The Structural Basis of the Blood-Aqueous Barrier in the Chicken Eye

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In order to identify the structural basis of the blood aqueous barrier in the chicken eye, the morphology of the blood vessels and epithelium of the ciliary body were examined with light microscopy, conventional electron microscopy, and the freeze-fracturing technique; the permeability properties of the vessels and epithelium were tested with intravascular injection of horseradish peroxidase (HRP). The ciliary body and iris of the adult chicken are supplied principally by a single temporal long posterior ciliary artery that, by dividing into two branches, gives rise to the great circle of the iris. From this circle multiple branches reach the iris, while a few run posteriorly to the ciliary body stroma. Most of the blood supply to the ciliary body stroma is derived from vessels that return from the iris, run in the valleys between ciliary processes, and are continuous, at the ora serrata, with the veins of the vortex system. Electron microscopy shows that the vessels of the ciliary body stroma differ from their counterpart in mammals in two respects: (1) the endothelial cells are joined by simple but continuous zonulae occludentes; (2) the openings in the endothelial lining (plasmalemmal vesicles, fenestrae, and transendothelial channels) are less numerous. The walls of these vessels retard, but do not prevent the diffusion of intravenously injected HRP into the surrounding connective tissue spaces. From the ciliary body stroma, HRP diffuses into the intercellular clefts of the ciliary epithelium, but its progression toward the posterior chamber is blocked by very complex zonulae occludentes between the nonpigmented cells. Thus, in chickens as in mammals tight junctions between the nonpigmented cells of the ciliary epithelium represent the structural equivalent of the blood-aqueous barrier. Invest Ophthalmol Vis Sci 24:326-338, 1983

In the recent past, studies on the tissue distribution of electron opaque tracers introduced into the bloodstream, complemented by high resolution conventional electron microscope and freeze-fracturing investigations on the identity and location of specialized intercellular junctions, have provided information on the morphologic basis of the blood aqueous barrier of mammals.1-6 These studies have demonstrated that in the ciliary body of the eye of all mammals studied so far, a barrier to circulating macromolecules is represented by zonulae occludentes that connect the lateral aspects of the nonpigmented cells of the ciliary epithelium.

Since no information is available in the literature on the ultrastructural counterpart of the blood aqueous barrier in birds, we carried out this study to establish whether, in the ciliary body of the chicken, a barrier is present that similarly excludes circulating macromolecules from the posterior chamber. The results of this study demonstrate that, in spite of the fact that in the chicken both the vessels of the ciliary body stroma and the zonulae occludentes of the ciliary epithelium possess special morphologic features, the structural counterpart of the blood aqueous barrier is essentially identical to that previously described in mammals. A preliminary account of this work has been published in abstract form.7

A better knowledge of the normal fine structure and physiologic characteristics of the anterior segment of the eye in birds is a necessary prelude to experimental studies on light-induced avian glaucoma.8

Materials and Methods

Animals

Male and female adult chickens (Gallus domesticus, White Plymouth Rock) were used for this study.

Whole-mount Preparations of the Anterior Segment of the Eye

Three birds were sedated with an intramuscular injection of Ketamine HCl (10 mg/kg) and anesthe-
tized with sodium pentobarbital intraperitoneum (60 mg/kg). Horseradish peroxidase (HRP; Sigma, Type II; 0.5 g/kg body weight) dissolved in 5 ml of phosphate buffered saline, pH 7.2, was injected slowly into the wing vein. The temporal and superior quadrants of the eyeball were marked with colored threads so that the orientation of the eye, in situ, would not be lost in subsequent procedures. Two, 10, and 20 min after the end of HRP injection, the animals were killed by decapitation, and the eyes were enucleated, cut at the equator, and immersed in the fixative fluid (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3 containing 0.2% CaCl₂).

