Scanning and Transmission Electron Microscopic Study on a Multilayered Basement Membrane in the Pineal Organ of the Ayu Plecoglossus altivelis*

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Summary. The pineal organ possesses highly fenestrated capillaries, and is devoid of the so-called blood-brain barrier. The present study indicated that the pineal epithelium of the teleost fish, ayu Plecoglossus altivelis, possesses an unusually thick and convoluted basement membrane (2.2-2.4 μm in width) which is visible even under the light microscope. This pineal basement membrane was observed by scanning and transmission electron microscopy and its detailed composition and relationships with the fenestrated capillaries and the perivascular space were investigated. As the basement membrane was composed of three to eight layers of basal laminae interspersed with laminae lucidae, we termed it the “multilayered basement membrane”. In consideration of our previous demonstration that macromolecules such as HRP are trapped by the basement membrane, it is suggested that this multilayered basement membrane may prevent foreign substances from reaching the pineal epithelium.

Unlike the brain tissue which is surrounded by a blood-brain barrier, the pineal organ has been known to possess highly fenestrated capillaries (Lee, 1971; Welsh et al., 1981; Bouchaud et al., 1986; Cervós-Navarroso et al., 1988). Due to this feature, the pineal organ is devoid of a barrier structure. In the teleost fish, the capillaries in the pineal organ are highly fenestrated, likely allowing the passage of foreign substances from the circulating blood to the pineal parenchyma. The structure of the basement membrane located in the perivascular space is the theme of the present study.

Usually a single layer of the basement membrane underlies the epithelial cells, whereas a double basement membrane has been reported in mammals (Lee, 1971; Kefalides et al., 1979). The usage of the terms “basement membrane” and “basal lamina” have been discussed by several authors. According to Kefalides et al. (1979), who preferred the term “basement membrane”, the basal lamina is the dense, amorphous matrix that lies between the lamina lucida and the fibrillar layer. Ichimura and Hashimoto (1984) and Inoue (1989), following the international Anatomical Nomenclature, used the term “basement membrane”, noting that this structure comprises three layers: lamina lucida, lamina basalis (or basal lamina), and lamina fibroreticularis (or reticularis). Many authors avoid the term “basement membrane”, because the structure in question has nothing to do with a lipid bilayer characteristic of cellular membranes (see Peters et al., 1991).

The present study of the pineal organ of the ayu demonstrated the existence of an unusually thick basement membrane, which is easily recognized even under a light microscope and does not seem to have been reported in any other fish species. Electron microscopic observation indicated that this thick and convoluted basement membrane contains several layers of basal laminae. Due to this peculiar characteristic, we named the basement membrane found in the pineal organ of the ayu the “multilayered basement membrane”.

The significance of the co-existence of highly fenestrated capillaries, well developed perivascular spaces, and multilayered basement membrane in the pineal organ of the ayu is discussed in this study.

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MATERIALS AND METHODS

Adult ayu were purchased from a fish supplier in Toyota (Aichi Prefecture). During the experiment, they were kept in laboratory tanks containing well aerated water under an artificial photoperiod (14L: 10D). All fish were anesthetized with 1/2000 FA 100 (Tanabe, Osaka) before each treatment.

Scanning electron microscopy

Eight fish, after an initial flushing with a phosphate buffer containing 1% heparin, were fixed by vascular perfusion with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.3. The whole brains were dissected and trimmed, immersed in the same fixative for 2 h, and immersed in the phosphate buffer containing 8% sucrose overnight. After treatment with 1% tannic acid, the brains were submitted to postfixation in 1% OsO₄, saturation in 15, 30 and 50% DMSO, and freeze-fracture on an aluminium plate previously cooled with liquid nitrogen. After dehydration in ethanol, the specimens were saturated in isoamyl acetate, critical point-dried and then sputter-coated with Pt/Pd. Finally, the specimens were examined with a Hitachi S-800 scanning electron microscope (SEM) at 10 kV.

Light and transmission electron microscopy

Four fish were fixed by vascular perfusion with 2% paraformaldehyde and 2% glutaraldehyde as mentioned above. The whole brains were dissected, immersed in the same fixative for 2 h, and immersed in 0.1 M phosphate buffer containing 8% sucrose overnight. After postfixation in cold 1% OsO₄, saturation in 15, 30 and 50% DMSO, and freeze-fracture on an aluminium plate previously cooled with liquid nitrogen. After dehydration in ethanol, the specimens were saturated in isoamyl acetate, critical point-dried and then sputter-coated with Pt/Pd. Finally, the specimens were examined with a Hitachi S-800 scanning electron microscope (SEM) at 10 kV.

