LABORATORY STUDY

Glycoproteins of trabecular meshwork, cornea and sclera

S.A. CHAPMAN, S. AYAD, E. O'DONOGHUE, R.E. BONSHEK

Abstract

Purpose **To analyse high-molecular-weight matrix glycoproteins in trabecular meshwork, cornea and sclera using SDS/PAGE and immuno- and lectin blotting.**
Method **Extracts of normal trabecular meshwork (TM), cornea and sclera were analysed under reducing conditions on SDS/PAGE. Western blots were stained for total protein, and major high-molecular-weight components were identified by immunoblotting with antibodies to fibronectin (FN) and type VI collagen. Lectin blotting with PSA, MPA and DSA identified some of the glycoprotein glycans.**
Results **FN antibody bound to the 240 kDa band in TM, cornea and sclera. Type VI collagen antibody bound more strongly to one band and less so to two other bands at ~200 kDa in normal TM and to a ladder of bands in cornea and sclera. PSA and DSA bound at 240, 200 and 140 kDa in TM, cornea and sclera. MPA bound at 240, 200 and 140 kDa in TM and at 240, 200 and ~120 kDA in cornea and sclera.**
Conclusions **FN is a component of the band at 240 kDA in TM, cornea and sclera. Normal TM was found to contain relatively more of one of the isoforms of the α3 (VI) chain whilst cornea and sclera contained all the α3 (VI) isoforms. Complex *N*-linked bi/tri-antennary glycans were localised in FN and the α1, α2 and α3 (VI) chains in TM, cornea and sclera. *O*-linked glycans (identified by MPA binding) were located in FN and α3 (VI) chains of TM, cornea and sclera.**

Key words Cornea, Extracellular matrix, Sclera, Trabecular meshwork, Type VI collagen

S.A. Chapman
R.E. Bonshek
Department of Pathological Sciences
University of Manchester
Manchester, UK

S.A. Chapman
R.E. Bonshek ✉
Department of Ophthalmology
University of Manchester
Manchester Royal Eye Hospital
Oxford Road
Manchester M13 9WH, UK
Tel: +44 (0)161 276 5568
Fax: +44 (0)161 273 6354
e-mail: richard.bonshek@man.ac.uk

S. Ayad
School of Biological Sciences
University of Manchester
Manchester, UK

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Trabecular meshwork (TM) is the principal regulator of aqueous flow in the human eye, and is considered to operate via complex interrelationships, as yet poorly understood, between extracellular matrix (ECM) and TM cells. The number of cells is known to decrease with age and this decrease occurs to a greater extent in glaucomatous TM.[1,2] Cell migration and adhesion, together with stabilisation of the ECM, are mediated through specialised and unique domains on ECM molecules. Matrix glycoproteins (GP) of the TM with known binding sites for ECM components and the cell surface include fibronectin (FN)[3–6] and type VI collagen.[7–10] These GP are also found in cornea and sclera – tissues that show very different physical properties and functions whilst sharing many of the same matrix components.[11]

FN binds with high affinity to cell surfaces, collagens and proteoglycans and mediates the attachment of cells to basement membranes and ECM. In the TM it is secreted by TM cells[12] and has been localised on trabecular beams by immunocytochemical methods.[13] In cornea, FN has been localised in epithelial basement membrane, stroma and Descemet's membrane.[14]

Type VI collagen is a microfibrillar protein that interacts with cells and ECM,[8,9,15,16] where it forms extensive networks.[17,18] Type VI collagen is heterotrimeric, consisting of three genetically distinct α chains, where the molecular mass of each of the α1 and α2 chains is 140 kDa, and that of the α3 chains is ~200 kDa (using non-collagenous standards).[18–20] Type VI collagen is believed to play a key role in the maintenance of connective tissue and an age-related increase in a 140 kDa protein in TM extracts has been demonstrated on polyacrylamide gel electrophoresis (PAGE).[21] Subsequently, immunoblotting identified the 140 kDa protein as α1/α2 chains of type VI collagen [α1/α2 (VI)].[22] It has also been identified immunocytochemically in normal TM.[23] Type VI collagen forms a major constituent of human cornea[24] and promotes adhesion and spreading of corneal fibroblasts.[25]

The characteristics and function of GP depend on the nature of the glycan component, so that altered glycosylation states can affect cell–matrix and matrix–matrix interactions. The microfibrillar structure of type VI collagen is believed to be due, in part, to the glycosylation of helical domains[26] where lateral aggregation is restricted, and it is known that the glycans of type VI collagen and FN include complex *N*-linked and *O*-linked saccharides.[26,27] In a lectin

histochemical study of glycan expression in normal TM[28] we localised high mannose, complex *N*-linked, bisected and non-bisected bi/tri-antennate glycans on Schlemm's canal endothelium and, to a lesser extent, on trabecular cells and in the cores of trabecular beams. *N*-acetyl lactosamine and *O*-linked sequences were also identified. When compared with glycan expression in glaucomatous TM[29] we observed a decrease in some *N*-linked glycans as shown by binding of PSA (*Pisum sativum* agglutinin). *O*-linked glycans, as shown by binding of MPA (*Maclura pomifera* agglutinin), and *N*-acetyl glucosamine oligomers, as indicated by DSA (*Datura stramonium* agglutinin), were significantly increased. The associated GP were not identified.