During the fixation, the vitreous body and the lens were carefully removed, the anterior segment was cut into two parts and the whole ciliary body and iris dissected from the sclera. Subsequently the tissues were processed as previously described. After bleaching of the melanin with H₂O₂ the specimens were either preserved at 4°C in 100% glycerol or dehydrated and mounted for light microscopy with Permount.

Conventional Electron Microscopy

In three animals, an overdose of anesthetic was administered intravenously, the eyes were enucleated, opened at the equator, and immediately immersed in fixative. The eyes of two of these chickens were fixed in the same fixative fluid used for the whole mount preparations, whereas the eyes of the third bird were fixed in a mixture of 3% glutaraldehyde, 0.5% paraformaldehyde, 1.2% tannic acid in 0.1 M Sörenson phosphate buffer, pH 7.4. In all eyes, after 10–15 min, the lens was removed, and the anterior segment was dissected from the corneoscleral tunic. The ciliary bodies were fixed for 2–3 hr at room temperature and washed overnight in buffer at 4°C. Subsequently, they were processed as previously reported. Micrographs were taken with a Jeol-100 CX electron microscope.

Tracer Experiments

With the animals in general anesthesia, HRP (Sigma, Type II; 0.5 g/kg body weight) dissolved in phosphate buffered saline, pH 7.2, was injected slowly into the wing veins of four chickens. After 2, 4, 10, and 20 min the eyes were enucleated, and the ciliary bodies fixed by immersion and processed for the ultrastructural demonstration of HRP.

Freeze-Fracturing

The eyes of two animals were fixed as for conventional electron microscopy. Subsequently, their anterior segments were trimmed into radially oriented wedges from which 250-μm sections were cut with a Smith-Farquhar tissue chopper. Chopper sections were equilibrated with 20% glycerol in cacodylate buffer and mounted on gold specimen carriers. The specimens were rapidly frozen in the liquid phase of partially solidified Freon-22 (monochlorodifluoromethane) and stored in liquid nitrogen. Specimens were fractured and replicated with platinum/carbon in a Balzers apparatus (BAF-301, Balzers High Vacuum Corp., Santa Ana, CA) operated at approximately 10⁻⁷ Torr with a stage temperature of —115°C. The tissues were transferred into methanol and digested in 5–6% sodium hypochlorite containing 10–15% potassium hydroxyde. Platinum/carbon replicas were rinsed with several changes of distilled water and mounted on Gilder grids (Marivac Services; Halifax, Nova Scotia, Canada). Micrographs were taken with a Jeol-100 CX electron microscope.

Morphometry

The following features of the ciliary body were quantified: (1) The junctional complexity of the zonulæ occludentes of the ciliary body vessels was estimated from replicas, by measuring the percentage of junctional length occupied by 1, 2, 3, 4, and more than 4 strands. (2) The surface density of plasmalemmal vesicles, fenestrae, and transendothelial channels in the endothelium of the vessels underlying the pigmented cells of the ciliary epithelium, was calculated in thin sections of the anterior, intermediate, and posterior region of the ciliary body. To this purpose, the ciliary body was serially sectioned and every fifth section analyzed at the electron microscope.
Fig. 2. Stroma of the chicken ciliary body, posterior region. The wall of this vessel is composed of a thin endothelium and an incomplete thin basal lamina (bl). CE, pigmented cells of the ciliary epithelium (×13,350).

Measurements of the endothelial length and counts of the endothelial openings* were made on photographic montages prepared from series of negatives taken at an original magnification of 10,000×. The density of the openings in the endothelium was finally referred to the movement of materials across the vessel wall is unknown. For this reason we will collectively refer to these structures as openings.

