Development of Astrocytes and Their Relation to Blood Vessels in Fetal Monkey Retina

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Purpose. To determine the development of astrocytes and their vascular relations in Macaca monkey retina.

Methods. Sections and wholemounts of retinas from fetal day (Fd) 65 to adult animals were analyzed immunohistochemically to detect glial fibrillary acidic protein (GFAP) and vimentin.

Results. Astrocytes appeared first near the optic disc, then subsequently further peripherally, but avoided the fovea. In the nerve fiber layer, round and ovoid cells extended processes parallel to ganglion cell axons. In the ganglion cell layer, ovoid and stellate cells exhibited anisotropic processes or a honeycomb network. The inner lamina of astrocytes developed ahead of the outer lamina, and both reached their final positions before birth. Astrocytes lay more peripherally than did developing blood vessels, and the growing edge of nerve fiber layer vessels lay between the two astrocytic layers. Spindle cells, which may be vascular precursor cells, often aligned along linear astrocytic processes. Occasional spindle-shaped cells containing GFAP or vimentin were identified as immature glia. Astrocytes and blood vessels coincided regionally during development, but astrocyte processes were typically not in register with the meshwork of growing blood vessels. Astrocyte–vessel associations increased during fetal life and postnatally.

Conclusions. During development, astrocytes display the same bilaminar pattern and morphologies present in adult retina. Astrocytes and blood vessels exhibit a similar regional distribution, but develop in distinct spatial patterns. Vessel investment by astrocytic processes increases during fetal life but is variable at all ages. Invest Ophthalmol Vis Sci. 1996;37:2367-2375.

Astrocytes have multiple functions in the retina and central nervous system that include nutritional and structural roles, intercellular signaling, potassium buffering, neurotransmitter metabolism, regulation of exchange between blood vessels and neural tissue, and involvement in pathologic processes. Several lines of evidence closely relate retinal astrocytes to blood vessel structure and function. Species without intrinsic retinal vessels lack intraretinal astrocytes, whereas species with vessels in a small portion of the retina have astrocytes restricted to the vascular region. Human, monkey, dog, cat, and rodent retinas contain widespread intraretinal vessels associated with astrocytes, except in primate fovea where both are absent. The dynamic relation of astrocytes and blood vessels is further documented by observations that astrocytes contribute to the glial limitans lining blood vessels, modulate the differentiation of vascular endothelial cells, and are involved in the formation and preservation of the blood-brain and blood-retinal barriers.

The appearance of astrocytes and blood vessel is approximately concurrent in rodent and feline retina, but there is little information on the temporal development of astrocytes in primate retina. This study determines the spatial and temporal developmental sequences of astrocytes in Macaca monkey and describes their relation to the emerging vasculature.

METHODS

Subjects

Details of animal usage and tissue processing have been published previously. All tissue was obtained...
in accordance with the Animal Use guidelines of the Regional Primate Research Center at the University of Washington and by the Association for Research in Vision and Ophthalmology.

Eyes were from Macaca monkeys ranging in age from fetal day (Fd) 65 (birth = Fd165 to 170 days) to adulthood at 5 years. Enucleated eyes were hemisected and fixed by immersion in 4% paraformaldehyde for 1 to 3 hours, cryoprotected, and serially frozen sectioned at 10 μm. A few eyes were studied that were injected whole with methyl Carnoy solution and then fixed overnight before being processed for paraffin embedding and sectioning.

Sections were stained by immunohistochemistry with antibodies against the astrocytic markers glial fibrillary acidic protein (GFAP; rabbit antihuman, 1:200 to 1:10,000; Sigma, St. Louis, MO) or vimentin (mouse antihuman, 1:2000; Boehringer-Mannheim, Indianapolis, IN). Müller glial cells also are labeled by vimentin, but are distinguished from astrocytes by cell body location within the inner nuclear layer and vertically oriented processes that span the retinal thickness. Microglial cells are not labeled by these markers. Antibody labeling was visualized by peroxidase–aniperoxidase and diaminobenzidine (DAB) reaction or immunofluorescence.