In this preliminary report we have analysed tissue extracts from normal TM, cornea and sclera using PAGE and Western blotting. Blots were stained for total protein and immunoblotted with anti-FN antibody and anti-type VI collagen antibody. Lectin blotting with PSA, MPA and DSA localised some of the glycans present to bands corresponding to the identified GP.

Materials and methods

Tissue

Normal TM was obtained from six whole globes, with no ocular pathology. Two of these were taken at autopsy within 12 h of death (ages 70 and 79 years). Four were obtained from orbital exenteration procedures for sebaceous carcinoma of the eyelid (ages 76, 85 and 68 years) and lacrimal gland carcinoma (age 84 years) where there was no ocular involvement. Cornea and sclera were also acquired from three of these eyes. Normal TM, cornea and sclera from seven time-expired corneas (age range 65–96 years) were obtained from the Eye Bank, Royal Eye Hospital, Manchester.

Preparation of tissue

Normal TM was excised from the three whole globes by one of two methods. After cutting the eye at the ora serrata and removing the lens, the cornea was positioned resting on the anterior surface and cut radially into segments. The tissue was immediately frozen and stored at −70 °C. The TM was readily dissected away from the sclera and cornea by use of a dissecting microscope after thawing, as required. In the second method[30] the attachments of the ciliary body were severed, and clean partial-thickness cuts were made in front of the scleral spur and behind Schwalbe's line. The TM could then be removed with fine forceps and pulled away, prior to freezing and storage at −70 °C.

The time-expired corneas included a rim of sclera and TM. After removing the TM with fine forceps, pieces of sclera and cornea (approx. 2 × 1 × 1 mm in size) were cut off and the tissue washed twice with 1 mM phosphate-buffered saline (PBS) pH 7.4 to remove culture medium.

Materials

Reagents used in the extraction procedures and PAGE, and the lectins PSA and DSA, were obtained from Sigma (Poole, UK). MPA was supplied by Vector Laboratories (UK). Non-collagenous molecular weight standards were obtained from Bio-Rad Laboratories (Hemel Hempstead, Herts., UK). Polyvinylidene fluoride (PVDF) membrane, Immobilon P, was obtained from Millipore (Watford, Herts., UK). Protein binding gold colloid (Protogold), gold-labelled antibiotin (20 nm) and silver-enhancing kit were obtained from British BioCell International (Cardiff, UK). Two polyclonal antibodies that recognise human type VI collagen were used. One (termed VIB[20]), with principal specificity for the α3 (VI) chain, was raised to bovine type VI collagen but reacts strongly with human type VI collagen. The second (termed VIG, supplied by Gibco BRL), with principal specificity for the α1, α2 (VI) chains, was solid phase-absorbed with normal human plasma proteins and had no cross-reactivity with human FN. Anti-human FN antibody (monoclonal) was obtained from Gibco BRL (UK) and biotinylated goat anti-rabbit and goat anti-mouse immunoglobulins were obtained from Dako (UK).

Tissue extraction

The GP were extracted from TM, cornea and sclera by adapting a method described previously.[21] Briefly, following two washes with PBS pH 7.4, whole TM was extracted into 200 μl of buffer containing 0.0625 M Tris-HCl, 2% (w/v) sodium dodecyl sulphate (SDS), 10% (v/v) glycerol, 8 M urea, 0.1 M dithiothreitol (DTT) and 0.02% (w/v) bromophenol blue, and heated at 100 °C for 5 min. Following centrifugation at 13 000 *g* for 15 min, the supernatant was removed, the pellet extracted again with the same volume of buffer and the combined supernatants stored at −20 °C.

The cornea and scleral tissues were cut into pieces measuring approximately 2 × 1 × 1 mm and washed twice with PBS. Each tissue fragment was extracted twice with a volume of sample buffer equivalent to 25 μl for each piece of tissue and the tissue extracts were pooled.

Protein concentration was estimated using a comparative dot-blotting technique with known concentrations of prepared standard solutions of bovine serum albumin (BSA). The dot-blotted proteins were visualised using Protogold, a colloidal gold sol that binds quantitatively to proteins blotted onto a membrane. The method used was that recommended by the manufacturers (British Biocell). The colour intensity of the dots was compared visually with that of the standards to give an approximate value of protein concentration.

Gel electrophoresis

The tissue extracts from individual eyes were each investigated separately and, where possible, extracts of

TM, cornea and sclera from each eye were analysed on the same gel.

