Immunohistochemical Characterization of Developing and Mature Primate Retinal Blood Vessels

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**Purpose.** To characterize developing retinal blood vessels with vascular markers and to relate the histochemical profile of maturing vessels to morphologic stages in retinal vascular development.

**Methods.** Vessels were examined in frozen and paraffin-embedded retinas and in wholemounts of *Macaca* monkeys ranging in age from fetal day 75 (F75) to adulthood. Endothelial cells were visualized immunohistochemically using antisera to von Willebrand's factor and CD31 with lectins *Ulex europaeus*, *Bandeiraea simplicifolia*, peanut agglutinin, *Ricinis communis*, and wheat germ agglutinin, and by ATPase and ADPase enzymatic histochemistry. Antibodies to vascular basement membrane and matrix markers laminin, fibronectin, and collagen types I and VIII, and antisera recognizing cell cycle-specific nuclear proteins (cyclin, Ki-67, Mib-1) also were used.

**Results.** Newly formed and mature vessels were reactive with reagents specific for CD31, von Willebrand's factor, types I and VIII collagens, laminin, fibronectin, *U. europaeus*, *R. communis*, and peanut agglutinin. Wheat germ agglutinin labeled vessels only after pretreatment with neuraminidase. All vascular markers appeared simultaneously, but some were distributed differentially between capillaries and larger vessels, along the central–peripheral extent of a vascular plexus, and among different vascular laminae. Markers of vessels failed to label spindle-shaped presumed vascular precursor cells lying peripheral to the advancing vessels during development. Spindle cells exhibited cyclin, Ki-67, and Mib-1 immunoreactivity.

**Conclusions.** Immature and mature vitread and sclerad vessels displayed histochemical profiles that were qualitatively similar but that had subtle quantitative differences. Results do not support identification of spindle-shaped cells as vascular precursors in the developing monkey retina and are discussed in relation to mechanisms of retinal vascularization.


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laminar vascular plexus development in primate retina using histologic methods. To analyze further the development of primate retinal vasculature, we have applied immunohistochemical, enzyme cytochemical, and lectin markers that recognize endothelial and basement membrane components of retinal vessels. The aims of these experiments were to determine whether spindle cells are characterized by vascular markers; to define a panel of markers that differentiates developing and mature vascular endothelium and basement membrane components; and to use these markers to compare inner retinal vessels, which form in the presence of putative precursor cells, to outer vessels, which form in the absence of these cells.

METHODS

Tissue

All subjects were *Macaca* monkeys ranging in age from fetal day 75 (F75; normal gestation, F165 to F170) to adult (5+ years), treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Fetuses were obtained from timed pregnancies with a precision of ±1 to 3 days, or were aged by comparison with laboratory and breeding colony standards (±5 days).

Collection of specimens from prenatal and postnatal subjects was previously described. Briefly, monkeys were enucleated, and the eyes were immersed in either phosphate-buffered 4% paraformaldehyde or methyl Carnoy fixative for 1 to 4 hours. Specimens fixed in paraformaldehyde were cryoprotected and frozen, and the posterior pole (between the inferior and superior arcade vessels) was sectioned serially at 10 μm parallel to the horizontal meridian. Specimens fixed in methyl Carnoy solution were embedded in paraffin and serially sectioned at 6 μm.

Immunohistochemistry

Antisera against known markers of vascular endothelial cells or their basement membrane and extracellular matrix proteins were used. The working dilutions and sources were: CD31 (also named platelet–endothelial cell adhesion molecule; mouse anti-human, 1:250; Dako, Carpinteria, CA), laminin (rabbit anti-mouse, 1:500; Gibco, Telios, San Diego, CA), fibronectin (rabbit anti-mouse, 1:1000; Chemicon, Temecula, CA), von Willebrand factor (rabbit anti-human, 1:500; Dako), and collagen types I and VIII (guinea pig anti-rat 1:250 and guinea pig anti-bovine 1:500, respectively). In addition, three cell-cycle specific nuclear antigens were used as markers for proliferating cells: cyclin (also named proliferating cell nuclear antigen, mouse anti-mouse, 1:100; Boehringer–Mannheim, Indianapolis, IN), Ki-67 antigen (rabbit anti-human, 1:300; Dako), and Mib-1 fragment (rabbit anti-human, 1:250; Amac, Westbrook, ME), which recognizes a Ki-67 epitope.

