Rabbit retinal glial cells at the vascular and internal limiting membranes : an electron microscopic study using extracellular stains and tracers
by Darryl Martin Espeland

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Zoology
Montana State University

Abstract:
An electron microscopic study has been made to define cell to cell and extracellular variations between glial cells in vascular and avascular portions of the fiber layer of the rabbit retina. Studies using ruthenium red, lanthanum, and ferritin as extracellular stains and tracers are correlated with fine structural results.

In avascular regions of the retina, the Muller cell is the dominant supporting pell, forming the internal limiting membrane and contributing to all of the extracellular channels, except when ganglion cells or their axons are present, Miill'er cells lack cell to cell junctional complexes making the extracellular channels and their large dilations continuous with the vitreous except for an intervening basal lamina. Extracellular stains and tracers introduced in fixative or into the vitreous stained the inner limiting membrane, the extracellular channels between Muller cells, Muller cell pinotytotic invaginations and vesicles, and large extracellular dilations. These results suggest that the Muller cell is specialized to take up materials from the vitreous via the patent extracellular channels.

In vascular regions of the retina, the astrocyte is the major supporting cell, forming the inner limiting and glial limiting membrane around blood vessels. These limiting membranes are characterized by numerous cell to cell junctional complexes, Extracellular stains and tracers did not penetrate these junctional complexes from the vitreous to enter the vascular fiber layer. Tracers in fiber layer blood vessels are stopped at endothelial junctional complexes. They are pinocytized by endothelium, but aren’t transported to or released into the perivascular space. These results suggest an astrocytic barrier system to materials from the vitreous, and- an endothelial barrier system to materials in the vasculature, The close association of astrocytes to blood vessels also suggests that these cells serve an intermediary role between the vessels and neuronal elements, in terms of metabolic support.

Tracers introduced in choroidal blood vessels were found in endothelial pinocytotic invaginations and vesicles, and outside of the endothelium, After longer periods, particles were found deep to Bruch’s membrane and between pigment epithelial microvilli, but not deeper. These results suggest a pigment epithelial barrier system between the choroid and the outer retina.

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RABBIT RETINAL GLIAL CELLS AT THE VASCULAR AND INTERNAL LIMITING MEMBRANES. AN ELECTRON MICROSCOPIC STUDY USING EXTRACELLULAR STAINS AND TRACERS

by

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A thesis submitted in partial fulfillment
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of

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in

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ABSTRACT

An electron microscopic study has been made to define cell to cell and extracellular variations between glial cells in vascular and avascular portions of the fiber layer of the rabbit retina. Studies using ruthenium red, lanthanum, and ferritin as extracellular stains and tracers are correlated with fine structural results.

In avascular regions of the retina, the Müller cell is the dominant supporting cell, forming the internal limiting membrane and contributing to all of the extracellular channels, except when ganglion cells or their axons are present. Müller cells lack cell to cell junctional complexes making the extracellular channels and their large dilations continuous with the vitreous except for an intervening basal lamina. Extracellular stains and tracers introduced in fixative or into the vitreous stained the inner limiting membrane, the extracellular channels between Müller cells, Müller cell pinotytotic invaginations and vesicles, and large extracellular dilations. These results suggest that the Müller cell is specialized to take up materials from the vitreous via the patent extracellular channels.

In vascular regions of the retina, the astrocyte is the major supporting cell, forming the inner limiting and glial limiting membrane around blood vessels. These limiting membranes are characterized by numerous cell to cell junctional complexes. Extracellular stains and tracers did not penetrate these junctional complexes from the vitreous to enter the vascular fiber layer. Tracers in fiber layer blood vessels are stopped at endothelial junctional complexes. They are pinocytized by endothelium, but aren't transported to or released into the perivascular space. These results suggest an astrocytic barrier system to materials from the vitreous, and an endothelial barrier system to materials in the vasculature. The close association of astrocytes to blood vessels also suggests that these cells serve an intermediary role between the vessels and neuronal elements, in terms of metabolic support.

Tracers introduced in choroidal blood vessels were found in endothelial pinocytotic invaginations and vesicles, and outside of the endothelium. After longer periods, particles were found deep to Bruch's membrane and between pigment epithelial microvilli, but not deeper. These results suggest a pigment epithelial barrier system between the choroid and the outer retina.

INTRODUCTION

All nerve cells within the central nervous system are surrounded, physically supported, and probably nourished by a complex system of glial cells. How these cells perform these functions and the total complement of functions is still not known. As a uniquely accessible and specialized extension of central nervous system parenchyma, the retina provides an opportunity to study the relationship between central nervous system glial cells, adjacent neuronal elements, vasculature, and other supporting tissues.

The Müller cell is a specialized glial cell, found only in the retina, which forms a coarse supporting skeleton for the neural elements. The presense of astrocytes, glial cells which form this skeleton in the central nervous system, is variable in the retina depending upon the species. When they are present in the retina, they are mainly associated with blood vessels and large fields of axons. Oligodendroglia, the other common central nervous system glial cell is only rarely present in the retina, and is always associated with myelinated nerve fibers.

