An immunohistochemical study of collagens in trachoma and vernal keratoconjunctivitis

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Abstract

Purpose To analyse the distribution and types of collagen in the conjunctiva of patients with trachoma and vernal keratoconjunctivitis (VKC).

Methods Conjunctival biopsy specimens were collected from 9 patients with active trachoma, 9 patients with scarred trachoma, 6 patients with active VKC and 9 control subjects. The presence and distribution of collagen was assessed microscopically with

immunohistochemical techniques and a panel of monoclonal and polyclonal antibodies directed against types I, III, IV and V collagen.

Results In normal conjunctiva, the staining for types I and III collagen was localised to the substantia propria. Type IV collagen was located in the epithelial, vascular endothelial and accessory lacrimal gland basement membranes. Staining for type V collagen was absent. New type V collagen deposition close to basement membranes was noted in active trachoma, scarred trachoma and VKC. The extent of deposition of type V collagen was markedly increased in scarred trachoma when compared with active trachoma. Staining for type IV collagen showed irregularly thickened epithelial basement membrane in active trachoma, and a marked increase in basement membrane type IV collagen was noted in scarred trachoma. Immunoreactivity of types I and III collagen increased in active trachoma and decreased in scarred trachoma. VKC conjunctiva contained increased amounts of types I, III and IV collagen due to marked increase in the thickness of vascular endothelial basement membrane and very prominent deposition of types I and III collagen around stromal vessels.

Conclusions Our data indicate new type V collagen formation in the conjunctiva from patients with active trachoma, scarred trachoma and VKC. Increased deposition of types I, III and IV collagen is noted in VKC and active trachoma. Our findings suggest that increased deposition of type IV collagen and new type V collagen formation contributes to the development of conjunctival fibrosis in scarred trachoma.

Key words Collagen, Conjunctiva, Trachoma, Vernal keratoconjunctivitis

The deposition of increased amounts of extracellular matrix components including collagen is a feature of many chronic inflammatory diseases and contributes to post-inflammatory fibrosis. Inflammatory mononuclear cells generate cytokines that affect fibroblast functions including stimulation of fibroblast chemotaxis, proliferation and collagen synthesis. These phenomena result in metabolic alterations in extracellular matrix components.¹

Chronic inflammatory conditions of the conjunctiva often lead to conjunctival scarring with subsequent visual disability. Trachoma, a chronic follicular conjunctivitis due to *Chlamydia trachomatis*, is the world's leading cause of preventable blindness.² The blinding complications of trachoma are associated with progressive conjunctival and subconjunctival fibrosis that may lead to dry eye syndrome, and inturning of the eyelid margin (entropion) and lashes (trichiasis). Vernal keratoconjunctivitis (VKC) is a chronic, seasonally exacerbated bilateral external allergic ocular inflammation characterised by tarsal conjunctival giant papillae and limbal thickening and opacification resulting from hyperplasia of conjunctival connective tissue.

There are no data available regarding the phenotypic expression of various collagens in the conjunctiva during the course of trachoma and VKC. In a previous immunohistochemical study we described alterations in conjunctival collagen in the conjunctiva of patients with active trachoma.³ The purpose of this study was to examine, with immunohistochemical techniques, the nature and distribution of types I, III, IV and V collagen in the conjunctiva from patients with scarred trachoma and VKC, and from normal individuals, and to compare these results with our previous findings in active trachoma.

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Table 1. Monoclonal and polyclonal antibodies used in this study

Antibody	Specificity	Working dilution	Source
Anti-type I collagen (pc)	Type I collagen	1:10	Campro
Anti-type III collagen (pc)	Type III collagen	1:10	Chemicon
Anti-type IV collagen	Type IV collagen	1:20	Euro-diagnostics
Anti-type V collagen (pc)	Type V collagen	1:20	Chemicon

Location of manufacturers: Campro, Veenendaal, The Netherlands; Chemicon, Tmecula, CA, USA; Euro-diagnostics, Malmö, Sweden. pc, polyclonal antibodies.

Patients and methods

Study subjects and specimen collection

Six patients with severe active VKC seen at the outpatient clinic of King Abdulaziz University Hospital were included in the study. Patients ranged in age from 7 to 15 years (mean 11 years). All had the palpebral form of the disease characterised by the presence of giant polygonal flat-topped cobblestone papillae affecting the upper palpebral conjunctiva. Nine children with active trachoma between 7 and 16 years of age (mean age 9.4 years) were included in the study. A detailed description of this group, who all attended a village school in the Eastern province of Saudi Arabia, has been reported previously.³ Nine patients with cicatricial inactive trachoma seen at the outpatient clinic of King Abdulaziz University Hospital and ranging in age from 52 to 72 years (mean age 60 years) were studied. A 2 mm × 2 mm upper palpebral conjunctival biopsy specimen was obtained from each patient after informed consent had been given. None of the patients was on topical therapy.