Sections were incubated in 70% methanol containing 3% hydrogen peroxide for 30 minutes at 4°C to reduce endogenous peroxidase activity, washed twice in cold phosphate-buffered saline (PBS), pH 7.6 and stored overnight in PBS containing 2% goat serum and 0.1% Tween-20. At this stage, sections being tested for Ki-67 and Mib-1 immunoreactivity were treated for 5 minutes at 90°C in 10 mM citrate buffer (pH 6.0) to unmask the epitope. All sections were overlaid with approximately 300 μl of primary antisera in PBS and were incubated in a humid chamber at room temperature for 2 to 5 hours, followed by four 10- to 15-minute washes in PBS. Incubation with the secondary antibody (biotinylated species-specific IgG 1:100, 300 μl) was performed in a humid chamber for 90 minutes at room temperature. The sections were then washed in PBS and subsequently incubated with avidin–biotin–complex (ABC, Vector Laboratories) for 30 minutes at room temperature before a final wash in PBS. The sections were reacted in diaminobenzidine and hydrogen peroxide (DAB kit; Vector, Burlingame, CA) for 2 to 30 minutes, then quenched with cold tap water for 10 minutes. For control sections, PBS replaced primary or secondary antibody. Selected sections were counterstained with cresyl violet, and all sections were coveredslipped with Permount (Fisher, Fair Lawn, NJ).

Biotinylated lectins (E–Y Laboratories, San Mateo, CA; except as noted) that have been shown to label blood vessels were used as follows: *Ricinus communis* agglutinin I (5 μg/ml), peanut agglutinin (10 μg/ml), wheat germ agglutinin (10 μg/ml), and *Banderaea* (Griffonia) simplicifolia I (20 μg/ml; Sigma, St. Louis, MO); for *Ulex europaeus* I (2 μg/ml; Sigma), we used fluorescein isothiocyanate-conjugated lectin. Sections were incubated in diluted lectin for 3 hours at room temperature, washed briefly, and reacted with avidin–biotin–complex (except for *U. europaeus*). For *B. simplicifolia*, 0.1 mM CaCl₂ and 0.1 mM MnCl₂ were added to phosphate buffer during incubation. In some experiments, neuraminidase (Sigma), dissolved in 0.05 M sodium acetate buffer (pH 5.2) and 2% bovine serum albumin at a concentration of 0.5 U/ml, was overlaid onto sections at room temperature for 1 hour before the lectin incubation step.

Retinal Wholemount Immunocytochemistry

The retina was separated from the underlying pigment epithelium, choroid, and sclera shortly after fixation. To enhance permeability, the tissue was immersed in 30% sucrose–PBS, frozen quickly with dry ice, and thawed; sucrose was removed by a thorough rinsing
in PBS. The retina was placed in 70% methanol containing 3% H$_2$O$_2$ for 1 hour at 4°C, washed twice for 30 minutes in PBS, and incubated in PBS–2% goat serum overnight at 4°C. Tissue was incubated with antisera to CD31 1:200 at 4°C for 1 week, followed by incubation with secondary antibody (horse anti-mouse IgG, 1:100) for 24 to 48 hours; each incubation was followed by a 24-hour wash with PBS. The tissue was then soaked in avidin–biotin–peroxidase complex solution for 4 to 6 hours, washed for 1 hour, and placed in 1 M DAB containing 0.003% H$_2$O$_2$ for 10 minutes. The reaction was subsequently stopped with distilled water.

