Light and Electron Microscopic Detection of Anionic Sites in the Rat Choroid Plexus

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Summary. Electron microscopy of ultrathin sections stained with cationic iron colloid revealed that, in the choroid plexus of the rat brain ventricles, the luminal surface and fenestral diaphragm of the capillary endothelium as well as the basement membranes of the endothelium and epithelium are strongly anionic or intensely negatively charged. The iron colloid reaction to these anionic sites was erased by treatment with hyaluronidase or digestions with chondroitinase ABC/heparitinase/keratanase. These results indicate that sulfated proteoglycans provide such anionic sites of the choroidal capillaries. Discussion suggested that the negative charge on the luminal surface of the capillary endothelium prevents the adhesion of blood cells to capillary walls and also prevents endothelial adhesion by their repelling each other. It was further discussed that the negatively charged endothelial fenestrae and basement membranes may act as a charge barrier to inhibit the passage of anionic molecules.

Recent electron microscopic studies of ultrathin sections stained with cationized ferritin, polyethyleneimine, or ruthenium red have revealed that the luminal surface of the capillary endothelium as well as the basement membranes of the capillary endothelium and epithelium in the rat choroid plexus are intensely negatively charged (Schurer et al., 1978; Dermietzel et al., 1983; Thurauf et al., 1983; Kirsch, 1986; Schmidley and Wissig, 1986a, b). Such negative charges of the choroidal capillaries have also been confirmed in our previous studies of mouse brain perineuronal sulfated proteoglycans stained with cationic iron colloid (Murakami et al., 1996).

The present study reinvestigates the negatively charged sites of the rat choroidal capillaries with cationic iron colloid staining, and shows that these sites consist of sulfated proteoglycans, whose stainability is eliminated after digestion with hyaluronidase or chondroitinase ABC/heparitinase/keratanase.

MATERIALS AND METHODS

Male Wistar rats weighing 200-300 g were anesthetized with ethyl ether, and their descending aorta was ligated after opening the thorax. The animals were perfused via the ascending aorta with Ringer’s solution and with a mixture of 2% paraformaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The choroid plexuses of the third ventricle were then isolated together with brain tissues, and refixed in the buffered paraformaldehyde/glutaraldehyde fixative for 3-4 h. They were embedded in paraffin. In some cases, the choroid plexuses were cut into small blocks (1 x 1 x 1 mm) prior to the refixation. These small blocks were embedded in LR-White resin, or were postfixed with 1% osmium tetroxide and embedded in epoxy resin.

Transmission electron microscopy

The epoxy resin-embedded specimens were cut into ultrathin sections, additionally stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope (H-700, Hitachi).

The LR-White resin-embedded specimens were also cut into ultrathin sections. They were incubated in a cationic iron colloid solution with a pH value of 1.5-2.0 for 20 min (Ohtsuka et al., 1993), additionally stained with uranyl acetate and lead citrate and observed with a transmission electron microscope.

Light microscopy

The paraffin-embedded specimens were cut into sections of 5-10 μm thickness. They were deparaffinized with xylene and treated as follows.

Some sections were incubated in the cationic iron
colloid solution with a pH value of 1.5-2.0 for 20 min at room temperature, and treated with a mixture of 1% K₄Fe(CN)₆ and 1% HCl for Prussian blue reaction for 5 min (MURAKAMI et al., 1986). They were counter-stained with nuclear fast red prior to the light microscopic observations. Some sections were stained solely with aldehyde fuchsin (GOMORI, 1950). Oxidation with potassium permanganate prior to aldehyde fuchsin staining was omitted (MURAKAMI et al., 1994).

Some sections were methylated with a mixture of HCl and methanol (FISHER and LILLIE, 1954). Some of these methylated sections were saponificated with a mixture of KOH and ethanol (SPICER and LILLIE, 1959). Methylated or methylated-saponificated sections were processed for cationic iron colloid staining (pH 1.5-2.0). Adjacent sections were immersed as controls in methanol not containing any HCl.

Some sections were rinsed in a mixture of sodium acetate and NaCl and treated with hyaluronidase (*Streptomyces hyalurolyticus*, Seikagaku Kogyo) (MURAKAMI et al., 1994). The sections were then incubated with cationic iron colloid (pH 1.5-2.0), treated for Prussian blue reaction, and stained with nuclear fast red. Controls consisted of sections treated with a sodium acetate/NaCl mixture containing no hyaluronidase.

