Localization of Laminin to Retinal Vessels of the Rat and Mouse Using Whole Mounts

David A. Belford, Glen A. Gole, and Robert A. Rush*

Using a whole mount procedure in adult and neonatal mice and adult rats, we have developed an immunohistochemical method for the localization of laminin-like immunoreactivity (LLIR) to the retinal vessels. LLIR was localized to the vascular basement membrane, permitting a clear three-dimensional view of the retinal vasculature. Positive stain was seen in the inner limiting membrane, in retracted capillaries, encasing pericytes, and in a banding pattern on retinal arterioles. The major findings with the whole mount preparations were confirmed using paraffin-embedded material, with the additional observation of LLIR in the lens capsule. In whole mounts of retinas from neonatal mice, LLIR was present from the earliest stages of capillary growth, indicating that laminin is likely to be secreted by endothelial cells during retinal angiogenesis. LLIR within the retinal nerve fiber layer does not precede capillary ingrowth, so no evidence was found that laminin acts as a tracker signal for retinal angiogenesis. Invest Ophthalmol Vis Sci 28:1761–1766, 1987

Laminin is a large (MW = 1×10^6), non-collagenous, matrix glycoprotein widely distributed in the basement membranes of a variety of human and animal tissues,[1–3] including the retina.[4,5] It is thought to play a role in the attachment of epithelial and endothelial cells to basement membranes,[6,7] and to be involved in vitreoretinal adhesion.[5] In culture, vascular endothelial cells secrete laminin into the extracellular matrix.[8,9]

In order to investigate the possible role laminin may play in retinal vascular development and maintenance, we have localized laminin-like immunoreactivity (LLIR) to the retinal vessels of adult rats and mice and to the developing retinal vessels of the mouse. Because three-dimensional analysis is difficult using conventional cross-sections, we first developed an immunohistochemical technique using retinal whole mounts. Localization of laminin in whole mounts was correlated with the findings from conventional cross-sections.

Materials and Methods

Ten adult rats, 8 adult mice, and 5 litters of developing mice aged from 1 to 9 days were used for this study. The protocols used conform to the ARVO Resolution on the Use of Animals in Research.

The following methods were employed.

Whole Mount Immunohistochemistry

The animals were killed by an overdose of halothane and the eyes immediately enucleated. The eyes were opened behind the limbus and the anterior segment, lens, and vitreous removed. Several radial cuts were made through the eye wall and retina and the specimen was transferred to a glass slide. Preliminary fixation was achieved in 0.5% paraformaldehyde in 15% saturated picric acid for 15 min. The retina was then excised at the disc and gently floated free of the sclera and onto the slide where a further period of fixation took place for a total of 1–2 hr.[10] Fixative was eluted by washing the specimen with phosphate-buffered saline (PBS) until the yellow color of the picric acid was removed. Retinas were then mounted on polyornithine (10 μg/ml; Sigma, St. Louis, MO) coated slides. In adult rats, antibody penetration was enhanced by digestion of the inner limiting membrane (ILM) in 0.4% pepsin in 0.01M HCl for 1 hr. This step was unnecessary in mice.

Immunohistochemical localization of laminin was achieved using a fluorescein isothiocyanate (FITC)-labelled double antibody technique. Whole mounts were pre-incubated for 1 hr in normal goat serum (NGS; Commonwealth Serum Laboratories, Melbourne, Australia) before overnight exposure to the primary antiserum (rabbit anti-laminin; Bethesda Research Laboratories, Gaithersburg, MD) diluted 1:800 in PBS/1% NGS. Following several PBS washes, FITC-labelled goat anti-rabbit antibodies

From the Departments of Ophthalmology and *Physiology and The Centre for Neuroscience, The Flinders University of South Australia, Bedford Park, Australia.

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Reprint requests: Dr. G. A. Gole, Department of Ophthalmology, The Flinders Medical Centre, Bedford Park, SA 5042, Australia.

(Wellcome, Sydney, Australia) diluted 1:200, were applied for 2 hr.

