Eye (2006) 20, 71–79

www.nature.com/eye

CLINICAL STUDY

Immunopathogenesis of conjunctival remodelling in vernal keratoconjunctivitis

AM Abu El-Asrar[1], S Al-Mansouri[1], KF Tabbara[1], L Missotten[2] and K Geboes[3]

Abstract

Purpose **To study the processes involved in mediating conjunctival remodelling in vernal keratoconjunctivitis (VKC) by investigating the expression of integrin receptors, epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and Ki67 antigen, which is a marker for cell proliferation.**

Methods **Conjunctival biopsy specimens from 16 patients with active VKC and nine control subjects were studied by immunohistochemical techniques using monoclonal and polyclonal antibodies directed against the integrin α3 and α6 subunits, EGFR, VEGF, TGF-β, bFGF, PDGF, and Ki67 antigen. The phenotype of inflammatory cells expressing growth factors was examined by double immunohistochemistry.**

Results **In the normal conjunctiva, very weak immunoreactivity was observed for EGFR and VEGF in epithelial cells, and for α3 and α6 integrin subunits on basal epithelial cells, and on vascular endothelial cells in the upper substantia propria. There was no immunoreactivity for the other antibodies. In VKC specimens, strong staining for α3 and α6 integrin subunits was observed on the membranes of basal and suprabasal epithelial cells, and all vascular endothelial cells. Immunoreactivity for Ki67 antigen was observed in the nuclei of the basal and suprabasal epithelial cells. Strong immunoreactivity was observed for EGFR in the deeper layers of the epithelium, and for VEGF in all epithelial cells. Inflammatory cells expressing EGFR, VEGF, TGF-β, bFGF, and PDGF were noted in 8, 9, 11, 10, and 10 specimens, respectively. The majority of inflammatory cells expressing growth factors were eosinophils (45±4%) and monocytes/macrophages (35±4%).**

Conclusions **Chronic conjunctival inflammation in VKC is associated with increased staining of α3, and α6 integrin subunits, EGFR, VEGF, TGF-β, bFGF, and PDGF that might mediate conjunctival remodelling.**

Eye (2006) **20,** 71–79. doi:10.1038/sj.eye.6701811; published online 4 March 2005

Keywords: allergy; conjunctiva; growth factors; integrins; Ki67 antigen

Introduction

Vernal keratoconjunctivitis (VKC) is a chronic, seasonally exacerbated bilateral external allergic ocular inflammation associated with remodelling of the conjunctiva. Characteristic features of conjunctival remodelling in VKC include hyperplasia of the epithelium with numerous epithelial ingrowths, and extensive deposition of extracellular matrix components, including types I, III, IV, V, and VII collagen, tenascin, and laminin in the substantia propria.[1–4] In addition, angiogenesis, the growth and proliferation of new blood vessels, is one of the histological hallmarks of tissue remodelling of the allergic inflammation.[5]

The mechanisms by which chronic conjunctival inflammation in VKC promotes conjunctival remodelling have not been clarified yet. Potential candidates are epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF, also known as vascular

[1]Department of Ophthalmology, College of Medicine, King Saud University, Riyadh, Saudi Arabia

[2]Department of Ophthalmology, University of Leuven, Belgium

[3]Laboratory of Histochemistry and Cytochemistry, University of Leuven, Belgium

Correspondence: Professor AM Abu El-Asrar, Department of Ophthalmology, King Abdulaziz University Hospital, Airport Road, PO Box 245, Riyadh 11411, Saudi Arabia
Fax: 966 1 4775724/4775741.
E-mail: abuasrar@KSU.edu.sa

Received: 10 November 2004
Accepted: 12 November 2004
Published online: 4 March 2005

permeability factor), transforming growth factor-β (TGF-β), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF). EGFR, a member of the receptor tyrosine kinase family, plays a role in epithelial cell migration, proliferation and differentiation, and enhanced survival.[6,7] VEGF plays a central role in the process of angiogenesis and increases vascular permeability so that plasma proteins can leak into the extravascular space, which leads to oedema and profound alterations in the extracellular matrix.[8–10] TGF-β is able to stimulate fibroblast proliferation and increase the synthesis by fibroblasts of many components of the extracellular matrix.[11] bFGF is a potent mitogen for many cell types including fibroblasts, and endothelial cells.[12] PDGF is a potent mitogen for fibroblasts.[13]

Integrins are a family of heterodimeric cell surface receptors, which consist of α and β subunits. They mediate interactions of cells with different components of the extracellular matrix, and cell–cell interactions.[14] Integrin $\alpha3\beta1$ interacts with laminin, fibronectin, and collagen, and integrin $\alpha6\beta1$ is a receptor for laminin.[14] Integrin $\alpha6\beta4$, which is expressed primarily on the basal surface of most epithelia, is defined as an adhesion receptor for most of the known basement membrane laminins. A primary function of $\alpha6\beta4$ integrin is to maintain the integrity of epithelia. This critical role for $\alpha6\beta4$ derives from its ability to mediate the formation of stable and rigid adhesive structures known as hemidesmosomes on the basal cell surface that link the cytokeratin filament network with laminins in the basement membrane.[15] Several studies have demonstrated the involvement of $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha6\beta4$ integrins in epithelial wound healing and in the migration of epithelial cells and epithelial-derived carcinoma cells.[16–20] In addition, several studies suggested that these integrins are essential participants in new vessel growth and remodelling.[21,22]

Recent studies have demonstrated that growth factors regulate the expression of $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha6\beta4$ integrins, modulate integrin-mediated cell adhesion and motility, and their receptors share proteins that mediate intracellular signalling with integrin receptors.[18–20,22–29] The crosstalk between these receptors is thought to play a relevant role in the migration of epithelial cells and epithelial-derived carcinoma cells.[28] Collectively, these data suggest that both growth factors and $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha6\beta4$ integrins affect tissue remodelling.

