Histogenesis of the Intravitreal Membrane and Secondary Vitreous in the Mouse

Masataka Ito, Misako Nakashima, Nobuo Tsuchida, Junko Imaki, and Masabiko Yosbioka

PURPOSE. The intravitreal membrane (IVM) is a membranous structure between the primary and secondary vitreous bodies in developing mammalian eyes. In this study, for the first time the histogenesis of the IVM and the relationship between the hyaloid vasculature and the IVM was characterized in newborn mice.

METHODS. Eyes of mice less than 12 days old were fixed and embedded. From these, serial paraffin-embedded sections were made for lectin histochemistry, immunohistochemistry, and picrosirius red (PSR) staining, and ultrathin sections were made for transmission electron microscopy (TEM). Eight biotinylated lectins and antibodies for laminin and type IV collagen were used.

RESULTS. Among the eight lectins tested, concanavalin A (Con A) agglutinin, Ricinus communis agglutinin I, and wheat germ agglutinin demonstrated strong positive staining in the IVM and vitreous fibrils of the primary and secondary vitreous bodies. They also bound to the internal limiting membrane (ILM) of the retina. At postgestational day 4, the secondary vitreous first appeared between the ILM and the vasa hyaloidea propria (VHP). Immunohistochemical staining revealed that the IVM consists of extracellular matrix components including laminin and type IV collagen, whereas PSR staining and TEM showed that collagen fibrils in the IVM are bundled and continuous with the basement membrane of hyaloid capillaries or the VHP.

CONCLUSIONS. Lectin histochemistry and immunohistochemistry provided good methods for visualizing the structures of the IVM and vitreous fibrils. These results suggest that the IVM is separated from the basement membrane of the retinal ILM along with the vascular network of the VHP when the secondary vitreous begins to form. (Invest Ophthalmol Vis Sci. 2007; 48:1923–1930) DOI:10.1167/iovs.06-0325

The vitreous body is a delicate, transparent tissue occupying the largest part of adult mammalian eyes. It contains a gel-like material consisting of extracellular matrix (ECM) components, >99% of which is water, and cellular components such as fibroblasts, macrophages, and a small amount of vascular tissues of hyaloid vessels during immature stages. Vitreous ECM components are mainly hyaluronic acid molecules and collagen fibers that construct the three-dimensional architecture of the vitreous gel. The structure of the vitreous body changes along with the development of the eye.

During the early organogenesis of eyes, the primary vitreous first appears between the neural retina and the lens, mainly from a mesenchymal component and partly from an ectodermal component. By the 10-mm stage, or the fourth week of human gestation, and by the 11th day in mice, mesenchymal cells enter the optic cup through the fetal fissure together with hyaloid vessels and through the space between the anterior edge of the developing optic cup and the lens vesicle. Ectodermal components giving rise to the primary vitreous are derived from extracellular matrices produced by the inner surface of the optic cup (neuro-ectodermal origin) and by the posterior surface of the lens vesicle (surface ectodermal origin). Because the primary vitreous contains hyaloid vessels such as hyaloid arteries and the vasa hyaloidea propria (VHP), it is also called the vascular vitreous. The primary vitreous is replaced gradually by the secondary vitreous (or avascular vitreous) that forms in the cortical zone of the vitreous body. In humans at the end of the sixth week of gestation, and in mice on the fourth day after birth, the avascular secondary vitreous appears from the inner surface of the retina. The boundary between the primary and secondary vitreous bodies is a fibrous structure called the intravitreal membrane (IVM) or intervitreous condensation. As the primary vitreous is compressed centrally around the hyaloid artery by the developing secondary vitreous, this membranous structure forms the wall surrounding Cloquet's canal, a tubular structure in the vitreous running from the optic disc to the area behind the lens with a remnant of the hyaloid artery and primary vitreous confined in it. However, the histogenesis and structural detail of the IVM, along with the developmental mechanism of the secondary vitreous, have remained unclear.

