood. The present study examines the role of VCAM-1 and the molecular mechanism of its expression in nasal fibroblasts.
Methods: Nasal fibroblasts were isolated from human nasal polyps and after four passages, the cells were stimulated with TNF-α and VCAM-1 expression was examined by ELISA, flow cytometry, and RT-PCR. The activation of NF-κB induced by TNF-α was determined by electrophoretic mobility shift assays and the influence on the expression of VCAM-1 was investigated.
Results: VCAM-1 protein and mRNA were expressed in unstimulated controls and remarkably increased by TNF-α stimulation. NF-κB activity was enhanced by TNF-α stimulation and remarkably suppressed by NF-κB proteasome inhibitor.
Conclusions: The present study discovered that nasal fibroblasts produce VCAM-1 protein and mRNA and that production is increased by TNF-α stimulation. Furthermore, VCAM-1 expression in nasal fibroblasts is induced through an NF-κB-dependent pathway. These findings might provide a rationale for using NF-κB inhibitors as a treatment for nasal inflammatory diseases such as polyps.

Keywords: Nasal polyp; Eosinophil infiltration; Adhesion molecule; Signal pathway

1. Introduction

Nasal polyps composed of epithelial cells, fibroblasts, and immunocompetent cells (such as macrophages, mast cells, and eosinophils) are characterized by hyperplastic and swollen mucosa arising from the middle meatus [1,2]. Eosinophils comprise over 60% of the cell population [3] and 80–90% of nasal polyps are characterized by abundant eosinophils [4]. These findings suggest that eosinophil accumulation is a key factor in the pathogenesis of nasal polyps. However, the role of eosinophils and the mechanism of their infiltration of nasal polyps remain unknown.

Eosinophil infiltration is regulated by numerous chemokines and adhesion molecules such as eotaxin, regulated on activation normal T cell expressed and secreted (RANTES), and vascular cell adhesion molecule (VCAM)-1. To infiltrate sites of inflammation, eosinophils leave the bloodstream and pass through the endothelium in four steps, namely rolling, adhesion, transendothelial migration, and chemotaxis [5]. Adhesion molecules such as VCAM-1 play an important role during adhesion to endothelial cells. Originally identified on the surface of endothelial cells after exposure to inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1-beta (IL-1β), VCAM-1 is a 90- to 110-kDa glycoprotein [6]. Studies of the signal transduction pathways leading to VCAM-1 mRNA expression through TNF-α and IL-1β receptors in endothelial cells such as human umbilical vein endothelial cells (HUVEC)

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[6,7] have discovered that TNF-α stimulates endothelial cells, which activates nuclear factor kappa B (NF-κB) that subsequently activates the VCAM-1 mRNA promoter [6].

Fibroblasts in the lungs and synovium express VCAM-1 [8,9] and produce extracellular matrix proteins as well as pro-inflammatory cytokines that are associated with the repair and remodeling of inflamed tissues [10]. Fibroblasts constitute one of the major cellular components of nasal polyps and they produce several chemokines and adhesion molecules such as eotaxin, RANTES, and intercellular adhesion molecule (ICAM)-1 [11,12]. Thus, VCAM-1 might also be produced by nasal fibroblasts and play a role in eosinophil infiltration and retention in the connective tissue of nasal polyps. However, little is known about VCAM-1 production in nasal fibroblasts and the molecular mechanism involved in the expression of VCAM-1 in nasal fibroblasts has not been studied [11].

The present study examines the production of VCAM-1 protein and mRNA from nasal fibroblasts to clarify the role(s) of these cells in the eosinophil infiltration into nasal polyps. We also investigated NF-κB activation after TNF-α stimulation and discuss the mechanism of VCAM-1 expression in nasal fibroblasts.

2. Materials and methods

2.1. Cell cultures

Cultured human nasal fibroblasts were derived from nasal polyps obtained at polypectomy. Samples of nasal polyp were obtained from six patients and each cell culture of nasal fibroblasts was established from each sample of the nasal polyp. 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) supplemented with 10% fetal bovine serum (FBS). In phase-contrast microscopically, there was no contamination of epithelial cells and leukocytes. Further, the cells were characterized by flow cytometry using an anti-human Thy-1 antibody (Dianova, Hamburg). The purity of fibroblasts was more than 99%. Cells were used throughout the study after four passages. The present study was approved by Institutional Review Board of Kagoshima University, Faculty of Medicine.

2.2. Enzyme-linked immunosorbent assay (ELISA)

After reaching confluence in 24-well plates, the DMEM was removed and the fibroblasts were exposed to serum free DMEM with various concentrations (0.1, 1, 10, and 100 ng/ml) of recombinant human TNF-α (CHEMICON International Inc., Temecula, CA) for 24 h. The negative control consisted of cells cultured without stimulation. The culture supernatants were collected and soluble VCAM-1 (sVCAM-1) concentrations were determined using a human VCAM-1 ELISA kit (BioSource International Inc., Camarillo, CA).

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

After reaching confluence in 6-well plates, the culture supernatants were removed and the fibroblasts cells were exposed to various concentrations of TNF-α diluted in serum free DMEM for 6 h. Total mRNA was isolated using a High Pure RNA Isolation kit (Roche, Mannheim, Germany). One microgram of total RNA was reverse-transcribed with 200 units of transcriptase (SuperScript II, Invitrogen Corp., Carlsbad, CA), 500 μg oligo (dT)12–18 (Invitrogen Corp., Carlsbad, CA), 0.5 mol/l 2′-deoxyribonucleoside-5′-triphosphates (dNTPs:Pharmacia, Piscataway, NJ), and 10 mmol/l DTT in a total volume of 20 μl according to manufacturer's instructions. PCR was performed using 1 μl template complementary DNA (cDNA), 0.5 U Taq polymerase (Stratagene, La Jolla, CA), 1.25 μmol/l oligomer primers, and 0.75 mmol/l dNTPs in a total volume of 20 μl. VCAM-1 message was amplified by 30 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 15 s in an automatic thermocycler (Pekin-Elmer Cetus DNA thermocycler, New walk, CA). The PCR products were resolved by electrophoresis on a 1% agarose gel and visualized with ethidium bromide. PCR proceeded using specific primer sets for VCAM-1 and β-actin as the internal control. The primer combinations were: 5′-GTCTGCA-TCCTCCAGAAATTCC-3′ and 5′-TAAAATCGAGAC-CACCCC-3′ for VCAM-1 (predicted 433 bp fragment) and 5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CTCC-TTAATGTCACGCACGATTTC-3′ for β-actin (predicted 540-bp fragment).

