Asymmetric Distribution of Charged Domains on the Two Fronts of the Endothelium of Iris Blood Vessels

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The authors have studied the distribution of anionic and cationic sites on both luminal and abluminal endothelial aspects of iridial vessels in Macaca mulatta and Macaca fascicularis. With the animals in general anesthesia, anionic ferritin (AF) and cationic ferritin (CF) were either injected intravenam or perfused at known intraocular pressure (15–20 mmHg) through the anterior chamber. AF introduced intravenam was retained in the vessels' lumen. The tight junctions between the endothelial cells were impermeable and the plasmalemmal vesicles did not transport tracer to the iridial stroma. In contrast, when perfused through the anterior chamber, AF was present in the vessels' lumen. Here again the tight junctions between the endothelial cells were impermeable, but AF was contained within a great number of plasmalemmal vesicles. Iridial vessels were impermeable to CF perfused into the lumen, but a continuous layer of CF particles was found to adhere to the luminal plasma membrane. When perfused through the anterior chamber, CF was bound to the proteoglycans associated with collagen fibrils of the iridial stroma and basal laminae of stromal, pericytic, and endothelial cells but was never found in the vessels' lumen. These results indicate that different electrical charges are associated with the plasmalemmal vesicles on the luminal and abluminal fronts of iridial vessels. The authors suggest that in these vessels a unidirectional vesicular transport is responsible for the selective movement of anionic organic substances from the tissues of the eye to the bloodstream. Invest Ophthalmol Vis Sci 26:597–608, 1985

We have previously shown that when horseradish peroxidase (HRP) is introduced into the bloodstream, it is retained in the lumen of iridial vessels, but that when this tracer is perfused through the anterior chamber, it reaches the lumen of the same vessels by a transendothelial vesicular transport.1 Recent studies2–3 on the topographic distribution of electric charges on the capillary endothelium and on structures involved in transendothelial transport (plasmalemmal vesicles, transendothelial channels, and fenestrae) have indicated that biochemically differentiated microdomains are present on the cell surface. These microdomains are characterized by the preferential distribution of anionic sites, and of some glycoproteins and proteoglycans, and correspond to the structures involved in endocytosis and transcytosis. These new findings suggest that the capillary endothelium has differentiated pathways and that the electric charge and chemical composition of macromolecules may be important for their interaction with the endothelial features responsible for their uptake and transport across the capillary lining. In the vessels of the iris the unidirectional transport of HRP may be governed by an asymmetric distribution of chemical residues and electric charges on the luminal and abluminal fronts of the endothelial cells. In this article we tested this hypothesis with an anionic probe, anionic ferritin (AF), and a cationic probe, cationic ferritin (CF), introduced either into the bloodstream or perfused through the anterior chamber. Our results demonstrate that there is indeed an asymmetric distribution of charges on the luminal and abluminal fronts of iridial vessels.
Materials and Methods

Animals

A total of five *Macaca fascicularis* and four *Macaca mulatta* were used for these experiments. The animals were adults of either sex and they were treated according to the ARVO Resolution on the Use of Animals in Research.

Chemicals

Anionic ferritin (AF), pi 4.5, (ferritin, 66.2 mg/ml) and cationic ferritin (CF), pi > 10.5, (ferritin, 11 mg/ml) were purchased from Miles Laboratories (Elkhart, IN). Before use they were dialyzed for 48 hr at 4°C against 0.15 M NaCl. The Stokes-Einstein radius of ferritin is 6 nm. Oxypherol-ET, perfluorochemical perfusion emulsion, was obtained from Alpha Therapeutic Corporation (Los Angeles, CA).

Intravenous Injection of AF

One *M. fascicularis* was injected intravenously with AF. With the animal under general pentobarbital anesthesia, 20 ml of AF (4 ml/kg body weight) was slowly injected into the small saphenous vein. After 30 min and 1 hr, the globes were removed and opened with an equatorial incision. The anterior segment was immersed in osmium tetroxide and stained en bloc with uranyl acetate. Tissue blocks were finally embedded in Epon-Araldite. Thin sections were stained with uranyl acetate and lead citrate. Micrographs were taken with a JEOL 100CX electron microscope at 60 KV (JEOL USA, Electron Optics Division; Peabody, MA).

