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Cytokines in the conjunctiva of acute and chronic mucous membrane pemphigoid: an immunohistochemical analysis

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Abstract. The aim of this study was to evaluate the potential role of certain soluble factors in conjunctival scar tissue formation of pemphigoid patients. Epibulbar conjunctival biopsy specimens were taken from patients with acute ulcerative ($n=4$), subacute ($n=8$) and chronic ($n=8$) mucous membrane pemphigoid and from twelve age-matched healthy individuals. The tissues were embedded in glycol methacrylate and analysed by immunohistochemical methods. Interleukin-2 (IL-2), interferon-γ, transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), tumour necrosis factor-α and proliferating cells (as identified with the antibody Ki-67) were found in both pemphigoid patients and normal controls. Interleukin-4 was not found with this method in either normal or diseased conjunctiva. Significant differences between normal and diseased conjunctiva were found for TGF-β and for proliferating cells, which were both increased in the acute disease group. More intense staining was found in the subacute disease group for IL-2, bFGF and PDGF. Our findings showed that a variety of cytokines were present in normal and diseased bulbar conjunctiva. Acute conjunctival disease in mucous membrane pemphigoid may indicate active scar tissue formation, implied by an increase in TGF-β and the presence of proliferating fibroblasts.

Introduction

Many conjunctival disorders can be associated with cicatricial changes [53]. Most of these conditions may be characterized by an acute phase of tissue injury with subsequent scar tissue formation. Alternatively, a chronic progressive course can be found in mucous membrane pemphigoid and other anti-basement membrane antibody-mediated conjunctivitides. Sequelae of chronic progressive conjunctival cicatrization include obstruction of lacrimal and meibomian glands, tear film alteration, trichiasis, keratopathy and blindness [14, 15, 34, 53].

The demonstration of antibody and/or complement deposition at the epithelial basement membrane of affected conjunctiva is regarded as diagnostic in mucous membrane pemphigoid [15]. It can be found in up to 67% of the patients [15]. However, similar findings have been reported in linear immunoglobulin A (IgA) disease [31] and drug-induced pemphigoid [29, 32, 40]. These conditions can, therefore, be indistinguishable from mucous membrane pemphigoid [33].

The mechanisms involved in chronic progressive conjunctival cicatrization are not well understood, and therapy for this condition is often of limited usefulness. Recent immunohistochemical studies analysing the cellular infiltrate in pemphigoid patients have shown a predominance of T cells in subacute and chronic disease [4, 42, 45] and a marked increase in the number of neutrophils and macrophages in acute ulcerative disease [4]. Other chronic conjunctival disorders show characteristics in the composition of the cellular infiltrate [16, 24, 26] that resemble those in patients with chronic and subacute ocular pemphigoid. This suggests that it is not primarily the composition of the cellular infiltrate, but rather differences in the secretory activity of these cells, which determine the clinical characteristics of these diseases.

The aim of this study was to assess the role of certain soluble factors and study the proliferative cellular response in the bulbar conjunctiva of patients with mucous membrane pemphigoid. The presence or absence of interleukin-2 (IL-2), interleukin-4 (IL-4) and interferon-γ (IFN-γ) may determine the function of infiltrating T cells [25, 36]. Investigation of the fibrogenic cytokines, transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), basic fibroblast growth factor

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(bFGF) and tumour necrosis factor-α (TNF-α) may provide evidence as to whether these factors play the same critical role in conjunctival scar tissue formation as in other fibrosing conditions [27, 28, 52]. The cellular response to growth factors can be assessed by studying actively proliferating cells. The commercially available monoclonal antibody to the proliferation-associated antigen Ki-67 discriminates between actively cycling cells and quiescent (G_0) cells [8, 18, 19]. Previous reports [4] have shown that the composition of the subepithelial infiltrate of mucous membrane pemphigoid varies with disease activity, and there is evidence that rapid progression of cicatrization occurs during or after manifest inflammation [35]. Patients with acute ulcerative or subacute disease might therefore be expected to show features relating more closely to cicatrization than patients with less active inflammation. The study of these groups may help to elucidate mechanisms relating to disease activity and consequently identify potential therapeutic strategies.

