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LABORATORY STUDY

Expression of growth factors in the conjunctiva from patients with active trachoma

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Abstract

Purpose **The blinding complications of trachoma are associated with progressive conjunctival fibrosis due to excessive accumulation of extracellular matrix (ECM) components. We studied the processes involved in the regulation of fibrosis in trachoma by investigating the expression of the fibrogenic and angiogenic connective tissue growth factor (CTGF) and basic fibroblast growth factor (bFGF), the angiogenic vascular endothelial growth factor (VEGF), the angiogenesis-associated endothelial cell marker CD105 (endoglin), and the ECM protein tenascin in the conjunctiva.**

Methods **Conjunctival biopsy specimens from six patients with active trachoma, and six control subjects were studied by immunohistochemical techniques using monoclonal and polyclonal antibodies directed against CTGF, bFGF, VEGF, CD105, and tenascin.**

Results **In the normal conjunctiva, weak immunoreactivity for VEGF was observed in epithelial cells. There was no immunoreactivity for the other antibodies. In all trachoma specimens, immunoreactivity for CTGF and bFGF was localized in monocytes/macrophages, positive for the CD68 marker. Strong immunoreactivity for VEGF was observed in epithelial cells and on vascular endothelial cells. CD105 immunoreactivity was observed on vascular endothelial cells. Immunoreactivity for tenascin was noted in the upper substantia propria.**

Conclusions **These findings suggest that macrophages play an active role in conjunctival scarring, upregulated local production of CTGF, bFGF, and VEGF contributes to both fibrous tissue growth and angiogenesis, vascular endothelial cells are activated and are undergoing active angiogenesis, and deposition of tenascin reflect remodelling of the conjunctiva in trachomatous conjunctivitis.**

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Introduction

Trachoma is a chronic follicular keratoconjunctivitis caused by repeated reinfection with the ocular serovars A, B, Ba, and C of *Chlamydia trachomatis*. It is the leading infectious cause of blindness worldwide, affecting an estimated 300–500 million people of whom 5.9 million are blind. The blinding complications of trachoma are associated with progressive conjunctival and subconjunctival scarring that may lead to dry eye syndrome, entropion, trichiasis, and corneal blindness.[1] The pathological mechanisms involved in chronic progressive conjunctival scarring in trachoma that lead to blindness remain incompletely defined. However, in previous immunohistochemical studies we have demonstrated that the tissue damage might result from immunological mechanisms involving T lymphocytes, macrophages, gelatinase B, and cytokines released by resident conjunctival cells, and by inflammatory cells infiltrating the tissue.[2–4]

Growth factors regulate many of the processes crucial for normal repair after tissue injury, including chemotactic migration of inflammatory cells and fibroblasts, mitosis of cells, neovascularization, and synthesis of extracellular matrix (ECM) components.[5,6] However, prolonged production of these

cytokines can lead to excessive matrix accumulation and chronic fibrosis, often resulting in loss of tissue structure and function. Among these growth factors, connective tissue growth factor (CTGF), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF, also known as vascular permeability factor) are of major importance in this process.

CTGF is a cysteine-rich secretory protein of 36–38 kDa, which is composed of 349 amino-acid residues, and its gene belongs to the CCN family. It functions as a downstream mediator of transforming growth factor-β (TGF-β) action on connective tissue cells, stimulating cell proliferation and ECM synthesis. CTGF acts as a fibroblast chemoattractant and mitogen, and also stimulates ECM production, cell attachment, cell survival, and angiogenesis.[6,7] bFGF is a potent mitogen for many cell types including fibroblasts and endothelial cells. It is a potent angiogenic factor and is involved in tissue remodelling and regeneration.[8] VEGF is a specific mitogen for vascular endothelial cells and plays a central role in the process of angiogenesis, and increases vascular permeability. It acts on receptors such as Flt-1 and KDR, which are expressed selectively on endothelial cells.[9]