Wholemounts
Retinas at Fd82d, Fd88d, F134d, F150d, and adult were freed from the underlying pigment epithelium and choroid shortly after enucleation, radial cuts were made to allow flattening, and the tissue was fixed for 3 hours in 4% paraformaldehyde at 4°C. Retinas were cut into quadrants and were processed for immunohistochemistry. Free-floating mounts were exposed to 3% hydrogen peroxide in 70% methyl hydroxide for 45 minutes, washed, and incubated overnight in 2% goat serum. The incubation solution in all steps was 0.1 M glycerol in phosphate buffer. Blood vessels were identified in wholemounts by phase microscopy or by the weak DAB reaction product in erythrocytes. The temporal retina and fovea were not available for ages Fd82d and F134d.

RESULTS

Astrocyte Morphology and Distribution During Development

In retinal sections and whole mounts, immunoreactivity corresponding to GFAP and vimentin was present within the nerve fiber layer (NFL) and ganglion cell layer (GCL) at all ages examined. Astrocytes first appeared within the NFL near the optic nerve head and then spread radially in all directions at progressively older ages. At Fd65, astrocytes labeled for GFAP or vimentin were present in the optic nerve and within a retinal zone extending 500 μm around the optic nerve head (Fig. 1A). This retinal zone did not contain blood vessels, although they were present in the optic nerve. Between Fd65 and Fd75, astrocytes in the NFL could be detected 2 to 3 mm from the optic nerve. By Fd88, the NFL contained astrocytes over much of the retina (Fig. 1B), with occasional fine GFAP-immunoreactive fibers reaching to within 500 μm of the ora serrata.

Astrocytes in the GCL also developed in a radial direction from the optic nerve. Except in the perifoveal region (see below), GCL astrocytes lagged posteriorly to NFL astrocytes at all ages and by Fd88, extended only to the midperiphery (Fig. 1C). The two astrocytic laminae are not distinguished easily in the periphery where the NFL is thin and the GCL is irregular.

In whole mounts at Fd82 to Fd88, NFL astrocyte cell bodies generally were round, ovoid, or elongated, with two to five processes running mainly in radial directions (Fig. 2A). Between Fd88 and Fd150, these processes collected into increasingly thick and distinct parallel bundles that followed the course of ganglion cell axons (Fig. 2B). Parallel bundles were interconnected periodically by astrocytic processes that crossed over to an adjacent bundle. As well, single astrocytic cell bodies appeared to send processes into multiple bundles (Fig. 2B). The NFL astrocyte cell bodies often occurred in groups of two to four cells situated between parallel bundles of astrocyte processes (Fig. 2B). In adult retina, these bundles of astrocytes were less distinct, and in general, the number of astrocyte cell bodies appeared to be less than at Fd150. Astrocytes in the perifoveal area and in the retinal periphery by Fd150 maintained astellate distribution of processes (Fig. 2D).

At all ages, most astrocytes in the GCL were round or ovoid, but smaller stellate astrocytes also were seen (Fig. 2C). The GCL astrocytes emanated processes in all directions that did not form bundles. At Fd82 and Fd88, GCL astrocyte processes were sparse and often orthogonal to the orientation of ganglion cell axons (Fig. 1C). The GCL astrocytic processes tended to increase with age and formed either an anisotropic net-
FIGURE 1. (A) Astrocytes in peripapillary retina at Fd65 to Fd70 are labeled with antibodies against vimentin. Note spindle-shaped astrocytes (arrowheads) peripheral to blood vessels (arrows) and the lack of labeled Müller cells. Cresyl violet counterstain. Magnification, ×75. (B) Parallel radially oriented glial fibrillary acidic protein (GFAP) immunoreactive astrocyte fibers in the nerve fiber layer of an Fd88 wholemount. Note many astrocytic processes are colinear with the long axis of spindle-shaped cells seen after counterstaining with toluidine blue (arrowheads). No ganglion cell layer (GCL) astrocytes are present in this micrograph, which shows the most central part of temporal avascular retina. Magnification, ×150. (C) Retinal region approximately 1.5 mm posterior to that in B, before counterstaining, shows peripheral extent of sparse anisotropic GCL astrocyte processes reactive with anti-GFAP antibodies. Magnification, ×75.

work or, in areas of higher density, a honeycomblike meshwork. Both NFL and GCL astrocytic processes occasionally projected vertically between the two layers. Infrequently, astrocytic processes extended to the inner plexiform layer and rarely reached the inner nuclear layer; these were more common in posterior retina.

At all ages, the number of astrocyte cell bodies and processes in both NFL and GCL was higher near the optic nerve and then decreased into the periphery along all meridians. This gradient might be exaggerated during development because immunoreactivity for GFAP increases with age, and presumably, peripheral astrocytes are less mature than are more central cell bodies.

