

The potential role of interleukin-13 in eosinophilic inflammation in nasal mucosa

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Background Recent studies have revealed that interleukin (IL)-13, as well as IL-4, causes *de novo* surface expression of vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells of the umbilical vein and accelerates selective eosinophil migration. However, its role in allergic rhinitis remains to be clarified. Of particular interest is whether IL-13 upregulates VCAM-1 expression in human mucosal microvascular endothelial cells (HMMECs), to which eosinophils adhere in nasal mucosa.

Methods To understand the potential role of IL-13 in eosinophilic inflammation in nasal mucosa, we examined the effects of IL-13 on the adhesiveness between HMMECs and eosinophils.

Results IL-13 increased VCAM-1 expression in HMMECs, the adhesiveness of endothelial cells to eosinophils, and the transendothelial migration. On the other hand, IL-13 decreased the adhesiveness of eosinophils to HMMECs, and, as a result, accelerated eosinophil infiltration. Those effects are more potent than was those of IL-4. In addition, we also report that the amount of IL-13 in nasal mucosa was higher than that of IL-4.

Conclusions These results strongly indicate that IL-13, as well as IL-4, may be important in eosinophilic inflammation in the nasal mucosa.

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Key words: adhesion molecule; endothelial cells; eosinophil; interleukin-13; nasal allergy; vascular cell adhesion molecule-1.

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Activated eosinophils are believed to be involved in nasal swelling and hyperreactivity to nonspecific stimuli in nasal mucosa of nasal allergy patients (1–3). We have been studying the mechanisms of eosinophil recruitment in the hope that new information on this issue may reveal a potential target for the therapy of nasal allergy. There is increasing evidence that interactions between cytokines released by inflammatory cells and vascular endothelial cells are of major importance in maintaining the chronicity of allergic inflammation. A novel cytokine, interleukin-13 (IL-13), shares many biologic properties on B cells and monocytes with IL-4 (4–9). Recent studies have revealed that IL-13, as well as IL-4, causes *de novo* surface expression of vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells of the umbilical vein and acceler-

ates selective eosinophil migration (10, 11). This is because eosinophils express the $\alpha 4$ integrin ligand for VCAM-1, but neutrophils do not (12, 13). However, their role in allergic rhinitis remains to be clarified. Of particular interest is whether IL-13 upregulates VCAM-1 expression in human mucosal microvascular endothelial cells (HMMECs), to which eosinophils adhere in nasal mucosa. If so, throughout the migration of eosinophils from blood vessels to the nasal cavity via the lamina propria of nasal mucosa, eosinophils would repeatedly adhere to and subsequently detach themselves from endothelial cells, the intercellular matrix, and epithelial cells. To understand the potential role of IL-13 in eosinophilic inflammation in the nasal mucosa, we tested the effect of IL-13 on the adhesiveness between eosinophils and HMMECs, as well as on the eosinophil transmigration through HMMECs.

We show that the effects of IL-13 on VCAM-1 expression in HMMECs, on the adhesiveness of HMMECs to eosinophils, and on the transendothelial migration are stronger than those of IL-4, and that IL-13 decreases the adhesiveness of eosinophils to HMMECs, and, as a result, accelerates

Abbreviations. HMMECs: human mucosal microvascular endothelial cells; ICAM-1: human intercellular adhesion molecule-1; IL: interleukin; MFI: mean fluorescence intensity; RANTES: regulated upon activation, normal T expressed and presumably secreted; TNF- α : tumor necrosis factor-alpha; VCAM-1: vascular cell adhesion molecule-1.

eosinophil infiltration. In addition, we also report that the amount of IL-13 in nasal mucosa is higher than that of IL-4. These results strongly indicate that IL-13, as well as IL-4, may be important in eosinophilic inflammation in the nasal mucosa.

