Fibronectin in the Trabecular Meshwork: Immunofluorescent and Immunoelectron Microscopic Findings

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We examined the fibronectin (FN) secretion of cultured trabecular meshwork (TM) cells in a normal human eye by indirect immunofluorescent technique using mouse anti-human FN monoclonal antibody and FITC-conjugated goat anti-mouse IgG. To localize FN on frozen sections of normal TM, which were obtained from 7 enucleated eyes owing to traumatic eyeball rupture, the same indirect immunofluorescent method was used. Immunoelectron microscopy was applied to demonstrate the distribution pattern of FN in the normal TM of 2 human eyes using an avidin-biotin-peroxidase complex method.

In the tissue culture of TM, the TM cell walls and extracellular matrices showed an intense staining with antibody to FN. Indirect immunofluorescent staining of FN on frozen sections of TM showed strong positive reactions in the subendothelial region. There was no reaction in the central core of the trabecular beam. Immunoelectron microscopy revealed the reaction products to FN in the areas lining the trabecular endothelial cells.

Key words: fibronectin, trabecular meshwork, tissue culture, indirect immunofluorescence, immunoelectron microscopy.

INTRODUCTION

It is generally accepted that in primary open-angle glaucoma the increase of intraocular pressure is mainly caused by the increase of aqueous outflow resistance in the trabecular meshwork (TM). Although the mechanism of increase of aqueous outflow resistance remains controversial, TM cells and extracellular matrices have been implicated in many reports.1-6 The macromolecular components of the extracellular matrix of the TM are several types of collagen, elastin, proteoglycan, laminin, and fibronectin.7 Of these, fibronectin, an extracellular glycoprotein, has been shown to play a role in the cellular attachment to the basement membrane and cell-matrix interaction.8,9 Recently, some histochemical attempts to demonstrate fibronectin in the aqueous outflow pathway in human eyes have been made.10-14 However, since the identity and distribution of fibronectin in the TM have been inconsistent among previous reports, and fibronectin may have the potentially important role in the aqueous outflow resistance, additional investigations are needed. We have studied the fibronectin secretion by cultured TM cells and the localization of fibronectin in the human TM by
immunofluorescent and immunoelectron microscopic examinations. We are reporting our findings in a series of nonglaucomatous eyes of young patients.

**MATERIALS AND METHODS**

We used nonglaucomatous human trabecular meshworks from 10 eyeballs, which were enucleated owing to traumatic rupture of the eyeballs. The 10 patients were all males, and their ages ranged from 16 to 40 years. There was no history of glaucoma, and no evidence of glaucomatous cupping was noted on gross examination of all the eyes. Three types of experiments were performed.

**Tissue culture of TM explants and immunofluorescent staining**

The eyeball of a 17-year-old patient was used within one hour of enucleation. We opened the globe at the ora serrata and removed the anterior segment. After removal of the lens, the iris, and the ciliary body, the anterior segment was divided into 12 segments with a razor blade under a stereomicroscope. Two parallel cuts were made in the trabecular tissue, one in front of the scleral spur and the other just posterior to the Schwalbe's line. The TM was gently lifted away from the Schlemm's canal. These segments of TM were used as explants after examination by phase-contrast microscopy. The explants on the sterile glass coverslips were placed into a 6-well plate (Nunclon, Denmark), which was filled with culture medium consisting of Dulbecco’s modified Eagle’s Medium (Flow, U. K.), 10% fetal calf serum (Flow, U. K.), 250 ng/ml fibroblast growth factor (Chemicon, U. S. A.), 50 mg/L gentamicin, and 1.25 mg/L amphotericin B. Explants were incubated at 37°C in 5% CO₂, and media were changed twice a week for 3 weeks. The specimens were examined by phase-contrast microscopy.

At 45 days in culture, indirect immunofluorescent staining was done on the monolayer culture of the TM cells after fixation in acetone. Mouse anti-human fibronectin antibody (BioGenix, U. S. A.) was used as the primary antibody and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Atlantic, U. S. A.) was used as the secondary antibody. The staining was observed with a fluorescence microscope (Nikon, Japan).