In our previous study on the regression of the hyaloid vessels in mice, the VHP branched from the proximal portion of the hyaloid artery and approached the posterior surface and equatorial region of the lens. During the first several days after birth, the capillaries of the VHP adhered to the surface of the retina and then they dissociated and began to regress. They were almost completely atrophied by 10 days after birth. In this study, we characterize the histogenesis of the IVM and the relationship between the VHP and the IVM of newborn mice by using lectin histochemistry, immunohistochemistry for laminin and type IV collagen, picrosirius red (PSR) staining, and transmission electron microscopy (TEM). PSR staining is positive on thick bundles of fibrous collagen molecules and shows specific high birefringence when observed with polarized light microscopy but is negative on sparse, randomly arranged collagen fibers or proteoglycans. It is often used to visualize and quantify the area of collagen fibers in connective tissues.
MATERIALS AND METHODS

Animals

Because the mechanism and time course of the regression of hyaloid vessels in the postnatal period were best described using ICR strain mice in our previous study, the same strain of mice was used in the present study.12 Male and female ICR mice bred and raised in our laboratory were mated, and the newborn litters obtained were used in the experiments. The age of the newborn mice was expressed in postgestational days. The day of birth was defined as postgestation day (P)0. The newborn mice were killed on P0, P4, P8, and P12; at least four animals were used for observation at each age. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Serial Paraffin-Embedded Sections

Each mouse was deeply anesthetized with an intraperitoneal injection of a pentobarbital sodium solution (60 mg/kg body weight) and perfused with phosphate-buffered saline (PBS; pH 7.4) followed by phosphate-buffered 4% paraformaldehyde. The eyes were enucleated, and each cornea was removed at its limbus. The eyeballs were then immersed in the same fixative overnight at 4°C. The samples were dehydrated in a graded ethanol series and embedded in paraffin. Serial sagittal and frontal sections (5-μm thickness) were made for lectin histochemical and immunohistochemical and PSR staining. All sections of eyes shown in the figures are frontal sections unless otherwise indicated.

Lectin Histochemistry

Lectin histochemical staining was performed on the paraffin-embedded sections. The following biotinylated lectins were used: concanavalin A agglutinin (Con A), *Dolichos biflorus* agglutinin (DBA), *Phaseolus vulgaris* leukoagglutinin (PHA-L), peanut agglutinin (PNA), *Ricinus communis* agglutinin I (RCA-I), soybean agglutinin (SBA), *Ulex europaeus* agglutinin I (UEA-I), and wheat germ agglutinin (WGA; Vector Laboratories, Burlingame, CA). The plant source of these lectins, their specific sugar residues, and the inhibitory hapten sugars used are listed in Table 1. After sections were routinely deparaffinized by xylene and rehydrated through a series of graded ethanol solutions, they were dewaxed sections were washed for 10 minutes in running water and then stained for 30 minutes in 0.1% PSR solution (0.1% Sirius red in saturated aqueous picric acid). The stained sections were viewed with a light microscope with a polarizing filter.

Transmission Electron Microscopy

Each mouse was perfused with a solution of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate (pH 7.4; Karnovsky's fixative) through the left ventricle while under general anesthesia. The eyes were enucleated and immersed in the same fixative overnight at 4°C. Ultrathin sections were cut as previously described12 and were examined with an electron microscope (model 1010; JEOL, Tokyo, Japan).

RESULTS

Lectin Histochemistry

The summary of the findings obtained with lectin histochemical staining is shown in Table 2. The lectins DBA and PNA did not bind to any examined ocular structure of P0 (Figs. 1G, 1H) or the older stage mice.

Postgestational Day 0. On P0 (day of birth), the VHP is attached to the internal surface of the retina.12 The vitreous chamber is filled with primary vitreous; the secondary vitreous has not been formed. The primary vitreous fibrils in P0 mice were stained by Con A, RCA-I, and WGA and weakly stained by SBA (Figs. 1A–C, 1E arrowheads), whereas PHA-L, UEA-I, DBA, and PNA did not bind to them (Figs. 1D, 1F–H). The lectins that were positive in the primary vitreous also bound to the capillary walls of the VHP (Figs. 1A–C, 1E arrows). Con A and WGA also bound to the retinal ILM in P0 (Figs. 1A, 1C, thick arrows).

