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The 67-kd Laminin Receptor Is Preferentially Expressed by Proliferating Retinal Vessels in a Murine Model of Ischemic Retinopathy

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Endothelial cell association with vascular basement membranes is complex and plays a critical role in regulation of cell adhesion and proliferation. The interaction between the membrane-associated 67-kd receptor (67LR) and the basement membrane protein laminin has been studied in several cell systems where it was shown to be crucial for adhesion and attachment during angiogenesis. As angiogenesis in the pathological setting of proliferative retinopathy is a major cause of blindness in the Western world we examined the expression of 67LR in a murine model of hyperoxia-induced retinopathy that exhibits retinal neovascularization. Mice exposed to hyperoxia for 5 days starting at postnatal day 7 (P7) and returned to room air (at P12) showed closure of the central retinal vasculature. In response to the ensuing retinal ischemia, there was consistent preretinal neovascularization starting around P17, which persisted until P21, after which the new vessels regressed. Immunohistochemistry was performed on these retinas using an antibody specific for 67LR. At P12, immunoreactivity for 67LR was absent in the retina, but by P17 it was observed in preretinal proliferating vessels and also within the adjacent intraretinal vasculature. Intraretinal 67LR immunoreactivity diminished beyond P17 until by P21 immunoreactivity was almost completely absent, although it persisted in the preretinal vasculature. Control P17 mice (not exposed to hyperoxia) failed to demonstrate any 67LR immunoreactivity in their retinas. Parallel *in situ* hybridization studies demonstrated 67LR gene expression in the retinal ganglion cells of control and hyperoxia-exposed mice. In addition, the neovascular intra- and preretinal vessels of hyperoxia-treated P17 and P21 mice labeled strongly for 67LR mRNA. This study has characterized 67LR immunolocalization and gene expression in a murine model of ischemic retinopathy. Results suggest that, although the 67LR gene is expressed at high levels in the retinal ganglion cells, the mature receptor protein is preferentially localized to the proliferating retinal vasculature and is almost completely absent from quiescent vessels. The differential expression of 67LR between proliferating and quiescent retinal vessels suggests that this laminin receptor is an important and novel target for future chemotherapeutic intervention during proliferative vasculopathies. *(Am J Pathol 1998, 152:1359–1365)*

Laminin, as an integral component of vascular basement membranes, is vital for endothelial cell adhesion, spreading, differentiation, and phenotypic stabilization under physiological conditions.[1,2] Integrin and nonintegrin receptor interactions with this noncollagenous, extracellular matrix glycoprotein have been recognized for many years in several cell types[3] and constitute an intimate relationship between the cell and its substrate with a major influence on behavior and function. Among the many laminin-binding proteins, a high-affinity, nonintegrin laminin receptor that migrates at 67 kd after post-translational modification of a ~34-kd precursor protein (designated p40),[4,5] has been identified in several cell types, including vascular endothelial cells. The gene encoding this receptor (designated 67LR) has been cloned and the nucleotide sequence determined.[6–8]

Several experimental and correlative studies have implicated the interaction between 67LR and laminin-rich substrates as having a major role in metastasis and tumor progression[9,10] as well as in normal and embryonic development of neural structures.[11,12] However, it has also become clear that 67LR is expressed in vascular tissue where it may have a crucial function in angiogenesis, allowing proliferating endothelial cells to attach and form new blood vessels.[1,10,13,14] The importance of 67LR in endothelial function has also been demonstrated by use of a synthetic antagonist peptide derived from the murine epidermal growth factor (EGF) amino acid sequence 33 to 42 (EGF_{33-42}), a region that shares homology with the

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67LR-binding domain (found in the EGF motif of laminin B1 chain $aa_{925-933}$). The peptide EGF_{33-42} can inhibit endothelial cell motility and angiogenesis *in vitro* and *in vivo*.[15,16]

Angiogenesis in the pathological settings of proliferative diabetic retinopathy, retinopathy of prematurity, and age-related macular degeneration is the major cause of blindness in the Western world. Several strategies with potential for therapeutic intervention are at early, preclinical stages of investigation. These include antagonism of the $\alpha 5\beta 3$ (vitronectin) receptor and inhibition of vascular endothelial growth factor (VEGF), which have both yielded successful prevention of proliferative retinopathy in animal models.[17,18] Nonetheless, there remains a need to identify clear targets on proliferating endothelial cells, antagonism of which will leave the quiescent retinal vasculature unharmed.

