Topographic Variations in the Rabbit and Primate Internal Limiting Membrane

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The internal limiting membrane (ILM) and cortical vitreous of the rabbit and primate were studied with transmission electron microscopy following staining with the cationic dye, Alcian blue GX. An unusual feature of the cortical vitreous collagen fibril was its displacement from the ILM: it did not insert into the lamina densa. The separation between vitreal collagen and the ILM was especially noticeable in the rabbit eye, which possessed an extremely strong vitreal retinal attachment in the posterior fundus. The lack of fibril insertion was observed in rabbit tissue that had been fixed by quick freezing on a helium-cooled copper block. The similarity in the appearance of tissue fixed by glutaraldehyde, glutaraldehyde supplemented with Alcian blue, or by quick freezing suggested that the lack of collagen fibril insertion into the ILM was an accurate representation of the relationship between collagen and the ILM. It was found that these two animal species had radically different ILM's; the rabbit ILM was a thin basement membrane throughout all areas of the posterior fundus, whereas the ILM of the cynomolgus monkey was a thick basement membrane in the peripapillary region and a thin basement membrane in the region of the fovea centralis. The topographic variations in the primate ILM thickness and appearance followed the pattern observed in human eyes. Like man, the thickening of the cynomolgus ILM in the posterior fundus was age related. The similarity between the cynomolgus and human ILM suggests that this animal would be more suitable than the rabbit for studying age-related changes or alterations in the strength of vitreal attachment following trauma. Invest Ophthalmol Vis Sci 25:71-82, 1984

The vitreous body is a hydrated gel composed of collagen and the glycosaminoglycan, hyaluronic acid. It is attached to the inner surface of the posterior chamber, with the strongest attachment being in the area overlying the pars plana, ora serrata, and peripheral retina. Vitreoretinal attachment is weaker in the posterior fundus, so that with trauma or aging, the vitreous can become detached from the inner limiting membrane (ILM) of the retina, a condition known as posterior vitreous detachment (PVD).

Transmission electron microscopy was used to study the anatomical features of vitreal attachment to the ILM. This report differs from earlier ultrastructural studies because we used the cationic dye, Alcian blue GX, to demonstrate the complex sugars that make up the ILM and cortical vitreous. Traditional fixation procedures employing glutaraldehyde and paraformaldehyde do not reveal these substances. Newer fixation and staining techniques, however, can be used to demonstrate glycoproteins and glycoasminoglycans. By using such a new fixation technique, Rentsch showed that hyaluronic acid can be retained and visualized with ruthenium red. Alcian blue GX is similar to ruthenium red in that it stains the extracellular space and the glycoasminoglycans in a similar manner. We extended Rentsch's work by comparing two species of animals that have markedly different ocular structures with striking differences in the strength of vitreal adherence to the posterior fundus. By taking advantage of this species difference, we were able to demonstrate specific anatomical features that are associated with strong vitreal attachment.

As a control for artifacts induced by chemical fixation, “slam freezing” fixation was used for preserving vitreal structure. This technique involved the rapid freezing of living tissue, so that the changes in vitreal structure induced by glutaraldehyde-paraformaldehyde fixation can be determined. With this frozen material, water was replaced by substitution with acetone at -70°C. The direct replacement of frozen water with acetone provided an additional control in evaluation of the effects of the dehydrating agents on vitreal structure.
Materials and Methods

Preparation of Tissue for Electron Microscopy

We studied six young adult rabbits and seven cynomolgus monkeys whose ages ranged from 7 months to 16 years. All animals were anesthetized with an intravenous injection of pentobarbital and, while under deep anesthesia, were fixed by vascular perfusion with 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2-7.4.

Following perfusion, the eyes were enucleated and the corneas removed. The eyes were fixed in the perfusion solution overnight and dissected the next morning. Portions of the posterior fundus and the pars plana were fixed overnight in either fresh fixative or in fixative supplemented with 0.1% Alcian blue GX. Following a brief rinse in phosphate buffer, the tissue was postfixed in 1.0% osmium tetroxide and dehydrated in a series of graded ethanol. Tissues were embedded in either Medcast (Polysciences, Warrington, PA) or Polybed 812 (Polysciences), thin sectioned, and stained with uranyl acetate and lead citrate.