Patients and methods

Patients

Epibulbar conjunctival biopsies were taken from 20 patients with ocular cicatricial pemphigoid. The patient population consisted of 7 men and 13 women, of whom 19 were white and one was black. They ranged in age from 48 to 87 years (mean 73 years). All patients underwent a thorough clinical evaluation, which included documentation of disease course and previous therapy, staging of disease activity and documentation of ocular changes, immunofluorescent investigation for immunoglobulin and complement deposition at the conjunctival basement membrane and an assessment of extraocular mucous membrane involvement. The patients were classified according to the ocular disease activity as having acute ulcerative ($n=4$), subacute ($n=8$) or chronic ($n=8$) pemphigoid. Acute pemphigoid was clinically defined as conjunctival ulceration with severe hyperaemia, conjunctival swelling and subconjunctival fibrosis of less than 12 months duration prior to the biopsy. Subacute pemphigoid was defined as moderate to severe conjunctival inflammation in the absence of conjunctival ulceration and after control of secondary causes of inflammation such as trichiasis, blepharitis and dry eye. Patients in the chronic disease group had minimal signs of active conjunctival inflammation. All patients showed symblepharon formation with at least 25–50% forniceal shrinkage and fell clinically into stage 3 according to Foster [15] and into stage 2 according to Mondino [34]. The extraocular involvement was assessed by a dermatologist (J.N.L.). Twelve of the 20 patients had extraocular mucous membrane involvement and one had additional skin involvement. The diagnosis of mucous membrane pemphigoid was confirmed in 16 of 20 patients by the presence of immunoglobulin and/or complement in the ocular or extraocular mucous membranes. Twelve patients were untreated when the biopsy was taken, seven were on topical steroids, one was on systemic steroids and two were on sulfones; no patient was on systemic cytotoxic therapy.

Conjunctival biopsy specimens were also obtained from twelve age-matched controls (four men and eight women) with no known ocular inflammatory disease who were undergoing cataract surgery.

Biopsy specimens

The biopsy specimens were obtained after topical instillation of amethocaine 1% and subconjunctival injection of 2% lignocaine. Specimens of 3 × 3 mm were taken from bulbar conjunctiva close to the limbus. Each specimen was bisected for immunohistochemical investigation and immunofluorescent analysis.

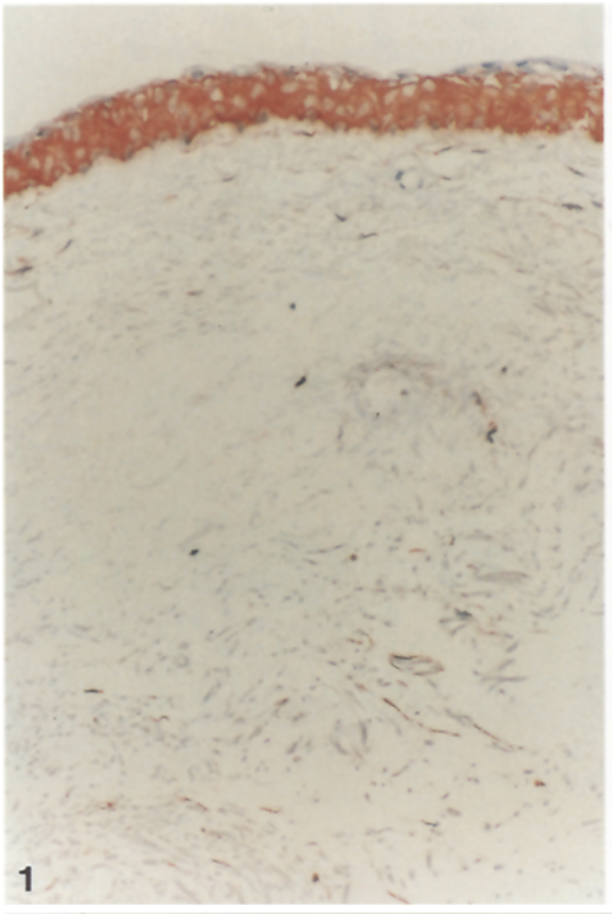
Specimens for immunohistochemical investigation were immediately fixed in acetone plus inhibitors (2mM phenylmethylsulfonylfluoride and 20 mM iodoacetamide) and stored overnight at −20° C. They were then post-fixed for 15 min in acetone and dehydrated for the same time using methylbenzoate. The specimens were then placed for 6 h in glycol methacrylate (JB-4 embedding kit solution A, Polysciences SA, Warrington, UK) plus 5% methylbenzoate and subsequently embedded in the resin (glycol methacrylate, JB-4 embedding kit, solution A; JB-4 embedding kit, component B; benzoyl peroxide, JB-4 embedding kit, component C). The blocks were stored at −20° C.

