Immunofluorescent Studies of Fibronectin and Laminin in the Human Eye

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The topographic distribution of fibronectin and laminin in young and old human eyes was determined by indirect immunofluorescent techniques. These two glycoproteins may play a role in the attachment of the vitreous to the internal limiting membrane (ILM) and the internal limiting membrane to the Mueller cell processes. A double-laminated pattern of fluorescence for both glycoproteins was frequently found at the ILM of the posterior retina of aged eyes. This pattern of fluorescence, which was rarely seen in young eyes, may represent senescent changes in the ILM which could predispose the eye to posterior vitreous detachment.


Posterior vitreous detachment (PVD) occurs with age and in association with many ocular diseases, and plays an important role in the pathogenesis of vitreoretinal disorders, including rhegmatogenous retinal detachment, traction retinal detachment, and proliferative diabetic retinopathy. The mechanism of PVD development remains unknown, in part due to the lack of experimental models, and in part because the basis of the vitreous adhesion to the internal limiting membrane (ILM) is not understood. Although the mechanisms of vitreoretinal adhesion are unknown, it is reasonable to assume that the vitreous fibrils are attached to the basement membrane of the retina via chemical interactions between components of the two tissues.

Recent attempts to unravel the mechanism of cell-cell and cell-substratum adhesion have implicated two extracellular glycoproteins, fibronectin and laminin, as important substances which mediate the attachment of cells to their substrata. Fibronectin is produced by many different cell types; it is found on the surface of most cells and in most extracellular matrices within the body, as well as in plasma and other body fluids. Fibronectin binds with high affinity to interstitial collagens, proteoglycans, hyaluronic acid, and mesenchymal cells. Laminin is restricted primarily to basement membranes, and binds most strongly to basement membrane (type IV) collagen of epithelial cells.

Fibronectin and laminin appear to be ideal candidates as molecules which could stabilize the vitreoretinal attachment. Several brief reports on the presence of these substances in ocular tissues have appeared in the literature; however, no attention has been given to the vitreoretinal interface. In a previous report, fibronectin and laminin were found in the ILM and fibronectin was also found in the vitreous cortex of the bovine eye. This article describes the distribution of these glycoproteins at the interface between the vitreous and its adjacent tissues, as well as their distribution in selected tissues of human eyes of normal subjects ranging from 4–83 years of age.

Materials and Methods

Human Ocular Tissues

In order to elucidate possible age-related changes in the distribution of fibronectin and laminin, we grouped the eyes we examined into young (six eyes of humans 4–37 years of age), middle-aged (eight eyes of humans 42–59 years of age) and old (10 eyes of humans 61 to 83 years of age). The eyes were obtained through the Lions Doheny Eye Bank within 24 hr of death.

The unfixed eyes were cut in half in the horizontal plane and carefully examined with a dissecting microscope for the presence of posterior vitreous detachment (PVD) or other macroscopic changes. Total PVD was present in 6 of the 10 aged eyes; no PVD was present in young or middle-aged eyes. Optic atrophy, retinal tears, and some dot hemorrhages in the equatorial retina were present in three aged eyes. Five eyes in the young and middle-aged groups exhibited some focal pathological changes.
The central part of the vitreous body was carefully resected to avoid artificial separation of the vitreous cortex from the retina in subsequent manipulations. Small specimens were taken from the posterior retina with the optic nerve, the equatorial retina, and the peripheral retina with the pars plana of the ciliary body. Specimens were also taken from the cornea, lens, and ciliary body. These unfixed specimens were embedded in OCT embedding medium (Miles Laboratories, Elkhart, IN) and then frozen in liquid nitrogen. Serial frozen sections (8 μm thick) were cut at −20°C with a cryostat and dried at 35°C for 10 min. Adjacent sections were used for indirect immunofluorescence for fibronectin, laminin, and control tests.

Antibodies
Rabbit anti-human plasma fibronectin was purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, MD) and rabbit anti-laminin was purchased from EY Laboratories, Inc. (San Mateo, CA); the secondary antibodies, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit gamma globulins, were purchased from Cappel Laboratories (Westchester, Pennsylvania). The specificity of the antisera to each antigen was established by the companies from which they were obtained using immunoelectrophoresis and/or enzyme-linked immunosorbent assay. All antisera were diluted with calcium- and magnesium-free phosphate buffered saline (CMF-PBS).

Indirect Immunofluorescence
Indirect immunofluorescence techniques used for detecting fibronectin and laminin were described in detail in a previous report. The frozen sections were reacted with rabbit anti-human fibronectin (1:10 dilution with CMF-PBS) or rabbit antilaminin (1:15 dilution), washed, stained with FITC-conjugated goat anti-rabbit gamma globulin (1:20 dilution), washed again, and mounted on glass slides with 0.1% bovine serum albumin in CMF-PBS.

