Eosinophil granule proteins expressed in ocular cicatricial pemphigoid

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Abstract

Background—Blister formation and tissue damage in bullous pemphigoid have been attributed to the release of eosinophil granule proteins—namely, to eosinophil derived cationic protein (ECP) and major basic protein (MBP). In the present investigation these eosinophil granule proteins were studied in the conjunctiva of patients with ocular cicatricial pemphigoid (OCP).

Methods—Conjunctival biopsy specimens obtained from patients with subacute (n=8) or chronic conjunctival disease (n=13) were analysed histologically and immunohistochemically using antibodies directed against EG1 (stored and secreted ECP), EG2 (secreted ECP), MBP, CD45 (common leucocyte antigen), CD3 (pan T cell marker), and HLA-DR (class II antigen).

Results—Subepithelial mononuclear cells, mast cells, and neutrophils were detected in all specimens. The number of mononuclear cells, neutrophils, CD45+ cells, CD3+ cells, and the HLA-DR expression were significantly higher in the subacute than in the chronic disease group. Some eosinophils were found in specimens from five of eight patients with subacute OCP, but in none of the patients with chronic disease. The eosinophil granule proteins (ECP and MBP) were found in the epithelium and substantia propria in patients with subacute conjunctivitis.

Conclusions—Subepithelial cell infiltration in the conjunctiva greatly differs between subacute and chronic ocular cicatricial pemphigoid specimens. The findings suggest that eosinophil granule proteins may participate in tissue damage in acute phase of inflammation in OCP.

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Ocular cicatricial pemphigoid (OCP) is a rare disease leading to shrinkage of the conjunctiva.^{1,2} Conjunctival fibrosis may cause severe entropion, trichiasis, symblepharon formation, dry eye, and epithelial ulceration.^{1,3} Without treatment the cicatrizing disease progresses in 75% of the patients,⁴ eventually resulting in blindness by complications to the cornea. The disease course may be chronic, subacute, or acute, with exacerbation of severe conjunctival inflammation. While systemic immunosuppression stops progression of cicatrization in most patients,⁵⁻⁷ it fails in approximately 10% of them. Further investigation is

required to characterise the pathogenetic steps of this disease more exactly.

There is compelling evidence that cicatricial pemphigoid is an autoimmune disease. Auto-antibodies directed against antigens in the lamina lucida of the epithelial basement membrane have been demonstrated in genetically susceptible individuals.^{1,2,8,9} Marked inflammatory cellular infiltrations in the epithelium and substantia propria are typically found during exacerbation.^{2,10}

It has been shown recently that eosinophils, by their highly proinflammatory activity and the release of toxic products, contribute to the tissue damage in various inflammatory diseases, including ocular allergies and orbital inflammation.¹¹⁻¹⁴ The four major proteins found in eosinophil granules and released upon eosinophil stimulation are eosinophil cationic protein (ECP), eosinophil protein X/eosinophil derived neurotoxin (EPX/EDN), major basic protein (MBP), and eosinophil peroxidase.

It has been shown recently that activated eosinophils and their granules contribute to the development of dermal lesions in bullous pemphigoid of the skin.¹⁵ While neutrophils and lymphocytes are abundant in OCP lesions, eosinophils are rarely detected by classic histological techniques even in the acute stage of disease.^{1,16,17} However, eosinophils might not be found histologically after degranulation. In the present study we investigated the presence of eosinophils and their granule proteins by immunohistochemical techniques in cicatricial pemphigoid affecting the conjunctiva in an attempt to study the pathogenic role of eosinophils in this inflammatory disorder.

Materials and methods

PATIENTS

Twenty one patients with bilateral progressive OCP were enrolled in this study. The records and photographs of all patients were reviewed. Each patient's comprehensive medical history, including prior ocular surgery, and treatments with systemic immunosuppressive drugs were noted.