CD105 (endoglin) is a proliferation-associated protein abundantly expressed in angiogenic endothelial cells. It is expressed on the cell surface as a 180 kDa transmembrane glycoprotein. CD105 is a receptor for TGF-β1 and TGF-β3 and modulates TGF-β signalling by interacting with TGF-β receptors I and/or II. The levels of CD105 protein, mRNA, and promoter activity are upregulated by hypoxia and by TGF-β1 in vascular endothelial cells.[10]

Tenascin is a large oligomeric ECM glycoprotein that is abundantly expressed during embryogenesis especially at epithelial–mesenchymal junctions, and in developing brain tissue. During adulthood, tenascin expression is markedly decreased in normal tissues, but reappears during wound healing, tissue remodelling, and tumorigenesis. Tenascin is believed to play important roles in tissue development, wound healing, and repair, because it mediates several cellular activities including cell adhesion and antiadhesion, migration, proliferation, and differentiation.[11]

Understanding the control mechanisms involved in conjunctival scarring in trachoma should facilitate the development of new therapeutic strategies to control this aberrant wound healing. Therefore, using immunohistochemical methods we examined the expression of CTGF, bFGF, and VEGF, the proliferative endothelial cell marker CD105, and tenascin in the conjunctiva from patients with active trachoma and from normal individuals.

Materials and methods

Study population

Six patients with intense follicular trachomatous inflammation[1] ranging in age from 5 to 17 years (mean age 7.7 years) were included in the study. Upper palpebral conjunctival biopsy specimen was obtained from each patient after informed consent. None of the patients was on topical therapy. In addition, six upper palpebral conjunctival biopsy specimens were obtained from patients of the same age group who underwent cataract extraction or strabismus surgery without obvious inflammation served as controls, in full compliance with the tenets of the Declaration of Helsinki.

The conjunctival biopsy specimens were immediately fixed for 2–3 h in B5 fixative composed of: (A) 90 ml distilled water, 6 g mercuric chloride, 2.074 g sodium acetate; and (B) 37% formaldehyde solution, pH 5.7. Since the reagents can react among each other, 9 ml of A and 1 ml of B were mixed immediately before use. The specimens were then embedded in paraffin.

Immunohistochemical staining

After deparaffinization, endogenous peroxidase was abolished with 2% hydrogen peroxide in methanol for 20 min, and nonspecific background staining was blocked by incubating the sections for 5 min in normal swine serum. For CTGF, bFGF, CD105, and tenascin detection, antigen retrieval was performed by boiling the sections in 10 mM Tris-EDTA buffer (pH 9) for 30 min. For CD68 (KP1) detection, the sections underwent trypsinization for 10 min at 37°C using a mixture of 0.1% trypsin (Sigma-Aldrich, Bornem, Belgium) and 0.1% $CaCl_2$ at pH 7.8. Subsequently, the sections were incubated with the monoclonal and polyclonal antibodies listed in Table 1. Optimum working concentration and incubation time for the antibodies were determined earlier in pilot experiments. For bFGF, CD105, and tenascin immunohistochemistry, the sections were incubated for 30 min with goat anti-rabbit or anti-mouse immunoglobulins conjugated to peroxidase-labeled dextran polymer (EnVision$^+$; Dako, Carpinteria, CA, USA).