Material and methods

mAbs and cytokines

IL-13 and IL-4 were purchased from Genzyme (Boston, MA, USA). Tumor necrosis factor- α (TNF- α) was purchased from R&D Systems (Minneapolis, MN, USA). Mouse mAb recognizing IL-13 (clone: 32116,11) was purchased from R&D Systems. Mouse mAb recognizing leukocyte function-associated antigen-1 (LFA-1) (TP1/32, IgG1) was purchased from Upstate Biotechnology (New York). Mouse mAb recognizing Mac-1 (D12*, IgG2a) and mouse mAb recognizing very late antigen-4 (VLA-4) (CD49d, 9F10, IgG1) were purchased from Becton-Dickinson (San Jose, CA, USA) and PharMingen (San Diego, CA, USA), respectively. Monoclonal mouse anti-human intercellular adhesion molecule-1 (anti-human ICAM-1), vascular cell adhesion molecule-1 (anti-human VCAM-1), and endothelial cell-leukocyte adhesion molecule-1 (anti-human ELAM-1) were all purchased from Genzyme (Cambridge, MA, USA). CD16 microbeads were purchased from Miltenyi Biotec (Sunnyvale, CA, USA).

Peripheral blood eosinophil purification

Eosinophils were isolated from peripheral blood obtained from patients with allergic rhinitis (average age 28.1 ± 4.4 years). The diagnosis of allergic rhinitis was based on a typical history of perennial symptoms of allergic rhinitis, eosinophilia in nasal smears, and positive radioallergosorbent test (RAST) to house dust and/or house-dust mites.

The leukocyte fraction was obtained by dextran sedimentation. Eosinophils were then isolated by negative immunomagnetic selection with CD16 microbeads and the MACS system (Miltenyi, Biotec, Germany) (14). The cells were collected and washed twice in RPMI with 10% FCS and resuspended by the same medium.

For this study, we used isolated materials in which eosinophil purity (based on examination by Hinkelman stain) was $>97\%$ and viability (based on the trypan blue test) was $>98\%$.

Collection of mucosa from the inferior turbinate

Turbinal mucosa samples were collected from patients with allergic rhinitis sensitized to house

dust and/or house-dust mites (average age 21.1 ± 6.0 years). The patients had moderate to severe nasal obstruction. Inferior turbinal mucosa samples were obtained at the time of surgery for submucosal resection. Informed consent was obtained after the objective and procedures of the present study had been adequately explained.

Purification and determination of HMMECs

HMMECs were isolated from human nasal mucosa specimens by the method of Fukuda et al. (15). Surgically removed mucosa specimens were washed three times with Ca^{2+} - and Mg^{2+} -free phosphate-buffered saline (PBS) solution containing 500 U/ml penicillin, 500 $\mu\text{g/ml}$ streptomycin, and 6 $\mu\text{g/ml}$ fungizon.

Subsequently, the mucosal tissue specimens were cut into 4-mm² sections and was incubated separately for 1 h at 37°C in PBS containing 0.05% collagenase (type II) and 1% EDTA. These sections were placed in Dulbecco's modified Eagle's medium (DMEM) containing 20% FBS in a culture dish after the specimens had been washed three times with PBS. Next, these samples were pressed with a mucosa elevator from the center of each dish toward the periphery to release the endothelial cells. The cells were collected by centrifugation (400 g, 3 min) and resuspended in DMEM with added 20% FBS and antibiotics.

The cells were then placed onto a 60-mm diameter plastic culture dish coated with type I-rich collagen (Sigma, St Louis, MO, USA) and incubated at 37°C under a humidified atmosphere of 5% CO_2 and 95% air. After incubation for 1 h, the medium was discarded and the plate washed three times with DMEM to remove floating cells. The HMMECs were cultured in MCDB107 medium (GIBCO RBL, Life Technologies, New York) containing 5% fetal calf serum (FCS) and 75 $\mu\text{g/ml}$ endothelial cell growth supplement (Collaborative Research, Bedford, MA, USA). According to the morphology and positive reactions for anti-factor VIII antibody (Dakopatts) in immunofluorescence staining tests, more than 95% of the cells were shown to be endothelial cells. Materials contaminated with fibroblasts were excluded from the following experiments. After two or three passages, we used them for the experiments.