**Immunofluorescent fibronectin staining of TM**

To identify and localize the fibronectin in nonglaucomatous human TM, we used fresh specimens of 7 normal human eyes. After careful dissection of the enucleated eyes, TM blocks were embedded in Tissue-Tek, rapidly frozen at −70°C and sectioned at 20 μm with a microtome. Indirect immunofluorescent staining of fibronectin on frozen sections of TM was carried out with the same antibodies used in the culture staining, and the staining was observed with a fluorescence microscope.

**Immunoperoxidase fibronectin staining of TM**

Two normal human eyes were used to demonstrate the distribution pattern of fibronectin in TM. The dissected TM blocks were prefixed with 0.5% glutaraldehyde and 2% paraformaldehyde. The sections were incubated with mouse anti-human fibronectin antibody (Chemicon, U.S.A.), biotinylated goat anti-mouse IgG (BioGenix, U.S.A.), and horseradish peroxidase (HRP)-labeled avidin, one by one. Diaminobenzidine (Sigma, U.S.A.) was applied as a chromogen, and the sections were postfixed with 1% osmium tetroxide, dehydrated in ethanol, and embedded in Epon 812. The Epon blocks were cut at 1 μm. After detecting the reaction area by light microscopy, the blocks were sectioned into ultrathin pieces at 600 Å, and the sections were observed with a transmission microscope (Hitachi 600, Japan).

**RESULTS**

**Tissue culture of TM and secretion of fibronectin by cultured TM cells**

All initial explants of the TM showed the characteristics of the trabecular beams (cords and sheets) by phase-contrast microscopy (Fig. 1). In the tissue culture, initial outgrowth of the TM cells around the explants appeared in about 3 weeks. At 29 days, many round active cells were floating in the culture, and the somewhat elon-
gated cells were adhering to the culture plate (Fig. 2). The edge of the monolayer culture showed a dendritic configuration at the margin of the elongated flattened cells at 45 days in culture (Fig. 3). In indirect immunofluorescent staining of the TM confluent culture, the TM cell walls and extracellular matrices showed intense staining with antibody to fibronectin (Figs. 4-6).

Localization of fibronectin in TM by immunofluorescent staining

Indirect immunofluorescent staining on frozen sections of TM showed strong positive reactions in the subendothelial region. There was no reaction in the central core of the trabecular beam (Figs. 7-10).

Localization of fibronectin in TM by immuno electron microscopic examination

Reaction products to fibronectin were localized to the area lining the trabecular endothelial cells. There was no reaction product in the central core region (Figs. 11, 12).

DISCUSSION

TM cells may play a major role in the pathogenesis of glaucoma. The cells have a variety of functions, including the synthesis and degradation of extracellular materials and the phagocytosis of extracellular debris. Human TM cells have been studied in cell, tissue, and organ cultures, and cultured TM cells have previously been shown to produce extracellular matrices. Explant culture preserves the cell position on the trabecular beams. However, cells are not exposed to the normal one-directional flow of fluid as occurs in vivo, and Alvarado et al. report that there are some differences in the cultured TM cells compared with uncultured TM cells. Although the condition of explant culture is not completely the same as that in vivo, we believe that the use of explant culture to evaluate the secretion of fibronectin by TM cells may be justified.

It is very important to demonstrate that the cultured TM cells are truly of trabecular origin. The human TM is small and delicate, and the dissected TM can be easily contaminated with keratocytes, corneal endothelium, scleral fibroblasts, and iris melanocytes. To prevent contamination, we washed the eyeball, dissected the trabecular tissue very carefully under a stereomicroscope by the Tripathi and Tripathi method and checked the dissected tissue by phase-contrast microscopy.

We used a monoclonal fibronectin antibody on frozen sections to identify and localize the fibronectin of the nonglaucomatous human TM. Our study showed a distinct localization of fibronectin to the narrow area of the subendothelial region of the trabecular beam. Studies of Rodrigues et al. suggest that fibronectin may be one of several glycoproteins found in the peripheral portion of the trabecular beams in aged eyes. Murphy et al. also find subendothelial localization of fibronectin in the TM of relatively young individuals. In contrast to these studies, Floyd et al. report that fibronectin staining of the TM is minimal to non in the majority of aged eyes. They pointed out that the difference might be due to a difference between methodologies.

