Fibronectin, an extracellular glycoprotein, has been shown to play a role in the cellular attachment to basement membrane and cell–matrix interaction. This protein has previously been shown to be produced by human trabecular meshwork cells in culture by us and by other investigators. However, the presence of fibronectin on the extracellular surface of trabecular meshwork cells and endothelial cells lining the Schlemm’s canal has not been demonstrated by routine histochemical staining.

A recent publication of immunohistologic studies by Rodrigues et al has shown that fibronectin as well as type IV collagen and laminin are present in the subendothelial regions of the trabecular meshwork beams and also in Schlemm’s canal. These investigations reported that these substances are increased in glaucomatous eyes as compared with age-matched controls.

We speculate therefore that an increased amount of fibronectin in the corneoscleral meshwork may play a role in the resistance of aqueous outflow.

We have developed a sensitive immunoassay which utilizes avidin-biotinylated enzyme complex, (ABC) to detect fibronectin in aqueous drainage channels of human eyes. We are reporting our findings in a series of nonglaucomatous eyes of elderly patients.

Materials and Methods

Subjects

Eighteen human eyes were obtained from 13 donors (9 men and 4 women) from the San Diego Eye Bank. All eyes were enucleated within 5 hr postmortem. The ages of these donors ranged from 54 to 84 yr with an average of 71 yr (men, 72 yr; women, 69 yr). Medical records were available for nine of the 13 donors. No history of glaucoma, elevated intraocular pressure, or changes in the optic nerve head were recorded. In addition, no evidence of glaucomatous cupping was noted on gross examination of all the eyes. Four of these eyes showed pathologic findings including pseudophakia (OS-228), aphakia (OS-326), and background diabetic retinopathy (OD-317 and OS-317).

Antisera

Rabbit antihuman fibronectin antibody was purchased from Cappel Laboratory (Westchester, PA). For control serum, rabbit antiserum to herpes simplex virus (HSV) was obtained from an adult male rabbit 3 mo after corneal inoculation with 200 plaque-forming units of HSV type I. This serum has an enzyme immunofiltration assay anti-HSV titer of 1:12,000. All animals used in this study were handled in accordance with the ARVO Resolution of the Use of Animals in Research.

Purification and Biotinylation of Antisera

Both antisera were purified and biotinylated in our laboratory by the following methods. Two milliliters of serum was allowed to pass through a 5-ml affinity column of agarose-staphylococcal protein A (Zymed,
Inc.; Burlingame, CA). The column effluent was monitored for absorbance at 280 nm. When all of the non-IgG fractions had passed through, the IgG fraction was eluted with 0.1 M glycine HCl, pH 3.0. The IgG fraction was neutralized with 0.1 N NaOH and analyzed for protein concentration. The IgG fraction was dialyzed overnight at 4°C against 0.1 M NaHCO₃ at pH 8.4. After dialysis, the purified IgG fraction was adjusted to a concentration of 1 mg/ml in 0.1 M NaHCO₃, pH 8.4.

Each purified antibody was biotinylated by using N-hydroxysuccinamide ester of biotin (1.1 mg/ml) (Calbiochem; La Jolla, CA) dissolved in dimethylsulfoxide. One hundred microliters of the biotinylation solution was added to each milliliter of antibody and incubated at room temperature for 2 hr. One hundred microliters of 1.0 M NH₄Cl was added to each milliliter of antibody to block unreacted ester and incubated for an additional 10 min. One milliliter of phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) was added to each sample to stabilize the antibody. The sample was then dialyzed overnight at 4°C against PBS containing 0.01% thimerosal to remove the reactants.

Immunohistologic Studies

Biotinylated rabbit antihuman fibronectin was injected into the anterior chamber of 13 human eyes obtained from the eye bank and allowed to perfuse through the aqueous drainage pathways for 1 hr at 37°C. Three age-matched control eyes were injected with biotinylated rabbit anti-herpes simplex virus antibody. In addition, one eye was injected with 1% bovine serum albumin (BSA) and one eye received no intracameral injection. The anterior chamber of each eye, including cornea, angle structure, iris, and ciliary body was dissected from the whole globe and immediately frozen in isopentane at -30°C and embedded. Frozen sections were cut at 15 μm with an American Optical Cryostat and were mounted on gelatin-coated microscope slides.

