Vascularization of the Human Fetal Retina: Roles of Vasculogenesis and Angiogenesis

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PURPOSE. To characterize the topography of and the cellular processes that underlie vascularization of the human retina.

METHODS. The vasculature of human eyes obtained from fetuses ranging in age from 14 to 38 weeks of gestation (WG) was examined in Nissl-stained, whole-mount preparations and by anti-CD34 immunohistochemistry.

RESULTS. The first event in retinal vascularization, apparent before 15 WG, was the migration of large numbers of spindle-shaped mesenchymal precursor cells from the optic disc. These cells proliferated and differentiated to produce cords of endothelial cells. By 15 WG, some cords were already patent and formed an immature vascular tree in the inner retinal layers that was centered on the optic disc. These processes are consistent with vessel formation by vasculogenesis. Angiogenesis then increased the vascular density of this immature plexus and extended it peripherally and temporally. Maturation of the plexus was characterized by substantial remodeling, which involved the withdrawal of endothelial cells into neighboring vascular segments. The outer plexus was formed as a result of the extension of capillary-sized buds from the existing inner vessels, a process that began around the incipient fovea between 25 and 26 WG.

CONCLUSIONS. These observations suggest that the formation of primordial vessels in the central retina is mediated by vasculogenesis, whereas angiogenesis is responsible for increasing vascular density and peripheral vascularization in the inner retina. In contrast, the outer plexus and the radial peripapillary capillaries are formed by angiogenesis only. These mechanisms of retinal vascularization appear similar to those of vascularization of the central nervous system during development. (Invest Ophthalmol Vis Sci. 2000;41:1217–1228)

Vascular development involves vessel formation either by vasculogenesis or angiogenesis, vessel withdrawal, and vessel maturation. Vasculogenesis, the de novo formation of vessels by the differentiation of endothelial precur-

or cells that give rise to primitive vessels, is responsible for formation of the major vessels and the vessels of endoderm-derived organs. Angiogenesis, the formation of vessels by budding or sprouting from existing vessels, plays an important role in vascularization of the central nervous system (CNS) and kidney.¹

The retina, embryologically an extension of the diencephalon, is an excellent model in which to study vascular develop-

ment in the CNS. The retinal vasculature consists of inner and outer layers that are joined by fine capillaries. The thin laminar structure of the retina renders it suitable for whole-mount preparations that allow visualization of the entire forming vasculature in situ. An understanding of normal retinal vascularization is particularly important, given that several retinopathies are caused by abnormal vessel growth. The developing retinal vasculature of premature infants is extremely vulnerable, and perturbation of the normal developmental processes can result in retinopathy of prematurity (ROP), the leading cause of infant blindness in the Western world. The incidence of blindness resulting from ROP increases markedly for infants born before 26 weeks of gestation (WG).² However, relatively little is known about the normal state of human retinal vascularization at this developmental stage or of the reasons for its pronounced susceptibility. Such information would be invaluable to ophthalmologists and neonatologists treating premature infants, as well as to clinicians participating in the National Institutes of Health multicenter trial “Stop ROP” for evaluation of the benefits of supplemental oxygen therapy as a noninvasive treatment for ROP.³,⁴

Vascular development in the retina has been examined in several species. Initially, spindle-shaped cells are apparent mi-

grating ahead of the developing inner vasculature. These cells are characterized by their labeling with Grifonia simplicifolia isolectin and their distribution as revealed by Nissl staining in the cat⁵ as well as by their ATPase⁶ and ADPase⁷ activities in the dog. They subsequently coalesce to form solid vascular cords, which in turn give rise to patent vessels,⁸,⁹ suggesting that vasculogenesis contributes to formation of the inner retinal plexus.⁵ Further vascularization of the cat retina occurs by
angiogenesis. Our previous studies led us to suggest that formation of retinal vessels is promoted by the increased metabolic demand of neurons, which results in local tissue hypoxia, or "physiological hypoxia," and that this effect is mediated by vascular endothelial growth factor (VEGF), a potent angiogenic protein induced by hypoxia.