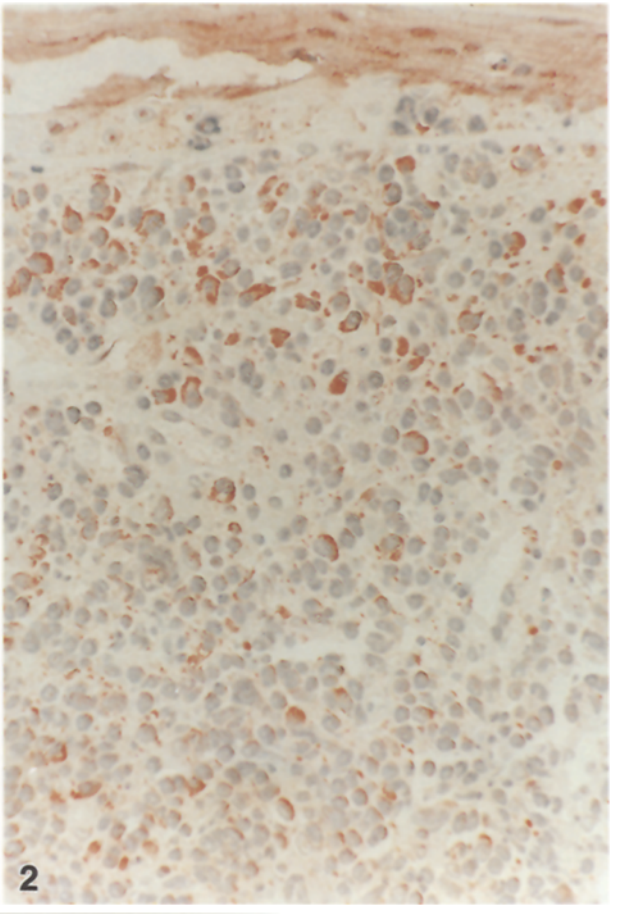
Serial sections 1.5 μm thick were cut using an ultramicrotome (Reichert-Jung Ultracut E, Leica UK Ltd, Milton Keynes, UK), mounted on APES-coated slides (APES = 3-Amino propyl triethoxy silane, Sigma Ltd.) and air dried. The endogenous peroxidase was inhibited for 30 min using a solution of 0.1% sodium azide and 0.3% hydrogen peroxide. The slides were washed twice in Tris-buffered saline (TBS) and incubated for 30 min with culture medium (20% fetal calf serum, 1% bovine albumin), after which 100 μl of appropriately diluted anti-human antibody was placed on each tissue section. These were then incubated in a moist chamber for 18–20 h. The antibodies used are listed in Table 1. The production and characterization of these antibodies have been described elsewhere [7, 11, 17]. After the primary incubation, the slides were washed

Table 1. Antibody specificities, working solutions and suppliers

Antibody and reference(s)	Dilution	Supplier
Antibodies to cytokines		
Anti-IL-2 [17]	1/100	Genzyme Diagnostics
Anti-IL-4 [17]	1/50	Genzyme Diagnostics
Anti-IFN-γ [17]	1/50	Genzyme Diagnostics
Anti-PDGF [33, 51]	1/1000	British Bio-technology Ltd
Anti-bFGF [22, 51]	1/2000	British Bio-technology Ltd
Anti-TGF-β [51]	1/50	British Bio-technology Ltd
Anti-TNF-α [17]	1/3000	Genzyme Diagnostics
Antibody to assess cell proliferation		
Ki-67 [8, 18, 19, 23]	1/25	DAKO Ltd

See reference list for suppliers' addresses
IL-2, Interleukin-2; IL-4, interleukin-4; IFN-γ, interferon-γ; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; TGF-β, transforming growth factor-β; TNF-α, tumour necrosis factor-α