The urea/SDS/DTT-solubilised extracts (10 μl samples, containing approximately equal amounts of protein) were separated by SDS/PAGE on either 6.5% (w/v) polyacrylamide gels or ready-prepared 4–20% (w/v) gradient gels (BioRad) using the BioRad mini-Protean system (gel size 10 × 8 mm) adapted from the method of Laemmli.[31] Electrophoresis was carried out in a Tris/glycine buffer system, pH 8.3 (0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS), at a constant voltage of 200 V at 60 mA for 40 min. A mixture of biotinylated protein standards was included with each electrophoretic analysis.

The separated proteins were electroblotted onto 0.45 μm PVDF membrane using a tank transfer system (Hoefer Mighty Small Transphor unit) with the same transfer buffer as was used in the SDS/PAGE but with 20% (v/v) methanol included. Transfer was carried out at 100 V and 400 mA for 1.5 h.

Total protein staining

The blotted membrane was blocked with 0.3% (v/v) Tween-20 in PBS for 3 h at room temperature (RT), washed in distilled water (2 × 2 min) and stained with Protogold for 2–4 h. Excess Protogold was rinsed off with two 5 min washes.

Immuno- and lectin blotting

For immunoblotting, the membrane was blocked with 5% BSA (w/v) overnight at 4 °C and incubated with primary antibody for 3 h at RT. Polyclonal type VI collagen antibodies were diluted 1:1000 and monoclonal antibodies 1:500 in PBS containing 1% BSA (w/v), 1% (v/v) normal serum of the species in which the secondary antibody was raised and 0.05% Tween 20 (v/v). After three 10 min washes in PBS containing 0.1% (w/v) BSA, the membrane was incubated for 3 h at RT with biotinylated secondary goat antibody to rabbit immunoglobulins (following a polyclonal primary antibody) or mouse immunoglobulins (following a monoclonal primary antibody). After three 10 min washes the membrane was incubated overnight with gold-labelled anti-biotin at RT and finally, after following the same washing procedure, the gold label was silver-enhanced. This was done by incubating the membrane in a solution containing equal quantities of initiator and enhancer (Biocell) for 15–20 min, after which the membrane was rinsed for 3 min with water.

A negative control was included where the primary antibody was replaced with buffer or non-immune serum. BSA and normal serum from the species of the secondary antibody were included to block non-specific binding. A type VI collagen-containing extract from cartilage and a purified FN extract were run simultaneously on one gel to act as positive controls for the type VI collagen and FN antibodies.

For lectin staining, the membrane was blocked overnight in Tris-buffered saline (TBS, 0.05 m, pH 7.6) with 1 mM calcium chloride and 0.05% (v/v) Tween 20. The blotted membranes were incubated with biotinylated lectin (concentration 1 μg/ml), diluted in the blocking solution, for 2 h at RT. Following three 10 min washes in the same buffer, the membrane was incubated with anti-biotin gold diluted 1:100 in the same buffer

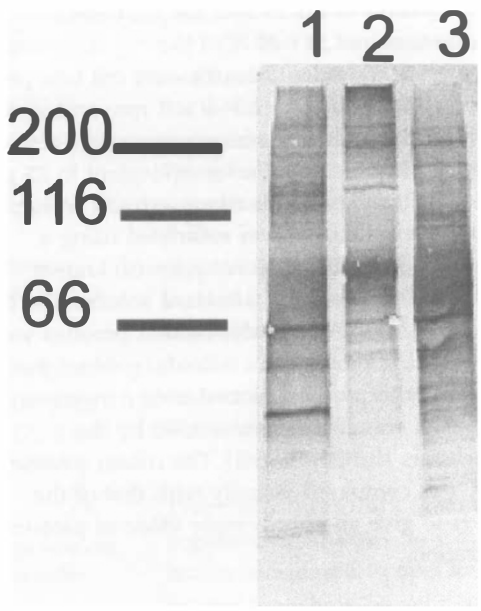


Fig. 1. *Analysis of protein in urea/SDS extracts from sclera (lane 1), cornea (lane 2) and trabecular meshwork (TM; lane 3) on Western blots visualised with Protogold (4–20% gel). High-molecular-weight components at ~240, 200 and ~130 kDa are visible in all three lanes.*

