Three-dimensional organization of the perivascular glial limiting membrane and its relationship with the vasculature: A scanning electron microscope study

By

Kota WATANABE, Hiroyuki TAKEISHI, Toru HAYAKAWA and Hiroshi SASAKI

Department of Anatomy, School of Medicine, Tokyo Women’s Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo, 162-8666, Japan

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Summary: To examine the three-dimensional structure of the perivascular glial limiting membrane (Glm) and its relationship with the vasculature in rat/mouse cerebral cortices, serial ion-etched plastic sections were observed under the scanning electron microscope and their images were reconstructed. In the case of arterioles and venules close to the pial surface, cord-like principal processes predominantly formed the endfeet; whereas in the case of capillaries and venules, sheet-like secondary processes chiefly formed Glm. Moreover, it was found that several plate-like structures protruded from the basement membrane surrounding the arterioles to penetrate into the astrocytic somata. The perivascular Glm was formed by monolayers of astrocytic processes and/or somata irrespective of the type of blood vessel. However, the thickness of the perivascular Glm, varied greatly according to the type of blood vessel. The thickness of Glm decreased in the order of arterioles, venules and capillaries. The outer surface of the perivascular Glm was extremely irregular, and sheet-like processes arising from this Glm infiltrated into the surrounding neuropil.

Introduction

As is well known, astrocytes have long processes reaching the blood vessels or the pial surface as endfeet to form the perivascular or subpial glial limiting membrane (Peters et al., 1991). As viewed by electron microscopy, nearly all of the intracerebral blood vessels are ensheathed by astrocytic processes irrespective of the type of blood vessel. In studies that investigated the perivascular Glm, however, classifications of intracerebral blood vessels into arterioles, capillaries, and venules have scarcely been attempted. Moreover, in a long history of morphological studies on neuroglial cells, excluding the work of Wolff and Bär (1976), little effort has been placed in determining whether differences in morphological features of the perivascular Glm could be discerned between capillaries and large vessels (arterioles or venules). According to these authors, the perivascular Glm at large vessels of the rat cerebral cortex resembles the subpial Glm. Astrocytic processes border a connective tissue space via basal lamina and tend to become fibrillar, whereas the pericapillary endfeet are formed by very thin lamellar processes. On the other hand, based on our morphological analysis of the anuran astrocytes (Sasaki and Mannen, 1981), we concluded that anuran astrocytic principal processes attach to the subpial basal lamina entering the brain parenchyma together with arteries or veins, whereas they encounter the capillary basal lamina in a mode of juxtaposition. Accordingly, in the present study, we have attempted to address this question by scanning electron microscopy combined with serial ion-etched semi-thin sections and by light and confocal microscopic studies.

Materials and Methods

The present experiments were approved by the Animal Care and Use Committee of Tokyo Women’s Medical University and conformed to the guidelines for the care...
and use of laboratory animals (NIH).

Tsuiiyama method for staining macroglial cells

Brain tissue was obtained from one adult cat that was administered an overdose of sodium pentobarbital by intraperitoneal injection and immersed into 10% formalin fixative for several days. The protocols used were those described by Tsuiiyama (1977). The brain tissue was sectioned at a thickness of 25 μm on a cryostat, the sections were washed in distilled water, transferred into 5% potassium cyanide solution for 30 min, and washed twice in distilled water. These sections were incubated in 10% silver nitrate solution or Cajal’s silver solution (12 ml of 2% silver nitrate solution, 7 ml of 100% ethanol, and 6 drops pyridine) for 30 min and washed in distilled water. Each section was soaked in 1–5% potassium cyanide solution for a few seconds and transferred into distilled water after which it was impregnated in the following solution for 30 s. A mixture of ammonia and pyridine that had been previously prepared at the ratio of 6 drops of pyridine to 2 ml of ammonia was added drop by drop to 10 ml of 10% silver nitrate solution until the solution became opalescent. Then, 10 ml of 5% sodium carbonate solution was added to the obtained solution, which was then reduced in 2% formalin and washed in distilled water. Impregnated sections were toned in 0.1% gold chloride solution and fixed in 5% sodium thiosulfate solution. After washing in distilled water, they were dehydrated in graded alcohol, cleared in xylene, and mounted in balsam.