It is important to obtain intact specimens. In most cases, trabeculectomy specimens or donor eyes from the eye bank were used. Trabeculectomy specimens have certain limitations, including the possibility of operative artefact, relatively small tissue size and possible effects of ocular medication or laser treatment before trabeculectomy. TM tissue from the cold-stored eye is somewhat different from the normal tissue in situ. We therefore used only fresh specimens, although we could not obtain many.

Methods of staining varied among the studies. We used indirect immunofluorescent staining of fibronectin on the frozen section of TM, and to do more sensitive immunoassay, avidin-biotinperoxidase complex(ABC) was used in the immuno electron microscopic examination. The ABC method is known to be a sensitive technique in localizing the presence of fibronectin in human aqueous drainage channels.

We found that fibronectin was specifically localized to the area lining the trabecular endothelial cells by immunoelectron microscopy. Actin-like filaments in the cytoplasm of trabecular endothelial cells may play a role in the binding of fibronectin to the subendothelial matrix. We think that fibronectin lining the trabecular endothelial cells may influence the resistance of
aqueous outflow by interaction with other extracellular matrices surrounding the trabecular beam such as glycosaminoglycan and collagen. TM cells have phagocytic activity. TM cells which are actively engaged in phagocytosis in vivo often detach from the trabecular beam, and consequent loss of TM cells may be a factor in the pathogenesis of the aging and the glaucoma. Fibronectin may act as a glue-like molecule between the TM cells and extracellular matrices and help prevent cellular detachment from the trabecular beam in the presence of stress, such as fluid pressure and phagocytosis.

In this way, changes of fibronectin in the TM may possibly lead to trabecular dysfunction and an increase of intraocular pressure as observed in the aging and in glaucoma. Our findings provide a standard for comparison with aged and glaucomatous TM tissues.

REFERENCES

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LEGENDS FOR FIGURES

Fig. 1. The initial explant of human trabecular meshwork shows uveal (arrowheads) and corneoscleral (arrows) trabecular beams (phase-contrast micrograph, ×400).

Fig. 2. Culture of the trabecular meshwork cells is seen after 29 days in culture. The round active cells are floating, and the somewhat elongated cells adhere to the culture plate (phase-contrast micrograph, ×100).

Fig. 3. The edge of the monolayer culture of the trabecular meshwork cells shows a dendritic configuration at the margin of the elongated flattened cells after 45 days in culture (phase-contrast micrograph, ×100).

Fig. 4. Immunofluorescence of fibronectin staining in confluent culture of human trabecular meshwork cells is shown. Fluorescent staining is in a reticular pattern corresponding to the border of the cells (fluorescence micrograph, ×400).

Fig. 5. The fibronectin produced by human trabecular meshwork cells in culture is seen. Fluorescent staining is faint in the cytoplasm (solid arrow) but strong in the cell walls and extracellular matrices (open arrow) (fluorescence micrograph, ×400).

Fig. 6. Immunofluorescence of fibronectin staining can be seen in human trabecular meshwork cells in culture. Note the strong fluorescence in the cell walls and extracellular matrices (arrows) (fluorescence micrograph, ×400).

Fig. 7. A frozen section of uveal meshwork is stained with fibronectin antibody. There are strong positive reactions in the subendothelial region (small arrows) of the trabecular beam and no reaction in the central core region (large arrow) (fluorescence micrograph, ×400).

Fig. 8. Corneoscleral meshwork is stained with fibronectin antibody. Fibronectin is also localized to the subendothelial region (small arrows) of the trabecular beam, and the central cores (large arrows) are not stained (fluorescence micrograph, ×400).

Fig. 9. Subendothelial localization of fibronectin takes place in the uveal (U) and corneoscleral (CS) meshwork (fluorescence micrograph, ×400).

Fig. 10. Cribriform meshwork is stained with fibronectin antibody. Fibronectin is densely distributed in the subendothelial region (small arrows) and absent in the central core region (large arrows) (fluorescence micrograph, ×400).

Fig. 11. Immunoelectron micrograph of trabecular meshwork stained with fibronectin antibody can be seen. Reaction products (arrows) are localized to the area lining the trabecular endothelial cells. Note the absence of reaction product in the central core region (C) (×8,000).

Fig. 12. Immunoelectron micrograph shows the trabecular meshwork stained with fibronectin antibody. There are many reaction products (arrows) in the area lining the trabecular endothelial cells (E) and no reaction product in the central core region (C) (×12,000).