Perifoveal Development

The fovea was studied in sections at Fd65, Fd88, Fd95, Fd130, Fd140, and adult and in wholemounts at Fd88.
FIGURE 3. Age Fd88 wholemount with focus on nerve fiber layer (NFL) (A) and ganglion cell layer (GCL) (B) glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes extending toward the fovea (right). Perifoveal vessels, poorly seen because they are in a plane between those shown in A and B, are outlined with dashed lines in B. Vessels do not extend as far centrally as do astrocytes and are not in register with linear NFL or honeycomb GCL astrocyte meshworks. Magnification, ×75. (C) Stellate astrocytes (arrowheads) labeled for GFAP in the nerve fiber and GCLs approach the nasal foveal edge, but do not invade the fovea (f) at Fd140. Vertical fold in the fovea is artefactual. Magnification, ×100. (D) At Fd150, stellate astrocytes closest to the fovea form a radial pattern around the fovea (f), whereas slightly less central astrocytes are arranged circumferentially. Further temporally, in the left half of the figure, astrocyte processes from superior and inferior retina meet or overlap minimally along the horizontal raphe (arrowheads). The GCL astrocyte processes near the raphe form a honeycomb meshwork barely visible beneath the curvilinear NFL processes. Dark subretinal areas are adherent retinal pigment epithelium, and faint Müller cell GFAP immunoreactivity in the fovea. Magnification, ×4. (E) Astrocyte processes labeled by GFAP immunofluorescence (upper right) at temporal foveal edge at Fd130. Note faint Müller cell labeling. Magnification, ×100.

Fd150, and adult to determine the distribution of astrocytes during development in this specialized retinal region. In all fetal specimens, astrocytes encroached on the fovea from its nasal border, but remained absent from the foveal center (Fig. 3). In wholemounts, the NFL and GCL astrocytes were found roughly equidistant from the fovea, but perifoveal astrocytic processes were less dense in the NFL compared to the GCL (Figs. 3A, 3B, 3C). Foveal and perifoveal Müller cells exhibited significant immunoreactivity for GFAP in many fetal specimens (Figs. 3D, 3E), and lower levels of labeling persisted in the adult perifoveal and far peripheral retina.

The size of the perifoveal astrocyte-free zone decreased over development. It was approximately 2 mm in vertical height at Fd88 and approximately 500 to 750 μm in diameter at Fd150, when its size was similar to that in adult retina. The shape of this zone changed...
as well. At Fd88, astrocytes were lacking in a roughly semicircular area centered on the fovea that was continuous with a band along the temporal horizontal meridian or raphe. The NFL astrocytes nearest to the nasal, superior, and inferior borders of the fovea assumed a radial orientation with respect to the center of the fovea (Fig. 3A), whereas GCL astrocytes in the perifoveal region formed a honeycomb meshwork (Fig. 3B). By Fd140 to Fd150, sparse stellate astrocytes encroached on the foveal center and completely surrounded the fovea temporally in a concentric pattern (Figs. 3C, 3D, 3E). The temporal horizontal raphe became by Fd150 filled in with NFL astrocyte processes from superior and inferior retina that met along the raphe (Fig. 3D, arrowheads). The GCL astrocytes at the raphe remained in a honeycomb meshwork with no apparent change or rearrangement that marked the raphe.

Astrocyte–Vascular Relations During Development

Spindle-shaped cells were identified at the edge of advancing vascularization from Fd65 to approximately Fd95. A few of these cells were reactive with antibodies to vimentin (Fig. 1A). Spindle-shaped astrocytes labeled with GFAP antibodies also were noted (Fig. 2A) but were not preferentially distributed at the advancing vascular front. Spindle-shaped cells were located in the vitread NFL and usually were oriented with their long axis parallel to ganglion cell axons. Many spindle cells were aligned along astrocytic fibers, similar to a bead on a string (Fig. 1B). Antivimentin antibodies reacted primarily with astrocytes in early fetal life (Fd65 to Fd80; Fig. 1A), but in older retinas, Müller cells reacted with vimentin antibodies with increasing intensity and specificity.