Confocal laser scanning microscopic study

The cerebral cortices of two young male ICR mice (25–30 g body weight; CREA Japan, Tokyo, Japan) were used in this study. The animals were deeply anesthetized with intraperitoneal sodium pentobarbital (Nembutal, 40 mg/kg body weight). The animals were transcardially perfused with 20–50 ml of Ringer’s solution, followed by 100–250 ml of a fixative containing 2% glutaraldehyde and 3% paraformaldehyde solution in 0.1 M PBS (pH 7.4) at room temperature. After perfusion, the cerebral cortices were dissected out and serially sectioned in the tangential plane from the pial surface to the white matter at a thickness of 200 μm with a tissue sectioner. The slabs were further trimmed into 3 × 3 × 0.2 mm blocks. The small blocks were fixed with the same fixative used for perfusion for 6 h at room temperature. The blocks were macerated with 0.1% osmium tetroxide in acetate-veronal buffer (pH 7.5) for two days at 20°C and then with 0.1% osmium tetroxide in distilled water for two days at 20°C. Tissues were stained en bloc with 1% uranyl acetate for two days, dehydrated in graded ethanol, and flat-embedded in Epon. Serial thick sections were tangentially cut with an ultramicrotome and mounted on silicon wafers. Judging from the interference color, sections were 0.1–0.2 μm in thickness. Sections were ion-etched for 5 min with an ion bombarder (Hitachi VAI5: power of the high frequency radio wave, 1 W) to remove the embedded plastic. This apparatus produces plasmatic argon ions by exciting low-pressure (0.1 torr) argon gas with low-power, high-frequency radio waves. The argon ions selectively etch away epoxy resin. The ion-etched sections were then coated with osmium and observed by a scanning electron microscope (SEM) (Hitachi S-800, S-4300) at a magnification of ×5,000 and at an accelerating voltage of 10 KV. A photographic montage was prepared for each section utilizing Photoshop (ver. 6.0, Adobe) and covered with tracing paper. Identified astrocytic processes were marked with red ink on the tracing paper. The digitalization of the serial drawings and the computer-aided reconstructions were performed with a commercially available software program, TRI/3D-VOL (ver. 3.0, Ratoc System Engineering, Tokyo, Japan).
**Morphometric study**

To measure the thickness of the perivascular Glm, 20 consecutive sections were used, and the ratio of the volume of the perivascular Glm to the surface area of the outermost surface of the vessel was calculated using a commercially available software program (TRI/3D-VOL).

**Results**

To determine whether the perivascular Glm could be morphologically varied between capillaries and large blood vessels such as arterioles or venules, three-dimensional (3-D) reconstructions of the perivascular Glm were produced from serial ion-etched epoxy resin-embedded sections. Prior to describing the SEM results, we first present light and confocal microscopic observations.

**Light microscopy**

To show astrocytes at the level of light microscopy, we adopted the Tsujiyama macroglial staining method (Tsujiyama, 1977). Several stellate astrocytes were found. Several straight processes arise from the somata into the surrounding neuropil, some of which attached to the wall of one large blood vessel. Judging from the caliper and mode of bifurcation, this vessel was identified as a vein (arrows in Fig. 1A). In sharp contrast, astrocytic processes rarely ended on the wall of capillaries. The capillaries could be identified based of the presence of erythrocytes arranged in a line within the vessels (Fig. 1B).

**Confocal microscopy**

After fixation and processing with immunohistochemistry to visualize the location of GFAP, mouse cerebral cortices were studied by confocal microscopy. By forming rosette-like, whirl-like, loop-like, or coil-like endfeet, astrocytic processes attached to the large blood vessels (Figs. 2A and 2B). These vessels could be described as arterioles or venules based on their caliber. In sharp contrast, astrocytic processes rarely ended on the capillary in this mode of attachment. In most cases, a clear contact was not discernible between an individual GFAP-immunoreactive process and the surface of the capillary (Fig. 2C).