Intravenous Injection of CF

Upon intravenous injection, CF binds rapidly to plasma proteins and free tracer cannot be seen in the vessels’ lumen. For this reason, injection of CF was performed in one *M. mulatta* directly from the heart after washing the vascular tree with a plasma substitute. With the animal in deep pentobarbital anesthesia, the thoracic cage was opened and the heart exposed. The left ventricle was cannulated, the descending thoracic aorta and axillary arteries were clamped, and the neck and head were perfused with 500 ml of Oxypherol. At the beginning of the perfusion, the auricle of the right atrium was opened. The perfusion pressure was 100 mmHg and the temperature of Oxypherol was 37°C. As soon as blood-free plasma substitute emerged, the right auricle was clamped and 20 ml of CF was introduced through the left ventricle. The heart continued to beat throughout the perfusion of CF. Fifteen and 30 min later, the eyes were enucleated, opened at the equator, fixed by immersion, and processed in the same manner as described for the animal intravenously injected with AF.

Perfusion through the Anterior Chamber with AF or CF

With the animals anesthetized with sodium pentobarbitone, the anterior chamber of both eyes of five monkeys (3 *M. fascicularis* and 2 *M. mulatta*) were perfused with AF. Both eyes of one *M. fascicularis* and one *M. mulatta* were perfused with CF. The perfusion was performed with the same perfusion apparatus we had used for the perfusion of the anterior chamber with HRP. In these experiments, the separating chamber and tubing were filled with perfusate alone, while AF or CF were contained in a separate loop of tubing joined in parallel with the main circuit. The anterior chamber was perfused with mock aqueous humor for 20 min at 15–20 mmHg at a rate of 25 µl/min, after which either AF or CF (3 ml) was introduced to the anterior chamber at the same rate. Introduction of AF or CF into the anterior chamber of these macaque monkey eyes did not cause any appreciable change in the intraocular pressure (IOP).

Fixation of the Perfused Eyes

However rapidly tissue fixation is achieved, changes in ocular fluid distribution inevitably occur on death. It was considered desirable to lessen these changes by maintaining the IOP at its experimental level during fixation. Fixation by perfusion through the anterior chamber in vivo is impracticable because of the immediate irritant effect of the chemicals, causing gross reflex uveal vasodilation, and so creating an even more abnormal physiologic state, irrespective of whether the IOP is allowed to increase concomitantly or is kept artificially normal. Moreover, intravascular or intracameral perfusion of fixative will tend to wash away the tracer, which in this experiment, was not desired. Fixation was therefore carried out by immersion. The eyes were enucleated immediately after death with the needles left in situ and the IOP set to the required level. Under these conditions, the entire eyeball was immersed in fixative for 30 min to 1 hr at constant IOP, after which the needle was discon-
Fig. 1. Macaca fascicularis injected through the small saphenous vein with AF. Ferritin molecules are present in the lumen of an iridial vessel where they are uniformly distributed in the plasma. No particles are found in the plasmalemmal vesicles of the endothelium and in the connective tissue spaces of the iridial stroma. P: pericyte (original magnification, X87,000).

Intravenous Injection of AF

The blood vessels of the iris have a continuous endothelium surrounded by a discontinuous covering of pericytes. Freeze-fracture analysis of these vessels has shown that the endothelial cells are joined to one another by complex zonulae occludentes that seal the intracellular spaces. After intravenous injection, AF appeared uniformly dispersed in the vessels' lumen. Ferritin particles were never seen to be attached singly or to aggregate in clusters on the luminal plasma membrane of the endothelial cells. Plasmalemmal vesicles opening on the luminal front of the endothelium contained no ferritin molecules (Fig. 1), and the interendothelial clefts were closed by junctions impermeable to the circulating tracer. As a result, even 1 hr after intravenous injection, the tracer was retained in the lumen of the iridial vessels, and no AF was present in the basal lamina or iridial stroma.