As a control for chemical fixation, we preserved the ocular structure by freezing. The medullary ray region of a pigmented rabbit was placed on a Polaron slam freezer and rapidly frozen on a helium-cooled copper block. Dehydration was performed by freeze substitution at −70°C with acetone containing 1% osmium tetroxide. Once dehydrated, tissues were warmed to room temperature and embedded in Medcast and stained with uranyl acetate and lead citrate.

Areas of the Eye Studied

The portions of the eye studied included the pars plana region isolated from the superior meridian and two areas of the posterior fundus. In the rabbit, an area 2 mm above the optic nerve head was removed as a sample of avascular retina. In addition, a sample was taken from an area 2 mm nasal or temporal to the optic nerve head, ie, the medullary rays; this represented the vascularized area peculiar to rabbit retina. In the primate, the avascular retina was represented by the fovea centralis, while the vascularized retina was represented by an area 2 mm superior to the optic nerve head. The latter is referred to in our studies as the superior peripapillary region.

Results

Rabbit ILM

General description: The retinal inner limiting membrane is a basement membrane that separates the cortical vitreous from the processes of the Mueller cells. Glutaraldehyde fixation, followed by osmium tetroxide postfixation, reveals its three-layered structure (Fig. 1A). There is a 30-nm thick electron-dense layer, the lamina densa (LD), composed of fine filaments; separating this layer from the processes of the Mueller cells is an electron-clear layer, the lamina rara interna (LRI); vitreal to the lamina densa is the lamina rara externa (LRE).

Fixation with Alcian blue GX revealed the ILM in greater detail (Fig. 1B) as the filamentous composition of the lamina densa was more evident and the entire structure stained more intensely. The most striking feature was the increased retention of material within the lamina rara interna. This material consisted partly of an amorphous mass while other components had the appearance of posts or strands joining the cells to the basement membrane.

Vitreoretinal attachment at the pars plana: In the pars plana, the cortical vitreous had numerous collagen fibrils organized in bundles or sheets. In oblique sections of the ILM, some of the collagen appeared to be inserted directly into the basement membrane (Fig. 2A). The lamina densa of the pars plana was approximately 40 nm thick. There were no breaks in the...
Fig. 2. Electron micrographs of the rabbit retina following Alcian blue fixation. A. The basement membrane (between opposed arrows) lines the inner surface of the nonpigmented epithelial cells of the pars plana. This micrograph shows the basement membrane cut in an oblique section and the collagen fibrils (CF) appear to insert within the lamina densa. Thin material can be seen joining neighboring fibrils (arrows) (x70,400). B. The rabbit avascular retina following Alcian blue fixation. Within the vitreous cortex, collagen fibrils run parallel to the retinal surface. The lamina rara interna contains strands or filaments that connect the ILM (opposed arrows) to the Mueller cell endfeet (arrowhead) (x72,450). C. The rabbit medullary ray following Alcian blue fixation. The ILM lamina rara interna contains material that spans the space (opposed arrows) between the retina and lamina densa. This material appears as either an amorphous precipitate (asterisk) or as strands (arrowheads) connecting the ILM to the Mueller cell endfeet. Note the collagen fibrils of the vitreous do not penetrate the lamina densa. Thin strands of Alcian blue stained material may join the collagen fibrils to the ILM (x80,000).

surface of the ILM, and it had a smooth, even appearance throughout. Alcian blue staining of this region made the collagen fibrils more evident; a dense precipitate surrounded the fibrils, and in some sections, thin strands of material could be seen joining neighboring fibrils (arrows, Fig. 2A). Over the peripheral
Fig. 3. A high magnification electron micrograph of the rabbit medullary ray. Collagen fibrils (CF) have thin wisps of materials that extend into the vitreous. Occasionally there are connections between neighboring collagen fibrils (arrow) ($\times 68,850$).

