Conjunctival and Episcleral Blood Vessels are Permeable to Blood-borne Horseradish Peroxidase

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The vessels of the conjunctival and episcleral plexuses of *Macaca mulatta* eye are of the continuous type. Most of the vessels in the conjunctival plexus have the diameter of capillaries, while the vast majority of the vessels in the episcleral plexus are venules. Both types of vessels have a simple wall, which consists of an endothelium and a discontinuous layer of pericytes. The aim of this study was to establish their permeability properties to blood-borne horseradish peroxidase (HRP). After intravenous injection of HRP, in 20 μm chopper sections of the anterior segment of the eye examined with the light microscope, the subconjunctival and episcleral tissues appear intensely and diffusely stained by the reaction product. The electron microscope shows that HRP escapes from the vessels lumen by crossing the interendothelial clefts and, in addition, a great number of pinocytotic vesicles loaded with HRP are present on the luminal, tissue front and in the cytoplasm of the endothelial cells. HRP, which rapidly penetrates the loose connective tissue of the region, reaches the spaces between the cells of the conjunctival epithelium where it is finally blocked by the zonulae occludentes that connect the most superficial epithelial cells. A slow diffusion into the compact tissue of the cornea and of the sclera was also observed. Thus, under normal conditions, blood-borne macromolecules can freely diffuse into the subconjunctival and episcleral loose connective tissues. On the other hand, one can equally expect that the aqueous humor that reaches the episcleral and conjunctival blood plexuses through the canal of Schlemm and collector channels can freely diffuse into the subconjunctival spaces across the walls of these permeable vessels. Invest Ophthalmol Vis Sci 24:725–736, 1983

The bulbar conjunctival and episcleral plexuses of blood vessels are vascular networks lying on the surface of the most anterior part of the sclera, next to the limbus. These plexuses, which anastomose with each other, receive most of the aqueous humor leaving the anterior chamber and, therefore, are of special importance for the maintenance of the normal intraocular pressure. In spite of such a crucial physiologic role, very few studies exist on the fine structure of these vessels and these are limited to the conjunctival capillaries of the human limbus and fornix. Furthermore, the permeability properties of both conjunctival and episcleral vessels after intravenous injection of a physiologic electron opaque tracer such as horseradish peroxidase (HRP) are still unknown.

The aim of this study was an analysis of the fine structure and permeability properties of conjunctival and episcleral vessels in the rhesus monkey. It shows that both conjunctival and episcleral blood vessels possess a continuous endothelium but that they are highly permeable to intravenously injected HRP.

Materials and Methods

Morphology of the Conjunctival and Episcleral Vessels

Five adult *Macaca mulatta* monkeys of either sexes were used. In four monkeys under general anesthesia the eyes were enucleated, cut at the equator, and immersed for 2 hrs in a fixative fluid consisting of a mixture of glutaraldehyde and formaldehyde in cacodylate buffer. In the fifth animal, 2 liters of fixative fluid were perfused through the heart at a pressure of about 100 mmHg. At the end of the perfusion the head, with the eyes in situ, was left overnight in fixative at 4 C. With this procedure mechanical injuries to the periocular structures before fixation were avoided. Furthermore, in contrast with blood vessels, which had an empty lumen, lymphatic vessels contained coagulated lymph and therefore could be identified easily with both the light and electron microscopes. During enucleation of the eyeball, great care...
was taken to preserve intact the bulbar conjunctiva. The anterior segment of the eye was radially cut into wedges, washed overnight in buffer, and postfixed for 2 hrs at 4°C with 1% osmium tetroxide and 1.5% potassium ferrocyanide in distilled water. After dehydration with ethanol, specimens were embedded flat in an Epon-Araldite mixture. For light microscopy, sections 1–2 μm in thickness were stained with toluidine blue. For electron microscopy, thin silver sections were stained with uranyl acetate and lead citrate and examined either in a RCA-3G or a 100 CX Jeol electron microscope.

**Experiments with HRP**

Three adult *Macaca mulatta* were anesthetized and injected through the small saphenous vein with HRP (Sigma, Type II, 500 mg/kg body weight) dissolved in 5 ml phosphate buffered saline. Two different procedures were used to fix the eyeball: (1) in two monkeys the eyes were enucleated, 5, 10, 15, and 20 min after the beginning of the injection of the tracer and immediately immersed in the fixative fluid; (2) in the third animal, fixation of the tissue was performed with perfusion of the fixative fluid from the heart 10 min after the injection of HRP.