For CTGF, VEGF, and CD68 (KP1) immunohistochemistry, the sections were incubated for 30 min with the biotinylated secondary antibody and reacted with the avidin-biotinylated peroxidase complex (Dako). The reaction product was visualized by incubation for 10 min in 0.06% 3.3′-diaminobenzidine (Sigma) and 0.01% hydrogen peroxide resulting in brown immunoreactive sites. The slides were faintly counterstained with Harris hematoxylin. Finally, the

Table 1 Monoclonal and polyclonal antibodies used in the study

Primary antibody	*Dilution*	*Incubation time (min)*	*Source*[a]
● Anticonnective tissue growth factor (L-20) (pc)	1 : 20	120	Santa Cruz Biotechnology, Inc.
● Antivascular endothelial growth factor (A-20) (pc)	1 : 100	30	Santa Cruz Biotechnology, Inc.
● Antibasic fibroblast growth factor (147) (pc)	1 : 20	30	Santa Cruz Biotechnology, Inc.
● Anti-CD105 (Clone SN6h[1]) (mc)	1 : 20	120	Dako
● Anti-tenascin (Clone T2H5) (mc)	1 : 20	120	Neomarkers
● Anti-CD68 (Clone KP1) (mc)	1 : 50	30	Dako

[a]Location of manufacturers: Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; Dako, Glostrup, Denmark; Neomarkers, Westinghouse Dr., Fremont, CA, USA.
pc = polyclonal; mc = monoclonal.

Table 2 Summary of staining results

	Control specimens (n = 6)				*Trachoma specimens* (n = 6)			
	Epithelium	*Vascular endothelium*	*Macrophages*	*Stroma*	*Epithelium*	*Vascular endothelium*	*Macrophages*	*Stroma*
CTGF	−	−	−	−	−	−	++	−
bFGF	−	−	−	−	−	−	++	−
VEGF	+	−	−	−	+++	++	−	−
CD105	−	−	−	−	−	++	−	−
Tenascin	−	−	−	−	−	−	−	++

CTGF, connective tissue growth factor; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor.
−, No staining; +, weak staining; ++, intense staining; +++, very intense staining.

sections were rinsed in 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. Sections from patients with colorectal carcinoma and breast cancer were used as positive controls. The sections from the control patients were obtained from patients treated at the University Hospital (University of Leuven, Belgium).

All sections were examined by two independent observers (AMA, KG) and the staining was graded on the basis of the presence or absence of immunoreactivity, intensity of immunoreactivity (+, weak staining; ++, intense staining; +++, very intense staining), and localization of immunoreactivity.

Results

The routine histology and immunohistochemical findings of these biopsy specimens were previously reported.[2,4] Briefly, the inflammatory infiltrate in the epithelium consisted of large numbers of macrophages, T lymphocytes, polymorphonuclear leucocytes, and dendritic cells. The stromal inflammatory infiltrate was organized as lymphoid follicles and as a diffuse infiltrate. The lymphoid follicles consisted of B lymphocytes. In the follicular centre, large macrophages and few T lymphocytes were observed. In the area between the follicles and the epithelium, as well as in areas of diffuse infiltrate, a mixed population of T lymphocytes, macrophages, dendritic cells, B lymphocytes, and polymorphonuclear leucocytes was observed. A band of plasma cells was situated directly underneath the epithelium in which IgA^+ cells were most prevalent. In addition, the superficial conjunctival epithelial cells infected with *C. trachomatis* expressed major histocompatibility complex class II antigens. Immunoreactivity for gelatinase B was localized in macrophages. In the centre of the lymphoid follicles, the macrophages with gelatinase B immunoreactivity were large and some were multinucleated giant cells.

A summary of the results is given Table 2. There was no staining in the negative control slides and when the chromogen alone was applied (Figure 1a). In normal conjunctiva, patchy weak intracytoplasmic perinuclear immunoreactivity for VEGF was observed in the epithelium. In addition, some epithelial cells showed perimembranous immunoreactivity for VEGF. There was no immunoreactivity for CTGF, bFGF, CD105, and tenascin.