Vitreoretinal attachment of rabbit avascular retina: Most of the rabbit retina was avascular, and in these regions, the retinal cells were supplied by the choriocapillaris. In the region 2 mm superior to the optic nerve head, there was no evidence of either intravitreal or intraretinal vessels. Mueller cell endfeet were directly juxtaposed underneath the ILM and provided a smooth, contoured surface for this basement membrane (arrowhead, Fig. 2B). After Alcian blue GX staining, the lamina densa was seen to be 30 nm thick. The lamina rara interna contained a dense precipitate or thin filaments connecting the ILM to the Mueller cell endfeet (Fig. 2B). The appearance of vitreal collagen following Alcian blue GX staining was the same as that observed for collagen in the region of the medullary ray.

Vitreoretinal attachment at the medullary rays: The regions nasal and temporal to the optic nerve head contained the vascularized medullary rays. These areas consisted of bundles of myelinated ganglion cell axons coursing through the retina before exiting the globe through the optic nerve. Internal to the ILM were intravitreal vessels. Non-Mueller glial cell processes were occasionally seen surrounding the vessels. The inner limiting membrane had a thin lamina densa, 30-nm thick, and a lamina rara interna (Fig. 2C). Thin Alcian blue-stained filaments (arrowheads) traversed the lamina rara interna and joined the ILM to the Mueller cell endfeet. In tissue prepared with Alcian blue GX, an electron-dense material frequently sur-
rounded the collagen fibrils, giving them an amorphous coat (Fig. 3). Collagen fibrils were observed in close proximity to the preretinal vessels of the medullary ray (Fig. 4A). Most of the fibrils did not insert into the lamina densa surrounding the intravitreal vessel but ran parallel to the surface of the basement lamina (Fig. 4B). Filaments near the retinal surface were not inserted into the lamina densa.

Slam freezing of vascular retina: Slam freezing fixation of the medullary rays resulted in a tissue whose...
appearance (Figs. 5A and B) was similar to that of Alcian blue GX-fixed material (see Figs. 1B and 2B). Collagen fibrils (insert, Fig. 5B) were identifiable as thin, 16-nm filaments without bands; no precipitated material surrounded the fibrils. The ILM had an obvious two-layered appearance with a lamina rara interna and a lamina densa. In the lamina rara interna, thin wisps of material joined the lamina densa to the base of the Mueller cell endfeet. The collagen fibrils did not extend into the substance of the ILM but ran parallel to the retinal surface (insert, Fig. 5B). Ice crystal damage was evident in the deeper retinal regions; however, areas closest to the freezing block, ie, the ILM and cortical vitreous, were not damaged.

**Primate ILM**

**General description:** The primate ILM consists of the three basic layers described for the rabbit: a lamina densa, a lamina rara interna, and a lamina rara externa. However, in this species, the ILM appearance varied so greatly with the position of the eye being studied that no generalized description can be made.

**Topographic variations in the ILM: pars plana:** The pars plana region of the primate eye has a dual layer of epithelial cells with NPE cells internal to the PE cell layer. The surface of the eye in this region was roughened, and there were indentations in the NPE cell layer (Fig. 6A). Crypts or valleys were found within this area, and there were numerous breaks in the ILM, so NPE cells were exposed to the vitreal space (arrow, Fig. 6A).

**Topographic variations in the ILM: avascular retina, fovea centralis:** The fovea centralis does not have a retinal vascular supply, so its cells, like those of the rabbit retina, receive their nutrients and oxygen from the choriocapillaris. In the foveal pit (Fig. 6B) the ILM was extremely thin, with a lamina densa only 30 nm thick. The ILM thickened outside of the pit (Fig. 6C) and resumed its normal size and appearance in the parafoveal regions (Fig. 6D). The stacking of ganglion cell bodies just outside the fovea did not interfere with the appearance of the ILM.