Intravenous Injection of CF

After 15 and 30 min exposure of the vascular tree of the iris to CF, the luminal aspect of the endothelial cells was decorated by a continuous layer of CF molecules (Fig. 2). Binding was uniform and represented by three or more tiers of overlapping molecules. Small islands of unlabeled plasma membrane or of membrane decorated by a single tier of molecules were rarely seen. When the band of CF particles was tangentially cut, the tracer molecules appeared organized in a very precise geometric lattice (Fig. 2, inset). The plasmalemmal vesicles open to the lumen only occasionally contained CF. Large vacuoles, up to 300 nm in diameter, located next to the luminal plasmalemma did contain CF, but no tracer was detected in
Fig. 2. *Macaca mulatta* perfused through the heart with Oxypherol, followed by infusion with CF. Tracer particles are bound to the luminal aspect of the endothelium in a compact band composed by multiple layers of ferritin molecules. Only seldom does one find short regions of the plasma membrane not covered by the tracer (arrow). CF penetrates into the interendothelial clefts but its progression toward the iridial stroma is blocked by tight junctions (arrowhead). No CF particles are present outside the vessel's wall. *Inset*, in grazing sections of the luminal coat, molecules of CF appear organized in a very orderly lattice as though no spaces are left between adjacent molecules. P: pericyte; BL: basal lamina. *(original magnification, ×88,000; inset, original magnification, ×195,000).*
the cisterns of the Golgi apparatus. CF was also present in the intercellular clefts leading to the junctional regions of the endothelial cells, but the zonulæ occludentes were always intact, effectively sealing the intercellular spaces (Fig. 2). As a result, after 15 and 30 min of exposure to CF, no tracer was ever found in plasmalemmal vesicles located in the abluminal portion of the endothelial cells, or in the basal lamina or iris stroma.

Perfusion of AF through the Anterior Chamber

Examination of the iris of eyes in which AF had been administered intracameraly revealed that the tracer had penetrated the anterior surface of the iris and had spread throughout the stroma. Its progression towards the posterior chamber was blocked by the zonulæ occludentes connecting the lateral aspects of the cells of the posterior iridial epithelium. AF was not bound to connective tissue cells or to collagen fibrils; it diffused across the vessels' basal lamina and did not preferentially label the abluminal aspect of the endothelial plasmalemma. Tracer was consistently found inside the vessels' lumen (Fig. 3). At high magnification, the clefts between adjacent endothelial cells were always closed by typical tight junctions, represented by fusion points between the outer leaflets of the adjacent plasma membranes (Fig. 4). A great number of plasmalemmal vesicles containing AF molecules was present in the cytoplasm of the endothelial cells. These vesicles were 70–80 nm in diameter and mostly located toward the abluminal surface of the endothelial cells. On the luminal aspect of the endothelium, only a few vesicles containing AF could be seen fused with the cell membrane, presumably in the process of discharging their contents into the lumen (Fig. 3). Frequently, in the cytoplasm of the endothelial cells, two or more plasmalemmal vesicles containing ferritin could be seen fusing with one another. Vesicles were seen near the Golgi apparatus, but the cisterns of this organelle never contained AF. Tracer was found in the lumen of both large and small vessels, and the number and distribution of AF-labeled vesicles were similar in all segments of the iridial vascular tree.

Perfusion of CF through the Anterior Chamber

In contrast to the distribution of AF after intracameral perfusion, CF was never found in the lumen of iridial vessels. The most striking feature of the distribution of CF was the selective labeling of stromal collagen fibrils and of the basal lamina. Individual collagen fibrils appeared decorated by transverse bands of ferritin, with a repeat unit of about 60 nm (Fig. 5). Where collagen fibrils were aggregated in bundles, the CF decorations of the various fibrils appeared in register with one another, and thus, collagen fiber bundles displayed a regular striation. The CF decorating bands encompassed the entire thickness of individual collagen fibrils and, in addition, extended over short distances beyond their margins. These lateral projections connected to one another the individual fibrils aggregated in bundles. The basal laminae surrounding endothelial cells, pericytes, Schwann cells, and the cells of the iridial epithelium were labeled by clusters of ferritin molecules (Fig. 6). The distance between the clusters varied in different regions of the iris, probably because of uneven tracer penetration. Oblique sections through the basal laminae showed that the clusters were located at regular intervals from one another, but their arrangement never conformed to a precise geometric pattern. In sections perpendicular to the vessels' wall, it was not possible to identify and separate in the basal lamina, a lamina rara externa, a lamina densa, and a lamina rara interna. The entire subendothelial space was occupied by aggregates of CF, which in places merged with the abluminal plasma membrane of the endothelial cells without interposition of a lucent space (Figs. 5, 6). As a rule, plasmalemmal vesicles in the cytoplasm of endothelial cells did not contain ferritin molecules. On rare occasions, however, CF was seen in plasmalemmal vesicles exclusively located on the abluminal front of the endothelial cells. Finally, large aggregates of tracer were concentrated in phagosomes of the macrophages that are normally scattered throughout the iridial stroma (Fig. 5).