* In the endothelium of capillaries, plasmalemmal vesicles, fenestræ, and transendothelial channels are thought to represent a dynamic system adapted to the transendothelial exchange of water soluble molecules. The relative contribution of each of these structures to the movement of materials across the vessel wall is unknown. For this reason we will collectively refer to these structures as openings.
to \( \mu \text{m}^2 \) of vessel wall by assuming an average section thickness of 60 nm. Statistical evaluation was performed by means of nonparametric analysis of variance, tests of Friedman, Kruskal-Wallis with the application of Dunn’s multiple comparison.\(^{1,2}\) (3) The population of endothelial openings was also evaluated on replicas. Vessels were selected regarding the position near the pigmented layer of the ciliary epithelium. Since the number of openings counted in thin sections was approximately the same throughout the ciliary body, the quantitative analysis on replicas was limited to the intermediate region.

All measurements were made with a MOP-3 Digital Image Analyzer System (Carl Zeiss Inc.).

**Protein Determination**

Total protein content was calculated from six samples of aqueous humor each one from one eye and plasma from three chickens. The aqueous humor was obtained from a needle puncture of the anterior chamber of anesthetized birds and plasma was obtained from blood from the wing veins. Protein concentrations were measured using the spectrophotometric method of Lowry et al.\(^{13}\)

**Results**

**Gross Morphology**

The analysis under the dissecting microscope of the anterior segment of the adult chicken eye demonstrates that in this animal species the ciliary body can be divided into three regions: (1) an anterior region represented by 80 prominent ciliary process heads that are closely apposed to the periphery of the lens; the ciliary processes are continuous anteriorly with the iris. (2) An intermediate region, in which each ciliary process divides into three or four shallower ridges. These ridges are about 300 in number and are longer than the ciliary processes of the anterior region. (3) A posterior region occupied by numerous branching thin, low ridges. These are particularly evident on the temporal side of the eyeball and they end at the ora serrata. A pars plana is absent in the ciliary body of the adult chicken.

Examination of whole mount preparations of the anterior segment of the eye of chickens intravenously injected with HRP shows that the ciliary body and iris are chiefly supplied by a single long posterior ciliary artery, that penetrates the sclera lateral to the optic nerve and runs in the outer choroid following the temporal position of the horizontal meridian of the eye. At the boundary between ciliary body and iris, this artery divides into two branches that form the great circle of the iris. From this circle, multiple branches enter the iris with an undulating course, while a few recurrent branches run posteriorly to supply the ciliary body stroma. Blood from the iris is drained by vessels that are located in the valleys between the ciliary processes and become continuous at the ora serrata with the veins of the vortex system (Fig. 1).
Fig. 4. The zonula occludens between the endothelial cells of the ciliary body stroma vessel (arrows) consists of strands which, in this freeze-fracture replica range in number from one (asterisk) to three. Inset: Enlargement of a zonula occludens formed by a network of strands represented by discontinuous rows of particles on the P-face (PF) complemented in the E-face (EF) by grooves which contain a few particles. A gap junction (arrow) is inserted within the tight junctional meshwork (×30,000; Inset ×66,750).

**Electron Microscopy**

The vessels of the ciliary body stroma located immediately beneath the pigmented cells of the ciliary epithelium have variable diameters, ranging from 10 to 70 μm. Their walls consist of a thin endothelium, a basal lamina, a discontinuous row of pericytes and an adventitia, composed primarily of fibroblasts and...
Table 1. Complexity of the zonulae occludentes in the vessels of the ciliary body stroma

<table>
<thead>
<tr>
<th>Number of strands</th>
<th>% Total length*</th>
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<tbody>
<tr>
<td>1</td>
<td>51.2</td>
</tr>
<tr>
<td>2-3</td>
<td>42.3</td>
</tr>
<tr>
<td>4-more than 4</td>
<td>6.5</td>
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* Total junctional length measured: 112.21 μm.

bundles of collagen fibrils (Fig. 2). No smooth muscle cells or elastic lamina were observed in these vessels. The basal lamina of the endothelium changes gradually in structure from the ciliary processes, where it is continuous and about 200 nm thick, toward the ora serrata, where it is incomplete and about 100 nm in thickness.