In all trachoma specimens, cytoplasmic immunoreactivity for CTGF (Figure 1b) and bFGF (Figure 1c) was noted in large inflammatory mononuclear cells. These cells were located in the dense inflammatory infiltrate in the upper substantia propria in

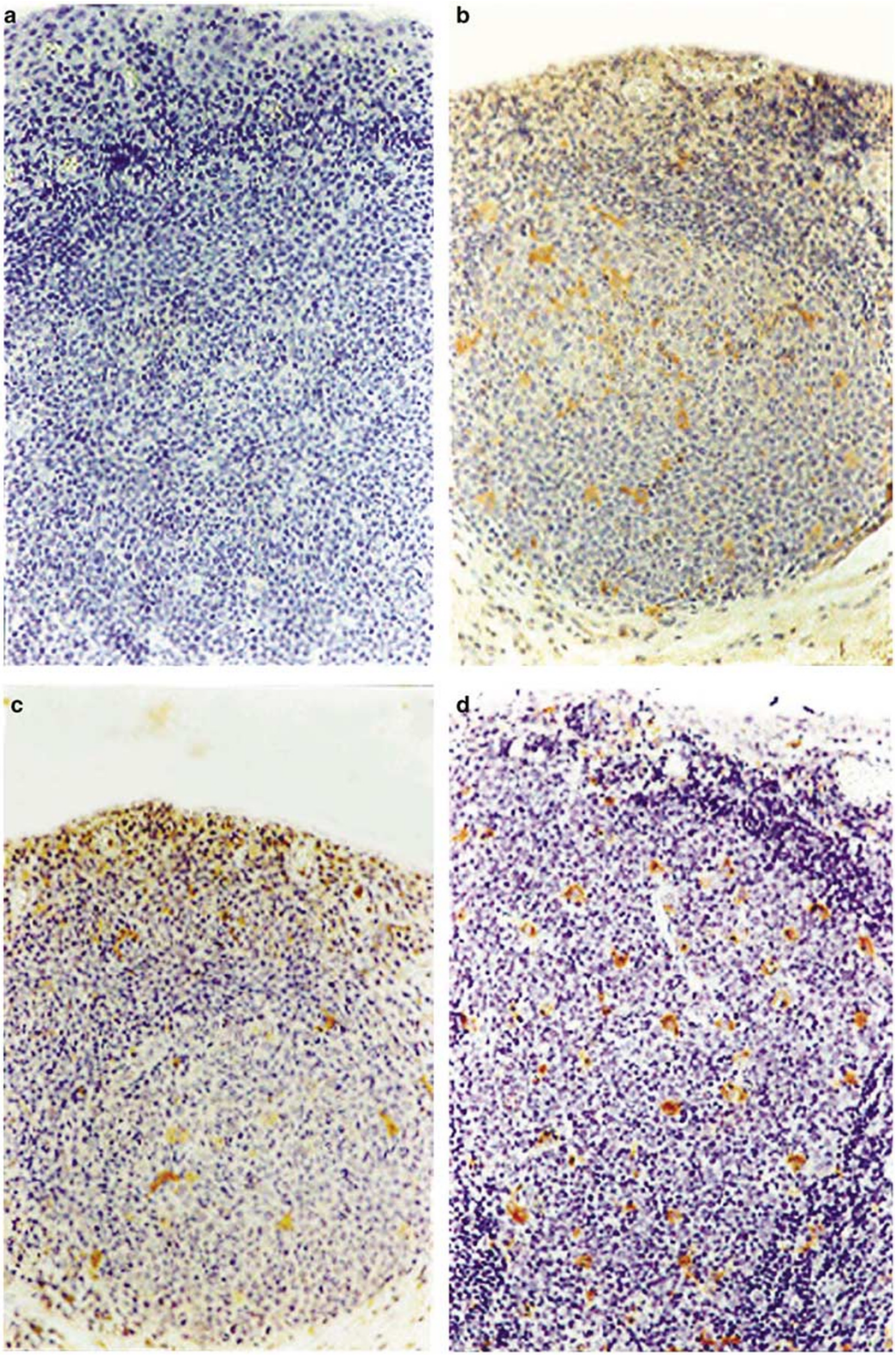

Figure 1 Immunohistochemical staining of trachomatous conjunctiva. (a) Negative control slide that was treated identically with the omission of the primary antibody showing no staining. (b) Staining for connective tissue growth factor showing immunoreactivity in inflammatory mononuclear cells. (c) Staining for basic fibroblast growth factor showing immunoreactivity in inflammatory mononuclear cells. (d) Staining for the monocyte/macrophage marker (CD68) (original magnification $\times 25$).