Four upper palpebral conjunctival biopsy specimens without obvious inflammation were obtained from patients undergoing strabismus surgery and served as controls. These patients were in a similar age group to the patients with VKC and active trachoma. In addition, conjunctival biopsy specimens from patients with scarred trachoma were compared with conjunctival biopsy specimens that were obtained from 5 age-matched controls with no known ocular inflammation who were undergoing cataract surgery.

Immunohistochemical staining

The conjunctival biopsy specimens were immediately snap-frozen in tissue-Tek optimum cutting temperature (OCT) compound (Miles Laboratories, Indiana, IL) and

maintained at 80 °C until use. For immunohistochemistry, 5 µm serially cut cryostat sections were dried overnight at room temperature, fixed in absolute acetone for 10 min and stained with a three-step avidin/biotin peroxidase-labelled complex procedure. Rehydrated slides were incubated for 30 min with the monoclonal and polyclonal antibodies listed in Table 1, which were diluted to an optimum concentration. The secondary and tertiary antibodies consisted of biotin-conjugated rabbit anti-mouse immunoglobulin and the avidin/biotin peroxidase-labelled complex, respectively, both of which were purchased from Dakopatts (Copenhagen, Denmark). All incubations were carried out for 30 min at room temperature, then washed in three changes of phosphate-buffered saline at pH 7.2 for 15 min. The reaction product was developed by addition of diaminobenzidine and hydrogen peroxide, resulting in brown immunoreactive sites. The slides were faintly counterstained with Harris haematoxylin. Finally, the sections were rinsed with distilled water and coverslipped with glycerol. Controls, which were invariably negative, consisted of omission of the primary or secondary antibody and use of chromogen alone.

All sections were examined by two independent observers and the staining was graded qualitatively on the basis of: presence or absence of immunoreactivity, intensity of immunoreactivity in the same section, thickness of the staining, localisation of the staining, and the homogeneous or heterogeneous character of the staining.

Results

A summary of the results is given in Table 2, including data obtained in active trachoma and normal controls that were previously reported.³ The two control groups showed similar findings.

Table 2. Summary of collagen staining

Collagen	Controls (n = 9)				VKC (n = 6)				Active trachoma (n = 9)				Scarred trachoma (n = 9)			
	US	SV	SLG	BM	US	SV	SLG	BM	US	SV	SLG	BM	US	SV	SLG	BM
I	+	+	+		+++	+++	+		+++	+	+		+	+	+	
III	+	+	+		+++	+++	+		+++	+	+		+	+	+	
IV																
E				+				++				++				+++
VE				+				+++				++				++
LG				+				+				+				+++
V	-	-	-		+++	+++	++		+	+	+		+++	++	+++	

VKC, vernal keratoconjunctivitis; US, upper stroma; SV, around stromal vessels; SLG, stroma around accessory lacrimal glands; BM, basement membrane; E, epithelium; VE, vascular endothelium; LG, accessory lacrimal glands.

-, no staining; +, thin and/or patchy discontinuous staining; ++, thicker homogeneous continuous staining; +++ very thick dense staining.

Fig. 1. Frozen section immunohistochemical staining of vernal keratoconjunctivitis. (a) Thick, irregularly arranged collagen fibres between inflammatory cells in the upper substantia propria (arrows) with the antibody to type III collagen. (b) Very broad, intense staining around stromal vessels (arrows) with the antibody to type III collagen. (c) Very thick vascular endothelial basement membrane with the antibody to type IV collagen (arrows). (d) Thick, continuous immunoreactivity in upper substantia propria (arrows) and around stromal vessels (arrowheads) with the antibody to type V collagen. Three-step avidin/biotin peroxidase-labelled complex procedure; $\times 300$.