The retina in vertebrates is located between the choroid (a vascularized connective tissue layer) outside, and the vitreous (a gelatinous mass) inside (Fig. 1). The outer layer of the retina, adjacent to the choroid, is the pigment epithelium. The pigment epithelium is

a single layer of pigmented cells joined by specialized cellular junctions. The rods and cones, the light sensitive elements of the retina, lie just vitreal to the pigment epithelium. These photoreceptors synapse with a variety of intermediate neuronal elements (Fig. 1) which in turn synapse with the ganglion cells. The ganglion cell axons cross the inner layer of the retina, the fiber layer, to converge to form the optic nerve at the optic disc. The fiber layer consists of ganglion cell axons clustered between glial cells. The inner limiting membrane (ILM), an apparent barrier between the fiber layer and the vitreous, is variously defined as a basal lamina and the plasma membrane of the glial cell end feet[27], or as the basal lamina only[53]. The presense or lack of blood vessels in the fiber layer determines whether the retina is considered vascular or avascular, which is species dependent. Functional differences between glial cells in vascular and avascular retinas would be expected due to their supportive role.

Vascular retinas have blood vessels in the fiber layer, and sometimes in the vitreous just inside of the fiber layer. These vessels probably supply the inner half of the retina, while the choroid supplies the outer half via diffusion. Avascular retinas have no blood vessels in the fiber layer and supposedly would be dependent entirely upon the choroid for nutrition and gas exchange. Examples of animals with vascular retinas are the rat, mouse, and human[34]. The gecko

lizard and grass snake have avascular retinas[34]. Morphological studies in these animals have shown that differences in glial specializations exist when avascular and vascular species are compared[34]. However, interpretation of such differences is complicated by the fact that irregardless of vascularity, the retinal glial cells differ between species. The rabbit retina offers a unique model to study glial specializations reflecting the functional differences between vascular and avascular retinas. Although most of the rabbit retina is avascular, there are two vascular regions of tissue that form horizontal bands just lateral and medial to the optic disc. These bands are supplied by branches of the central retinal arteries which enter the eye through the optic nerve and form a system of capillaries within the fiber layer. There is also a system of superficial capillaries in the vitreous[33], adjacent to the fiber layer. These vascularized bands are also the only part of the rabbit retina with myelinated, rather than unmyelinated axons in the fiber layer. As a consequence, the vascular fiber layer is often referred to as the medullated ray.

This study was undertaken to define cell to cell and extracellular structural variations between glial cells in vascular and avascular parts of the rabbit retina using standard electon microscopic procedures, cell surface stains, and extracellular tracers. This information will contribute to an understanding of the role of glial cells in the functional support and nutrition of the retina and of nervous

tissue in general, their relationship to adjacent tissues, and the possible role of the vitreous in exchanging materials with the retina. This information will also contribute to the interpretation of studies which utilize the retina to model extracellular space for the rest of the central nervous system[51,53].

The understanding of glial and vascular structural specializations would also help provide an understanding of the blood-retinal barrier, the functional barrier system between the retina and its blood supply. It is likely there are three separate components to the barrier. 1. The pigment epithelial cells lie between the outer retina and the choroid. 2. Endothelial cells form the walls of blood vessels in the vascular part of the fiber layer, and are surrounded by glial elements. 3. The ILM, an apparent barrier formed by glial cell specializations, lies between the retinal fiber layer and the vitreous. A fourth barrier may exist between the vasculature and the vitreous.

Alterations or deficiencies in the blood-retinal barrier have been implicated in some pathological conditions. One of the earliest changes known to occur in diabetic retinopathy is an abnormal leakage of intravascular fluorescein into the vitreous[7]. In cystoid macular edema, retinal failure results when abnormal leakage from capillaries destroys retinal structures[8]. An understanding of the normal blood-retinal barrier could lead to important diagnostic and preventive procedures in retinal vascular disease.

LITERATURE REVIEW

Neural tissue appears morphologically to have relatively little extracellular space in contrast to most other body tissue. Estimates of intercellular volume range from 5 to 35% of the tissue volume, depending on the technique used[43]. Electron microscopic studies of chemically fixed central nervous system tissues reveal 100 to 200 Å extracellular spaces between glial cells[19]. When freeze-substitution fixation is used, it is estimated that 25.5% of the central nervous tissue is extracellular space, and that these extracellular spaces average 200 to 250 Å in width[46]. Ferritin and horseradish peroxidase, both documented extracellular tracers[3,11], when injected into the cerebrospinal fluid are able to pass through the ependyma into this parenchymal extracellular space[4]. The pinocytotic uptake of materials in extracellular channels by glial cells has been noted in the central nervous system[4].

The Müller cell, as the predominant supportive cell in the avascular retina, is in contact with most of the retinal extracellular space. The Müller cell processes in the fiber layer have been characterized as containing numerous 250 to 320 Å glycogen granules and occasional structures that appear to be smooth endoplasmic reticulum[25,30]. In some preparations, 100 Å filaments appear to pack the processes[31], while other organelles are lacking[25,31]. Müller cell

Pinocytotic invaginations and vesicles have not been described except in one preliminary study from this laboratory[31].

Retinal extracellular space in vertebrates has not been well studied. Most comments about it in the literature are made in passing. In the toad, ferrocyanide stain introduced into the vitreous penetrates the 120 to 250 Å extracellular space as deep as the bipolar layer (inner ½ of the retina)[19]. In the cat, thorium and ferritin introduced into the vitreous were located in 150 to 200 Å wide spaces through the retinal layers as deep as the external limiting membrane (almost the entire thickness of the retina) where their movement was stopped[43]. However, attempts to duplicate this experiment as a part of this study were unsuccessful.

Large extracellular dilations in the fiber layer are not mentioned except in the preliminary study from this laboratory[31]. It is of interest, however, that such dilations can be seen in light microscopic studies, although they are not commented on[17]. Extracellular space size in the outer retina has been shown to be dependent on the osmolarity of the cellular environment[51]. Standard electron microscopic fixation procedures cause slight shrinkage of cellular elements but only minimal distortion of extracellular space and cell to cell relationships[52].