Discussion

Intravenous Injection of AF

We have observed that when AF is introduced into the systemic circulation, it appears uniformly dispersed throughout the plasma in the lumen of iridial vessels. The endothelial cells of these vessels represent a barrier to the diffusion of the tracer. This barrier is based on a two-fold mechanism: first, the clefts between endothelial cells are closed by tight junctions that effectively prevent paracellular diffusion; and second, there is no transcellular vesicular transport, i.e., plasmalemmal vesicles do not pick up and transport AF to the interstitial front of the vessels. Previous investigations, in which AF was used as an electron-opaque probe in vessels of different diameters and belonging to different tissues, have reached contrasting conclusions. In segments of guinea pig aorta and vena cava incubated in vitro with AF, the molecule was neither taken up from the lumen nor transported...
across the endothelium. In contrast, in isolated frog mesenteric capillaries, which appear structurally similar to continuous capillaries of mammals, AF appeared to be transported by plasmalemmal vesicles across the endothelium. Isolated endothelial cells from rabbit myocardium, incubated with AF in protein-free medium, showed extensive labeling of plasmalemmal vesicles. In the presence of additional plasma proteins, the number of labeled vesicles decreased, and on this basis the authors concluded that endothelial vesicles seem to favor the uptake and transport of anionic proteins.

Equally at variance are the results obtained on fenestrated vessels when AF was present in the lumen. From their study on blood capillaries of the mouse intestinal mucosa, Clementi and Palade concluded...
that AF leaves the lumen mainly through the fenestrations, and that transport by plasmalemmal vesicles seems less efficient. More recently, however, it has been reported that in these same vessels, and in the vessels of the pancreas, AF did not bind to any endothelial structure but did have access to plasmalemmal vesicles, permeating only a small fraction of the fenestral diaphragms. It was found in detectable amounts in the pericapillary spaces 10 min after the injection. In striking contrast, studies on fenestrated capillaries of the brain showed that AF had no affinity for the luminal endothelial membrane and was not transported by plasmalemmal vesicles. The discrepancy between these latter results was interpreted as being due to differences in the electrophysiologic or structural properties of the two types of fenestrated vessels.

It is clear that the affinity of plasmalemmal vesicles for AF is remarkably variable and is not necessarily correlated with the type of vessel, continuous or fenestrated. We have shown that plasmalemmal vesicles of iridial vessels do not pick up and transport AF out of the lumen; in this respect, they resemble the vesicle population of large vessels and that of the fenestrated vessels of the brain.

Intravenous Injection of CF

CF was introduced by Danon et al to visualize negative charges on the surface of cells. The application of this tracer to the study of vascular permeability has provided contrasting results when applied to either continuous or fenestrated vessels. In segments of aorta and vena cava of guinea pig and rabbit, Skutelsky and Danon have demonstrated an even distribution of negative charges over the luminal surface. Incubation in vitro in a medium containing CF resulted in a clustering of CF molecules, endocy-
Fig. 5. *Macaca mulatta*. CF perfused through the anterior chamber at 20 mmHg. No tracer is found in the lumen of the vessel. Collagen fibrils of the stroma show a periodic decoration (arrows); large phagosomes in a macrophage contain great amounts of CF, and the vessel's basal lamina shows patchy aggregations of ferritin molecules. E: endothelium; BL: basal lamina; P: pericyte; M: macrophage; CF: collagen fibrils (original magnification, ×38,000).

tosis, and discharge of the tracer on the abluminal front. In isolated mesenteric capillaries of the frog perfused with CF, Clough\(^\text{16}\) reported a continuous layer of molecules adhering to the luminal surface of the capillary endothelium. Vesicles everywhere in the endothelial cytoplasm were seen to be labeled after perfusions lasting 60 sec or longer. In isolated perfused rat lung,\(^\text{17}\) CF was seen to decorate about one half of the plasmalemmal vesicles open to the lumen. CF was taken up by vesicles and after 60 min was discharged on the abluminal side of the endothelium. Different results were obtained by Simionescu and Simionescu\(^\text{18}\) in the endothelium of mice lung capillaries. In the vesicular area of these endothelial cells, the plasma membrane was homogeneously decorated by CF, with the exception of the membrane of plasmalemmal vesicles, transendothelial channels, and their associated diaphragms. Translocation across the endothelium of CF-labeled vesicles was found neither in vivo nor after plasma substitution with PBS.