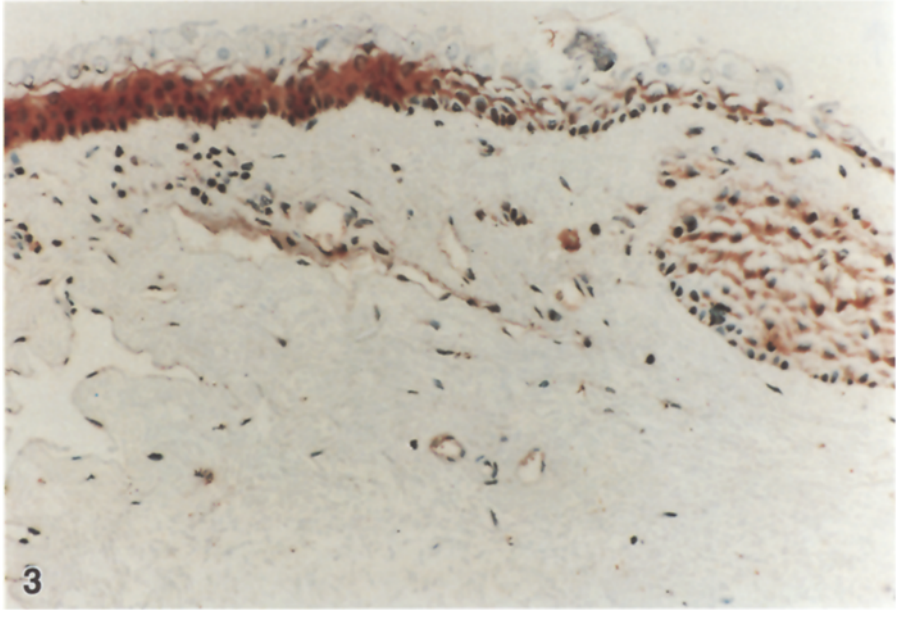


Fig. 1. Normal conjunctiva, staining for interleukin-2 (IL–2). Note the positive staining in the basal layer of the epithelium. (anti-IL–2 antibody, immunoperoxidase reaction, magnification × 188)

Fig. 2. Subacute ocular pemphigoid, staining for IL–2. Abundance of lymphocytes in the subepithelial substantia propria, extracellular and cellular staining for IL–2. Note the squamous metaplasia of the epithelium. (anti-IL–2 antibody immunoperoxidase reaction, magnification × 375)

Fig. 3. Normal conjunctiva, staining for platelet-derived growth factor (PDGF). Positive staining is mainly found in the basal layer of the epithelium (Anti-PDGF-antibody, immunoperoxidase reaction, magnification × 188)

three times in TBS, after which 100 µl of biotinylated rabbit anti mouse immunoglobulins (1:200) (DAKO Ltd, High Wycombe, Bucks, UK) were placed on the section which was incubated for two hours. The slides were washed three times with TBS, 100 µl of avidin-biotin complex (DAKO Ltd) was placed on each section, and the specimens were incubated for 2 h. They were again washed three times in TBS and developed for 20 min in peroxide substrate solution containing 3-amino-9-ethylcarbazole, dimethylformamide and hydrogen peroxide in 0.1–M acetate buffer. The specimens were rinsed in tap water, counterstained with Meyer's haematoxylin and mounted with Glycergel mounting medium (DAKO Ltd). Sections without the primary antibodies (negative controls) and tissue sections of lymphoid tissue (positive controls) served as experimental controls.The immunostaining technique for glycol methacrylate-embedded tissues has been published elsewhere [6].

Table 2. Staining intensity in the conjunctival epithelium. + + +, Very intense staining; + +, intense staining; +, slight staining; -ve, staining absent

	Normals ($n=12$)	Acute CP ($n=4$)	Subacute CP ($n=8$)	Chronic CP ($n=8$)
Anti-IL-2	+ +[a]	+ +[a]	+ +[a]	+ +[a]
Anti-IL-4	-ve	-ve	-ve	-ve
Anti-IFN-γ	-ve or +[a]	-ve to + +[a]	-ve to + +[a]	-ve to + +[a]
Anti-PDGF	+	+ +	+ +	+ +
Anti-bFGF	+ +	+ +	+ +	+ +
Anti-TGF-β	-ve	-ve	-ve	-ve
Anti-TNF-α	+ + +	+ + +	+ + +	+ + +
Antibody to assess proliferation				
Anti-Ki-67	+[a]	+[a]	+[a]	+[a]

CP, Cicatricial pemphigoid
[a] Staining of the basal cells

Table 3. Staining intensity in the extracellular substantia propria. + + +, Very intense staining; + +, intense staining; +, slight staining; -ve, staining absent