The presence or absence of junctional complexes between Müller cells has not been commented upon in the literature. Astrocytic and

Müller cell junctional complexes should be expected since in most central nervous system tissues such complexes are characteristic of astrocytes around perivascular spaces and in limiting membranes[29], and since Müller cells are generally considered to be modified astrocytes[26,32,33].

The patency of extracellular spaces may play a role in the blood-retinal barrier system and thus in inner retinal nutrition and metabolism. It has been suggested that the exposure of cells to extracellular space indicates a role by these cells in active water and ion transport[19]. The role of the vitreous in retinal nutrition is not known, although one biochemical study[1] suggests that the glucose content of the vitreous adjacent to the retina is less than that of the remainder of the vitreal body, and that in the rabbit, there is a preferential flow of vitreous in the posterior direction. An active exchange of water between the vitreous and surrounding retina has been demonstrated with tritiated water[1]. These studies suggest that the vitreous might serve as an intermediary between the blood and the retina.

Early investigators speculated that anaerobic glycolysis occurs in the inner retinal layers[1,22]. This possibility is supported by biochemical studies showing a relatively greater activity of glucose-6-phosphotase and lactate dehydrogenase in the inner retinal layers when compared to the rest of the retina[25]. Glycogen is probably an

important energy reserve for the retinal tissues since ischemia produced by pressure on the internal carotid artery produces rapid glycogen depletion in the retina[49]. Incubation of retinal tissues in tritiated glucose for electron microscopic radioautography[25] indicates that glial elements, and particularly the Müller cells, are performing most of the glucose to glycogen conversion. The vitread portions of the Müller cells are richer in glycogen than the outer portions[25]. This might be explained by the fact that the outer retina requires less stored carbohydrate since the plentiful choroidal vessels provide easy access to nutrients. These studies suggest an active role of the Müller cells in retinal nutrition.

Clues to the nature of the blood-retinal barrier may be derived from the structure of fiber layer blood vessels and the adjacent glial cells. In a review article, Raviola [36] cites studies which demonstrate continuous tight junctions or zonula occludens between retinal fiber layer endothelial cells, like those seen in central nervous system vasculature. Zonula occludens in central nervous system vessels consist of five ultrastructural layers: two cytoplasmic dense leaflets (the inner leaflet of each trilaminar membrane), two electron lucent leaflets (the middle lipid layer of each trilaminar membrane), and a single median dense leaflet (representing intimate opposition if not fusion between the outer leaflets of adjacent cell membranes)[5]. These zonula occludens are considered to be the site of maximum

constraint to movement along intercellular spaces[9]. In the central nervous system, these endothelial tight junctions are impermeable to horseradish peroxidase[5,6,14,15,37], ferritin[6], and injected dyes[36]. It has also been shown that horseradish peroxidase can't pass through these tight junctions from the tissue side of central nervous system blood vessels into the lumen[41]. These endothelial cells do, however have numerous invaginations and vesicles that accumulate intravascular ferritin[6] and horseradish peroxidase[37]. Pinocytotic activity in fiber layer blood vessels of the retina has not been discussed in the literature.

The extracellular stains and tracers used in this study were ruthenium red, lanthanum, and ferritin. Ruthenium red is an inorganic, synthetically prepared, crystalline compound containing the moderately heavy element ruthenium[12]. Ruthenium red in fixative is generally used as a cell surface stain[12]. When used to bathe peripheral nerves, ruthenium red penetrates into the periaxonal space and stains apparent pinocytotic invaginations in Schwann cells[42]. It has been noted penetrating 25 to 30 Å spaces in myelin figures generated from lecithin[24]. It is believed that ruthenium red has a staining affinity for highly polymerized acid mucopolysaccharides[23]. Ruthenium red staining may have functional significance since recent studies indicate that anionic side chains of acid mucopolysaccharides facilitate the utilization and transfer of various ions and metabolites, and bind large amounts of

water[27]. Ruthenium red has sufficient electron density to be easily seen on electron microscopic sections not post-stained with lead citrate and uranyl acetate. Ruthenium red may be a cell poison[42], which complicates interpretations of its use as a stain. The extent of extracellular staining may be limited by heavily bound stain impeding the movement of unbound stain[24].

Lanthanum is described as an extracellular tracer or stain[38]. It is a relatively heavy element, which exists as two nm wide colloidal particles that may be used prior to or during fixation[12]. It is assumed that tissue spaces filled with aggregates of lanthanum are occupied by interstitial fliud or water under physiological conditions[12]. It is generally found in narrow extracellular spaces[38], but can be washed out of tissues during processing[12,16,38]. Studies with peripheral nerve myelin suggest that lanthanum is likely to penetrate into preexisting spaces and leave their sizes unaltered rather than cause a separation of normally opposed layers[39]. Lanthanum particles can pass through 18 Å wide cardiac "tight junctions", but can not pass through true zonula occludens[38]. Lanthanum does not appear loose in the cytoplasm of undamaged cells[16,38], but can be taken up pinocytotically by the axons of certain insect neurons[18]. Lanthanum is toxic to living tissues, although many studies utilize it before fixation[12].

Ferritin is a nontoxic, protein-iron complex, 100 Å in diameter[12], which occurs naturally in the brain[3,5]. It has been used as an extra-

cellular tracer in brain parenchyma after introduction into the cerebrospinal fluid and in the vasculature[3,4,5]. Ferritin is applied to tissues both before and after fixation[3,4].

