Development of the Mouse Retinal Vasculature: Angiogenesis Versus Vasculogenesis

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PURPOSE. The inner vasculature of the retina develops as a spreading network, which is preceded by spindle-shaped cells. These cells are alleged to be vascular precursor cells (angioblasts). This study was designed to test whether such angioblasts exist in neonatal mouse retina.

METHODS. In situ hybridization and immunohistochemistry on mouse retinal wholemount preparations were used to visualize specific vascular cell types.

RESULTS. In situ hybridization with an RNA probe against vascular endothelial growth factor receptor (VEGFR)-2 (a marker for endothelial cells and angioblasts) labeled the vascular network but failed to label the spindle-shaped cells in front of it. A probe against VEGFR1, a marker for endothelial cells only, revealed the same staining pattern. Pericytes, visualized with a probe against platelet-derived growth receptor (PDGFR)-β, were spread over the entire vessel network, but not beyond it. However, in situ hybridization with a probe against PDGFRα (a marker for retinal astrocytes) labeled spindle-shaped cells preceding the vessel network.

CONCLUSIONS. These observations imply that in the mouse retina the spindle-shaped cells preceding the forming vasculature are immature retinal astrocytes and not vascular precursor cells and that the primary vascular network in the retina develops by angiogenesis (budding from existing vessels) and not vasculogenesis (assembly of dispersed angioblasts). (Invest Ophthalmol Vis Sci. 2002;43:522–527)

The formation of blood vessels can occur by various mechanisms. The first major vessels, such as the dorsal aorta or the cardinal veins, are formed by a process called vasculogenesis. The term vasculogenesis describes the de novo formation of vessels from vascular endothelial precursor cells (angioblasts), which migrate to or differentiate at the location of future vessels, coalesce into cords, differentiate into endothelial cells, and ultimately form patent vessels. The term angiogenesis describes a different process of blood vessel formation in which no angioblasts are involved and in which proliferating endothelial cells from preexisting vessels extend the vascular network.

In the retina, both angiogenesis and vasculogenesis are reported to participate in vasculization. The retinal vasculature is a good model system for study of the development of blood vessels in general, because its vasculature is restricted to two dimensions, which simplifies the study of a vascular plexus in its entirety. In addition, development of the retinal vasculature is important in the context of retinopathies in which abnormal vessel growth in the retina can ultimately lead to blindness. Although mice offer the added benefits of well-established genetic modification techniques the mouse retinal vasculature is little used as a model system. This study attempts to establish the mouse retina as a model system for vascular development by characterizing the basic events of mouse retinal vasculization.

In general, the mouse retinal vasculature develops as it does in humans. In both species the first vessels originate at the optic nerve head and spread over the inner surface of the retina, forming a dense network. These vessels are preceded by a network of astrocytes that also spreads from the optic nerve head. Initially, retinal vessels seem to follow this network of retinal astrocytes. After the vascular network has spread across the entire retina, vessels start to sprout downward, into the inner plexiform layer, where they establish a second vascular network parallel to the first. The second vascular network is not associated with retinal astrocytes. It is a widely held view that the primary vascular development across the inner surface of the retina occurs by vasculogenesis, whereas the establishment of the secondary network in the inner plexiform layer occurs by angiogenesis. Evidence for the occurrence of vasculogenesis during the primary vascularization of the retina is based on identification of angioblasts spreading across the retina before the appearance of endothelial cells. Nissl staining of wholemount retinas has revealed a population of spindle-shaped cells spreading across the retina before the primary vascular network and even before glial fibrillary protein (GFAP)-positive retinal astrocytes are detectable. It has been said that these spindle cells are angioblasts. However, all studies demonstrating such angioblasts in the retina relied on methods that are not universally accepted, to identify angioblasts unequivocally, such as Nissl staining, labeling with Griffonia simplicifolia isoelectrin, or determination of adenosine triphosphatase (ATPase) activity.