2.4. Flow cytometry

Fibroblasts cultured in 6-well plates were stimulated with TNF-α (10 ng/ml) for 6 h. Then the cells were trypsinized with 0.53 mM EDTA 4Na and 0.05% trypsin (Invitrogen Corp., Carlsbad, CA) and detached from cell culture dishes. After washing twice with phosphate buffered saline (PBS), the cells were resuspended in 200 μl of PBS and incubated with mouse monoclonal anti-human VCAM-1 antibody (DakoCytomation, Produktionsvej, Denmark) on ice for 30 min. The cells were washed and incubated on ice with fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody (DakoCytomation, Produktionsvej, Denmark) for 30 min. The cell surface expression of VCAM-1 was analyzed using immunofluorescence flow cytometry (Beckman Coulter Inc., Fullerton, CA).

2.5. Electrophoretic mobility shift assay (EMSA)

The activation of NF-κB was assessed by EMSA. Nasal fibroblasts were stimulated with 10 ng/ml of TNF-α for 30 min. After the stimulation, cells were harvested by using of cell scraper (CORNING, Acton, MA) and then nuclear proteins were isolated as described [13]. In brief, the harvested cells were lysed in buffer A (0.6% Nonidet-P40; 10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 3.5 mM PMSF; 1 mM DTT) on ice for 15 min with occasional vortex mixing. Nuclei were collected by centrifugation at 10,000 × *g* for 4 min and washed once with buffer A. The pellets were suspended in buffer C (20 mM HEPES pH 7.9; 0.4 M KCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF) and incubated on ice for 30 min with occasional vortex mixing. After centrifugation at 10,000 × *g* for 30 min, the supernatants containing nuclear protein were stored at −80 °C. Protein concentrations were measured using a protein assay kit (Bio Rad, Hercules, CA). Equivalent amounts of nuclear proteins (1–3 μg) were resolved by electrophoresis in a gel shift assay system (Promega, Madison, WI) according to the manufacturer's protocol, with a few modifications. The double-stranded NF-κB-oligonucleotide probe supplied with the assay system was end labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase. Nuclear proteins were incubated with 30,000 cpm of NF-κB probe at room temperature for 30 min. Complexes of DNA-protein were resolved by electrophoresis on non-denaturing 6% polyacrylamide gels in Tris-boric acid/EDTA buffer, then the gels were dried under vacuum and exposed to Kodak X-ray film overnight at −70 °C. For the competition experiment, nuclear extracts were preincubated with unlabeled competitors for 20 min prior to adding the radio-labeled oligonucleotide. For the supershift experiment, nuclear extracts were preincubated with 1 μg of polyclonal antibody on ice for 30 min prior to adding the radiolabeled oligonucleotide.

2.6. Effects of a proteasome inhibitor specific to NF-κB

Nasal fibroblasts were incubated with 0.3 and 1 μM of MG132 (EMD Bioscience Inc., USA), a proteasome inhibitor of Z-Leu-Leu-Leu-H which have been shown to prevent IκB degradation and thereby specifically inhibits NF-κB activation [14], for 4 h and then stimulated with TNF-α (10 ng/ml). The expression of VCAM-1 and of NF-κB activation were then investigated as described above.

2.7. Statistical analysis

The soluble VCAM-1 (sVCAM-1) concentrations measured by ELISA are expressed as means ± standard error (S.E.). Data were statistically analyzed using the two-way analysis of variance (ANOVA). Values of $p < 0.05$ were considered significant.

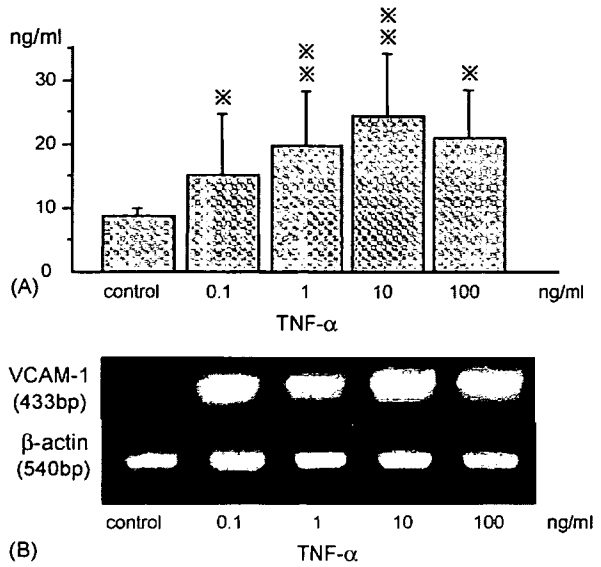

Fig. 1. (A) Mean concentrations of soluble VCAM-1 (sVCAM-1) in culture supernatants of human nasal fibroblasts detected by ELISA. Production of sVCAM-1 was dose-dependently increased by TNF-α stimulation, and reached a plateau at 10 ng/ml of TNF-α. Bars represent S.E. of six experiments in each group. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control group. (B) Expression of VCAM-1 mRNA after stimulation with TNF-α. Expression in control group and enhanced by TNF-α stimulation.