**Topographic variations in the ILM of the vascularized retina: superior peripapillary region:** In this area of the primate eye, the Mueller cell endfeet approached the surface of the ILM and separated the bundles of nonmyelinated ganglion cell axons (Fig. 6E). The Mueller cell endfeet reached the inner surface of the retina and then splayed out, forming a layer underneath the ILM. Their internal surface had complex folds that matched the ridges of the lamina densa. In these regions, the lamina densa was thickened, ranging from 100 nm to 2000 nm. Glutaraldehyde fixation (not shown) revealed the lamina densa to be a homogeneous matrix with occasional breaks in its substance. There was no stainable material within the lamina rara interna, which appeared as an electron-transparent layer between the Mueller cell endfeet and the lamina densa. In contrast, Alcian blue GX stained the lamina densa with much greater intensity (Fig. 6E). There was a retention of material within certain regions of this structure and deposits ran through the lamina rara interna, adjacent to the Mueller cells (arrowheads, Fig. 6E).

Within the superior peripapillary region, ILM thickness and appearance was altered in areas where large retinal vessels approached the inner surface of the eye. The complex external folds of the lamina densa were lost and the Mueller cell endfeet became attenuated in these areas (Fig. 7). The presence of the vessels limited the thickness of the Mueller cell endfeet at the inner surface of the eye.

**Age variations in the posterior fundus: peripapillary region of the cynomolgus monkey:** There was no difference in ILM thickness of monkeys 12 and 16 years old compared to that of young adult monkeys, whose ages ranged from 6 to 8 years; the only difference was found between the 7-month-old and the adult animals. The youngest monkey had an ILM that was 110 nm thick and possessed a smooth regular external and internal face (Fig. 8A). Occasionally, small indentations were found in the Mueller cell endfeet; however, the complex folds characteristic of the adult eye were not observed (Fig. 8B). In the adults, the ILM varied in thickness from 100 nm to 2000 nm.

**Comparison of rabbit and monkey ILM:** The size and appearance of the rabbit ILM was similar in the regions of the pars plana (Fig. 9A), medullary rays (Fig. 9B), and avascular retina (Fig. 9C); it had a thin basement lamina with a lamina densa, lamina rara interna, and lamina rara externa. In the posterior fundus, collagen fibrils ran parallel to the retinal surface and did not insert into the lamina densa. This is in contrast to the pars plana, where the fibrils appeared to insert directly into the lamina densa.

In contrast, the primate ILM showed a topographic variation in size and appearance. The ILM was a thin basement membrane in the pars plana region (Fig. 9D) and in the pit of the fovea (Fig. 9E) but was a thick basement membrane in the superior peripapillary (Fig. 9F) and parafoveal regions. Primate vitreal collagen was not inserted into the lamina densa of the ILM. The majority of the fibrils did not penetrate into the substance of the ILM, but ran parallel to the retinal surface.

**Discussion**

The most dramatic difference between the rabbit and primate vitreoretinal junction is the uniform appearance of the rabbit ILM compared to the regional
Fig. 5. A. Light micrograph of the rabbit medullary ray fixed by slam freezing. There is some ice crystal (IC) formation within the Mueller cell cytoplasm (MC), but ganglion cell axons (AX) and endothelial cells are well fixed (×1,134). B. Transmission electron micrograph of the medullary ray following slam freezing. Intravitreal vessels are present, and their endothelial cells do not display gross ice crystal damage (×3,360). Insert: a higher magnification shows the collagen fibrils running parallel to the retinal surface. (MC) Mueller cell (×81,000).
Fig. 6. Electron micrographs of the cynomolgus retina following Alcian blue fixation. A. The pars plana region has a roughened inner surface. A crypt can be seen in this micrograph, and there is a break (arrow) in the thin basement membrane lining the inner surface of the nonpigmented epithelial cell. (VC) vitreous cortex (X3,500). B. In the pit of the fovea the ILM (opposed arrows) is a thin basement membrane (X27,500). C. The ILM (opposed arrows) gradually becomes thicker outside the pit of the fovea (X27,500). D. In the parafovea, the ILM (opposed arrows) is a thick basement membrane (X27,500). E. In the superior peripapillary region, the ILM is a thick basement membrane (opposed arrows) with complex external folds and a smooth inner surface. A dense precipitate (arrowhead) lies within the lamina rara interna joining the lamina densa to the Mueller cell (X7,000).