	Normals ($n=12$)	Acute CP ($n=4$)	Subacute CP ($n=8$)	Chronic CP ($n=8$)
Anti-IL-2	-ve to +	+	+	(+)
Anti-IL-4	-ve	-ve	-ve	-ve
Anti-IFN-γ	-ve to (+)	-ve to + +	-ve to +	-ve to +
Anti-PDGF	-ve to +	+ +	+ +	+
Anti-bFGF	-ve to +	+	+ +	+
Anti-TGF-β	-ve	-ve or +	-ve or +	-ve
Anti-TNF-α	+ + +	+ + +	+ + +	+ + +
Antibody to assess proliferation				
Anti-Ki-67	–	–	–	–

Analysis of staining intensity

The positive red reaction in the epithelium and intercellular space was analysed in ten representative high-power fields (× 400) from pemphigoid and normal specimens. It was classified as very intense (+ + +), intense (+ +), slight (+) or absent (–). Where possible (TGF-β and proliferating cells) cell staining in the subepithelial substantia propria was quantified by cell counts in ten high-power fields (× 400). In cases of intense (diffuse) intercellular staining where it was not possible to attribute the positive red reaction to the cell surface, the quantification was done as for epithelial and intercellular staining. A 10 × 10 mm grid was used with an Olympus BH-2 microscope for each assessment, and all quantifications were performed masked. Where applicable, means and standard errors of the mean were calculated. Nonparametric statistics (Mann-Whitney *U*-test) were used to analyse differences between the means. These analyses were carried out using Stat View 512 software (Brain Power, Calabasas, Calif., USA) on a Macintosh microcomputer.

Results

Staining for IL-2, IFN-γ, TGF-β, PDGF, bFGF, TNF-α and proliferating cells as identified with the antibody Ki-67 was found in specimens from both pemphigoid patients and normal controls. Staining for IL-4 was absent in the majority of specimens, but there was a slight (cross-?) reactivity with neutrophils when these were present. The results are shown in detail in Tables 2–4.

Epithelium

The epithelium of both pemphigoid and control specimens showed a consistently positive reaction for IL-2 and IFN-γ. Staining intensity was greatest in the basal layer and showed no differences between normal and diseased conjunctiva (Figs. 1, 2). Staining for IL-4 was absent, with the exception of neutrophilic granulocytes in some specimens.

There was diffuse and localized epithelial staining for PDGF (Fig. 3) and bFGF in most cases of pemphigoid and in most normal controls. Epithelial staining for TGF-β (Figs. 4, 5) was absent in all cases, but intense staining for TNF-α was found in all the specimens.

Proliferating cells were found in the basal cell layer of the epithelium in most cases of pemphigoid and most normal controls (Fig. 6).

Fig. 4. Normal conjunctiva, staining for transforming growth factor-β (TGF-β). Staining is absent in normal conjunctiva. (Anti-TGF-β antibody, immunoperoxidase reaction, original magnification × 188)

Fig. 5. Acute pemphigoid, staining for TGF-β. Note the slight intercellular staining and the positive reaction of macrophages and plasma cell-like cells (Anti-TGF-β antibody, immunoperoxidase reaction, original magnification × 235)

Fig. 6. Normal conjunctiva, staining for proliferating cells. Note the positivity of the basal cell layer in the epithelium (Anti-Ki-67 antibody, immunoperoxidase reaction, original magnification × 375)