Retinal neovascularization sufficient for morphological studies has been produced in experimental animals, although these models have often been inconvenient, expensive, and inconsistent. Smith et al[19] have described a model of hyperoxia-induced retinopathy in neonatal mice that produces retinal ischemia leading to consistent neovascularization and has proved useful in the study of the neovascular process.[20] As in proliferative diabetic retinopathy and retinopathy of prematurity, neovascularization in this model is a direct result of vascular insufficiency and inner retinal ischemia with the new retinal vessels penetrating the internal limiting membrane to proliferate in the preretinal space.

In the current investigation, we have characterized 67LR gene expression and immunolocalization in the murine model of oxygen-induced ischemic retinopathy and demonstrated differences between expression in quiescent (normal) and proliferating retinal vasculature.

Materials and Methods

Mouse Model of Retinal Neovascularization

The murine oxygen-induced retinopathy model characterized by Smith et al[19] was used in this study. Briefly, 7-day-old litters of C57-BL/6J mice, together with their nursing dams, were exposed to 80% oxygen for 5 days. Oxygen flow was maintained at 1.5 L/minute in an incubator regulated at 23°C. On postnatal day 12 (P12) the animals were returned to room air, and pups were sacrificed between P12 and P21.

The extent of retinal neovascularization and reproducibility of the model was assessed by use of fluorescein isothiocyanate (FITC)-dextran perfusion. Before sacrifice, the mice were heavily anesthetized (Hypnorm, 0.01 ml/g, intraperitoneally; fentanyl, 0.315 mg/ml; fluanisone, 10 mg/ml; and midazolam, 5 mg/ml) and perfused with FITC-dextran (molecular weight, 2×10^6) (Sigma Chemical Co., Poole, UK) dissolved in phosphate-buffered saline (PBS). On enucleation, the eyes were fixed in 4% paraformaldehyde for 2 hours. The anterior segment and lens were removed and the posterior eye cup mounted onto silane-coated microscope slides in a Maltese cross configuration after four radial, full-thickness cuts. Photomicrographs were taken on a Leitz Ortholux II microscope with fluorescent attachment.

Eyes to be processed for immunohistochemistry and *in situ* hybridization were enucleated under deep anesthesia and fixed in 4% paraformaldehyde for 2 hours. After fixation, the lens was removed and the eyes washed in PBS before embedding in wax for standard histological sectioning.

Treatment of the mice throughout this study was conducted in accordance with the ARVO regulations on the Use of Animals in Ophthalmic and Vision Research.

Antibody Preparation

A polyclonal antibody to the extracellular carboxyl-terminal domain of the 67LR laminin receptor (PTEDWSAQ PATEDWSAAPTA, peptide Pro-20-Ala)[21] was raised in rabbits as described earlier.[22]

Western Blotting Analysis of Retinal Microvascular Endothelial Cells

Bovine retinal microvascular endothelial cells (RMECs) were isolated as previously described.[23] RMECs were grown in 10% fetal calf serum. While they where subconfluent, plasma-membrane-rich fractions were prepared by scraping the RMEC monolayers in ice-cold PBS, centrifuged at $500 \times g$ for 5 minutes, and disrupted with a tight Dounce homogenizer in a solution of PBS containing 1 mmol/L EDTA and a protease inhibitor cocktail consisting of 1 mmol/L benzamidine, 5 ng/ml pepstatin, 2 mmol/L phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin (all obtained from Sigma). Nuclei and cell debris were removed after centrifugation at $1100 \times g$ for 10 minutes. A crude membrane fraction was removed from the initial supernatant by centrifugation at $15{,}000 \times g$ for 20 minutes at 4°C. The resultant supernatant was subsequently centrifuged at $27{,}000 \times g$ for 2 hours at 4°C. The pelleted membrane extract was then resuspended in PBS, and the protein content was determined using the BCA protein assay method (Pierce, Rockford, IL).