the lymphoid follicles and in the diffuse infiltrate outside the follicles. In serial sections, the distribution of mononuclear cells expressing CTGF and bFGF was similar to the distribution of mononuclear cells that expressed the CD68 marker for monocytes/macrophages (Figure 1d). In the centre of the lymphoid follicles, the monocytes/macrophages with CTGF and bFGF immunoreactivity were larger and some were multinucleated giant cells. The morphology of these cells varied from round to dendritic. Upregulation of VEGF expression was noted in the epithelium. Immunoreactivity for VEGF (Figure 2) and CD105 (Figure 3) was observed on vascular endothelial cells in the upper substantia propria in the area of dense inflammatory infiltrate. Band-like immunoreactivity for tenascin was noted in the upper substantia propria just underneath the epithelium. In addition, immunoreactivity for tenascin was noted in the perivascular areas in the upper substantia propria (Figure 4). There was a close association between tenascin immunoreactivity and the inflammatory infiltrate.

Discussion

Macrophages play a central role in normal wound healing and in the pathogenesis of fibrotic disorders associated with chronic inflammatory states. After activation, macrophages produce many cytokines that chemoattract leucocytes and mesenchymal cells, such as fibroblasts, trigger the proliferation of fibroblasts, stimulate the production of ECM components, and modulate angiogenesis. Thus, macrophages are capable of influencing both fibrous tissue regrowth and capillary growth, and thus is a major regulator of the repair response.[12] There is evidence that macrophages are activated during *C. trachomatis* infection. In previous immunohistochemical studies, we demonstrated the presence of increased numbers of activated macrophages in the conjunctival biopsy specimens from patients with active trachoma.[2,3] These macrophages showed cytoplasmic expression of the fibrogenic cytokines interleukin-1α, interleukin-1β, tumor necrosis factor-α, and platelet derived growth factor.[3] In the normal conjunctiva, there was no immunoreactivity for CTGF and bFGF. In contrast, immunoreactivity for CTGF and

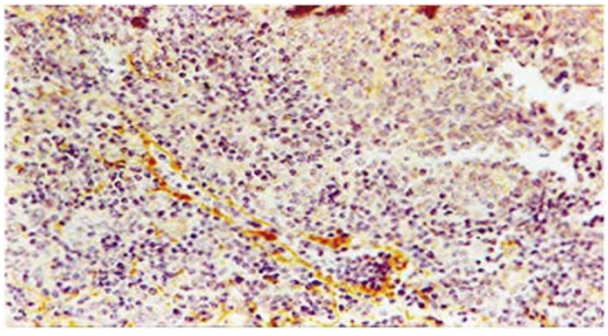

Figure 2 Trachoma. Immunohistochemical staining for vascular endothelial growth factor showing immunoreactivity on vascular endothelial cells (original magnification $\times 40$).

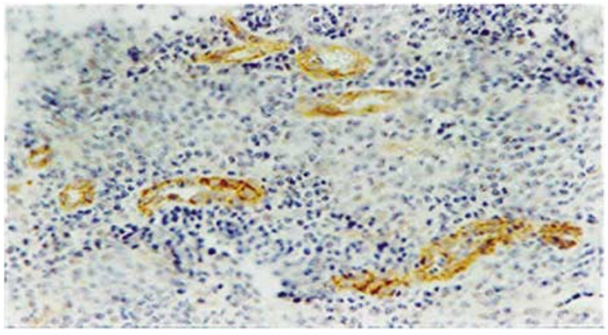

Figure 3 Trachoma. Immunohistochemical staining for CD105 showing immunoreactivity on vascular endothelial cells (original magnification $\times 40$).