The diagnosis of cicatricial pemphigoid was based on clinical criteria published elsewhere.^{1,2} Progressive symblepharon formation, fornix foreshortening, and subconjunctival fibrosis were found in all patients. According to the Foster staging system,¹ the patients had stage 3 disease. The inflammatory activity was graded on a scale of 0 to 4+, based on conjunctival redness and mucous discharge. In agreement with a previous study the patients were clinically divided into a subacute and a

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Table 1 Mononuclear cell infiltration and eosinophil granule proteins in the conjunctiva of patients with ocular cicatricial pemphigoid

Primary monoclonal antibodies			
Antibody	Dilution	Specificity	Source
Mononuclear cell infiltrate:			
Anti-CD45	1/20	Common leucocyte antigen	Dako, Hamburg, Germany
Anti-CD3	1/20	T cell lineage, thymocytes	Dako, Hamburg, Germany
Anti-HLA-DR	1/50	HLA-DR (class II histocompatibility antigen)	Dako, Hamburg, Germany
Eosinophil granule proteins:			
EG1	1/200	ECP (stored and secreted)	Kabi Pharmacia, Uppsala, Sweden
EG2	1/200	ECP (secreted), EPX/EDN	Kabi Pharmacia, Uppsala, Sweden
BMK-13	1/50	Major basic protein	Biodesign, Dunn, Asbach, Germany

ECP = eosinophil cationic protein; EPX = eosinophil protein X syn; EDN = eosinophil derived neurotoxin.

chronic disease group.¹⁰ The clinical course of disease was either chronic, typically with low levels of conjunctival inflammation ($\leq 2+$), or subacute with rapid disease progression within a short course and with an inflammatory activity of 3+ or more. Conjunctival or corneal epithelial ulcerations have not been seen in any of the patients.

METHODS

After obtaining informed consent, the conjunctival biopsies were taken by subconjunctival anaesthesia, as published in detail elsewhere.¹ Tissues were processed for routine histological and immunoperoxidase studies.

Specimens for light microscopy were fixed in Karnovsky's solution (1% paraformaldehyde, 1.25% glutaraldehyde, 0.13% sucrose, and 25 mM sodium phosphate in 0.2 M sodium cacodylate buffer, pH 7.2), rinsed in cacodylate buffer, dehydrated with ethanol, and embedded in glycol methacrylate (LKB Histo-resin, LKB Produkter, AB, Bromma, Sweden). Sections of 2 μ m were stained with standard staining procedures with haematoxylin and eosin and alkaline Giemsa.

For immunohistochemistry, specimens were snap frozen, embedded in Tissue Tek OCT compound (Ames Company, Miles Laboratory, Elkhart, IN, USA) and stored at -70°C . Tissues were stained with the avidin-biotin-immunoperoxidase technique. The various primary monoclonal antibodies applied are listed in Table 1. All antibodies were mouse IgG anti-human preparations.¹⁸⁻²⁰

Briefly, 4 μ m cryostat sections were mounted on poly-L lysin (Sigma, Munich, Germany) coated slides. The sections were fixed in acetone and, after incubating with bovine serum albumin (1:20 in PBS) for 20 minutes, the sections were stained with the primary antibodies in a moist chamber at 20°C for 20 minutes. The tissue was then blocked for endogenous peroxidase using 3% hydrogen peroxide in phosphate buffered saline (PBS)

and rinsed with PBS. This was followed by an incubation with a secondary biotinylated rabbit anti-mouse antibody (1:10 in PBS; Dako, Hamburg, Germany) for 20 minutes, and the sections were rinsed in PBS again. Avidin-biotin peroxidase complexes (peroxidase conjugated streptavidin, 1:500; Dako) were applied for 20 minutes. The reactions at sites of binding were developed in peroxidase substrate containing 3-amino-9-ethylcarbazole substrate (Sigma, Munich, Germany) and hydrogen peroxide in 0.1 M acetate buffer. Specimens were then fixed in formalin (4%, in acetate buffer), counterstained with Gills No 3 haematoxylin (Sigma), and coverslipped with Aquatex (Merck, Darmstadt, Germany).

All results were independently interpreted by two investigators without knowledge of the patients' identity. Total inflammatory cells, neutrophils, mast cells, and eosinophils were analysed on the haematoxylin and eosin stained slides in the subepithelial layers of the substantia propria. Giemsa staining aided in enumerating the mast cells. The cell numbers per high power field were counted. The significance of the differences in cell numbers from the chronic and the subacute pemphigoid tissues were determined by the unpaired Student's *t* test.