The immunologic staining was performed as illustrated in Figure 1. The slides were washed gently with three changes of phosphate buffered saline (PBS), pH 7.4, and then incubated with either BSA buffer (PBS containing 1% BSA) only, or biotinylated anti-fibronectin or anti-herpes simplex virus antibody in BSA buffer at 37°C for 1 hr. The slides were washed again with PBS and incubated with avidin-biotinylated horseradish peroxidase complex (ABC) (Vector Laboratory; Burlingame, CA) for 30 min at 37°C. Then the slides were washed again with PBS and incubated with the peroxidase substrate solution. The substrate solution contained 0.12% 3-amino-9-ethylcarbazole (AEC), 0.03% H₂O₂, 69.85% 0.05 M acetate-acetic acid buffer, pH 5.0, and 30% N,N-dimethylformamide. The latter solution was filtered prior to use. These slides were washed and coverslipped with Aquamount (Lerner Laboratory; New Hampshire, CT) and observed using a light microscope. After immunostaining, some representative sections were stained with Mayer’s hematoxylin for comparison.
Results

Immunoperoxidase Staining after Perfusion Alone

In 12 of the 13 eyes in which antifibronectin was perfused into the anterior chamber and the sections were stained without reincubation with antifibronectin, staining was noted only in Schlemm's canal, the collecting channels, and aqueous veins. Little or no staining was noted in the trabecular meshwork area. Only one eye (OS-228) showed pronounced staining in the corneoscleral (juxtacanalicular) trabecular meshwork (Fig. 2). This eye (OS-228) was obtained 7 mo following cataract extraction with intraocular lens implantation. Pronounced staining was not observed in the other aphakic eye (OS-326). The latter eye had cataract extraction surgery 10 yr previously and had no intraocular lens implantation.

No staining of Schlemm's canal, the collecting channels, and aqueous veins was seen in eyes perfused with either anti-herpes simplex virus antibody or 1% BSA.

Immunoperoxidase Staining after Perfusion with Reincubation

In all 13 previously perfused eyes when sections were reincubated with antifibronectin prior to reaction with ABC, the staining pattern was different from that seen with perfusion alone. Not only did the aqueous drainage channels (ie, Schlemm's canal, collecting channels, and aqueous veins) show staining, but faint staining was also noted in adjacent tissues as well (Fig. 3). The latter included trabecular meshwork, sclera, surface of ciliary muscle cells (Fig. 4), subendothelial basement membrane of the conjunctiva (Fig. 5), and cornea. However, fibronectin staining was more abundant in the endothelial region of Schlemm's canal, connecting channels, and aqueous veins rather than in the adjacent trabecular meshwork and sclera (Table 1). Fibronectin staining was typically greater in the subepithelial basement membrane of the conjunctiva than in the cornea. A similar overall staining pattern was observed in the eye perfused...
The amount of fibronectin staining was quite variable from eye to eye in the 15 experimental eyes (Table 1). This variation did not seem to correlate with either age or sex. Two of the 13 eyes that were perfused with antifibronectin had background diabetic retinopathy. The staining pattern of these two eyes did not show significant alteration from that seen in normal eyes. The intensity of staining was most striking in the pseudophakic eye (OS-228). In that eye, increased staining of the juxtacanalicular meshwork was noted, in sections reincubated with antifibronectin as when sections were immunostained without reincubation (Figs. 5, 6). In addition, we also observed fibronectin at the corneoscleral wound only in that pseudophakic eye (Fig. 5).

Sections from the three eyes perfused with rabbit anti-herpes simplex virus antibody showed no staining whether staining was performed with or without reincubation with the same antibody. Thus, this control antiserum showed no nonspecific staining by either method of antibody incubation.

**Discussion**

Intraocular pressure is regulated by the aqueous outflow system; the rate of aqueous outflow is decreased with age. The cause of this decrease of outflow with age is unknown. Until now, the histologic clues to this mystery had been limited to light microscopy and electron microscopy. Recent advances in immunohistochemistry and cell culture techniques have permitted the study of fibronectin and other structural proteins that might play a role in the regulation of outflow.

