Chemokines in the limbal form of vernal keratoconjunctivitis

Ahmed M Abu El-Asrar, Sofie Struyf, Soliman A Al-Kharashi, Luc Missotten, Jo Van Damme, Karel Geboes

Abstract

Background/aims—Chemokines are a family of low molecular weight cytokines that attract and activate leucocytes. The CC chemokines act on eosinophils, basophils, monocytes, and lymphocytes, suggesting that they play an important part in allergic diseases. The aims of this study were to investigate the expression of the CC chemokines, RANTES, eotaxin, monocyte chemotactic protein (MCP) 1, MCP-2, and MCP-3 in the conjunctiva of patients with vernal keratoconjunctivitis (VKC) and to determine the cellular source of these chemokines.

Methods— Conjunctival biopsy specimens from nine subjects with active VKC, and six control subjects were studied by immunohistochemical techniques using a panel of monoclonal and polyclonal antibodies directed against RANTES, eotaxin, MCP-1, MCP-2, and MCP-3. The phenotype of inflammatory cells expressing chemokines was examined by sequential double immunohistochemistry.

Results—In the normal conjunctiva, superficial epithelial cells showed a constitutive, weak cytoplasmic expression of eotaxin. Few inflammatory cells in the perivascular areas expressed RANTES, MCP-1, MCP-2, and MCP-3. In VKC specimens, the epithelium showed intense cytoplasmic eotaxin staining in all cells, and cytoplasmic RANTES staining mainly in the superficial layers. Furthermore, RANTES and eotaxin were expressed on the vascular endothelium mainly in the upper substantia propria. Compared with normal controls, VKC specimens showed significantly more inflammatory cells expressing RANTES, eotaxin, MCP-1, and MCP-3 (p < 0.001, 0.0028, 0.0092, and < 0.001, respectively). In VKC specimens, the numbers of inflammatory cells expressing RANTES were significantly higher than the numbers of inflammatory cells expressing eotaxin, MCP-1, and MCP-2 (all p values < 0.001). Colocalisation studies revealed that the majority of inflammatory cells expressing chemokines were CD68 positive monocytes/macrophages.

Conclusions—These results demonstrate an increase in the expression of RANTES, eotaxin, MCP-1, and MCP-3 in the conjunctiva of patients with VKC compared with control subjects. These data suggest a potential role for these chemokines in the pathogenesis of VKC. Antagonists of chemokine receptors may provide new therapeutic modalities in VKC.
Chemotactic protein; (pc) = polyclonal antibodies; (mc) = monoclonal antibodies.
RANTES = regulated upon activation, normal T cell expressed and secreted; MCP = monocyte
Abingdon, UK; Dakopatts A/S, Copenhagen, Denmark; Dako, CA, USA.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Working dilution</th>
<th>Source*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-human RANTES (mc)</td>
<td>RANTES</td>
<td>1:20</td>
<td>Pepro Tech</td>
</tr>
<tr>
<td>Anti-human eotaxin (pc)</td>
<td>Eotaxin</td>
<td>1:20</td>
<td>Pepro Tech</td>
</tr>
<tr>
<td>Anti-human MCP-1 (mc)</td>
<td>MCP-1</td>
<td>1:20</td>
<td>R &amp; D systems</td>
</tr>
<tr>
<td>Anti-human MCP-2 (mc)</td>
<td>MCP-2</td>
<td>1:20</td>
<td>R &amp; D systems</td>
</tr>
<tr>
<td>Anti-human MCP-3 (mc)</td>
<td>MCP-3</td>
<td>1:20</td>
<td>Pepro Tech</td>
</tr>
<tr>
<td>CD68 (mc)</td>
<td>Macrophages</td>
<td>1:1000</td>
<td>Dakopatts</td>
</tr>
<tr>
<td>CD3 (mc)</td>
<td>Pan T cell</td>
<td>1:400</td>
<td>Dakopatts</td>
</tr>
<tr>
<td>Factor VIII related antigen</td>
<td>Endothelial cells</td>
<td>1:20</td>
<td>Dako</td>
</tr>
</tbody>
</table>

*Location of manufacturers: Pepro Tech, Rocky Hill, NJ, USA; R & D systems Europe Ltd, Abingdon, UK; Dakopatts A/S, Copenhagen, Denmark; Dako, CA, USA.