Cellular and extracellular staining of the eosinophil granule proteins was graded on a scale from 0 to 3+ in representative high power fields ($\times 450$) with a 10×10 mm grid similar to the technique described elsewhere¹⁴; a 10×2 mm grid was used for the epithelial counts. The positive staining was graded as follows: 0, no staining; 1+, few foci (10–30% of the area) containing light amounts of staining; 2+, scattered foci (30–50% of the area) with intense staining or larger areas ($\geq 50\%$ of the area) with light staining; 3+, intensive staining throughout large areas ($\geq 50\%$ of the area). The significance of differences from the granular protein staining of chronic and subacute inflammation was determined by the Mann-Whitney U test.

Results

Cicatricial conjunctivitis was subacute in eight patients and chronic in 13. In all cases, conjunctival inflammation was present at the time of biopsy. The conjunctival biopsies fulfilled the histological and immunohistochemical characteristics of ocular cicatricial pemphigoid. Immunoglobulin and/or

Table 2 Conjunctival histology of patients with ocular cicatricial pemphigoid; subepithelial cell infiltration

	Chronic (n=13)	Subacute (n=8)
Total inflammatory cells	32.5 (19.2)	145.6 (43.7)*
Neutrophils	21.5 (11.5)	65.4 (24.7)*
Mast cells	13.6 (2.4)	16.3 (4.5)
Eosinophils	0 (0)	9.2 (7.75)*

Cell numbers/high power field ($450\times$); mean (SEM).

* $p < 0.001$ by Student's *t* test comparing subacute and chronic disease.

Table 3 Mononuclear cell infiltration and eosinophil granule proteins in the conjunctiva of cicatricial pemphigoid

Antibodies	Epithelium		Subepithelium	
	Chronic	Subacute	Chronic	Subacute
Mononuclear cell infiltrate§:				
CD45	27.2 (15.8)	39.6 (13.7)	48.3 (15.4)	62.9 (14.3)
CD3	9.7 (6.5)	15.6 (4.5)	20.3 (3.7)	37.2 (5.6)**
HLA-DR	34.1 (9.3)	42.2 (14.6)	39.3 (6.2)	76.8 (11.9)**
Human eosinophil granule proteins†:				
EG1	0.01 (0.01)	1.44 (0.44)*	0.13 (0.08)	1.75 (0.4)**
EG2	0.14 (0.06)	0.88 (0.2)*	0.39 (1.07)	1.5 (0.19)**
BMK-13	0.08 (0.08)	0.88 (0.38)*	0.17 (0.1)	2.25 (0.48)**

§ Cell numbers/high power field (450×); mean (SEM).

† Cellular and extracellular staining within high power field (× 450): 0 = no staining; 1+ = few foci containing light amounts of staining; 2+ = scattered foci with intense staining or larger area with light staining; 3+ = intensive staining throughout large areas.

* $p < 0.05$; ** $p < 0.005$ by Mann-Whitney U test comparing subacute and chronic disease. EG1=antibody directed against stored and secreted eosinophil cationic protein; EG2=antibody directed against secreted eosinophil cationic protein or eosinophil derived neurotoxin; BMK-13 = antibody directed against major basic protein.

complement depositions were found at the epithelial basement membrane of the conjunctiva in all patients included in this study, substantiating the clinical diagnosis of cicatricial pemphigoid.^{1,2}

Figure 1 Pattern and location of eosinophil granule proteins in the conjunctival tissue from patients with subacute cicatricial pemphigoid; immunoperoxidase staining (magnification × 450). (A) Eosinophil cationic protein, stored and secreted (EG1 antibody). (B) Eosinophil cationic protein (secreted), eosinophil protein X syn, eosinophil derived neurotoxin (EG2 antibody). (C) Major basic protein.

In the patients with chronic conjunctivitis, the mean age was 70.5 years (range 40–92 years); there were nine women and four men. The duration of disease before biopsy was 2 to 13 years (mean 6 (SEM 3.4) years). In the group of patients with subacute conjunctivitis, the mean age was 71.2 years (range 56–86 years); seven were female, one was male. In this second group of patients, the duration of conjunctivitis before biopsy was less than 1 year in six cases, and was 2 years in a further two. Extraocular manifestations were present only in two patients with subacute conjunctivitis. None of the patients had a history of ocular allergies or atopy. All patients were newly diagnosed as cicatricial pemphigoid cases who had never been on immunosuppression. At the time of biopsy, one patient with subacute conjunctivitis was taking azathioprine, one taking oral prednisone, and another taking a non-steroidal anti-inflammatory drug. Four of these patients received artificial tears, three were using topical antibiotics, and another patient was treated with topical β blockers. One patient with chronic conjunctivitis was on dapsone; lubricants were given in nine cases, and antibiotic eyedrops in another four patients.