On the basis of this, we hypothesized that EGFR, VEGF, TGF-β, bFGF, and PDGF and the integrins $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha6\beta4$ might play a role in the pathophysiology of VKC, especially in conjunctival remodelling. To examine this hypothesis, we studied, with immunohistochemical techniques, their expression in conjunctival biopsy specimens from patients with active VKC, and from normal individuals. In addition, we examined the expression of Ki67 antigen, which is a marker for cell proliferation.[30]

Materials and methods

A total of 16 consecutive patients with active VKC presenting to the outpatient clinic of King Abdulaziz University Hospital were included in this study. The patients were 11 males, and five females, with a mean age of 14.5 ± 6.11 (range, 8–25 years). The symptoms in all patients included itching, redness, photophobia, and tearing. All patients had the limbal form of the disease characterized by broad gelatinous infiltrates of the limbus. A clinical score (0–4: 0 = absent; 4 = severe) was given considering the severity of the following eye symptoms and signs: itching, redness, photophobia, tearing, conjunctival erythema, conjunctival chemosis, discharge, limbal infiltrates, and corneal epithelial disease. All the patients had severely active VKC. A limbal conjunctival biopsy specimen was obtained from each patient. None of the patients was on topical or systemic therapy before the biopsy. In addition, nine limbal conjunctival biopsy specimens were obtained from patients undergoing strabismus surgery without obvious inflammation, who served as controls. The controls were from the same age group, and were five males, and four females. This study was approved by the Research Center, College of Medicine, King Saud University, the patients and controls were admitted to the study and their guardians gave informed consent.

Immunohistochemical staining

The conjunctival biopsy specimens were embedded in OCT (optimum cutting temperature compound, Tissue-Tek, Miles Laboratories, IN, USA), immediately snap frozen in liquid nitrogen and maintained at -80°C until use. For immunohistochemistry, 5 μm serially cut cryostat sections were fixed in absolute acetone for 10 min and then treated with 2% hydrogen peroxide in methanol for 3 min to block endogenous peroxidase activity. After rinsing three times in phosphate-buffered saline (PBS) at pH 7.2 for 15 min, the slides were incubated for 30 min with the monoclonal and polyclonal antibodies as listed in Table 1. Three sections were stained per antibody for each patient. Optimal conditions and concentrations of all antibodies used were determined in pilot experiments, including staining of cryostat sections immediately after being prepared or following drying overnight at room temperature. After a wash with PBS, the sections were incubated for 30 min with EnVision$^+$, peroxidase, Rabbit, or EnVision$^+$,

Table 1 Monoclonal and polyclonal antibodies used in this study

Primary antibody	*Dilution*	*Source*[a]
Anti-α3 subunit of $\alpha 3\beta 1$ integrin (CD49c) (mc)	1 : 20	Novocastra Laboratories Ltd.
Anti-α6 subunit of $\alpha 6\beta 1$ and $\alpha 6\beta 4$ integrins (CD49f) (mc)	1 : 20	Novocastra Laboratories Ltd.
Anti-Ki67 antigen (MIB-1) (mc)	1 : 10	DAKO
Anti-EGFR (EGFRI) (mc)	1 : 10	Amersham International PIC
Anti-VEGF (A-20) (pc)	1 : 100	Santa Cruz Biotechnology, Inc.
Anti-TGFβ1/2/3 (H-112) (pc)	1 : 100	Santa Cruz Biotechnology, Inc.
Anti-bFGF (147) (pc)	1 : 100	Santa Cruz Biotechnology, Inc.
Anti-PDGF (Ab-1) (pc)	1 : 10	Oncogene Research Products
Anti-eosinophil peroxidase (Ab-1) (mc)	1 : 100	Oncogene Research Products
CD68 (KP1) (mc)	1 : 1000	DAKO

[a]Location of manufacturers: Novocastra Laboratories Ltd, Newcastle, UK; DAKO, CA, USA; Amersham International PIC, Buckinghamshire, UK; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; Oncogene Research Products, Cambridge, MA, USA.
mc = monoclonal; pc = polyclonal; EGFR = epidermal growth factor receptor; VEGF = vascular endothelial growth factor; TGF-β = transforming growth factor-β; bFGF = basic fibroblast growth factor; PDGF = platelet-derived growth factor.

peroxidase, Mouse (DAKO, CA, USA). These are goat anti-rabbit or anti-mouse 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 visualization. The slides were washed again with PBS and the reaction product was visualized by incubation for 10 min in 0.05 M acetate buffer at pH 4.9, containing 0.05% 3-amino-9-ethylcarbazole (Sigma-Aldrich, Bornem, Belgium) 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, and staining with chromogen alone were used as negative controls. Normal oesophageal and skin biopsies as well as biopsies from oesophageal cancer and different forms of inflammatory bowel and skin diseases were used as positive controls.

Double Immunohistochemistry

To examine the phenotype of inflammatory cells expressing growth factors, cryostat sections were studied by sequential double immunohistochemistry. Colocalization studies were performed in VKC specimens from three patients. After rinsing the slides with PBS, they were incubated for 30 min with the first antibody and rinsed again with PBS. Subsequently, the sections were incubated for 30 min with EnVision$^+$, peroxidase, Mouse (DAKO, CA, USA) and washed again with PBS. The reaction product was visualized by incubation for 10 min in 0.05 M acetate buffer at 4.9, containing 3-amino-9-ethyl-carbazole 0.05% (Sigma-Aldrich, Bornem, Belgium) and hydrogen peroxide 0.01%, resulting in red immunoreactive staining. Subsequently, the sections were rinsed in acetate buffer, washed with tap water, rinsed in PBS and incubated for 30 min with the second antibody. After a wash with PBS, the sections were incubated for 30 min with EnVision$^+$, alkaline phosphatase, Rabbit (DAKO, CA, USA). The blue reaction product was developed using 5-bromo-4-chloro-3-indoxyl phosphate and nitro blue tetrazolium chloride (BCIP/NBT) (DAKO, CA, USA) for 30 min. No counterstain was applied.