Table 1 Monoclonal and polyclonal antibodies used in this study

Monokines in the limbal form of vernal keratoconjunctivitis

1361

All the patients were males. The mean age was 12.4 (SD 2.2) years (range 10–17 years). The symptoms mentioned by all the patients were itching, redness, photophobia, and tearing. Each patient underwent complete ophthalmic examination, and the corneal and conjunctival changes were noted and recorded. All patients had the limbal form of the disease characterised by broad gelatinous infiltrates of the limbus. Limbal conjunctival biopsy specimens were obtained from each patient. None of the patients was on topical therapy before obtaining the biopsy. In addition, six limbal conjunctival biopsy specimens were obtained from patients undergoing strabismus surgery without obvious inflammation and served as controls. The controls were from the same age group, and were four males, and two females.

IMMUNOHISTOCHEMICAL STAINING

The conjunctival biopsy specimens were immediately snap frozen in Tissue-Tek optimum cutting temperature (OCT) compound (Miles Laboratories, IN, USA) and maintained at −80°C until use. For immunohistochemistry, 5 µm serially cut cryostat sections were dried overnight at room temperature, fixed in absolute acetone for 3 minutes, and then treated with 2% hydrogen peroxide in methanol for 3 minutes to block endogenous peroxidase activity. After rinsing three times in phosphate buffered saline (PBS) at pH 7.2 for 15 minutes, the slides were incubated for 30 minutes with the monoclonal and polyclonal antibodies listed in Table 1. Optimal concentrations of all antibodies used were determined in pilot experiments. After a wash with PBS, the sections were incubated for 30 minutes with EnVision+, Peroxidase, Rabbit, or EnVision+, Peroxidase, Mouse (Dako, CA, USA). These are goat anti-rabbit or antimouse immunoglobulins conjugated to peroxidase labelled dextran polymer. The products react with rabbit immunoglobulins or with mouse immunoglobulins of all classes and minimally with human immunoglobulins thus allowing better visualisation. The slides were washed again with PBS and the reaction product was visualised by incubation for 10 minutes in 0.05M acetate buffer at pH 4.9, containing 0.05% 3-amino-9-ethylcarbazole (Sigma) and 0.01% hydrogen peroxide, resulting in bright red immunoreactive sites. The slides were faintly counterstained with Harris haematoxylin. Finally, the sections were rinsed with distilled water and coverslipped with glycerol.

Omission or substitution of the primary antibody with an irrelevant antibody of the same species was used as a negative control.

DOUBLE IMMUNOHISTOCHEMISTRY

To examine the phenotype of inflammatory cells expressing chemokines, cryostat sections were studied by sequential double immunohistochemistry. In addition, sequential double immunohistochemistry was used to confirm the expression of RANTES and eotaxin by vascular endothelial cells. Colocalisation studies were performed in three VCK specimens. After rinsing the slides with PBS, they were incubated for 30 minutes with the appropriate...
monoclonal antibody to determine cellular phenotype (CD68, CD3, and factor VIII related antigen) and rinsed again in PBS. Subsequently, the sections were incubated for 30 minutes with Envision +, Peroxidase, Mouse (Dako, CA, USA) and washed again with PBS. Then, the reaction product was visualised by incubation for 10 minutes in 0.05 M acetate buffer at pH 4.9, containing 0.05% 3-amino-9-ethylcarbazole (Sigma) and 0.01% hydrogen peroxide, resulting in red immunoreactive staining. Afterwards the sections were rinsed with PBS, washed with distilled water, and incubated for 30 minutes with the monoclonal anti-human RANTES antibody. After a wash with PBS, the sections were incubated for 30 minutes with a biotin labelled rabbit antimouse antibody, followed by a monoclonal anti-biotin-alkaline phosphatase conjugate (Sigma). The blue reaction product was developed using fast blue BB salt (4-benzoylamino-2,5-dioxohydrobenzene-diazonium chloride) (Sigma-Aldrich, Bornem, Belgium) for 5 minutes.