Recent advances in our understanding of vascular development have revealed gene products that can be used as more specific markers for angioblasts. For example, vascular endothelial growth factor receptor (VEGFR)-2 (also known as flk-1) is expressed by endothelial cells but is also reportedly the earliest marker for endothelial cell precursors and is even expressed in hemangioblasts, a common precursor of blood cells and angioblasts. In this study, I tested for the existence of angioblasts during primary vascularization of the retina by visualizing gene expression of the angioblasts marker VEGFR2, using wholemount in situ hybridization. In addition, the spatial distribution of endothelial cells, pericytes, and retinal astrocytes was studied. Thus, this work may provide a basis for further studies attempting to characterize cellular interactions leading to retinal vascularization.

MATERIALS AND METHODS

Tissue Preparation

All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Retinal wholemounts were prepared as follows. After a brief fixation (3–5 minutes) in 2% (wt/vol) paraformaldehyde (PF) in phosphate-
buffered saline (PBS) the sclera was dissected from eyes in PBS and lens and vitreous were removed. Retinae were flattened and stored in methanol at −20°C. Before use, retinas were postfixed in 4% PF in PBS for 10 minutes. Nissl staining with cresyl violet was performed as previously described.2,3 Hoechst 33342 stain (1 μg/mL in PBS; Sigma, Poole, UK) was used to label cell nuclei.

**Immunohistochemistry**

After a brief wash in PBS, retinal wholemounts were incubated in blocking buffer (PBS containing 1% fetal calf serum and 0.1% Triton X-100) for 1 hour at room temperature. Incubations in antibodies (diluted 1:100 in blocking buffer) were performed overnight at 4°C in the case of primary antibodies and for 2 hours at room temperature in the case of secondary antibodies. Antibodies used were rabbit anti-mouse collagen type IV (Biogenesis Ltd., Poole, UK), mouse anti-GFAP (clone G-A-5; Sigma), FITC-conjugated mouse anti-rabbit smooth muscle actin (ASMA; Sigma), tetrahydroamino isothiocyanate (TRITC)-conjugated anti-rabbit IgG (Sigma) and FITC-conjugated anti-mouse IgG (Sigma).

**In Situ Hybridization on Retinal Wholemounts**

After the post fixation step in 4% PF in PBS, retinas were washed twice in PBS containing 0.1% Tween-20 (PBT) and digested briefly with proteinase K (in PBT), followed by fixation in 4% PF and 0.2% glutaraldehyde in PBS for 5 minutes and two washes with PBT. After 15 minutes of prehybridization in hybridization buffer22 at 64°C, retinas were hybridized at 64°C overnight with RNA probes diluted in hybridization buffer. Probe labeling with digoxigenin-UTP (Roche Molecular Biochemicals, Indianapolis, IN) and visualization of RNA hybrids with alkaline phosphatase-conjugated anti-digoxigenin antibodies (Roche) was performed according to the manufacturer’s instructions using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as a color reagent. In combined in situ hybridization and immunohistochemistry, the antibody labeling was performed as described, after the in situ hybridization protocol was completed. Nuclear labeling of in situ-hybridized tissue was performed with Hoechst 33342 (1 mg/mL in water). Template for PDGFRα, PDGFRβ, and GFAP RNA probes were gifts from William D. Richardson (University College London, London, UK), Christer Betsholtz (University of Göteborg, Göteborg, Sweden), and James Cohen (King’s College London, UK), respectively. Other templates were obtained by reverse transcription of mRNA from 6-day-old mouse retinas and PCR amplification of specific gene fragments, using nested primer pairs (VEGFR2: 5′-tg-gccaaataaacccttcag-3′, 5′-tctcgagaaaaggtgtgctg-3′; VEGFR2 nested: 5′-ctctcgagaaaaggtgtgctg-3′, 5′-cgatggagacaccacacgcg-3′; VEGFR1: 5′-gccgccaggccacacacgcg-3′; 5′-gccgcttcctgaaccttaca-3′; and VEGFR1 nested: 5′-agggaaattcttacaactc-3′, 5′-agggaaattcttacaactc-3′). All pictures were taken with a digital camera (Hamamatsu Corp., Hamamatsu City, Japan) mounted on a microscope (Axioplan; Carl Zeiss, Oberkochen, Germany).