Conjunctival specimens from VKC patients

The substantia propria in VKC patients showed diffuse, fine fibrillar staining with antibodies directed against types I and III collagen. The intensity of the staining was markedly increased in very broad areas around stromal vessels, which were increased in number and size. Very thick, irregularly arranged collagen fibres that showed immunoreactivity with antibodies directed against types I and III collagen were noted between inflammatory cells in the upper part of the substantia propria. Staining with antibody against type IV collagen revealed irregular thickening of the epithelial basement membrane, and very thick vascular endothelial basement membranes that showed a marked increase in staining intensity. Staining with antibody directed against type V collagen demonstrated band-shaped, thick, continuous immunoreactivity in the upper substantia propria just beneath the epithelium. In addition, thick, intensely stained continuous immunoreactivity was noted around stromal vessels and the acini of accessory lacrimal glands (Fig. 1).

Conjunctival specimens from patients with scarred trachoma

Staining with antibodies directed against types I and III collagen in conjunctival specimens from patients with scarred trachoma showed loss of the fine fibrillar staining pattern seen in normal conjunctiva. In addition, the more intense band-shaped area located in the upper substantia propria just beneath the epithelium seen in normal conjunctiva was replaced by thick, irregularly arranged collagen fibres. Slightly more intense staining was observed in the perivascular areas in the upper substantia propria and around the acini of accessory lacrimal glands. The epithelial and accessory lacrimal glands basement membranes were very thick and showed strong immunoreactivity for anti-type IV collagen antibody. The same antibody also demonstrated fibrillar staining around the acini of accessory lacrimal glands. The vascular endothelial basement membrane was thickened. Staining with the antibody against type V collagen showed strong stromal immunoreactivity in the form of continuous, thick, band-shaped areas in the upper substantia propria just beneath the epithelium, around

(a)

(b)

(c)

(d)

Fig. 2. Frozen section immunohistochemical staining of scarred trachoma. (a) Very thick, intensely stained epithelial basement membrane (arrow) and thick vascular endothelial basement membrane (arrowheads) with the antibody to type IV collagen. (b) Very thick, intensely stained accessory lacrimal glands basement membrane (arrows) and fine, fibrillar immunoreactivity around acini of accessory lacrimal glands (arrowheads) with the antibody to type IV collagen. (c) Thick, continuous immunoreactivity in upper substantia propria (arrow) and around stromal vessels (arrowheads) with the antibody to type V collagen. (d) Thick, continuous immunoreactivity (arrows) and fine, fibrillar immunoreactivity (arrowheads) around acini of accessory lacrimal glands with the antibody to type V collagen. Three-step avidin/biotin peroxidase-labelled complex procedure; $\times 300$.

acini of accessory lacrimal glands and around upper stromal vessels. In addition, there was fibrillar staining around the acini of accessory lacrimal glands (Fig. 2).

Discussion

The collagens, which are the major proteins of connective tissue, provide the extracellular framework for all multicellular organisms. A subclass of this family comprises collagens I, II, III, V and XI, which form banded fibrils. They have thus been called the fibrillar collagens, to distinguish them from other collagens unable to aggregate into these highly ordered fibrils. Collagens I, II and III are the most abundant and have therefore been studied far more than the quantitatively minor collagens V and XI. The function of type I collagen is to give tissue tensile strength. Type II collagen is principally present in cartilage and type III collagen is associated with tissues and organs that require a motile structural scaffolding such as uterus, arteries, skin, intestines and lung. Type IV collagen is a non-fibrillar collagen found only within basement membranes.^{4,5} In the present study, the localisation of types I, III and IV collagen in normal conjunctiva was consistent with that reported by Dake *et al.*⁶

Histochemically, we observed that the conjunctival substantia propria from patients with VKC, active trachoma and scarred trachoma contained type V collagen. The extent of deposition of type V collagen was markedly increased in scarred trachoma when compared with active trachoma. On the other hand, type V collagen was not detected in the normal conjunctiva. To our knowledge, this is the first study to demonstrate the deposition of type V collagen in conjunctival inflammatory disorders. New deposition of type V collagen was noted close to the basement membranes of the conjunctival epithelium, stromal vascular endothelium and accessory lacrimal glands. This is consistent with previous immunolocalisation data that demonstrated localisation of type V collagen in the immediate vicinity of basement membranes, suggesting an anchoring function for this type of collagen between basement membranes and stromal matrix.⁴ Some studies showed a preferential pericellular localisation of type V collagen.⁷ Due to its pericellular localisation type V collagen may interact with cell surfaces and modulate cellular activities such as adhesion, differentiation, migration or synthesis. Several cell lines including fibroblasts were found to adhere and spread on native