Despite insights gained from animal studies, our knowledge of the normal development of the human retinal vasculature is incomplete. Previous human studies have examined ink-perfused retinas (in which only patent vessels are apparent), digested tissue (in which the normal relations of vessels with neighboring structures are destroyed), or transverse sections (which provide little information on the topography of vessel formation) or were restricted to small numbers of specimens. The importance of studying the human retina is highlighted by comparative data showing that, although retinal vascularization in humans resembles that in other mammals, there are significant differences. In addition, formation of the human retinal vasculature occurs in utero, where arterial oxygen tension is <30 mm Hg, whereas substantial portions of the cat and rat retinal vasculatures are formed after birth, at markedly higher arterial oxygen tensions.

We have now examined a series of Nissl-stained, retinal whole-mount preparations from human fetuses at 14 to 26 WG for evidence of vascular precursor cells and have mapped changes in their topographical distribution with maturation. In addition, we have applied immunohistochemistry with an antibody to human CD34, a protein that is expressed by hematopoietic precursor cells and capillary endothelial cells, to visualize the formation of blood vessels from 15 to 38 WG. Our study thus represents the most complete description to date of both the cellular and topographical features of the forming retinal vasculature in humans.

**METHODS**

**Collection, Age Determination, and Preparation of Human Fetal Eyes**

Human fetal eyes, ranging in age from 14 to 38 WG, were collected in China in accordance with the guidelines set forth in the Declaration of Helsinki; the study was also approved by the human ethics committee of the University of Sydney. Fetuses older than 20 WG had died of natural causes after premature or difficult deliveries. Younger fetuses were obtained after water bag- or prostaglandin-induced abortions, which are permitted up to 20 WG. The age of each fetus was determined from charts of crown-rump length and crown-heel length.

After enucleation of the eyes, the anterior segment and vitreous were removed, and the eyeball was fixed at 4°C for a minimum of 2 days with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The retina was then dissected as previously described. A small series of Nissl-stained (1% cresyl violet), human retinal whole-mounts prepared previously by J. Proviss also was examined.

**Anti-CD34 and Anti-GFAP Immunohistochemistry**

The vasculature was visualized by immunohistochemistry with a monoclonal antibody (QBEND/10, 1:50 dilution; Serotec, Oxford, UK) to CD34, a single-chain transmembrane glycoprotein with a molecular mass of 110 kDa that binds L-selectin and is selectively expressed on human lymphoid and myeloid hematopoietic progenitor cells as well as on the filopodial extensions and the luminal membrane of endothelial cells. Astrocytes were visualized with rabbit polyclonal antibodies (1:2 dilution; Biogenex, San Ramon, CA) to glial fibrillary acidic protein (GFAP). Retinas were labeled with these antibodies as previously described.

**Mapping of the Outer Limit of the Retinal Vasculature**

The outer limit of spindle-shaped presumed vascular precursor cells and the outer limit of vascular cords were determined in three specimens aged 14 to 15, 18, and 21 WG that had been subjected to Nissl staining. In addition, maps of the extent of retinal vascularization at various ages were prepared from a series of retinas subjected to anti-CD34 immunohistochemistry. Retinal boundaries, the outer limit of CD34 vessels in both inner and outer layers of the vasculature, and the radial peripapillary capillaries (RPCs) were mapped for each retina with a 1-mm grid incorporated into the 10X eyepiece of an Olympus Vanox microscope (Tokyo, Japan), as previously described.

**RESULTS**

**Role of Vasculogenesis in Early Vascularization of the Human Retina**

**Invasion of Spindle Cells from the Optic Disc.** Nissl-stained, whole-mount preparations of the human fetal retina revealed substantial numbers of spindle-shaped cells emanating from the optic disc. These cells were apparent at 14 to 15 WG (Fig. 1A), the youngest age examined, in a superficial plane, located mostly between nerve fiber bundles and with their long axis oriented along the path of apparent migration toward the retinal periphery (Figs. 1B, 1C). More centrally in the same preparations, the spindle cells aggregated to form putative vascular cords (Figs. 1D, 1E), resulting in a region of dense Nissl staining corresponding to the leading edge of vessel formation (Fig. 2). Immediately central to the outer limit of the vascular cords, patent vessels containing red blood cells were apparent (Fig. 1F). The presence of spindle cells, their aggregation to form cords, and the subsequent development of patent vessels are consistent with vessel formation by vasculogenesis.