RESULTS

Under the light and scanning electron microscopes, the pineal organ typically displays an epithelium with many folds, which cause a lobular configuration to the parenchyma (Figs. 1a, 2b). It possesses a cerebrospinal fluid-filled lumen in open communication with the third ventricle. The proximal third of the pineal organ is in contact with another circumventricular organ named the “dorsal sac” (or “saccus dorsalis”) (Fig. 1a).

Capillaries and perivascular spaces

A rich network of blood vessels was seen to irrigate the pineal organ. The sagittally sectioned pineal organ in this study displayed a large number of blood vessels both on the dorsal and ventral sides (Figs. 1a, 2a, b). Usually, capillaries were more numerous found on the ventral side. The tridimensional image of large capillaries obtained with SEM revealed many apertures on the endothelial wall, which represented vascular branchings.

The capillaries were lined with a single layered endothelium, and their lumina were usually large. The endothelial cells possessed many fenestrae ranging from 50 to 70 nm in diameter (Fig. 3a, b). A diaphragm closed each fenestra, separating the capillary lumen from the perivascular space.

The surrounding connective tissue displayed interwoven fibrous elements (Fig. 1b). This was relatively thick and contained cellular and fibrous elements including fibroblasts, macrophages, fibrils and/or fibers. Irregularly shaped cells, probably macrophages, were also observed in the perivascular space (Fig. 1b). Under the TEM, the fibrous elements revealed periodical cross-striations.

Multilayered basement membrane

A very thick and convoluted basement membrane (2.2–2.4 μm in width) underlay the epithelial cells. It was easily seen even under the light microscope (Fig. 2). This structure was termed, in this study, the “multilayered basement membrane”, due to the presence of many layers of basal laminae. The present SEM and TEM study revealed that, in certain areas of the pineal epithelium, the basement membrane possessed many folds, which in turn contained several invaginated layers of basal laminae (Figs. 1b, 3a, b). The TEM observations indicated that this structure constituted many—three to eight—layers of basal laminae interspaced with laminae lucidae, although the latter could not be seen under the SEM. Even in the areas where the basement membrane ran rather straight (1.2–1.4 μm in width), we could find two or more layers of basal laminae which extended to cover also the invaginated areas (Fig. 3b).

Under the TEM and SEM, a delicate meshwork of fibrils and/or fibers was observed between the layers of basal laminae and also in the perivascular space (Figs. 1b, 3a, b). Many fibrils seemed to be connected to the angles formed in those areas where the
Fig. 1. Scanning electron micrographs of a sagittally sectioned pineal organ of the ayu. a. Pineal organ (P) lying on the telencephalon (T) and in contact with the dorsal sac (DS). Arrows indicate capillaries. x 165. b. Scanning electron micrograph of the perivascular space. Arrows show cellular elements intermingled in the fibrous elements. C capillary lumen. x 18,500. c. Multilayered basement membrane (arrows) filled with fibrous elements. P pineal parenchyma. x 22,000
multilayered basement membrane was folded (Fig. 3a, b). These fibrillar elements were oriented in various directions, at times crossing each other or linking two segments of basal laminae. Thin fibrils, resembling elastic fibrils, were also found spanning parallel segments of the basal laminae, or a collagen fibril and a segment of basal lamina (see the inlet of Fig. 3b).

**DISCUSSION**

The pineal organ is one of the circumventricular organs, which usually possess fenestrated capillaries surrounded by perivascular spaces and lack a blood-brain barrier (Lee, 1971; Welsh et al., 1981; Bouchaud and Bosler, 1986; Cervós-Havarros et al., 1988). This study revealed that the pineal organ of the teleost fish, the ayu, has a well developed perivascular space surrounding highly fenestrated capillaries. The connective tissue filling the perivascular space and enveloping the organ of the ayu, contains the usual connective tissue elements, such as fibroblasts, macrophages, and collagen fibrils.

Many capillaries were found both on the dorsal and the ventral sides of the pineal organ in the ayu. Under the TEM, the endothelial cells of the pineal capillaries showed many fenestrae, characteristic of "leaky type" capillaries (see Holash and Stewart, 1993). The presence of this type of endothelium suggests the ample exchange of substances through the capillary wall (Bouchaud and Bosler, 1986). Minute foreign particles may also be able to penetrate the pineal parenchyma through the capillary wall in the ayu.