In both cases the fixative fluid was the mixture of glutaraldehyde and paraformaldehyde previously reported for conventional electron microscopy. Subsequently, wedges of the anterior segment of the eye were sectioned radially with a Smith-Farquhar tissue chopper, and sections 200 μm in thickness were reacted for the histochemical demonstration of HRP. Part of these sections were dehydrated in alcohol, cleared in xylenes, and mounted with Permount for light microscope examination. The remaining chopper sections were osmicated, dehydrated, and embedded as previously reported for conventional electron microscopy.

**Results**

**Morphology of the Conjunctival and Episcleral Vessels**

*Light microscopy:* In radial sections of the anterior segment of the eye, 1–2 μm in thickness, the region between bulbar conjunctiva and sclera has the shape of a wedge with the apex corresponding to the corneoscleral limbus. With the light microscope it is possible to recognize, immediately beneath the conjunctival epithelium, a band of loose connective tissue, the subconjunctival layer. Deep to it, is a layer of dense connective tissue (Fig. 1) that corresponds to both the attachment of the Tenon’s capsule and to the episcleral tissue. Here, it will be simply referred to as episcleral layer. Finally, deep to this layer the sclera is seen, easily identified by the compact texture of its connective tissue fibers.

In the subconjunctival layer both blood and lymphatic vessels are found. As a rule, the subconjunctival blood vessels are small and are particularly numerous in the region near the limbus, where they give rise to the limbal or peripheratic plexus. They often contact the basement membrane of the conjunctival epithelium, and their wall consists of an endothelium with a few surrounding pericytes. In the vicinity of these vessels, the connective tissue fibers maintain their irregular course and do not give rise to a distinct adventitial layer. In sections of specimens fixed by perfusion, numerous lymphatic vessels are seen under the conjunctival epithelium along the entire extent of the bulbar conjunctiva. The lymphatic vessels have an irregular lumen and a very attenuated endothelium; the nuclei of the endothelial cells are spaced at a greater distance from one another than in the lining of blood vessels. Lymphatic and blood vessels often occur very close to one another (Fig. 2).

In the underlying, dense episcleral layer, blood vessels are less numerous than in the subconjunctival layer and their diameters are much larger. In spite of their size, the majority of these vessels have a very simple wall, consisting of an endothelium and a discontinuous layer of pericytes; they can be easily classified as venules. Only a small proportion of the vessels of the episcleral plexus belong to the class of arterioles. Venules and arterioles can be found in close association with one another.

Within the sclera, blood vessels are rare and have a large diameter. Collector channels can be recognized easily when originating from Schlemm’s canal. Their endothelium is very attenuated, contains sparse nuclei, and is intimately adherent to the surrounding, dense connective tissue. In this respect, the morphology of their walls is very similar to that of the scleral wall of Schlemm’s canal.

*Electron microscopy:* The small conjunctival vessels and the venules of the episcleral plexus have identical morphology and therefore will be described together. Their wall consists of a continuous endothelium and a discontinuous layer of pericytes (Fig. 3, 4). The endothelial cells are irregular in shape, being provided with blunt luminal protrusions and slender basal leaflets or digitations; in this respect they are similar to the endothelial cells of the iridial vessels in the same animal species. Adjacent endothelial cells often overlap extensively and interendothelial clefts follow an unusually tortuous, irregular path (Fig. 4). In most cases the interendothelial clefts are open throughout their length (Fig. 4); only occasionally the adjacent plasma membranes approach each
other focally at one or more points, thus occluding the intercellular space. The cytoplasmic organelles of the endothelial cells are unremarkable. A large number of 7 nm filaments permeate the entire cytoplasm; these filaments are associated in bundles, which in cross sectioned vessels appear longitudinally, obliquely, or perpendicularly cut, indicating that they traverse the endothelial cytoplasm in random directions. Weibel-Palade bodies are of common occurrence in the cytoplasm of the endothelial cells and crystalloid inclusions, associated with the rough endoplasmic reticulum, are occasionally found (Fig. 4,
Fig. 3. A cujmaui ui me vuuiui,uvai  JJICAUS pusscsscs a very simpie wan composea oy a continuous endothelium and a pericyte. The endothelial cells have a rather irregular outline and cytoplasmic luminal digitations (arrows). The interendothelial clefts follow an irregular path (END: endothelium ×11,000).

inset): these organelles are identical to those previously reported in a variety of adult and developing ocular blood vessels in Macaca mulatta.\textsuperscript{6-7} Within the endothelial cells, a large number of plasmalemmal vesicles, 60–75 nm in diameter, are present either isolated in the cytoplasm or opening into the lumen, tissue front, or intercellular clefts. A small number of coated vesicles is found also.