Quantitation

Inflammatory mononuclear cells showing immunoreactivity were counted in five representative fields. Only cells containing a clearly identifiable nucleus were counted. Counting was performed by two independent observers (AMA and KG). One of them (KG) was unaware of the origin of the specimens. In case of disagreement, the results obtained by the blinded observer were used. We used an eyepiece calibrated grid with $\times 40$ magnification. With this magnification and calibration, we counted the cells present in an area of 0.33×0.22 mm. For the colocalization studies, inflammatory cells expressing both growth factors and eosinophil peroxidase, or CD68 were counted and expressed as a percentage of cells expressing growth factors.

Statistical analysis

All data are presented as mean $\pm$ SD. The data were analysed using the Kruskal–Wallis nonparametric test for one-way analysis of variance (ANOVA). Program 3 S from the BMDP Statistical Package was used to conduct

the ANOVA. Post-ANOVA pairwise comparisons were based on the Z-test.

Results

There was no staining in the negative control slides, and when the chromogen alone was applied.

Integrins: In normal conjunctiva, very weak immunoreactivity for the integrin α3 subunit was localized at the cell membranes of the basal epithelial cells, and on vascular endothelial cells in the upper substantia propria (Figure 1). Very weak membranous immunoreactivity for the integrin α6 subunit was observed at the basal aspect of the basal epithelial cells, and on vascular endothelial cells in the upper substantia propria (Figure 2).

In all VKC specimens, strong membranous immunoreactivity for the integrin α3 (Figure 3) and α6 (Figure 4) subunits was observed on the basal and suprabasal epithelial cells, except the most superficial cells. The staining pattern of the integrin α6 subunit at the basal aspect of the basal epithelial cells appeared as a thick linear irregular band. A marked upregulation of the integrin α3 and α6 subunits expression was noted on superficial and deep stromal vascular endothelial cells. Inflammatory cells expressing pronounced cytoplasmic integrin α3 and α6 subunits were noted in the substantia propria in only four specimens.

Ki67 antigen: MIB-1 staining was negative in the normal conjunctiva. All VKC specimens showed nuclear immunoreactivity for MIB-1 in the basal and suprabasal epithelial cells (Figure 5).

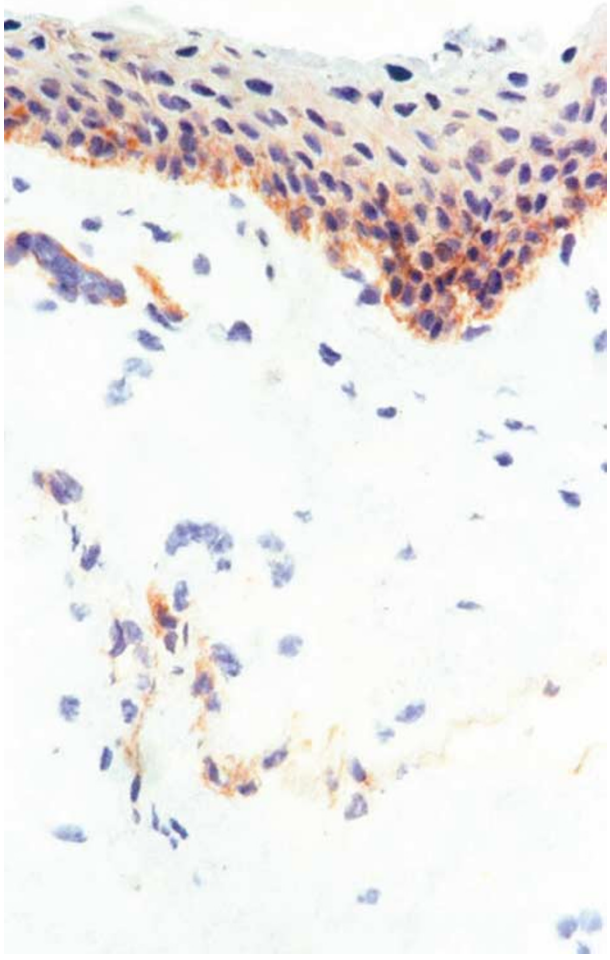

Figure 1 Immunohistochemical staining for the integrin α3 subunit of conjunctiva from a normal control subject showing weak immunoreactivity at the cell membranes of the basal epithelial cells, and on vascular endothelial cells in the upper substantia propria (original magnification × 40).

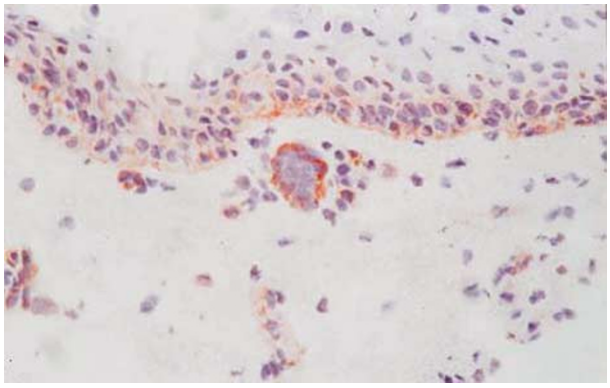

Figure 2 Immunohistochemical staining for the integrin α6 subunit of conjunctiva from a normal control subject showing weak immunoreactivity at the basal aspect of the basal epithelial cells, and on vascular endothelial cells in the upper substantia propria (original magnification × 40).

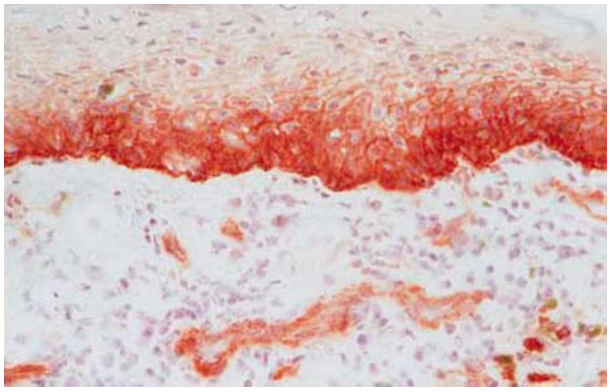

Figure 3 Vernal keratoconjunctivitis. Immunohistochemical staining for the integrin α3 subunit showing strong membranous immunoreactivity on the basal and suprabasal epithelial cells and on vascular endothelial cells (original magnification × 40).