**Topography of Spindle Cell Spread, Astrocyte Spread, and Vascular Cord Formation.** The distributions of spindle cells and astrocytes differed markedly at similar stages of human retinal development (Fig. 3). At 18 WG, astrocytes were confined to the central two thirds of the retina, being absent from the fovea (Fig. 3B). In contrast, even from 14 to 15 WG, the spindle cells showed an extensive four-lobed distribution, extending farthest temporally and superiorly and corresponding to the paths of the future artery–vein pairs of the adult human retina (Fig. 3A). Initially, the vascular cords showed a similar topography, but their distribution was less extensive. They later spread into the regions pioneered by spindle cells. Likewise, at 14 to 15 WG, the CD34 vessels displayed a four-lobed topography (Fig. 4A). The similarities in the topographies of spindle cell invasion, cord formation, and early patent vessels support the conclusion that the primordial vessels of the inner plexus of the human retina are formed by vasculogenesis.
By 25 WG, astrocytes had almost reached the retinal periphery but were not present at the fovea. In contrast, at 21 WG, spindle cells were no longer evident in the retina; thus, substantial areas of the human retina appeared never to be invaded by the spindle-shaped presumptive mesenchymal precursor cells (Fig. 3A). Unlike astrocytes, neither spindle cells nor vascular cord formation were detected in the region of the retina temporal to the optic disc—that is, the site of the temporal raphe and incipient fovea—or in the peripheral retina. Given that spindle cell invasion and vascular cord formation are thought to represent the initial stages of vasculogenesis in the retina, our observations suggest that vasculogenesis...
mediates early vessel formation in the inner plexus but is not responsible for vessel formation in the temporal and peripheral regions of the human retina.

**Role of Angiogenesis in Vascular Spread and in Increasing Vascular Density in the Inner Retinal Plexus**

**Contribution of Angiogenesis to Vascular Spread.**

The antibody to CD34 labeled the vasculature but did not label the spindle cells. At 15 WG, patent radial vessels, with few interconnecting segments, were apparent emanating from the optic disc (Fig. 4A). This vascular pattern suggests the existence of only a low level of metabolite exchange. As the retina matured, capillary networks became apparent, linking the radial vessels. At 17 to 18 WG, discrete netlike vascular formations (Figs. 4B, 4C) were observed at the leading edge of vessel formation. Localized exuberant capillary meshes also were evident (Fig. 4D) both nasally and temporally, and these became more widespread by 21 WG (data not shown). From 25 WG, as the vasculature approached the retinal periphery, the leading edge of vessel formation was characterized by the presence of vascular shunts between terminal artery–vein pairs (Fig. 4E).

The antibody to CD34 binds to the filopodial extensions of vascular endothelial cells, which are indicative of angiogenesis. From 18 to 30 WG, filopodia often were present at the leading edge of vessel formation (Figs. 4F, 4G). They were apparent extending into regions where spindle cells were never observed, indicating that angiogenesis is responsible for the vascularization of these regions. The presence of red blood cells at the outer limit of CD34 immunoreactivity (Fig. 4G) revealed that these newly formed vessels were patent.

**Contribution of Angiogenesis to the Increase in Vascular Density.**

In addition to its contribution to the spread of vessels peripherally, angiogenesis also was responsible for increasing the vascular density of the primordial plexus formed by vasculogenesis. Initially, capillary networks were rare (Figs. 4A, 5A). However, by 18 WG, regions of active sprouting began to give rise to substantial capillary networks within the existing vascular tree (Fig. 5B). As the retina increased in size and the radial vessels spread peripherally, the distance between these vessels became greater, and it was in these avascular spaces that sprouting was most pronounced. Filopodia extended, established contact with other filopodia or vessels, and subsequently dilated to form vascular segments (Figs. 5C, 5D). Moreover, sprouting was evident even from the edges of larger preformed vessels (Fig. 5E) and was especially marked near and along veins. By 21 WG (Fig. 5F), exuberant immature
capillary plexuses were apparent throughout the vascular tree. Thus, angiogenesis augments the initial radial vessels by increasing capillary density. Such a prominent role for angiogenesis in increasing the vascular density of the inner plexus of the retina has not been described in other species.