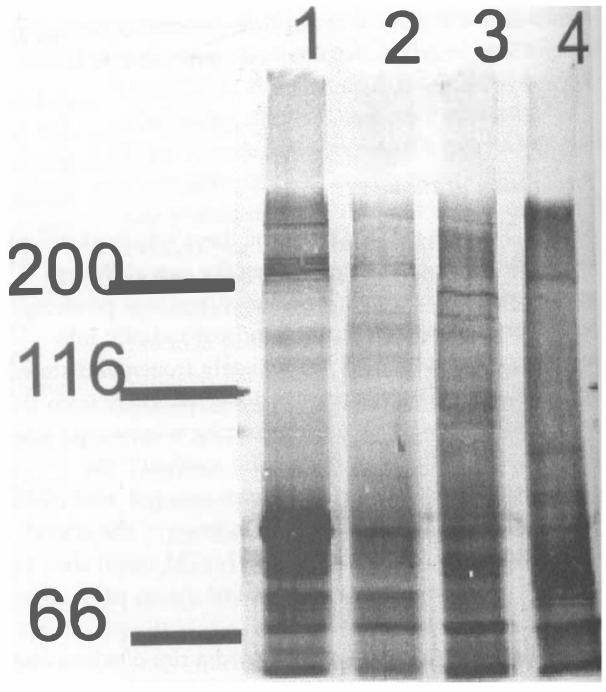


Fig. 2. *Analysis of protein in urea/SDS extracts from cornea (lane 1), sclera (lane 2) and TM (lane 3) from the same eye and TM from a different eye (lane 4) on Western blots visualised with Protogold (6.5% gel). In normal TM there is one main component at ~200 kDa. In cornea and sclera a ladder of bands is visible at ~200 kDa.*

overnight at RT. After three 10 min washes the gold label was enhanced with silver. For a negative control, the lectin was replaced by TBS. Blots were analysed using the Phoretix 1D Analysis system (Phoretix International, Newcastle, UK).

Lectin specificity

The specificity of the lectin binding was confirmed by incubating the lectin with the appropriate inhibiting sugar prior to lectin blotting. PSA (major specificity α-D-mannose in non-bisected bi/tri-antennary *N*-linked

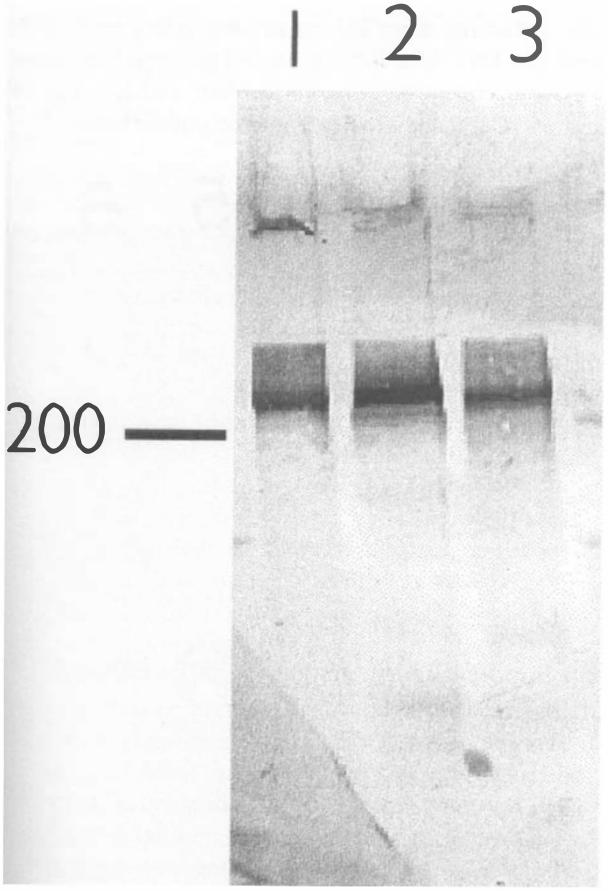


Fig. 3. *Identification of fibronectin (FN) in urea/SDS extracts from TM (lane1), cornea (lane 2) and sclera (lane 3) from the same eye. Immunoblot with anti-FN antibody.*

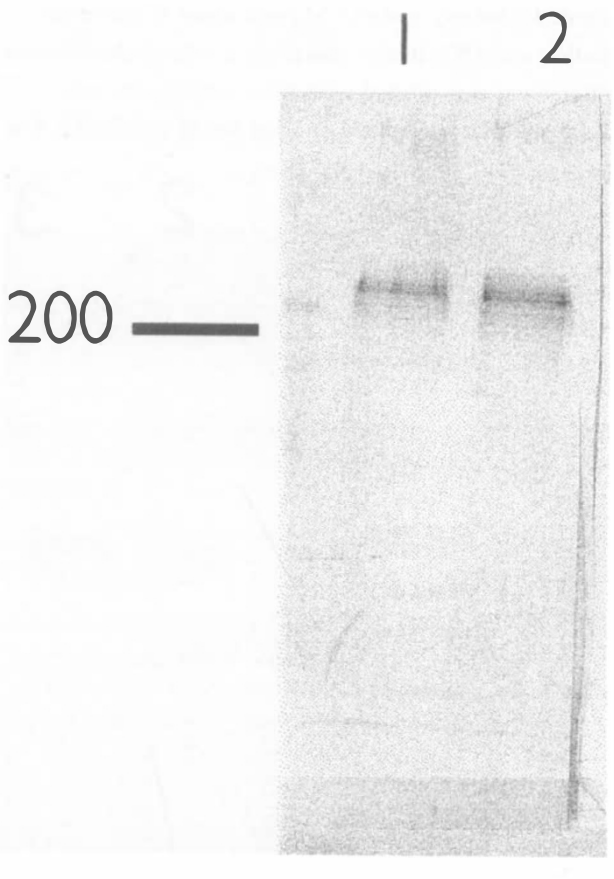


Fig. 4. *Identification of FN in urea/SDS extracts from TM (lane 1) and sclera (lane 2). Immunoblot with anti-FN antibody. The band at 240 kDa is visible as a doublet.*