3. Results

3.1. VCAM-1 expression induced by TNF-α stimulation

The production of sVCAM-1 and of VCAM-1 mRNA observed in controls was dose-dependently and significantly increased by TNF-α stimulation (Fig. 1A and B). The levels of sVCAM-1 shown in Fig. 1A are the average of six experiments and the results of RT-PCR shown in Fig. 1B are one of the representatives of those experiments. These results suggest that VCAM-1 is constitutionally expressed in nasal fibroblasts and that its expression was upregulated by stimulation with TNF-α. Since the characteristics of clinically isolated samples of fibroblast are heterogeneous, the discrepancy between the results shown in Fig. 1A and B might be due to the difference in the expression of VCAM-1 mRNA as well as the production of sVCAM-1 in each sample. The same reason might explain the discrepancy between the expression of mRNA in 1.0 ng/ml TNF-α-stimulated cells and that in 0.1 ng/ml TNF-α-stimulated cells shown in Fig. 1B. Further, the stimulations with 0.1 and 1.0 ng/ml of TNF-α might not be optimum condition.

3.2. VCAM-1 expression on the surfaces of nasal fibroblasts

Flow cytometry showed that constitutional VCAM-1 expression on the surfaces of nasal fibroblasts was enhanced by stimulation with TNF-α (Fig. 2). The mean channel fluorescence value for binding VCAM-1 on nasal fibroblasts without TNF-α stimulation was 1.27 ± 0.13 (gray line). In

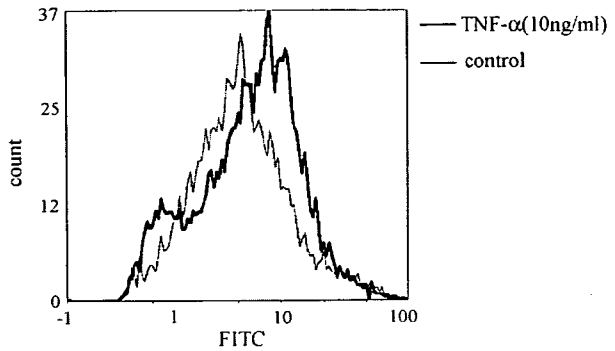

Fig. 2. VCAM-1 expression on the surfaces of nasal fibroblasts. Flow cytometry showed that constitutional VCAM-1 expression on the surfaces of nasal fibroblasts was enhanced by stimulation with TNF-α. The mean channel fluorescence value for binding VCAM-1 on nasal fibroblasts without TNF-α stimulation was 1.27 ± 0.13 (gray line:cont.). On the other hand, mean fluorescence value for binding VCAM-1 on nasal fibroblasts with TNF-α stimulation was 2.28 ± 0.45 (black line). The difference was statistically significant ($p < 0.05$).

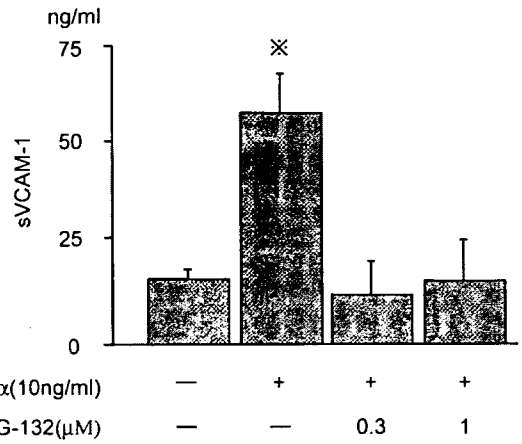

Fig. 4. Effects of NF-κB inhibitor, MG-132, on sVCAM-1 production in human nasal fibroblasts. Incubating nasal fibroblasts with MG132 significantly reduced subsequent production of sVCAM-1 induced by TNF-α. Bars represent S.E. of four experiments in each group. $^{*}p < 0.01$ vs. control cells.

contrast, mean fluorescence value for binding VCAM-1 on nasal fibroblasts after TNF-α stimulation was 2.28 ± 0.45 (black line). The difference was statistically significant ($p < 0.05$). These results are consistent with the ELISA and RT-PCR data and suggest that sVCAM-1 detected in culture supernatants was shed from the surface of nasal fibroblasts as suggested by Gao and Issekutz [15].

3.3. NF-κB activation by TNF-α stimulation

Nuclear factor-κB is a transcriptional factor that regulates the gene expression of many proteins associated with cell growth, inflammation and immune responses. The EMSA results revealed that the NF-κB activation in nasal fibroblasts was remarkably increased by TNF-α (Fig. 3). The specificity of NF-κB band was confirmed by oligo competition assay and supershift assay (Fig. 3A).

3.4. Effects of proteasome inhibitor specific to NF-κB on VCAM-1 expression

The production of sVCAM-1 enhanced by TNF-α was remarkably and dose-dependently suppressed by the NF-κB proteasome inhibitor MG-132 (Fig. 4). The expression of