**Formation of the Perifoveal and Temporal Raphe Vessels by Angiogenesis.** The incipient fovea is avascular at 25 WG (Figs. 6A, 6B). The avascular zone is oval in shape, with a diameter of 500 to 600 μm. Because spindle cells do not invade the region of the temporal raphe or the perifoveal region, these areas must be vascularized by angiogenesis alone. Unlike other areas of the retina, the perifoveal region appears to be fed predominantly by arterioles, which could be distinguished by their caliber, the presence of a capillary-free space, and the arterial nature of the vessels feeding them (Fig. 6A). Even when first formed at 21 WG, the capillaries were more uniform in density and caliber (Fig. 6C) than were those apparent in other regions of the retina at similar eccentricities (Fig. 6D). Thin, tortuous vascular strands were evident, extending into the avascular region (Fig. 6B); however, our time points were not sufficiently frequent to determine whether these strands were regressing or forming vessels.

**Formation of RPCs by Angiogenesis.** Fine RPCs were evident in the nerve fiber layer, extending from the inner vasculature, from 21 WG (data not shown). RPCs in the region of the optic nerve head of 25- and 26-WG retinas are shown in Figures 6E and 6F, respectively. These vessels were located superficially in the nerve fiber layer and extended radially from...
FIGURE 5. Anti-CD34 immunohistochemical analysis of vascular sprouting in regions central to the leading edge of vessel formation during development of the human retina. (A) At 18 WG, the vascular density in the central retina was low, with the vasculature consisting predominantly of near-radial main vessels emanating from the optic disc. (B) Vascular sprouting was evident in regions of the 18-WG retina central to the leading edge of vessel formation. (C) High-magnification view of vascular sprouts in a midperipheral region of the retina at 26 WG. Filopodia extended in numerous directions and then expanded to form vessel segments. (D) Sprouting near the vascular periphery at 18 WG. (E) Filopodia extending from larger retinal vessels at 21 WG. (F) Selection of major channels, including veins (v), and formation of the capillary-free space surrounding arteries (a) were evident at 21 WG.

FIGURE 4. Immunohistochemical labeling of human retinal vessels with an antibody to CD34 at various stages of gestational development. (A) The full extent of CD34<sup>+</sup> vessels at 15 WG. (B, C) The fishnet-like appearance of the vasculature at the leading edge of vessel formation in 17- and 18-WG retinas, respectively. (D) The vascular front at the junction of the superior and inferior vascular lobes of the nasal retina at 18 WG. (E) The leading edge of vessel formation in a 25-WG retina. Each artery-vein pair terminated in a minor vascular meshwork. (F, G) Progressively higher magnification views of the filopodial extensions that were apparent in various directions at the leading edge of vessel formation at 18 and 25 WG, respectively. In (G), the presence of various red blood cells (arrowheads) immediately central to the region of filopodial extension shows that these vascular segments are patent.
the optic nerve head. The timing and extent of their formation suggest that they function to satisfy the metabolic requirements of the thick nerve fiber layer that surrounds the optic nerve head.

Retraction of Excess Vascular Segments

The immature vascular meshes of the inner plexus are subsequently remodeled to form sparser vascular trees, a process that is not complete at birth. Vessel retraction was especially marked in regions of higher tissue oxygenation, such as along arteries, contributing to the formation of a peri-arterial capillary-free space (Figs. 5E, 7A, 7B). Retraction also was apparent in close proximity to newly formed vessels, in regions where functional circulation recently had been established, including those immediately central to the terminal vascular shunts (Fig. 4E), and in areas where the outer plexus was formed or forming (Fig. 7C). The proximity of angiogenesis and vascular retraction (Fig. 7A) indicated just how localized these processes can be. Anti-CD34 immunohistochemistry revealed the rounding up of vascular endothelial cells, resulting in the sev-

![Image of figure 6 showing anti-CD34 immunohistochemical analysis of perifoveal vessel and RPC formation.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932907/.../April%202000,Vol.41,No.5)
erance of their normal junctions with neighboring cells (Figs. 7B–7D). A basement membrane was all that remained after retraction of such endothelial cells into a neighboring capillary segment (Figs. 7A–7D).