Fluorescent Microscopy
Stained sections were examined with a Zeiss photomicroscope III equipped with an epi-illuminator and photographed using Kodak (Rochester, NY) Tri-X Pan film. Although bright autofluorescence was evident in the retinal pigment epithelial cells, it was easily distinguishable from FITC fluorescence. The specimens were also observed by phase contrast microscopy.

Controls
Antisera to fibronectin or to laminin were substituted with CMF-PBS or normal rabbit serum as controls.

### Table 1. Distribution of fibronectin and laminin in normal human ocular tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fibronectin</th>
<th>Laminin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornea epithelium</td>
<td>(+) diffuse</td>
<td>-</td>
</tr>
<tr>
<td>BM* of epithelium</td>
<td>+ laminar/linear</td>
<td>-</td>
</tr>
<tr>
<td>Bowman’s layer</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>stroma</td>
<td>+ laminar</td>
<td>-</td>
</tr>
<tr>
<td>Descemet’s membrane</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>endothelial cells</td>
<td>+ diffuse</td>
<td>-</td>
</tr>
<tr>
<td>Lens fiber</td>
<td>(+) meshwork</td>
<td>-</td>
</tr>
<tr>
<td>epithelial cells</td>
<td>+ meshwork</td>
<td>-</td>
</tr>
<tr>
<td>capsule: inner surface</td>
<td>+ diffuse</td>
<td>+ linear</td>
</tr>
<tr>
<td>intermediate zone</td>
<td>-</td>
<td>+ diffuse</td>
</tr>
<tr>
<td>outer surface</td>
<td>+ linear</td>
<td>+ linear</td>
</tr>
<tr>
<td>Zonular Fiber</td>
<td>(+) diffuse</td>
<td>-</td>
</tr>
<tr>
<td>Vitreous body</td>
<td>(+) diffuse</td>
<td>-</td>
</tr>
<tr>
<td>vitreous cortex</td>
<td>(+) fibrillar</td>
<td>-</td>
</tr>
<tr>
<td>Ciliary Body</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BM of nonpigmented epithelium</td>
<td>(+) diffuse + linear/band</td>
<td></td>
</tr>
<tr>
<td>nonpigmented epithelium</td>
<td>(+) diffuse</td>
<td>-</td>
</tr>
<tr>
<td>BM of pigment epithelium</td>
<td>+ band</td>
<td>+ band</td>
</tr>
<tr>
<td>vessel wall</td>
<td>+ band</td>
<td>+ band</td>
</tr>
<tr>
<td>stroma</td>
<td>+ meshwork</td>
<td>-</td>
</tr>
<tr>
<td>Retina</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ILM**, peripheral region</td>
<td>(+) linear</td>
<td>+ band</td>
</tr>
<tr>
<td>equatorial region</td>
<td>(+) linear</td>
<td>+ band</td>
</tr>
<tr>
<td>posterior region</td>
<td>+ linear/laminar</td>
<td>+ linear/laminar</td>
</tr>
<tr>
<td>vessel wall</td>
<td>+ band</td>
<td>+ band/laminar</td>
</tr>
<tr>
<td>Optic Disc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ILM</td>
<td>+ band</td>
<td>+ band</td>
</tr>
<tr>
<td>vessel wall</td>
<td>+ band</td>
<td>+ laminar</td>
</tr>
<tr>
<td>lamina cribrosta</td>
<td>+ linear</td>
<td>+ linear</td>
</tr>
</tbody>
</table>

+: definite
(+): detectable, although faint
+: not detectable
* BM: basement membrane
** ILM: internal limiting membrane

Additionally, some sections were treated with antisera to fibronectin or to laminin followed by unconjugated and then FITC-conjugated secondary antibodies. Only autofluorescence was observed in these sections.

### Results
In the control experiments, whether the primary antibodies were substituted with CMF-PBS or with normal rabbit serum, only autofluorescence of the retinal pigment epithelial cells was observed. Fluorescence could be totally blocked by the use of the unconjugated secondary antibodies followed by the FITC-conjugated secondary antibodies.

Since we used only unfixed tissue, the morphology was not well-preserved, and occasionally tissues such as the internal limiting membrane and blood vessel walls detached from adjacent tissues and exposed their...
surface aspects, giving rise to excessive accumulation of fluorescence in these areas, which was recognized as an artifact. Table 1 summarizes the results of our findings without taking into account differences due to age.

