Rhinology, 44, 145-150, 2006

Eosinophilic nasal polyps are a rich source of Eotaxin, Eotaxin-2 and Eotaxin-3*

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SUMMARY

Introduction: *The CC-chemokine eotaxin plays a key role in the pathologic mechanism of tissue eosinophilia in nasal polyposis. In thisstudy, we investigated a possible role of eotaxin-2 and eotaxin-3, the recently discovered members of the eotaxin family.*

Methods: *Nasal polyps from 24 patients (non allergic/ allergic/ aspirin-intolerant patients) and turbinate tissue from 8 controls were investigated. Chemokine protein content (eotaxin, eotaxin-2, and -3) of tissue homogenates was measured by ELISA. Paraffin sections of samples were stained to determine the extent of eosinophilia.*

Results: *Protein expression of eotaxin, eotaxin-2 and eotaxin-3 was significantly higher in nasal polyps than in controls. There was a direct correlation between the protein concentrations of all three eotaxins. Further, protein levels of all chemokines were significantly correlated to the amount of eosinophilia. In aspirin-sensitive polyps the number of eosinophils was significantly higher than in the other patient groups and they had significantly higher eotaxin, eotaxin-2, and -3 protein levels than non-allergic and significantly higher amounts of eotaxin-3 compared with allergic patients.*

Conclusions: *Our findings suggest, that all members of the eotaxin family are involved in the pathogenesis of nasal polyposis. The results are more likely indicative of a complex cooperation between all members of the eotaxin family than of a specific role in the development of eosinophilia and nasal polyposis.*

Key words: eotaxin, eotaxin-2, eotaxin-3, eosinophils, polyposis nasi

INTRODUCTION

Recent cytokine and chemokine research has rapidly expanded our understanding of inflammatory responses. Most new insights on acute viral and allergic rhinitis as well as on chronic sinusitis and nasal polyposis have been gained in the last 10 years [(1-19)]. Specific cytokines and chemokines that appear to play a central role in the pathogenesis of nasal polyposis have now been identified [(2,3,5,6,7-10,12-19)]. In most bilateral nasal polyps, tissue eosinophilia is a striking finding which is believed to play a central role in the pathogenesis. Eosinophils initiate tissue damage by the release of cytotoxic substances like major basic protein, eosinophil cationic protein and autocrine production of chemokines that cause a selfsubstained inflammatory process. Eosinophilia may be explained by increased migration and prolonged eosinophil survival. Recent findings on specific eosinophil chemotactic factors have led various authors to propose selective recruitment mechanisms to describe the in vivo situation [(20-29)]. Not only the cytokines IL-1, IL-4, IL-5 and IL-8, but also and most importantly the chemokines eotaxin (CCL11) and RANTES (CCL5) have been ascribed a chemotactic potency. Chemokines are more specific activators of target cells than cytokines. Moreover, chemokines play a key role in selective cell migration, and they have cell-selecting and activating properties. Four classes of chemokines, CXC, CC, C and CXXXC, have been defined by the arrangement of the amino acid cysteine in the amino terminal region. Even though there are no specific chemokines for the different cell types, one group of CC-chemokines, the eotaxin family, has a preference in the activation of eosinophils via a single CC-chemokine receptor, the CCR3 [(30-32)]. Apart from the eotaxins, the CCR3 ligand family also includes RANTES (CCL5), monocyte chemoattractant protein (MCP)-2 (CCL8), MCP-3 (CCL7) and MCP-4 (CCL13), each with its own characteristic affinity for the receptor [(30-34)]. After identification of eotaxin as a relatively selective chemokine for eosinophils [(23,25)], eotaxin-2 (CCL24) was cloned and showed chemotactic activity for eosinophils in a similar range as eotaxin [(30)]. More recently a new member of the