**Formation of the Outer Vascular Plexus**

Capillary sprouting from the inner vascular plexus was first evident at the fovea between 25 and 26 WG. Capillary-sized buds were observed descending into the inner nuclear and outer plexiform layers, giving rise to small vascular segments in a deeper plane (Figs. 7E, 7F). With maturation, a confluent outer plexus became apparent.

**Topography of Formation of the Inner and Outer Vascular Plexuses**

Formation of the inner vascular plexus had begun by 14 to 15 WG. These early vessels were centered on the optic disc and showed a four-lobed topography (Figs. 4A, 8). In the following
weeks, the inner vascular plexus extended peripherally, curving around the location of the incipient fovea (Fig. 8). By 32 WG, the inner plexus had reached its outer limits, leaving a narrow rim of avascular tissue at the periphery of the retina. In contrast, the formation of the outer vascular plexus began in the perifoveal region at about 25 to 26 WG and subsequently spread with an elongated topography along the horizontal meridian (Fig. 8).

DISCUSSION

Roles of Vasculogenesis and Angiogenesis in Human Retinal Vascularization

Our results are consistent with the conclusion that vasculogenesis contributes to the formation of the inner plexus of the human retina. We have extended previous observations in this tissue by examining Nissl-stained, whole-mount preparations and studying the timing and topography of the spread of spindle-shaped cells that were present from 14 to 15 WG. These cells never entered the peripheral or temporal retina, supporting the conclusion that most are mesenchymal precursor cells rather than astrocytes. These presumed mesenchymal cells aggregated to form solid vascular cords, consistent with descriptions of vasculogenesis in cat, rat, dog, and primate retinas.

The earliest vessels detected in the human retina were radial vessels that were present at low density. The early vasculature exhibited the same four-lobed pattern as that of the spindle cells and vascular cords, leading us to conclude that it is formed by vasculogenesis. Angiogenesis was responsible for increasing vascular density in regions previously pioneered by vasculogenesis, and, given that spindle cells were not detected after 21 WG, it also appeared to be solely responsible for formation of the vessels at the raphe and in the perifoveal and peripheral regions as well as for that of the outer vascular plexus and the RPCs. We propose that vasculogenesis provides a mechanism for rapid formation of a rudimentary vascular plexus in the regions previously invaded by vascular precursor cells and that this plexus is then expanded by angiogenesis to satisfy the increasing metabolic needs of the developing retina.

The mechanism of retinal vascularization is thus similar to that of vascularization of the brain during development. The primordial vascular bed on the surface of the neuroepithelium is derived from migratory vascular precursor cells and is thus formed, at least in part, by vasculogenesis. New vessel segments sprout from these preexisting vessels and grow tangentially by angiogenesis into the neuroepithelium. These similarities are not unexpected given that the retina is an extension of the CNS during embryological development.

Roles of “Physiological Hypoxia” and VEGF in Angiogenesis in the Human Retina

We previously proposed that the “physiological hypoxia” that results from the increasing metabolic demands of maturing neurons is the driving force for retinal vascularization. Earlier evidence suggests that the formation of vessels in response to physiological hypoxia is mediated by VEGF expressed by neuroglia. VEGF expression is spatially and temporally correlated with ocular neovascularization, is closely

FIGURE 8. Schematic representations of the outer limits of the inner and outer vascular plexuses as well as that of the RPCs at various times during development of the human fetal retina.
associated with vessel formation during retinal development,\textsuperscript{12,32} and is downregulated by hyperoxia,\textsuperscript{12} which inhibits retinal vessel formation.\textsuperscript{31} However, several observations in our present study have led us to refine our original hypothesis: We now propose that, rather than all retinal vascular formation being driven by hypoxia-induced VEGF expression, only vessel formation by angiogenesis (not that by vasculogenesis) is mediated in this manner.