We found immunostaining using the avidin-biotinylated enzyme system to be a useful technique in localizing the presence of fibronectin in human aqueous drainage channels. Our findings in elderly eyes are consistent with those of other investigators who showed that fibronectin is found in the endothelial region of the Schlemm's canal and collecting...
channels and in the aqueous veins. Staining was more prominent in the inner wall of Schlemm's canal than in the outer wall. This fact suggests that fibronectin was bound or associated with the glycosaminoglycans located in that area of the trabecular meshwork. Glycosaminoglycans have been shown to be present in greater amounts in the inner walls of Schlemm's canal than in its outer wall. Furthermore, fibronectin as well as glycosaminoglycans may be components of the electron dense amorphous material which has been observed to accumulate in human trabecular meshwork and in the endothelial lining of Schlemm's canal. Some of these investigators have suggested that these deposits are more prominent in the trabecular meshwork of aged eyes and glaucomatous eyes.

In contrast to the normotensive eyes in the study of Rodrigues and co-workers, fibronectin staining of the trabecular meshwork was minimal to none in the majority of aged eyes we examined. The difference between our findings and theirs is probably due to a difference between the methodologies. We used intact angle structures rather than trabeculectomy specimens. The source of our antifibronectin antibody was commercial and different from theirs. The perfusion technique and the immunoperoxidase (ABC) method we used in our study have not been reported in previous studies of fibronectin in human eye tissues.

We observed a different immunologic staining pattern in the sections from eyes that were perfused with antibody prior to sectioning than from those in which the slides were reincubated with antifibronectin after sectioning. The different staining pattern might be due to the difference in exposure to antibody. During the perfusion incubation, the antibody could only reach the antigenic sites exposed along the lumina of the drainage channels; whereas when the antibody was applied to the sections for reincubation, all fibronectin deposits were exposed to the antibody and could be stained.
Fig. 5. Increased fibronectin staining is seen in the corneoscleral (juxtacanalicular) trabecular meshwork (arrowhead) and corneal wound (arrow) in an eye 7 mo post-cataract extraction (OS-228). This eye was perfused with antifibronectin and the section has been reincubated with the same antibody. Staining is also seen in the endothelial basement membrane of the conjunctiva (B), Schlemm’s canal (S), anterior chamber (A) (not counterstained, X140).

The observation that increased staining was also demonstrated in the aqueous drainage channels of the two eyes that were not perfused with antifibronectin indicates that there are higher levels of fibronectin along the drainage channels, and it is not an artifact of the perfusion technique. Previous studies

Table 1. Distribution of fibronectin in human aqueous drainage channels and adjacent tissues

<table>
<thead>
<tr>
<th>Human eye no.</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Trabecular meshwork</th>
<th>Schlemm’s canal</th>
<th>Aqueous veins (Anterior ciliary v.)</th>
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<th>Basement membrane of cornea</th>
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NE: Not examined.
* Pseudophakia.
† Background diabetic retinopathy.
‡ Aphakia.
of Yamada and Olden\textsuperscript{15} suggested that fibronectin is present in the basement membrane of all blood vessels. Therefore, we speculate that the staining seen in our sections was probably due to fibronectin both in the basement membrane and on the exposed cell surfaces of the aqueous drainage channels. However, we cannot exclude that some of the staining along these drainage channels is contributed by plasma fibronectin, since the antibody we used binds with both forms. We feel that this latter explanation is not likely, since there was no staining along the external surfaces of trabecular beams or the endothelial surface of the corneal endothelium, both of which were also exposed to the plasma fibronectin present in the aqueous humor.

Recent surgical trauma and inflammation associated with intraocular lens implantation may have played a role in the increased concentration of fibronectin found in the angle and in the surgical wound of the pseudophakic eye. Increased fibronectin staining may be transient, as in the case 10 yr after cataract surgery in which there was no increased concentration of fibronectin staining at the wound. Studies of fibronectin deposition at wound sites in rabbit corneas support this view.\textsuperscript{16}

These results add information to the quest to uncovering the pathogenesis of chronic open-angle glaucoma. The findings also suggest a role for fibronectin in glaucoma secondary to inflammation associated with surgical trauma. Finally, these studies in older age eyes can serve as a basis of comparison for our future studies in eyes from patients with chronic open-angle glaucoma.

Key words: fibronectin, trabecular meshwork, immunoperoxidase staining, human

Acknowledgments

The authors thank Dr. Allen F. Ryan and Charles Graham for their technical advice.

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