*Received for publication: June 27, 2005; accepted: December 30, 2005

eotaxin family, eotaxin-3 (CCL26) has been cloned [32,35]. The special feature of the eotaxins is that they interact exclusively with the CCR3 receptor, which is relatively selectively expressed on eosinophils [31,35]. Whereas several groups have investigated and described the key role of eotaxin in the pathogenesis of nasal polyposis, the relative roles of eotaxin-2 and eotaxin-3 remain poorly defined. Based on the available data, eotaxin-3 has not been detected in nasal polyp tissue until now. Results of recent chemokine research indicate, that possibly all members of the eotaxin-family are involved in the development of nasal polyps. So the objective of the present study was to investigate the protein concentrations of eotaxin, eotaxin-2 and eotaxin-3 in nasal polyps in a precisely characterized patient population, with special attention to etiological factors and the degree of eosinophilia and to compare the results with turbinate nasal mucosa of control subjects and within the patient group.

PATIENTS AND METHODS

Patients

Nasal polyps from 24 patients with chronic sinusitis (mean age: 44.5 years; 15 male, 9 female) and tissue samples taken from the inferior turbinate of 8 control subjects during routine endonasal surgery (mean age 41.3 years, 5 male, 3 female) were investigated.

Evaluation of patients and controls

Nasal polyposis was diagnosed by anamnesis of chronic sinusitis [36], endoscopic investigation and CT-scan. Patients were classified into 3 groups: non-allergic patients (n=8; no allergy, no aspirin-sensitivity), allergic patients (n=8; no aspirin-sensitivity) and aspirin-intolerant patients (n=8; no allergy). Subjects were identified as allergic by means of history, skin prick test results and specific IgE levels. Aspirin-sensitivity was diagnosed from the patients´ history and results of nasal or oral provocation test. None of the patients were treated with antibiotics, corticosteroids, anti-histamines or anti-leukotrienes at least 2 weeks before surgery. Only patients with tissue eosinophilia were included in this study. Samples of inferior nasal turbinates of patients with no history of sinus disease, allergies, aspirin-sensitivity, asthma and previous sinus surgery were used as controls. Only control subjects with negative prick test results and without any eosinophils in histological examination were included in the study. The study conformed to the declaration of Helsinki and was performed with the approval to the Charité ethics committee and the patients' informed consent.

Quantification of eosinophils

One part of samples was fixed in 10% formalin and embedded in paraffin wax processed routinely and eosinophils were counted at 400x magnification (5 high power-fields). The other part was frozen in liquid nitrogen and stored at –80°C until further processing.

Preparation of the sample fluids for ELISA

The biopsies were weighed and cut into small pieces. The tissue was homogenized in a pre-cooled glass-teflon homogenizer in ice-cold buffer of 5 ml PBS, supplemented with a cocktail of protein inhibitors (Complete, Roche Diagnostics, Mannheim), centrifuged at 4°C at 3000 rpm for 10 minutes. Supernatants were stored in aliquots of 250 µl at -80°C for later experiments. The chemokine concentrations were measured by using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA.). Standard curves were prepared according to the manufacturer´s instructions. The minimum detectable dose of the ELISA kits were eotaxin: 5 pg/ml, eotaxin-2: 0,8 pg/ml and eotaxin-3: 0,87 pg/ml.

Measuring of the total protein concentration

The total protein concentration of the samples was measured by the method of Biuret in a Protein Assay Reagent Kit (Pierce Chemical Co., Rockford, IL, USA). With the dilution series from standard or from supernatants of the samples (50 µl, 1:1, 1:5, 1:100) buffered protein base (200 µl) was added into the wells. After incubation at 37°C the intensity of the color was measured on a plate reader. Chemokine concentrations were normalized to total protein concentration. The results are given in pg /mg of total protein

Statistics

Statistical analyses were performed using SPSS for Windows (Release 11.5.1, Copyright SPSS Inc. 1989-2002). To cope with outliers and/or skew distributions, differences between interesting groups of individuals were analyzed using nonparametric statistical tests (Mann-Whitney-U test for two independent groups or Wilcoxon test for paired observations). Associations between the protein concentrations of all investigated chemokines and the number of eosinophils and between the protein concentrations of all three eotaxins were analyzed using Spearman correlation. Significance was assessed at $p < 0.05$, exact, two-sided. Boxplots display summary statistics for the data. The box contains the values for 50% of cases, from the 25th (Q1) to the 75th (Q2) percentile, and the length of the box corresponds to the interquartile range (IQ). The line through the box is the median value. A point beyond an inner fence (Q1-1,5 x IQ; Q2+1,5 x IQ) on either side is considered a mild outlear ("o"). A point beyond an outer fence (Q1-3 x IQ; Q2+3 x IQ) is considered an extreme outlier.