Flow-cytometric analysis

HMMECs were cultured in type I collagen-coated, 12-well tissue-culture plates (Sumitomo Bakelite, Tokyo, Japan). After reaching confluence, the cells were incubated in the presence or absence of the indicated concentration of IL-13 or IL-4 at 37°C.

Eosinophils in RPMI containing 10% FCS were also cultured in the presence or absence of the indicated concentration of IL-13 or IL-4 at 37°C. Then, the cells were washed with FACS buffer (PBS containing 0.1% of NaN₃ and 1.0% of BSA). The cell suspensions were incubated with saturating concentrations of IgG murine mAb-recognizing ICAM-1, VCAM-1, E-selectin, VLA-4, Mac-1, LFA-1, or an irrelevant control mAb, respectively, for 30 min at 4°C. Then, they were washed free of unbound antibodies, and incubated with the 1:50 dilution of FITC-conjugated anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL, USA) in PBS for 30 min at 4°C. After washing, the cells were suspended in 0.5 ml of FACS buffer. Labeled cells were analyzed by using flow cytometry (FACScan, Becton-Dickinson, San Jose, CA, USA).

Eosinophil adhesion assay

The procedure for eosinophil adhesion assay has been described previously (16). Briefly, HMMEC monolayers were seeded in collagen type I-coated, 96-well culture plates (Sumitomo Bakelite Co). At confluence, HMMECs were incubated in the presence or absence of the indicated concentration of IL-13 or IL-4 at 37°C. For examination of the effects of IL-13 and IL-4 on eosinophil adhesiveness to HMMECs, the eosinophils were preincubated at 37°C in the presence or absence of an indicating concentration of IL-13 or IL-4 before their addition to HMMECs. The viabilities of these cells were determined by the trypan blue exclusion test after the preincubation, and were noted to be more than 98%. The eosinophils were labeled with ³⁵S by incubating them with [³⁵S]

Protein Labeling Mix (Du Pont, Biotechnology Systems, DE, USA) in RPMI without methionine and cysteine (GIBCO RBL) for 3 h at 37°C. Labeled eosinophils in an enriched medium (RPMI 1640 with methionine and cysteine (GIBCO RBL) were then added. Then, they were allowed to adhere to the monolayers of HMMECs for 60 min at 37°C. Monolayers with eosinophils were rinsed by PBS, and then lysed by the addition of 0.5% Triton X-100 (Sigma). After 30 min had elapsed, a liquid scintillator (SuperMix™, Wallac, Finland) was added, and ³⁵S radioactivity was determined by Micro Beta™ (Pharmacia Biotech, Sweden). Previous studies have shown that eosinophils take up ³⁵S at different rates, depending on the stimulation. Therefore, the numbers of adherent eosinophils were calculated with each standard curve.

Eosinophil transmigration assay

We used the Coster Transwell double chamber system (6.5-mm diameter polycarbonate membrane with 5.0-μm pores, Coster Corp., Cambridge, MA, USA) (17). HMMECs were pretreated for 18 h in the presence or absence of IL-13 and IL-4. TNF-α was used as a positive control. ³⁵S-labeled unstimulated eosinophils were added to the chamber before immersion of the Transwell chamber. After a 3-h incubation, eosinophils that had migrated and fallen into the lower chamber were combined with those from the undersurface of the upper chamber. ³⁵S radioactivity was determined by Micro Beta.

Inhibition assay

To confirm that the changes in the expression of adhesion molecules, eosinophil adhesion and transmigration induced by IL-13 were IL-13 specific, we performed an inhibition assay. The HMMECs or eosinophils were preincubated for 30 min at 37°C in the presence or absence of a saturating concentration of mAb for IL-13 (10 μg/ml) or VCAM-1 (10 μg/ml) before stimulation by IL-13. The viabilities of these cells were determined by the trypan blue exclusion test after the preincubation, and were noted to be more than 98%. After two washes, the cells were used in each experiment.