Fig. 7. Acute pemphigoid. Cluster of fibroblasts staining positively for proliferating cells. (anti-Ki-67 antibody, immunoperoxidase reaction, original magnification × 938)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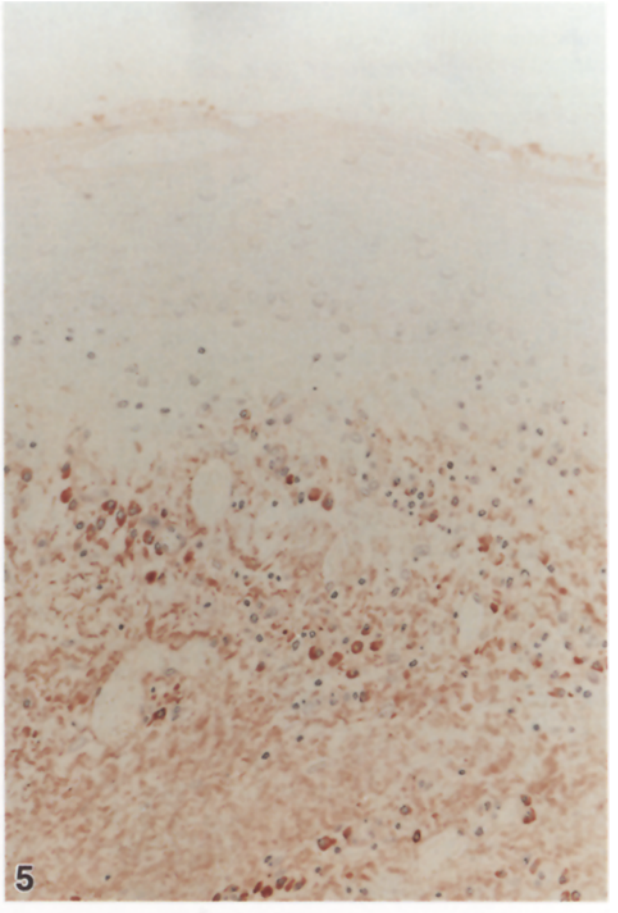
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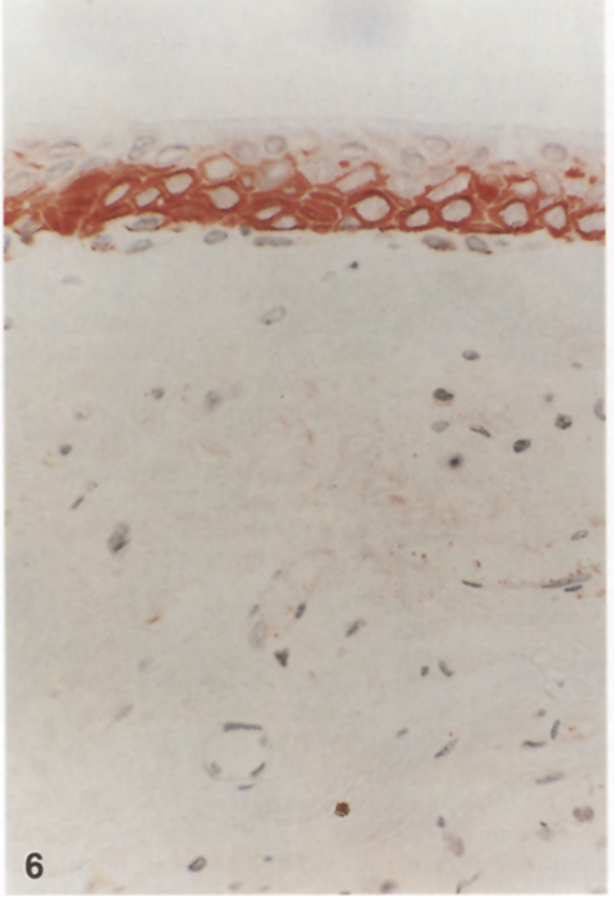
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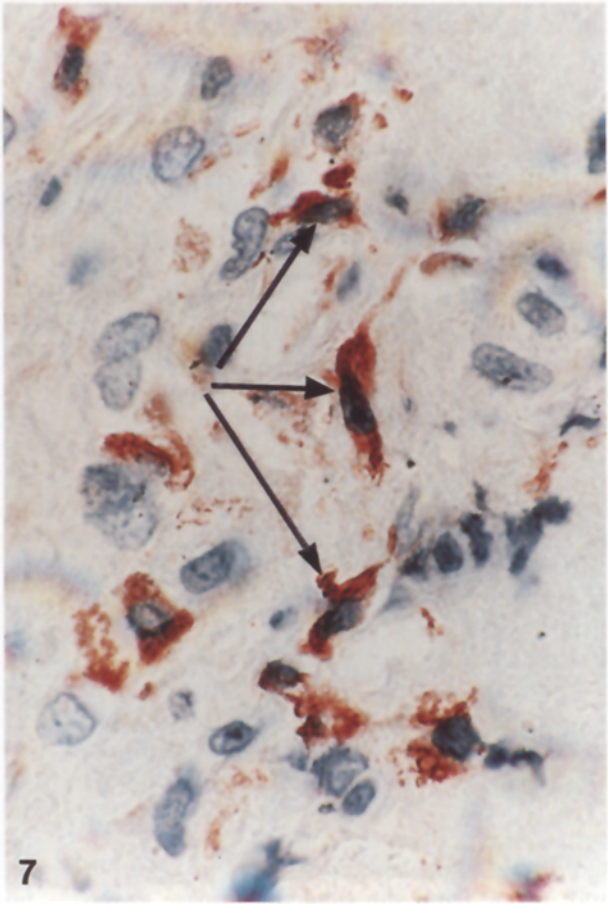
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Table 4. Cellular staining in the subepithelial substantia propria. Where applicable mean cell counts/mm² with standard error of the mean, otherwise semiquantitative assessments. + + +, Very intense staining, + +, intense staining; +, slight staining; -ve, staining absent