In thin sections, endothelial cells are joined by tight junctions, represented by multiple fusion points of the outer leaflets of the adjacent membranes (Fig. 3). In freeze-fracture replicas, the junctions between endothelial cells are represented by simple but continuous zonulae occludentes (Fig. 4). On the P-face or inner leaflet of the plasma membrane, rows of particles are found that are aligned on the top of low ridges; on the E-face or outer leaflet, shallow grooves are present. A few particles lie along the linear depressions of the E-face. Small gap junctions, represented by aggregates of particles on the P-face and by arrays of pits on the E-face, are occasionally found inserted within the tight junctional network (Fig. 4 inset). Table 1 summarizes the complexity of the endothelial zonulae occludentes in the vessels of the ciliary body stroma.

The zonulae occludentes of the endothelial cells of the ciliary body stroma consist of from one to five strands. Approximately one half (51.2%) of the length of the zonula occludens has a single strand and nearly the other half (42.3%) has two or three strands. Only 6.5% of the junctional length has more than three strands.

The endothelial cells of the vessels of the ciliary body stroma possess a rather regular luminal surface and a variable population of coated and uncoated plasmalemmal vesicles, fenestrae, and transendothelial channels. Plasmalemmal vesicles that open on either the luminal or abluminal front of the cells, possess stomata closed by a single diaphragm, in which the structure of the unit membrane cannot be recognized (Fig. 5A). Fenestrae and transendothelial channels closed by single or double diaphragms respectively, are usually localized in the attenuated regions of the endothelium that abut on the ciliary epithelium (Figs. 5B, C).

In spite of the fact that in thin sections the endothelial openings possess diverse morphology, they all look similar in freeze-fracture replicas. They appear as vallate papillae (round depressions) on the P-face of the endothelial cell membrane and as shallow vol-

Fig. 5. Endothelial wall of a ciliary body stroma vessel. bl, basal lamina. A, Plasmalemmal vesicle stomata (arrows) facing the lumen and closed by electrodense diaphragms; B, fenestra closed by a single diaphragm (arrow); C, transendothelial channel closed by a double diaphragm (arrow) (×102,000).
canoes (round elevations) on the E-face, with diameters ranging from 50 to 70 nm (Fig. 6).

The density of vesicle stomata, fenestrae, and transendothelial channels was estimated in the portion of the vessel wall that faces the ciliary epithelium. This count was done in the three different regions of the ciliary body. The results are shown in Table 2. The table shows that there is no significant difference in the number of vesicle stomata and fenestrae among the three regions of the ciliary body (test of Friedman $X^2 = 0.73$ and $X^2 = 2.77$). The transendothelial channels are significantly more numerous in the intermediate region as compared with the posterior region (test of Friedman $X^2 = 11.23$, $P < 0.01$, critical value of Dunn's multiple comparison = 10.98). When the openings are compared in each region, the vesicle stomata are significantly more numerous than the fenestrae and transendothelial channels (test of Kruskal-Wallis, in the anterior region—$H = 29$, $37$, $P < 0.001$, critical value of Dunn's multiple comparison = 10.86; in the intermediate region—$H = 37.98$, $P < 0.001$, critical value = 11.58; in this region all openings differs significantly from each other; posterior region—$H = 24.7$, $P < 0.001$, critical value = 9.65).

The estimated density of the endothelial openings in freeze-fracture replicas of the intermediate region of the ciliary body gave 4.64 openings per $\mu m^2$ (3,520 openings in 757.22 $\mu m^2$).