Detection of cytokine proteins in nasal mucosa

Inferior nasal turbinate mucosae were homogenized in 1 ml of ice-cold PBS by Polytron (Kintematica, Sweden). The supernatants were separated by centrifugation at 2000 g for 15 min at 4°C. The

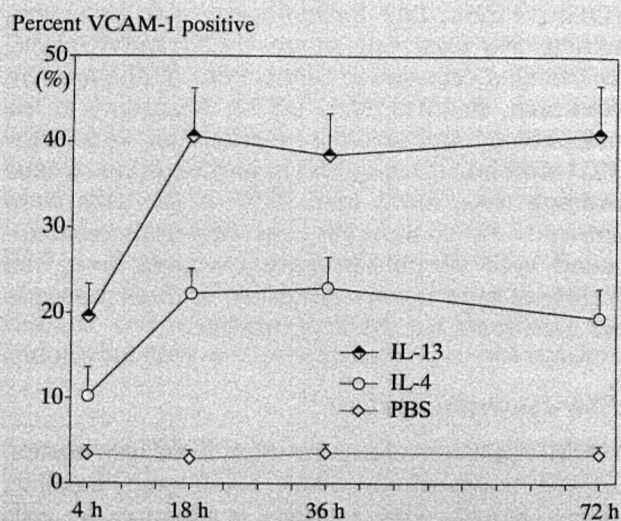


Fig. 1. Time course of VCAM-1 expression in HMMECs induced by 100 ng/ml of IL-13 and IL-4 ($n=6$).

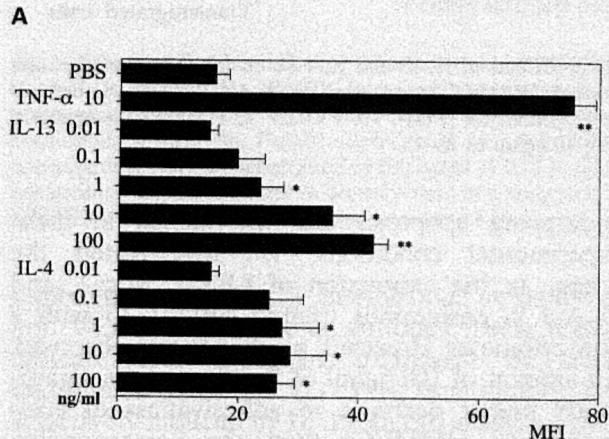
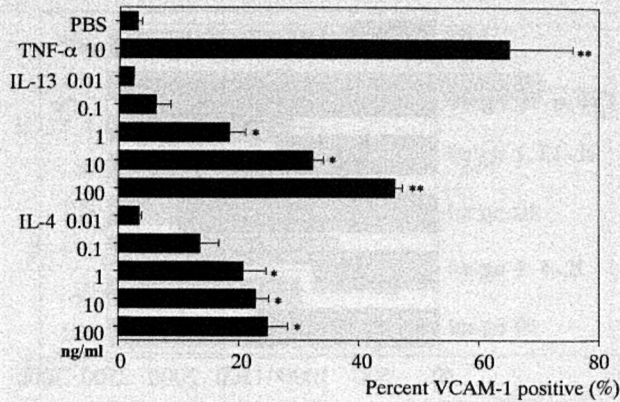


Fig. 2. Effects of IL-13 and IL-4 on VCAM-1 expression in HMMECs; A) expressed as percent VCAM-1-positive cells; B) expressed as MFI ($n=6$; * $P<0.05$, ** $P<0.01$ vs unstimulated HMMECs [PBS]). Volumes of 10 and 100 ng/ml of IL-13 caused significantly higher increase in percent VCAM-1-positive cells and MFI than corresponding concentrations of IL-4 ($P<0.05$).

concentration of IL-4 was measured by ELISA using Cytoscreen, USTM, purchased from BioSource International (Camarillo, CA, USA). The minimum detectable dose of human IL-4 is 65 fg/ml. The concentration of IL-13 was measured by ELISA using IL-13 purchased from Bender Medsystems, Vienna, Austria. This test has a sensitivity of 1.5 pg/ml.