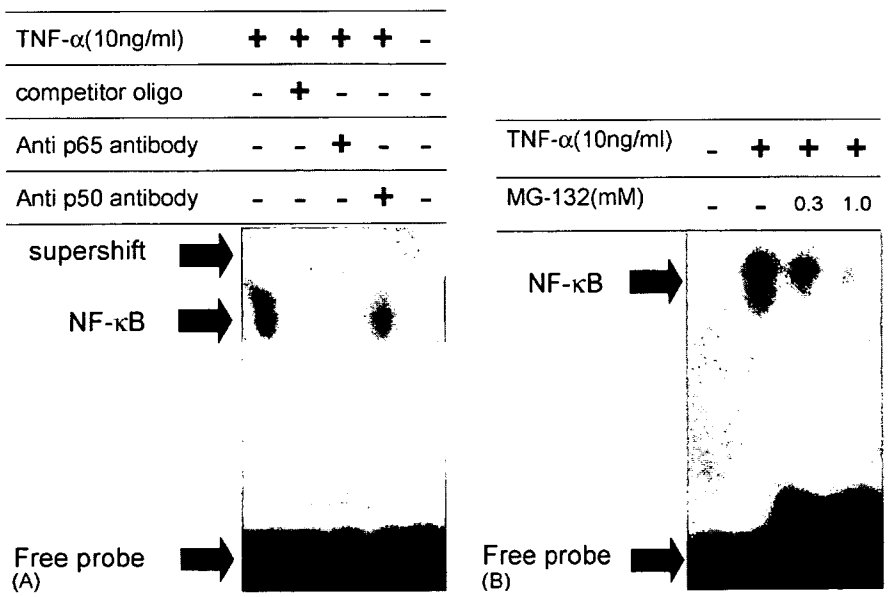

Fig. 3. Activation of NF-κB induced by stimulation with TNF-α (10 ng/ml) and effects of NF-κB inhibitor, MG-132, on NF-κB activation in human nasal fibroblasts. (A) The specificity of the NF-κB band was confirmed by oligo competition assays and supershift assays. Arrows indicate specific bands of NF-κB DNA complexes, supershift, and free probe, respectively. (B) NF-κB was constitutively activated and significantly enhanced by TNF-α stimulation. Incubation with MG132 reduced activation of NF-κB induced by TNF-α in nasal fibroblasts in dose dependent manner. Inhibition of p50/p65 heterodimer NF-κB complex was remarkable. Arrows indicate specific bands of NF-κB DNA complexes and free probe, respectively.

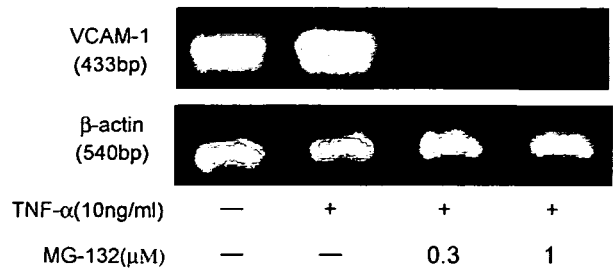

Fig. 5. Effects of NF-κB inhibitor, MG-132, on VCAM-1 mRNA expression in human nasal fibroblasts. NF-κB was constitutively activated and significantly enhanced by TNF-α stimulation. Arrows indicate specific NF-κB DNA complexes. Incubation with MG132 remarkably suppressed subsequent VCAM-1 mRNA expression in nasal fibroblasts.

VCAM-1 mRNA induced by TNF-α stimulation was suppressed by MG-132 to below control levels, suggesting that MG-132 reduced the constitutional expression of VCAM-1 (Fig. 5). Moreover, EMSA showed that MG-132 reduced the activation of NF-κB induced by TNF-α and blocked binding of the NF-κB p50/p65 heterodimer (Fig. 3B).

4. Discussion

The present study demonstrated that VCAM-1 at both the protein and mRNA levels is constitutionally produced by nasal fibroblasts and dose-dependently enhanced by stimulation with TNF-α. Epithelial and immunocompetent cells such as mast cells, macrophages, and eosinophils produce TNF-α [16,17], levels of which are obviously increased in nasal secretions of patients with chronic sinusitis [18]. Kramer and Rasp [4] reported that eosinophils are the major source of TNF-α in nasal polyps. These findings suggest that TNF-α increases VCAM-1 expression in nasal fibroblasts and activates the transmigration of eosinophils, which induce further production of TNF-α and accelerate the accumulation of eosinophils in nasal mucosa. Such a vicious circle might be formed in inflamed nasal mucosa and associated with intractable and recurrent nasal polyps. In fact, increased VCAM-1 expression is correlated with eosinophil infiltration in nasal polyps [19].

Soluble VCAM-1, the result of shedding from the cell surface, is upregulated in chronic inflammatory diseases and in some types of cancer [20,21]. The results in the present study obtained by ELISA and flow-cytometry supported these findings. Silvestri et al. [11] investigated the VCAM-1 expression in nasal polyp fibroblasts by flow cytometry and found the constitutional expression of VCAM-1. However, they did not show that VCAM-1 was up-regulated by TNF-α stimulation in nasal polyp fibroblasts. In contrast, they demonstrated using the same technique that TNF-α increases VCAM-1 expression in human fetal lung fibroblasts [8]. In the present study, ELISA and RT-PCR detected increased expression of VCAM-1 in nasal polyp fibroblasts at the protein and mRNA levels in response to TNF-α, respectively. The characteristics of the fibroblasts and the methods used to examine VCAM-1 expression might explain why the results of these studies differed.

Although the role(s) of sVCAM-1 and VCAM-1 expressed on nasal fibroblast surfaces is not yet fully understood, VCAM-1 as well as sVCAM-1 is supposed to be associated with eosinophil activation in the connective tissue of nasal mucosa. Nagata et al. [22] reported that eosinophil binding to vascular endothelial cells mediated by VCAM-1 activates eosinophils. Moreover, sVCAM-1 is present in inflamed tissues and synergistically with TNF-α induces angiogenesis [23]. These findings indicate that VCAM-1 produced by nasal fibroblasts plays a relevant role in eosinophil retention and activation in the connective tissue of nasal polyps and affects the pathogenesis of this disease.

The regulation of VCAM-1 by NF-κB was originally implicated in studies of the VCAM-1 promoter in HUVEC, which demonstrated the importance of two adjacent NF-κB binding sites to the TNF-α responsiveness of the VCAM-1 promoter [6]. The present study found that NF-κB is constitutionally activated in human nasal polyp fibroblasts and is further activated by TNF-α stimulation. The proteasome inhibitor MG-132, which is a potent inhibitor of NF-κB through blocking IκB-α degradation [24], reduced the sVCAM-1 production as well as the expression of VCAM-1 mRNA enhanced by TNF-α to levels lower than those in the controls. VCAM-1 production is increased by many stimulatory factors including TNF-α, IL-1β, IL-4, and IL-13 [10]. However, some of the pathways of VCAM-1 production triggered by these cytokines are independent of NF-κB. For instance, VCAM-1 expression induced by IL-4 does not require NF-κB activation [25]. These findings suggest that constitutional and TNF-α-stimulated VCAM-1 production in nasal fibroblasts is NF-κB dependent, since VCAM-1 expression was almost completely blocked even when the inhibition of NF-κB by MG-132 was incomplete.