Specimens were mounted in buffered glycerol (pH 8.3) and examined using a Leitz microscope equipped with epifluorescence optics.

Some sections were counterstained with hemotoxylin and eosin (H & E), following removal of the glycerol with PBS washes and dehydration via an ascending series of alcohols (50, 70, 80, 90 and 100% ethanol). This allowed the distribution patterns of nuclei of endothelial and, in the case of arterioles, smooth muscle cells in the vessel walls to be examined.

Corroboration of Results From Flat Mounts With Cross-Sections

Some eyes were enucleated, and fixed in 10% buffered formalin, dehydrated, and embedded in paraffin wax by routine procedures. Paraffin-embedded 5 μm cross-sections were de-waxed in xylene, and rehydrated by suspending the slides for 2 min each in 90%, 80% and 70% aqueous alcohol, then in distilled water. In a humid chamber, the specimens were immersed in a solution of 0.4% pepsin in 0.01M HCl at 37°C for 1 hr, washed three times in PBS, and preincubated for 1 hr in NGS. The specimens were incubated overnight in rabbit anti-laminin serum (1:800), followed by further PBS washes, and application of FITC-labelled goat anti-rabbit serum (1:200 dilution) for 1 hr. After further PBS washes, the specimens were mounted in buffered glycerol and examined with the fluorescence microscope.[11]

Protocol For Oxygen Exposure

In an attempt to confirm whether retracted capillaries stained for LLIR, 5-day-old mice were placed in an atmosphere of 98% oxygen for 5 days[12] in order to produce retraction of existing capillaries. The retinas were then processed as described in "Whole Mount Immunohistochemistry" above.

India Ink Injection

Several neonatal mice were given an intracardiac injection of 10% India ink in normal saline to aid in the identification of growing intra-retinal capillaries. The retinas were then treated as in "Whole Mount Immunohistochemistry" above.

Controls

Several control experiments were performed. Preabsorption of the primary antiserum was carried out with purified laminin (1.1 mg/ml) and with fibronectin (1 mg/ml; BRL, Gaithersburg, MD). The experiments were repeated with the primary antiserum replaced by non-immune or an alternate hyper-immune (anti-nerve growth factor) rabbit serum.

Results

In preliminary experiments, it was found that fixation with formaldehyde concentrations greater than 0.5% reduced specific immunofluorescence and increased background fluorescence in whole mount preparations but not in cross-sectioned retinas.

Adult Rats

Using the whole mount technique, LLIR was demonstrated on the basement membrane of retinal vessels, permitting a clear three-dimensional view of these structures. Exposure to pepsin enhanced vessel immunofluorescence but was found to be difficult to control and often created holes in retinal vessels because of overdigestion. In specimens where the inner limiting membrane persisted, LLIR on this structure reduced immunofluorescence on the underlying vessels.

Thin lines of LLIR bridging small vessels were seen (Fig. 1A). In most cases such bridging lines were seen to arise from globular cells on the external aspect of small vessels.

A transverse banding pattern of LLIR seen over the retinal arterioles suggested increased LLIR between smooth muscle cells (Fig. 1B, C), because the banding matched the distribution pattern of the nuclei of the smooth muscle cells in the arteriolar walls (Fig. 1D).

The photoreceptor layer showed diffuse staining for LLIR which appeared to be in the interphotoreceptor matrix and not restricted to either rods or cones (Fig. 2).

Adult Mice

The retinal vessels of adult mice stained for LLIR, and a similar transverse banding pattern to that seen in adult rats was observed (Fig. 3A). The intercapillary bridges were also seen. Cross-sections showed staining for LLIR on the inner limiting membrane (Fig. 3B).