QUANTITATION
Cells were counted in five fields that were chosen on the basis of the presence of an adequate number of inflammatory cells. We ignored fields in which no positively stained cells were present. We used an eye piece calibrated grid with 25× magnification. With this magnification and calibration, we counted the cells present in an area of 0.155 × 0.155 mm. For the colocalisation studies, inflammatory cells expressing both chemokines and CD68, or CD3 were counted and expressed as a percentage of cells expressing chemokines.

STATISTICAL ANALYSIS
Means (SD) were calculated for each cell type in VKC and control specimens. The t test was used to analyse the statistical significance of differences between mean numbers of cells stained with different chemokine antibodies in patients and controls. One way analysis of variance (ANOVA) was used to analyse the statistical significance of differences between mean numbers of cells stained with different chem-

**Figure 1**  (1) Vernal keratoconjunctivitis control slide, treated identically, using an irrelevant monoclonal antibody showing no staining (×300). (2) Immunohistochemical staining for eotaxin of conjunctiva from a normal control subject showing weak cytoplasmic expression in the superficial epithelial layers (×500). (3) Vernal keratoconjunctivitis. Immunohistochemical staining for eotaxin showing intense cytoplasmic staining in all epithelial cells and staining on the vascular endothelium (arrows) (×500).
Chemokines in the limbal form of vernal keratoconjunctivitis

Results

There was no staining in the negative control slides (Fig 1(1)). In normal conjunctiva, the surface epithelial cells demonstrated weak cytoplasmic staining for eotaxin (Fig 1(2)). Conjunctival epithelial cells of controls were negative for RANTES, MCP-1, MCP-2, and MCP-3. Few inflammatory cells in the perivascular areas showed cytoplasmic staining for RANTES, MCP-1, MCP-2, and MCP-3. No immunoreactivity was seen on the vascular endothelium.

 Conjunctival specimens from VKC patients showed intense cytoplasmic staining for eotaxin (Fig 1(3)). Patchy cytoplasmic RANTES staining was seen in the epithelium which was most intense in the superficial epithelial layers (Fig 2(4)). The epithelial cells did not express MCP-1, MCP-2, and MCP-3. Eotaxin (Fig 1(3)) and RANTES (Fig 2(4)) staining was observed on the vascular endothelium expressing factor VIII related antigen mainly in the upper substantia propria adjacent to the epithelium. The vascular endothelium did not express MCP-1, MCP-2, and MCP-3. In the substantia propria, inflammatory cells expressing cytoplasmic RANTES, eotaxin, MCP-1 (Fig 2(5)), MCP-2, and MCP-3 were noted.

VKC specimens showed statistically significant higher counts than control specimens for inflammatory cells expressing RANTES, eotaxin, MCP-1, and MCP-3 (Table 2). Statistically insignificant higher numbers of inflammatory cells expressing MCP-2 were noted in VKC specimens compared with control specimens. In VKC specimens, the numbers of inflammatory cells expressing RANTES were higher than the numbers of inflammatory cells expressing eotaxin, MCP-1, MCP-2, and MCP-3. The mean values of the five groups differed significantly (p <0.001, ANOVA). Furthermore, post-ANOVA pairwise multiple comparisons showed that the numbers of inflammatory cells expressing RANTES were
**Table 2** Number of inflammatory cells expressing chemokines in VKC and control specimens (mean (SD))

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Control (n=6)</th>
<th>VKC (n=9)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RANTES</td>
<td>2.0 (1.8)</td>
<td>16.3 (3.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>0.0 (0.0)</td>
<td>6.6 (3.9)</td>
<td>0.0028</td>
</tr>
<tr>
<td>MCP-1</td>
<td>2.8 (1.6)</td>
<td>9.0 (4.4)</td>
<td>0.0092</td>
</tr>
<tr>
<td>MCP-2</td>
<td>1.0 (1.7)</td>
<td>5.7 (5.5)</td>
<td>0.2968 (NS)</td>
</tr>
<tr>
<td>MCP-3</td>
<td>1.2 (1.3)</td>
<td>11.1 (4.0)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

VKC = vernal keratoconjunctivitis; RANTES = regulated upon activation, normal T cell expressed and secreted; MCP = monocyte chemotactic protein.