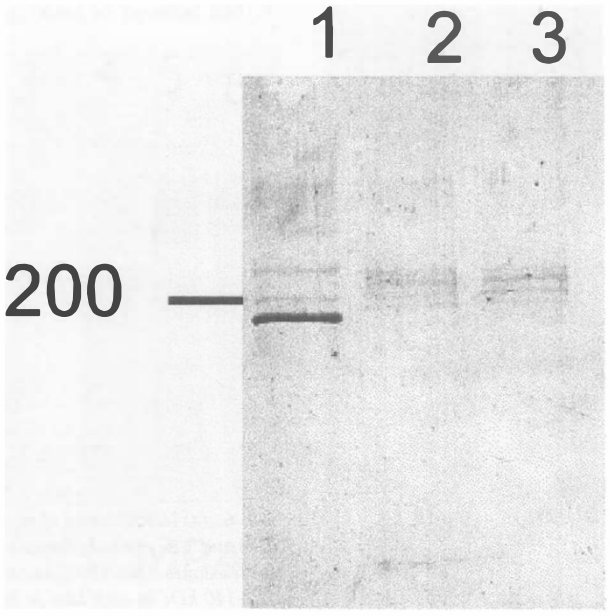


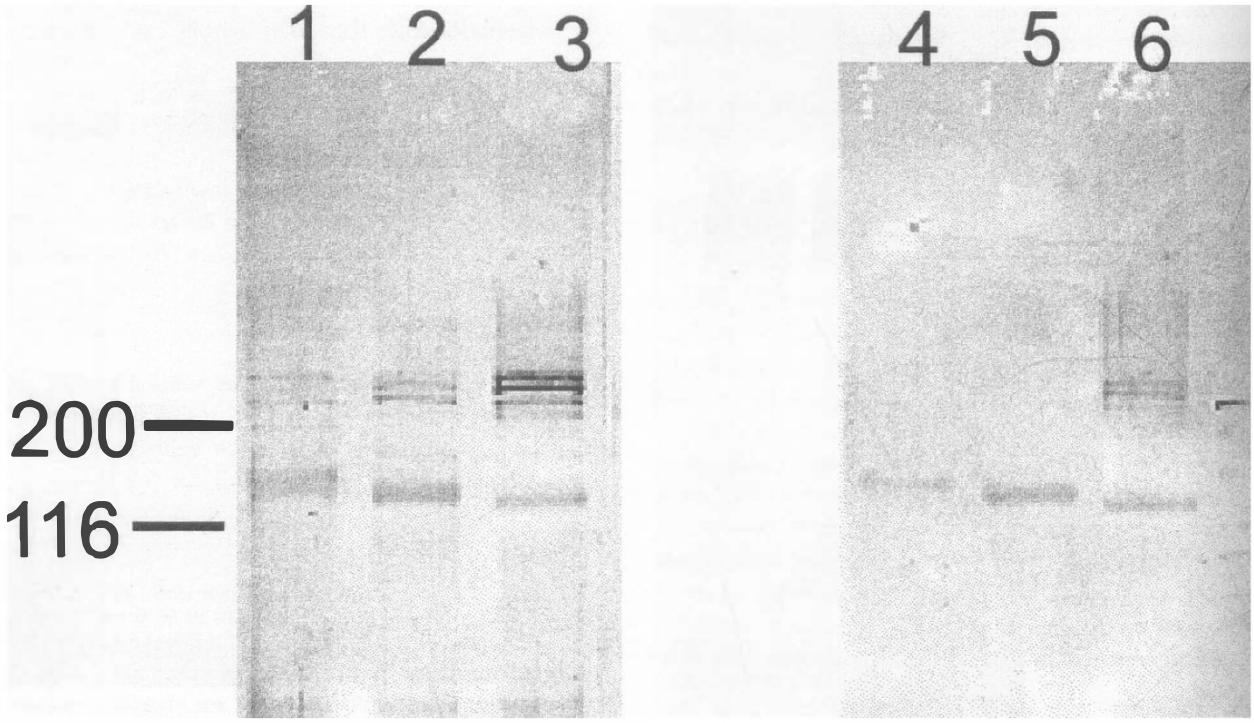
Fig. 5. *Identification of type VI collagen with VIB antibody in TM (lane 1), sclera (lane 2) and cornea (lane 3) from the same eye.*

glycans) was diluted to 1 μg/l with 0.5 M α-methyl mannopyranoside in 0.05 M TBS with 1 mM calcium chloride and 0.05% Tween 20. MPA (major specificity Galβ1, 3GalNAc-, where Gal is galactose and GalNAc is *N*-acetyl galactosamine, on *O*-linked glycans) was diluted similarly with 0.5 M D-galactose in the same buffer, and DSA (major specificity *N*-acetyl glucosamine oligomers) was diluted with D-*N*-acetylglucosamine. Each solution was mixed and left for 30 min at RT. The inhibited lectins were each used in a parallel lane and compared with the uninhibited lectin on the same blot.