MATERIALS AND METHODS

Animals

Weanling New Zealand white rabbits, .5 to .7 Kg. in weight, were used for the intravascular ferritin and ruthenium red perfusion studies in order to minimize the volume of perfusate required. Mature 2.0 to 4.2 Kg. rabbits were used in all other experiments.

Anesthesia

All animals were anesthetized with a sodium pentobarbital solution. The initial dose was 30 mg/Kg body weight given in the marginal ear vein. Successive doses were given until the animal failed to reflexively respond to Achilles tendon pinch and/or corneal touch.

Enucleation and Tissue Preparation

At the time of sacrifice, the medial and lateral palpebral commissures were cut for better access. Scissors were used to penetrate the conjunctiva and cut extrinsic eye muscles. Using the superior rectus tendon as a hold, the eyes were pulled down and forward, and the optic nerve severed with scissors. The eyes were then removed by cutting any tissues still attached. With the eye on a flat surface, the sclera was cut along a line approximately corresponding to the ora serrata, and the anterior eye and vitreal body discarded. The rest of the eye was immediately submerged in fixative. This total

process, from conjunctival penetration to submersion, was accomplished within approximately 20 seconds. The retina was then dissected from the sclera by blunt teasing in fixative, and was divided into optic disc, medullated ray, and avascular retina. Optic nerve tissues left with the eye were also dissected out. All tissues were then diced into approximately one mm^2 pieces for further fixation.

Perfusion Technique

The anesthetized animals were given 1000 units of heparin intraperitoneally. Nitroglycerine tablets (.6 mg total dose) for vasodilation were placed under the tongue with water 2 to 5 minutes before sacrifice. At the time of perfusion, an incision was made to expose the xiphoid process of the sternum which was used as a handle to expose the diaphragm by upward tension. Incisions were made in the diaphragm to enter the thoracic cavity, and parasagittal cuts through the rib cage approximately 4 to 5 cm from the midline followed. The rib cage was lifted by a hemostat which also clamped the internal thoracic arteries, and the pericardial sac was opened and slipped out of the way. A 13 gauge needle was inserted into the left ventricle and passed into the aorta, then its position verified by feeling the needle's location from the posterior side of the vessel. Upon insertion of the needle into the aorta, the right atrium was cut and the fixative was allowed to perfuse at approximately 100 to 120 mm Hg pressure. The elapsed time between the first incision into the

thoracic cavity and the flow of perfusate was in the range of 30 to 45 seconds. The perfusion was continued for 10 to 15 minutes after initiation.

Experiments

EXTRACELLULAR SPACE STUDIES

Ruthenium Red Immersion

To stain cell coats, the eyes were enucleated, the anterior eye removed, and the remaining posterior cup immersed in fixative containing ruthenium red.[24]

Lanthanum Hydroxide Immersion

To use lanthanum as an extracellular tracer, the eye was enucleated, the anterior eye and vitreal body removed, and the posterior eye cup immersed in fixative containing 1% lanthanum hydroxide.[38]

Lanthanum Hydroxide Vitreal Exposure

Approximately 40 cc of a 2% solution of lanthanum hydroxide in Ringer's solution was introduced into the posterior vitreal cavity through a slit made in the posterior superior quadrant of the living eye in order to observe lanthanum movement in the intact retina. Enucleation and fixation occured after 30 minutes of exposure.

INTRAVASCULAR STUDIES

Intravascular Lanthanum Hydroxide

To demonstrate lanthanum movement in the retinal vasculature, 2 cc of a 40% lanthanum hydroxide solution was slowly injected into the marginal ear vein of anesthetized rabbits. The animals were then perfused with fixative and the eyes removed after 2, 15, and 28 minutes of exposure to the intravascular stain. Intravascular injection of lanthanum hydroxide tended to stop the animal's respiration, suggesting some possible poisoning. In the 2 minute infusion study, breathing stopped shortly before the perfusion. In the 15 minute infusion study, breathing was artificially maintained for the last 5 minutes before perfusion.

Ruthenium Red Perfusion

The anesthetized animal was perfused with fixative containing 0.2% ruthenium red to observe ruthenium red staining associated with the retinal vasculature. Following the perfusion, the eyes were removed and fixed further in the same fixative.

Intravascular Ferritin

To use ferritin as an extracellular tracer, 2 cc of Ringer's solution containing 200 mg of ferritin[3] was injected into the marginal ear vein. The animal was perfused with fixative and after 20 minutes, the eyes removed.

Fixatives and Buffer Washes

See tables 1 and 2.

Dehydration and Embedding

After dicing, the tissue was left in fixative for 2 to 3 hours, followed by 4 to 5 rinses in buffer wash, 10 to 15 minutes per wash. Postfixation in OsO_4 followed for 2 to 2.5 hours. Next, the tissues were dehydrated in an ascending series of ethanol solutions (50, 70, 95, and 100%). All of the above steps utilized solutions at 4°C, while the balance of the procedure was carried out at room temperature. Three more changes of 100% ethanol were made, followed by three 10 to 15 minute changes in propylene oxide. The tissues were then placed on a rotating wheel in a 1:1 mixture of propylene oxide-maraglas[10] for 1 to 1.5 hours, and then in a 1:4 mixture of propylene oxide-maraglas for 20 to 30 minutes. The tissue was then infiltrated with undiluted maraglas for two, 1 hour periods and embedded in #00 plastic capsules. Polymerization took place in a 60°C vacuum oven for 72 to 96 hours.