	Normals ($n=12$)	Acute CP ($n=4$)	Subacute CP ($n=8$)	Chronic CP ($n=8$)
Anti-IL-2	-ve to +	+	+ +	+
Anti-IL-4	-ve	-ve	-ve	-ve
Anti-IFN-γ	-ve to (+)	+ to + +	-ve to +	-ve to +
Anti-PDGF	+	+	+ +	+
Anti-bFGF	-ve to +	+	+ +	+
Anti-TGF-β	0.6 (0.4)	20.3 (14.2)*	5.1 (5.1)	5.2 (2.3)
Anti-TNF-α	+ + +	+ + +	+ + +	+ + +
Antibody to assess proliferation				
Anti-Ki-67	0.5 (0.3)	26.5 (16.1)*	2.9 (2.0)	0.9 (0.7)

* $P<0.05$ by the Mann-Whitney U-test in comparison with normal controls

Staining in the extracellular substantia propria

Slight staining for IL-2 was seen mainly in the acute and subacute disease group (Fig. 2). IFN-γ was found in few specimens, and staining for IL-4 was consistently absent.

A positive reaction for PDGF and bFGF was present in diseased and normal conjunctiva, but the staining for PDGF was clearly more intense in the acute and subacute disease groups than in the controls. The same applied to bFGF, but the differences were less obvious. Slight intercellular staining for TGF-β was found in a few specimens of the acute and subacute disease group, but clear diffuse staining was present for TNF-α in all the specimens.

Cellular staining in the substantia propria

Positive staining for IL-2 was found mainly in the subacute disease group. Since there was also intercellular staining and the cells were unevenly stained, it was not always possible to attribute the positive red reaction to the cell surface. Cells staining positively for IL-2 were mainly lymphocytes (Fig. 2). A positive red reaction for IL-4 was consistently absent, with the exception of neutrophilic granulocytes in specimens from patients with acute or subacute disease. Staining for IFN-γ varied from negative or slightly positive reactions to intense staining present in one specimen from a patient with acute disease.

A positive reaction for PDGF and bFGF was found in all sections of normal and diseased conjunctiva. It was diffuse in the majority of sections and quantification was not possible, but the staining intensity seemed to correlate with the infiltrating cell number. Cells staining for transforming growth factor-β (TGF-β) were macrophages, fibroblasts and plasma cell-like cells. Strong positive extracellular and cellular staining was found in two sections from the acute and in one from the subacute disease group. Staining intensity for TNF-α was very high in both diseased conjunctiva and normal controls.

Proliferating cells in the substantia propria were only present in a few specimens. Cells staining for Ki-67 were some macrophage-like cells and fibroblasts. Clusters of proliferating fibroblasts were found in two sections from the acute and in one from the subacute disease group (Fig. 7). In one section they were situated in the area of the basement membrane.

Discussion

Previous immunohistochemical studies of the cellular infiltrate in normal and diseased conjunctiva [4, 16, 24, 26, 42, 44, 45] have suggested that it is not primarily the cellular composition, but rather differences in the secretory pattern of these cells, which determine the clinical characteristics of conjunctival disease. The present study analyses the potential role of certain cytokines in conjunctival scar tissue formation of pemphigoid patients. Although the immunostaining technique for glycol methacrylate-embedded tissue allows use of thin sections with good morphology, quantification of soluble factors remains difficult, as shown in this study. Nevertheless, it has been possible to suggest some possible mechanisms of conjunctival disease based on our results.