Protein samples were diluted in lysis buffer containing 2% β-mercaptoethanol and separated on a denaturing 7.5% SDS gel (Bio-Rad, Richmond, CA). The gels were then transferred onto nitrocellulose membrane (Bio-Rad) and subsequently probed with a polyclonal antibody to 67LR. After incubation with a horseradish-peroxidase-conjugated secondary antibody (Dako, Glostrup, Denmark), immunoreactivity was detected using enhanced chemiluminescence (Amersham, Little Chalfont, UK).

Immunohistochemistry

Sections of mouse eyes were dewaxed and then rehydrated in PBS, and the endogenous peroxidase activity was quenched in 3% hydrogen peroxide. Sections were then washed in PBS and blocked in 5% normal goat serum (20 minutes) in an antibody diluent buffer (PBS

containing 1% bovine serum albumin, 0.01% Triton X-100). The 67LR antibody was diluted in antibody diluent (1:200) and added to the sections overnight at 4°C in a humidified chamber. For controls, rabbit IgG (Sigma) was added at similar concentrations to the primary antibody, or the primary antibody was omitted. After extensive washing, biotinylated anti-rabbit (Fab fragment; Dako) was added to the sections at a 1:200 dilution for 1 hour and followed by washing in PBS. Streptavidin was added in the form of the avidin-biotin complex (Vector Laboratories, Burlingame, CA) for 1 hour, and subsequent detection was carried out by development in 3-amino-9-ethylcarbazole (Vector Laboratories), which yielded a red reaction product after ~15 minutes at 37°C. After stopping the reaction development by immersion in water at an appropriate stage, the sections were briefly washed, counterstained with 0.02% fast green, and mounted in Glycermount (Dako).

In Situ *Hybridization*

PCR was used to amplify a 350-bp fragment of the 67LR gene from cDNA from human umbilical vein endothelial cells. The PCR product was cloned into the pGEM-T vector (Promega, Madison, WI), and its identity was confirmed by ABI Prism terminator cyle sequencing (Perkin Elmer Applied Biosystems, Norwalk, CT). The recombinant plasmid was linearized with appropriate restriction enzymes (Gibco BRL, Gaithersburg, MD), and sense and antisense riboprobes were transcribed using T7 or SP6 RNA polymerase respectively, incorporating digoxigenin-UTP (Boehringer Mannheim, Mannheim, Germany).

Sections of eyes were dewaxed and then rehydrated into water. After fixation in 4% paraformaldehyde, the sections were washed in PBS and the proteins denatured in 200 mmol/L HCl for 10 minutes. The sections were then treated with proteinase K (20 μg/ml in PBS/50 mmol/L EDTA) for 30 minutes at 37°C followed by washing in PBS. The riboprobes were then hybridized to the sections (~20 ng/100 μl of buffer) for 18 hours at 42°C in a saline sodium citrate (SSC) hybridization buffer consisting of 10% dextran sulfate, 10 mmol/L dithiothreitol (DTT), 0.02% sodium dodecyl sulfate (SDS), 50% formamide, and 10 mg/ml salmon sperm DNA. After hybridization, the sections were washed in descending SSC solutions at room temperature and placed in PBS. Anti-digoxigenin alkaline phosphatase antibody (Boehringer Mannheim) was then added for 2 hours followed by washing in PBS. Hybridized probes were then detected using nitroblue tetrazolium solution (NBT and BCIP, 75 mg/ml, in dimethylformamide; Boehringer Mannheim) dissolved in a Tris buffer (100 mmol/L, pH 9.5) containing NaCl (100 mmol/L), $MgCl_2$ (50 mmol/L), and levamisole (2 mg/ml). Sections were then counterstained with 0.02% fast green, washed, and mounted with Glycermount (Dako).

Results

Fluorescein Dextran Perfusion

In comparison with retinas from mice that had not been exposed to hyperoxia, experimental mice demonstrated closure of the central retinal vasculature at P12 (compare Figure 1, A and B). At P17, there was evidence of recanalizing intraretinal vessels and a clear proliferative response with fronds of new vessels observed on the FITC-dextran-perfused retinal flat mounts (Figure 1C). These vessels persisted until P21, after which they appeared to regress. Histological sections of the retinas revealed the preretinal nature of new vessels between P17 and P21 (Figure 1D).