This procedure was followed in all experiments except two of the lanthanum hydroxide experiments. In one study, the time the tissue was in the dehydration steps was cut down. In the other, the ethanol and propylene oxide dehydration steps were replaced by immersion of the fixed tissue into activated 2.2-dimethoxypropane

Table 1. FIXATIVES AND BUFFER WASHES

Extracellular Label Studies

Experiment	Fixative (pH= 7.4)	Buffer Wash (pH= 7.4)	OsO_4
Control Immersion	3% glutaraldehyde in .67M cacodylate buffer	.67M cacodylate buffer	1% OsO_4 in .67M cacodylate buffer
Control Perfusion	3% glutaraldehyde in .1M phosphate buffer	.1M phosphate buffer	1% OsO_4 in .1M phosphate buffer
Ruthenium Red Immersion	3% glutaraldehyde in .67M cacodylate buffer with .2% ruthenium red	.67M cacodylate buffer with .2% ruthenium red	1.66% OsO_4 in .67M cacodylate buffer with .1% ruthenium red
Lanthanum Hydroxide Immersion	3% glutaraldehyde in .67M cacodylate buffer with 1% lanthanum hydroxide	.67M cacodylate buffer with 1% lanthanum hydroxide	1% OsO_4 in .67M cacodylate buffer with 1% lanthanum hydroxide
Lanthanum Hydroxide Vitreal Exposure	3% glutaraldehyde in .67M cacodylate buffer	.67M cacodylate buffer	1% OsO_4 in .67M cacodylate buffer

Table 2. FIXATIVES AND BUFFER WASHES

Intravascular Label Studies

Experiment	Fixative (pH= 7.4)	Buffer Wash (pH= 7.4)	OsO_4
Intravascular lanthanum Hydroxide	3% glutaraldehyde in .1M phosphate buffer	.1M phosphate buffer	1% OsO_4 in .1M phosphate buffer
Ruthenium Red Perfusion	3% glutaraldehyde in .1M phosphate buffer with .2% ruthenium red	.1M phosphate buffer	1% OsO_4 in .1M phosphate buffer
Intravascular Ferritin	3% glutaraldehyde in .1M phosphate buffer	.1M phosphate buffer	1% OsO_4 in .1M phosphate buffer

for 30 minutes. This dehydrant was prepared by adding 1 drop of concentrated HCl to 60 ml of 2,2-dimethoxypropane.[28] These variations in procedure were performed to minimize the possibility of lanthanum washing from the tissue during the length of the run up.

Sectioning and Staining

2 micron sections were cut with glass knives, stained with basic toluidine blue, examined by light microscopy for study and orientation. Selected areas were sectioned for EM on a LKB Ultrotome III, and mounted on uncoated copper grids. Sections were examined and photographed in a Zeiss EM9S-2 electron microscope. Some secitons were post-stained before viewing with 1% lead citrate[47] and saturated uranyl acetate.[50] Others were left unstained as controls for the specific experimental stains.

RESULTS

CONTROL FIBER LAYER

Avascular Fiber Layer

The fiber layer of the avascular retina consists chiefly of Müller cell end feet and scattered bundles of ganglion cell axons (Fig. 2). Müller cells were readily identified by their dense appearance which is due to numerous glycogen granules and filaments. The glycogen granules measured 250 to 300 Å in diameter, and were found as single granules, or occasionally in rossettes. There were variations in glycogen concentration between processes and between areas of the same process (Fig. 3). Scattered throughout the cytoplasm were vesicles and tubules of apparent smooth endoplasmic reticulum (Fig. 3). Mitochondria, dense bodies, ribosomes, or other organelles were not seen.

The Müller cell end feet membranes and the 700 to 900 Å wide overlying basal lamina formed a 3-layered structure at the vitreal interface (Fig. 4). The outer membrane of the Müller cell end feet stained as a thin, electron dense line. The vitreal side of the basal lamina appeared as a less densely stained line while between these two lines, the basal lamina had a relatively clear appearance. The basal lamina had fine filamentous material embedded in its vitreal side. The basal lamina, along with the Müller cell end foot

membrane will be referred to as the internal limiting membrane (ILM) in this paper.

Between Müller cell end feet, 150 to 250 Å wide, extracellular spaces opened toward the vitreous at the basal lamina. There, the spaces expanded to 350 to 500 Å in width. The basal lamina was continuous over these openings (Fig. 3). Deeper into the fiber layer extracellular spaces were generally narrowed to 100 to 200 Å width in most areas. These channels were sometimes in continuity with large dilations of the extracellular space (Figs. 3, 6). Some of these dilations contained membranous material (Fig. 6), and some contained lightly stained particulate material similar to the vitreous (Fig. 3). Junctional complexes between Müller cell processes were not seen. At various depths, invaginations of the Müller cell membranes were seen (Fig. 5). The plasma membrane lining the extracellular space at these invaginations was thicker and denser than seen elsewhere, and appeared similar to coated membranes associated with pinocytotic formations. Apparent pinocytotic vesicles were seen in the Müller cell cytoplasm (Figs. 3, 5).

Just choroidal to the fiber layer was the inner plexiform layer, characterized by numerous intertwined axons, dendrites, synapses, and highly branched Müller cell processes.

Vascular Fiber Layer

The fiber layer of the vascular medullated ray consisted of

large bundles of myelinated axons supported by glia. Capillaries could be found scattered throughout the fiber layer. Capillaries and larger vessels were also common on the vitreal surface of the vascular retina. The predominant glial cell type was the astrocyte, although occasional oligodendroglia were also found. Only scattered areas of Müller cell cytoplasm, mainly near the inner plexiform layer were intensely stained. Transitional areas at the borders between the medullated ray and avascular retina contained both astrocytes and Müller cells. Astrocytes and their processes were characterized by numerous filament bundles, an electron lucid cytoplasm and scattered 300 to 600 Å in diameter glycogen particles, arranged both singly and in rosettes (Fig. 7). Astrocyte processes, unlike Müller cell processes, also contained rough endoplasmic reticulum and mitochondria.