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Plant Source</th>
<th>Major Sugar-Binding Specificity</th>
<th>Hapten Sugar</th>
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<tbody>
<tr>
<td>Con A</td>
<td><em>Canavalia ensiformis</em></td>
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<td>RCA I</td>
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<td>DBA</td>
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<td>β-Gal</td>
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Fuc, fucose; Gal, galactose; GalNac, N-acetylgalactosamine; GlcNac, N-acetylglucosamine; Man, mannose; NeuAc, neuraminic acid.

Immunohistochemistry

For immunohistochemistry, anti-laminin antiserum (Cosmo Bio; LSL, Tokyo, Japan) and anti-type IV collagen antibody (Cedarlane Laboratories, Burlington, Ontario, Canada) were used at dilutions of 1:200 and 1:500, respectively, in antibody diluent (DakoCytomation, Glostrup, Denmark). Deparaffinized sections were blocked for endogenous peroxidase activity as described earlier. Antigens were retrieved by the treatment of sections in 1% trypsin in PBS for 30 minutes at room temperature. Primary antibodies were incubated overnight at 4°C. Subsequently, peroxidase-conjugated secondary antibody (ImmunPRESS reagent; Vector Laboratories) and DAB were used according to the manufacturer's instructions. Sections were counterstained with hematoxylin.

PSR Staining

The PSR staining procedure is based on that of Sweat et al.14 Briefly, dewaxed sections were washed for 10 minutes in running water and then stained for 30 minutes in 0.1% PSR solution (0.1% Sirius red in saturated aqueous picric acid). The stained sections were viewed with a light microscope with a polarizing filter.

<table>
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Fuc, fucose; Gal, galactose; GalNac, N-acetylgalactosamine; GlcNac, N-acetylglucosamine; Man, mannose; NeuAc, neuraminic acid.
Postgestational Day 4. In the primary and secondary vitreous of P4 mice, all lectins investigated except PNA and DBA were positive at least weakly (Fig. 2). Histochemical staining of Con A, RCA I, and WGA showed fine meshworks of secondary vitreous (Figs. 2A–C, asterisks) newly appearing between the detached capillary network (Figs. 2A–C, arrowheads) and the retina in the sagittal sections of P4 eyes. In the anterior part of the vitreal cortex near the ciliary body of this stage, the vascular network of

<table>
<thead>
<tr>
<th>Lectin</th>
<th>ILM of Retina</th>
<th>VHP</th>
<th>IVM</th>
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<th>2° Vitr.</th>
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ILM, internal limiting membrane; VHP, basement membrane and/or endothelial cell of the vasa hyloidea propria; IVM, intravitreous membrane; 1° Vitr., primary vitreous fibril; 2° Vitr., secondary fibril; –, negative; ±, weakly positive; +, positive.

**FIGURE 1.** Lectin histochemical staining of hyaloid vessels and the primary vitreous at P0. Lectins ConA, RCA I, WGA, and SBA bound to primary vitreous fibrils (A–C, E, arrowheads). Lectins ConA and WGA bound to the retinal ILM (A, C, thick arrows). The capillary wall of the VHP, which is located on the inner surface of the retina, was stained by the same four lectins (A–C, E, arrows). Other lectins bound only weakly to the vitreous body, VHP capillary, or retinal ILM (D, F–H). Negative control (I) on which no biotinylated lectin was applied showed weak background in the retina and VHP capillaries. Magnification, ×400.
the VHP was not detached from the retina and the secondary vitreous had not been formed yet (Figs. 2A–C, thick arrows).

The meshwork of the secondary vitreous fibrils was finer than that of the primary ones, while the primary vitreous was more granular than the secondary one. In this stage, the IVM was observed as the condensation of vitreous fibrils between the primary and secondary vitreous bodies in some lectins (Figs. 2D, 2H, 2I, arrows). The VHP was located on the IVM. All lectins that showed positive staining in the primary or secondary vitreous were positive in the retinal ILM (Figs. 2D–I, arrowheads).

