Retinal Vascular Development Is Mediated by Endothelial Filopodia, a Preexisting Astrocytic Template and Specific R-Cadherin Adhesion

Michael I. Dorrell, Edith Aguilar, and Martin Friedlander

PURPOSE. A neonatal mouse retina developmental model was used to study endothelial cell guidance and subsequent formation of vascular patterns. Since most diseases that cause catastrophic loss of vision do so as a result of abnormal ocular angiogenesis, a better understanding of events regulating normal retinal vascular growth may provide insight into pathologic angiogenesis.

METHODS. Development of the retinal vasculature at various postnatal and embryonic time points was analyzed by collagen IV immunohistochemistry and staining with isolectin *Griffonia simplicifolia*. GFAP-GFP transgenic mice were used to evaluate the relationship between developing vessels and retinal glial cells. Immunolocalization of R-cadherin and intravitreous injection of R-cadherin–specific antibodies was performed to determine the role of R-cadherin during patterning of the superficial and deep retinal vascular plexuses.

RESULTS. The characteristic honeycomb pattern of vessel formation observed in the superficial layer is a result of endothelial cell migration over a preexisting astrocytic template. Filopodial extensions associate with underlying astrocytes by protruding from the tips of endothelial cells at the migrating vascular front. Branching of vessels in the primary vascular plexus, as well as appropriate localization of the deep vascular network is mediated by R-cadherin, an adhesion molecule known to be involved in neuronal cell guidance. Injection of antibodies directed against R-cadherin prevents the normally extensive collateralization observed during formation of the superficial network. Injection of anti-R cadherin antibodies also dramatically affects vessels of the deep network. These vessels migrate beyond the normal turning point, penetrating into the deeper photoreceptor layer.

CONCLUSIONS. These studies suggest that angiogenesis and formation of vascular patterns in the retina may use many of the same developmental cues used by neurons in both the central and peripheral nervous systems. Furthermore, retinal vascular endothelial cell guidance mediated by filopodial extensions and neuronal guidance cues may represent a novel conceptual framework within which to study the establishment of vascular patterns in a variety of angiogenic systems. (*Invest Ophthalmol Vis Sci.* 2002;43:3500–3510)

From the Department of Cell Biology, The Scripps Research Institute, La Jolla, California.

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Corresponding author: Martin Friedlander, The Scripps Research Institute, 10550 North Torrey Pines Road, MB28, La Jolla, CA 92037; friedlan@scripps.edu.

In the normal adult, angiogenesis (defined as the growth of new blood vessels from preexisting capillaries) is tightly regulated and limited to wound healing, pregnancy, and uterine cycling. Our understanding of the molecular events involved in the angiogenic process has advanced significantly since the purification of the first angiogenic molecules more than two decades ago.[1] This process, under physiologic conditions, may be turned on by specific angiogenic molecules such as basic and acidic fibroblast growth factor (FGF),[2] vascular endothelial growth factor (VEGF),[3] angiogenin,[4] transforming growth factor,[5] interferon,[6] tumor necrosis factor-α,[7] and platelet-derived growth factor.[8] Angiogenesis can also be suppressed by inhibitory molecules, such as interferon-α,[9] thrombospondin-1,[10] angiostatin,[11] endostatin,[12] hemopexin-like domain of MMP-2 (PEX),[13] pigment epithelium–derived factor,[14] and fragments of tryptophan tRNA synthetase.[15,16] It is the balance of these naturally occurring stimulators and inhibitors that tightly controls the normally quiescent capillary vasculature.[17] When this balance is upset, as in certain disease states, capillary endothelial cells are induced to proliferate, migrate, and form new blood vessels. In most eye diseases that result in loss of vision, the cause is abnormal angiogenesis. It is the unregulated growth of retinal or choroidal vasculature in the adult eye that leads to extravasated fluid (macular edema), frank hemorrhage, and even tractional retinal detachment, resulting in the loss of vision associated with such diseases as diabetic retinopathy, age-related macular degeneration, retinopathy of prematurity, and rubeotic glaucoma.

Although recent work has identified several factors involved in neovascularization, relatively little is known about the guidance mechanisms that determine the pattern of complex vascular networks during developmental or pathologic angiogenesis. One conceptual approach has focused on the role of cell adhesion molecules, such as integrins and cadherins, and extracellular matrix molecules in regulating the relationship between proliferating endothelial cells and their environment. Most of these studies have been performed with culture systems in which the relationship between cell migration, adhesion molecules, and the extracellular environment (both cells and extracellular matrix) has been examined at the cellular and molecular level. In vivo correlates to these observations remain obscure.

The process of formation of the primary vascular layer in the retina is intimately associated with underlying astrocytes that emerge from the optic nerve[18,19] and are thought to migrate just ahead of the developing vascular network.[20] During the development of the brain, neurons have been shown to migrate along a previously established radial glial fiber system,[21] using interactions with receptor systems including $\alpha 3$ and αv integrins and astrotactin.[22–25] Although several cytokines have been hypothesized to be mediators of retinal vessel formation and endothelial cell migration, the precise molecular interaction between the developing retinal vasculature and other cellular components of the retina is not clear.

To further investigate the mechanism of endothelial cell guidance during angiogenesis and the potential role of the

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astrocytes in mediating formation of vascular patterns, we used an in vivo murine model of developmental ocular angiogenesis. In this study, the superficial vascular network in the neonatal mouse retina was found to form according to a preexisting astrocytic template, and both the superficial and deep vascular layers were shown to use endothelial cell filopodial extensions and R-cadherin cell adhesion molecules as guidance cues. In a broader context, these studies suggest that angiogenesis and formation of the vascular pattern in the retina may use many of the same developmental cues used by neurons in both the central and peripheral nervous systems. Furthermore, unique filopodial extensions are observed on migrating endothelial cells, facilitating directional migration during formation of the retinal vasculature.

Materials and Methods

Retinal Wholemounts

All protocols involving the use of mice adhered to the regulations set forth in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice of various ages were killed and the eyes enucleated. After 10 minutes in 4% paraformaldehyde (PFA), the cornea, lens, sclera, and vitreous were excised and the retina isolated. Retinas were then soaked in methanol for 15 minutes on ice, followed by blocking in 20% fetal bovine serum (FBS; GibcoBRL, Grand Island, NY) and 20% normal goat serum (NGS; Jackson ImmunoResearch Laboratories) for 1 hour. Unless stated otherwise, staining was performed by incubating the retinas in 1:200 dilution of primary antibody in buffer (10% FBS and 10% NGS in PBS) for 18 hours at 4°C. After washes, retinas are incubated with the appropriate fluorescence-conjugated secondary antibody (1:200 dilution in buffer; Molecular Probes, Eugene, OR) for 2.5 hours at room temperature followed by washes and mounting on slides with slow-fade medium. Antibodies and molecular markers included rabbit anti-mouse collagen IV (AB756P; Chemicon, Temecula, CA), fluorescence-conjugated isolectin *Griffonia simplicifolia* (GS lectin, I-21413; Molecular Probes), fluorescence-conjugated phalloidin (A-12381; Molecular Probes), mouse monoclonal anti-glial fibrillary acidic protein (GFAP, G-3893; Sigma Chemical Co., St. Louis, MO), and monoclonal anti-R-cadherin (primary incubation for 3 days at 4°C, 1:100 dilution; C38020; Transduction Laboratories, Lexington, KY). In each case, preimmune IgG and secondary control incubations were performed to determine specificity of staining.