In the vascular fiber layer the ILM was formed by astrocytic end feet (Fig. 8) instead of Müller end feet except in areas of transition where some Müller cell end feet were found. The morphology of the astrocytic processes forming the ILM was characterized by an accumulation of filaments and a very apparent submembrane density where the processes abutted the basal lamina (Fig. 8). The basal lamina was similar to that in avascular retina except that the vitreal filaments embedded in it were more obvious.

The extracellular space of the vascularized fiber layer was wider than that in the avascular regions, ranging from 160 to 900 Å

(Fig. 8). Adjacent astrocytes usually had definitive cell to cell membrane specializations, especially near the limiting membranes (Figs. 7, 13). Most of these junctions had associated intracellular densities and an intercellular spacing of 200 to 250 Å, and correspond best to puncta adherans (Fig. 7). Others appeared similar to zonula occludens (Fig. 13). No particular junctional specializations were apparent between Müller cells and astrocytes, and the intervening extracellular spaces were similar to those between Müller cells.

Fiber Layer Blood Vessels

Fiber layer blood vessels, which were mostly capillaries and venules, were only encountered in the medullated ray tissues. They were characterized by a layer of endothelium surrounded by a perivascular space. Outside of this space was a layer of astrocytic cell processes which separated this space from the fiber layer axons (Fig. 9). Transitional areas could also be found with both astrocyte and Müller cell processes against the perivascular space (Fig. 10). On closer examination, five anatomical layers separated the lumina of the blood vessels from the neural elements: 1. endothelium; 2. the endothelial basal lamina; 3. the perivascular space with collagen (and perivascular cells when present); 4. the basal lamina of the glia; and 5. the glia themselves (Fig. 11).

The endothelium was irregular in thickness with numerous invaginations and vesicles (Figs. 10, 11). At the junctions between endothelial cells (Fig. 12), zonula occludens were present. The endothelial basal lamina was an amorphous, light staining layer, 300 to 1500 Å wide. The perivascular space contained collagen, with generally more present in the optic disc area and less in the transitional areas between vascular and avascular retina. The astrocytic basal lamina was a uniformly dense amorphous layer 400 to 1100 Å thick, covering the densely stained astrocytic cell membrane (Fig. 11). When Müller cells formed part of the perivascular layer, the basal lamina was similar, but the cell membrane was not as intensely stained.

Choroidal-Retinal Interface

The choroid contained both large and small arteries and veins, often stacked upon one another. The vessels in the plane directly against the retina were thin capillaries with 280 to 430 Å wide fenestrations on the retinal side (Fig. 14). All fenestrations were closed by diaphragms. Apparent pinocytotic invaginations and intracellular vesicles were common. Zonula occludens were observed between endothelial cells. The endothelial basal lamina was a 450 to 1100 Å wide amorphous layer. Vitreal to the capillary zone was an electron lucid perivascular space containing collagen. Apposed to the perivascular space was the 420 to 1800 Å thick pigment

epithelial basal lamina. The endothelial basal lamina, the perivascular collagen space, and the pigment epithelial basal lamina correspond to the classical Bruch's membrane.[2] Numerous microvilli of the pigment epithelial cells were against the basal lamina. They ranged from 280 to 1300 Å in diameter and comprised a layer .5 to 1,3μ thick. The pigment epithelial cell body was characterized by large mitochondria, abundant smooth endoplasmic reticulum, and dense phagosomes (Figs. 14-16). Junctional complexes, consisting of zonula occludens and zonula adherens, were noted between pigment epithelial cells (Fig. 15). Extracellular spaces between pigment epithelial cells were relatively narrow and inconspicuous. Rod outer segments were embedded in the vitreal side of the pigment epithelial cells (Figs. 15, 16).

Extracellular Label Studies

RUTHENIUM RED IMMERSION STAINING

Avascular Fiber Layer

Immersion of avascular retina in fixative containing ruthenium red produced intense staining at the vitreal-retinal junction. At the ILM there were two separate densities (Fig. 17). The vitreal portion of the basal lamina stained uniformly with 500 to 740 Å clumps of electron dense material outside the basal lamina and against the vitreal surface. These clumps were also seen loose in the vitreous, and appeared to be membrane bound (Figs. 17, 19). Just choroidal to

this dense layer, the basal lamina was stained but much less densely. The inner basal lamina against the membranes of the Müller cell end feet also stained intensely, but with smaller 50 to 250 Å ruthenium red particles.

Ruthenium red entered extracellular spaces between Müller cell end feet in most areas (Fig. 17). In sections without post-staining, the ruthenium red clearly stained the cell coats of the Müller cells and revealed the outlines of all the Müller cell processes (Fig. 19). Closer examination confirmed that the stain was bound to the extracellular cell coat since the relatively unstained extracellular space of 250 to 350 Å in width separated two adjacent stained membranes (Fig. 17). The ruthenium red was in 180 to 260 Å clumps between cells. Extracellular dilations were not as apparent as in control tissues, but when present, contained 400 to 1100 Å wide electron dense particles (Fig. 18). In axonal fields, the ruthenium red was in the extracellular spaces between the axons and the Müller cells (Figs. 19, 20). Ruthenium red coated invaginations of the Müller cell membrane, in continuity with the extracellular spaces, indicate possible pinocytotic uptake (Figs. 21, 22). Vesiculated ruthenium red was also noted within Müller cell cytoplasm (Fig. 23, 24). Free, cytoplasmic ruthenium red was not observed in any cells. Other retinal layers only occasionally stained with ruthenium red, but never as intensely as the Müller

cell membranes. These findings would indicate that there is no vitreal-retinal barrier to ruthenium red in the avascular retina.