Statistical assessment

The results of the study were expressed as the mean value $\pm$ SE. The statistical significance of within-group differences was determined by the paired t -test for normally distributed data and the Wilcoxon signed rank test for data without a normal distribution. The normality was confirmed by the Shapiro-Wilks test. Statistical significance for intergroup comparison was determined by the Mann-Whitney U test. Differences were considered significant only when the P value was less than 0.05.

Results

Effect of IL-13 on expression of adhesion molecules in HMMECs

The kinetics of IL-13-induced expression of VCAM-1 expression was similar to that seen with IL-4, that is, IL-13, like IL-4, induced a significant, sustained increase in VCAM-1 expression, evident at 18 h, and persisting for 72 h (Fig. 1).

The percentage of positive cells and mean fluorescence intensity (MFI) are shown in Fig. 2. IL-13, as well as IL-4, induced a significant increase in VCAM-1 expression in a dose-dependent manner. Volumes of 10 and 100 ng/ml of IL-13 caused a significantly higher increase in the percentage of VCAM-1-positive cells and MFI than the corresponding concentrations of IL-4 ($P<0.05$). They had no significant effect on expression of ICAM-1 and E-selectin (data not shown).

The pretreatment with anti-IL-13 monoclonal antibody canceled IL-13-induced VCAM-1 expression, as shown in Fig. 3.

Effect of IL-13 on adhesiveness of HMMECs to eosinophils

To confirm that the induction of the expression of VCAM-1 by IL-13 and IL-4 was associated with an increased adhesiveness of HMMECs, we performed an adhesion assay using eosinophils purified from peripheral blood. As shown in Fig. 4, IL-13 and IL-4 increased the adhesiveness of HMMECs to eosinophils in a dose-dependent manner. A concentration of 100 ng/ml of IL-13 caused a significantly higher increase in the adhesiveness of HMMECs to eosinophils than the corresponding concentration of IL-4. The IL-13-induced increase in the adhesiveness of HMMECs was almost completely inhibited by the anti-IL-13 monoclonal antibody or the anti-VCAM-1 monoclonal antibody (data not shown).

These results indicate that VCAM-1 expressed on HMMECs is functional and is selectively induced by IL-13.

Effect of IL-13 on transendothelial migration of eosinophils

IL-13 and IL-4 increased the transendothelial migration of eosinophils in a dose-dependent manner (Fig. 5). Again, 10 ng/ml of IL-13 caused a significantly higher increase in eosinophil transmigration than IL-4. The IL-13-induced increase in transendothelial migration of eosinophils was almost completely inhibited by anti-IL-13 mAb or anti-VCAM-1 mAb (data not shown).

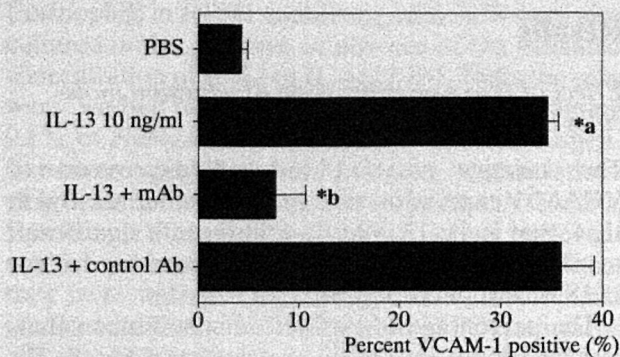


Fig. 3. Effect of mAb to IL-13 on IL-13-induced VCAM-1 expression ($n=3$; *a, $P<0.05$, vs unstimulated HMMECs [PBS], *b, $P<0.05$, vs HMMECs stimulated with IL-13).