RESULTS

Simultaneous detection of eotaxin, eotaxin-2 and eotaxin-3 in patients and controls

All of the investigated CC-chemokines were present in the patients as well as in the controls. Quantitative differences in the protein concentration and the number of positive samples for each cytokine were detected. Overall, protein expression of the CC-chemokines eotaxin, eotaxin-2 and eotaxin-3 was significantly higher in the nasal polyp group than in the control

Table 1. Protein concentrations (pg/mg) of eotaxin, eotaxin-2 and eotaxin-3 in control subjects, non-allergic patients, allergic patients, aspirin-intolerant patients and all patients. Values are expressed as medians of all positive samples, n= number of positive samples in each group.

	Eotaxin	Eotaxin-2	Eotaxin-3
Control subjects	15.0	20.6	16.7
n	4	3	5
Non-allergic patients	111.5	124.6	161.3
n	5	4	6
Allergic patients	120.5	244.3	120.5
n	7	6	7
Aspirin-intolerant patients	225.4	346,4	250.0
n	8	8	8
All patients	180.3	298.1	202.2
n	20	18	21

group. The protein concentrations and the number of positive samples of eotaxin, eotaxin-2 and eotaxin 3 in the control group and in the patient groups with different underlying diseases are summarized in Table 1. In the patient group, the rank order of chemokine protein concentrations was eotaxin-2 > eotaxin-3 > eotaxin. The differences between eotaxin and eotaxin-2 (p=0.012) and eotaxin and eotaxin-3 (p=0.007) were significant. Moreover, correlation analysis also revealed a significant, two-sided positive correlation between the protein concentrations of all three eotaxins: eotaxin / eotaxin-2 ($p < 0.01$), eotaxin-2 / eotaxin-3 ($p < 0.01$) and eotaxin-2 / eotaxin-3 ($p < 0.01$).

Increased eotaxin, eotaxin-2 and eotaxin-3 protein synthesis in patient group - differences among groups with different underlying diseases

Eotaxin: Samples from patients with nasal polyps contained significantly higher amounts of eotaxin protein (12.4-times higher) than control subjects (p=0.001). Within the patient group eotaxin concentrations of samples from non-allergic patients were significantly decreased compared with aspirin-intolerant patients ($p = 0.005$). Eotaxin protein synthesis did not show significant differences between the other diseases (Figure 1).

Eotaxin-2: The eotaxin-2 protein concentrations in the samples of the patient group were significantly elevated (13.1-times higher), when compared with control subjects ($p = 0.007$). Samples from non-allergic patients contained significantly decreased amounts of eotaxin-2-protein than aspirin-intolerant patients ($p = 0.009$). The other values did not differ significant between the groups (Figure 2).

Eotaxin-3: Eotaxin-3 concentrations were significantly higher in samples from patients with nasal polyps (13.7-times higher), than in control subjects ($p = 0.001$). Within the patient group samples of aspirin-intolerant patients have shown a significantly higher amount of eotaxin-3 compared with non-allergic patients ($p = 0.037$) and allergic patients ($p = 0.028$) (Figure 3).