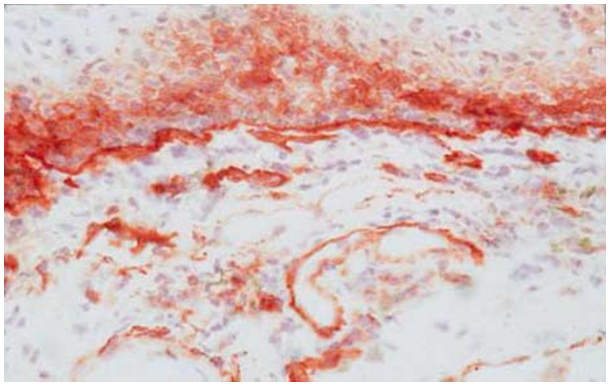

Figure 4 Vernal keratoconjunctivitis. Immunohistochemical staining for the integrin $\alpha 6$ subunit showing strong membranous immunoreactivity on the basal and suprabasal epithelial cells and on vascular endothelial cells (original magnification $\times 40$).

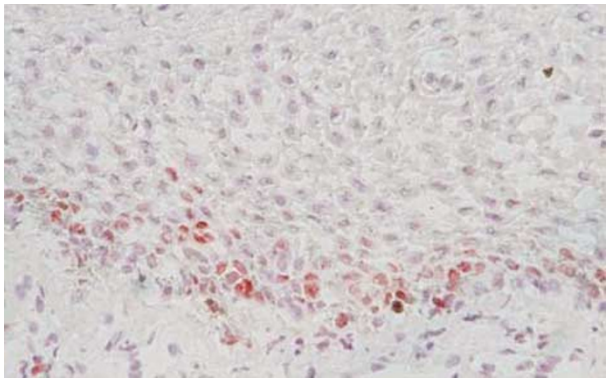

Figure 5 Vernal keratoconjunctivitis. Immunohistochemical staining for Ki67 antigen showing nuclear immunoreactivity in the basal and suprabasal epithelial cells (original magnification $\times 40$).

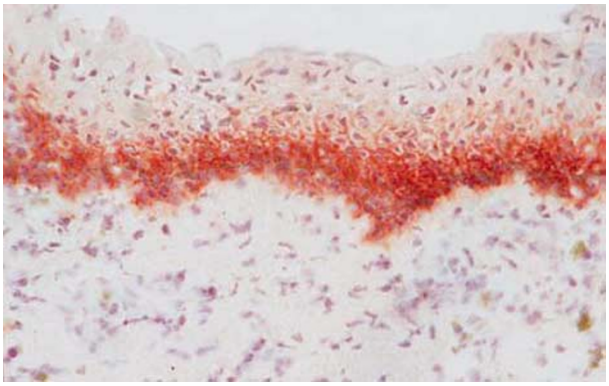

Figure 6 Vernal keratoconjunctivitis. Immunohistochemical staining for EGFR showing strong immunoreactivity in the deeper layers of epithelium (original magnification $\times 40$).

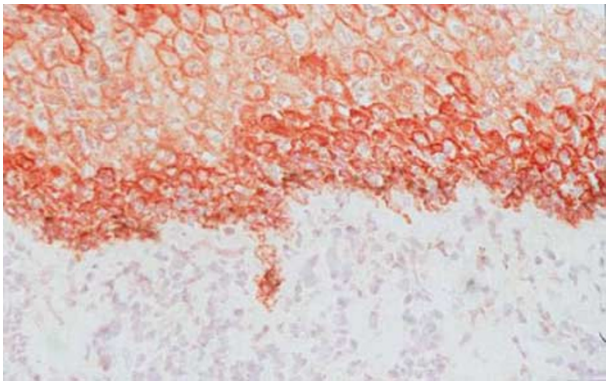

Figure 7 Vernal keratoconjunctivitis. Immunohistochemical staining for VEGF showing strong immunoreactivity in the epithelium (original magnification $\times 40$).

Growth factors: In normal conjunctiva, there was very weak cytoplasmic immunoreactivity for VEGF and EGFR in the epithelium. There was no immunoreactivity for TGF-β, bFGF, and PDGF.

All VKC specimens showed strong membranous and cytoplasmic immunoreactivity for EGFR (Figure 6) and VEGF (Figure 7) in the epithelium. Immunoreactivity for VEGF was observed in all epithelial layers and was more intense in the deeper layers, whereas immunoreactivity for EGFR was observed only in the deeper layers. Inflammatory cells expressing pronounced cytoplasmic VEGF, EGFR, TGF-β (Figure 8), bFGF, and PDGF were noted in the substantia propria. The immunohistochemical appearance of these inflammatory cells was similar for all the growth factors studied. $EGFR^+$ inflammatory cells were observed in eight specimens, $VEGF^+$ inflammatory cells were observed in nine specimens, TGF-β^+ inflammatory cells were observed in 11 specimens, $bFGF^+$ inflammatory cells were observed in 10 specimens, and $PDGF^+$ inflammatory cells were observed in 10 specimens. The cell counts are presented in Table 2. The mean values of the five groups did not differ significantly ($P = 0.6920$, ANOVA). Double immunohistochemistry to confirm the phenotype of TGF-β-positive inflammatory cells showed that most inflammatory cells expressing TGF-β were eosinophils (mean $\pm$ SD, $45 \pm 4\%$, $N = 3$) (Figure 9) and monocytes/macrophages (mean $\pm$ SD, $35 \pm 4\%$, $N = 3$).