**RESULTS**

**Development of the Primary and Secondary Vascular Network in Mouse Retina**

Nissl staining, Hoechst staining, and immunohistochemistry with antibodies against collagen type IV, ASMA, and GFAP were used to document the progress of retinal vascularization in mouse (Fig. 1). Collagen type IV is part of the basal lamina secreted by endothelial cells and is a useful marker for retinal vessels,23 because it can be used in combination with in situ hybridization protocols (see later description). ASMA is expressed by pericytes associated with developing retinal arteries,24 and GFAP is expressed by retinal astrocytes. In the newborn (postnatal day [P]0) mouse pups, vascular sprouts had emerged from a ring-shaped vessel around the optic nerve head (Fig. 1A). The primary vascular network had spread approximately halfway across the inner surface of the retina by P4 (Fig. 1B) and had reached the periphery approximately 1 week after birth (Fig. 1C). At this stage, arteries and veins strictly alternated and vascular sprouts (Fig. 1C, arrows in inset) started to grow from areas around veins into the inner and outer plexiform layers of the retina, where they estab-

**FIGURE 1.** Progress of retinal vascularization visualized by fluorescence immunohistochemistry on retinal wholemounts using anti-collagen type IV (red, A–C, F), anti-ASMA (green, A–C), and anti-GFAP (green, F) antibodies. In retinas from P0 (A) mice the vasculature appeared as an undifferentiated network, whereas at P4 (B) and P8 (C) arteries could be distinguished from veins by ASMA expression. Spindle cells in P0 retinas, as revealed by Nissl-staining (D) or Hoechst 33342 staining (E) had a different morphology than the GFAP-positive, star-shaped retinal astrocytes (green, F) at P8. In P8 retinas secondary sprouting into the inner layers of the retina began around veins. Because these secondary sprouts grew perpendicular to the plane of the retina, they could be seen only as bright, circular thickenings of vessels (C, inset, arrows). (C, D, E, insets) Higher magnifications of boxed areas in (C, D, E). Scales bars, 100 μm.
staining with an antibody against collagen type IV revealed a good correlation between collagen type IV distribution and VEGFR1-expressing cells (Fig. 2B, 2C, 2E, 2F). This confirms that collagen type IV is secreted by all endothelial cells in the retina, even at the growing edge of the developing vascular network, and opens up the possibility of comparing the distribution of endothelial cells, as determined by collagen type IV staining, with other in situ hybridization probes. An RNA probe against VEGFR2 is expected to label not only endothelial cells but also angioblasts.16–19 However, in whole-mount preparations of P0 and P5 mice, VEGFR2-positive cells were associated closely with collagen type IV and their distribution appeared to be no different from the distribution of VEGFR1 (Fig. 3). There was no evidence for the presence of VEGFR2-positive cells (i.e., angioblasts) in front of collagen IV–positive vessels. This raises the question of the true nature of the spindle-shaped cells, previously thought to be angioblasts.

Pericytes

Pericytes invade the retina together with endothelial cells.24 To check whether they could account for spindle cells, an RNA probe against PDGFRβ was used to visualize pericytes. PDGFRβ is known to be expressed by pericytes during early

Endothelial Cells and Angioblasts

To investigate whether the spindle-shaped cells are angioblasts, in situ hybridization was performed on retinal whole-mount preparations. An RNA probe against VEGFR1 was used to visualize endothelial cells,26 and a probe against VEGFR2 was used to label potential angioblasts.16,17 In retinal whole-mounts of P0 and P5 mice, the distribution of VEGFR1-positive endothelial cells appeared uniform across the entire vascular network (Fig. 2). Even though the dark in situ hybridization signal slightly quenched immunofluorescent signals, double
PDGFRβ (pericytes)

FIGURE 4. A combination of in situ hybridization and fluorescence immunohistochemistry with an RNA probe against PDGFRβ and an anti-collagen type IV antibody on retinas from P0 (A, B) and P5 (C) mice revealed retinal pericytes within the context of the developing vasculature. Pictures were taken by combining bright-field and fluorescence microscopy. (B) Higher magnification of the boxed area in (A). Scale bar, 100 μm.