Medullated Ray Fiber Layer

Immersion of medullated ray retina in fixatives containing ruthenium red produced little staining at the ILM (Fig. 25). Ruthenium red was not seen in extracellular spaces in the fiber layer as verified by the study of sections without post-staining (Fig. 26).

Ruthenium red was not found associated with deeper fiber layer blood vessels. Stain particles, in 620 to 3100 Å clumps, were against the abluminal basal lamina of some superficial blood vessels in the vitreous (Fig. 27, 28).

Summary of Ruthenium Red Immersion Studies

In summary, the results from ruthenium red immersion studies imply that the ILM is different in the vascular and avascular areas. The avascular ILM was stained, while the vascular ILM was not, suggesting a biochemical difference between the basal lamina and glial end feet in these areas. In the avascular area the basal lamina and Müller cell membranes are specialized to allow ruthenium red to pass through the ILM and into the extracellular spaces between cells, whereas the basal lamina and astrocytes of the vascular retina appear to exclude this stain. Müller cells in the avascular retina

also had apparent pinocytotic invaginations and intracellular vesicles laden with ruthenium red particles, suggesting an active cellular uptake of materials from the extracellular space and vitreous.

LANTHANUM HYDROXIDE IMMERSION

The tissue fixation after immersion in fixative containing lanthanum hydroxide was generally less consistent than in control tissues, with some areas of membrane breakdown and swollen endoplasmic reticulum.

Avascular Fiber Layer

Immersion of avascular retina in fixatives containing lanthanum hydroxide resulted in 250 to 1100 Å clumps of electron dense lanthanum deposited in the vitreous and at the junction of the vitreous with the basal lamina (Fig. 29). The basal lamina and the Müller cell end feet membranes were uniformly stained (Fig. 29). However, the lanthanum particles in the vitreous were 250 to 1100 Å in diameter, while those against the Müller cell end feet were smaller, 200 to 370 Å in diameter particles. Lanthanum extended into extracellular spaces from the basal lamina to as deep as the inner plexiform layer (Fig. 30), but never deeper. Sections without post-staining also had the same extracellular distribution of stain (Fig. 31). Apparent pinocytotic invaginations filled with lanthanum were observed in Müller cells near the ILM (Fig. 32). One section

had a possible labeled vesicle within the Müller cell cytoplasm (Fig. 33).

Numerous spaces were present in the avascular retina. Some small intracellular spaces were within the Müller cells (Fig. 29). Other, dilated spaces appeared to be continuous with extracellular spaces. There were three general types of these larger spaces. The Type 1 spaces had irregular and broken linings, with membranous material within them (Figs. 29, 34). These spaces didn't contain stain even though stain was present in extracellular spaces immediately adjacent to the space. The Type 2 spaces (Figs. 30, 35-37) had a smoothly lined surface, contained no membranous material, and frequently were lined by lanthanum. Occasionally, these spaces were full of stain (Fig. 38). The Type 3 spaces (Figs. 29, 33, 39) contained 110 to 170 Å particles, similar to particles in the vitreous. These spaces showed some vacuolation and possible membrane disruption of the lining of the space. The Type 2 and Type 3 spaces corresponded best to spaces seen in the control avascular retina. Lanthanum staining could be seen in the extracellular spaces extending to all three types of spaces.

Lanthanum particles were never seen as free cytoplasmic inclusions.

Medullated Ray Fiber Layer

Immersion of medullated ray retina in fixatives containing

lanthanum hydroxide produced scattered staining of the ILM, similar to that seen in avascular areas (Fig. 40). Lanthanum particles were seen entering some extracellular spaces between astrocytes near the basal lamina (Fig. 40, 41). However, the staining of these extracellular spaces was shallow, being only as deep as 290 nm where they were stopped by astrocytic cell to cell specializations. Lanthanum particles were seen against astrocytic junctional complexes, but did not appear to penetrate them (Fig. 41). Lanthanum particles were not found associated with fiber layer blood vessels or their perivascular spaces.

Summary of Lanthanum Hydroxide Immersion Studies

In summary, the results from lanthanum immersion studies imply as did the ruthenium red studies, that the ILM is different in the vascular and avascular areas. In the avascular area, the Müller cell membranes are specialized to allow lanthanum to pass through the ILM and into the extracellular spaces between cells, whereas the astrocytes of the vascular retina are specialized to exclude this stain. Müller cells in the avascular retina also demonstrated apparent pinocytotic invaginations and vesicles laden with lanthanum particles, suggesting an active uptake of materials from the extracellular space and vitreous.

LANTHANUM HYDROXIDE VITREAL EXPOSURE

The preservation of this tissue was generally poor as

indicated by membrane disruption, numerous swollen and disrupted vesicles within the Müller cells, dilation of Müller cell endoplasmic reticulum, and myelin disruption.