Cornea

In aged eyes, the basement membrane of the corneal epithelium exhibited a definite fluorescent line of moderate intensity when reacted with antisera to fibronectin and laminin. Bowman's layer did not show any fluorescence (Fig. 1a). In some aged corneas, the surface of the corneal epithelial cells fluoresced weakly with antisera to fibronectin (Fig. 1a). The corneal stroma exhibited a laminar pattern of fluorescence for fibronectin in the interlamellar space (Figs. 1a and 1d) but no fluorescence was evident when reacted with antilaminin antibodies (Fig. 1b). Descemet's membrane exhibited detectable staining for fibronectin and laminin, although the staining patterns were different. A linear pattern of moderate fluorescence was evident on the endothelial surface of Descemet's membrane when stained with antisera to laminin (Fig. 1b), whereas the pattern of fluorescence for fibronectin was variable: diffuse (Fig. 1d), laminated (Fig. 1e), or linear (Fig. 1f). The endothelial cells exhibited positive fluorescence for fibronectin (Figs. 1e and 1f). Occasionally, in very old eyes, small excrescences of Descemet's membrane were seen which ex-

![Fig. 1. Fluorescent micrographs of the cornea stained with antisera to fibronectin (FN) or to laminin (LM) in human eyes. a, 81-year-old male. The basement membrane (arrow) of the corneal epithelium stains for fibronectin; however, Bowman's layer (B) does not. The stroma (S) exhibits a laminar pattern of fluorescence in the interlamellar space. The surface of the corneal epithelial cells (ep) fluoresces weakly with antifibronectin. b, 83-year-old female. The endothelial surface of Descemet's membrane (D) shows a fluorescent line for laminin (arrow). The corneal stroma (S) does not stain. c, 83-year-old female. Laminin is distributed at the undulating endothelial surface of Descemet's membrane (D). The small excrescences of Descemet's membrane (arrows), known as Hassall-Henle warts, are occasionally found in old eyes. d-f, Descemet's membrane (D) shows variable fluorescent pattern for fibronectin: diffuse (81-year-old male), laminated (72-year-old female), or linear (74-year-old female). The corneal stroma (S) exhibits a laminar pattern of fluorescence in the interlamellar space. The endothelial cells exhibit positive fluorescence for fibronectin (e and f, arrows). g, 4-year-old male. Descemet's membrane (D) shows a bi-laminate pattern of fluorescence for fibronectin with the stromal side staining more intensely (arrows). Occasionally a faint, diffuse stain for fibronectin was present on the surface of the endothelial cells. h, 4-year-old male. In the cornea, laminin is present only in Descemet's membrane (D), where it exhibits a bi-laminate pattern of two parallel faint bands of fluorescence (arrows). (Bar = 20 μm).](https://iovs.arvojournals.org)
Fig. 2. Fluorescent micrographs of the lens stained with antisera to fibronectin (FN) in the eye of an 82-year-old male (a), or to laminin (LM) in the eye of an 18-year-old female (b), and of a 65-year-old male (c). a, the outer surface (arrow) of the lens capsule (LC) reveals moderate fluorescence for fibronectin and the lens epithelial cells (ep) exhibit surface fluorescence for fibronectin. The degenerated lens fibers (l) fluoresce weakly with antifibronectin. b, In the young eye, laminin is present in the inner (arrow) and outer (double arrow) surfaces as well as in the intermediate zone of the lens capsule. No staining for laminin is evident in the lens epithelial cells (ep) or fibers. c, In the aged eye, the inner surface (arrow) of the lens capsule (LC) shows bright fluorescence for laminin, although the outer surface and the intermediate zone show little or no fluorescence. A part of the ciliary process with positive fluorescence at the basement membrane and in the stroma is seen at the upper left corner. (Bar = 20 μm).

Fig. 3. Fluorescent micrographs of the pars plana of the human ciliary body stained with antisera to fibronectin (FN) or to laminin (LM). a, 37-year-old female. Weak fluorescence for fibronectin is seen on the zonular fibers (zf) and nonpigmented ciliary epithelial cells (NPE) and strong fluorescence in the basement membrane (BM) of the pigment epithelium (PE) and the vessel walls in the stroma (S). b, 37-year-old female. Strong fluorescence for laminin is evident in the basement membranes (BM) of both nonpigmented (NPE) and pigment (PE) ciliary epithelium as well as in the vessel walls (S). c, 70-year-old female. Weak fluorescence for fibronectin is seen on the vitreous surface of the nonpigmented epithelium (arrow); the basement membrane of the pigment epithelium exhibits a strong band of fluorescence (double arrows). d, 70-year-old female. The internal (arrow) and external (double arrows) basement membranes of the ciliary epithelium reveal definite fluorescence for laminin; the fluorescent line follows the contours of the frequently invaginated basement membrane and the surface of the displaced pigment. v: vessel walls in the stroma. (Bar = 20 μm).
Fig. 4. Micrograph of immunofluorescent staining with antisera to fibronectin (FN) in the vitreous of a 29-year-old male, with the internal limiting membrane artificially detached from the peripheral retina, possibly the vitreous base. The peripheral vitreous (vit) shows fibrillar pattern of fluorescence for fibronectin and the internal limiting membrane (ILM) exhibits a band of fluorescence. (Bar = 20 μm).