**Scanning electron microscopy**

In this study, processes enwrapping blood vessels were regarded as astrocytic (Peters et al., 1991). Processes with exceedingly irregular, rugged contours and fine sheet-like processes frequently surrounding synapses were also regarded as astrocytic (Stensaas and Stensaas, 1968; Spacek, 1971, 1985). In addition, thin sheet-like processes were traced through serial sections to larger processes to confirm their identities. We did not pay attention to the presence of glial filaments for identifying astrocytes because they were not clearly discernible following osmium maceration (Sasaki, 1989). In principle, we referred to thick cord-like processes extending from the somata and their ramified branches as principal processes and to sheet-like spongiform offshoots decorating both principal processes and somata as secondary processes. We then classified intracerebral blood vessels into arterioles, venules, capillaries according to previously described criteria (Frederickson and Low, 1969; Dahl, 1973, 1986; Roggendorf and Cervos-Navarro, 1977; Bär, 1980). One representative ion-etched SEM photograph and the same photograph in which astrocytic processes are marked with red color are depicted in Figs. 3 and 4, respectively. The contours of astrocytic sheet-like secondary processes as well as of thick principal processes were clearly demarcated. It should be noted that individual processes forming the pericapillary Glm can be discerned. We thus reconstructed the perivascular Glm using serial ion-etched epoxy sections. The number of sections used for the 3-D reconstruction of each Glm and the number of lost or damaged sections are presented in Table 1. To facilitate comparison, several photographs were depicted with the same magnification.

**Capillary 1**

The astrocytic soma with an oval nucleus emitted two principal processes (AsP1 and AsP2 in Fig. 5A). AsP1 extended toward the upper left corner, followed a rather
Fig. 2. GFAP-immunostained astrocytes in the mouse cerebral cortex. With rosette-like, whirl-like, loop-like, or coil-like endfeet, astrocytic processes attach to the large blood vessels (A, B), while they rarely come in contact with capillaries with typical endfeet (C). Astrocytes were triply labeled by GFAP (green), lectin (red), and nucleic acid (blue) immunofluorescence in Figs. A and C, while those in B were doubly labeled with GFAP and lectin. Scale bars: 10 μm (A) and 5 μm (B, C).

Fig. 3. One representative ion-etched SEM photograph. Thirty-eighth section in the case of Capillary 2. A transversely sectioned capillary is found in the upper left corner, and one astrocytic soma is founded in the lower right corner of the figure. Mouse cerebral cortex. Scale bar: 5 μm.

Fig. 4. The same SEM photograph as shown in Fig. 3. In this figure, astrocytic processes are marked with red color, including processes surrounding the capillary. Note that most of them are irregular and rugged in contours. Scale bar: 5 μm.
straight trajectory in close apposition to two neuronal cell bodies, and then ramified into several finer processes. Passing by a capillary, this principal process issued stout short side-branches that enveloped almost the entire circumference of this capillary wall (Fig. 5B). The pericapillary Glm was composed of two processes (magenta and yellow in color). Mouse cerebral cortex. Scale bars: 5 μm.

### Table 1. Sections used for the 3-D reconstruction of the perivascular Glm

<table>
<thead>
<tr>
<th>blood vessel</th>
<th>total number of used sections</th>
<th>missing sections</th>
<th>species of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>capillary 1</td>
<td>54</td>
<td>No.28</td>
<td>mouse</td>
</tr>
<tr>
<td>capillary 2</td>
<td>81</td>
<td>No.26, 40, 71</td>
<td>mouse</td>
</tr>
<tr>
<td>capillary 3</td>
<td>59</td>
<td>No.50</td>
<td>mouse</td>
</tr>
<tr>
<td>arteriole</td>
<td>24</td>
<td>No.22, 23</td>
<td>rat</td>
</tr>
<tr>
<td>venule and capillary 4</td>
<td>51</td>
<td>–</td>
<td>rat</td>
</tr>
<tr>
<td>connection 1</td>
<td>36</td>
<td>No.22</td>
<td>rat</td>
</tr>
<tr>
<td>connection 2</td>
<td>25</td>
<td>No.3, 23</td>
<td>rat</td>
</tr>
</tbody>
</table>