Cytosolic NF-kB can be inactive (complexed to IkB inhibitory protein) or exist as homodimers or heterodimers of 50-(p50) and 65-kDa subunits. Activation of NF-κB induces the formation of p50/p50 homodimers and p50/p65 heterodimers through IκB phosphorylation [24]. The present study found that TNF-α stimulation caused formation of p50/p65 heterodimers. MG-132 inhibited p50/p65 heterodimers formation. NF-κB complexes activated by TNF-α stimulation differ among cell types [26]. The activation of p50/p65 NF-κB was predominant in human chondrocyte-like cells stimulated by TNF-α [27]. In contrast, TNF-α stimulation induces mainly the p50/p50 homodimer in fibroblast-like synoviocytes [8]. IκB-α and IκB-β have been characterized and they are associated with NF-κB activation. Most NF-κB activators including TNF-α rapidly phosphorylate IκB-α, resulting in its degradation through a proteasome-dependent pathway. IκB-β becomes degraded more slowly and is induced by agonists that cause persistent NF-κB activation [24,28]. Moreover, the activity of IκB-α and IκB-β binding to NF-κB complexes differs [26]. Such

diversity in the pathway leading to NF-κB activation might explain the differences in NF-κB complexes induced by TNF-α in the various cell types and the inhibitory effect of the IκB-α degradation inhibitor, MG-132, on the p50/p65 heterodimer in nasal fibroblasts as shown in the present study. Collectively, the data suggest that NF-κB plays a critical role in the TNF-α mediated expression of VCAM-1 in nasal fibroblasts.

We previously demonstrated that 14-membered macrolide antibiotics and steroids reduce the production of inflammatory cytokines such as IL-1β and IL-8 as well as NF-κB activation in nasal and adenoidal fibroblasts [13,29]. Cetrizine, an antihistamine drug, inhibits the TNF-α-induced hyperactivity of NF-κB. [30]. A strategy targeting NF-κB activation might be useful not only for inhibiting eosinophil infiltration but also for treating inflammatory diseases such as nasal polyps, chronic sinusitis, and nasal allergies.

In conclusion, our experiments demonstrated that TNF-α stimulation induces VCAM-1 protein and mRNA expression in human nasal fibroblasts. Both the constitutional and TNF-α stimulated production of VCAM-1 were induced through an NF-κB-dependent pathway. These findings provide a rationale for the use of pharmacological agents that inhibit NF-κB as a treatment for nasal inflammatory diseases.

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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

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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).

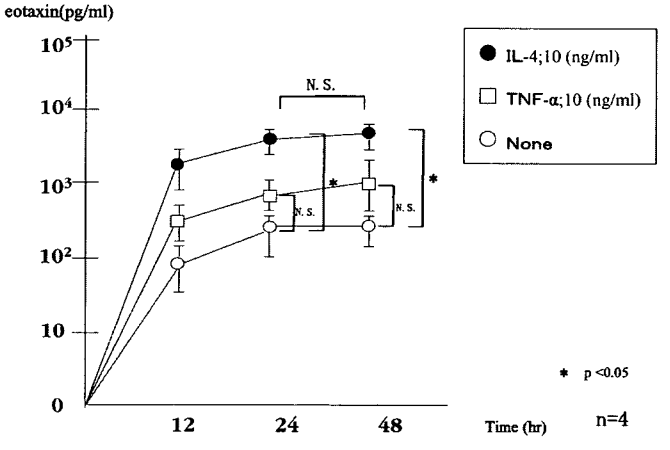

Figure 1. Time-dependent eotaxin secretion from fibroblasts in response to TNF-α and IL-4.

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

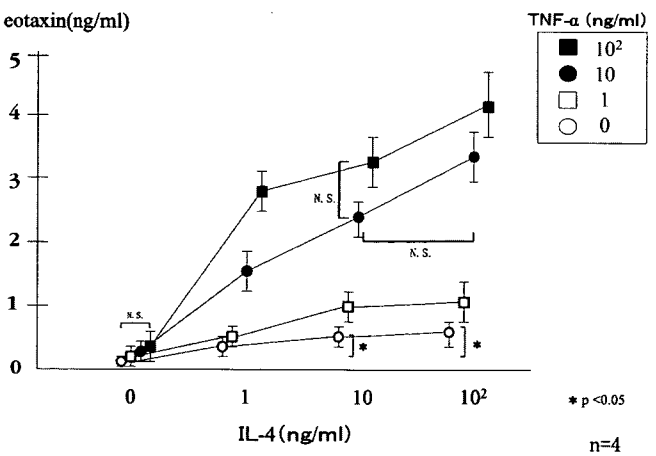

Figure 2. Dose-dependent eotaxin secretion from fibroblasts in response to IL-4 and TNF-α.

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.

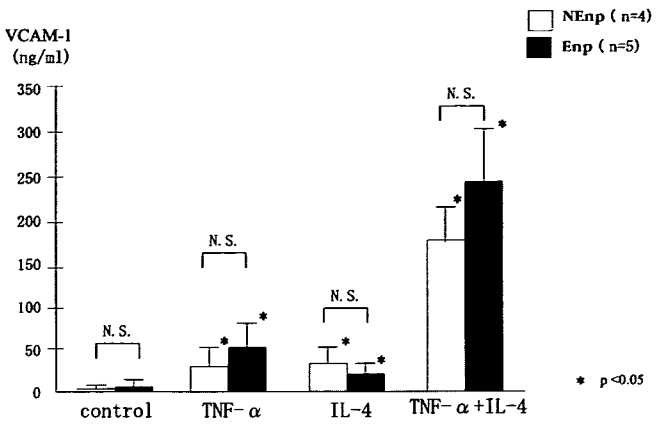

Figure 3. VCAM-1 secretion from nasal fibroblasts by stimulation with TNF-α and IL-4.
VCAM-1 secreted from fibroblasts was induced by TNF-α and by IL-4; the effects were enhanced when co-stimulation was performed. VCAM-1 secretion induced by co-stimulation with TNF-α and IL-4 was significantlyhigher 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.