NS = not significant.

significantly higher than the numbers of inflammatory cells expressing eotaxin, MCP-1, and MCP-2 (all p values <0.001, t test). Double immunohistochemistry to confirm the phenotype of chemokine positive inflammatory cells showed that the majority of mononuclear cells expressing RANTES were CD68 positive monocytes/macrophages (mean 83% (SD 7.3%), n = 3) (Fig 2(6)). Smaller numbers of inflammatory cells expressing RANTES were CD3 positive T cells (mean 7% (2.9%), n=3). Similarly, the majority of inflammatory cells expressing eotaxin, MCP-1, and MCP-3 were CD68 positive monocytes/macrophages (mean 58% (5%), 81% (8.1%), and 78% (6.5%), respectively, n = 3). Smaller numbers of inflammatory cells expressing eotaxin, MCP-1, and MCP-3 were CD3 positive T cells (mean 7% (3%), 5.3% (3%), and 8% (2%), respectively, n = 3). Other chemokine positive inflammatory cells were not identified by the two markers used.

Discussion

Eotaxin, a potent CC chemokine originally purified from the bronchoalveolar lavage fluid from allergen challenged guinea pigs, is a potent and selective eosinophil chemoattractant. In addition, eotaxin is a potent activator of the respiratory burst and actin polymerisation of eosinophils. Eotaxin, therefore, plays an important part not only by attracting eosinophils to the site of inflammation but also by damaging tissue by its capacity to induce the release of reactive oxygen species.

From clinical specimens, it has been previously demonstrated that eotaxin mRNA and immunoreactivity were constitutively expressed by bronchial epithelium, and nasal epithelium from normal individuals. It is suggested that the constitutive expression of eotaxin in healthy conditions regulates the physiological trafficking of eosinophils. In conjunctival biopsies from normal individuals, there was a constitutive, weak expression of eotaxin immunoreactivity in the epithelial layer. Compared with normal conjunctiva, the conjunctiva from patients with VKC showed strong expression of eotaxin immunoreactivity in the epithelial layer, stromal inflammatory cells, and vascular endothelial cells. Previous studies have documented the increased expression of eotaxin mRNA and immunoreactivity within the airways of asthmatic individuals, and in nasal biopsy specimens from individuals with allergic rhinitis. In subjects with allergic rhinitis allergic challenge of the nasal mucosa resulted in a local upregulation of eotaxin expression. In the airways of asthmatic individuals, the expression of eotaxin mRNA was significantly correlated with the numbers of eosinophils present. Our observations are consistent with previous data in bronchial and nasal biopsy specimens from patients with asthma and allergic rhinitis which suggested that the majority of cells expressing eotaxin mRNA and protein were epithelial cells, endothelial cells, and inflammatory cells and that the predominant eosinophils bearing inflammatory cells were CD68 positive macrophages.

RANTES is a potent chemoattractant for eosinophils, basophils, monocytes/macrophages, and CD45RO/CD4 memory T lymphocytes. In addition, it exhibits pleiotropic effects on a variety of target cell types, including activation of eosinophils, and T lymphocytes, induction of eosinophil transendothelial migration in the extracellular spaces in vitro that was potentiated by pre-exposure of eosinophils to IL-5, and induction of histamine release from basophils. Further, RANTES seems to stimulate IgE+ tonsillar B cells for IgE production. Thus RANTES may be involved in both acute and chronic stages of allergic inflammation.

In the present study, we have demonstrated that the conjunctival epithelial cells from patients with VKC showed cytoplasmic expression of RANTES. Normally, conjunctival epithelial cells do not express RANTES. Furthermore, we found that in VKC vascular endothelial cells and inflammatory cells in the substantia propria expressed RANTES. The majority of these inflammatory cells were CD68+ monocytes/macrophages. Similarly, positive immunohistochemical staining for RANTES was demonstrated in nasal epithelial and vascular endothelial cells of bronchial biopsy tissues obtained from asthmatic patients, and in epithelial cells, vascular endothelial cells, and mononuclear cells of nasal polyps. After local allergen provocation of the nasal mucosa, the majority of RANTES mRNA+ cells were macrophages.