Both astrocytes and intraretinal blood vessels have the same developmental pattern in that they grow radially from the optic nerve toward the ora. At Fd65 to Fd70, peripapillary NFL astrocytes lay just peripheral to the earliest-appearing intraretinal vessels. At all subsequent ages, astrocytes labeled for either GFAP or vimentin were detected more peripheral than the advancing vascular front (Figs. 1A, 1B) until vessels reach the ora serrata at Fd95 to Fd110. The GCL astrocytes were at all fetal ages slightly more centrally situated than advancing NFL astrocytes and typically were near the advancing vascular front. Astrocytes that approached the fovea and raphe during fetal life also lay ahead of advancing vessels (Fig. 3B).

The regional distribution of astrocytes and blood vessels was similar throughout development in that both avoided the fovea and a narrow strip at the ora. As shown earlier for blood vessels, astrocytes also invaded along the temporal horizontal raphe relatively late. However, the overall patterns of developing vessels and astrocyte processes differed in three respects. First, in Fd82 and Fd88 wholemounts, the NFL and GCL astrocytes and the advancing vascular meshwork were segregated into three laminae. Sequential deeper focus levels into the retina showed a mostly linear layer of astrocytes (Fig. 4A), a plexus of blood vessels but few astrocytes (Fig. 4B), and deeper sparse randomly oriented astrocytic processes (Fig. 4C).

Second, vessels and astrocytes generally exhibited different two-dimensional patterns. Throughout the retina, growing vessels extended in a chicken-wire or honeycomblike meshwork, whereas astrocytic processes were either radially aligned with ganglion cell axons in the NFL or were anisotropic in the GCL (Figs. 1C, 2A, 2B, 2D, 4). In the perifovea and along the temporal horizontal raphe, where developing GCL astrocytes did exhibit a honeycomb meshwork, this meshwork was not in register with the vascular network (Fig. 3B). The only exception was seen at Fd150 in far peripheral retina, where overlap was noted occasionally between meshwork patterns of astrocytes and adjacent vessels (Figs. 4D, 4E).

The third difference was seen during vessel remodeling. As retinal arterioles form within peripheral capillary meshwork, capillary density decreases around these larger vessels to form the periarterial avascular zone. A corresponding perivascular zone lacking astrocytes was not detected at any age.

A variety of astrocyte-vessel associations characteristic of adult retina were present by Fd132. Astrocyte processes crossed over or under vessels without any apparent morphologic change or contact; ended on vessel surfaces, often as a footlike extension; bifurcated and ran alongside the lateral vessel wall; bifurcated or arborized along the vitread or scleral vessel wall; and wrapped around the vessel wall in a coiled or spiral fashion (Fig. 5). The NFL astrocyte bundles arborized intermittently to send a small tuft of processes along the vitread wall of vessels that ran parallel to the bundles. The above relations occurred between astrocytes and arterioles, venules, and, to a lesser extent, capillaries. Astrocyte-vessel contacts were most common on the vitread vessel surface, less common along the lateral vessel walls, and infrequent on the scleral vessel surface (Figs. 5A, 5B, 5C).

The number of astrocyte processes associated with vessels increased from Fd132 to adult, despite an apparent decrease in astrocyte numbers throughout the retina after Fd150. Vessels within 1 to 2 mm of the fovea or the temporal horizontal raphe exhibited few or no contacts with astrocytes (Fig. 5D). In fetal retina, rare astrocytic processes extended as far as the inner nuclear layer, but vessels in the inner plexiform and inner nuclear layer lacked astrocytic investment at all ages.
DISCUSSION

Astrocyte Morphology and Distribution in Development

Astrocyte development in the primate follows an optic nerve-to-periphery vector described in other species. Retinal vascularization also begins at the optic nerve head, but cytogenesis, neuronal differentiation, and synaptogenesis spread radially from the fovea. The source of glial stem cells is within the optic nerve, unlike the precursors to other developmental processes that are intrinsic to retinal neurons.

Features of retinal astrocytes described in humans and monkeys include bilaminar distribution of astrocytes in the nerve fiber and ganglion cell layers; presence of two morphologic classes of astrocytes; decreasing density gradient of astrocytes from the disc into the periphery; and regional correspondence with intraretinal vasculature and ganglion cell axons. Our observations in developing primate retina indicate the above features are established from the earliest stages of development, and, with some exceptions, do not derive from later remodeling of an immature glial anatomy. Thus, astrocytes occupy relatively distinct laminae within the nerve fiber and ganglion cell layers soon after they appear within the retina. Further, astrocytes in these laminae differ morphologically, and their density decreased in a centrifugal direction through all stages of development in this study. In both fetal and mature retinas, astrocytes and vessels have a similar distribution, and NFL astrocytes align predominantly with ganglion cell axons.