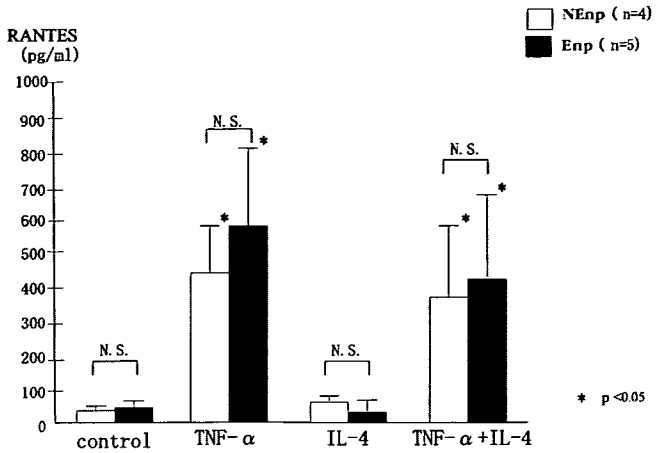

Figure 4. RANTES secretion from nasal fibroblasts by stimulation with TNF-α and IL-4.
RANTES secretion from fibroblasts was significantly enhanced by TNF-α, but not by IL-4. Co-stimulation with TNF-α and IL-4 did not enhance the secretion of RANTES. The level of RANTES did not differ significantly between 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

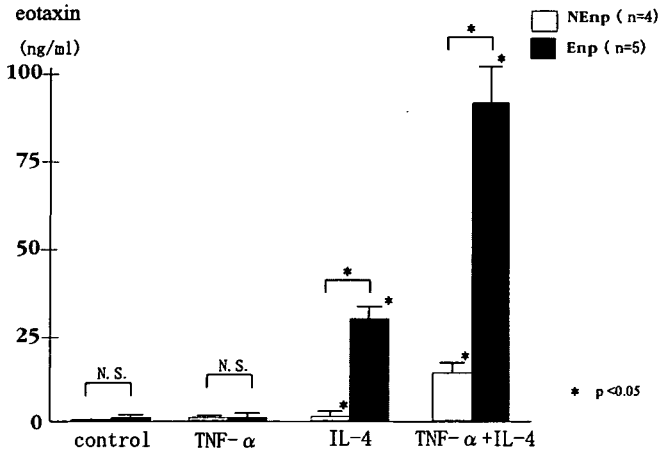

Figure 5. Eotaxin secretion from nasal fibroblasts by stimulation with TNF-α and IL-4.
Eotaxin secretion from fibroblasts was significantly increased by IL-4, but not by TNF-α. Secretion of eotaxin induced by co-stimulation with TNF-α and IL-4 was significantly higher than that by TNF-α or IL-4 alone ($p < 0.05$, 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.

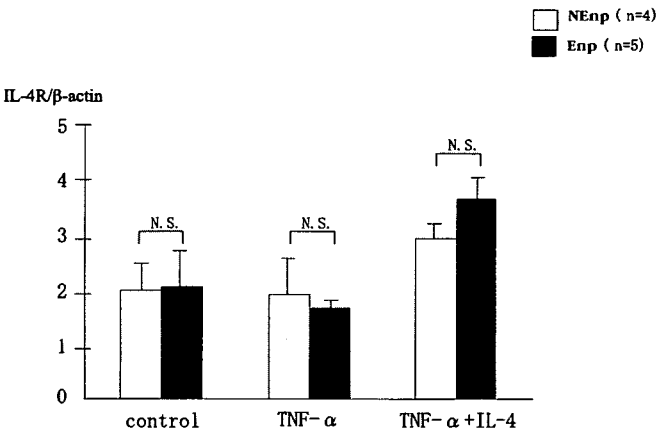

Figure 6. Expression of IL-4R mRNA.
The expression of IL-4R mRNA in nasal fibroblasts was quantitatively analyzed by real time RT-PCR. IL-4 mRNA expression was not enhanced by stimulation with TNF-α. The expression of IL-4R mRNA did not differ significantly between NEnp and Enp. N.S.: not significant.

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.

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IMMUNOLOGY ORIGINAL ARTICLE

Histamine H4 receptor agonists have more activities than H4 agonism in antigen-specific human T-cell responses

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Summary

Histamine not only mediates immediate allergic reactions, it also regulates cellular immune responses. H4R is the most recently identified histamine receptor (HR). In the present study, we examined the *in vitro* effect of histamine and H4R agonists on the responses of human T cells to purified protein derivative from *Mycobacterium tuberculosis* (PPD) and to Cry j1, the major allergen of *Cryptomeria japonica* pollen. Dimaprit, clobenpropit and clozapine, which are H4R agonists, dose-dependently blocked both PPD-induced interferon-γ and Cry j1-induced interleukin-5 production by both peripheral blood mononuclear cells (PBMCs) and antigen-specific T-cell lines. However, the addition of thioperamide, an H3R/H4R antagonist, as well as a mixture of *d*-chlropheniramine, famotidine and thioperamide, did not reverse the inhibition. Pretreatment of PBMCs with SQ22536 and 8-bromoadenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer, had varying abilities to reverse the inhibitory effects of H4R agonists, except for clobenpropit. Moreover, the addition of H4R agonists induced annexin-V expression on PBMCs, especially in $CD19^+$ and $CD4^+$ cells. cDNA microarray analysis revealed that, among 16 600 genes tested, increased expression following treatment with clozapine was seen in 0·8% of the genes, whereas decreased expression was seen in 3·0% of the genes. These results suggest that H4R agonists inhibit antigen-specific human T-cell responses, although H4R does not appear to be important for this effect. In addition, the present study indicated that there may be orphan receptors or HR subtypes which can bind dimaprit, clobenpropit and clozapine, and that can exert an inhibitory effect on antigen-specific cellular responses via a cAMP/cAMP-dependent protein kinase-dependent, apoptotic pathway.