Discussion

In normal conjunctiva, expression of epithelial integrin $\alpha 3$ and $\alpha 6$ subunits was weak, and largely confined to the basal layer. The integrin $\alpha 6$ subunit was expressed at the basal aspect of the basal epithelial cells, whereas the integrin $\alpha 3$ subunit was expressed at the cell membrane of the basal epithelial cells. Our observations are consistent with reports that $\alpha 3$, $\alpha 6$, $\beta 1$, and $\beta 4$ integrin

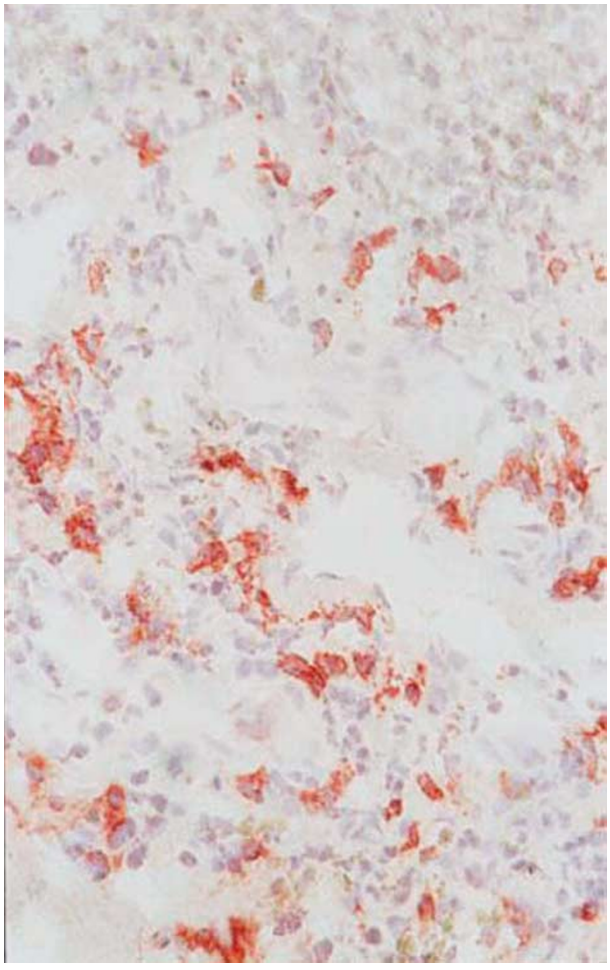

Figure 8 Vernal keratoconjunctivitis. Immunohistochemical staining for TGF-β showing strong immunoreactivity in inflammatory cells (original magnification $\times 40$).

Table 2 Numbers[a] of inflammatory cells in VKC specimens ($n = 16$)

Cell type	*Mean ± SD*	*Range*	*No. of specimens with detectable expression*
EGFR	10.5 ± 14.9	6–53	8
VEGF	11.8 ± 20.5	4–70	9
TGF-β	22.8 ± 32.6	7–126	11
bFGF	16.8 ± 24.6	3–80	11
PDGF	14.5 ± 23.6	4–74	10

[a]Cells counted in an area of 0.33×0.22 mm.
EGFR = epidermal growth factor receptor; VEGF = vascular endothelial growth factor, TGF-β = transforming growth factor-β; bFGF = basic fibroblast growth factor; PDGF = platelet-derived growth factor.

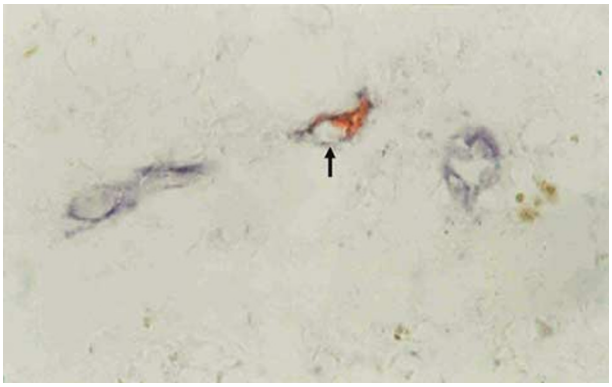

Figure 9 Vernal keratoconjunctivitis. Double immunohistochemical staining for TGF-β (red) and eosinophil peroxidase (blue) showing TGF-β-positive cell coexpressing eosinophil peroxidase (arrow). No counterstain was applied (original magnification $\times 100$).

subunits were expressed only on the basal layer of proliferating keratinocytes in normal skin,[16,31–34] and on the basal epithelial cells in normal cornea.[35–37] In fact, the integrin α6 subunit (CD49f) is known to be associated with hemidesmosomes of basal keratinocytes.[32] Compared to normal conjunctiva, the conjunctiva from patients with active VKC showed strong expression of the integrin α3 and α6 subunits on the basal and suprabasal epithelial cells. Similarly, aberrant expression of integrins on suprabasal keratinocytes has been observed in hyperproliferative epidermis in wound repair,[16,31,33] and psoriasis.[33,38] In addition, suprabasal integrin expression is seen in response to corneal epithelial injury,[39] after anterior keratectomy,[35] in keratoconus cornea,[36] and in superior limbic keratoconjunctivitis.[40] Carroll *et al*[41] demonstrated that suprabasal integrin expression in transgenic mice is a cause of abnormal keratinocyte behaviour including epidermal hyperproliferation and skin inflammation.