Astrocytes avoid the fovea and perifoveal area during development, but the size and shape of this astrocytic region changes markedly during fetal life. At Fd88, it is relatively large and includes a horizontal band extending temporally along the horizontal raphe. By late gestation, the horizontal raphe contains astrocytes, and the anastrocytic foveal region is smaller and circular. Interestingly, development of retinal vasculature obeys this same pattern with respect to the fovea and the temporal horizontal raphe. This suggests a connection between development of the temporal raphe region and early processes of foveation.

Factors relating to avoidance of perifoveal retina by migrating astrocytes might include the relative thinness of the nerve fiber layer centrally, different oxygen tension in the macula resulting from variable distance to the choriocapillaris, and differing blood flow in the macula. Alignment of nerve fiber layer astrocytes with ganglion cell axons in both fetal and adult retina suggests the orientation of perifoveal and temporal raphe axons relates to the distribution of perifoveal and raphe astrocytes. Mere presence of axons does not determine the plan of astrocytic orientation in other species, because astrocytes develop nor-
FIGURE 5. Astrocyte associations with same vessel segment viewed at three retinal depths (F8132): (A) glial fibrillary acidic protein-immunoreactive astrocyte processes in the inner nerve fiber layer pass over a large vessel without apparent contact, or arborize or terminate on vitread vessel surface. (B) Astrocyte processes course along lateral vessel wall. (C) Sparse astrocyte processes along sclerad aspect of vessel. Some of these processes coil around the vessel one or more times. Magnification, ×100. (D) In adult retina, astrocyte contacts with vessel (right) terminate abruptly as vessel narrows and approaches foveal avascular zone (toward the left). Magnification, ×120.

Nonetheless, anatomic associations between astrocytes and vessels during early vascularization were infrequent. Both at the edge of vascular growth and posteriorly where formed vessels exist, inner retinal vessels lay in a plane sandwiched between the two astrocytic laminae. In other species, developing astrocytic and vascular meshworks show striking correspondence. However, the chicken-wire meshwork pattern of growing primate blood vessels typically is not in register with patterns of astrocytic processes, which are linear in the nerve fiber layer and anisotropic in the ganglion cell layer. Our findings extend the conclusion of Rungger-Brandl et al who state that the relative association of astrocyte processes with blood vessels or neuronal elements is species-dependent in mature retina.

We occasionally observed spatial overlap of developing astrocytic and vascular meshworks in peripheral retinas of older fetal specimens, an age during which vascular growth diminishes and remodeling occurs.
Spatial overlap might indicate a relation between vascularization and astrocyte development, as suggested in other species. Alternatively, the two cell populations may form similar two-dimensional networks in response to similar mechanical factors of the local milieu. Biophysical factors have been implicated in the formation of cellular networks.

Immunocytochemical methods used in this study identify anatomic associations, but not cellular contact, between astrocytes and vessels. Such associations might therefore be underestimated, especially in the case of capillaries and fine astrocytic processes. At Fd82 and Fd88, astrocyte-vessel associations were rare, both at the advancing edge of vascularization and further posteriorly. At Fd132, vessel-astrocyte relations existed in all categories described in adult monkey by Distler et al., but were uncommon. By Fd150, these relations were more evident. In adult retina, the frequency of astrocyte-vessel relations increased further, despite a reduction in the overall density of astrocytes. Thus, astrocytic influences on early vascularization might be mediated with minimal contact; for instance, by paracrine actions of growth factors. Later interactions may occur through the numerous, although intermittent and variable, contacts that increase during fetal life and postnatally.

Because retinal vessels surrounding the inner nuclear layer and vessels encroaching on the macula lack astrocytic investment, and because astrocyte associations with vessels are sporadic, astrocyte input may be insufficient for creation or maintenance of the blood-retina barrier, which is present in all intraretinal vessels. It has been suggested that the second class of retinal macroglia, Müller cells, share glial functions with astrocytes. It will be of interest to determine whether Müller cells associate with perivascular and inner nuclear layer vessels in a manner complementary to astrocytes. Microglial cells also have been characterized in mature and developing retina, whether these immune-related cells interact with retinal macroglia or vasculature is unknown.

**Key Words**

angiogenesis, fovea, glia, glial fibrillary acidic protein, primate

**References**

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