Keywords: cytokine; histamine; H4R; human studies; T cell

Introduction

Histamine has numerous physiological effects, including the induction of allergic responses and gastric acid secretion. These effects are mediated by several histamine receptors (HRs). To date, four subtypes (H1R, H2R, H3R and H4R) of HRs have been identified and cloned. All belong to the seven-transmembrane domain G-protein-coupled receptor family, and coupled G proteins and subsequent activated intracellular signals have been characterized.[1] Of these, H4R was most recently identified, and this receptor is expressed at high levels in mast cells and leucocytes.[2,3]

Histamine not only mediates immediate airway hyperresponsiveness, but also regulates cellular immunity by

Abbreviations: Ag, antigen; APC, antigen-presenting cell; DC, dendritic cell; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HR, histamine receptor; IFN-γ, interferon-γ; IL, interleukin; 4-MH, 4-methylhistamine; α-MH, alpha-methylhistamine; PBMC, peripheral blood mononuclear cell; 2-PEA, 2-pyridylethylamine; PKA, protein kinase A; PPD, purified protein derivative of *Mycobacterium tuberculosis*; PMA, phorbol 12-myristate 13-acetate; RT–PCR, reverse transcription–polymerase chain reaction; SD, standard deviation; TCC, T-cell clone; TCL, T-cell line; Th1, T helper 1; Th2, T helper 2.

controlling the production of pro-inflammatory cytokines and chemokines, the expression of adhesion molecules, and the migration of inflammatory cells, such as eosinophils.[1,4,5] The maturation and activity of dendritic cells (DCs) is also affected by histamine.[6] In addition, histamine regulates T-cell function.[7,8] For example, histamine can enhance T helper 1 (Th1)-type responses by stimulating H1R, whereas both Th1- and T helper 2 (Th2)-type responses are negatively regulated by H2R.[7] At present, there is one report that $CD8^+$ T cells produce interleukin (IL)-16 in response to histamine activation of H4R.[9] However, despite the high expression of H4R on both antigen-presenting cells and T cells, it is not known whether signals through H4R affect antigen-specific human T-cell responses.[3] Therefore, in the present study, we investigated the roles of H4R agonists in antigen-specific human T-cell responses. The findings presented here may help to identify new therapeutic approaches for using HR agonists to treat allergic diseases.

Materials and methods

Antigen and reagents

The purified protein derivative of *Mycobacterium tuberculosis* (PPD) was purchased from Nihon BCG Seizo Co. (Tokyo, Japan). Cry j1 was purified from the crude extracts of *Cryptomeria japonica* pollen using a well-established procedure.[10] The protein concentration was determined using a bicinchoninic acid assay (Pierce, Rockford, IL), according to the manufacturer's instructions. Endotoxin contamination was considered to be negligible because of a negative Endospec^{TM} ES test result (Seikagaku Kogyo Corporation, Tokyo, Japan). Histamine was purchased from Nacalai Tesque Inc. (Kyoto, Japan). 2-Pyridylethylamine (2-PEA; H1R agonist) and 4-methylhistamine (4-MH; H2R agonist) were provided by GSK (Welwyn, Garden City, UK). Dimaprit (H2R/H4R agonist), clobenpropit (H4R agonist/H3R antagonist) and thioperamide (H3R/H4R antagonist) were purchased from Tocris (Ellisville, MO). Clozapine (H4R agonist) was purchased from MP Biomedicals (Irvine, CA). α-Methylhistamine (α-MH; H3R agonist) was a gift from Professor J. C. Schwartz (INSERM, Paris, France). *d*-Chlropheniramine (H1R antagonist) and famotidine (H2R antagonist) were provided by Yamanouchi Pharmaceutical Co. Ltd. (Tokyo, Japan). SQ22536 was purchased from Sigma (St Louis, MO). RP-8-Br-cAMP was purchased from BIOLOG Life Science Institute (Bremen, Germany).

Isolation and culture of peripheral blood mononuclear cells (PBMCs)

All experiments were approved by the Institutional Review Board in the affiliated hospital of Okayama University Graduate School of Medicine. Informed consent was obtained from each volunteer. Twelve Japanese subjects (five men and seven women; age 20–52 years; mean age 39·5 years) with positive tuberculin skin tests, and 12 Japanese patients (seven men and five women; age 19–59 years; mean age 37·4 years) with Japanese cedar pollinosis showing a positive skin scratch test to Japanese cedar pollen, were examined. PBMCs were isolated and cultured as described previously.[11] The culture medium used throughout the study was RPMI-1640 (Sigma) supplemented with 10% human AB serum (ICN Biomedicals, Aurora, OH), 100 U/ml of penicillin, 100 μg/ml of streptomycin (Sigma) and 20 mM L-glutamine (Gibco BRL, Grand Island, NY). In brief, PBMCs (1×10^6/ml) were incubated in the presence or absence of 2 μg/ml of PPD or 10 μg/ml of Cry j1, together with histamine and/or HR-selective agonists or antagonists, for 72 hr in 24-well plates (Corning Inc., Corning, NY) at 37° in a 5% CO_2/air mixture. To determine the involvement of adenylate cyclase and cAMP-dependent protein kinase A (PKA) in the action of HR agonists, PBMCs were pretreated for 1 hr at 37° with 5 mM SQ22536 and 5 mM RP-8-Br-cAMP, respectively.[12] After this incubation, the cells were washed twice with culture medium and then cultured as described above.

Generation and culture of antigen-specific T-cell lines

The $CD4^+$ PPD^- and Cry j1-specific T-cell lines (TCLs) used were generated using a procedure described previously.[10] In flat-bottomed 96-well microtiter plates (Corning Inc.), 2×10^4 TCL were mixed with 1×10^5 irradiated autologous PBMCs (PBMCx) as antigen-presenting cells (APCs). Following this, the cells were cultured in the presence or absence of antigen (Ag), in 0·2 ml of culture medium, together with HR-selective agonists, for 65 hr.

To determine adenylate cyclase activity, TCLs and/or APCs were incubated with SQ22536 at 37° in 24-well plates for 1 hr. Following this, the cells were washed with culture medium three times, after which they were mixed and cultured in the same manner described above.