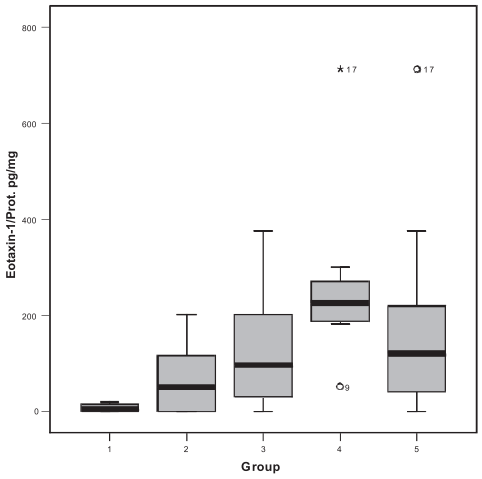

Figure 1. Eotaxin-protein concentrations in groups 1-5

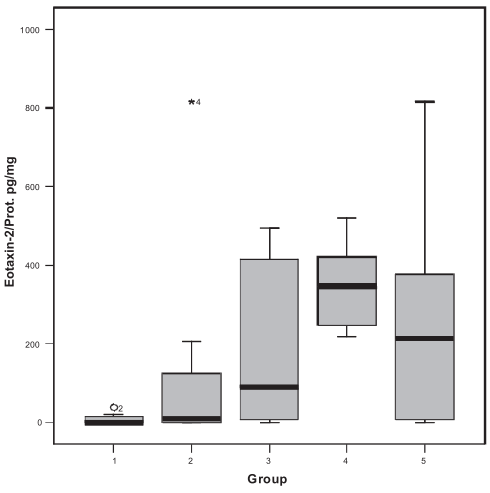

Figure 2. Eotaxin-2 protein concentrations in groups 1-5

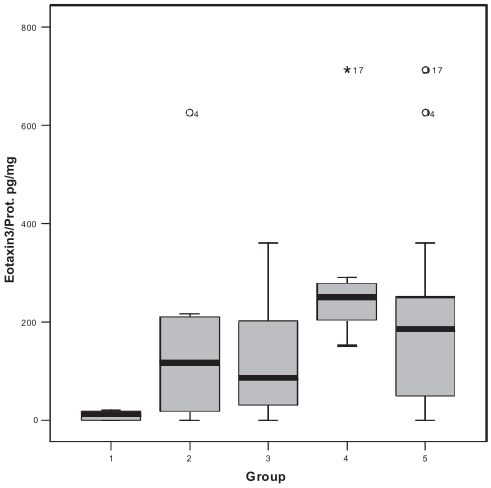

Figure 3. Eotaxin-3 protein concentrations in groups 1-5

1 = control subjects

2 = non allergic patients

3 = allergic patients

4 = aspirin-intolerant patients

5 = all patients

Direct correlation of eotaxin, eotaxin-2 and eotaxin-3 protein levels with tissue eosinophilia

Results of correlation analysis have shown a significantly direct correlation between the number of eosinophils and the tissue concentrations of all the investigated chemokines. For eotaxin, the two-sided significance level for correlation with the number of eosinophils was determined to be $p < 0.01$, for eotaxin-2: $p = 0.002$ and for eotaxin-3: $p = 0.003$. To further analyze tissue eosinophilia, the eosinophils in tissue samples from the individual subgroups were counted at 400x magnification in 5 high power-fields. The findings showed that the differences between all the investigated groups with respect to the number of eosinophils in polyp tissue were highly significant. The number of eosinophils in nasal polyps of allergic ($p = 0.003$) and asprin-intolerant patients ($p < 0.001$) was significantly increased compared with non-allergic patients. Additionally samples of aspirin-intolerant patients ($p < 0.001$) contained significantly more eosinophils than samples from allergic patients.