Some sections were treated with chondroitinase ABC (*Proteus vulgaris*, Seikagaku Kogyo), heparitinase (*Flavobacterium heparinum*, Seikagaku Kogyo), or keratanase (*Pseudomonas* sp., Seikagaku Kogyo) (MURAKAMI et al., 1994). Some sections were also treated successively with these three enzymes. Some other sections were digested solely with neuraminidase (*Streptococcus 6646K*, Seikagaku Kogyo) or heparinase (*Flavobacterium heparinum*, Seikagaku Kogyo). Controls consisted of sections treated with the respective buffers containing no enzymes. The sections, including the controls, were incubated in cationic iron colloid (pH 1.5-2.0), and treated for Prussian blue reaction.

**RESULTS**

**Electron microscopy**

Transmission electron microscopic observations of ultrathin sections embedded in epoxy resin showed that the rat choroid plexus appeared as a series of tightly packed villous folds which essentially consisted of trilayered structures, i.e., a vascular core of choroidal capillaries, perivascular spaces, and a single row of epithelia (Figs. 1, 2). The choroidal capillaries were lined with highly-fenestrated endothelial cells which had pores closed by a diaphragm, and surrounded by a thin basal lamina (endothelial basement membrane) (Fig. 2). The endothelial cells were occasionally provided with some pericytes. The perivascular space contained leptomeningeal cells, fibroblasts and bundles of collagen fibrils (Figs. 1, 2), and was delimited by a thin basal lamina (epithelial basement membrane), to which was attached a row of low columnar epithelial cells (Fig. 2).

The epithelial cells extended many microvilli into the ventricle (Fig. 1). The side and base of the cells were thrown into numerous tortuous cytoplasmic processes, forming the basolateral folds (Figs. 1, 2). The adjacent epithelial cells were interdigitated by the basolateral folds. Near their apexes, the cells were joined by a zonula adherens and true tight junctions (zonula occludens) (Fig. 1).

Electron micrographic images from LR-White resin embedded sections stained with iron colloid at pH 1.5-2.0 and with uranyl acetate and lead citrate were obscurely contrasted (Figs. 3, 4). It was, nevertheless, confirmed that the luminal surface of the endothelium and the interstitial surface of the endothelial and epithelial basement membranes showed preferential deposits of the clustered colloid particles, which are typically aligned in a stepping stones fashion (Fig. 4). Approximate intervals of the colloid clusters on the ultra-thin sections were: 33±5 nm (mean±SD) at the endothelial luminal surface, 134±18 nm at the endothelial basement membrane, and 39±5 nm at the epithelial basement membrane. The diaphragm of the endothelial fenestrae accompanied smaller-sized depositions of the particles (Fig. 4). Such colloid deposits were not noted inside the endothelial and epithelial cells nor in the connective tissues spaces, except for insignificant solitary deposits of iron particles.

**Light microscopy**

Light microscopic observations revealed that the luminal and abluminal (contraluminal) aspects of the capillaries and the basal aspect of the epithelial cells were strongly reactive to the cationic iron colloid even at a low pH value of 1.5-2.0 (Figs. 5, 6). The same sites were also reactive to aldehyde fuchsin (Fig. 7). No blood vessels reacted to the iron colloid in adjacent brain tissues (Fig. 5).

The reactions of the choroidal capillaries and epithelial basal site to the cationic iron colloid were obliterated by methylation (Fig. 8A) and not reversed by saponification (Fig. 8B). Such obliteration was never noted in control sections.

 Digestion with hyaluronidase thoroughly eliminated
Fig. 1. Survey transmission electron micrograph of a ultrathin section of the rat choroid plexus, embedded in epoxy resin and stained with uranyl acetate and lead citrate. The choroid plexus comprises a series of packed villous folds, which consist of a vascular core of choroidal capillaries (Ca), a layer of perivascular space (PS) containing leptomeningeal cells (Im) and fibroblasts (fb), and a row of epithelium (Ep) with the apical microvilli (mv) and some basolateral foldings (large arrows). The adjacent epithelial cells are interdigitated by the basolateral folds, and attached by apical tight junctions (small arrows). VL ventricular lumen. ×8,000
Fig. 2. Closer view of a part of Figure 1. Endothelium (En) with fenestrae (arrows) ensheathed by a thin endothelial basement membrane (large arrowheads), perivascular space housing a leptomeningeal cell (lm) and a fibroblast (fb), and epithelium (Ep) attached to the thin epithelial basement membrane (small arrowheads) are observable. The basolateral side of the epithelium is intensively infolded as basolateral folds. CaL capillary lumen. ×40,000