Antibody Recognition

Western blotting analysis of RMEC plasma membranes revealed that the 67LR antibody detected a 67-kd band (Figure 2). When the antibody was properly titrated, there was no recognition of a band that could correspond to other low molecular weight products of the 67LR gene, ie, p40.

Immunohistochemistry

Sections of hyperoxia-exposed and room air control retina at P12 showed only weak 67LR immunoreactivity (67LR-IR) in the retinal vasculature (Figure 3A). At p17, room air control mice also failed to show any 67LR-IR (Figure 3B). However, hyperoxia-exposed P17 mice demonstrated intense 67LR-IR in recanalizing vessels of the central retina (Figure 3C) and in preretinal new vessels. Hyperoxia-exposed P21 animals showed weak 67LR-IR throughout the intraretinal vasculature but demonstrated labeling in the preretinal vessels (Figure 3D). The retinal vasculature of age-matched, room air control mice demonstrated only weak 67LR-IR. All controls produced negative staining throughout the retina.

In Situ *Hybridization*

As determined by *in situ* hybridization, 67LR gene expression in room air control retina at P12 to P21 was observed largely in the ganglion cells (Figure 4A) whereas lower expression was evident at the innermost aspect of the inner nuclear layer. Hyperoxia-exposed retinas demonstrated similar levels of 67LR gene expression in the neural retina (Figure 4B). Noncomplementary, sense riboprobes revealed no significant areas of labeling throughout the retina (Figure 4C). In the P17 to P21 hyperoxia-exposed animals, 67LR gene expression was observed in the intraretinal vasculature and the preretinal new vessels (Figure 4D).

Discussion

Endothelial cells are known to have an inherent ability to switch from a quiescent to proliferative phenotype in