Taken together, these reports suggest that suprabasal expression of integrins, and expression of $\alpha 6\beta 4$ integrin at nonhemidesmosomal sites in epithelial tissues is associated with changes in cell proliferative capability toward a high potential for proliferation. The occurrence of Ki67-positive cells in the basal and suprabasal layers of the conjunctival epithelium in VKC supports this hypothesis. Upregulation of epithelial integrins in VKC is to be expected because of the function of these molecules in epithelial wound healing. During wound healing, migrating keratinocytes express $\alpha 3\beta 1$, $\alpha 6\beta 1$, and $\alpha 6\beta 4$ integrins,[16] and migrating corneal epithelial cells express $\alpha 6\beta 4$ integrin.[20] Lotz *et al*[17] demonstrated that the $\alpha 3\beta 1$, $\alpha 6\beta 1$, and $\alpha 6\beta 4$ integrins mediate epithelial cell migration that is requisite for resealing of disruptions in the mucosal lining of the gastrointestinal tract. Pouliot *et al*[19] demonstrated a critical role for $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrin receptors in laminin-10-mediated migration of colon cancer cells. In a previous report,[4] we showed

immunoreactivity for laminin among the basal epithelial cells in VKC suggesting that this pathway is also mediating epithelial remodelling in VKC. The integrin $\alpha6\beta4$ also has a significant impact on signalling molecules that stimulate migration and invasion.[15]

In the normal conjunctiva, vascular endothelial cells showed very weak immunoreactivity for the integrin $\alpha3$ and $\alpha6$ subunits. In contrast, the conjunctiva from patients with active VKC showed strong expression of these integrins on vascular endothelial cells. Our results are in agreement with those of others, who have also observed that microvascular endothelial cells express at their surface $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha6\beta4$ integrins.[22] Members of the integrin family of adhesion receptors are essential participants in blood vessel growth and remodelling.[21,22] Enenstein *et al*[21] demonstrated in neonatal foreskin that $\alpha6\beta4$ integrin was consistently found along the capillary loops and the distal ends of presumed sprouts, suggesting an important role for the $\alpha6\beta4$ integrin in new vessel growth. Kelin *et al*[22] showed that bFGF-stimulated microvascular endothelial cells expressed increased levels of $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha6\beta4$ integrins and adhered better to laminin. In a previous study,[4] we demonstrated intense immunoreactivity for laminin, the ligand for $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha6\beta4$ integrins,[14,15] around stromal vessels in VKC. Similarly, studies of induced angiogenesis in corneas have detected laminin at the tips of newly forming vessels.[42] Collectively, these data suggest that the interaction between the upregulated integrin receptors on endothelial cells and laminin might participate in the process of angiogenesis in VKC conjunctiva.

In the present study, EGFR immunoreactivity was strongly expressed in the deeper layers of the conjunctival epithelium in VKC. Similarly, several studies showed EGFR upregulation in the epithelium of asthmatic airways at both mRNA and protein levels.[6,43,44] In addition, *in vitro* studies showed that epidermal growth factor (EGF), and EGFR may play an important role in bronchial epithelial repair in asthma. EGFR-selective inhibition was found to inhibit both EGF-stimulated and basal wound closure.[6] It is possible that the strong expression of EGFR in the conjunctival epithelium in VKC may reflect the repair of damage incurred by the chronic inflammation. However, overhealing of the damage might be one of the mechanisms of conjunctival epithelial remodelling in VKC.

The results of the present study demonstrate that the expression of VEGF was upregulated in the conjunctiva of VKC patients compared with that of control subjects. Epithelial cells and inflammatory cells including eosinophils and monocytes/macrophages were the major cellular sources of VEGF. Our observations are in agreement with previous studies in asthma showing increased immunoreactivity for VEGF in the airways of asthmatic subjects that was expressed by eosinophils and macrophages.[5] Moreover, there was a significant correlation between the increased vascularity of the bronchial mucosa and the numbers of VEGF-positive cells.[5] Recently, Anthony *et al*[45] demonstrated that antigenic stimulation induces VEGF release in bronchial airway epithelial cells. In addition, a recent study reported overexpression of VEGF in a murine model of asthma, and inhibition of VEGF almost completely prevented the pathophysiological changes of asthma.[46]

In the present study, the expression of the fibrogenic growth factors TGF-β, bFGF, and PDGF was increased in VKC conjunctiva compared with expression in normal conjunctiva. Our data are in agreement with those of Leonardi *et al*[3] for inflammatory cells. Eosinophils and monocytes/macrophages were the major cellular sources of TGF-β, bFGF, and PDGF. However, in our study, TGF-β, PDGF, and bFGF were not expressed by the conjunctival epithelium, endothelial cells, and extracellular conjunctival stroma. This difference can be explained by differences in the antibodies used.

Growth factors can modulate the level of expression and function of integrins in several cell types. TGF-β enhances the expression of $\alpha3\beta1$ integrin by epithelial cells,[24,25] and fibroblasts,[23] and $\alpha6\beta1$ integrin by epithelial cells.[29] bFGF stimulation of endothelial cells induces an increase in $\alpha3\beta1$, $\alpha6\beta1$, and $\alpha6\beta4$ integrin expression.[22] EGF increases $\alpha3\beta1$ integrin expression in epithelial cells,[25] and $\alpha6\beta4$ integrin expression in epithelial cells,[20] and cancer cells.[19] The results from integrin inhibition experiments indicate that migration of EGF-stimulated cancer cells on laminin-10 is mediated by both $\alpha3\beta1$ and $\alpha6\beta4$ integrins.[19] Robinovitz *et al*[18] demonstrated that EGF-stimulated chemotaxis of squamous carcinoma cells on laminin-1 was associated with mobilization of $\alpha6\beta4$ integrin from hemidesmosomes and their redistribution to actin-rich protrusions. Several studies examined the mechanisms that induce disassembly of hemidesmosomes and inactivation of the ability of $\alpha6\beta4$ integrin to mediate stable adhesion to basement membrane during epithelial migration. It was demonstrated that EGFR combines with the hemidesmosomal integrin $\alpha6\beta4$ and that activation of the EGFR causes tyrosine phosphorylation of the $\beta4$ cytoplasmic domain and disruption of hemidesmosomes.[18,26] The interaction between growth factors and cell integrin receptors was also highlighted by Falcioni *et al*,[28] who demonstrated that $\alpha6\beta1$ and $\alpha6\beta4$ integrins are associated with tyrosine kinase receptors in tumour cells. Tumour cells overexpressing both receptors showed enhanced proliferation rates.[28] In agreement with these data, the deeper layers of conjunctival

epithelium in VKC coexpressed both EGFR and integrin receptors that might enhance the hyperproliferative capability of these cells. In addition, integrins can regulate growth factor expression. Recently, Chung *et al*[27] demonstrated that $\alpha6\beta4$ integrin can enhance VEGF translation in carcinoma cells.