hhibited an undulating irregular fluorescent line for fibronectin and laminin at the endothelial surface (Fig. 1c). These alterations are Hassall-Henle warts.

In young eyes (4-year-old), fibronectin (Fig. 1g) and laminin (Fig. 1h) are present only in Descemet’s membrane, where they exhibit two parallel bands of fluorescence. The staining for fibronectin is more intense on the stromal side than on the endothelial cell side; the staining for laminin is faint on both sides. Occasionally the endothelial cells exhibit a faint surface fluorescence for fibronectin.

Ciliary Body

In young eyes, the zonular fibers and the nonpigmented ciliary epithelial cells showed weak fluorescence for fibronectin (Fig. 3a). When treated with antilaminin antibodies, a bright fluorescent band was observed on the basement membrane of the nonpigmented epithelium, but not in the zonular fibers (Fig. 3b). In aged eyes, the nonpigmented and pigment epithelia appeared to be degenerating, and pigment materials were dispersed and displaced into the stroma. Weak fluorescence for fibronectin was seen on the vitreous surface (Fig. 3c) and intercellular space of the nonpigmented epithelium; the basement membrane of the nonpigmented epithelium exhibited an intense fluorescent band for laminin (Fig. 3d). The basement membrane of the pigment ciliary epithelium as well as the walls of the stromal vessels exhibited a bright fluorescent band for fibronectin as well as laminin (Fig. 3).

Vitreous Body

In most cases the vitreous body was not well preserved. When some vitreous was preserved, a fine filamentous distribution of fluorescence for fibronectin was occasionally detected. The condensed peripheral vitreous adjacent to the retina and pars plana of the ciliary body also showed weak fluorescence for fibronectin (Fig. 4). There was no fluorescence for laminin except for a faint, linear fluorescence at the edge of the vitreous cortex. No difference in fibronectin distribution was observed between young and old eyes, even in six aged eyes which had PVD.

Retina

The walls of the retinal vessels showed a dense fluorescent band for both fibronectin and laminin (Figs. 5, 6, 7). Fibronectin and laminin were also present on the ILM of the retina, although the intensity of fluorescence was weaker than that of the vessel walls and varied in different regions of the retina. Occasionally, some retinal cells fluoresced weakly with antifibronectin antibodies (Fig. 5a).

In the peripheral and ora serrata regions of the retina of young eyes, fibronectin staining was very faint in the ILM (Fig. 5a); the dense vitreous cortex with its tightly adherent ILM (vitreous base) was also fluorescent (Fig. 4). In this region, the ILM fluoresced more intensely for laminin than for fibronectin; the vitreous cortex, however, did not stain for laminin (Fig. 5b).

In aged eyes stained with antifibronectin, the condensed vitreous and the peripheral retina exhibited diffuse, weak fluorescence while the ILM fluoresced intensely (Fig. 5d). When stained with antilaminin, the ILM revealed an intermittent fluorescent line; the reti-
In the equatorial retina of young eyes, fibronectin was barely detectable in the ILM. In both young and aged eyes, laminin exhibits a fine fluorescent line (Fig. 6a). In aged eyes, the intensity of fluorescence for fibronectin at the equatorial ILM appeared brighter than in young eyes, exhibiting a coarse fluorescent band (Fig. 6b).

In the posterior retina of young eyes, the ILM exhibited a thick band of fluorescence for fibronectin and laminin (Figs. 7a and 7b), which was occasionally bi-laminated (3 out of 14 young and middle-aged eyes for fibronectin and 2 out of 14 for laminin). This bi-laminated band of fluorescence with a smooth inner (vitreal) and irregular outer (retinal) surfaces was detected only in the posterior retina. The ILM of the posterior retina of all aged eyes exhibited this bi-laminar pattern of fluorescence (Figs. 7c and 7d).