In fenestrated capillaries of mouse pancreas and jejunum perfused in vivo with CF, Simionescu et al\(^\text{13}\) showed high density of high-affinity anionic sites conferred by sulfated proteoglycans on the luminal aspect of the fenestral diaphragms. These authors suggested that the great density of negative charges on these diaphragms may be of importance in excluding the passage of anionic plasma components into the extravascular space. Equally remarkable was the lack of decoration with CF of the endothelial plasmalemmal vesicles and channels that have otherwise been implicated in transendothelial transport of macromolecules. In contrast, Dermietzel et al\(^\text{14}\) observed vesicular uptake and release of CF on both the luminal and abluminal sides of fenestrated capillaries of the rat brain. Bankston and Milici,\(^\text{19}\) who...
studied rats injected intravenam with CF for 30 sec to 1 min, found considerable variations in the affinity of fenestral diaphragms for CF in different organs. In fetal and adult intestine, pancreas, pituitary, and renal peritubular capillaries, nearly all the fenestral diaphragms were labeled with a tuft of particles, whereas only a fraction of the fenestrae in the thyroid, adrenal cortex, and fetal kidney glomerulus bound CF. The authors concluded that there is considerable variation in the distribution of negatively charged groups on the fenestrae of different capillary beds.

In the continuous vessels of the iris, we have observed that plasmalemmal vesicles do not transport CF to the tissue front and, thus, they behave like the plasmalemmal vesicles of mouse lung capillaries and those of the fenestrated vessels of pancreas and jejunum. Therefore, plasmalemmal vesicles belonging to vessels of different tissues appear to possess different physicochemical characteristics. These differences do affect the transendothelial transport of macromolecules with different charges and may reflect the very specialized metabolic needs of various organs and tissues.

**Perfusion of AF through the Anterior Chamber**

When perfused through the anterior chamber, AF permeated the iris stroma. Moreover, it was present in the lumen of the iridal vessels in rather high concentration. Although a similar penetration into these vessels of a variety of tracers, including indigocarmine, carbon, thorotrast, inulin, and dextrans up to 80,000 daltons, had been previously reported (see Raviola and Butler for review of literature), their route of entry had never been identified. The present experiments illustrate one possible mechanism for such transendothelial transport. We have seen that AF crosses the vessel walls contained in plasmalemmal vesicles, whereas the tight junctions between the endothelial cells appear impermeable, thus, suggesting a transcellular rather than paracellular pathway.

Fig. 6. *Macaca fascicularis*. CF perfused through the anterior chamber at 18 mmHg. The basal lamina which surrounds the vessel's endothelium and the pericyte does not show a clear-cut stratification. Small aggregates of CF are rather evenly distributed around the vessel and the pericyte, especially in the region indicated by the arrows. RBC: red blood cell; END: endothelium; BL: basal lamina of the vessel (original magnification, ×84,000).
Neither the unidirectionality nor the modality of transport of AF across the walls of the iridal vessels are unique to this vascular tree. The endothelium of brain vessels too, can be crossed by interstitially injected tracers, while a blood-brain barrier blocks the passage of intravenously injected AF in the opposite direction. Thus, the vessels of the iris and brain show similar behavior in relation to intravenously or interstitially injected AF.