Histochemistry

Selected F71, F105, F134, and postnatal year 9 (P9) eyes were processed for ADPase or ATPase cytochemistry of wholemounts and frozen sections.$^{5,6,8,29}$

RESULTS

Spindle Cells

This cell population was not consistently labeled by any of the antibodies to vascular markers, nor was it labeled by ADPase cytochemistry. The lack of labeling clearly distinguished them from the adjacent labeled endothelial cells. Spindle cells were reactive with antibodies to cell-cycle specific nuclear antigens (see below).

Formed Vessels in Sections

The endothelial markers CD31 and von Willebrand’s factor specifically labeled both newly formed vessels and more mature vessels in all retinal layers (newly formed vessels are found in the peripheral-most vascularized retina; Figs. 1A to 1G). No qualitative differences in labeling for CD31 and von Willebrand’s factor were noted between vessels of different laminae or vessels at different eccentricities in a given section. Both reagents reacted with endothelial cell membranes at the leading edge of vascularization in the NFL (Fig. 1A) and in the earliest vessels extending toward the INL. $R$. communis lectin bound to retinal vessels in both paraffin and frozen sections (Fig. 1D), and the intensity of labeling increased slightly from F83 to F157 (not shown). In comparison to capillaries, larger arterioles and venules exhibited less staining with $R$. communis. Pretreatment with neuraminidase did not alter $R$. communis vascular staining. Peanut agglutinin lightly labeled all vessels and more intensely labeled cone outer segment sheaths. A peanut agglutinin (+) band in the INL might correspond to Müller cells (not shown). After pretreatment of sections with neuraminidase, peanut agglutinin binding increased in intensity, but the distribution was unchanged. Wheat germ agglutinin did not react with vessels but did stain rod outer segment sheaths and a band in the INL. In contrast, wheat germ agglutinin bound to vessels treated with neuraminidase in both the NFL and the GCL but not in the INL (Fig. 1E). $U$. europaeus reacted with newly formed and mature vessels at all ages in frozen sections and revealed multiple thread-like processes extending peripherally from the distal-most primordial vessels in the NFL. $B$. simplicifolia reacted with all cellular elements in developing and mature retina and thus exhibited a nonspecific labeling pattern.

Collagen types I and VIII showed different distributions in immature and mature retinal vessels in only paraffin sections. Collagen type VIII labeled the most peripheral and presumably the most recently formed vessels in the NFL and the vascular extensions into the INL more intensely than collagen type I. Collagen type I labeled larger venules and, to a lesser extent, larger arterioles more intensely than capillaries, whereas the opposite was true for collagen type VIII (Figs. 2A, 2B).

Laminin and fibronectin were localized by immunohistochemical staining to blood vessels at all ages and of all sizes and layers. More mature vessels tended to exhibit more intense labeling than newly formed vessels (Figs. 2C, 2D, 2E). Laminin and fibronectin immunoreactivity coincided in adjacent sections, and neither marker was seen peripheral to growing vessels.

ATPase and ADPase enzymatic histochemistry was used on frozen sections of tissues from all ages. In postnatal retinas, ATPase activity was confined to vascular elements, and all vessels in all laminae reacted darkly. In fetal specimens, a more complex staining pattern was found (Fig. 3). Between F75 and F145, recently formed vessels at the leading edge of vascularization exhibited sporadic, light staining, whereas more centrally located vessels were more darkly stained. Staining was not specific for blood vessels at these ages because neuronal and glial cells in the NFL and ganglion cell layer also showed ATPase activity. Light staining of horizontal fibers in the NFL was seen from F75 to F150. The orientation of these fibers in bundles within the retina and optic nerve, and labeling of globular cell bodies in the NFL, suggest that fetal glia exhibit significant ATPase activity. Occasional spindle-shaped cells in the NFL exhibited ATPase activity; however, these cells usually were located central to the leading edge of vascularization, making their designation as vasculogenic precursor cells doubtful. Beginning at approximately F140, ATPase activity in blood vessels increased progressively, whereas it decreased in nonvascular elements (Fig. 3). Therefore, by late gestation and after birth, ATPase was an increasingly specific vascular marker. ADPase
FIGURE 1. (A) Recently formed nerve fiber layer (NFL) vessels in fetal day 88 retina are labeled by anti-von Willebrand's factor antibodies. Note the absence of labeling distal to most peripheral vessel (arrow; identified by phase contrast and counterstaining of adjacent sections). (B) Adult retina exhibits von Willebrand's factor labeling in all four vascular laminae (in the NFL, ganglion cell layer, and at inner and outer edges of the inner nuclear layer (INL, designated by asterisks). (C) CD31-immunoreactivity in all vessels of adult retina. (D) *Ricinis communis* binding of vessels in fetal day 158 retina. (E) Wheat germ agglutinin binding after neuraminidase treatment identifies vessels in the NFL and ganglion cell layer but not in the INL. (A,B,C) Frozen sections. (D,E) Paraffin sections. Magnification, ×60.