variations in ILM thickness in the cynomolgus monkey. In the rabbit, the ILM is a thin, smooth basement lamina in all regions of the posterior fundus. In contrast, the primate ILM is a thick membrane with complex external folds in the superior peripapillary region and a thin basement membrane in the pit of the fovea.

The simple uniform appearance of the rabbit ILM is but one characteristic that distinguishes this animal's retina from that of man. It is noteworthy that the rabbit possesses numerous anatomical features that are not found in the human eye. For example, the rabbit retina is avascular and contains tracts of my-
elinated ganglion cell axons that run nasal and temporal to the optic nerve head, ie, the medullary rays. Internal to the surface of these rays are intravitreal vessels that are attached to the retinal surface by glial cell processes.

In spite of its unusual ocular morphology, the rabbit eye is commonly used in experimental studies on ocular injury. For example, it is a suitable animal for demonstrating the role that various cell types play in traction retinal detachment, and several investigators have induced total retinal detachment in this animal after intravitreal cell injections. However, the progression of events leading to complete retinal detachment is different from that of man in that the earliest manifestation of detachment is elevation of the rabbit medullary rays. Apparently vitreal adherence is so strong in this region that there is an anterior-posterior directed vitreal traction. Strong vitreal attachment in this region is consistent with the difficulty of performing a complete vitrectomy on the rabbit and the rarity of finding PVD following controlled surgical injuries.

The results of our ultrastructural studies correlate with the clinical observations of strong vitreal attachment in the region of the medullary rays. The presence of the preretinal vessels in this area of the rabbit eye provides an additional surface for vitreoretinal interaction and the potential for an increased strength in vitreoretinal adhesion. Surprisingly, the interaction between the vitreal collagen fibrils and the internal limiting membrane is not evident in this region of the rabbit retina. Collagen fibrils do not directly insert into ILM, but run parallel to it. In material that stained with Alcian blue GX, there are occasionally thin wisps of noncollagenous material, which interconnect col-

Fig. 7. An electron micrograph of the superior peripapillary region of the monkey showing a vessel approaching the inner surface of the retina. The endfeet processes of a Mueller cell (MC) as well as the ILM (opposed arrows) are thinner in the region immediately over the vessel (×17,500).
Fig. 8. Electron micrographs of the vitreoretinal junction in the superior peripapillary region of the cynomolgus monkey, following Alcian blue-glutaraldehyde fixation. A. The ILM (opposed arrows) of a 7-month-old monkey is a relatively thin basement membrane without complex external folds (×25,100). B. In contrast, the ILM (opposed arrows) of an adult cynomolgus monkey is dramatically thicker and possesses complex external folds (×31,000).

Collagen fibrils to their neighbors or to the ILM. The demonstration of an indirect connection between the fibrils to the ILM is suggestive that an intermediate compound may serve as a structural link between collagen fibrils and retina. It is uncertain from our studies whether these Alcian blue-stained strands accurately reflect a structural attachment or are a precipitation artifact lying between the collagen fibrils and ILM. It should be noted that an adherence of the vitreous to this region of the rabbit eye does not require a direct interaction between vitreal collagen and retina. The preretinal vessels provide a complex surface topography in which vitreous can be entrapped between vessel and retina. Given the gel-like consistency of the vitreous, vitreal shrinkage could act upon the preretinal vessels, which, in turn, could transmit this force of the retina.