Retinal sections

Retinas were dissected, laid flat with four radial relaxing incisions, placed in 4% PFA, and incubated at 4°C overnight. Retinas were then placed in 20% sucrose for 2 hours and embedded in optimal cutting temperature compound (OCT; Tissue-Tek; Sakura FineTech, Torrance, CA). Cryostat sections (10 μm) were postfixed (Prefer; Anatech Ltd., Springfield, Va), rehydrated in PBS, and blocked with 5% BSA-2% NGS in PBS at room temperature for 2 hours. Blood vessels were visualized with rabbit anti-mouse collagen IV (5 μg/mL), and R-cadherin was visualized with rabbit anti-mouse R-cadherin polyclonal antibodies (4 μg/mL; sc-7941; Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer overnight at 4°C. After washes in PBS, the sections were visualized by incubation with fluorescently labeled secondary antibodies.

Transgenic Mice

GFAP-GFP (FVB/N-TgN(GFAPGFP)14mes) transgenic mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Males homozygous for the transgenic phenotype were mated with wild-type BALB/c females to outbreed the *rd* phenotype characteristic of FVB mice. Neonatal mice were tested at various days after birth for expression of green fluorescent protein (GFP) by analyzing thin sections of brain for astrocyte fluorescence. Retinas were dissected from pups positive for the transgene and analyzed by confocal microscopy. To obtain embryonic mice, males homozygous for the transgene were placed with females in the morning and females were checked for evidence of mating in the evening. Females were killed at appropriate days after mating and embryos obtained and the retinas dissected.

Microarray Analysis

Retinas were dissected in RNase-free medium, transferred directly into RNA extraction reagent (TRIzol; Gibco BRL), and frozen at −80°C for less than 1 month. Thirty retinas from each time point were pooled, and total RNA was isolated according to the manufacturer's protocol. Isolated RNA was further purified (RNeasy kit; Qiagen, Valencia, CA). Integrity of the RNA was verified before reverse transcription by visualization of the 28S and 18S ribosomal RNA bands. After reverse transcription, cDNA was transcribed in vitro, with incorporation of labeled ribonucleotides. cRNA was then fragmented and hybridized to microarray gene chips (Mu74a; Affymetrix, Santa Clara, CA). Data were analyzed on computer (GeneSpring software; Silicon Genetics, Redwood City, CA).

Intravitreous Antibody Injections

Intravitreous injections were performed as previously described.[16] Briefly, an eyelid fissure was created in postnatal day (P)2 or P7 mice to expose the globe for injection. Antibodies against extracellular regions of R-cadherin (sc-6456 or sc-7941; Santa Cruz Biotechnology) were injected in 0.2-mg/mL concentrations (P2, 0.5 μL/eye; P7, 1.5 μL/eye) with a syringe (Hamilton, Reno, NV) fitted with a 32-gauge needle. The injection was made between the equator and the corneal limbus. During injection, the location of the needle was visualized through a dissecting microscope to ensure that it was in the vitreous cavity. After the injection, the eyelids were repositioned to close the fissure. Three days later, the retinas were dissected as described, and the vessels and astrocytes visualized by immunohistochemistry. Quantification of peripheral vascularization, vascular length, and vascular area was achieved by imaging noninjected, preimmune injected, and anti-R-cadherin antibody–injected retinas under the same microscopy settings. Numbers were then generated by computer (LaserPix software; Bio-Rad, Richmond, CA), with noninjected control littermates used for baseline normalization of the extent of retinal vascularization.

Confocal Microscopy

Retinal wholemounts and retinal sections were examined with a multiphoton confocal microscope (model MRC1024MP; Bio-Rad). Three-dimensional vascular images and projected images were produced from a set of Z-series images by using the accompanying software (Confocal Assistant; Bio-Rad).

Results

Developmental Angiogenesis in the Mouse Retina

Immediately after birth (P0), spokelike peripapillary vessels were observed growing radially from the optic nerve head, becoming progressively interconnected by a capillary plexus that formed between them. This superficial retinal plexus grew in area, volume, and complexity, as a monolayer within the nerve fiber layer during the first 10 days after birth (Figs. 1A, 1B). The second phase of formation of retinal vessels began between P7and P9 when collateral branches sprouted from capillaries of the superficial plexus. These sprouts branch and migrate toward the outer retina where they anastomose laterally to form a planar secondary, deep, vascular plexus at the outer edge of the inner nuclear layer (Fig. 1C). This is followed by the formation of an intermediate vascular plexus between the primary and secondary vascular layers during the third postnatal week (Fig. 1D). The reproducibility of this process and its easy accessibility in postnatal animals provide an opportunity for experimental manipulation and a chance to cor-

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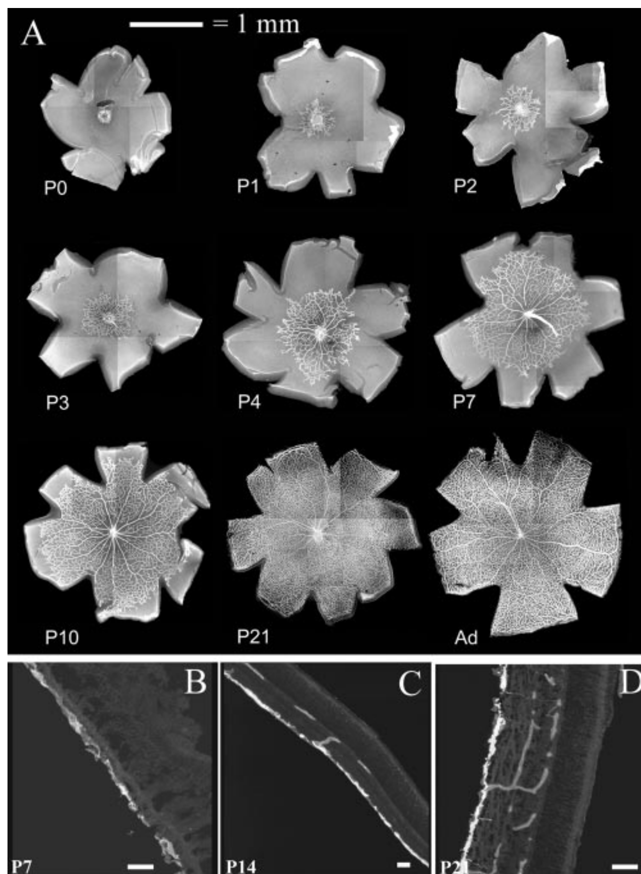

FIGURE 1. Neonatal mouse retinal vascular development. (**A**) Visualization of the retinal vasculature from BALB/c mice by collagen IV immunostaining. At birth (P0), mouse retinal vasculature is absent. During the first three postnatal weeks, an adult vascular pattern is formed as endothelial cells migrate and proliferate from the central retinal artery toward the retina periphery. (**B–D**) Collagen IV staining of blood vessels in retinal cross sections illustrate the formation of the deep vascular networks. (**C**) Vessels branch and migrate from the superficial vascular layer and form the secondary, deep vascular network at the outer edge of the inner nuclear layer (INL) between P7 and P10. (**D**) A tertiary, intermediate vascular plexus then forms between the superficial and deep vascular networks at the inner edge of the INL during the third postnatal week. Scale bars: (**A**) 1 mm; (**B–D**) 40 μm.

relate the developmental events of normal angiogenesis with the spatial and temporal appearance of critical angiogenic factors.