Fig. 3. Electron micrograph of an ultrathin section from the rat choroid plexus, embedded in LR-White resin and stained with cationic iron colloid at pH 1.8. The image shows a trilayered structure consisting of central capillaries (Ca), perivascular spaces (PS), and a row of epithelial cells (Ep). mv Microvilli, v venule, large arrows basolateral folds, small arrows junctional apparatus. ×7,700
the cationic iron colloid staining of the choroidal capillaries and the epithelial basal site (Fig. 9A). Such elimination was not observed in the control sections (Fig. 9B).

Successive digestions with chondroitinase ABC, heparitinase, and keratanase considerably reduced the iron colloid staining of the negative-charged sites (Fig. 10A). No reduction in the colloid staining was noted in the control sections (Fig. 10B). It was difficult to eliminate the iron colloid reactions with one or two of these enzymes.

Digestion with neuraminidase or heparinase did not interfere with cationic iron colloid staining of the sites (data not shown).

**DISCUSSION**

Previous electron microscopy of ultrathin sections stained with cationized ferritin, ruthenium red, polyethylenimine, and other cationic probes have revealed that the luminal surfaces of capillaries in various organs, including the fenestrated ones in the rat choroid plexuses, are negatively charged (De Bruyn et al., 1978; Simionescu et al., 1981a, b; Dermietzel et al., 1983; Thurauf et al., 1983; Schmidley and Wissig, 1986a, b; Koshly and Avasthi, 1987). Similarly, it has been also revealed that the capillary endothelial and epithelial basement membranes of various organs, including those of the brain choroid plexus, are intensely negatively charged (Schurer et al., 1978; Kanwar and Farquhar, 1979; Simionescu et al., 1981a, b; Charonis and Wissig, 1983; Dermietzel et al., 1983; Thurauf et al., 1983; Krisch, 1986; Schmidley and Wissig, 1986a, b; Koshly and Avasthi, 1987). However, it is still obscure whether these negative chargings are caused by sulfated proteoglycans (Kanwar and Farquhar, 1979; Simionescu et al., 1981a, Koshly and Avasthi, 1987) or sialoglycoproteins (De Bruyn et al., 1978; Simionescu et al., 1981a; Koshly and Avasthi, 1987).

The present study has confirmed that the luminal
surface of the endothelial cells as well as the interstitial surfaces of the endothelial and epithelial basement membranes in the rat choroid plexus abundantly possess strongly anionic sites reactive to the cationic iron colloid at a low pH value of 1.5-2.0 and aldehyde fuchsin. Moreover, the present light microscopic study shows that the colloid reactions to these anionic sites are obliterated by methylation, and not reverted by saponification. It further shows that the anionic sites are digested with hyaluronidase or chondroitinase ABC/heparitinase/keratanase, but not with neuraminidase or heparinase. These facts indicate that, in the rat choroid plexus, the anionic sites of the endothelial luminal surface as well as those of the endothelial and epithelial basement membranes consist of sulfated (chondroitin/heparan/keratan) proteoglycans. Similar sulfated proteoglycans have been described as the perineuronal extracellular matrix in the brains and spinal cords of man and other mammals, including the rat; sulfate groups and core proteins of the proteoglycans are stained with cationic iron colloid and aldehyde fuchsin, respectively (MURAKAMI et al., 1994, 1995).

The enzyme digestion study of the articular cartilage has shown that proteoglycans attached to collagen fibrils are not easily removed by hyaluronidase...
or chondroitinase treatment, but that collagenase pretreatment is necessary for their removal with those enzymes (YOSHIKAWA et al., 1997). However, the present enzyme digestion study has shown that proteoglycans on the basement membrane can be removed by hyaluronidase or chondroitinase/heparitinase/keratanase digestion with no prior collagenase treatment. The cartilage tissue contains mainly type II collagen (KÜHN, 1987), and the basement membrane contains type IV collagen (GLANVILLE, 1987; OHTSUKA et al., 1992). Furthermore, it is suggested that in the cartilage tissues, type IX collagen is involved in the connection of proteoglycans and collagen fibrils (MÜLLER-GLAUSER et al., 1986). The difference in the enzyme digestion of proteoglycans in situ may be due to the manner of attachment to collagens or other molecules.