Measurement of cytokine production

The levels of interferon-γ (IFN-γ), IL-5 and IL-10 in culture supernatants were measured using Opt EIA^{TM} sets (BD Biosciences, San Jose, CA), according to the manufacturer's instructions. The detection limits for IFN-γ, IL-5 and IL-10 in these assays were 20, 20 and 1·9 pg/ml, respectively.

Analysis of apoptosis by annexin V staining

PBMCs (1×10^6/ml) were incubated with 100 μM histamine or HR-selective agonists for 24 or 72 hr. The cells were then harvested, and apoptotic cells were detected

using an Annexin V-FITC Apoptosis Detection KitI (BD Biosciences), according to the manufacturer's protocol. Stained cells were analysed using a fluorescence-activated cell sorter (FACScan) with CELLQUEST software (BD Biosciences).

cDNA microarray analysis

cDNA microarray analysis was performed using IntelliGene HS Human expression chips, containing about 16 600 probe sets (Takara, Tokyo, Japan). PBMC (1×10^6/ml) were incubated with 2 μg/ml of PPD, in the presence or absence of 100 μM clozapine, for 12 hr. Total cellular RNA was extracted by the RNeasy™ mini kit (Qiagen, Tokyo, Japan), according to the manufacturer's instructions. Four micrograms of total RNA from PBMCs treated with and without clozapine were labeled using the RNA Transcript SureLABEL Core Kit (Takara) with Cy-5 UTP and Cy-3 UTP (Amersham Bioscience Corp., Piscataway, NJ), respectively, in each paired case. Labeled samples were hybridized to IntelliGene HS Human expression chips, according to the manufacturer's instructions. After hybridization for 16 hr at 65°, the slides were washed and then scanned for Cy-5 and Cy-3 fluorescence using the Affymetrix 428 scanner (Affymetrix Japan, Tokyo, Japan). The signal intensity of hybridization was evaluated photometrically by the IMAGENE computer program (BioDiscovery K. K., Tokyo, Japan). A gene expression ratio (Cy-5/Cy-3) of $> 2{\cdot}0$ and $< 0{\cdot}5$ was considered significant.

Real-time quantitative polymerase chain reaction

PPD- and Cry j1-specific TCLs were immediately soaked in RNAlater™ RNA stabilization reagent (Qiagen) and stored at −30° until use. Total cellular RNA extraction, reverse transcription to generate cDNA, and real-time quantitative polymerase chain reaction (PCR) assays were performed as described previously.[10] In brief, the assays were performed using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) with QuantiTect SYBR Green PCR (Qiagen). The PCR primer sequences and product sizes were as follows: H1R, forward 5′-AAGT CACCATCCCAAACCCCCAAG-3′ and reverse 5′-TCAGG CCCTGCTCATCTGTCTTGA-3′ (195 bp); H2R, forward 5′-AGGAACGAGACCAGCAAGGGCAAT-3′ and reverse 5′-GGTGGCTGCCTTCCAGGAGCTAAT-3′ (197 bp); H3R, forward 5′-TGCAAGCTGTGGCTGGTAGTGGAC-3′ and reverse 5′-AGCTCAGGATGGCTGGTCCGTACA-3′ (202 bp); H4R, forward 5′-CCGTTTGGGTGCTGGCCTTCTTAG-3′ and reverse 5′-GATCACGCTTCCACAGGCTCCAAT-3′ (204 bp); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5′-ACCACAGTCCATGCCATCAC-3′ and reverse 5′-TCCACCACCCTGTTGCTGTA-3′ (452 bp).[9] The expression level of H1R, H2R, H3R and H4R was estimated by dividing each signal into the signal for GAPDH. As a positive control for H3R, the testicular cell line, NEC14, was used.[13]

Statistical analysis

Statistical comparisons were performed by the non-parametric Mann–Whitney *U*-test and Wilcoxon's signed-rank test. Differences were considered significantly different at a *P*-value of $< 0{\cdot}05$. Values are given as means ± standard deviation (SD).

Results

Effect of histamine on PPD-induced IFN-γ production

PBMCs from subjects with positive tuberculin skin tests produced IFN-γ in response to PPD, whereas there was negligible IFN-γ production by PBMCs in the absence of PPD. Histamine inhibited the PPD-induced production of IFN-γ in a dose-dependent manner. Typical results are shown in Fig. 1(a). Using 12 subjects, the mean concentration of IFN-γ induced by PPD was 10 479 ± 10 783 pg/ml. IFN-γ production was significantly inhibited to a mean of 4697 ± 4657 pg/ml (54·3 ± 35·8% inhibition; $P = 0{\cdot}010$ by Wilcoxon's signed-rank test) upon exposure of the cells to 100 μM histamine (Fig. 1b). PBMCs did not produce IL-10 in response to PPD, and the addition of histamine did not alter IL-10 production (data not shown).

Effect of HR-selective agonists on PPD-induced IFN-γ production

Next, we examined the effect of HR-selective agonists on PPD-induced IFN-γ production. H4R agonists, including

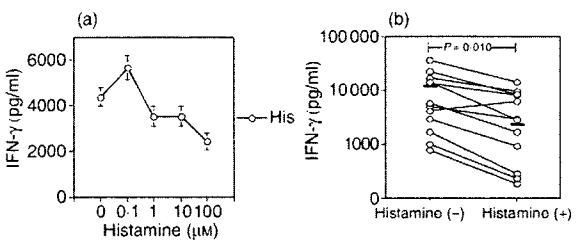

Figure 1. Inhibition of purified protein derivative of *Mycobacterium tuberculosis* (PPD)-induced interferon-γ (IFN-γ) production by histamine. (a) Peripheral blood mononuclear cells (PBMCs) were cultured with 2 μg/ml of PPD in the presence or absence of serial dilutions of histamine. Results are expressed as the mean concentrations ± standard deviation (SD) from triplicate cultures. Data are representative of four separate experiments. (b) PBMCs from 12 subjects with positive tuberculin skin tests were stimulated with 2 μg/ml of PPD in the presence or absence of 100 μM histamine. The *P*-value was obtained using Wilcoxon's signed-rank test. Vertical bars represent the mean concentrations for each group.