Guidance of Retinal Angiogenesis

Previous studies have demonstrated a relationship between the developing retinal vasculature and retinal astrocytes,[26–29] largely based on immunohistochemical analysis with anti-glial fibrillary acidic protein (GFAP) antibodies. These antibodies generally are directed against epitopes found in polymerized GFAP (characteristic of more mature astrocytes) and may not recognize the GFAP monomers that are present in less mature astrocytes. To gain better understanding of the relationship between astrocytes and the developing retinal vasculature, we examined mouse retinas at various times of development, by using both GFAP-specific antibodies and GFAP-GFP transgenic mice. This allowed us to visualize earlier astrocytic events, because both GFAP monomers and polymerized GFAP can be observed in transgenic GFAP-GFP mice.

When GFAP-GFP transgenic mouse retinas were evaluated, retinal astrocytes were observed significantly ahead of the migrating vascular front. In fact, fluorescent cells were visible nearly to the retinal periphery by birth. Embryonic mouse retinas from GFAP-GFP mice were examined to determine when astrocytes first appeared on the retina. We observed only minimal GFAP-positive staining of the optic nerve head at 15 days after conception (E15). However, by E19 GFAP-positive astrocytes had emerged from the optic nerve head onto the retina. GFAP-positive cells continued to spread peripherally and established a complex interconnected astrocytic network by birth (Figs. 2A–D). The final staining pattern for the astrocytes strongly resembled that later observed with collagen IV staining of retinal endothelial cells. Costaining for GFAP and collagen IV showed a strong correlation between the pattern of retinal vessels and retinal astrocytes. Although there were regions positive for astrocytes and negative for endothelial cells, the converse was not true. Endothelial cells were never observed in regions without underlying astrocytes. This astrocytic template for endothelial cell growth was observed throughout the development of the superficial vascular plexus (Figs. 2E, 2F).

Once the vascular plexus had formed and the vessels began to mature, astrocytes became solely associated with endothelial cells. In mature vessels from 6-week-old mice, the astrocytes surrounded each vessel, and GFAP-positive, endothelium-negative regions were no longer observed (Fig. 2G). During initial formation of the deeper vascular plexuses, no GFAP-positive cells were observed in the deeper plexus regions. However, by P42, GFAP-positive cells resembling Müller cells, a glial cell type with processes that project to both the nerve fiber and photoreceptor cell layers, were observed at the interface of the inner nuclear and outer plexiform layers where the deep vascular plexus had formed (Fig. 2H).

Morphology of the Tips of Migrating Endothelial Cells

Because collagen IV is an extracellular matrix component that becomes a part of the vascular basement membrane relatively late in angiogenesis, we decided to use another marker that directly binds to the endothelium as the vessels form. Fluorescently labeled GS lectin is a relatively early endothelial cell marker and has the advantage of consistently staining all regions of endothelium, although it also stains other cell types including retinal microglia.[30] In general, the staining pattern of GS lectin was very similar to that observed with collagen IV. However, at the tips of the growing vessels, significant differences were observed. Fingerlike processes were observed at the tips of the growing endothelial cells during development of both the superficial and deep vascular plexuses (Fig. 3). Filamentous actin, a feature consistent with filopodia-like cellular extensions, was observed in these processes by using phalloidin staining (Fig. 3D). These three-dimensional filopodia-like processes were observed extended from the tips of endothelial cells at the developing vascular front and within regions behind the vascular front where the complex vascular interconnections were forming (Figs. 3A, 3B). Filopodial extensions were also observed at the tips of migrating endothelial cells as the vessels branched, dived, and subsequently formed the complex interconnections of the deep vascular plexuses (Fig. 3C). Similar to the results obtained with collagen IV staining, GS lectin and GFAP costaining of developing retinas demonstrated that endothelial cells were always associated with the underlying astrocytic template. A significant observation was that filopodial extensions at the tips of endothelial cells were also associated with underlying astrocytes (Figs. 3E, 3F). Long, extended filopodia nearly always colocalized with an underlying astrocytic process, whereas the significantly fewer number of filopodia observed in an astrocyte-negative region appeared shorter and much less extended.

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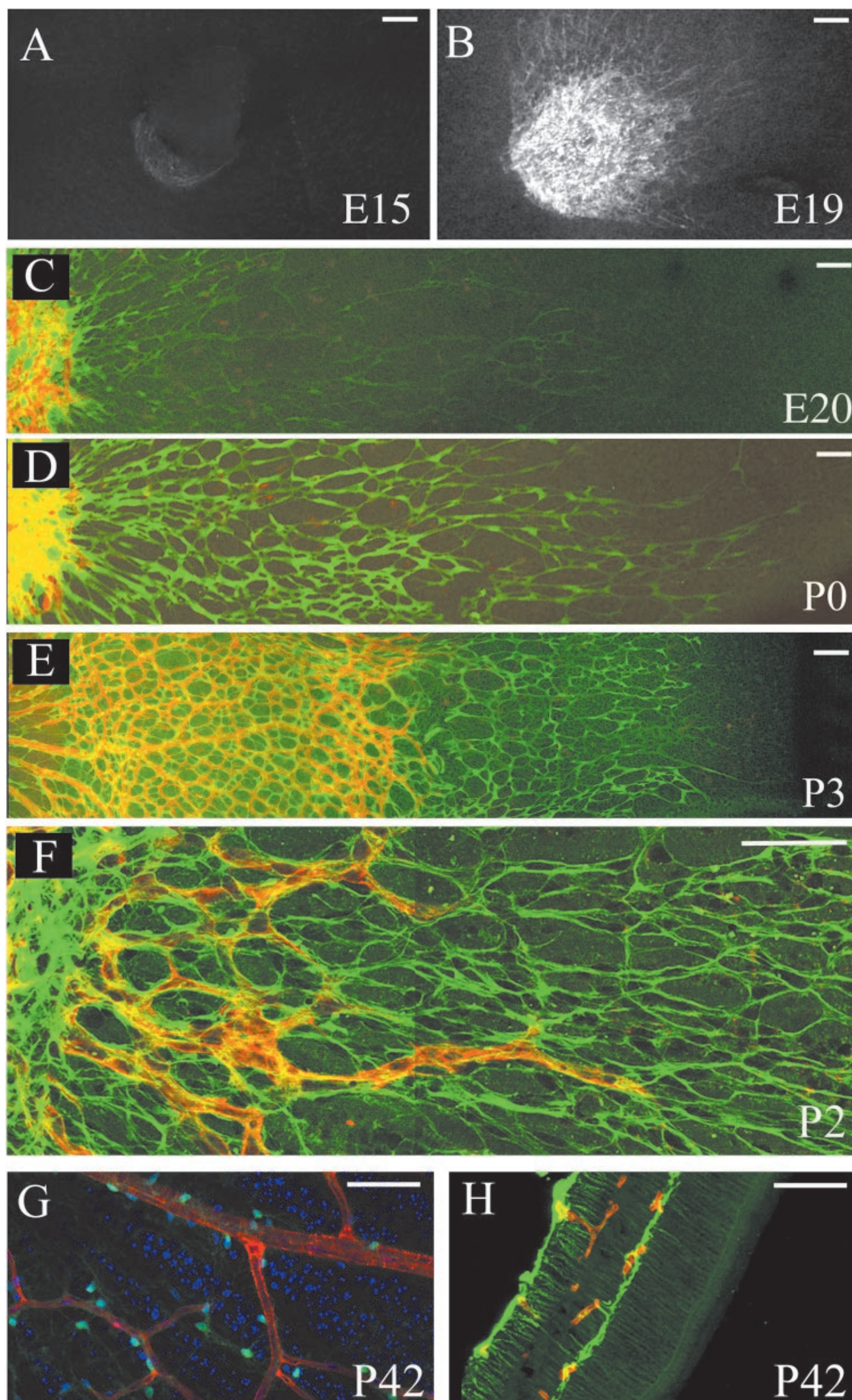