Factors regulating the expression of eotaxin and RANTES by conjunctival epithelial cells, and vascular endothelial cells in VKC are incompletely understood. Other cytokines may be involved in regulating eotaxin and RANTES production. Several studies demonstrated the production of eotaxin, and RANTES by bronchial epithelial cells stimulated by IL-1β and tumour necrosis factor α (TNFα). RANTES production was demonstrated in nasal epithelial cells and endothelial cells stimulated by IL-1β, and TNFα. In addition, TNFα induced RANTES production in a conjunctival epithelial cell line. Possible sources for these cytokines include macrophages which are present in abundance in VKC, mast cells, and basophils. Our data indicate that con-
Chemokines in the limbal form of vernal keratoconjunctivitis

The conjunctival epithelium is not only a structural barrier but it is also a source of chemokines able to modulate inflammation. Conjunctival epithelial cells produce eotaxin and RANTES that recruit a pattern of cells including eosinophils, T lymphocytes, basophils, and monocytes that are known to migrate to the conjunctiva in VKC. It is noteworthy that the greatest accumulation of inflammatory cells is seen adjacent to the epithelium.

In the present study, we detected cytoplasmic expression of MCP-1, MCP-2, and MCP-3 by inflammatory cells in the substantia propria. The majority of these inflammatory cells were monocytes/macrophages. However, the numbers of MCP-2-inflammatory cells in VKC specimens and normal conjunctival biopsy specimens did not differ significantly. MCP-1, MCP-2, and MCP-3 have originally been identified as potent monocyte chemotactic proteins. MCP-1 is a potent histamine releasing factor for basophils, but does not attract or activate eosinophils. MCP-3 causes eosinophil and basophil chemotaxis, and stimulates histamine release from human basophils. MCP-2 has been found to share the bioactivity profile with MCP-3 rather than with MCP-1. However, it is a weaker inducer of chemotaxis and release responses in basophils and eosinophils than MCP-3. Several clinical studies demonstrated that the bronchial-alveolar lavage fluid from patients with asthma contained elevated concentrations of MCP-1. Allergen provoked a significant increase of mRNA* cells for MCP-3 which paralleled the kinetics of early eosinophil response in the skin of atopic subjects. Furthermore, increased mRNA expression for MCP-3 was detected in bronchial biopsies in asthmatic patients.

All chemokines act via G protein coupled, 7-transmembrane-domain receptors. The cellular targets of a chemokine are determined by the recognition of one or more receptors, and whether these are functionally expressed on different leukocyte populations. The CC chemokine receptor 3 (CCR3) used by eotaxin is found on eosinophils, basophils, and TH2-type lymphocytes. Unlike other members of the CC chemokines which generally act on several receptors, eotaxin only signals via the CCR3 explaining the high specificity of eotaxin for eosinophils. Other CC chemokines—for example, RANTES, MCP-3, and MCP-4 also bind to the CCR3 but with lower affinity. In light of its prominent role in eosinophil, basophil, and TH2-type lymphocyte chemotaxis, CCR3 is a promising target for the development of new anti-allergic drugs to block selectively the accumulation of the effector cells of allergic inflammation.

In conclusion, this study has demonstrated that increased expression of the CC chemokines RANTES, eotaxin, MCP-1, and MCP-3 is evident in the conjunctiva from patients with VKC, and that conjunctival epithelial cells are capable of synthesising RANTES and eotaxin. The expression of these chemokines may in part be responsible for the conjunctival accumu-

mulation of eosinophils, basophils, and mononuclear cells observed in VKC. Small molecule antagonists of chemokine receptors may therefore be desirable in modulating eosinophil recruit-

tment to conjunctiva, and thereby prevent the tissue damage mediated by eosinophilic toxic granule proteins and reactive oxygen species.

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1366

Abu El-Arar, Struyf, Al-Kharashi, et al