The pericytes of these vessels do not differ from those described elsewhere in the eye\textsuperscript{6,8-13} or in other organs.\textsuperscript{14-17} They are ensheathed by a basal lamina and are provided with long cytoplasmic processes that occasionally contact the basal aspect of the endothelial cells without interposition of the basal lamina (Fig. 4). The nucleus of the pericytes is surrounded by a thin rim of cytoplasm, and at the edges of the
nucleus the thickness of the cell declines abruptly. The cytoplasm contains a variable complement of filaments, especially concentrated in the cytoplasm subjacent the adluminal plasmalemma (Fig. 4). Furthermore, as already noted in limbal human capillaries as well as in the retina, myocardium, and iris, the distribution of the plasmalemmal vesicles at the cell surface is asymmetric: they are commonly present on the abluminal aspect of the cell and are quite rare on the opposite cell surface. This asymmetric distribution of the plasmalemmal vesicles is especially pronounced in the cell processes, whereas in the region containing the nucleus, the number of vesicles is usually the same on both fronts of the cells. Around the pericytes a distinct adventitial layer is absent: the bundles of collagen fibrils immediately adjacent to the vessel walls imperceptibly merge into the surrounding connective tissue.

Arterioles represent a very small component of the vessel population in the episcleral tissue; they are characterized by the presence of two to three layers of smooth muscle cells in their wall. Conjunctival lymphatic vessels differ from blood vessels in their irregular lumen, attenuated endothelium, discontinuous basal lamina, and absence of pericytes. The endothelium is continuous but the interendothelial clefts are open (Fig. 5). The cytoplasmic organelles are not different from those of the blood capillaries and include occasional Weibel-Palade bodies (Fig. 5, inset) as it has been observed in lymphatic vessels belonging to other regions of the body.
Very frequently cross-sectioned bundles of unmyelinated axons ensheathed in Schwann cell processes run in close proximity of blood vessels, especially of arterioles and venules of larger diameter.

Experiments with HRP

Light microscopy: Five minutes after intravenous injection of HRP, the subconjunctival and episcleral
tissues appear intensely and diffusely stained by the reaction product (Fig. 6). Ten to 20 min after the injection, the tracer had begun to invade the periphery of the cornea and to diffuse deeply into the sclera.

*Electron microscopy:* With the electron microscope the tracer appears to escape the lumen of the blood vessels by crossing the interendothelial clefts (Fig. 7). Furthermore, a number of pinocytotic vesicles loaded with reaction product is present on both the luminal and tissue fronts of the endothelial cells as well as free in their cytoplasm. After fixation by perfusion through the heart, HRP is washed away from the lumen of the vessels, and the number of pinocytotic vesicles filled with HRP on the luminal front of the endothelium decreases. After this fixation procedure, the interendothelial spaces of the vessels remain filled with reaction product throughout their entire length (Fig. 8). HRP, which has escaped from the vessels, permeates the surrounding connective tissue spaces, and it outlines, in negative contrast, connective tissue cells and collagen fibrils. Five minutes after the injection the tracer has already reached the intercellular spaces of the conjunctival epithelium, where it is blocked by the zonulae occludentes that connect the most superficial epithelial cells (Fig. 9). Only later, does HRP diffuse into the connective tissue spaces of the cornea and the compact tissue of the sclera.

Ten minutes after intravenous injection, HRP appears in the lumen of the lymphatic vessels of the conjunctiva (Fig. 10).