The Structural Equivalent of the Blood-Aqueous Barrier

The vessels of the ciliary body stroma are permeable to blood-borne HRP (Fig. 7). The tracer leaves the lumen of the vessels by crossing the openings in their walls, whereas the tight junctions block its movement along the interendothelial clefts (Fig. 8). Two and 4 min after injection only a small amount of HRP is found outside the endothelium. However, in birds killed 20 min after the injection, HRP has diffused throughout the ciliary body stroma and into the spaces between the pigmented cells of the ciliary epithelium. It penetrates the intercellular clefts between...
pigmented and nonpigmented cells and is finally blocked by tight junctions that connect the lateral aspects of the nonpigmented cells, near their apices (Fig. 9 inset). In thin sections, these tight junctions appear as numerous points of fusion of the outer leaflets of the adjoining epithelial cell membranes (Fig. 10). In freeze-fracture replicas, they are very elaborate and consist of a network of branching and anastomosing strands. The complexity of the zonulae occludentes increases from the ciliary process tips toward the ora serrata and the number of strands varies between 5 and 17. Gap junctions are inserted fre-
Proteins of the Aqueous Humor and Plasma

The mean total amount of protein in the aqueous humor of the adult chicken is 34.9 ± 3.3 mg/100 ml, in the same birds the mean total plasma protein is 7.06 ± 0.71 g/100 ml.

Discussion

Gross Morphology

The ciliary body of the chicken possesses approximately the same number of ciliary processes as the ciliary body of primates, but in the chicken the ciliary processes branch posteriorly, forming a great number of shallow ridges and a pars plana is absent. This condition is different from the ciliary body of fishes and amphibians where the ciliary processes are absent or poorly developed. The close contact between ciliary processes and lens pad in the chicken is a condition also present in rabbit and human embryos.

The vascular supply of the anterior segment of the eye in the adult chicken differs from that of adult mammals by the presence of a single long posterior ciliary artery. This condition was first described in a number of birds by Maggiore, but this author maintained that in Gallus domesticus the single long posterior ciliary artery runs along the superior meridian of the eye. In our material we consistently found this artery on the temporal side of the eyeball, as is the case for an early stage of development of the human eye (embryos of 18 mm), when the ophthalmic artery gives off the common ciliary artery that passes on the temporal side of the optic stalk. From the concavity of the great circle of the iris, the supply to the iris comes from tortuous vessels, and in the chicken they are less numerous than in primates. The vessels to the ciliary body stroma originate both from the convexity of the great circle of the iris and from vessels returning from the iris, as described in other species of birds.

The Vessels of the Ciliary Body Stroma

In the chicken, as in mammals, the vessels located immediately underneath the ciliary epithelium have been traditionally classified as capillaries because of the simple structure of their walls, although their diameter is quite large. The endothelium of these vessels is characterized by the presence of openings that, as is the case for the monkey and rabbit, are mostly located on the epithelial aspect of the endothelial wall. In the vessels of the ciliary body of the chicken, however, a proportion of openings is represented by trans-endothelial channels whose presence has never been reported in mammals. Since at present the relative...
Fig. 9. Chicken ciliary epithelium and stroma. HRP freely diffuses in the stroma and in the clefts between the cells of the pigmented epithelium (PE). INSET. The dense reaction product of the histochemical demonstration of HRP permeates the space between pigmented epithelium (PE) and nonpigmented cells (NPE) and is blocked by the zonula occludens that connects the lateral aspects of nonpigmented cells near their apices (arrow) (×13,400; Inset ×52,000).

The importance of fenestrae and transendothelial channels in the permeability properties of vascular walls is unknown, the significance of this observation is unclear. It is interesting to note however, that the number of openings in the walls of the blood vessels of the chicken ciliary body is approximately 14 times less than the number of openings in the visceral capillaries of mammals.21 More importantly, the total...
Fig. 10. The zonula occludens between the lateral surfaces of two nonpigmented cells (NPE) consists of multiple points of fusion of the outer leaflets of the plasmalemma (arrows). A pigmented cell (PE) and a nonpigmented cell are connected by a gap junction (GJ) (×225,900).

number of plasmalemmal vesicles, fenestrae, and transendothelial channels in the vessels of the ciliary body stroma of the chicken is much smaller, per unit length of the endothelium, than the number of fenestrae in adult man and monkey. In the monkey, endothelial openings are as scarce as in the chicken only in the first stages of embryonal development.