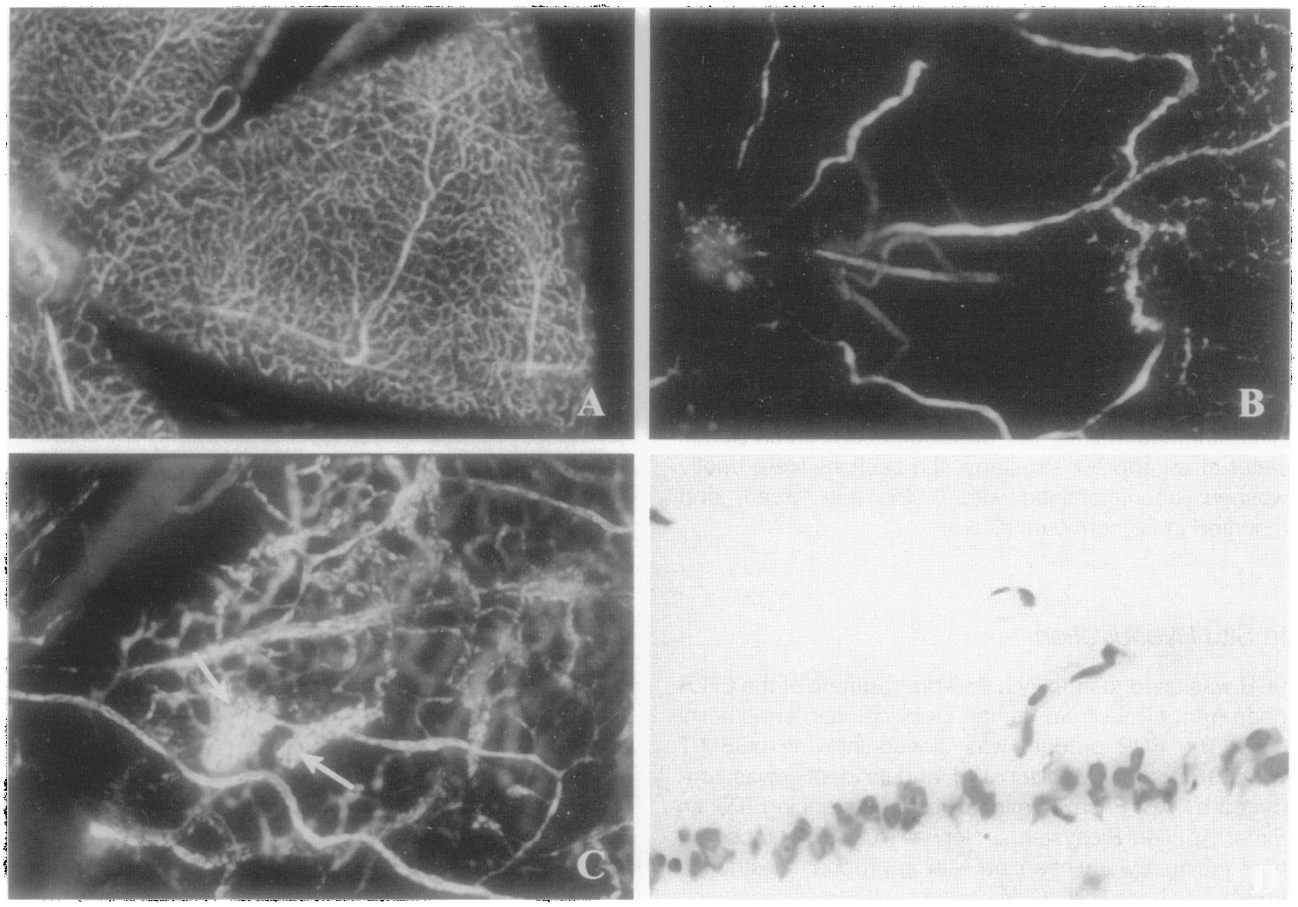

Figure 1. Demonstration of the murine hyperoxia-induced retinopathy model. **A:** FITC-dextran-infused retinal vasculature of a control P12 mouse. There is a dense vascular network throughout the central and peripheral retina. Original magnification, ×50. **B:** Hyperoxia-exposed P12 mouse infused with FITC-dextran. There is closure of the central retinal capillary network with only the large retinal vessels remaining perfused. Note that the peripheral capillary vasculature remains perfused. Original magnification, ×80. **C:** Hyperoxia-exposed P21 mouse infused with FITC-dextran. In comparison with the P12 mouse (**B**) there is reperfusion of the central retinal capillary bed. There is also a clear neovascular response observed with dense tufts of preretinal new vessels (**arrows**). Original magnification, ×80. **D:** H&E-stained section through the retina of a hyperoxia-exposed P21 mouse. The preretinal nature of the neovascular response is clearly seen, with new vessels breaching the internal limiting membrane and penetrating the vitreous body. Original magnification, ×400.

Figure 2. Western blotting analysis of RMEC membrane extract using a 67LR polyclonal antibody. A strong band is detected at 67 kd.

response to a multitude of environmental triggers.[24] The complex process of endothelial migration, invasion, and proliferation, all of which are operative in neovascularization, demand the expression of many genes that are not normally expressed under quiescent conditions.[25] These genes and their products are vital to sustain growth, migration, attachment, spreading, and the ability to repair vascular injury or potentiate new blood vessels. In the current investigation, we have demonstrated that proliferating retinal vascular endothelial cells produce markedly increased levels of the laminin receptor 67LR. By contrast, nonproliferating, quiescent retinal vascular endothelium appears to produce extremely low levels of this protein. We have also confirmed that our antibody recognizes 67LR on the plasma membrane of retinal microvascular endothelial cells where it may have an important role in attachment of proliferating cells during ischemic retinopathies.

Attachment of vascular cells to a substrate is vital for normal cell function. This can take the form of a constitutive-type interaction, with the cell constantly requiring substrate-derived cues to remain attached and viable.[26,27] Such communication is also important for prolif-