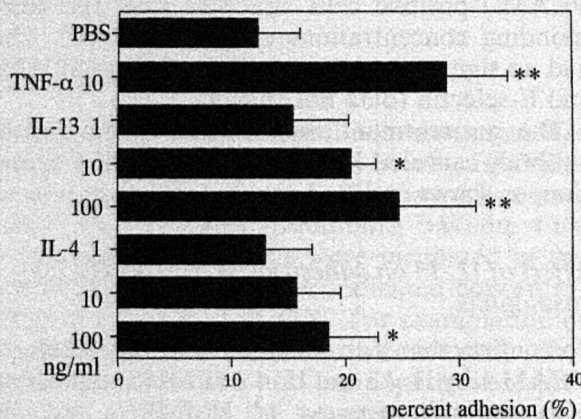


Fig. 4. Effects of IL-13 and IL-4 on adhesiveness of HMMECs to eosinophils ($n=6$; * $P<0.05$, ** $P<0.01$ vs unstimulated HMMECs [PBS]). Concentration of 100 ng/ml of IL-13 caused significantly higher increase in adhesiveness of HMMECs to eosinophils than corresponding concentration of IL-4.

Effect of IL-13 on adhesiveness of eosinophils to HMMECs

Next, we investigated the effects of IL-13 and IL-4 on eosinophil and endothelial cell adhesion when eosinophils were treated with IL-13 and IL-4. Eosinophils were preincubated with the indicated concentration of IL-13 or IL-4 for 18 h and were allowed to adhere to unstimulated HMMECs for 1 h. As shown in Fig. 6, more than 10 ng/ml IL-13 significantly reduced eosinophil adhesion to HMMECs. Because it was possible that the cause of this phenomenon was that IL-13 and IL-4 were affecting eosinophil viability, we examined eosinophil viability at the end of culture by using the trypan blue exclusion test. We also tested whether eosinophils had undergone apoptosis with an *in situ* cell-death detection kit (Boehringer Mannheim), which detects apoptosis cells. In this experiment, we did not observe any change in the eosinophil viability. Moreover, the percentage of eosinophils

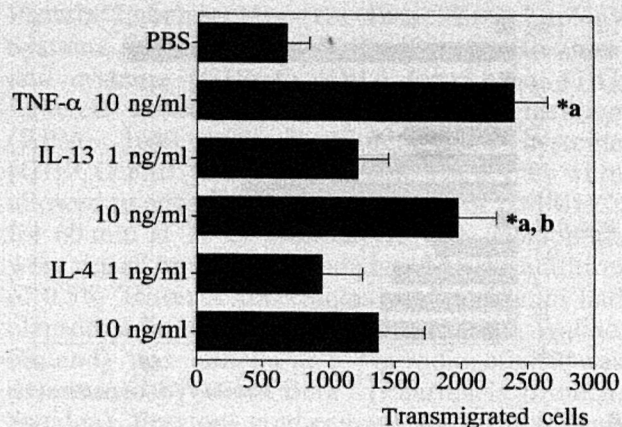


Fig. 5. Effects of IL-13 and IL-4 on eosinophil transmigration through HMMEC monolayers ($n=6$; *a, $P<0.05$, vs unstimulated HMMECs [PBS], *b, $P<0.05$, vs HMMECs stimulated with 10 ng/ml of IL-4).

undergoing apoptosis did not change in these experimental conditions. We investigated the change in the expression of LFA-1, Mac-1, and VLA-4 in eosinophils treated with IL-13 with a flow cytometer. However, no change was observed. An amount of 100 ng/ml of IL-13 caused a significantly higher decrease in adhesiveness of eosinophils to HMMECs than the corresponding concentration of IL-4.

Comparison of the amount of IL-13 and IL-4 in nasal mucosa

Fig. 7 shows the amount of IL-13 and IL-4 in supernatants obtained from homogenized nasal mucosa in patients with nasal allergy. The amount of IL-13 in nasal mucosa was also predominant.