Connection 1: Connection between two capillaries and one venule
Connection 2: Connection between one capillary and one venule

Capillary 3

One cross-sectioned capillary with one pericytic soma (p) was observed in the middle part. Two somata of oligodendrocytes (o) are shown, and the one in the left corner issued one thick process downward (Fig. 7A). The pericapillary Glm was mainly composed of thin sheet-like processes, and from this Glm emanated several secondary processes that infiltrated into the surrounding neuropil (arrows in Fig. 7B). Primarily, sheet-like secondary processes filled in the neuropil and surrounded this capillary wall.

Arteriole

Two astrocytic somata attached to the surface of an arteriole in a face-to-face mode, although one was devoid of its nucleus within the range of reconstruction (Fig.
Several thin plate-like structures protruded from the basement membrane surrounding the arteriole to penetrate into the astrocytic somata (arrowheads in Fig. 8C). It should be noted that these plate-like basement membranes were never observed in any other type of blood vessel. The lower left soma issued five principal processes, and the upper right soma issued three (AsP1–AsP8 in Fig. 8B). These processes were decorated with sheet-like secondary processes that were fully adapted to the surrounding structures of the neuropil. On the other hand, the neuropil was filled with numerous sheet-like secondary processes.

**Venule**

The abluminal surface of this venule was covered by five astrocytic processes (Fig. 9). In the upper right...
corner, one astrocytic soma projected two thick, short principal processes (AsP1 and AsP2); AsP1 attached to the vessel wall and AsP2 ran upward. In the lower right corner, one thick principal process (AsP3) appeared to make direct contact with this vessel wall. The process (light blue in color) adjacent to this process had one principal process (AsP4). The other processes (AsP5 and AsP6) attached to the vessel wall did not appear to have principal processes within the range of observation. Instead of principal processes, thin secondary processes appeared to surround the capillary wall (Fig. 9B).

Connection between capillaries and a venule
1) Two capillaries joining one venule
Two longitudinally sectioned capillaries joined and merged into a cross-sectioned venule (Fig. 10). Glm of the venule was composed of six astrocytic processes. Four of the six processes had a rather thick principal process. On the other hand, very fine processes connected the pericapillary Glm and the rather thick processes close to the capillary (arrows in Fig. 10C). Glm of the venule was clearly thicker than that of the capillary. Several thick astrocytic processes were observed near the capillary wall (arrowheads in Fig. 10B), but none of them made direct contact with the capillary wall.

2) One capillary joining one venule
One longitudinally sectioned capillary joined a cross-sectioned venule (Fig. 11A). At the junctional site, a pericyte was found (p in Fig. 11C). Glm of the

Fig. 8. Glm ensheathing an arteriole in the rat cerebral cortex. A. The periarterior Glm is composed of two astrocytic somata in a face-to-face manner. B. The lower left soma issues five principal processes and the upper right soma issues three principal processes (AsP1–AsP8). C. Several thin plate-like structures emanate from the basement membrane surrounding the arteriole to protrude into the astrocytic somata (arrowheads). It should be noted that these plate-like basement membranes were never observed at any other type of blood vessel. Rat cerebral cortex. Scale bars: 5 μm.
A venule was composed of eight astrocytic processes. Three of the eight processes appeared to have a thick principal process (AsP1, AsP2, and AsP5 in Fig. 11B). In sharp contrast to these thick principal processes reaching the venule wall, this type of process was not found on the wall of the capillary.

Thickness of the vascular Glm

The thickness of the perivascular Glm of each vessel reconstructed in this study is shown in Table 2 and Fig. 12. The perivascular Glm thickness is described as the ratio of the volume of the perivascular Glm to the surface area of the outer surface of the blood vessel. It is clear from these data that the thickness of the perivascular Glm increased step-by-step in the order of capillary, venule, and arteriole.