activity, in contrast, was localized to vessels at all ages and increased in intensity from early fetal specimens to adult retinas (not shown).

Spindle-shaped cells found adjacent and just peripheral to advancing NFL vessels invariably were labeled by antibodies to cyclin, Ki-67, and Mib-1 (Fig. 4A). Occasional labeled globular cell nuclei interspersed among the spindle cell clusters were also reactive with Ki-67 antibodies; these may represent spindle cells, whose long axes are neither aligned with the meridian of the section nor with a separate cell population (Fig. 4A). A relatively small percentage of endothelial cells was reactive for cyclin, Ki-67, and Mib-1 in the NFL and, less often, in the outer plexuses, of F80 to F157 retinas (Fig. 4B). Labeled endothelial cells were scattered along the entire central-to-peripheral extent of vascularized retina in fetal sections, including at the leading edge. After birth, endothelial cell labeling with these markers was rare. Immunoreactivity for cyclin also was observed in a band within the fetal INL that corresponds to the position of the Müller cell bodies.

**Retinal Wholemounts**

Because spindle cells are only occasionally seen in monkey retinal section, wholemounts were labeled by ADPase and ATPase histochemistry and with antibodies to CD31 to detect this population. Even in
Vascular Markers in Primate Retinal Development

FIGURE 2. (A) Antibodies to collagen type I label larger vessels more intensely than capillaries (arrows) in postnatal day 1 frozen section. The opposite pattern is seen for collagen type VIII (B) in postnatal week 6 retina, in which a larger vessel (arrow) is unlabeled. (C) Anti-laminin antibodies label peripheral-most vessels in fetal day 83 retina (peripheral direction is toward left of figure). At fetal day 127 (D), newly formed vessels of the inner inner nuclear layer (INL) lamina (arrows) and extension of nerve fiber layer vessel toward the INL (arrowhead) are stained less intensely than more mature NFL vessels. (E) By fetal day 157, vessels are more uniformly and darkly labeled for laminin. Laminin was associated only with formed vessels at all ages. Magnifications, ×30 (C); ×60 (A, B, D).

wholemounts, spindle or other cells peripheral to the leading edge of vascularization were not labeled by either marker at F71 or F105 (Fig. 5A). Wholemounts more convincingly demonstrated multiple CD31-positive, filament-like processes extending from the most peripheral endothelial elements. In some cases, these processes extended for 500 μm or more into avascular retina (Fig. 5B). No labeled spindle cells were seen along the course of these processes. Light counterstaining with toluidine blue revealed spindle-shaped cells in the innermost NFL (vitread to the advancing vessels) peripheral to formed vessels. Some spindle cells were arranged linearly, but they were not necessarily associated with the filamentous CD31-positive fibers (Fig. 5C). All immunohistochemical, lectin and enzymatic cytochemical results are summarized in Table 1.

DISCUSSION
Blood Vessel Cell and Matrix Labeling During Development

Growth of blood vessels requires extracellular matrix degradation, endothelial cell proliferation and migration, basement membrane synthesis, lumen formation, addition of mural cells, and remodeling to form mature vascular networks. In each step, changes occur in the expression of certain proteins by vascular cells.