FIGURE 5. In situ hybridization with an RNA probe against GFAP revealed retinal astrocytes in retinas from P0 (A, B) and P5 (C–E) mice. At P0, GFAP mRNA levels were high in the area of blood vessels (A, arrows), but were only faintly detectable in retinal astrocytes that were not in contact with blood vessels (B). At P5 GFAP mRNA levels were highest in the center of the retina, decreasing toward the periphery (C). Fluorescence immunohistochemistry with anti-collagen type IV antibody demonstrated the spatial relationship between blood vessels and retinal astrocytes containing high levels (D) and low levels (E) of GFAP mRNA. Higher magnifications of boxed areas: (B) area in (A) and (D, E) areas in (C). Scale bars, 100 μm.

stages of vessel formation.27,28 In situ hybridization on retinal wholemounts from P0 and P5 mice showed clearly that pericytes were confined to the collagen type IV-positive vascular network (Fig. 4). In fact, they appeared to lag slightly behind the leading edge of the spreading vascular network (Fig. 4B). Note that pericytes were distributed uniformly across the entire vascular network and were not restricted to arteries, as might have been suggested by ASMA staining (Figs. 1B, 1C).

Retinal Astrocytes

Retinal astrocytes are spreading as a proliferating cell population across the retina before the primary vessel network extends.10,11,23 Immunohistochemistry with an antibody against GFAP visualizes retinal astrocytes from the center to the periphery in P5 retinas, although the staining slightly weakens toward the periphery (Fig. 1F). In P0 animals GFAP immunofluorescent staining was barely detectable beyond the central area where the primitive vascular network is located (not shown). In situ hybridization with a probe against GFAP revealed a similar picture (Fig. 5). In P0 retinas GFAP mRNA was strongly expressed in the area of the blood vessel network (Fig. 5A; arrows), but was only just detectable beyond the vessels (Figs. 5A, 5B). In P5 mice GFAP mRNA was most abundant in the center of the retina and became more sparse toward the periphery (Fig. 5C). At higher magnification, star-shaped outlines typical for retinal astrocytes could be seen associated with blood vessels (Fig. 5D, arrow). Note that the GFAP mRNA signal significantly decreased beyond the leading edge of the collagen type IV-positive vascular network (Fig. 5E).