The results of the histological studies are summarised in Table 2. The subepithelial infiltration mainly consisted of mononuclear cells and lymphocytes. The number of inflammatory cells was significantly higher in patients with subacute disease than in the others. The subepithelial and perivascular mast cells in patients with subacute disease outnumbered the counts in patients with chronic disease; however, the difference was not statistically significant. Eosinophils were not detected in any of the tissues obtained from patients with chronic cicatricial pemphigoid. In contrast, some eosinophils were found in the substantia propria in five of eight patients with subacute conjunctivitis, but these cells were not detected in the epithelium.

The mononuclear cell infiltrations were counted (Table 3) in order to show that the study groups and the subacute and chronic groups of previous publications^{2,10} are the same. Cells expressing the common leucocyte antigen were found in the epithelium, and were especially abundant in the subepithelium and in close proximity to the vessels. The cell

numbers in subacute and chronic conjunctivitis did not differ significantly. The subepithelial numbers of CD3+ cells in patients with subacute disease were significantly higher compared with the chronic disease group. HLA-DR expression within the inflamed tissues was increased in both patients with subacute and chronic cicatricial pemphigoid. However, the highest intensity of class II antigen expression in the subepithelium and at the vessels was detected in patients with subacute disease.

While strongly positive cellular and extracellular staining with antibodies directed against the various eosinophil granular proteins was detected in the tissue sections of patients with subacute OCP, these granular proteins were not found in the tissues of patients with chronic disease (Table 3), or in the conjunctiva obtained from healthy individuals during cataract surgery (data not shown). The staining patterns were both cellular and diffuse in the majority of subacutely inflamed conjunctival specimens. Therefore, enumeration of cells alone was insufficient, and the extracellular staining was also quantified. Staining with the ECP antibodies (EG1 and EG2) in general was less intense than with the antibodies directed against MBP (BMK-13). The eosinophil granule proteins were abundant in the subepithelium and around the vessels; only small amounts of these eosinophil contents were observed in the epithelium (Fig 1).

Discussion

The pathogenic mechanisms in ocular cicatricial pemphigoid are incompletely understood. Sacks *et al*²¹ have shown an abundance of T cells in chronic cicatricial pemphigoid of the conjunctiva. Rice and Foster² studied the mononuclear cell infiltrate in acute ocular pemphigoid; the subepithelial infiltrate showed significantly increased numbers of T cells (CD3+, CD5+), T helper cells (CD4+), T suppressor cells (CD8+), macrophages (CD14+, MAC-1+), and dendritic cells (CD1+, HLA-DR+). The authors suggested that the conjunctival process in cicatricial pemphigoid may develop as a consequence of a primary autoimmune reaction with autoantibodies directed against a BMZ component.²² Bernauer *et al*¹⁰ found that the composition of the subepithelial cellular infiltrate varied with the disease activity. Acute disease was characterised by an abundance of macrophages and neutrophils, while the number of T cells was raised in all the disease groups, which is confirmed by our findings. Mast cell participation in ocular cicatricial pemphigoid has been disclosed by Hoang *et al*.²³ The total mast cell numbers observed in acute and chronic ocular pemphigoid did not differ significantly, and this corresponds with our observations.

Mast cells, T cells, macrophages, eosinophils, and fibroblasts apparently interact intimately in the cicatrising conjunctival process. But eosinophils have not been studied in detail in previous studies on OCP conjunctiva. However, complement fixation to tissue bound autoantibodies may lead to infiltration and

degranulation of various effector cells, including eosinophils, resulting in tissue injury and clinical inflammation.

After differentiation from a bone marrow derived stem cell, eosinophils migrate into the tissue. Eosinophil degranulation is especially activated by complement factors or immunoglobulins bound to surfaces,²⁴⁻²⁶ features typical of ocular cicatricial pemphigoid. Upon degranulation of the eosinophil, there is a local release of the granular proteins into the surrounding tissue.²⁷ The eosinophil specific granules contain enzymes (EPO) and non-enzymatic basophilic proteins (ECP, MBP, EPX/EDN). The eosinophil granule proteins have multiple properties. Besides defence against infections,²⁸⁻²⁹ eosinophil granular proteins are toxic to tumour cells and to many mammalian cells²⁴⁻³⁰⁻³¹ including corneal epithelial cells.³² They cause histamine release from basophils and mast cells, and they stimulate neutrophils.