Certain differences are recognized when the ayu is compared with other fish, such as the rainbow trout (see Omura et al., 1990; Omura, 1991). The pineal endothelia of the ayu are endowed with a larger number of fenestrae and the pineal epithelium is underlaid with a "multilayered basement membrane". In contrast, the pineal organ of the rainbow trout, which showed an extremely high uptake of injected molecules such as 3H-labelled 5-hydroxytryptophan, ferritin, and HRP (Oguri et al., 1968; Omura et al., 1985), possesses a thin single-layered basement membrane and poorly fenestrated endothelia. In the pineal
Fig. 3. Transmission electron micrographs of the multilayered basement membrane invaginated into many folds (a), and running rather straight (b). Arrowheads, small arrows, and large arrows show basal laminae, collagen fibrils, and endothelial fenestrae, respectively. Inlet: Higher magnification of the square from b. Note the fibrils crossing between a basal lamina (BL) and a collagen fibril (CF). C capillary lumen, P pineal parenchyma. a and b: ×23,000, Inlet: ×57,000
lumen and perivascular space of the rainbow trout, a large number of macrophages were also observed after the peritoneal injection of HRP.

The pineal epithelium and the adjacent capillary endothelia are in contact with their respective basement membranes. The basement membrane underlying the pineal epithelial cells of teleost fish has been regarded as a usual single-layered type (Vollrath, 1981). While double layered basement membranes have also been reported in the circumventricular organs of mammals (Lee, 1971; Kefalides et al., 1979), the present study in the ayu pineal organ demonstrated a thick basement membrane consisting of several layers of basal laminae.

The basement membrane underlying the epithelial cells of the pineal organ of the ayu is very thick and convoluted and comprises many layers of basal laminae interspaced with laminae lucidae. The basement membrane of the pineal organ of the ayu thus deserves the name “multilayered basement membrane”.

In the folds of the pineal epithelium, the basement membrane was constituted of piles of basal laminae. In other areas, where the basement membrane ran straight, two or more layers of basal laminae were still present. Inoue (1989) reported the presence of double, thick basement membranes lining different epithelia and brain capillaries. However, such a thick and convoluted basement membrane found in the pineal organ of the ayu does not seem to have ever been reported to date.

A delicate meshwork of fibrils was observed between the dense layers of basal laminae and also in the perivascular space of the pineal organ of the ayu. Many fibrils seemed to be connected to the anchors formed in those areas where the multilayered basal lamina was folded. These fibrous elements were oriented in various directions, crossing each other and sometimes linking two segments of basal laminae. Due to the presence of cross-striations, most of the fibrils were regarded as collagen in nature.

The presence of a filamentous layer on the outer surface of the basal lamina has also been seen in other circumventricular organs of guinea pigs (Kefalides et al., 1979; Ichimura and Hashimoto, 1984; Inoue, 1989). This layer, named the lamina reticularis, is comprised of elastic fibrils. In the pineal organ of the ayu, structures resembling elastic fibrils were found among the layers of basal laminae.

According to Spector and Johanson (1989), the choroid plexus, one of the circumventricular organs, may actively transport waste products from the ventricular cerebrospinal fluid into the blood circulation. Based on this view, they compared the choroid plexus to the kidney, regarding the former as “a kidney of the brain”. In addition, Zheng et al. (1991) demonstrated that this organ is able to sequester and concentrate heavy metals and metalloids. In the pineal organ of fishes, as mentioned above, not only the precursor of pineal hormone “melatonin" but also large molecules such as ferritin and HRP were taken up and sequestered in the epithelial cells (see Omura et al., 1990). Aggregations of heavy metals and metallic crystals were demonstrated in the pineal organ of fish collected from a polluted pond (Srivastava et al., 1985; Srivastwa et al., 1989/1990).

As reviewed by Kefalides et al. (1979), the basement membrane of the renal glomerulus, located between the endothelium and the epithelium, functions as the main filter in the glomerular system. The particles that eventually leak through the capillary fenestrae may be filtered primarily by the basement membrane and take up by the epithelial cells. In the pineal organ of the ayu, the highly fenestrated, “leaky” capillary endothelium apparently allows the passage of foreign substances, including minute particles, into the pineal parenchyma. Thus, the multilayered basement membrane may possibly trap certain of the substances which leak through the capillary fenestrae. In the sense of this involvement in filtration, it is suggested that the multilayered basement membrane may function as a part of the blood-cerebrospinal barrier in the pineal organ of the ayu.

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