FIGURE 2. Endothelial cells grow onto a preexisting astrocytic template. GFAP-GFP transgenic mice were used to study the relationship between astrocytes and endothelial cells. (**A**) Astrocytes are absent from the retina at embryonic day (E)15. (**B**) Astrocytes have emerge from the optic nerve by E19, (**C**) continue to migrate toward the periphery, and (**D**) form a nearly complete astrocytic network by birth. (**D–F**) Endothelial cells (collagen IV, *red*) migrate and develop along the astrocytic template (GFAP, *green*) as the superficial vascular network forms during postnatal week 1. (**F**) High-magnification image of endothelial cells migrating across the astrocytic template in a P2 retina. (**G**) In a mature P42 mouse retina, astrocytic processes wrap around vessels and astrocytes (*green*) and are solely associated with retinal vasculature (*red*). *Blue* nuclei are stained with 4′,6′-diamino-2-phenylindole (DAPI). (**H**) In P42 retinas, cells resembling Müller cells (*green*) within the deep retina layers are GFAP-positive. Collagen IV–stained vessels are *red*. Scale bars, 40 μm.

Correlation of Expression of R-Cadherin with Development of Retinal Vasculature

To identify factors that may be involved in the guidance of endothelial cells as they migrate across the underlying astrocytic template, we used gene microarray chips (Affymetrix) to study the expression profiles of thousands of different genes at various times during retinal vascular development. Analysis of the gene expression profiles revealed R-cadherin, a member of the large family of calcium-dependent cell–cell adhesion molecules,[31,32] to be a gene with significant changes in expression levels, temporally correlated with retinal vascularization during postnatal development in the mouse. Expression of R-cadherin was high at P0 and P4 when the primary vasculature was developing. Expression levels became negligible at P8, as for-

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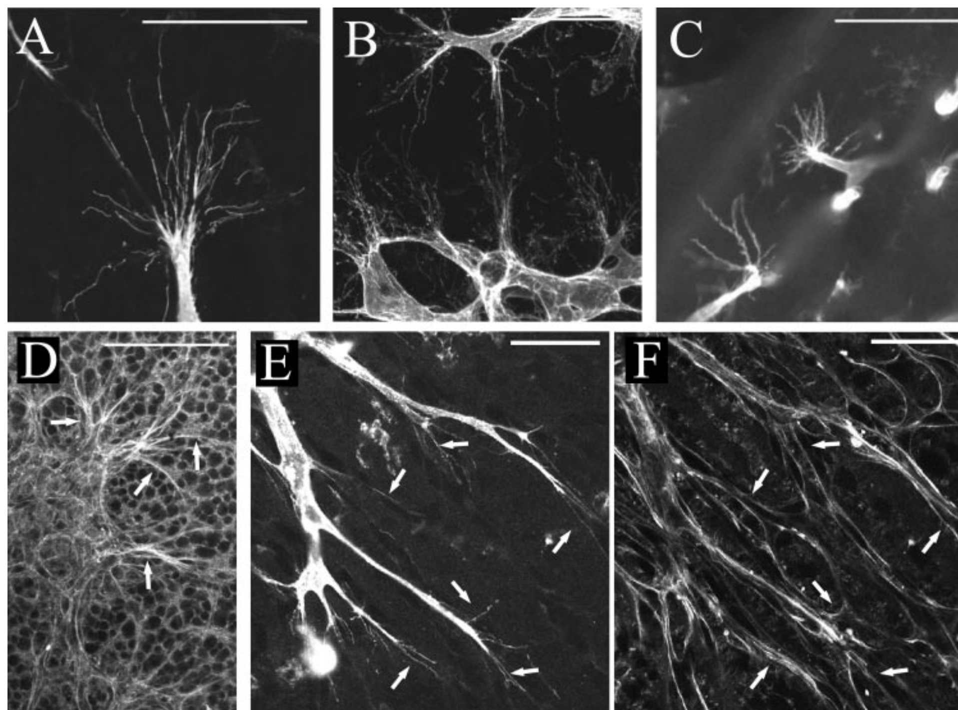
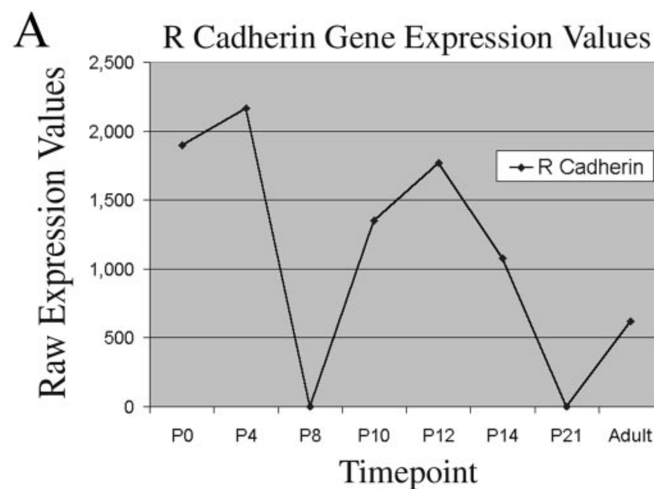
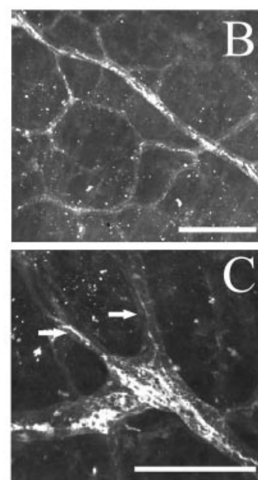


FIGURE 3. Endothelial cell filopodia are present at the tips of developing retinal vessels. GS lectin was used to label endothelial cells and visualize endothelial cell filopodia in postnatal BALB/c mouse retinas. (**A**, **B**) High-magnification projection images of a P4 wholemount showing (**A**) filopodial extensions from an endothelial cell at the leading edge of the growing superficial vascular network, and (**B**) filopodial extensions from endothelial cells forming complex capillary connections within newly vascularized regions. (**C**) Filopodial extensions from branching endothelial cells of a P8 mouse in the inner plexiform layer as they migrate toward the deep vascular plexus. (**D**) Phalloidin staining of filamentous actin in a P4 retina wholemount. *Arrows*: regions of filamentous actin localized within filopodia-like extensions. (**E**, **F**) A P4 retina stained with GS lectin (**E**) and GFAP (**F**) shows that most of the extended endothelial filopodia colocalize with the underlying astrocytic template. *Arrows*: regions of colocalization. Scale bars, 40 μm.

mation of the superficial vascular network neared completion. R-cadherin mRNA expression levels then increased during formation of the deep vascular layers, before again decreasing after vascular maturation in P21 and adult mice (Fig. 4A).