Postgestational Days 8 and 12. At P8 and P12, most of the VHP has regressed. Lectin histochemical staining of P8 and P12 sections showed, as in P4 sections, that vitreous fibrils and the IVM were stained by Con A, RCA-I, and WGA (Fig. 3). The lectins SBA and UEA-I were also positive in the vitreous body and the IVM in P8 and P12 (Table 1). Frontal (Figs. 3D–F) and sagittal (Figs. 3G–I) sections of P12 eyes stained by these lectins showed that the IVM was still located between the primary and secondary vitreous bodies (Figs. 3D–I, arrows). The staining pattern of the vitreous in P12 was, like that in P4, coarse granular in the primary vitreous and fine reticular in the secondary vitreous. Sagittal sections of P12 eyes stained by these lectins showed a funnel-shaped IVM (Figs. 3G–I, arrows) between the primary and secondary vitreous bodies.

Immunohistochemistry for Laminin and Type IV Collagen

Localization of laminin and type IV collagen was detected immunohistochemically in P0 to P12 eyes (Fig. 4). Generally,
laminin was strongly positive on the capillary walls of the VHP and primary and secondary vitreous (Figs. 4A, 4C, 4E, 4G), whereas type IV collagen was strongly positive on the capillary walls and somewhat weakly positive on the vitreous bodies (Figs. 4B, 4D, 4F, 4H).

The ILM of the retina was positively stained by laminin in all stages (Figs. 4A, 4C, arrowheads) and by type IV collagen in P4 (Fig. 4D, arrowheads) and the older stages (data not shown). Type IV collagen was not detected immunohistochemically in P0 (Fig. 4B). The IVM was stained by both laminin and type IV collagen in P4 and the older stages (Figs. 4C–H, arrows).

**PSR Staining**

In PSR-stained sections from P0, P4, and P8 eyes (Fig. 5), the retinal ILM in P0 and P4 eyes, and the IVM in P4 and P8 eyes, were shown as bright lines under a polarizing light microscope, suggesting that these structures contain bundles of collagen fibers. Particularly in P4, the IVM connected one capillary wall to an adjacent one (Fig. 5B), as seen in lectin histochemistry of the same stage (Fig. 2). There was no significant staining on the primary or secondary vitreous fibrils, which consist of thinner collagen bundles than those in the IVM or ILM.

**Ultrasound by Transmission Electron Microscopy**

In P0 (Figs. 6A, 6B), the VHP closely contacted the internal surface of the retina and its capillary basement membrane was associated with the retinal ILM by collagen fibers. At P4 (Fig. 6C), the VHP had detached from the ILM, and the IVM had appeared. The IVM consisted of bundles of collagen fibrils and separated the primary and secondary vitreous bodies (Fig. 6C, asterisk). Some collagen fibrils of the IVM were continuous with the basement membrane of the VHP. The VHP was located on the surface of the IVM.

**DISCUSSION**

The morphology of the boundary structure between the primary and secondary vitreous bodies has been observed by several researchers since the 1920s. However, its histogen-
esis and structural detail have not been elucidated to date. In this study, we successfully observed, for the first time, the histogenesis and structure of the IVM in relation to vitreous bodies and hyaloid vessels, by using lectin histochemistry and immunohistochemistry, PSR staining, and TEM.

Lectins are carbohydrate-binding proteins, mostly of plant origin. Lectin histochemical staining, using the specificity to certain sugar residues of glycoproteins, has been applied to most of the normal ocular tissues such as lens,\textsuperscript{16} iris, ciliary body,\textsuperscript{17} the anterior portion,\textsuperscript{18,19} retina,\textsuperscript{20,21} and primordium during organogenesis.\textsuperscript{22,23} However, there have been few lectin histochemical studies focusing on the ECM components in the vitreous bodies.\textsuperscript{24} Immunohistochemically, several ECM molecules such as collagens, glycosaminoglycans, fibronectin, and laminin\textsuperscript{25–27} have been detected in the human vitreous body.