Neonatal Mice

In the retinas of young air-raised mice, LLIR was seen from the earliest stages of capillary lumen formation (Fig. 4A). In mice injected with India ink, LLIR was present up to the patent tips of growing capillaries (Fig. 4B), but was not seen anterior to the tips of developing capillaries. No ink-filled vessels were seen without laminin staining. As the deep capillary net began to form at the age of 9 days, the

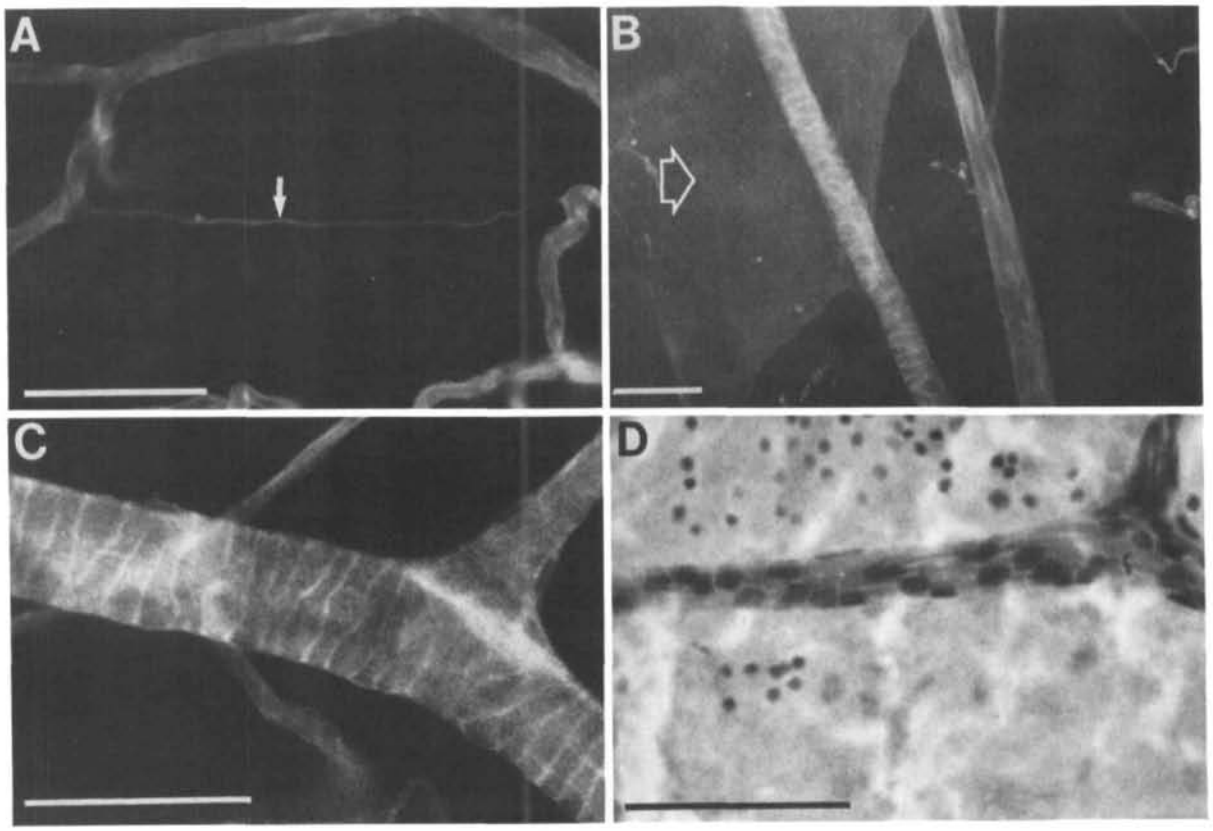

Fig. 1. (A) LLIR on adult rat capillaries showing intercapillary bridge (arrow) connecting two pericytes. Whole mount preparation (×368). Calibration bar = 50 μ. (B) LLIR on rat retinal arteriole (banded pattern) and vein (diffuse pattern). Note staining of inner limiting membrane (arrow). Whole mount preparation (×184). Calibration bar = 50μ. (C) Banding pattern of LLIR on rat retinal arteriole. Whole mount preparation (×442). Calibration bar = 50μ. (D) H & E counterstain of same arteriole as Fig. 1C showing distribution pattern of smooth muscle nuclei corresponding to banding pattern. Whole mount preparation (×442). Calibration bar = 50μ.

bulbous tips of the capillaries growing into the retina to form the deep vascular net stained for LLIR (Fig. 4C). Even in these young mice, the inner limiting membrane and the lens capsule stained for LLIR.