and denatured type V collagen.⁸ Cell-surface-associated heparan sulphate appears to mediate the attachment of cells to type V collagen.⁹ Our results are consistent with studies describing accumulation of type V collagen in conditions associated with tissue remodelling and new collagen synthesis. Increased content of type V collagen has been detected in other diseased tissues such as cardiac hypertrophy,¹⁰ neoplasia,¹¹ Crohn's disease,¹² atherosclerotic lesions,¹³ pseudointima of vascular grafts¹⁴ and chronically inflamed gingival tissue,¹⁵ suggesting that it may play an important role in the pathology of these diseases. A number of factors have been shown to induce expression of type V collagen, including transforming growth factor-beta 1,^{16,17} epidermal growth factor,¹⁸ hepatic fibrogenic factor,¹⁹ angiotensin II²⁰ and platelet derived growth factor.¹⁷

Scarred trachoma is characterised by marked increase in basement membrane type IV collagen. In contrast, immunoreactivity of types I and III collagen was markedly decreased when compared with active trachoma. These observations are in good agreement with the findings reported in wound healing. In the early phase of wound healing the expression of α_1 (I), and α_1 (III) collagen chains is coordinately increased. In later stages the gene expression is rapidly down-regulated.^{21,22} In addition, our findings agree well with immunohistochemical studies of kidney biopsies from patients with chronic renal disease. The severity of interstitial extracellular matrix accumulation was accompanied by a significant increase in the deposition of types IV and V collagen. On the other hand, the increase in interstitial abnormalities was not accompanied by a significant increase in the deposition of the interstitial types I and III collagen.²³ Our results in active trachoma and scarred trachoma are also in agreement with studies on experimental autoimmune uveoretinitis. Increased immunoreactivity of types I, III and V collagen was noted at the peak of inflammation. While a decrease in immunoreactivity for types I and III collagen but persistent immunoreactivity for type V collagen was observed in later stages.²⁴ Previous histopathological studies of conjunctival biopsies from patients with cicatricial entropion complicating trachoma showed a thick and compact subepithelial fibrous membrane.²⁵ Our data indicate that the increased deposition of type IV collagen in the epithelial basement membrane and new type V collagen formation in the immediate vicinity of the epithelial basement membrane might contribute to the formation of this subepithelial scar tissue. The additional increase in immunoreactivity for types IV and V collagen around the acini of accessory lacrimal glands might contribute to dysfunction of these glands leading to dry eye disease.

The results of the present study show that the conjunctiva from patients with VKC contains increased amounts of types I, III and IV collagen in addition to new formation of type V collagen. Of particular interest is the very prominent deposition of types I, III and V collagen around the stromal vessels and the marked increase in the thickness of the vascular endothelium basement

membrane. Previous biochemical studies demonstrated that the total amount of collagen was increased 3-fold in VKC conjunctival specimens compared with controls.²⁶

The collagen formation might be related to the type of inflammation occurring in VKC. The conjunctiva from patients with VKC contains increased numbers of mast cells.²⁷ Recent attention has focused on the potential role of mast cells and mast cell activation in the genesis of fibrosis in many chronic inflammatory disorders.²⁸⁻³¹ Mast cell activation including IgE-dependent activation can significantly enhance fibroblast proliferation and collagen production.³²⁻³⁴ Several studies suggest that mast cells may contribute to these pathological conditions by releasing mediators such as tryptase,³³ transforming growth factor-beta 1, tumour necrosis factor-alpha, interleukin-1³⁴ and basic fibroblast growth factor.³⁵ Mast cells may not be the only cell type involved. In a previous study we have demonstrated that the number of CD4⁺ lymphocytes was significantly increased in conjunctival specimens from VKC patients.³⁶ These lymphocytes belong predominantly to Th2 cells secreting interleukin-4.³⁷ Activated mast cells also release interleukin-4.³⁸ Stimulation by interleukin-4 induces a significant increase in total collagen production by fibroblasts³⁹ and mesangial cells.⁴⁰

In conclusion, new formation of type V collagen occurs in the conjunctiva from patients with active trachoma, scarred trachoma and VKC. This study highlighted the occurrence of extensive deposition of collagen in the conjunctiva of VKC patients. Basement membrane type IV collagen, and deposition of fibril-forming interstitial types I and III collagen, were markedly increased. In addition, increased deposition of basement membrane type IV collagen, and deposition of new fibril-forming type V collagen, seems more characteristic of conjunctival fibrosis associated with scarred trachoma than the deposition of types I and III collagen, which are prominent in active trachoma and VKC.

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