DISCUSSION

In the present study the protein concentration of the CC-chemokines eotaxin, eotaxin-2 and eotaxin-3 in nasal polyp tissue samples from patients was measured and compared with tissue samples obtained from the inferior turbinate of controls. Since patients with varying severities of tissue eosinophilia were included in the study, it was possible to analyze the CC-chemokine concentration relative to the degree of eosinophilia. The patients were additionally divided into numerically equivalent groups with and without allergy and aspirin-intolerance to test for etiological differences in chemokine patterns and to characterize and compare the effects of eosinophilia and etiology on the chemokine patterns. So far, this is the first systematic trial for simultaneous investigation of protein expression levels of all the aforementioned chemokines in patients with eosinophilic nasal polyps. Especially for eotaxin-3 protein, there have been no such studies in nasal polyposis until now. All of the investigated CC-chemokines were present in the patients as well as in the controls. Quantitative differences in the protein concentration and the number of positive samples for each cytokine were detected. Overall, protein expression of the CC-chemokines eotaxin, eotaxin-2 and eotaxin-3 was significantly higher in the nasal polyp group than in the control group. In the patient group, the rank order of chemokine protein concentrations was eotaxin-2 > eotaxin-3 > eotaxin. Correlation analysis also revealed a significant, two-sided positive correlation between the protein concentrations of all three eotaxins.

Various authors have demonstrated the specific chemotactic potency of all the eotaxins in vitro and in vivo [13,25,10-32,35,37]. Eotaxin is expressed by various kinds of cells, including nasal and dermal fibroblasts and epithelial cells [38]. Eotaxin-2 has been detected in the tissue of asthmatic lung- predominantly in cytokeratin-positive epithelial cells, $CD31^+$ endothelial cells, and $CD68^+$ macrophages [34] as well as in the skin patch test lesions of atopic patients [39]. Expression and production of the chemokines eotaxin and eotaxin-2 has furthermore been demonstrated in nasal polyp tissue from patients suffering from polyposis [11, 40]. Caversaccio et al. [4], who investigated eotaxin and eotaxin-2 protein levels in nasal polyps from polyposis patients, found that the levels measured in the patients were significantly different from those observed in the controls; moreover, the concentrations of eotaxin-2 were 20 times higher than those of eotaxin. In our own studies, we determined the difference to be a factor of 1.6. In our analysis, however, the data were related to the total protein concentration and not, as in Caversaccio's study, to the amount of sample.

In the year 1999, two independent groups succeeded in cloning a new member of the eotaxin family, eotaxin-3 [32,35]. Previously, this novel chemokine had only been identified at the mRNA level in human umbilical vascular endothelial cell line [35] as well as in the tissue of human heart and ovary [32]. At the individual cell level, eotaxin-3 mRNA expression had previously been demonstrated only in nasal and dermal fibroblasts [33, 40]. LightCycler PCR analyses showed that all three eotaxin ligands are constitutively expressed at the mRNA level in dermal fibroblasts [33]. Eotaxin-3 mRNA expression and protein concentrations in nasal polyps had not been investigated until now. By conducting the present study of patients with eosinophil nasal polyps, we were therefore the first group to identify eotaxin-3 at the protein level in patients and controls and to concurrently detect all three eotaxin species in tissue samples derived from nasal polyps as well as from the inferior turbinate of controls.

At any rate, the available data indicate that all three members of the eotaxin family are involved in the development of nasal polyps. However, little is known about the exact role of the various CCR3-binding eotaxins in the activation of eosinophils. The structural similarity of identical amino acids within the eotaxin family is low: sequence homology is only 39% between eotaxin-2 and eotaxin [30], 37% between eotaxin-3 and eotaxin, and 34% between eotaxin-3 and eotaxin-2 [35]. Nevertheless, a common feature of all three eotaxins is that, in their close evolutionary and genetic affiliation and their specific activity, they act exclusively via the CC-chemokine receptor CCR3 [30-32,35]. This, of course, raises the question of the biological relevance of the three eotaxins. Differences in relevance could arise due to differences in their affinity to the CCR3 receptor and in their chemotactic effects on eosinophils. Dulkys et al. [33], who investigated the affinity of members of the eotaxin family to CCR3, ranked the affinity as eotaxin > eotaxin-3 > eotaxin-2; the binding capacity of eotaxin-3 to CCR3 was determined to be approximately 10 times weaker than that of eotaxin. The exactly reverse rank order of eotaxin protein levels in the present study suggests that compensation for weaker binding at the receptor may occur and needs to be

tested. It is also possible that eotaxin-2 and eotaxin-3 may utilize other previously unidentified receptors. On the whole, the chemokine system can be characterized as an extremely redundant system with multiple ligand binding patterns for different receptors. The increased protein levels of all chemokines studied and the significantly demonstrated correlation between the protein concentrations of the individual chemokines is more likely indicative of a complex cooperation than of a specific role of an individual chemokine in the development of eosinophilia and nasal polyposis.