Angiogenesis in the developing human retina was localized and was coincident with unmet metabolic demand: (1) Angiogenesis was the only means of vessel formation in the raphe and perifoveal regions of the human retina, both of which are areas of high metabolic activity, given that they coincide with peak ganglion cell density and maturity. Angiogenesis also was responsible for the peripheral spread of the vasculature after 21 WG, which followed the central-Peripheral gradient of retinal ganglion cell maturation.\textsuperscript{24} VEGF is expressed in both these regions of angiogenesis.\textsuperscript{52} (2) Formation of the outer vascular plexus began between 25 and 26 WG, coincident with the peak period of eye opening, when the visually evoked potential, indicative of a functional visual pathway and photoreceptor activity, is first detectable in the human infant.\textsuperscript{33} Formation of the outer plexus also was centered around the fovea, rather than around the optic disc, thus mimicking the topography of photoreceptor maturation.\textsuperscript{54} (3) Formation of RPCs by angiogenesis was apparent by 21 WG, when the nerve fiber layer in the region of the optic disc becomes too thick to be adequately supplied by the inner retinal vessels. These observations are consistent with the hypothesis that physiological hypoxia induced by the metabolic demands of neurons stimulates angiogenesis.

Lack of Dependence of Vasculogenesis in the Retina on Hypoxia-Induced VEGF Expression

Although retinal angiogenesis appears to be driven by hypoxia-induced VEGF, several observations in the present study lead us to conclude that vasculogenesis in the human retina is independent of metabolic demand and hypoxia-induced VEGF expression: (1) Substantial vasculature in the human retina occurs in the absence of VEGF expression. At 18 WG, the inner plexus covered ~54% of the retinal area. However, VEGF mRNA was not detected in the human retina by in situ hybridization until 20 WG.\textsuperscript{52} (2) Given that formation of the inner plexus by vasculogenesis is well established by 14 to 15 WG, this process occurs before the differentiation of most retinal neurons.\textsuperscript{24} (3) Ganglion cell density and neuronal maturation are greatest at the perifoveal region of the human retina between 15 and 18 WG.\textsuperscript{24,35,36} If retinal vasculization were driven only by the metabolic needs of neurons, one would expect that vascular density and the extent of vascular spread would be maximal in this area. Instead, the raphe and perifoveal regions of the human retina were avascular between 15 and 18 WG. (4) Formation of the primordial vessels by vasculogenesis is centered around the optic disc; whereas neuronal maturation is centered around the fovea.\textsuperscript{24,35,36}

A comparative analysis of retinal vasculization in other species has shown that VEGF expression, tissue oxygen levels, and vasculization are not always correlated.\textsuperscript{37} The guinea pig retina is virtually anoxic and yet remains avascular,\textsuperscript{58} whereas overexpression of VEGF in the avian retina did not induce vasculization.\textsuperscript{39} Further evidence of the independence of vasculogenesis from VEGF is provided by VEGF knockout mice. In these animals, in which not only paracrine but also autocrine VEGF production is lost, vessels still form by vasculogenesis but are highly abnormal.\textsuperscript{30} Reduced VEGF expression in mice heterozygous for the VEGF null mutation is associated with the formation of vessels in the forebrain mesenchyme but not in the neuroepithelium.\textsuperscript{41} Given that the formation of vessels in the forebrain mesenchyme is thought to occur by vasculogenesis, whereas that within the neuroepithelium is thought to take place by angiogenesis, these observations provide further evidence that vasculogenesis is not dependent on hypoxia-induced VEGF expression.

Uniqueness of the Foveal Region

Spindle cells were not detected in the fovea region, leading us to conclude that vessels in this region could only be formed by angiogenesis. The fovea contains a high density of cones with a high concentration of mitochondria in their outer segments. If physiological hypoxia resulting from the increase in retinal metabolic activity is the main impetus for angiogenesis, then why does the fovea remain avascular? The absence of foveal vessels might be attributable to one of two distinct mechanisms: the lack of a stimulus for vascular formation or the retraction of vascular segments. We have previously shown that VEGF expression by astrocytes contributes to the formation of the inner plexus during retinal development.\textsuperscript{12} We have now shown that astrocytes do not enter the fovea during embryonic development of the human retina. It is therefore possible that angiogenesis does not take place in the fovea because of an absence of VEGF expression by retinal astrocytes.

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