Several findings have linked eosinophils to atopic dermatitis. Major basic protein has been demonstrated in eczematous skin,³³ and eosinophil cationic protein serum levels are increased in patients with severe atopic dermatitis.³⁴ Whereas eosinophils can be easily detected histologically in tissue by their characteristic granules, they are undetectable by classic staining after degranulation. It is interesting to note that eosinophils have not been found histologically in the cutaneous lesions from patients with chronic atopic dermatitis. However, significant accumulation of MBP and EDN/EPX has been detected by immunohistochemical techniques, supporting the concept of degranulation of eosinophils in the inflammatory lesions.³³⁻³⁵ Typically, within 2-8 hours after topical provocation with allergens, significant immigration with eosinophils is detected in the skin; mononuclear infiltration dominates after 24 hours, when most eosinophils have already degranulated and are histologically undetectable.³⁶ Deposition of granule proteins in the tissue is related to the disease activity in atopic dermatitis. Similar observations have been made in bronchial asthma. While eosinophils are not detected in the inflamed tissue, their granular contents have been demonstrated by electron microscopy and immunohistochemistry.³⁷ The secretion of granule proteins from blood eosinophils is increased in asthmatics,³⁸ significantly parallels disease activity, and returns to normal with immunotherapy.³⁹ Taken together, these findings provide evidence that eosinophils may play an important role in the propagation of inflammatory reactions including, perhaps, ocular cicatricial pemphigoid, as the findings in this report suggest.

Significant eosinophil invasion of the skin has been demonstrated in bullous pemphigoid and pemphigus vulgaris.⁴⁰⁻⁴¹ It has been suggested that activated eosinophils, releasing their granular products may be of importance for blister formation in bullous pemphigoid.¹⁵ The release of proteolytic enzymes of eosinophils to the lamina lucida in bullous pemphigoid, found by electron microscopy and immunohistochemistry,⁴² is believed to play a pathogenic role

during the initial stages of blister formation. We speculate that the contribution of eosinophils to the tissue damage in ocular cicatricial pemphigoid is greater than expected from previous immunopathological studies. Eosinophils are scanty in the conjunctival specimens by light microscopy.¹⁻¹⁶ Yet, our studies clearly demonstrated that significant amounts of eosinophil granule proteins are released into the highly inflamed conjunctival tissue in patients with subacute ocular cicatricial pemphigoid. It may be postulated that eosinophils migrate into the conjunctiva in the very early stage of OCP conjunctival lesions. Eosinophils, by their proinflammatory activity, may contribute to the amplification of mononuclear infiltration, seen in this and many previous studies. Eosinophils and eosinophil granule proteins, by their toxic effects to the epithelial cells, may eventually lead to the epitheliopathy³² developing in patients with ocular cicatricial pemphigoid. However, epithelial lesions were not found in any of the patients studied here.

An association between eosinophils and fibrosis is supported by numerous observations; by the elevated levels of MBP detected in patients with systemic sclerosis, by eosinophilia found with diffuse fasciitis, by the relation between eosinophilia or eosinophil degranulation with fibrosis detected by clinicopathological studies, and by the expression of transforming growth factors by eosinophils found during wound healing.⁴³⁻⁴⁷ Eosinophils and eosinophil granule proteins may also be involved in the cicatrizing process in OCP; however, eosinophil granular contents were not found in the conjunctival specimens from our patients with chronic disease.

Since our studies suggested that eosinophils may be involved in the early stages of the inflammatory cascades in ocular cicatricial pemphigoid, anti-inflammatory medication effective against these cells might exert a beneficial effect if employed early in the course of the disease. The activation and effector functions of eosinophils can be impaired by cyclophosphamide and corticosteroids. Our findings thus provide a rationale and support for the empirical observations of the superiority of broad spectrum, rather than cell specific immunosuppressive drugs, such as cyclosporin A. This is in agreement with previous therapeutic suggestions by others.^{6 10 48 49}

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