Oxygen-Treated Mice

In these animals, where widespread capillary retraction occurred, thin lines of LLIR were very prominent (Fig. 4D), indicating that basement membrane remnants of retracted capillaries contained laminin. The remnants of retracted capillaries seen in this model appeared, in many cases, identical to the intercapillary bridges seen in adult mice.

Controls

Pre-absorption of the primary antiserum with purified laminin was found to remove all immunoreactivity in both whole mounts (Fig. 5) and cross-sections. Pre-absorption with fibronectin had no effect on subsequent immunolocalization. No fluorescence was seen following replacement of the primary antiserum with non-immune or an alternate hyper-immune (anti-nerve growth factor) serum.

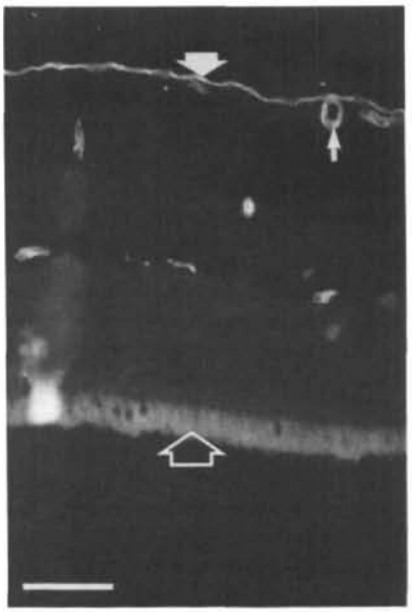

Fig. 2. Cross-section showing positive staining of inner limiting membrane (solid arrow), intraretinal vessels (small arrow), and photoreceptor layer (open arrow). Adult rat (×184). Calibration bar = 50μ.

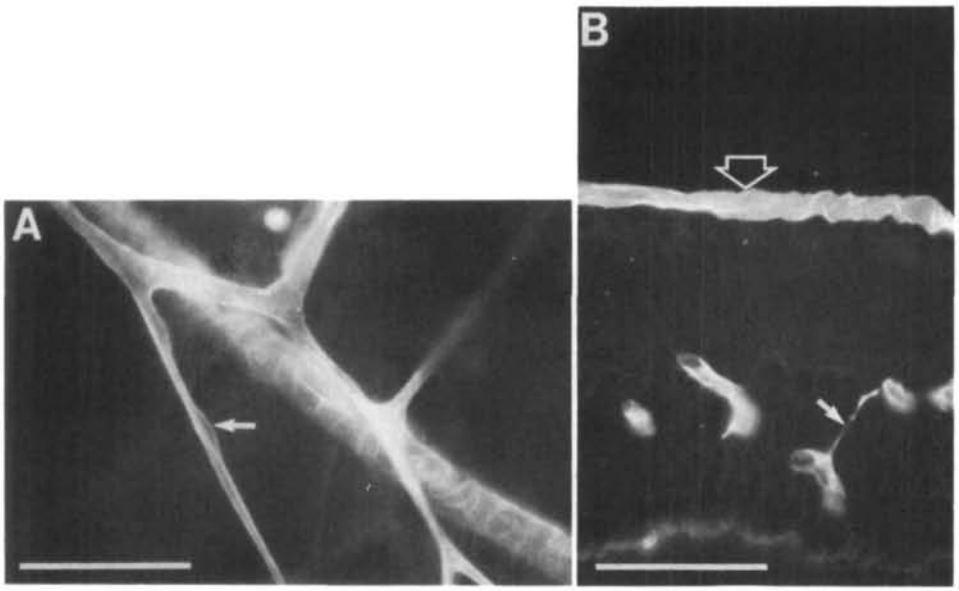

Fig. 3. (A) LLIR on the adult mouse retinal arteriole showing banding pattern. Capillary in foreground shows pericyte encased in laminin (arrow). Whole mount preparation (×368). Calibration bar = 50μ. **(B)** Cross-section showing positive staining of inner limiting membrane (open arrow) and intraretinal vessels. Note fortuitous sectioning through an intercapillary bridge (small arrow). Adult mouse (×368). Calibration bar = 50μ.