**Discussion**

*Morphology of the Conjunctival and Episcleral Plexuses of Blood Vessels*

In his classical description of the pericorneal blood vessels in humans, Maggiore 19 was able to identify by meticulous dissection four layers of vessels: (1) a conjunctival network of delicate, thin vessels, that surrounds the cornea at the limbus. These vessels form loops of variable shape and are very superficial, for they occur immediately beneath the conjunctival epithelium. Thus, they are visible in the living eye. (2) A subconjunctival network that is particularly prominent in the regions of the sclera intervening between the insertions of the recti muscles; after Maggiore, this layer corresponds to the region of Tenon's capsule. (3) An episcleral plexus, mostly composed by venous vessels that originate from the confluence of small venules draining the delicate conjunctival
plexus. (4) An intrascleral network, located in proximity of Schlemm's canal. This plexus does not have any direct communication with the canal. Schlemm's canal is connected to the intrascleral plexus by vessels of peculiar morphology, 20 to 30 in number, that Maggiore termed for the first time collector channels. Thus, Schlemm's canal belongs to a vascular plexus situated in the thickness of the limbus, mainly composed by vessels of venous type and, to a much lesser extent, by thin arterioles and capillaries. This description provided by Maggiore more than 60 years ago for the human is also valid for the eye of *Macaca mulatta*. In the present study, however, the subconjunctival and episcleral plexuses of Maggiore have been described together as episcleral vessels since in radial sections of the anterior segment of the eye it is often very difficult to distinguish the two vascular layers as clearly separated entities.

In spite of the fact that Maggiore's observations were essentially correct, Lee and Holze,20 prompted by the Chambers and Zweifach description21 of the microcirculation of the mesentery and omentum, claimed that the vascular bed of the human conjunctiva is a composite of structural units, each sharing as a basic architectural feature a central, capillary-like arteriovenous channel, of which the true capillaries are side branches. Subsequent observations on human eyes in vivo by Grafflin and Corddry22 did not confirm these results: no arteriovenous units were observed, and the vascular networks seemed to exhibit endless variations without a definite structural pattern. Bloch,23 also in human eyes, subsequently reported that the most superficial arterioles are situated usually deeper than their corresponding venules and that the course of the arterioles is straighter than that of their accompanying venules. More recently, Fenton et al.24 observed that the feeding arterioles of this network in the human eye have a very thin wall and an inside diameter of 10 μm, that the majority of the vessels in the conjunctival vasculature are either capillaries or postcapillary venules, and finally, that all vessels with diameters above 15 μm are venules.

The present study in the rhesus monkey eye con-

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Fig. 7. Animal injected intravenously with HRP. The eye was fixed by immersion 5 min after perfusion of the tracer. From the lumen of an episcleral venule HRP penetrates the interendothelial cleft following a tortuous path (arrow) and permeates the connective tissue spaces around the vessel. Note, in the pericyte, the great number of plasmalemmal vesicles loaded with tracer and preferentially located at the abluminal front of the cell (×34,500).
forms to the descriptions in humans: no arteriovenous unit can be discerned in the perilimbal plexus and, as it is now generally accepted, the majority of the vessels can be classified either as capillaries or venules.

With the electron microscope, both conjunctival and episcleral vessels are continuous and most of them are provided with very simple walls. In previous descriptions on human material a few fenestrated capillaries were reported in the conjunctiva at the limbus and in the region of the inferior fornix. In the present study, in spite of an accurate analysis of the same regions, no fenestrated vessels were encountered.

**Conjunctival and episcleral blood vessels are permeable to HRP:** It is known that perilimbal vessels are permeable to intravenously injected fluorescein. Maurice reported that when a high concentration of fluorescein is present in the blood stream of living rabbits, it is possible to observe the dye entering the cornea from the limbus. Furthermore, he described a decline in concentration around the edge of a stained cornea that he attributed to the return of the dye to the blood, thus implying that fluorescein can cross the vascular walls in either direction. Mitsui et al. described the diffusion of intravenously injected fluorescein out of the vessels in the limbus of normal human eyes. They observed that in an early phase, the dye had not yet leaked from the vessels and, thus, the fluorescence appeared as strands of cotton waste attached to the limbus. In a successive phase, fluorescein leaked from the vessels into the surrounding tissues. This leakage, however, was so diffuse that the authors were unable to identify the permeable vessels. In a final, postvascular phase, fluorescence disappeared from the blood vessels and gradually approached the center of the cornea.