Discussion

In this study, we examined the effects of IL-13 on the adhesiveness between HMMECs and eosinophils. As in previous studies using human umbilical vein endothelial cells (11, 12), IL-13 selectively induced concentration-dependent increases in the surface expression of VCAM-1 in HMMECs. The kinetics of IL-13-induced expression of VCAM-1 was similar to that seen with IL-4; that is, IL-13, like IL-4, induced a significant, sustained increase in VCAM-1 expression evident at 18 h, and persisting for 48 h. The pretreatment with anti-IL-13 monoclonal antibody canceled IL-13-induced VCAM-1 expression. The induction of the expression of VCAM-1 by IL-13 was associated with an increased adhesiveness of HMMECs to eosinophils.

The IL-13-induced increase in the adhesiveness of HMMECs to eosinophils was almost completely

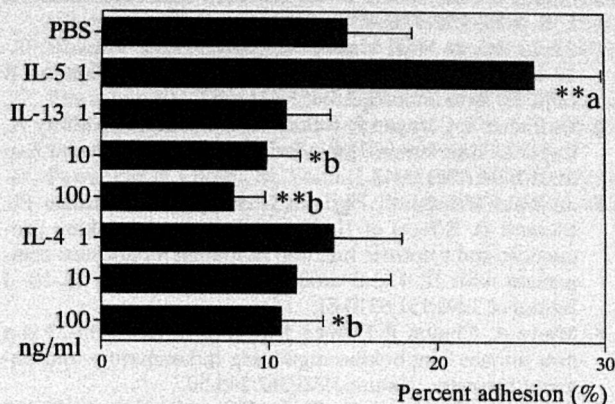


Fig. 6. Effects of IL-13 and IL-4 on adhesiveness of eosinophils to HMMECs ($n=6$; **a, $P<0.01$ (increased), vs unstimulated eosinophils [PBS], *b, $P<0.05$ (decreased), vs unstimulated eosinophils [PBS], **b, $P<0.01$ (decreased), vs unstimulated eosinophils [PBS]). Concentration of 100 ng/ml of IL-13 caused significantly higher decrease in adhesiveness of eosinophils to HMMECs than corresponding concentration of IL-4.

inhibited by anti-IL-13 monoclonal antibody or anti-VCAM-1 monoclonal antibody. These results indicate that VCAM-1 expressed on nasal endothelial cells by IL-13 is functionally and selectively induced by IL-13. In this study, we used eosinophils isolated from peripheral blood from patients with nasal allergy. In the preliminary study, we observed that IL-13 is less potent in eosinophils from healthy subjects. It is noteworthy that the response to IL-13 differed between the eosinophils of patients with nasal allergy and those of healthy subjects. It is well known that activated eosinophils from patients with allergic disease have increased capacity to secrete leukotriene C_4 by stimulation with the calcium ionophore, and to kill antibody-coated schistosomula. These results suggest that the eosinophils from patients with nasal allergy favor the induction of the adhesion molecules and the increase in adhesion to HMMECs by IL-13.

One novelty of our study was our demonstration that IL-13, as well as IL-4, decreases the eosinophil adhesiveness to HMMECs without changing the surface adhesion molecules such as LFA-1, Mac-1, or VLA-4. As IL-13 does not change eosinophil viability in these experimental conditions, it may change these adhesion molecules from a high- to a low-affinity state. It has been shown that granulocyte-macrophage colony-stimulating factor may alter the functional state of eosinophil-expressed VLA-4 from a low affinity to a high affinity (18). This modulation may involve both changes in extracellular domain affinity and changes in cytoplasmic integrin tail cytoskeleton interaction, either simultaneously or sequentially. It is known