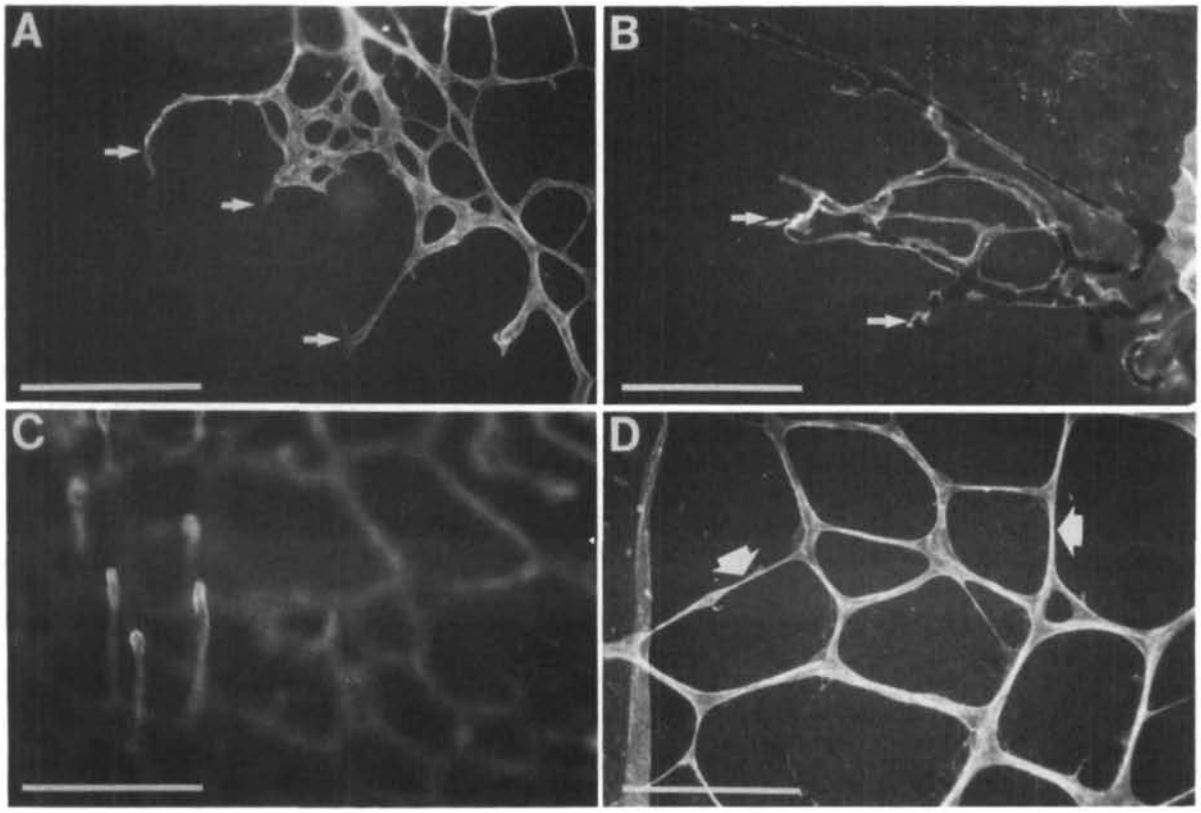

Fig. 4. (A) LLIR at advancing edge of developing retinal capillaries (arrows) in 2-day-old mouse. No stain is seen in nerve fiber layer in advance of growing capillary tips. Whole mount preparation (×368). Calibration bar = 50μ. **(B)** Edge of developing retinal vessels (arrows) in 2-day-old mouse given intracardiac injection of India ink to demonstrate patency of capillaries. Whole mount preparation (×368). Calibration bar = 50μ. **(C)** LLIR on capillaries growing from superficial capillary net (in background) to form deep capillary net. Nine-day-old mouse. Whole mount preparation (×368). Calibration bar = 50μ. **(D)** Retracted capillaries in oxygen treated mouse (arrows). Day 10. Whole mount preparation (×368). Calibration bar = 50μ.

