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

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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 isoelectric 

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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; Gibco BRL, 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 rinses and mounting on slides with slow-fade medium. Antibodies and molecular markers in 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 (GeneSpring software; Silicon Genetics, Redwood City, CA).

**Intravitreal Antibody Injections**

Intravitreal injections were performed as previously described. 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-64555 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 littersmates 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 P7 and 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-
Because both GFAP monomers and polymerized GFAP can be detected in mice. This allowed us to visualize earlier astrocytic events, examined mouse retinas at various times of development, by relating 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, 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 that had emerged from the optic nerve head into 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 vascularplexus (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 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. 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.
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, 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...
formation 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. 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, subs-

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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 intravitreally 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 vasculogenesis (Figs. 5A, 5B, 5E) compared with normal, non-injected retinas and retinas injected with control IgGs (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 vasculogenesis (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 intravitreally 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 primate35 and human36 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.57 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.30 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.26–25 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 vasculogenesis occurs.59–60 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.12–14 Earlier studies of astrocytes during early neonatal retinal vascular development
largely used immunohistochemical analysis with antibodies to GFAP. Our findings extend these studies significantly by demonstrating the presence of a nearly complete astrocytic tem-
plate covering the entire retina by birth. The use of GFAP-GFP transgenic mice provides the advantage of visualizing monoclonal GFAP, as well as polymerized GFAP fibrils, which would

**FIGURE 5.** R-cadherin function-blocking antibodies prevented normal formation of the superficial vascular plexus. (A–F) P5 wholemounts of retinas from mice injected intravitreally 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 the absence of interconnecting vascular capillaries in anti-R-cadherin–injected retinas and 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 littersmates (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.
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<sup>165/165</sup> mice that solely express the partially soluble VEGF<sup>165</sup> were found to be normally vascularized. However, VEGF<sup>120/120</sup> mice that solely express the more soluble VEGF<sup>120</sup> isoform had abnormal retinal vessels characterized by a decreased vascularization of the peripheral retina and numerous

**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.
dilated capillaries in the central retina that fail to form the normal vascular pattern. Because of the inclusion of extra heparin-binding domains, VEGF165 would remain closely associated with the astrocytes from which it was expressed, whereas VEGF120 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 VEGF165 expression, astrocytes may guide endothelial cells by promoting growth at regions of VEGF165 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. 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, the function of which is critical for normal retinal angiogenesis. Cross talk between integrins and cadherins has also been demonstrated. Thus, astrocytes may direct endothelial cell growth and guidance during retinal developmental angiogenesis by secretion of VEGF165 and the expression of selected adhesion molecules such as R-cadherin. Attracted by VEGF165 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.

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. Although cadherins preferentially form homodimers in vitro, functional heterodimers between N-cadherin and R-cadherin heterodimers have also been demonstrated. In the optic nerve of chickens, retinal axons expressing N-cadherin are guided along R-cadherin-expressing glia. Thus, different endothelial and glial cell subpopulations of hematopoietic stem cells (HSCs) can selectively target this astrocytic template and participate in the formation of a stable retinal vasculature. 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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References