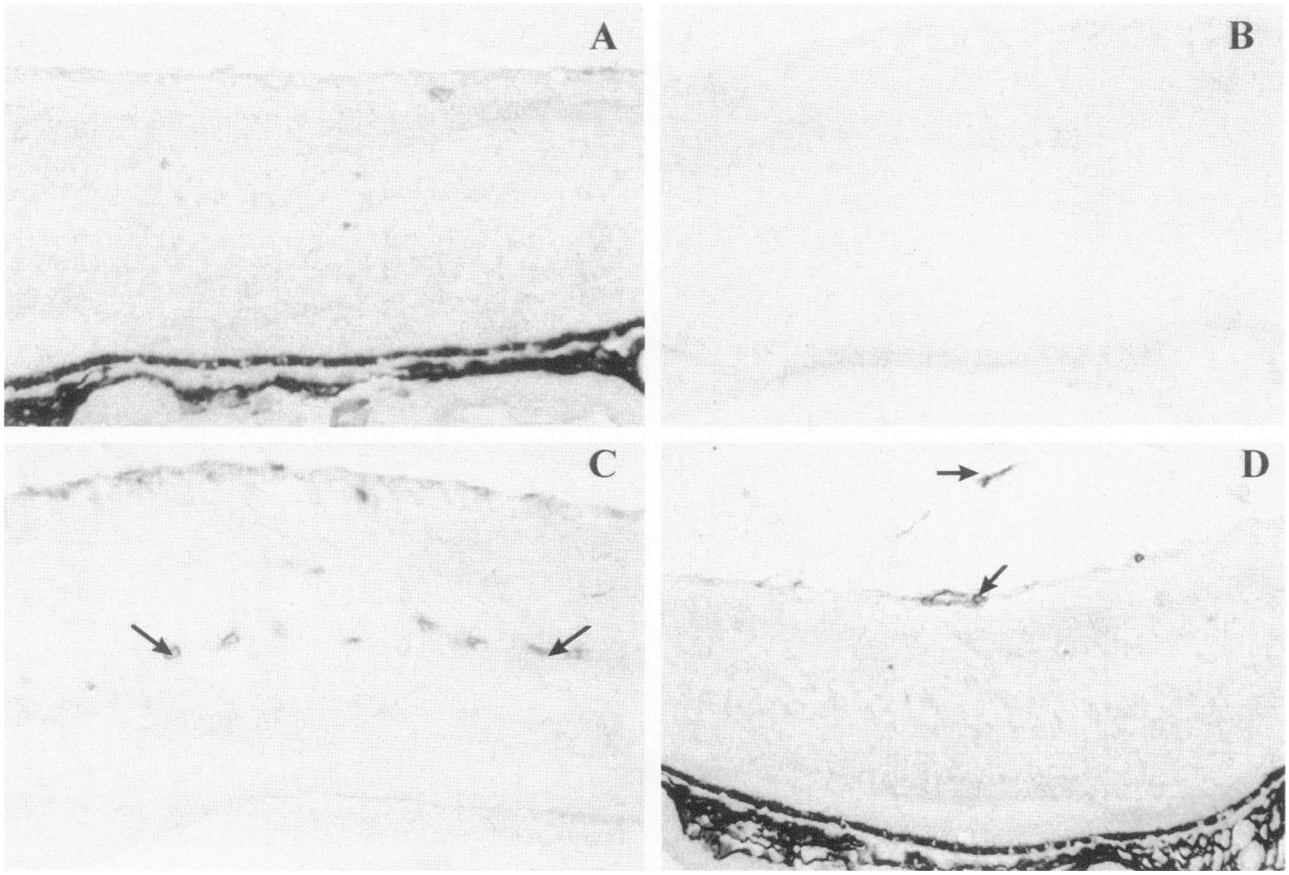

Figure 3. 67LR immunoreactivity in the retinal sections from experimental and control groups. **A:** Normal mouse retina (P12) stained for 67LR. There is a virtual absence of 67LR immunoreactivity. Original magnification, ×200. **B:** Normal mouse retina (P17). There is an absence of 67LR immunoreactivity throughout the retina. Original magnification, ×200. **C:** Hyperoxia-treated mouse (P17) stained for 67LR. There is immunoreactivity in the presumptive recanalizing, intraretinal vessels (**arrows**). Original magnification, ×200. **D:** Hyperoxia-treated mouse (P21). 67LR immunoreactivity is evident in the preretinal new vessels (**arrows**). There is little intraretinal immunoreactivity. Original magnification, ×200.