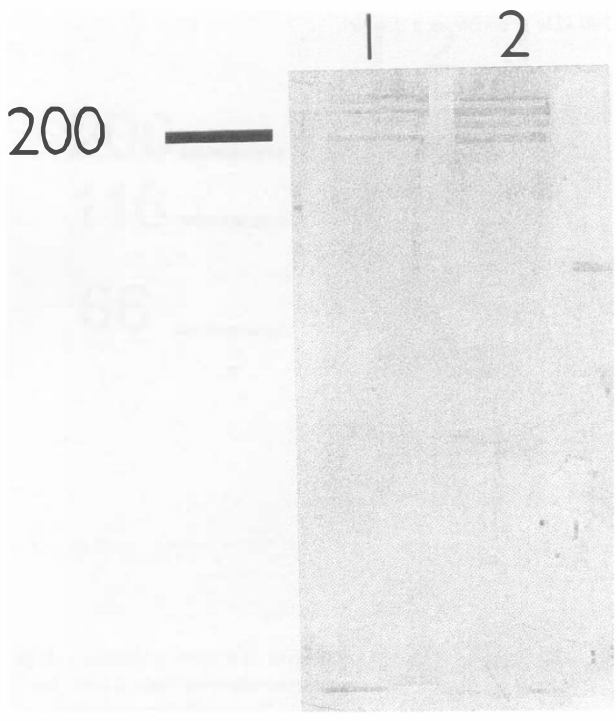
Results

Total protein staining

The extractions from TM, cornea and sclera with SDS/urea and DTT on 4–20% graduated gels produced major high-molecular-weight bands at ~240, 180–200, 125–130 and 66 kDa, together with numerous other faster-



(a)



(b)

Fig. 6. *(a) Identification of type VI collagen with VIB antibody (lanes 1–3) and VIG antibody (lanes 4–6) in TM (lanes 1, 4), sclera (2, 5) and cornea (lanes 3, 6). The tissue extracts are from the same eye. The band at ~140 kDa in each lane is the co-migrating α1/α2 (VI) chain. The bands at ~200 kDa in each lane are alternatively spliced forms of the α3 (VI) chain. (b) Type VI collagen-containing extract immunoblotted with VIB antibody. Four bands at ~200 kDa are visible.*

Table 1. *Lectin binding to components of trabecular meshwork (TM), cornea and sclera following analysis of SDS/PAGE*

	Relative molecular mass[a]							
	240	200	170	140	120	95	66	45
Normal TM								
PSA	++	+	+	+		+		+
MPA	+	+		+	+			
DSA	++	+	+	+		++		+
Cornea								
PSA	+	++		+			+	
MPA	+	+			+		+	
DSA	+	+		+			+	
Sclera								
PSA	+	++		+			+	+
MPA	+	++			+		+	+
DSA	+	+		+			+	+

PSA, *Pisum sativum* agglutinin; MPA, *Maclura pomifera* agglutinin; DSA, *Datura stramonium* agglutinin.
[a]Of the principal components observed following electrophoresis.
+, binding of the lectin to one band at the size indicated; ++, binding of the lectin to a doublet at the size indicated.

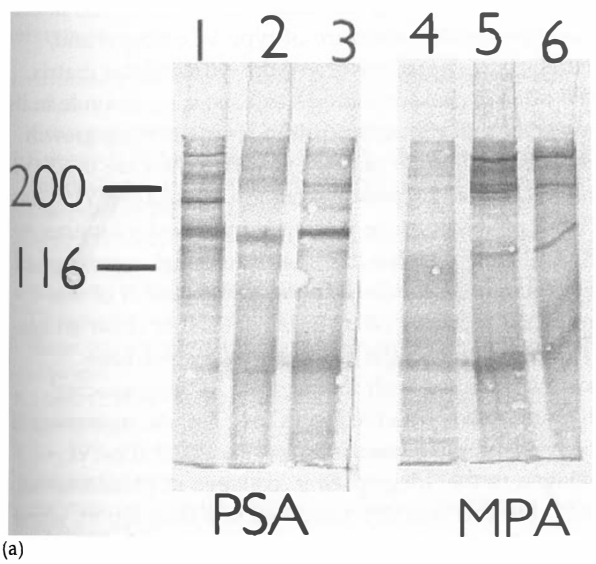
migrating bands. The pattern of major bands produced on analysis of each tissue was the same in individual eyes, with some minor variations in the relative intensities of individual bands. A band at ~100 kDa, together with other less distinct bands, was also observed in extractions from normal TM (Fig. 1). When a 6.5% PAGE gel was used, two bands at 140 and 130 kDa were seen in cornea and sclera (Fig. 2).