Discussion

The aim of the current study was to examine whether there were some morphological differences in the perivascular Glm between capillaries and large blood vessels (an arteriole or a venule). For this purpose, 3-D reconstruction of the perivascular Glm was made by SEM observation of serial ion-etched epoxy sections. As a result, we revealed some morphological differences in the perivascular Glm between these two types of blood vessels.

Comments on methods

In 3-D reconstruction using the transmission electron microscope (TEM), which has very high-resolution capability, a large number of serial sections must be
obtained by elaborate techniques without loss or damage of sections. In this context, the combination of semi-thin sections on SEM appears to be an efficient approach that overcomes the resolving power limitations of the light microscope and circumvents from the cumbersomeness of processing a great number of ultrathin serial sections for TEM. Thus, we adopted this method in the present study. This method was already used by some authors (Shimizu et al., 2001; Fujiwara et al., 2000). Using this method, Shimizu et al. reconstructed a newly formed blood vessel in the rabbit ear chamber. According to our experience, this method is positioned between light microscopy and TEM in terms of resolution, although it is rather closer to the latter method. The method adopted in this study has several advantages: (1) one can reconstruct any structures of interest with the present method from a smaller number of serial sections in comparison with TEM and (2) no laborious or time-consuming techniques are needed for SEM reconstruction. However, this method has some limitations, namely it was very difficult to reconstruct tangentially cut astrocytic thin processes with this method. As noted by Spacek (1985), it is almost impossible to trace dendritic spines, even using ultrathin serial sections. We agree with this author.

Fig. 10. Two capillaries simultaneously join one venule. 
A, B. Glm of the venule is composed of six astrocytic processes. Four of the six processes have rather thick principal processes. Glm of the venule is clearly thicker than that of the capillary. Several thick astrocytic processes are observed near the capillary wall (arrowheads in Fig. 10B), but none of them make direct contact with the capillary wall. C. Very fine processes connect the pericapillary Glm and rather thick processes close to the capillary (arrows). Rat cerebral cortex. Scale bars: 5 μm.
In our previous study (Sasaki and Mannen, 1981), based on the observations made with astrocytes in the bullfrog spinal cord, we suggested that mammalian astrocytes attach to the subpial basement membrane as well as to the basement membrane, which is equivalent to the subpial basement membrane, and surround the capillary merely via juxtaposition. Consequently, we reconstructed the mammalian perivascular Glm using serial sections using an SEM in this study. By reconstructing astrocytic processes of the rabbit brain at the electron microscope level, Wolff and Bär (1976) revealed that the perivascular Glm was morphologically different between the capillary and large vessels such as arterioles or venules.

3-D reconstruction of the perivascular Glm by means of SEM with serial ion-etched plastic sections

Table 2. Thickness of the Glm.

<table>
<thead>
<tr>
<th>blood vessel</th>
<th>thickness of Glm (μm)</th>
<th>volume/area</th>
<th>sections for reconstruction</th>
</tr>
</thead>
<tbody>
<tr>
<td>a cap 1</td>
<td>0.13</td>
<td>7.5/56.9</td>
<td>No.30–49</td>
</tr>
<tr>
<td>b cap 2</td>
<td>0.33</td>
<td>24.0/73.5</td>
<td>No. 1–20</td>
</tr>
<tr>
<td>c cap 3</td>
<td>0.26</td>
<td>20.5/80.0</td>
<td>No.40–60</td>
</tr>
<tr>
<td>d cap 4</td>
<td>0.28</td>
<td>21.3/76.9</td>
<td>No. 1–20</td>
</tr>
<tr>
<td>e connection 1</td>
<td>0.83</td>
<td>346.0/414.5</td>
<td>No. 1–20</td>
</tr>
<tr>
<td>f connection 2</td>
<td>0.88</td>
<td>282.0/322.1</td>
<td>No. 1–21</td>
</tr>
<tr>
<td>g venule</td>
<td>0.92</td>
<td>383.9/417.0</td>
<td>No. 1–20</td>
</tr>
<tr>
<td>h arteriole</td>
<td>2.66</td>
<td>960.9/360.8</td>
<td>No. 1–21</td>
</tr>
</tbody>
</table>

The thickness is expressed as the ratio of the volume of Glm to the surface area of the outer surface of the blood vessel. Twenty serial sections were used for measurements. For these measurements, principal and secondary processes associated with the flattened process in direct contact with the vessel wall were omitted. In the cases of connections 1 and 2, only a part of the venule was measured.