Our microarray results confirmed previous studies in which in situ hybridization studies of cadherin expression demonstrated similar expression patterns of R-cadherin in the neural neonatal mouse retina. Honjo et al.[33] showed expression of R-cadherin mRNA in the ganglion cell layer at P0 and P3 and in the inner and outer regions of the inner nuclear layer at P7 and P14. This corresponds precisely to sites of formation of a superficial vascular layer in the ganglion cell layer and, subse-





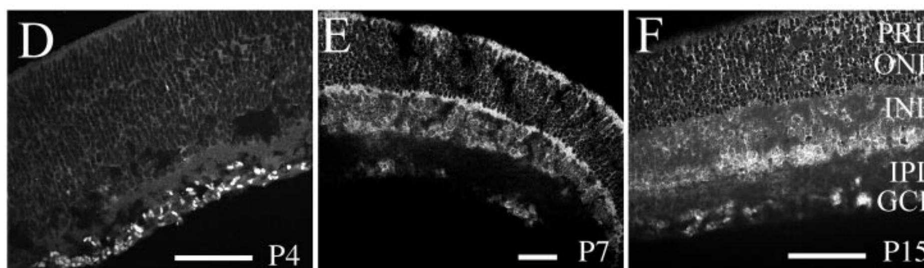


FIGURE 4. Expression of R-cadherin during retinal development in the mouse. (**A**) Gene expression analysis using microarray chips. R-cadherin expression was high at P0 and P4, negligible at P8, and increased again from P10 to P14 during formation of the deeper vascular plexuses. (**B**, **C**) R-cadherin expression in retina wholemounts. (**B**) R-cadherin antibodies label P5 retina vessels in a punctate, skeletal-like fashion resembling potential cadherin mediated cell–cell adhesion clusters between endothelial cells and associated astrocytic processes. (**C**) R-cadherin staining at the leading edge of vascular growth. *Arrows*: cadherin staining just ahead of an endothelial cell at the leading vascular edge. (**D**–**F**) Immunohistochemical localization of R-cadherin protein expression in mouse retinal sections. (**D**) In cross sections of P4 mouse retinas, R-cadherin is expressed solely in the ganglion cell layer. (**E**) In P7 mouse retinal sections, most R-cadherin expression was localized in the outer region of the inner nuclear layer where the deep vascular plexus soon forms. (**F**) In P15 mouse retinal sections, R-cadherin protein expression was localized to the inner region of the inner nuclear layer where the intermediate tertiary vascular plexus soon forms. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer photoreceptor nuclei; PRL, photoreceptor layer. Scale bars, 40 μm.

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quently, to the sites of formation of the deeper vascular networks. We confirmed this finding at the protein level with immunohistochemical analysis of P4, P7, and P15 retinal sections (Figs. 4D–F). Thus, expression of R-cadherin protein correlated both spatially and temporally with sites of retinal vascularization. Immunohistochemical analysis of R-cadherin on P5 retinal wholemounts, showed distinct staining of the vasculature, characterized by punctate, skeleton-like staining along vessel ridges. This staining pattern is consistent with the formation of cadherin clusters between adjacent cells, and the skeleton-like staining pattern observed along the vessels is very similar to GFAP staining of astrocytic processes wrapped around new vessels (Fig. 4B). At high magnification, faint staining of endothelial tips at the vascular front were observed, suggesting that small R-cadherin clusters may be present at sites of filopodial extension (Fig. 4C). When preimmune mouse IgGs were used in the same staining conditions, no significant staining was observed.

Effect of Injection of R-Cadherin–Blocking Antibodies on Retinal Vascular Development

To test whether R-cadherin is important in development of normal retinal vascularization, polyclonal antibodies specific for the N terminus of R-cadherin were injected intravitreously into P2 mice. These antibodies bind to a region within the extracellular cadherin domain 1 known to be important for cadherin transdimerization.[34] The mice were analyzed 3 days later (P5) and compared with littermates injected with preimmune IgG serum. Injection of R-cadherin antibodies during formation of the primary vascular layer caused incomplete vascularization (Figs. 5A, 5B, 5E) compared with normal, noninjected retinas and retinas injected with control IgG (Figs. 5C, 5F). Vessels in anti-R-cadherin–treated retinas failed to extend as far to the periphery. These vessels also appeared atrophic, were characterized by the absence of normal complex capillary connections, and generally had increased numbers of microglia surrounding vascularized regions. These effects were quantified by measuring the distance vascularization had proceeded from the central retinal artery, the total length of vessels, and the total area of vascularization (Fig. 5G). Measurements from noninjected control littermates were used to normalize test and control-injected retinas to 100%, indicating normal vascularization. The astrocytic template did not appear to be affected by injection of R-cadherin antibodies, even in regions of abnormal vascularization (Fig. 5D), suggesting that R-cadherin antibodies may mask guidance cues required for endothelial cells to follow the pattern already established by astrocytes.

We also investigated the potential role of R-cadherin in the formation of the deep vascular plexuses. R-cadherin antibodies or control preimmune IgG serum was injected intravitreously at P7, just before the superficial vessels branched and began formation of the deep vascular plexus. Retinal sections and wholemounts were subsequently analyzed at P10, by which time most of the secondary network had ordinarily formed. In control IgG-injected eyes, retinal vascular development was essentially normal. Vessels branched from the superficial vascular network and migrated to the outer edge of the inner nuclear layer where they turned and formed a secondary vascular plexus parallel to the superficial network. In contrast, eyes injected with R-cadherin antibody contained many regions where vessels continued to migrate past the region of the normal deep vascular plexus into the photoreceptor layer (Fig. 6). Filopodial processes were observed extending from the vessels migrating beyond the normal deep vascular plexus into the photoreceptor layer (Fig. 6C), suggesting that the endothelial cells continue to search the surrounding area for guidance cues. This effect was quantified by using confocal microscopy, focusing within the photoreceptor region, and counting the number of vessels that appeared beyond the normal deep vascular region (Fig. 6E). With this method of quantification, a small number of vessels were observed beyond the normal deep vascular plexus in control retinas that were most likely due to mechanical dissection and mounting artifacts. We have never observed any vessels in the photoreceptor layer in cross-sections of retinas from mice injected with preimmune serum. When the R-cadherin antibody solution is mixed with a 1:1 ratio of a blocking peptide corresponding to the antibody antigen before injection, the number of abnormal, penetrating deep vessels is reduced by 60%. Injection of polyclonal antibodies against N-cadherin had no effect, and retina vasculature was similar to that of control-injected retinas (data not shown). It is significant to note that, during extensive studies of this mouse retinal development model, we have never before observed vessels migrating beyond the normal secondary plexus.