In this study, the three lectins Con A, RCA-I, and WGA showed relatively strong positive staining in these ocular tissues. Extracellular matrix components most abundant in both primary and secondary vitreous bodies are hyaluronic acid and collagens.\textsuperscript{28} Because hyaluronic acid molecules contain a repeating disaccharide unit consisting of D-glucuronic acid and N-acetylgalactosamine (GlcNAc), it is stained by Con A and WGA by binding with its GlcNAc residue. RCA-I is thought to bind to the N-acetylgalactosamine (GalNAc) residue on some vitreal proteoglycans.\textsuperscript{28} Rhodes\textsuperscript{24} reported that UEA-I bound to the vitreous fibrils of various animals in the adult stage; however, younger animals have not been studied. In the present study, lectin histochemical staining of UEA-I was more intense in the older mice. This result is consistent with that obtained by Rhodes. UEA-I is also known as a marker of vascular endothelium;\textsuperscript{29} however, the histochemical stain by UEA-I on VHP was not significant compared with other lectins such as Con A and WGA. In our present study, neither PNA nor DBA was detected in the ILM, IVM, and vitreous body of the newborn mouse. However, Russell et al.\textsuperscript{30} reported that both PNA and DBA were positive in the human ILM from midfetal to adult stages. The distribution of glycoconjugates apparently differs between species.

The ILM of the retina is the basement membrane of retinal Müller cells. Its inner surface forms the boundary between the vitreous and the retina. Among the ECM components of the ILM in the adult mammals, type II collagen,\textsuperscript{31} type IV collagen, and laminin\textsuperscript{32} are well known. Peterson et al.\textsuperscript{33} reported that both laminin and type IV collagen are expressed in the space between the optic vesicle and the lens placode during oculogenesis in the macaque. In the present study, expression of type IV collagen on the ILM occurs postnataally, whereas lami-
nin is already expressed at P0. The expression pattern of type IV collagen may also differ between species. Electron microscopic studies have shown vitreous collagen fibrils to be inserted into the retinal ILM.31,34 Bremer35 reported that the basement membrane of the capillaries of the VHP in P1 mice is continuous with the retinal ILM. These findings are consistent with our present observations.

We previously reported that the VHP was attached to the inner surface of the ILM at P0 and later dissociated from it.12 In the present study, we further clarified that this dissociation occurred by P4 and was correlated with the generation of the secondary vitreous and the IVM. It suggests that the IVM is derived from the inner surface of the ILM when capillaries of the VHP detach from the ILM. The secondary vitreous is formed in the space between the retinal ILM and the IVM on

**FIGURE 5.** PSR staining on P0 to P8 eyes. The PSR staining of the retinal ILM and the IVM at P0 (A), P4 (B), and P8 (C). Sirius red bound collagen fibers in the retinal ILM and the IVM are shown as glittering lines when observed by polarized light microscopy (A–C, arrow). The retinal ILM at P0 and P4 and the IVM of P4 and P8 eyes, were stained by PSR. Magnification, ×400.

**FIGURE 6.** TEM of P0 and P4 eyes. TEM of the vitreoretinal junction area of P0 (A, B) and the IVM of P4 (C) eyes shows the close contact of the VHP and the ILM, and the VHP and the IVM, respectively. A migrating hyalocyte was located in the vitreous body (A, arrow). (B) Higher magnification of boxed area in (A). The IVM consists of an aggregation of collagen fibrils between the primary and secondary vitreous bodies (IVM in C). Scale bars: (A) 10 μm; (B) 1 μm; (C) 2 μm.
which the capillary network of the VHP is located. The chemical and histologic structures of the IVM are therefore mostly the same as those of the retinal ILM. However, the mechanism of the detachment of VHP from the retina is still unclear regarding whether the secondary vitreous pushes the IVM and VHP capillary network centrally or whether the increasing volume of the eyeball causes a tear between the retina and the basket-like VHP capillary network. The function of the IVM has not been presented, but results from the present study suggest that the IVM sustains the capillary network of the VHP until it becomes completely atrophied. The VHP is still functioning at P4 and regresses nearly completely by between P8 and P10.\textsuperscript{12}

It supplies blood flow, along with the hyaloid artery, to nourish the developing lens. The IVM may provide a physical backbone for the capillaries hanging in the vitreous gel. Sagittal sections in this study showed that the IVM is a funnel-shaped ECM sheet that consists mainly of collagen fibers. We have not confirmed how the IVM becomes a wall of Cloquet’s canal, but this funnel-shaped sheet may be stretched along the anterior-posterior axis to form a canal in the later developmental stages.

References