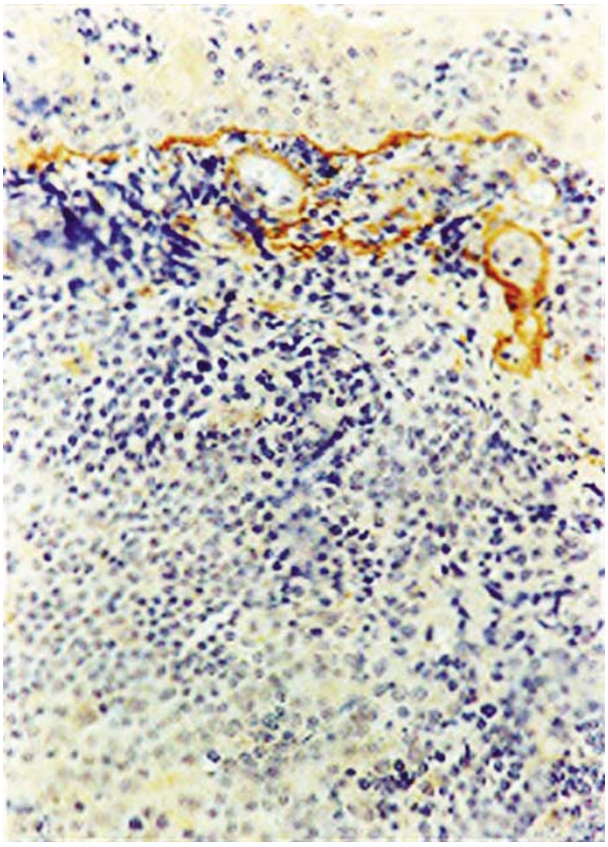

Figure 4 Trachoma. Immunohistochemical staining for tenascin showing immunoreactivity in the upper substantia propria and in the perivascular areas (original magnification $\times 40$).

bFGF was detected in trachoma specimens and was specifically localized in macrophages, particularly in the lymphoid follicles. Similarly, CTGF[5,13] and bFGF[14] were expressed in inflammatory mononuclear cells in other inflammatory and fibrotic disorders. CTGF has recently received much attention as a possible key determinant of progressive fibrosis and excessive scarring in several fibrotic diseases.[7,13,15–19] In addition, an increased expression of CTGF was demonstrated in conjunctiva of patients with ocular cicatricial pemphigoid[20] and in pterygium tissues[21] suggesting that CTGF is one of the molecules involved in conjunctival fibrosis.

Metabolic alterations of ECM components and collagen metabolism occur in the conjunctival tissue from patients with trachoma. In previous studies, we demonstrated new type V collagen formation and increased types I, III, and IV collagen content in the conjunctiva from patients with active trachoma.[22] Increased deposition of type IV collagen and new type V collagen formation was noted in scarred trachoma.[23] In the present study, trachoma specimens showed a close association between the inflammatory infiltrate and tenascin immunoreactivity in the upper substantia propria and in the perivascular areas close to the epithelial cells. It has been suggested that tenascin acts as an immuno-modulator,[24] promotes the transition of endothelial cells from a resting to a sprouting phenotype,[25] and increases the expression of gelatinase B by macrophages.[26]

Many studies indicate that CTGF can regulate the production of ECM components including types I, III, and IV collagen, fibronectin, fibromodulin, and tenascin-c in several cells.[27–29] Such findings are consistent with those indicating that subcutaneous injection of CTGF into NIH Swiss mice results in the formation of granulation tissue and fibrosis at the site of injection.[27] Therefore, it is likely that CTGF may regulate the expression of ECM components in conjunctiva of patients with trachoma. Since CTGF is profibrotic, inhibition of this growth factor may be a new molecular target for therapeutic intervention in fibrotic diseases. It is conceivable that inhibition of CTGF might block the profibrotic effects of TGF-β, without affecting TGF-β's antiproliferative, anti-inflammatory, and immunosuppressive effects.[6,7]