In conclusion, we have demonstrated significant overexpression of $\alpha3$ and $\alpha6$ integrin subunits, EGFR, VEGF, TGF-β, bFGF, PDGF, and Ki67 antigen in VKC lesions. These results suggest a possible contribution of integrins, EGFR, and growth factors in mediating conjunctival remodelling in VKC. Further *in vitro* studies on the interactions between integrins, growth factors, growth factor receptors, and components of the extracellular matrix may elucidate the pathophysiology of conjunctival remodelling that characterizes VKC.

Acknowledgements

This work was supported in part by the College of Medicine Research Centre, King Saud University. We thank Ms Christel Van den Broeck for technical assistance, and Ms Connie B Unisa-Marfil for secretarial work.

References

1 Abu El-Asrar AM, Van den Oord JJ, Geboes K, Missotten L, Emarah MH, Desmet V. Immunopathological study of vernal keratoconjunctivitis. *Graefe's Arch Clin Exp Ophthalmol* 1989; **227**: 374–379.
2 Abu El-Asrar AM, Geboes K, Al-Kharashi SA, Al-Mosallam AA, Tabbara KF, AL-Rajhi AA *et al*. An immunohistochemical study of collagens in trachoma and vernal keratoconjunctivitis. *Eye* 1998; **12**: 1001–1006.
3 Leonardi A, Brun P, Tavolato M, Abatangelo G, Plebani M, Secchi AG. Growth factors and collagen distribution in vernal keratoconjunctivitis. *Invest Ophthalmol Vis Sci* 2000; **41**: 4175–4181.
4 Abu El-Asrar AM, Meersschaert A, Al-Kharashi SA, Missotten L, Geboes K. Immunohistochemical evaluation of conjunctival remodelling in vernal keratoconjunctivitis. *Eye* 2003; **17**: 767–771.
5 Hoshino M, Takahashi M, Aoike N. Expression of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin immunoreactivity in asthmatic airways and its relationship to angiogenesis. *J Allergy Clin Immunol* 2001; **107**: 295–301.
6 Puddicombe SM, Polosa R, Richter A, Krishna MT, Howarth PH, Holgate ST *et al*. Involvement of the epidermal growth factor receptor in epithelial repair in asthma. *FASEB J* 2000; **14**: 1362–1374.
7 Davies DE, Polosa R, Puddicombe SM, Richter A, Holgate ST. The epidermal growth factor receptor and its ligand family: their potential role in repair and remodelling in asthma. *Allergy* 1999; **54**: 771–783.
8 Senger D, Van De Water L, Brown LF, Nagy JA, Yeo KT, Yeo TK *et al*. Vascular permeability factor (VPF, VEGF) in tumor biology. *Cancer Metastasis Rev* 1993; **12**: 303–324.
9 Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 1995; **146**: 1029–1039.
10 Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 1983; **219**: 983–985.
11 Kovacs E, Di Pietro L. Fibrogenic cytokines and connective tissue production. *FASEB J* 1994; **8**: 854–861.
12 Burgess WH, Maciag T. The heparin-binding (fibroblast) growth factor family of proteins. *Annu Rev Biochem* 1989; **58**: 575–606.
13 Bonner JC, Osornio-Vargas AR, Badgett A, Brody AR. Differential proliferation of lung fibroblasts induced by the platelet-derived growth factor - AA, - AB, and - BB isoforms secreted by rat alveolar macrophages. *Am J Respir Cell Mol Biol* 1991; **5**: 539–547.
14 Heino J. Biology of tumor cell invasion: interplay of cell adhesion and matrix degradation. *Int J Cancer* 1996; **65**: 717–722.
15 Mercurio AM, Rabinovitz I, Shaw LM. The $\alpha6\beta4$ integrin and epithelial cell migration. *Curr Opin Cell Biol* 2001; **13**: 541–545.
16 Larjava H, Salo T, Haapasalmi K, Kramer RH, Heino J. Expression of integrins and basement membrane components by wound keratinocytes. *J Clin Invest* 1993; **92**: 1425–1435.
17 Lotz MM, Nusrat A, Madara JL, Ezzell R, Wewer UM, Mercurio AM. Intestinal epithelial restitution: involvement of specific laminin isoforms and integrin laminin receptors in wound closure of a transformed model epithelium. *Am J Pathol* 1997; **150**: 747–760.
18 Rabinovitz I, Toker A, Mercurio AM. Protein kinase C-dependent mobilization of the $\alpha6\beta4$ integrin from hemidesmosomes and its association with actin-rich cell protrusions drive the chemotactic migration of carcinoma cells. *J Cell Biol* 1999; **146**: 1147–1159.
19 Pouliot N, Nice EC, Burgess AW. Laminin-10 mediates basal and EGF-stimulated motility of human colon carcinoma cells via $\alpha3\beta1$ and $\alpha6\beta4$ integrins. *Exp Cell Res* 2001; **266**: 1–10.
20 Song QH, Singh RP, Trinkaus-Randall V. Injury and EGF mediate the expression of alpha6beta4 integrin subunits in corneal epithelium. *J Cell Biochem* 2001; **80**: 397–414.
21 Enenstein J, Kramer RH. Confocal microscopic analysis of integrin expression on the microvasculature and its sprouts in the neonatal foreskin. *J Invest Dermatol* 1994; **103**: 381–386.
22 Kelin S, Giancotti FG, Presta M, Albelda SM, Buck CA, Rifkin DB. The fibroblast growth factor modulates integrin expression in microvascular endothelial cells. *Mol Biol Cell* 1993; **4**: 973–982.
23 Heino J, Ignotz RA, Hemler ME, Crouse C, Massagué J. Regulation of cell adhesion receptors by transforming growth factor-β. Concomitant regulation of integrins that share a common $\beta1$ subunit. *J Biol Chem* 1989; **264**: 380–388.
24 Sheppard D, Cohen S, Wang A, Busk M. Transforming growth factor β differentially regulates expression of integirn subunits in guinea pig airway epithelial cells. *J Biol Chem* 1992; **267**: 17409–17414.
25 Vignola AM, Bonsignore G, Siena L, Melis M, Chiappara G, Gagliardo R *et al*. ICAM-1 and $\alpha3\beta1$ expression by bronchial epithelial cells and their *in vitro* modulation by