Molecular weight estimation of GP on SDS/PAGE is more accurate on gradient gels than on uniform gels; the latter tend to overestimate molecular weight because of the glycan content in the GP. It was assumed that the 125–130 kDa component on 4–20% gradient gels is the same as the 140 kDa component on a 6.5% gel.

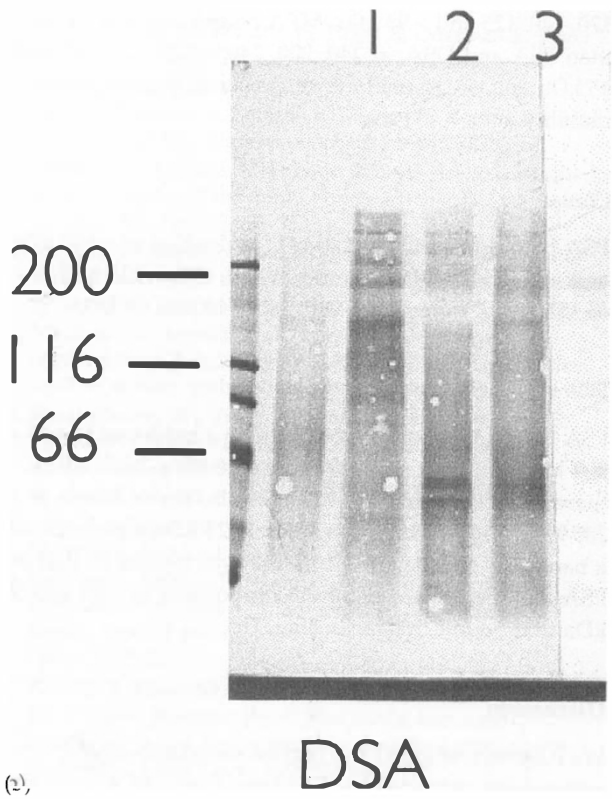
The main differences observed between the extracts of TM, sclera and cornea were as follows: The single band at ~200 kDa in normal TM was seen as a doublet or as multiple bands in cornea and sclera (Figs. 1, 2). The 140 kDa band in cornea and sclera was relatively stronger than the corresponding band in normal TM. A band at ~30 kDa was variable in intensity and was seen prominently in some extracts of sclera, but much less so in others.

Immunoblotting

Anti-FN antibody bound to the 240 kDa band in normal TM, cornea and sclera (Fig. 3) and was sometimes seen as a doublet (Fig. 4). The control was negative. The antibody bound extensively to the FN standard and degradation products. The VIB collagen antibody, with principal specificity for the α3 (VI) chain, bound to three bands at



(a)



(b)

Fig. 7 *(a) Localisation of glycans present in SDS/urea extracts from TM (lanes 1, 4), sclera (lanes 2, 5) and cornea (lanes 3, 6) from the same eye with lectins PSA (lanes 1–3) and MPA (lanes 4–6). (b) Localisation of glycans present in TM (lane 1), sclera (lane 2) and cornea (lane 3) with lectin DSA.*

~200 kDa in normal TM (Figs. 5, 6a). In six of the normal TMs the antibody binding was much stronger to one of the bands compared with the other two (Fig. 5). A prominent band in the same position was observed on blots of normal TM that were stained for total protein. Extracts of cornea and sclera from the same eyes (on the same blot), showed a ladder of four closely spaced bands at ~200 kDa (Figs. 5, 6a). Fig. 6b shows an immunoblot of the type VI collagen-containing extract and the VIB antibody where the four components are visible. Extracts of TM, cornea and sclera from one eye also each showed a band at ~140 kDa, with a slightly differing mobility, that was immunoreactive with both type VI collagen antibodies (Fig. 6a). The VIG antibody with specificity for α1α2 (VI) bound to the 140 kDa component in TM, cornea and sclera (Fig. 6a).

Lectin blotting

The binding specificity of the lectins was confirmed by the inhibition experiments, in which the binding of PSA and DSA was totally eliminated and that of MPA was very much reduced. Replacement of the lectin with saline gave a negative result with gold-labelled antibiotin. PSA and MPA binding were in general better defined than that of DSA, which was more diffuse. The results of lectin blotting are summarised in Table 1. An example of lectin blotting of TM, cornea and sclera is shown in Fig. 7.

Normal TM

PSA and DSA bound most strongly to bands at 240, 200, 170, 140, 125 and ~95 kDa. MPA bound, but less strongly than PSA and DSA, at 240, 200, 140, ~125, 97, ~70 and 45 kDa and occasionally to other bands with a greater mobility.

Cornea

PSA bound to bands at 240 kDa, a doublet at ~190 kDa, and at 140 kDa. MPA bound at 240, 190, ~125 and 66 kDa. DSA bound at ~240, 200, 140 and 66 kDa.