Discussion

We have analyzed an in vivo developmental model of physiological angiogenesis in which normal mice are born without retinal vasculature. The normal adult vascular pattern forms over a 3-week period as endothelial cells emerge from the central retinal artery and vascularize the retina through the process of angiogenesis, analogous to human retinal vascular development in the third trimester. The reproducibility of this model allowed us to analyze factors involved in normal physiological angiogenesis with the entire environmental context intact. From this analysis, we have developed a paradigm that may also be relevant to other developmental and pathologic angiogenic systems. Although the use of a preexisting astrocytic template to determine the developmental pattern of angiogenesis in the retina may be unique to this tissue, other components of this system may be relevant to angiogenesis in general, and the identification of unique filopodia-mediated cell–cell interactions provide a conceptual framework within which to analyze directed cell migration during the establishment of vascular patterns.

Brief reference to filopodia-like processes at vascular tips has been made in previous studies of primate[35] and human[36] retinal vasculature development. Numerous studies have also focused on endothelial cell migration, and the role of filopodia during migration of many different cell types in vitro. In these studies, lengths and stability of the filopodial processes have been shown to be dependent on adequate access to patterning information.[37] Guidance cues are also important for endothelial cell migration in vitro. Properties of endothelial cell migration can vary greatly with growth on various substrates, and specific adhesion molecules have been shown to play an important role in endothelial cell polarization and migration.[38] This is similar to our findings in vivo in which long, extended, stabilized filopodia colocalize with underlying astrocytes, and formation of normal vessel patterns appears to be dependent on sufficient guidance cues.

A relationship between astrocytes and the retinal vasculature has been established.[26–29] In fact, only species with vascularized retinas are known to express retinal astrocytes and within the retinas of those species, astrocytes are observed only in regions in which vascularization occurs.[39,40] Other groups have characterized the role of astrocytes in maintaining vascular integrity and postulate that they may serve to prevent migration of retinal vasculature into the vitreous.[41] Astrocytes may also serve to promote retinal angiogenesis through hypoxia-induced expression of VEGF.[42–44] Earlier studies of astrocytes during early neonatal retinal vascular development

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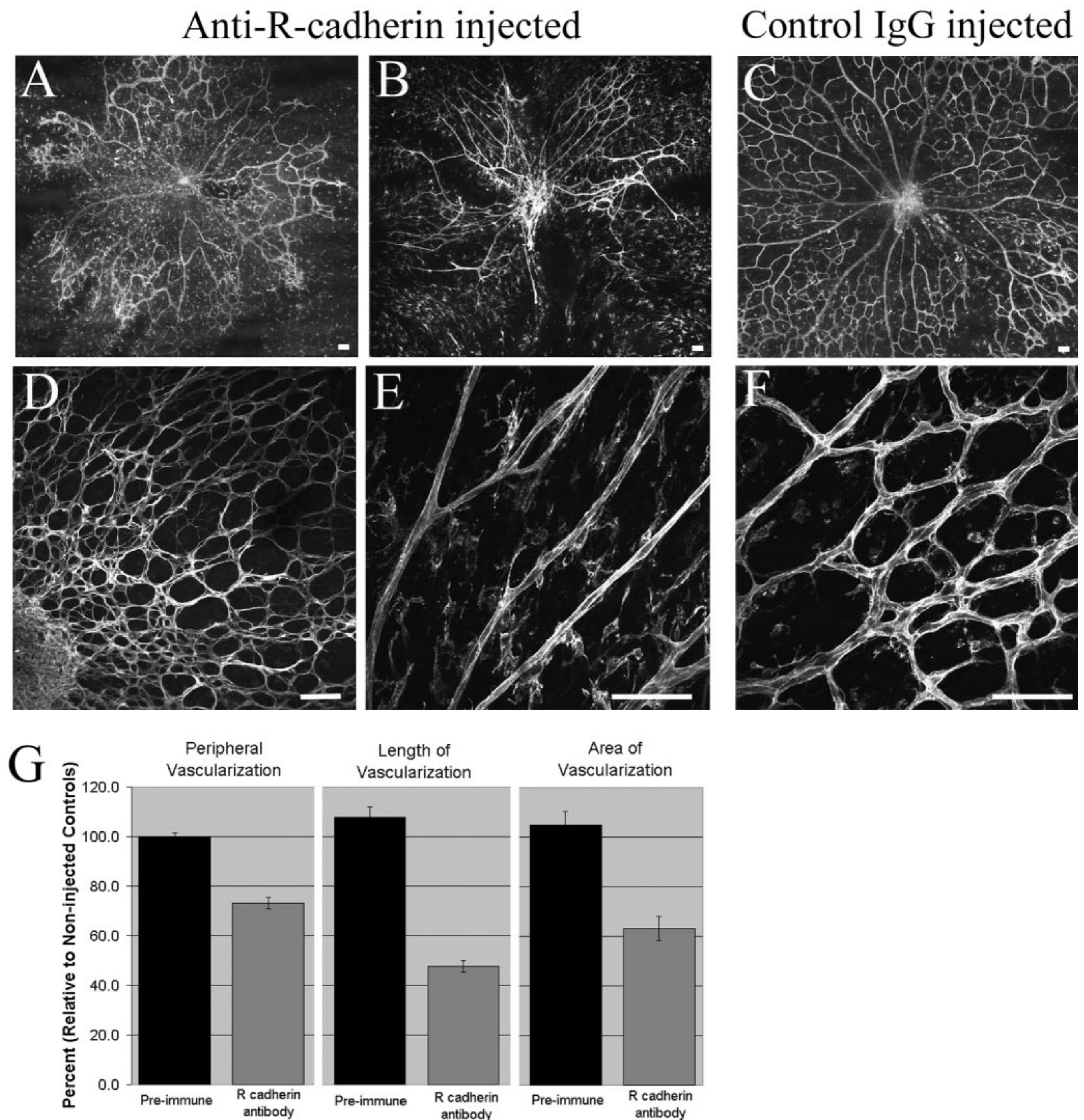