Angiogenesis is an important component of fibrous tissue growth, as it provides the essential nutrient support for cellular proliferation.[12] In addition to producing cytokines that directly influence capillary endothelial cells, macrophages may modulate angiogenesis through the production of matrix metalloproteinases that effectively alter the ECM through degradation.[12] In a previous study, we demonstrated increased activity of gelatinase B (matrix metalloproteinase-9) in conjunctival biopsy specimens from patients with active trachoma. Gelatinase B was localized in macrophages indicating that macrophages were primarily responsible for production of gelatinase B.[4] Gelatinase B functions to promote angiogenesis by regulating endothelial cell attachment, proliferation, migration and tube formation.[30,31] In addition, gelatinase B cleaves denatured collagens (gelatins) and other matrix proteins[32] that may contribute to progressive breakdown of conjunctiva by degrading minor constituents of the ECM. Recently, there is strong evidence supporting a role for CTGF in the regulation of endothelial cell function and angiogenesis. CTGF can promote endothelial cell growth, migration, adhesion, and survival *in vitro*. CTGF is also active *in vivo* assays for angiogenic activity.[33] In addition, CTGF contributes to the secretion of other angiogenic molecules such as gelatinase B[34] and bFGF,[28] which are upregulated in the conjunctiva from patients with active trachoma. Therefore, it is likely that CTGF influences angiogenesis in trachoma through other indirect mechanisms by increasing the expression of gelatinase B and bFGF in macrophages. These findings suggest that CTGF, bFGF, and gelatinase B produced by macrophages interact in mediating conjunctival angiogenesis and ECM remodelling in trachoma.

In the present study, we demonstrated that VEGF was constitutively expressed in conjunctival epithelial cells and was upregulated in trachoma. Our results are in agreement with previous studies that demonstrated constitutive cytoplasmic VEGF mRNA and protein expression in several epithelia,[35] including the conjunctival epithelium.[36] In addition, VEGF was expressed by vascular endothelium in the conjunctiva from patients with active trachoma. This finding is consistent with previous studies that demonstrated expression of VEGF mRNA and protein in stimulated human vascular endothelial cells. On the other hand, VEGF was not detected in endothelial cells under quiescent conditions.[37] As endothelial cells are known to express the high-affinity VEGF receptors, these cells in trachoma appear to include the requisite elements for an autocrine pathway that may augment and/or amplify the paracrine effects of VEGF in stimulating angiogenesis and increasing vascular permeability and oedema. Similarly, in the pterygium tissue, an abnormal mass of fibrovascular tissue that extends from the conjunctiva onto the cornea, several reports described immunoreactivity for VEGF in the vascular endothelium.[21,36]

In addition to VEGF, the vascular endothelium in trachoma expressed the proliferative endothelial marker CD105. The CD105 antibody binds preferentially to the activated endothelial cells that participate in angiogenesis, but expression is weak/or negative in vascular endothelium of normal tissues. Evidence that

CD105 is involved in angiogenesis and vascular development, in maintaining vessel wall integrity, and in tumor progression has recently accumulated.[10] Consistently, elevated levels of CD105 expression were detected on activated vascular endothelial cells in tissues undergoing active angiogenesis, such as wound healing, inflammation, and tumors.[38,39] Moreover, the assessment of neovascularization by CD105 staining was found to represent a potential predictor of prognosis in different solid malignancies.[39] Collectively, these data suggest that vascular endothelial cells in conjunctiva from patients with active trachoma are actively proliferating and are undergoing angiogenesis. These findings might explain vascular infiltration of the cornea (pannus) seen in patients with active trachoma.

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