Because GFAP does not seem to be uniformly expressed by the entire retinal astrocyte population, an RNA probe against PDGFRα was used as an alternative marker for retinal astrocytes. PDGFRα is expressed by retinal astrocytes29 and is involved in the mitogen-driven expansion of the retinal astrocyte population.23 Unlike GFAP mRNA, PDGFRα mRNA was detected strongly in all retinal astrocytes, including those that were not in contact with the vascular network (Fig. 6). In P0 mice, retinal astrocytes covering more than half of the retinal surface were detected far beyond the central vascularized area (Fig. 6A). This distribution correlates with the distribution of spindle cells stained with cresyl violet or Hoechst 33342 (Figs. 1D, 1E). At the leading edge of the PDGFRα-positive network, cord-like structures were observed (Figs. 6B, 6C). These cords are formed by spindle-shaped cells (Fig. 6B) and have previously been described.4,7,30 Double labeling with Hoechst demonstrated that all cells labeled with the PDGFRα probe had a spindle-shaped nucleus (Figs. 6C–E). It is obvious from Figures 6D and 6E that these cells not only possessed spindle-shaped nuclei (Fig. 1D; arrows) but also have a spindle-shaped morphology, suggesting that PDGFRα-positive cells are the same as the spindle-shaped cells revealed by cresyl violet. At P5 PDGFRα-positive cells (retinal astrocytes) were seen clearly labeled up to the periphery of the retina (Fig. 6F) but their bipolar morphology, apparent at P0, had changed to a more star-shaped morphology (Figs. 6G, 6H). Retinal astrocytes beyond the collagen type IV-positive vessel network were clearly labeled by the PDGFRα probe (Fig. 6H). At this age, the nucleus of retinal astrocytes was not as elongated as at P0, but was more ellipsoid (Fig. 6I, arrows).
that the PDGFRα/H9251 cell is the location of endothelial cells. In the current study, with in situ hybridization. However, collagen type IV appears to be an ideal vessel marker in combination with the in situ hybridization procedure well. Therefore, collagen expression in the retinal vasculature. The epitopes recognized into disease processes, such as diabetic retinopathy or retinopathy of prematurity. I developed a combination of whole mount in situ hybridization and immunohistochemistry to study gene expression in the retinal vasculature. The epitopes recognized by the anti-collagen type IV antibody used in this study survive the in situ hybridization procedure well. Therefore, collagen type IV appears to be an ideal vessel marker in combination with in situ hybridization. However, collagen type IV is an extracellular matrix component secreted by endothelial cells, and it had not been clear to what extent its distribution represents the location of endothelial cells. In the current study, collagen type IV clearly colocalized with VEGFR1 mRNA on a cellular level, thus confirming the validity of collagen type IV as a retinal vessel marker.

To investigate whether the primary vascular network in the mouse retina is based on differentiation of dispersed angioblasts (i.e., vasculogenesis) the mentioned combination of whole mount in situ hybridization and immunohistochemistry was used to identify distinct cell populations in retinal whole mount preparations. The spindle-shaped cells preceding the forming vessel network failed to label with the angioblast marker VEGFR2. In fact, no VEGFR2-positive cells at all were detected beyond the leading edge of the collagen type IV-positive vessel network at P0 and P5. It could be argued that retinal angioblasts downregulate VEGFR2 and are therefore not detectable with a VEGFR2 RNA probe at P0. However, this is unlikely, because the vascular endothelial cell lineage has been shown to be highly dependent on VEGF signaling throughout development.31–32 and VEGF is expressed in the retina during retinal vascular development.14 That a PDGFRα probe strongly labeled spindle-shaped cells at P0 and retinal astrocytes at P5 suggests that the spindle-shaped cells are not angioblasts but retinal astrocytes giving further weight to the view that, in the mouse, the primary retinal vessel network forms by angiogenesis and not by vasculogenesis. Confirming this view, in a recent study spindle cells were also identified as retinal astrocytes and angioblasts were not detected in the mouse retina using the markers Tie-2, CD31, and CD34.33 Previous studies in human34,35 or monkey25 retina have also suggested that spindle cells are glial cells and not vascular precursors.

Because the spindle-shaped, PDGFRα-positive retinal astrocytes identified in this study expressed little GFAP and their bipolar morphology significantly differed from the stellar shape of more differentiated retinal astrocytes, they could be described as retinal astrocyte precursors. The fact that GFAP mRNA is present at low levels in retinal astrocytes located beyond the vessel network could explain why immunohistochemistry failed to detect GFAP-positive retinal astrocytes at P0. Only after a few days (P5) enough GFAP accumulated to allow detection of retinal astrocytes with an anti-GFAP antibody. It is interesting that GFAP mRNA in retinal astrocytes was detectable at much higher levels in areas where blood vessels were present. It is possible that endothelial cells produce a retinal astrocyte differentiation factor that induces higher levels of GFAP mRNA. Previous studies have shown that retinal astrocytes can induce endothelial cell differentiation, thus demonstrating signaling from astrocytes to endothelial cells.36–37 The reverse, signaling from endothelial cells to astrocytes, could be the basis for increased levels of GFAP mRNA in vascularized areas.

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