We found that the most immature fetal vessels, whether in inner or outer vascular laminae, express qualitatively similar immunocytochemical, enzymatic, and lectin labeling patterns compared to more mature vessels. Only one distinction was found between NFL-GCL and INL vessels: Wheat germ agglutinin binding after neuraminidase pretreatment lightly labeled NFL-GCL vessels but not plexuses that border the INL. Our selection of markers was not exhaustive, but our data indicate
that as soon as vessels form, they contain many of the same markers exhibited by adult vessels.

Quantitative differences were more frequent. Larger vessels and capillaries exhibited differential intensity of labeling by collagen types I and VIII. *H. communis*, laminin, and fibronectin labeling, and ATPase and ADPase activity, increased in intensity over time so that differences were seen between fetal and postnatal periods. These differences might relate to progressive thickening of vascular basement membrane in development. Subtle variations in the level of expression of other markers in this study during development could occur that escape detection because histochemical results are not reliably quantifiable.

Our results are comparable to immunohistochemical investigations of developing or adult retina in other species. However, a few differences are apparent. Fibronectin is increased just distal to peripheral tips of developing rodent retinal vessels, and chick brain vessel sprouts extend in a fibronectin-rich environment devoid of laminin. The current study did not detect fibronectin in advance of vessels in fetal primate retina. In previous lectin-binding studies of human and rodent retina in which fixation and embedding techniques were different from ours, lectin labeling was dissimilar in some respects to that in our sections. *B. simplicifolia* labeled retinal vessels and spindle cells in cat and...
Vascular Markers in Primate Retinal Development

Figure 4. (A) Ki-67 immunoreactivity in fetal day 90 paraffin section shows specific staining of a cluster of spindle and globular nerve fiber layer cells in a region just left (peripheral) of the peripheral-most vessel in this section. Note granular nuclear reaction product. No nerve fiber layer (NFL) cells are labeled peripheral to the cells shown. (B) Ki-67 in fetal day 127 paraffin section shows occasional labeling of endothelial cells in NFL and ganglion cell layer vessel of mid-peripheral retina. Light Cresyl violet counterstain; magnifications, ×120 (A); ×60 (B).

**Spindle Cells**

Spindle-shaped cells in the NFL of immature retina have been implicated in vasculogenic vessel formation. They are thought to act as precursors that migrate from the optic nerve or hyaloid vessel region, or as angioblasts that arise and differentiate in situ at the leading edge of vascularization. In cat retina, the lectin *B. simplicifolia* reacts with spindle cells and endothelial cells. In the dog, angioblasts stain for ATPase activity, as do blood vessels, and in serial sections they appear to align and form cellular cords continuous with advancing vessels. In primates and other species, markers specific for vascular endothelial cells have not been reported to label spindle cells, although in humans spindle cells may acquire von Willebrand's factor immunoreactivity as they mature into presumed vascular precursor cells. Generally, in primate retina, their identification as vascular precursors has been based on morphologic descriptions.

This study challenges the concept that a vasculogenic growth mechanism involving precursor cells is a major contributor to primate NFL vascularization. Four lines of evidence support this conclusion. First, neither spindle cells nor any other cells in advance of growing vessels are labeled consistently by any of the 13 vascular markers we employed. The lectin *B. simplicifolia* labeled vascular endothelial precursor cells in cat retina, and antibodies to CD31 have been shown to bind to endothelial cell precursors at stages in vascular development as early as blood island formation. *B. simplicifolia* and CD31 failed to label monkey spindle cells, but CD31 did identify peripheral endothelial cells and the processes arising from them. However, we cannot exclude completely the possibility that spindle cells are precursors of endothelial cells because we may not yet have identified the correct marker for this population. Second, growing fetal vessels in the INL plexuses that arise in the absence of spindle cells exhibited the same labeling pattern as do retinal vessels in monkey, but it exhibited only non-specific labeling in the current study.