We have observed that the number of plasmalemmal vesicles labeled with AF was consistently higher in the abluminal than in the luminal region of the cytoplasm. Such a modality of AF movement is inconsistent with the commonly accepted view of transport by complete translocation of individual vesicles, with their contents, from one front to the other. It may agree, however, with an alternative model for vesicle transport, in which the steady-state transfer of macromolecules occurs by transient fusions between adjacent vesicles in the cytoplasm with progressive dilution of the tracer. 21

Although plasmalemmal vesicles loaded with tracer occasionally could be found in the proximity of the Golgi apparatus, AF was never observed in the cisterns of this organelle. Plasmalemmal vesicles appear to traverse the endothelial cytoplasm without interacting with other cell organelles or compartments; they carry substances through the endothelium by a shortcut between endocytosis and exocytosis, a process that has been termed transcytosis. 11

Perfusion of CF through the Anterior Chamber

The most striking results after CF perfusion of the anterior chamber were the decoration of the collagen fibrils of the stroma and the binding of the molecule to the basal laminae. The decoration of the collagen fibrils with highly regular spacing is similar to that described in the lung 18 and in the pancreas. 9 The distribution and binding of proteoglycans along the collagen fibrils must be of importance in producing the final morphologic pattern of a periodic structure, but the precise chemical nature and molecular interactions involved in this cross-linking are poorly understood. 22

The basal lamina of the iridal vessels, of the Schwann cells, and of the iridal epithelium were decorated by clusters of CF molecules. In sections in which the vessels’ basal lamina was tangentially or obliquely cut, the distribution of the clusters displayed a certain degree of order, but in cross sections of the vessel wall, a clear-cut separation into a lamina rara externa, interna, and lamina densa was not evident. With the exception of this feature, the clustering of CF particles in the basal lamina was very reminiscent of that described by Caulfield and Farquhar 23 and Kanwar and Farquhar 24 in renal glomerular capillaries, and similar anionic sites have been reported in other capillary beds. These anionic sites were shown to be conferred by heparan sulfates, 25-27 hyaluronic acids, 28 and entactin. 29 Since they are sensitive to pronase digestion, they appear to be chemically related to proteoglycans and/or glycoproteins.

Significance of the Transport of AF from the Interstitium into the Lumen of Iridial Vessels

It has been known for some time that anionic molecules can be selectively transported out of the eye. Rabbit iris–ciliary body preparations can accumulate a variety of substances from an incubation medium in vitro, and this accumulation has been related to in vivo transport functions. Two systems have been reported which are capable of accumulating iodide or iodopyracet in vitro and transporting these substances out of the eye. 30-31 An active transport of fluorescein by retinal vessels has been demonstrated by direct visualization of the tracer injected into the vitreous body. 32 This transport was partially inhibited by iodopyracet, benzyl-penicillin, and probenecid, suggesting an analogy with the organic anion transport system in the kidney and liver. Fluorescein perfused into the anterior chamber of rhesus monkeys was readily seen to penetrate the iris vessels, which thus seemed to behave, with regard to this organic anion, in a fashion similar to that observed in retinal vessels. 33-34 Radioactively labeled prostaglandins, introduced into the vitreous body, were rapidly transferred into the circulating blood. 35 In experiments in vivo, this absorptive transport was limited to the posterior segment of the eyeball. The ciliary processes, retinal vessels, and pigment epithelium of the retina were considered to be the most probable candidates for this transport, but in experiments in vitro, iris–ciliary body preparations also accumulated labeled prostaglandins. More recently it has been demonstrated that in primates, as in rabbit, beta-lactam antibiotics, such as penicillins and cephalosporins, introduced into the vitreous body are actively transported out of the eye across the retina. This transport pump was partially inhibited by probenecid and by inflammation. 36 Taken together, these data clearly indicate that there is an active transport of anionic substances out of the eye, and that the interocular vessels of the retina and iris are the most likely sites for this transport. Both sets of vessels behave in the same way in the presence of fluorescein, and we have previously observed an identical unidirectional transport of HRP in retinal and iridal vessels. 1,37 We have not yet established whether the transport of AF is...
when CF reaches the abluminal front after intracameral infusion, it does not penetrate the endothelium. In clear contrast, AF, like HRP, is transported rapidly into the lumen (Fig. 7). This transport takes place by plasmalemmal vesicles and, thus, must be carrier-mediated and saturable. These findings strongly suggest that organic anions of different molecular weight may be removed from the ocular tissues by the same morphologic mechanism.

**Key words:** monkey, iris, electron microscopy, anionic ferritin, cationic ferritin, blood-aqueous barrier, vessels, plasmalemmal vesicles

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**References**