Discussion

Within the retina, we demonstrated LLIR on blood vessels, on the inner limiting membrane, on fine bridge-like structures connecting small vessels, as well as in the photoreceptor layer.

Our results indicate that laminin is present from the earliest stages of capillary tube formation in the retina and is therefore likely to be secreted by the endothelial cells making the capillary. It has been suggested that laminin acts as a tracker signal for neurite growth,[13] but the absence of LLIR in advance of the tips of growing capillaries in the retina does not support a role for laminin as a tracker signal for capillary growth, nor does it support the intra-retinal production of laminin by angiogenic precursor cells such as mesenchymal cells. In corneal new vessels, laminin has also been shown to be laid down from the earliest stages of capillary growth.[14]

In the human retina, intercapillary bridges have been previously described in PAS-stained trypsin retinal digests,[15] and are thought to be the remnants of retracted capillaries.[16] We believe the intercapillary bridges staining for LLIR, seen in our adult animals, are also the remnants of retracted capillaries. The increased numbers of intercapillary bridges staining for LLIR in the oxygen-treated animals, where one expects to find many retracted capillaries, supports this interpretation. Further support is provided by the morphologic similarity between the intercapillary bridges seen in adult animals and retracted capillaries seen in oxygen-treated neonatal mice.

Other important findings were a transverse banding pattern of LLIR over the arterioles in adult animals and the detection of globular cells, coated by LLIR, on the external surface of small vessels. These globular cells frequently gave rise to the thin lines of LLIR which connected capillaries.

The globular cells, encased in laminin, are pericytes because of their location on the external aspect of capillaries. Pericytes are known to be encased in basement membrane,[17] of which laminin is a ubiquitous component. The observation that the intercapillary bridges frequently arise from these putative pericytes is consistent with the hypothesis that pericytes are endothelial cells which have migrated from retracting capillaries.[18] In human retinal whole mounts, using a primary antibody raised against glomerular basement membrane, pericytes have been observed to have a similar globular shape, although in that preparation there were no intercapillary bridges seen.[19]

In the retinal whole mounts, the demonstration of a banding pattern on the retinal arterioles, similar in distribution to smooth muscle cell nuclei, suggests a role for laminin in intercellular adhesion as distinct from it acting as a cell attachment protein.

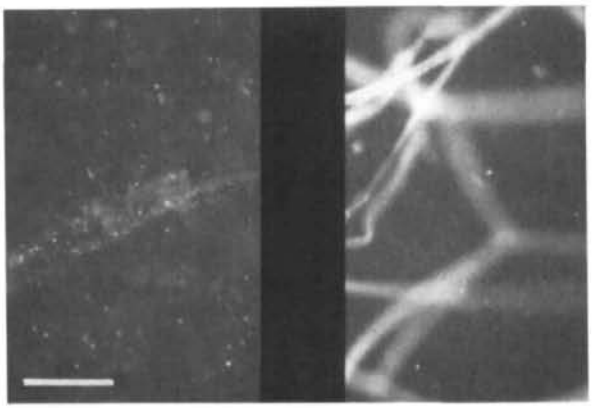

Fig. 5. Control—Adult mouse retina. Left panel—primary (anti-laminin) antibody adsorbed with laminin. Right panel—staining for laminin. Exposure time for both panels—25 sec. Whole mount preparations (×184). Calibration bar = 50μ.

The retinal whole mount preparation is ideal for allowing the three-dimensional study of matrix components of blood vessels, during various physiological and pathological states.

Key words: laminin, retinal vessels, angiogenesis, pericyte, basement membrane

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