Figure 5. R-cadherin function-blocking antibodies prevented normal formation of the superficial vascular plexus. (**A–F**) P5 wholemounts of retinas from mice injected intravitreously at P2 with R-cadherin–blocking antibodies (**A**, **B**, **D**, **E**) or preimmune control IgG serum (**C**, **F**). (**A–B**) Representative images of two separate retinas injected with antibodies specific for R-cadherin and prepared for wholemount GS lectin staining. Effects on treated retinas ranged from abnormal vessels without normal complex interconnections (**A**) to the near complete obliteration of retinal vascularization (**B**). In general, retina vessels from anti-R-cadherin–injected mice appeared atrophic, numbers of microglia were increased, vascularization failed to extend to the normal peripheral region, and vessels with normal interconnected capillaries were markedly decreased. (**C**) The superficial vascular plexus of control preimmune IgG-injected retinas formed normally with an extensive interconnected vascular network. (**D**) GFAP-stained astrocytes appeared normal in anti-R-cadherin–injected retinas with abnormal vascularization. (**E**, **F**) High-magnification images showing (**E**) the absence of interconnecting vascular capillaries in anti-R-cadherin–injected retinas and (**F**) normal vascular connections in a control preimmune IgG-injected retina. (**G**) Quantification of the extent of peripheral vascularization, total vessel length, and total area of vascularization in control preimmune IgG-injected or R-cadherin antibody–injected retinas normalized to vascularization of noninjected control littermates (100%). $P < 0.0001$ for each method of quantification. Error bars, SEM. Data were obtained from 15 control retinas and 25 R-cadherin–injected retinas. Scale bars, 40 μm.

largely used immunohistochemical analysis with antibodies to GFAP. Our findings extend these studies significantly by demonstrating the presence of a nearly complete astrocytic template covering the entire retina by birth. The use of GFAP-GFP transgenic mice provides the advantage of visualizing monomeric GFAP, as well as polymerized GFAP fibrils, which would

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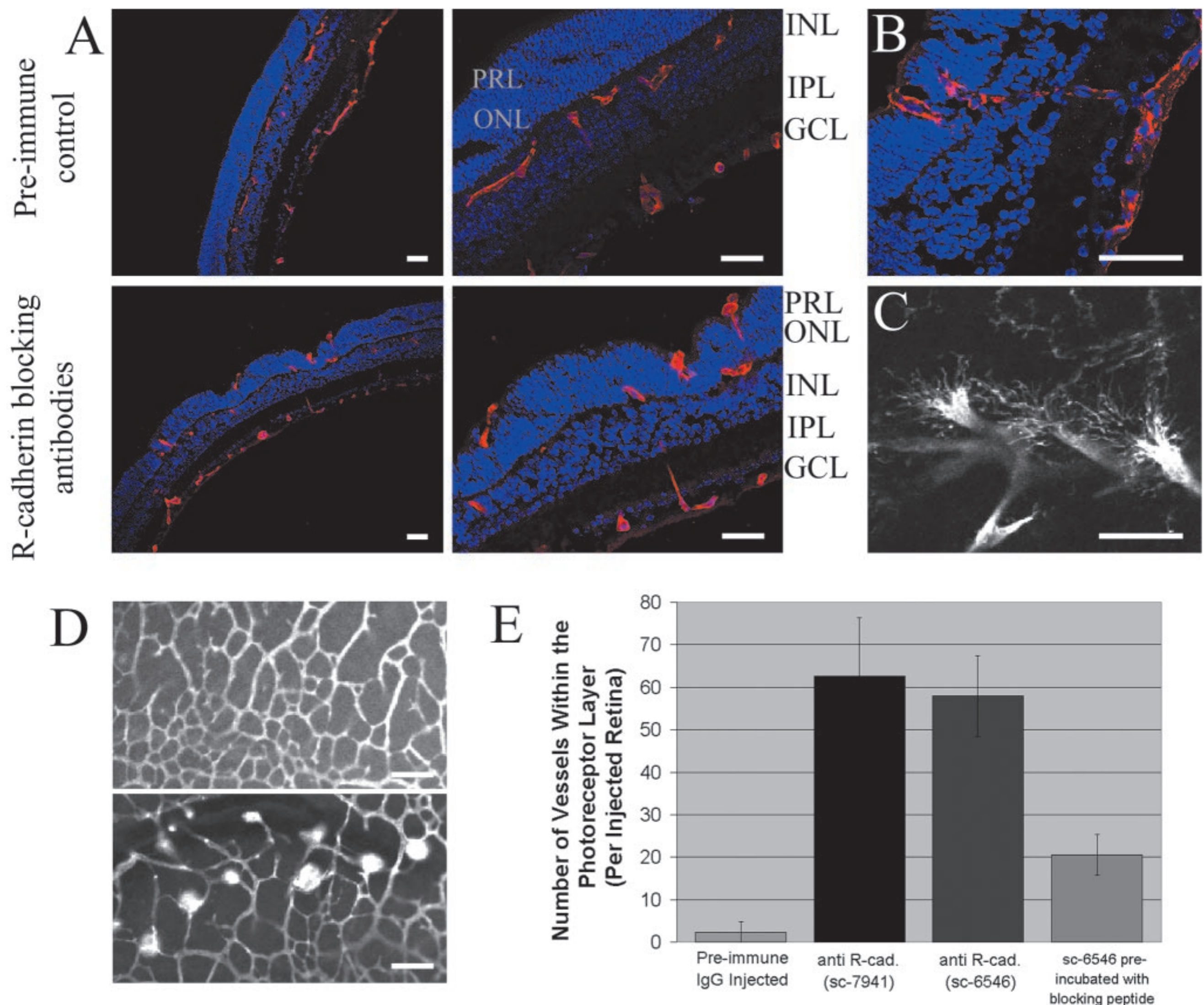

FIGURE 6. Results from P7 intravitreous injections of R-cadherin function-blocking antibodies. (**A**) Cross sections of preimmune IgG-injected, or R-cadherin antibody–injected retinas during formation of the secondary vascular network stained for nuclei with 4′,6′-diamino-2-phenylindole (DAPI; *blue*) and blood vessels with anti-collagen IV (*red*). In control-injected sections, the deep vascular plexus formed normally at the outer edge of the inner nuclear layer. In retinas injected with R-cadherin antibodies, vessels migrated past the normal vascular plexus and were observed in the normally avascular photoreceptor layer. (**B**) High-magnification image of a cross section from a P10 retina injected at P7 with R-cadherin antibodies. Collagen IV staining of the vessel (*red*) and DAPI staining of retinal nuclei (*blue*) showed a blood vessel that had branched from the superficial network and continued to migrate past the normal vascularization plexus into the photoreceptor layer. (**C**) High magnification of GS lectin-labeled vessels from a wholemount retina within the photoreceptor layer. Filopodia extended toward the RPE in the back of the retina from vessels that continued to migrate past the normal secondary plexus and deep into the photoreceptor layer. (**D**, **E**) Quantification of the number of vessels that migrate past the deep vascular plexus into the photoreceptor layer per injected retina. (**D**) Focusing just below the normal deep vascular plexus allowed vessels migrating into the photoreceptor layer to be visualized as vascular tufts (*bottom*) compared with regions with normal deep vascular development (*top*). (**E**) The number of vessels migrating into the photoreceptor layer per injected retina is reported for retinas from control eyes injected with preimmune IgG serum, eyes injected with anti-R-cadherin (sc-7941), eyes injected with anti-R-cadherin (sc-6546), and eyes injected with anti-R-cadherin (sc-6546) preincubated with a 1:1 ratio of blocking peptide corresponding to the antibody's antigen. Error bars, SEM. $n = 10$ retinas for each column. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer-photoreceptor nuclei; PRL, photoreceptor layer. Scale bars, 40 μm.

explain the apparent difference between our observations and those previously reported.