In early fetal specimens (F75 to F140), ATPase activity appeared to be a mixed astrocytic and vascular marker because it labeled the optic nerve and horizontal processes and globular cells in the NFL, and its reactivity in vessels was intermittent. Beginning in late gestation, blood vessels acquired increasing ATPase activity as the staining of glial elements diminished, and after birth ATPase was predominantly a vascular marker. Previous studies of canine vascular development showed that blood vessels and spindle cells specifically exhibit ATPase activity in the newborn retina. This result prompted us to designate spindle cells as angioblasts. In fetal primate retina, however, ATPase staining does not distinguish glial from vascular elements at the critical stages when such a marker is needed.
FIGURE 5. CD31 immunoreactivity in mid-peripheral retina at fetal day 105 (A,B,C; magnifications, ×30, ×120, and ×100, respectively). Red blood cells (arrowheads, B,C) can be visualized as biconcave intraluminal cells close to the edge of vascularization by slight defocusing of the microscope. The advancing edge of growing nerve fiber layer vessels consists of fine processes that extend into avascular retina in B (arrows). Spindle-shaped cells are not labeled by CD31 antibodies but are revealed in the inner nerve fiber layer in the vicinity of the peripheral-most extending vessels by a toluidine blue counterstain. Many of these are indicated by arrows in C.

NFL vessels with the markers we used. Third, although spindle cells were noted by counterstaining just peripheral to growing vessels, they were not consistently aligned with the meshwork pattern or the processes of growing vessels. This result would be expected if spindle cells formed vasculogenic cords directly. Finally, the proliferative potential of some endothelial cells at growing vessel fronts demonstrated by cyclin and Ki-67 immunoreactivity, and with nucleotide labeling, indicates that an angiogenic mechanism is feasible in inner and outer retinas.

Although our findings question the hypothesis that spindle cells are a major source of vascular endothelial cells, spindle cells must play some role in vascular or retinal development because of their temporal and spatial relationship to the leading edge of growing vessels in the NFL. It is possible that they are related to concurrent developmental processes, such as astrocyte and microglial development, pericyte formation, nourishment of the vascular vanguard or of avascular retina, or maturation of ganglion cell axons. Some spindle cells are labeled for glial fibrillary acidic protein, a protein characteristically expressed by astrocytes. Because glial fibrillary acidic protein is not expressed by very immature glia, it is possible that many spindle cells are in fact astrocytes.

It is also possible that spindle cells appear transiently during development to facilitate vascularization. Examples of cell populations in the brain that have transient roles during development are subplate neurons and radial glia. Alternatively, in conjunction with glia and the extending immature vascular network, spindle cells might modulate the mechanical properties of peripheral NFL. Vernon and colleagues have shown that cultured endothelial and other cell lines form intercellular tension lines along which the cells align, acquire a fusiform shape, and migrate to form a planar network similar to the immature vascular meshwork in retinal NFL. If these biophysical events occur in developing inner retina, spindle cells could contribute to the mechanical milieu of
TABLE 1. Summary of Immunohistochemical, Lectin Binding, and Enzyme Cytochemical Results

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<th>Marker</th>
<th>SC</th>
<th>NFL/GCL</th>
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SC = spindle cells; NFL/GCL = nerve fiber and ganglion cell layer vessels; IINL and OINL = inner and outer inner nuclear layer vascular plexuses, respectively; see text for other abbreviations. (-) = no specific labeling; (+) = labeling; (++) = intense labeling; (+/-) = light and/or intermittent labeling. See text for other abbreviations.

*With neuraminidase pretreatment.

†Vascular label was intense but sporadic, and varied with age; see text.

‡Labeling specificity varied with age; see text.

the growing vessels, or they may be bystanders that align along tension forces and thereby become spindle-shaped.

In conclusion, the similar immunohistochemical profile of all retinal vessels during development and the lack of labeling of spindle cells by vascular markers suggest that primate NFL vessels arise by a budding or angiogenic mechanism similar to the process in the INL.

Key Words
angiogenesis, CD31, Ki67, lectins, vasculogenesis

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