The correlations between the protein concentrations of the individual eotaxins in tissue, the potential etiological factors underlying nasal polyposis, and the number of eosinophils in nasal polyp tissue will now be analyzed. Patients with aspirin-intolerance had significantly higher eotaxin, eotaxin-2 and eotaxin-3 protein levels in polyp tissue than non-allergic patients. Additionally samples of aspirin-intolerant patients have shown a significantly higher amount of eotaxin-3 compared with allergic patients. These findings concur with those of Pods et al. [40], who observed increased eotaxin and eotaxin-2 expression and protein synthesis in eosinophil nasal polyps of patients with aspirin intolerance and asthma compared to the levels measured in patients without these concomitant conditions. To further analyze these differences, the numbers of eosinophils in tissue samples from the individual subgroups were determined. The findings showed that the differences between all the investigated groups with respect to the number of eosinophils in polyp tissue were highly significant. In terms of the number of eosinophils, the individual subgroups were ranked as follows: aspirin-intolerant patients > allergic patients > non-allergic patients. Since it would appear that the significantly increased number of eosinophils must be analyzed in connection with the chemokine protein results (the aspirin-intolerant patients, the group with the highest level of eosinophilia, also exhibited the highest protein levels for each of the investigated eotaxins), further tests were performed to investigate this correlation. In the case of eotaxin, eotaxin-2 and eotaxin-3, the two-sided significance level for correlation with the number of eosinophils was determined to be 0.01. Our results therefore point to a direct correlation between the number of eosinophils and the tissue concentrations of the individual chemokines. For the eotaxin family, this is presumably a result of their exclusive interaction with the chemokine receptor CCR3 and the relatively selective expression of this receptor on eosinophils. The correlation between the number of eosinophils and the protein concentrations of the chemokines as well as the significant difference between the groups with regard to eosinophilia permit the following conclusions:

There is apparently a mutual, close correlation between the number of eosinophils and the protein concentration of the individual chemokines in tissue. Patients with aspirin intolerance have the highest levels of eosinophilia; consequently, they also have higher protein levels of the corresponding chemokines than patients in the other subgroups. On the one hand, higher concentrations of these chemokines result in larger quantities of eosinophils migrating to the tissues. It is also known that eosinophils, themselves, are capable of producing chemokines such as eotaxin, MIP-1α, RANTES and IL-8 under certain conditions [41,42]. Although the corresponding studies on eotaxin-2 and eotaxin-3 are still lacking, they also count as potential candidates. So eosinophils could recruit their own replacements and additionally attract other cells, such as T cells, monocytes, neutrophils and basophils.

In summary, the results indicate that all members of the eotaxin family play a specific role in the pathogenesis of nasal polyposis. The data also suggest that there is a mutual close relationship between the number of eosinophils and the tissue concentrations of the individual chemokines. Simultaneous expression of all members of the eotaxin family or reaching a certain concentration threshold could possibly be prerequisites for stronger eosinophil migration. In this case, one can assume that the extent of tissue eosinophilia is not determined by individual chemokines fulfilling specific individual tasks, but rather is due to the complex interplay of multiple chemokines, chemokine receptors and cells. The actual starting mechanism that initially prompts the continuous accumulation of eosinophils and inflammation mediators and which ultimately leads to the chronic propagation of the disease is still unknown. Investigation of individual mediators is vital for improving the understanding of important steps in the pathogenesis of the disease. Moreover, elucidation of the individual components of the system is indispensable for the formulation of new therapeutic strategies.

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