In this study, retinal endothelial cells migrated across, and colocalized with, underlying retinal astrocytes in a pattern that was already established at birth. The exact mechanism by which the astrocytes facilitated endothelial cell migration is not clear. Colocalization of endothelial cells and astrocytes may be an indirect result of a third set of factors involved in guidance of both. In this case, astrocytes would not necessarily be directly involved in endothelial cell guidance. However, this seems unlikely, in light of several recent findings. In a report by Stalmans et al.,[45] mice expressing single isoforms of VEGF were tested for retinal neovascularization. Retinas from $VEGF^{165/165}$ mice that solely express the partially soluble $VEGF_{165}$ were found to be normally vascularized. However, $VEGF^{120/120}$ mice that solely express the more soluble $VEGF_{120}$ isoform had abnormal retinal vessels characterized by a decreased vascularization of the peripheral retina and numerous

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dilated capillaries in the central retina that fail to form the normal vascular pattern. Because of the inclusion of extra heparin-binding domains, $VEGF_{165}$ would remain closely associated with the astrocytes from which it was expressed, whereas $VEGF_{120}$ can diffuse further throughout the retina. Filopodia protruding from the tips of the endothelial cells may become stabilized by specific growth factors such as VEGF. During normal $VEGF_{165}$ expression, astrocytes may guide endothelial cells by promoting growth at regions of $VEGF_{165}$ expression near astrocytes, thus resulting in the formation of a distinct vascular pattern colocalized with the underlying astrocytes.

Retinal astrocytes have been shown to express R-cadherin.[46,47] We found that injection of R-cadherin antibodies prevented formation of a normal superficial vascular network, resulting in decreased peripheral vascularization and loss of normal collateralization and vascular patterning. This suggests that astrocytes may guide endothelial cell growth and migration through specific cell adhesion molecules including R-cadherin and the selective expression of VEGF isoforms. Growth factor receptors are coordinated with specific integrin receptors,[48] the function of which is critical for normal retinal angiogenesis. Cross talk between integrins and cadherins has also been demonstrated.[49-51] Thus, astrocytes may direct endothelial cell growth and guidance during retinal developmental angiogenesis by secretion of $VEGF_{165}$ and the expression of selected adhesion molecules such as R-cadherin. Attracted by $VEGF_{165}$ expression along astrocytes, endothelial cell filopodia may subsequently be stabilized by binding R-cadherin on the astrocytes causing the formation of adhesion complexes and directing endothelial cell growth along the preexisting astrocytic template. Cell adhesion molecules are known to participate in a similar process during axonal growth. For example, cell adhesion molecules expressed at the leading edge of axonal growth cones lead to changes in intracellular signaling. This results in cytoskeletal reorganization and, depending on the types of extracellular interactions and guidance cues encountered, results in directed neuronal migration.[52]

Endothelial cells are known to express N-cadherins, which may be involved in formation of the blood–brain or blood–retinal barriers through interactions with surrounding astrocytes and pericytes.[53] Although cadherins preferentially form homodimers in vitro, functional heterodimers between N-cadherin and R-cadherin heterodimers have also been demonstrated.[31,54,55] In the optic nerve of chickens, retinal axons expressing N-cadherin are guided along R-cadherin–positive glia.[46] Thus, endothelial cells could interact with, and potentially be guided by, astrocytes and other R-cadherin expressing cells through interactions between N- and R-cadherins. Another possibility, supported by immunolocalization of R-cadherin along endothelium in retina wholemounts, is that retinal endothelial cells express R-cadherin, making homodimeric interactions between adjacent endothelial and neuronal cells possible.

We found that R-cadherin also had temporal and spatial expression directly correlated to the development of deep mouse retinal vascularization. Antibodies against R-cadherin injected at P7, just before development of the deep vascular plexus, caused abnormal retinal vascularization as vessels migrated past the normal deep vascular plexus and into the photoreceptor layer. The invasion of retinal vessels into the photoreceptor layer and subretinal space has been observed in transgenic mice in which photoreceptors are induced to overexpress VEGF.[56] These findings are consistent with the proposal that deep vascular plexuses form in response to a cytokine gradient resulting from hypoxia within the retina. Our findings that R-cadherin antibodies also cause vessels to migrate beyond the normal deep vascular plexus and into the photoreceptor layer are also consistent with this hypothesis. Vessels are induced to migrate toward the deep vascular plexus. As they dive, they extend filopodia at the vessel front. When these filopodia encounter R-cadherin already expressed by cells at the outer edge of the inner nuclear layer, the vessels are induced to turn again and form the planar, deep vascular network. R-cadherin in this case could be a positive or negative guidance cue by either forming transcadherin adhesions and inducing endothelial cells to migrate within the R-cadherin–positive zone or by giving negative repulsion cues to the endothelial filopodia, thus preventing the vessels from passing into the photoreceptor layer. These guidance cues can be overcome by overexpression of the chemoattractant in the photoreceptor layer, or in our experimental system, by masking the R-cadherin adhesion molecules.

Blood vessels and nerves face many of the same challenges during development.[57] This is especially true in a complex striated tissue such as the retina, where the exact localization of different neuronal cell types and subsequent synapse formations, as well as localization of vascular beds, is critical for normal function. Abnormal vessel growth is associated with the loss of vision caused by many blinding diseases.[58,59] Thus, similar guidance mechanisms (including glial tracts and cadherins) may facilitate parallel requirements necessary for both neuronal and vascular pattern development. Glial processes are known to mediate directed neuronal migration in the developing brain and along the optic nerve. In this study we provide evidence that retinal astrocytes may also be involved in directing endothelial cell migration. In addition, cadherins are known to regulate development of the neural retina. Differential distributions of various cadherin members guide and restrict the different neuronal subtypes during formation of the complex striated retina.[33,60,61] These specific cadherin expression patterns may also be involved in creating the distinct vascularization patterns, allowing endothelial cells to use previously established neuronal guidance cues.

We have described a developmental angiogenic process where adjacent glial cells provide a template over which filopodial-mediated endothelial cell migration takes place, resulting in the establishment of a characteristic retinal vascular pattern. Recent work from our laboratory shows that, in addition to retinal endothelial cells, specific subpopulations of hematopoietic stem cells (HSCs) can selectively target this astrocytic template and participate in the formation of a stable retinal vasculature.[62] In fact, the interaction between the astrocytic template and developing vasculature appears to be restricted to retinal endothelial cells and these HSCs. Furthermore, HSCs, when injected intraocularly prior to the formation of the deep retinal vascular layers, migrate into the retina and localize to sites identical to those at which the deep retinal vascular layers will ultimately form. This suggests that signals necessary for proper localization of the developing vasculature are already in place prior to the time at which GFAP-expressing glia can be detected. The involvement of glia and R-cadherin, traditionally viewed as neuronal cues, suggests that common mechanisms may be operational during vascular and neuronal development. The well-known association of gliosis with many ocular neovascular processes suggests that our findings in the developing retinal vasculature may have applications to understanding pathologic neovascularization and may lead to the identification of additional factors common to neuronal and vascular development and diseases.

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