Avascular Fiber Layer

A 2% lanthanum hydroxide solution introduced into the posterior vitreal cavity *in vivo* stained the ILM much the same as when stain was used in the fixative. The basal lamina and Müller cell end feet membranes were uniformly stained (Figs. 42, 43). Lanthanum particles were present in the extracellular spaces between Müller cells (Figs. 42, 43). Because tissue preservation was poor, it was not possible to identify pinocytotic invaginations or vesicles, or clearly distinguish extracellular dilations. This staining was observed in, but not beyond the inner plexiform layer.

Medullated Ray Fiber Layer

Because the tissue was so disrupted by the treatment, it was difficult to interpret the extent of much of the stain. Lanthanum staining was observed as 470 to 1500 Å clumps at the ILM (Fig. 44). In contrast to the fixative introduced lanthanum, the stain was in extracellular spaces between astrocytes and between astrocytes and axons in heavily myelinated areas (Fig. 44, 45). In transitional areas, scattered lanthanum staining was seen in extracellular spaces between astrocytes, between astrocytes and axons, between astrocytes and Müller cells, and between Müller cells themselves (Fig. 46).

Lanthanum particles were not observed within glial or axonal cytoplasm. Lanthanum was also observed in perivascular spaces around fiber layer capillaries (Fig. 47). Stain was not observed within endothelial cells, within endothelial pinocytotic invaginations on vesicles, or within capillary lumina.

Intravascular Label Studies

INTRAVASCULAR LANTHANUM HYDROXIDE

(Two Minute Infusion Time)

Avascular Fiber Layer

Lanthanum particles were present in the vitreous and at the ILM, appearing as 130 to 900 Å wide particles or aggregates of particles (Fig. 48). Some of these particles were present in the extracellular spaces between Müller cells at the ILM (Fig. 48), but not deeper in the fiber layer.

Medullated Ray Fiber Layer

Lanthanum stain was not associated with the ILM in medullated ray tissue. Lanthanum stain was found in fiber layer capillary lumina as 180 to 400 Å wide dark, irregular particles or groups of particles. They were often found aggregated around erythrocytes (Fig. 49). Some medullated ray blood vessels had apparent pinocytotic invaginations in the endothelium containing lanthanum particles (Fig. 50). Vesiculated lanthanum within the endothelial

cytoplasm was also found (Fig. 51, 52). Lanthanum was not seen outside of the endothelium.

Choroidal-Retinal Interface

Lanthanum particles were found within the lumina of choroidal capillaries, but with one exception (Fig. 53), lanthanum was not found vesiculated within the endothelium and was never found outside of it.

(Fifteen Minute Infusion Time)

Avascular Fiber Layer

Lanthanum particles were in the vitreous and at the ILM, distributed in the same fashion as those seen in the two minute infusion.

Medullated Ray Fiber Layer

Lanthanum stain was not found associated with the ILM. Lanthanum staining of fiber layer blood vessels appeared similar to that in the 2 minute infusion study, with endothelial invaginations and vesicles containing stain (Figs. 55, 56). Lanthanum was not seen outside of the endothelium.

Choroidal-Retinal Interface

Endothelial pinocytotic invaginations contained lanthanum particles in the choroidal vessels (Figs. 57), and stain was found in cytoplasmic vesicles in the endothelium (Fig. 57, 58). In one section, a lanthanum particle was against the inside of an endothelial tight junction (Fig. 59). Lanthanum particles were found

in the choroidal perivascular spaces in post-stained sections (Fig. 59), and in unstained control sections (Fig. 60). Lanthanum was also found in the outermost extent of the spaces between the pigment epithelial microvilli (Fig. 61).

(Twenty Eight Minute Infusion Time)

Avascular Fiber Layer

The distribution of lanthanum at the ILM and avascular fiber layer was the same after 28 minutes as after 2 minutes (Fig. 62).

Medullated Ray Fiber Layer

Lanthanum stain was not found associated with the ILM. Lanthanum particles were seen in the spaces between endothelial cells, and adjacent to endothelial tight junctions in the medullated ray capillaries (Fig. 63). Lanthanum in endothelial invaginations and cytoplasmic vesicles was similar to that seen in the 2 and 15 minute infusions. As in the shorter infusions, lanthanum was not seen outside of the endothelium.

Choroidal-Retinal Interface

The lanthanum distribution at the choroidal-retinal interface was similar to that seen at 15 minutes except that there was a deeper penetration of stain between the pigment epithelial microvilli (Fig. 64). Some particles were also seen free within the pigment epithelial microvilli cytoplasm. Lanthanum particles were not seen in extracellular spaces between pigment epithelial cells, nor deep to the pigment epithelial cells proper.

Summary of Intravascular Lanthanum Studies

In summary, lanthanum hydroxide introduced into the vasculature in Ringer's solution resulted in particles entering the vitreous and being localized at the ILM in the avascular retina. The site of entrance of the lanthanum particles into the vitreous was not determined. In the vascular retina, lanthanum particles were pinocytized by endothelium, but they didn't cross the endothelium. At the choroidal-retinal interface, lanthanum particles crossed vessels, but their movement was stopped by the junctional complexes of the pigment epithelium.

RUTHENIUM RED PERFUSION

Ruthenium red introduced into the general circulation as an element of the perfusate was not visualized in the tissues, not even in the vasculature. It seem probable that higher concentrations are needed for such a stain to be used.

INTRAVASCULAR FERRITIN

Ferritin, in any sizable concentration, was not observed in avascular retinal areas when it was introduced into the vasculature. Ferritin particle size and density was similar to background material in the vitreous and extracellular spaces, thus it was not possible to accurately localize individual particles. Some fiber layer blood vessels contained electron dense ferritin particles, 60 to 90 $\mathring{A}$ in diameter. These particles were occasionally seen in endothelial