In this study IL-2 and IFN-γ were found in both normal and diseased conjunctiva. The main source for these cytokines are T cells. T cells are abundant in the conjunctiva of pemphigoid patients [4, 42, 45]. IL-2 is believed to be essential for the activation of T cells, and IFN-γ is known to induce expression of MHC class II molecules [20, 48]. A high number of MHC class II expressing cells are present in the conjunctiva of pemphigoid patients [4, 42, 45], indicating the potential to present local antigen to the CD4-positive cells. Recent investigations of the secretory pattern of CD4 cells in the mouse defined two CD4 subsets, Th1 and Th2 [37]. Th1 cells are characterised by IL-2 and IFN-γ secretion, whereas Th_2-cells secrete IL-4 [25, 36, 37]. The positive staining for IL-2, the absence of IL-4 and the finding of IL-2-expressing cells reported in previous studies of the conjunctiva of pemphigoid patients [4, 42, 45] may indicate that the T cells in the bulbar conjunctiva of pemphigoid patients are mainly of the Th1 subset. Th2 cells are believed to be important in allergic diseases [5, 25], and IL-4, also known as B-cell stimulatory factor-1, is involved in B cell activation [21]. The absence of IL-4 in our study is consistent with previous investigations [4, 42, 45] which showed no increase in the number of B cells of biopsy specimens. This suggests extraocular activation and maturation of B cells, with subsequent homing to target mucous membranes as "end-stage" B cells (i.e.

plasma cells) [15]. The role of T cells in the formation of scar tissue is not completely understood. It seems that they are not essential for scar tissue formation, as shown in experiments of T cell depletion [1] and in studies with nude rats, which have a profoundly impaired T cell system [2]. The role of T cells appears to be mainly in regulating the repair process [41].

Growths factors are regarded as crucial in fibrosing diseases and in the formation of scar tissue in wound healing [10, 27]. An important source of these fibrogenic cytokines are macrophages [10, 27], which are regarded as crucial for the promotion of inflammation to subsequent new tissue formation [10, 49]. Macrophages were shown to be increased in number mainly in acute and subacute conjunctival pemphigoid disease [4]. TGF-β was clearly shown in our study to be increased in acute disease. TGF-β is currently believed to be the most important stimulus for extracellular matrix production [50] and is able to stimulate autocrine PDGF secretion, thus also acting by indirect mechanisms [3]. Numerical quantification of the other growth factors was not possible, but staining intensity for PDGF and bFGF seemed to be correlated to the infiltrating cell number, which was highest in subacute disease [4]. PDGF stimulates fibroblast collagen deposition and biosynthesis and has chemotactic activities [12, 38, 39]. Many effects of bFGF have been reported, including fibroblast proliferation and angiogenesis [10, 47]. TNF-α seems to be abundant in normal and in diseased conjunctiva. This growth factor is also regarded as important in tissue repair mainly for its capacity of angiogenesis [30].

The cellular response to growth factors can be assessed by the number of actively proliferating cells [19]. The antibody Ki-67 preferentially labels *nuclei* of proliferating cells when cryostat sections are used. However, with mucous membranes the antibody can react with the cytoplasm, and this may depend on the amount of cellular damage caused by tissue fixation [9, 11, 46]. In this study proliferating cells were also identified by staining of the cytoplasm. Proliferating cells in the subepithelial substantia propria, however, were only rarely found and were only significantly increased in acute disease where there were clusters of proliferating fibroblasts. Acute conjunctival disease may thus represent active scar tissue formation. This confirms the clinical observation that rapid progression of cicatrization occurs during or after acute manifestations of conjunctival inflammation in pemphigoid patients [35]. The fact that we found a high number of proliferating fibroblasts in only three specimens may be due to the site of biopsy. Since biopsies in the forniceal and tarsal conjunctiva are contraindicated because of the possibility of promoting the disease process [13, 15, 53], our analyses were carried out in bulbar conjunctiva. Early signs of conjunctival involvement in cicatricial pemphigoid, however, are subepithelial fibrosis in the medial canthal region and tarsus with subsequent shrinkage of the fornices [15]. We therefore accept that, because of this, important findings may have been missed in this study.

Hyperproliferation of fibroblasts occurs at the end of a chain of events [43]. In this study we were able to elucidate some of the possible links in this chain. Further investigations are required to define the relative importance of each of the fibrogenic growth factors, and these may lead to new therapeutic regimens involving agents which block specific cytokines or their receptors.

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