It is unlikely that the separation of the vitreal collagen fibrils from the internal limiting membrane is a fixation artifact. The absence of collagen fibril insertion into the basement membrane is found in tissue fixed by rapid freezing. Although slam frozen tissue is also subject to artifact, ie, ice crystal damage, the similar appearance of the vitreoretinal interface in both freeze and chemical fixation indicates that we have accurately preserved this region of the eye. The presence of Alcian blue-stained filamentous precipitates between collagen fibrils, and between fibrils and ILM, indicates that the cortical vitreous is a matrix consisting of molecules surrounding the collagen filaments. Rhodes has shown that the mouse cortical vitreous contains sugars and mucoproteins that can be visualized by cationic dyes. Rentsch, by using ruthenium red, visualized hyaluronic acid strands in the vitreous. Our procedures using Alcian blue reveal similar material within the rabbit cortical vitreous.

Our electron microscopic studies could not definitively determine the molecular basis of vitreoretinal attachment in the rabbit. However, our topographic study of the rabbit and the cynomolgus monkey shows a dramatic difference in the ILM structure between these two species and similarity of the nonhuman primate ILM to that of man.

From Foos' studies, it is evident that the human ILM is different in the peripheral and posterior retina. It is a thin basement membrane in the peripheral retina and becomes a thicker basement in the posterior fundus. In the posterior fundus, the ILM possesses complex folds that match the convoluted surface of the Mueller cell endfeet. As stated earlier, the rabbit ILM lacks these features, is of uniform size, and possesses a simple trilaminar appearance throughout the posterior fundus. In contrast, the ILM of the cynomolgus is similar to that of man since it has a thick basement membrane with complex external folds, although in limited areas the cynomolgus ILM does becomes thinner, eg, in the pit of the fovea and those regions of the retina where
Fig. 9. Electron micrographs comparing the ILM at various regions in the rabbit (A, B and C) with the monkey (D, E and F). In all three regions of the rabbit retina, the ILM (opposed arrows) is of similar size and shape: A. pars plana; B. medullary ray; C. avascular retina (×57,000). In contrast to the rabbit, there is topographic variation in the ILM of the cynomolgus monkey. The ILM (opposed arrows) is a thin basement membrane in the pars plana region (D) and in the pit of the fovea (E) (×57,000). It is a dramatically thicker membrane in the superior peripapillary region (F) (×35,000).

The similarity of the cynomolgus ILM to that of man suggests that it would be a useful animal model for studying vitreal-retinal interactions following injury and preceding total retinal detachment. This is supported by the results of controlled experiments in which the larger retinal vessels approach the inner surface. These variations correspond to those previously described in human ILM.15,16
nonhuman primate vitreous completely separates from the retina in a manner analogous to human PVD.

In both man and the cynomolgus monkey, the thickening of the ILM is a developmental event. In young humans and our 7-month-old cynomolgus monkey, the ILM is a thin basement membrane, without complex external folds. In the adult, the juxtaposition of Mueller cell endfeet to the ILM suggests that there is an appositional deposition of material onto the ILM from the Mueller cell endfeet. Since vitreal adherence becomes weaker with age, the thickening of the ILM may play a crucial role in adjusting the strength of vitreal attachment. It is known that PVD is an event commonly associated with aging and is only rarely found in young human eyes. It is, thus, important that studies on the age variations in ILM and cortical vitreous be performed to investigate this phenomenon. In this respect, the cynomolgus monkey should prove to be a suitable animal model. It is noteworthy that the rabbit, with its strong vitreal retinal attachment, has an ILM that is uniformly thin in the posterior fundus.

A considerable body of work remains to be done on the mechanism of vitreal attachment. While the rabbit is useful for preliminary studies on ocular injury, its applicability to the human situation is clearly limited by its unusual vitreoretinal interface, as the unusually strong vitreal adherence to the posterior fundus influences the progression of events leading to retinal detachment. In contrast, the cynomolgus monkey has an ILM whose topographic variation is similar to that of the human ILM. This animal should thus be suitable for demonstrating age variations in the ILM and the events leading to traumatic as well as senile PVD.

Key words: internal limiting membrane, retina, vitreous, rabbit, primate, Mueller cells, Alcian blue, slam-freezing

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