The quasiperiodical depositions of clustered colloid particles show that proteoglycans are distributed like dots at regular intervals on the capillary luminal surface. Such periodicity might reflect the fact that polyanionic proteoglycans with membrane proteins float on the lipid bilayer of the plasma membrane and assume constant intervals by electrostatic repulsive force.

Some authors have revealed that cationized feritin with pI values of 7.8–10.0 in the rat choroid plexus preferentially labels the endothelial fenestrae,
especially their luminal side of the diaphragms (PERESS and TOMPKINS, 1981; DERMIETZEL et al., 1983; THÜRAUF et al., 1983; SCHMIDLEY and WISSIG, 1986a). SIMIONESCU et al. (1981a, b) also demonstrated that the fenestrated capillaries in the mouse pancreas and small intestine shows similar reactions to cationized ferritin (pI 7.8–10.0), and that these reactions are erased after in situ perfusion with heparinase. Thus, it was suggested that the negative charging of the fenestrae was brought about by heparan sulfate or heparin. The present results that our cationic iron colloid was scarcely deposited as small sized clusters on the fenestral diaphragm may indicate that such sulfated glycosaminoglycans are small in amount.

Ruthenium red staining of the heparinase- or chondroitinase ABC-digested specimens (KANWAR and FARQUHAR, 1979; SIMIONESCU et al., 1984) and immunostaining using the antibodies against heparan sulfate
or chondroitin sulfate proteoglycans (HASSEL et al., 1980; CHARONIS et al., 1983; AQUINO et al., 1984; LAULIE et al., 1987) demonstrated the presence of heparan sulfate and also chondroitin sulfate proteoglycans in the endothelial and epithelial basement membranes of a variety of rodent organs. The cationic tracers used in these studies were deposited as clusters of their particles at regular intervals. Similarly, our cationic iron colloid, as clustered particles, were distributed on the interstitial surface of the basement membranes.

It was well known that protein concentration is high in blood plasma (6000 mg/dl), but very low in cerebrospinal fluid (20 mg/dl) (GANONG, 1993). The existence of endothelial fenestrae is not necessarily correlated to transportation of macromolecules (MOLLER et al., 1978). An infusion-permeation study using various types of ferritins has shown that cationic ferritin penetrates into the perivascular space through the anionic one persists intravascularly (PERESS and TOMPKINS, 1981; DERMIETZEL et al., 1983). Moreover, it has been established in the rat and mouse that ferritin penetrates into the perivascular space through the fenestrae into the perivascular space (SIMIONESCU et al., 1983). The endothelial fenestrae with negative charging are thus suggested to act as a charge and size barrier to plasma proteins, which are anionic at a physiological pH, are discriminated against by fenestral diaphragms and transported across the endothelium via plasmalemmal vesicles and transendothelial channels in the choroid plexus and other organs (BECKER et al., 1967; SIMIONESCU et al., 1981b; SIMIONESCU, 1983). The endothelial fenestrae with negative charging are thus suggested to act as a charge and size barrier to plasma proteins, and allow water and small solutes to permeate through the fenestrae into the perivascular space (SIMIONESCU, 1983). Similarly, negative charging in the endothelial and epithelial basement membranes acts as a size and charge barrier (KANWAR et al., 1980; KELLY and CAVALLO, 1980).

It is interesting that anionic sites are arrayed more densely or with smaller intervals on the interstitial aspect of the epithelial basement membrane than on that of the endothelial one. These may act as two-step charged filters or sieves.

Another interesting role proposed by OHTSUKA and MURAKAMI (1994, 1997) is that negative charging on the free surface of coelomic mesothelium may reciprocally repel to prevent serosal adhesion or to maintain coelomic cavity. The same role may apply to the anionic charging on the luminal surface of the endothelial plasma membranes. The anionic charging on this surface of the capillary endothelium prevents the non-specific adhesion of blood cells to the capillary wall and also prevents the endothelial adhesion by the repelling of the endothelial cells.

REFERENCES