erating vascular cells that require suitable attachment to initiate spreading, leading to either repair of the endothelial barrier or formation of new blood vessels.[28] Cell association with laminin is essential to the cell's ability to bind to other extracellular matrix components, such as collagen[29] and heparin,[30] although it can also stimulate endothelial cell motility and modulate adherence to basement membranes.[2,10] Laminin is involved in the formation of new vessels during proliferative diabetic retinopathy[31–33] through laminin receptor-mediated interactions. Indeed, the laminin substrate is an integral component of cell migratory responses involving attachment to multiple binding domains across the molecule.[3,34] Initial binding appears to occur via the high-affinity 67-kd laminin receptor, which in turn initiates subsidiary, low-affinity attachments via integrin receptors leading to cell spreading.[22,35]

Although 67LR has been widely implicated in tumor cell adhesion and metastasis,[7,21,36,37] recent studies also suggest that it has a functional role in angiogenesis.[22,32] Antagonism of 67LR with the peptide fragment EGF_{33-42} can block both laminin and EGF-stimulated endothelial cell motility *in vitro* and inhibit angiogenesis in a chick vitelline membrane model.[16,22] Furthermore, the agonist peptide YIGSR, derived from the $LamB1_{929-933}$ chain, can induce tube formation by endothelial cells *in vitro*.[2,32]

The 67LR gene encodes a mRNA that translates to a 32.8-kd precursor protein (p40). This product can undergo post-translational modification to produce mature 67LR, which can localize cytoplasmically or to the plasma membrane.[7,38–40] p40 may itself be an important factor in protein synthesis as evidenced by its co-purification with ribosomes and the eIF-4A initiation factor.[41] Significantly, p40 could also have a vital role in embryonic development and differentiation of the neural retina[12,42] and an as yet unknown function in ganglion cells of the adult retina.[41] The results from *in situ* hybridization investigations conducted in the current study also confirm that 67LR mRNA is also expressed in the ganglion cells of the retina. Interestingly, the level of this expression did not vary according to the presence or absence of retinal ischemia in this layer. These findings, allied with the immunohistochemistry that recognized only 67LR and not p40, suggest that this gene produces proteins with multiple functions. We also suggest that proliferating retinal vascular endothelium can express this gene, which corresponds with our protein studies describing a marked up-regulation of mature 67LR. Indeed, it is likely that only