Sclera

PSA bound to a band at 240 kDa, to a ladder of bands at 200 kDa, and to a single band at 140 kDa. With MPA, there was binding at 240 kDa, to a ladder of bands at 200 kDa, and to a component at ~125 kDa together with a band at ~60 kDa. DSA binding was similar to that of PSA and there were also two components at ~60 and 50 kDa that bound this lectin (Fig. 7b).

Discussion

We have investigated the higher-molecular-weight components extracted by SDS/urea from TM, cornea and sclera. The component at 240 kDa, which was seen in normal TM, cornea and sclera was identified as FN on the basis of the immunoblotting results with an FN antibody. FN is a dimer of ~250 kDa disulphide-bonded subunits. Each subunit consists of a series of domains having specific functions that include binding to cells, heparin, fibrin and assembly of fibronectin fibrils.[32] FN is believed to play an important role in ECM remodelling as well as in cell attachment of trabecular cells to basement membrane of trabecular beams. An increased deposition of FN in TM in primary open angle glaucoma (POAG) has been postulated, although it has not been quantified.[33] In cornea, FN has been localised in Descemet's membrane and stroma[34] and has also been implicated in epithelial wound healing.

We have demonstrated by immunoblotting that, in reduced extracts of normal TM, the prominent band at ~200 kDa is the α3 (VI) chain. The ladder of bands in the range 180–240 kDa, which is visible in corneal and scleral extracts and binds anti-type VI antibody, is characteristic of the α3 (VI) chain and has been reported in type VI collagen extracts from numerous other tissues.[20,35] The band at ~140 kDa on 6.5% gels (125–130 kDa on 4–20% gels) observed in extracts of TM, cornea and sclera is identified as the co-migrating α1/α2 (VI) chains. Each of the three genetically distinct α chains consists of a short triple helical region with large N- and C-terminal globular domains.[19] The globular domains consist of repetitive sub-domains, and alternative splicing can occur in the C-terminal end of the α2 (VI) chain and in at least four subdomains of the N-terminal domain of the α3 (VI) chain.[36-38]

The ladder of bands observed in the type VI collagen standard and corneal and scleral extracts is therefore identified as the alternatively spliced forms of the α3 (VI) chain. It appears that they are produced in different proportions in normal TM, cornea and sclera, with normal TM producing relatively more of one variant, observed as a prominent band at ~200 kDa in blots of TM extractions stained for total protein. Two of the domains include cysteine residues and potential *N*-glycosylation sites, and hence alternative splicing could affect protein folding and function.[39] It may also affect the microfibrillar structure of type VI collagen and, thereby, the characteristics of the extracellular matrix. The α3 (VI) chain is thought to have a crucial role in its assembly and tissue deposition. Transforming growth factor beta (TGF-β) causes a selective increase in α3 (VI) mRNA and an increased secretion of collagen VI.[40] As TGF-β is known to be increased in aqueous humour in POAG[41] it is possible that the differential expression of α3 (VI) may be involved in the pathogenesis of this condition. Type VI collagen is believed to show an age-related increase in the TM but this has not been positively linked with the increase in 'long-spacing' (100 nm) that is observed in POAG TM. An immunogold study at the ultrastructural level localised type VI collagen to the 'long-spacing' collagen in glaucomatous TM,[42] but this was not confirmed in a later report, where it was identified in the fine network of trabecular cores and the juxtacanalicular network.[11]

Type VI collagen is a major component of cornea[24] and has been localised immunocytochemically at the

ultrastructural level in epithelial basement membrane, stroma and Descemet's membrane, though not in the 'long-spacing' collagen.[43] It is also one of the components of the interfibrillar matrix in sclera.[44]

The binding of PSA, DSA and MPA to FN of TM, cornea and sclera indicates the presence of complex, bi/tri-antennary *N*-linked glycans and some *O*-linked glycans. This concurs with reports of known carbohydrates in human FN which, depending on the tissue source, contain bi- or tri-antennary groups. The complex *N*-linked glycans identified on the α3 chains of type VI collagen are linked to asparagine residues in the triple helix where two potential *N*-linked acceptor sites are fully occupied[26] or to sites in the globular domain. The *O*-linked glycosides of the triple helix of VI collagen are linked to hydroxylysine and not to serine or threonine.[26] Since MPA binds only to glycans that are *O*-linked via serine or threonine, it is probably binding to sites on the globular domains of the α3 (VI) chain in TM, cornea and sclera. MPA binding to the component at 140 kDa in normal TM suggests that *O*-linked glycans are also present in the globular domains of the α1/α2 (VI) chains. These glycans are not present (or not available) in cornea and sclera, where MPA binds to a faster component.

Conclusion

Collagen VI isoforms in TM appear to differ in two respects from those of cornea and sclera. TM produces relatively more of one variant of the α3 (VI) chain, whereas cornea and sclera express all four variants in apparently similar proportions. There appears to be a greater availability of *O*-linked Gal on the α1/α2 (VI) chains of TM than in cornea and sclera. The variation in the expression of type VI collagen isoforms in TM compared with cornea and sclera may be a reflection of the function of type VI collagen in these tissues.

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