Such a difference between adult chicken and mammals can explain why, in the chicken, HRP injected intravenously diffuses out of the vessel lumen more slowly than in mammals.

It has been reported that in freeze-fracture replicas of visceral capillaries of mammals the stomata of plasmalemmal vesicles can be distinguished from the openings of the fenestrae because they have different size, the former being 20–40 nm in diameter and the latter 50–80 nm in diameter. In our freeze-fracture preparations, we were unable to detect any difference in size between the stomata of plasmalemmal vesicles, the fenestrae, and the transendothelial channels, since all openings had a diameter of about 50–70 nm.

The interendothelial clefts of the vessels of the chicken ciliary body stroma are sealed by tight junctions that exhibit a simple structure, but are impermeable to blood-borne HRP. In this respect, these vessels are different from those of the rabbit and monkey; in mammals, in fact, these intercellular junctions are characterized by creases or folds on the P-face and furrows on the E-face, which do not form continuous belts or zonulae around the entire cell perimeter.

Morphologic Equivalent of the Blood-Aqueous Barrier in the Chicken Eye

After intravenous injection of the fluorescent dye acriflavine, to localize the morphologic site of the blood aqueous barrier in the pigeon, Rodriguez-Peralta suggested that in birds the barrier corresponds to the basal lamina of the pigmented layer of the ciliary epithelium whereas in mammals it corresponds to the nonpigmented layer of the ciliary epithelium. We have demonstrated that in the chicken the vessels of the ciliary body stroma are different from their counterpart in mammals because the interendothelial clefts are closed by tight junctions and they possess a smaller population of openings. As a result the diffusion of blood-borne molecules into the ciliary body stroma is retarded, and a longer time interval after the injection is required before they reach the intercellular spaces of the ciliary epithelium. This may explain why, by using the same perfusion time in mammals and birds, the barrier was identified in the basal lamina of the avian ciliary epithelium. In contrast, the present study clearly shows that after an appropriate perfusion time, HRP diffuses out of the vessels of the ciliary body stroma, penetrates the...
Fig. 11. Junctional complex in the apical part of a nonpigmented epithelial cell (NPE) in the posterior portion of the ciliary epithelium. The tight junctional meshwork is formed by a great number of anastomosing strands in the inner leaflet of the plasmalemma (PF) complemented by grooves on the outer leaflet (EF). Gap junctions characterized by an assembly of particles on the P-face and arrays of pits on the E-face (arrows) are inserted within the meshwork (×52,300).

intercellular clefts between pigmented cells of the ciliary epithelium, and is finally blocked by the zonulae occludentes that connect the lateral surfaces of the nonpigmented cells. Thus, in birds as in mammals, the tight junctions between nonpigmented cells represent the morphologic equivalent of the blood-aqueous barrier. There is a difference, however, between mammals and birds: the mammalian zonula
occludens is rather simple and the ciliary epithelium can be classified as leaky, whereas in birds the junction is complex and the ciliary epithelium probably has the physiologic characteristics of a tight epithelium.

Plasma Proteins of the Aqueous Humor

It is generally accepted that in mammals the source of the small amount of plasma proteins normally present in the aqueous humor are the leaky vessels of the ciliary body stroma. Plasma proteins diffusing from these vessels penetrate the anterior portion of the ciliary body and enter the anterior chamber near the root of the iris, where an epithelial barrier is absent. Although in the chicken the walls of the vessels of the ciliary body stroma seem to retard the diffusion of macromolecules such as HRP, it was surprising to find that the total amount of plasma proteins in the aqueous humor is higher than in man and similar to that of the rabbit. Such a result indicates that the permeability properties of the vessels of the ciliary body stroma represent just one of a number of factors that contribute to the steady-state concentration of plasma proteins in the aqueous humor.

Key words: chicken, electron microscopy, freeze-fracturing, horseradish peroxidase, ciliary epithelium, blood-aqueous barrier, vessels

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