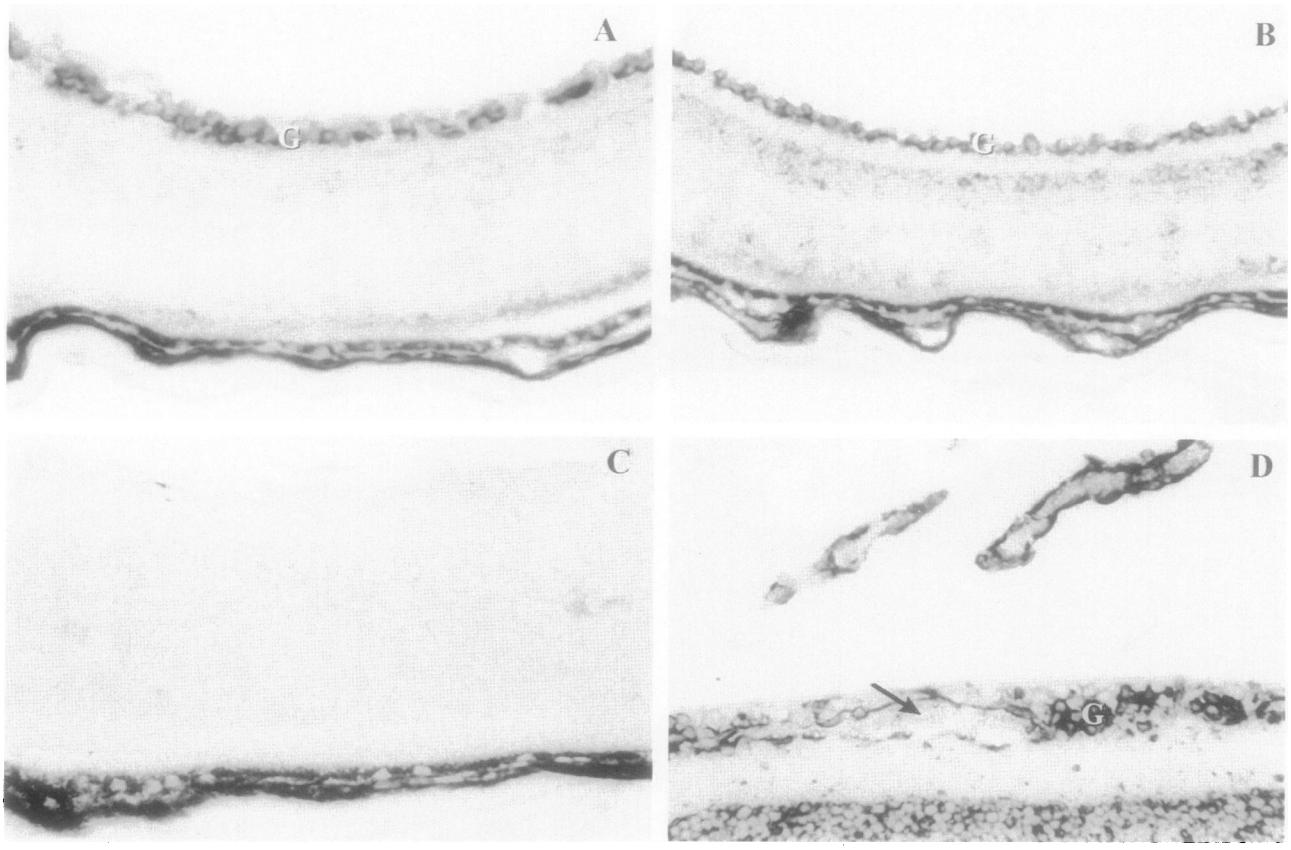

Figure 4. *In situ* hybridization showing 67LR gene expression in retinas from experimental and control groups. **A:** Retinal section from a normal mouse (P17). There is intense labeling in the retinal ganglion cells (G). Original magnification, ×200. **B:** Hyperoxia-exposed mouse retina (P17). mRNA labeling shows a similar labeling pattern and intensity as control mice. Original magnification, ×200. **C:** Sense labeling of hyperoxia-exposed mouse retina (P17) showing no hybridization to the retina. Original magnification, ×200. **D:** Hyperoxia-exposed mouse retina (P21). In addition to ganglion cell labeling (G), there is 67LR mRNA present in preretinal new vessels (**arrow**) and also in the endothelium of a large retinal vessel (**arrow**). Original magnification, ×320.

the proliferative endothelial cell phenotype present during neovascularization has the ability to post-translationally modify the p40 precursor to 67LR whereas quiescent retinal vascular cells may not. As p40 is a ribosome-associated protein, the strong expression observed in retinal ganglion cells may reflect their high ribosome content relative to other retinal cell types.

Retinal ischemia is known to stimulate increased gene expression of a multitude of paracrine growth factors from retinal glial and neural cells.[43] Among these, the potent endothelial mitogen VEGF has received considerable attention as it may have a major role in retinal neovascularization.[44,45] Secretion of VEGF is known to be up-regulated in response to retinal ischemia and is therefore an obvious target for development of anti-angiogenic therapy. Unfortunately, VEGF is also expressed at comparatively high levels in normal and nonproliferative diabetic retina where it may be a major survival factor for the retinal and choroidal vasculature.[46] This being the case, widespread inhibition of VEGF could possibly predispose the retinal vasculature to degenerative changes, especially in diabetic retinopathy where it is already considerably compromised. An alternative strategy of therapeutic intervention would be to block the locomotion of endothelial cells by interfering with their interaction with the extracellular matrix. For example, it has recently been shown that subcutaneous injection of peptide antagonists of vitronectin-specific integrin-type receptors inhibits retinal neovascularization.[17] Such a strategy has value as it would be effective regardless of the chemotactic growth factors involved. However, for this to be an effective strategy, there is a need to identify a target that occurs largely in proliferating vessels and will leave normal vessels unharmed.

The current study has identified a protein that distinguishes between quiescent and proliferating endothelial cells in the retinal microvasculature by its respective virtual absence and high expression between phenotypic forms. 67LR represents a novel and exciting target for future therapeutic interventions against proliferative retinopathies.

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