Previous studies with electron opaque tracers have yielded results of difficult interpretation. Thorium dioxide (10 nm diameter), when introduced intravenously in rats, was seen to diffuse from the limbal vessels, but this finding was attributed to the toxic properties of Thorotrast. Subsequently, however, the rat limbal vessels appeared impermeable to carbon (20 nm diameter), and this result was taken as a further evidence that the permeability of limbal vessels to thorium dioxide was an artifact due to the unphysiologic properties of the tracer. The present experiments demonstrate that HRP rapidly diffuses out of the lumen of conjunctival and episcleral vessels; thus, these vessels are at least permeable to molecules up to the size of this tracer (3 nm radius of an equivalent hydrodynamic sphere). Evidence that conjunctival and episcleral vessels are permeable comes also from another source: since these vessels represent the main route for the return of aqueous humor to
Fig. 9. Same experimental conditions as in Figure 8. HRP, after permeating the intercellular spaces of the conjunctival epithelium, is finally blocked by the zonulae oculudentes which join the lateral aspects of the most superficial epithelial cells (×24,500).

the blood stream, their permeability can be also tested by introducing tracers into the anterior chamber. Bill injected into the anterior chamber of dead Macaca irus radioactive diodone (0.5 nm diameter) and albumin (3.5 nm diameter). He reported that in the episcleral tissues and in the conjunctiva the diodone space was larger than the albumin space indicating that diodone leaked out of the episcleral and conjunctival vessels more easily than the larger albumin. Aniline blue injected into the anterior chamber of dead Macaca mulatta eyes readily appeared in the episcleral veins and rapidly diffused in the surrounding tissue. Cole and Monro injected fluorescein-labeled dextrans (FD) of different MW into the anterior chamber of living rabbits. After a 4½-min perfusion at 15–20 mmHg, the FD 40,000 (4.4 nm diameter) was contained in the lumen of the vessels of the limbal and conjunctival plexuses, and at the end of 10 min some leakage had begun into the surrounding tissues. This leakage was more rapid with FD 3,000 MW than with the larger dextrans 40,000 and 200,000 MW. In more recent experiments fluorescein was injected into the anterior chamber of living Macaca mulatta; the eyes were freeze-dried immediately after perfusion and sectioned for light microscopy. It was found that the episcleral vessels were leaky to fluorescein and that with longer perfusion times the dye had diffused throughout the entire limbus.

In conclusion, conjunctival and episcleral blood vessels have a continuous endothelium, but are permeable to tracers of different MW injected either into the blood stream or into the anterior chamber. From the behavior of these tracers it can be inferred that, under normal conditions, blood-borne macromolecules can freely diffuse from these vessels and permeate the subconjunctival connective tissue spaces. On the other hand, one can also expect that the aqueous humor that reaches the episcleral and conjunctival vessels from the lumen of Schlemm's canal...
and collector channels can freely diffuse from the vascular lumen into the subconjunctival spaces. Furthermore, it is reasonable to suppose that a certain amount of aqueous humor bypasses Schlemm's canal, freely diffuses across the sclera from the anterior chamber to the episcleral tissue, and finally enters the permeable vessels of this region. Thus, the composition of the subconjunctival fluid must considerably vary as a result of changes in both the osmotic or oncotic pressures of the plasma and the hydrodynamic conditions of the aqueous humor in the channels of the outflow system.

The fact that tracers, either injected intravenously or into the anterior chamber, permeate the corneal stroma, suggests that materials diffusing from the perilimbal vessels may contribute to the nourishment of the cornea. Furthermore, although the cornea receives most of its metabolites through its anterior and posterior surfaces, the contribution from the limbal vessels may be of special physiologic significance, because macromolecules such as proteins are more available in the subconjunctival fluid than in the aqueous humor or tears.

Lymphatic vessels: Since fluids and macromolecules can exit and reenter the permeable episcleral and conjunctival vessels, the significance of the lymphatic vessels located beneath the conjunctival epithelium is less clear. Possibly, they represent an emergency system that operates only when the connective tissue spaces of the episclera are overloaded.
with fluids. This might explain why, under normal conditions, the drainage of the aqueous humor through the lymphatic system is negligible.\textsuperscript{32} Lymphatic vessels, however, may be very important in pathologic conditions, for they represent the most direct anatomical link between the tissues of the eye and the immunocompetent cells located in the lymph nodes.

**Key words: Macaca mulatta, conjunctival blood vessels, episcleral blood vessels, conjunctival lymphatic vessels, horseradish peroxidase, electron microscopy, interendothelial junctions**

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**References**