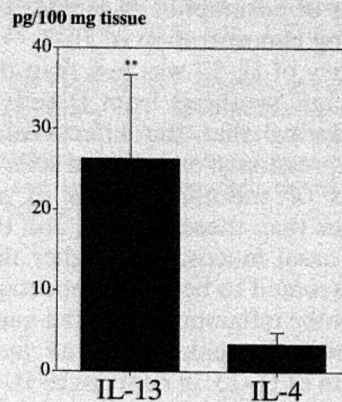


Fig. 7. Comparison of amount of IL-13 and IL-4 in nasal mucosa ($n=6$). Values represent means $\pm$ SE.

that an integrin, such as VLA-4, can exist in multiple functional states (19). While the exact mechanism by which IL-13 decreases the adhesiveness of eosinophils is at present unknown, it is possible that IL-13 causes a conformational change in the extracellular domain and/or in cytoplasmic integrin tail cytoskeleton interaction, and may change the affinity level from high to low.

After eosinophils have adhered to and transmigrated through HMMECs, they must detach themselves from HMMECs when they migrate into the lamina propria of the nasal mucosa. IL-13 may accelerate this step.

These results indicate that IL-13, as well as IL-4, may accelerate eosinophil infiltration by these mechanisms. When the eosinophils were in vessels, they were not affected here by IL-13 because the serum concentration of IL-13 was very low. In the lamina propria, IL-13 enhanced VCAM-1 expression and increased the adhesiveness of endothelial cells and then accelerated eosinophil transendothelial migration.

After this, IL-13 in the lamina propria promoted the detachment of eosinophils from endothelial cells, resulting in accelerating eosinophil migration to the lamina propria. IL-13 specifically activated human eosinophils, as determined by the expression of CD69 cell-surface protein and mRNA expression (20). Furthermore, IL-13 significantly prolonged eosinophil survival *in vitro* (20). These studies were then expanded to compare the effects of IL-13 with those seen with IL-4 with respect to the effects of VCAM-1 expression in HMMECs and the adhesiveness between HMMECs and eosinophils. IL-13 induced a significantly higher level of VCAM-1 than did IL-4. Accordingly, the effect of IL-13 on the increase of the adhesiveness between HMMECs and eosinophils was more potent than that of IL-4. In addition, 100 ng/ml of IL-13 caused a significantly higher decrease in the

adhesiveness of eosinophils to HMMECs than the corresponding concentration of IL-4.

The potency of IL-13 was less than that of IL-4 in inducing IgE synthesis from B cells (9). However, considering that the effects of IL-13 on VCAM-1 expression in endothelial cells, and on the adhesiveness of endothelial cells to eosinophils, were stronger than those of IL-4, and the amount of IL-13 in nasal mucosa was higher than that of IL-4, IL-13 seemed to be more important than IL-4 in eosinophilic inflammation in the nasal mucosa. This hypothesis coincides with the fact that the concentration of IL-13, in contrast to IL-4, strongly correlated with the eosinophil numbers in bronchoalveolar lavage found 18 h after allergen challenge (21).

The cell source of IL-13 in nasal mucosa has been reported by Pawankar et al. (22, 23) They demonstrated that IL-13 at the protein level was localized to lymphocytes and mast cells. Most recently, it has been revealed that eosinophils in nasal polyps also produce IL-13 (Nakano et al., unpublished observation). Eosinophils may contribute to paracrine interactions with T and B cells and, in autocrine fashion, locally participate in eosinophil differentiation, accumulation, and activation.

We conclude that IL-13, as well as IL-4, may be important in the pathogenesis of eosinophil-related inflammation, as in nasal allergy and bronchial asthma.

Most recently, a soluble IL-13-binding protein has been identified and purified (24). It binds IL-13 with a 100–300-fold higher affinity than does the cloned IL-13R α . In addition, unlike the cloned receptor, it acts as a potent inhibitor of IL-13 binding to its cell-surface receptor, suggesting that it may be used to modulate the effects of IL-13 *in vivo*, and that it may offer a tool in the therapy of nasal allergy.

Acknowledgment

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