In order to determine whether this bi-laminated pattern of fluorescence corresponds to structural changes of the ILM, light and electron microscopic examina-
tions were carried out on the posterior retina of the aged eyes. Epiretinal membranes were detected in only two eyes and a few cells were found on the ILM in three eyes; in the other eyes, no prominent morphologic alterations were evident.

**Optic Nerve**

Optic nerve fiber bundles exhibited no fluorescence; the surfaces of the pial septum, the pia mater, the arachnoid, and the dura showed a definite fluorescent line for both glycoproteins: diffuse fluorescence was also evident in these connective tissues when stained with antifibronectin (Fig. 8). With aging, the connective tissue of the optic nerve and its meninges become more abundant; however, the staining patterns were the same as those in younger eyes.

**Discussion**

By indirect immunofluorescent techniques, we examined the topographic distribution of fibronectin and laminin in post-mortem human eyes obtained from nondiabetic individuals of various ages. In general, the distribution pattern was similar in the aged human eye as in the young human eyes or bovine eyes.

A number of investigators have studied the localization of fibronectin in the cornea, but there remains...
controversy regarding the presence of fibronectin in the basement membrane of the corneal epithelium.\textsuperscript{18,19}

It has been demonstrated that the basement membrane of the normal corneal epithelium is positive for fibronectin in the human and bovine eye, and negative in the rabbit eye; this difference may be due to species difference.\textsuperscript{20} In our studies, the fluorescent pattern for fibronectin in Descemet's membrane was variable, in accordance with the results reported by Watanabe and Kumagai.\textsuperscript{21} Surface fibronectin on the corneal epithelial cells was occasionally detected in eyes from old patients. Although the corneal epithelial cells are known to synthesize fibronectin in vitro,\textsuperscript{22} its presence in vivo has not been reported previously.

The zonular fibers and the vitreous cortex showed positive fluorescence for fibronectin, but were negative for laminin, except for a faint, linear fluorescence at the edge of the vitreous cortex. This difference can be explained by the fact that fibronectin binds to various types of collagens, whereas laminin has a selective affinity to type IV (basement membrane) collagen.\textsuperscript{6}

In the posterior retina of young eyes, a thick, occasionally bi-laminated pattern of fluorescence for fibronectin and laminin was observed in the ILM (Fig. 7); the bi-laminated pattern of fluorescence was present in all aged eyes. Since we did not observe this pattern of fluorescence in the bovine retina,\textsuperscript{17} and in the human retina we observed it only in the posterior retina, it may be due to the thicker ILM of the posterior retina in the human eye.\textsuperscript{3}

Thickening of the ILM during aging has been reported in the human eye.\textsuperscript{23} Since in young primates the thickness of the posterior ILM is the same as that of the peripheral ILM,\textsuperscript{24} the thinning of the ILM in the posterior retina of adult eyes probably represents early aging changes. The increased staining intensity we have observed for fibronectin in the posterior ILM of eyes less than 40 years old may correspond to early senescent thickening of the ILM. The double-laminated pattern of fluorescence suggests that while thickening of the ILM may be present, additional abnormal changes of the ILM occur in the aged eye. We found no correlation between fibronectin and laminin distribution and occurrence of PVD, although PVD is common in the elderly. This suggests that PVD is a more complex phenomenon than simply thickening of the ILM, and that very likely quantitative, and possibly qualitative, changes in the biochemical composition of the ILM are a prerequisite to PVD.

Fibronectin staining in the ILM of the equatorial and peripheral retina was significantly less intense than that observed in the posterior region, although laminin staining was moderately strong in these areas. It is interesting that the attachment of the vitreous to its adjacent tissues has been thought to be stronger here than in the posterior regions.\textsuperscript{4}

The attachment of the vitreous to its adjacent tissues is mediated by the ILM of the retina, the basement membrane of Mueller cells, or by the basement membrane of the nonpigmented ciliary epithelium. We have demonstrated that fibronectin and laminin are present in these basement membranes, although there are regional differences in intensity and pattern of staining. Biochemically, both fibronectin and laminin have been found to have very high affinities for collagens and glycosaminoglycans; fibronectin binds with high affinity to collagen Type II and hyaluronic acid,\textsuperscript{10,25} two major components of the vitreous; laminin, on the other hand, binds strongly to collagen Type IV, a main component of basement membranes.\textsuperscript{12,13} The presence of fibronectin and laminin in the ILM suggests that these glycoproteins may play a role in the attachment of the vitreous to the ILM and the ILM to the Mueller cell processes and nonpigmented ciliary epithelial cells.
Key words: fibronectin, laminin, retina, internal limiting membrane, vitreoretinal adhesion, aging, indirect immunofluorescence

References