inflammatory and anti-inflammatory mediators. *Allergy* 2000; **55**: 931–939.
26 Giancotti FG. EGF-R signaling through Fyn kinase disrupts the function of integrin $\alpha 6\beta 4$ at hemidesmosomes: role in epithelial cell migration and carcinoma invasion. *J Cell Biol* 2001; **155**: 447–457.
27 Chung J, Bachelder RE, Lipscomb EA, Shaw LM, Mercurio AM. Integrin (alpha6beta4) regulation of eIF-4E activity and VEGF translation: a survival mechanism for carcinoma cells. *J Cell Biol* 2002; **158**: 165–174.
28 Falcioni R, Antonini A, Nistico P, Di Stefano S, Crescenzi M, Natali PG *et al*. Alpha6beta4 and alpha6beta1 integrins associate with ErB-2 in human carcinoma cell lines. *Exp Cell Res* 1997; **236**: 76–85.
29 Kumar NM, Sigurdson SL, Sheppard D, Lweduga-Mukasa JS. Differential modulation of integrin receptors and extracellular matrix laminin by transforming growth factor-beta 1 in rat alveolar epithelial cells. *Exp Cell Res* 1995; **221**: 385–394.
30 Gerdes J, Lemke H, Baisch H, Wacker H-H, Schwab U, Stein H. Cell cycle analysis of a cell proliferation—associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* 1984; **133**: 1710–1715.
31 Cavani A, Zambruno G, Marconi A, Manca V, Marchetti M, Giannetti A. Distinctive integrin expression in the newly forming epidermis during wound healing in humans. *J Invest Dermatol* 1993; **101**: 600–604.
32 Kanitakis J, Zambruno G, Vassileva S, Giannetti A, Thivolet J. Alpha-6 (CD49f) integrin expression in genetic and acquired bullous skin diseases. A comparison of its distribution with bullous pemphigoid antigen. *J Cut Pathol* 1992; **19**: 376–384.
33 Hertle MD, Kubler MD, Leigh IM, Watt FM. Aberrant integrin expression during epidermal wound healing and in psoriatic epidermis. *J Clin Invest* 1992; **89**: 1892–1901.
34 Häkkinen L, Westermarck J, Johansson N, Aho H, Peltonen J, Heino J *et al*. Suprabasal expression of epidermal $\alpha 2\beta 1$ and $\alpha 3\beta 1$ integrins in skin treated with topical retinoic acid. *Br J Dermatol* 1998; **138**: 29–36.
35 Päällysaho T, Tervo K, Tervo T, van Setten G-B, Virtanen I. Distribution of integrins $\alpha 6$ and $\beta 4$ in the rabbit corneal epithelium after anterior keratectomy. *Cornea* 1992; **11**: 523–528.
36 Ebihara N, Watanabe Y, Nakayasu K, Kanai A. The expression of laminin-5 and ultrastructure of the interface between basal cells and underlying stroma in the keratoconus cornea. *Jpn J Ophthalmol* 2001; **45**: 209–215.
37 Lauweryns B, Van den Oord JJ, Missotten L. The transitional zone between limbus and peripheral cornea. An immunohistochemical study. *Invest Ophthalmol Vis Sci* 1993; **34**: 1991–1999.
38 Pellegirni G, DeLuca M, Orecchia G, Balzac F, Cremona O, Savoia P *et al*. Expression, topography, and function of integrin receptors are severely altered in keratinocytes from involved and uninvolved psoriatic skin. *J Clin Invest* 1992; **89**: 1783–1795.
39 Stepp MA, Zhu L, Cranfill R. Changes in $\beta 4$ integrin expression and localization in vivo in response to corneal epithelial injury. *Invest Ophthalmol Vis Sci* 1996; **37**: 1593–1601.
40 Matsuda A, Tagawa Y, Matsuda H. TGF-$\beta 2$, tenascin, and integrin $\beta 1$ expression in superior limbic keratoconjunctivitis. *Jpn J Ophthalmol* 1999; **43**: 251–256.
41 Caroll JM, Romero R, Watt FM. Suprabasal integrin expression in the epidermis of transgenic mice results in developmental defects and a phenotype resembling psoriasis. *Cell* 1995; **83**: 957–968.
42 Jerdan JA, Michels RG, Glaser BM. Extracellular matrix of newly forming vessels—an immunohistochemical study. *Microvasc Res* 1991; **42**: 255–265.
43 Amishima M, Munakata M, Nasuhara Y, Sato A, Takahashi T, Homma Y *et al*. Expression of epidermal growth factor and epidermal growth factor receptor immunoreactivity in the asthmatic human airway. *Am J Respir Crit Care Med* 1998; **157**: 1907–1912.
44 Takeyama K, Fahy JV, Nadel JA. Relationship of epidermal growth factor receptors to goblet cell production in human bronchi. *Am J Respir Crit Care Med* 2001; **163**: 511–516.
45 Antony AB, Tepper RS, Mohammed KA. Cockroach extract antigen increases bronchial airway epithelial permeability. *J Allergy Clin Immunol* 2002; **110**: 589–595.
46 Lee YC, Kwak Y-G, Song CH. Contribution of vascular endothelial growth factor to airway hyperresponsiveness and inflammation in a murine model of toluene diisocyanate-induced asthma. *J Immunol* 2002; **168**: 3595–3600.