Fig. 11. One capillary joins a venule.
A, B. Glm of the venule is composed of eight astrocytic processes. Three of the eight processes appear to have thick principal processes (AsP1, AsP2, and AsP5 in B). On the other hand, this type of process is not found on the wall of the capillary. C. At the site of the junction, a pericyte is found (p). Rat cerebral cortex. Scale bars: 5 μm.
According to these authors, the pericapillary Glm was formed by one thin tube composed of several sheet-like processes. These sheet-like processes arose from processes situated close to the capillary. No thick cord-like processes were observed attaching to the capillary wall. They showed that the periarterial Glm was composed of astrocytic somata, but thick cord-like processes presumably participated in the Glm formation by producing “endfeet.” In our current study, we principally confirmed their observations and further found that several plate-like structures protruded from the basement membrane, ensheathing the arteriole to penetrate into the astrocytic somata. Similar structures have already been reported (Cancilla et al., 1972\textsuperscript{16}; Lafarga et al., 1991\textsuperscript{17}). The enfolding basement membranes are consistently found on the surface of the mammalian brain (Bondareff and McLone, 1973\textsuperscript{18}), and in this respect, the periarterial Glm exceedingly resembles the subpial Glm. Glm surrounding the large venule, on the other hand, was formed by one thick tube composed of several thick processes, as depicted in Fig. 9B. Three of five processes were found to have thick cord-like principal processes. This type of process presumably corresponds to those described in the classical literature as “vascular endfeet”. The present study revealed three morphological differences of Glm between small venules and capillaries: (1) in the former, cord-like thin processes predominantly compose Glm,
while in the latter, secondary processes predominantly form Glm; (2) Glm is clearly thicker in the former than in the latter; and (3) one astrocytic sheet-like process tends to cover a greater part of the entire circumference of the vessel wall in capillaries than in larger blood vessels. In spite of these morphological differences, Glms are quite similar between venules and capillaries. These results appear to represent quantitative differences rather than qualitative differences concerning the structure of Glm. According to Peters et al. (1991)\(^9\), the only essential difference between a vein and a capillary lies in the diameter of the lumen. On the other hand, our TEM experiences thus far demonstrate that attachment apparatus such as hemidesmosomes and/or dense plaques are not observed in the astrocytic processes directly surrounding the capillary wall. Postcapillary venules also revealed no presence of such an attachment structure. However, in the venules close to the pial surface, a perivascular space was recognizable, and the attachment apparatus appeared in the astrocytic processes attached to the wall of this vessel. Concerning the artery, attachment apparatus are consistently found (unpublished personal observations). These observations are in agreement with those published previously (Lange and Halata, 1972)\(^20\). Consequently, it is highly likely that at a certain transition site from a postcapillary venule to a large venule, the capillary-type Glm is transformed into a pial surface-type or artery-type Glm. In addition, several recent papers indicated that concerning the perivascular Glm, there are some morphological differences between the capillary and large vessels (Kacem et al., 1998\(^21\); Zaks and Wu, 2001\(^22\); Nedergaard, 2003\(^23\)). Regarding the thickness of the perivascular Glm, it varied greatly according to the type of blood vessel. The thickness of Glm decreased in a step-by-step manner in the order of arterioles, venules, and capillaries (Fig. 12). Immunohistochemically, GFAP was mainly located in the somata and principal processes with almost no GFAP found in the sheet-like processes (Eng and DeArmond, 1982\(^24\); Bailey and Shipley, 1993\(^25\)). Accordingly, the confocal microscope images reported in the present study reflect this distribution pattern of GFAP. Because the pericapillary Glm consists mainly of sheet-like processes, the outer surface of the capillary was rarely decorated with fluorescence.

**Functional difference of Glm between capillaries and large blood vessels**

To our knowledge, it is not known whether some functional difference could be present between capillaries and large vessels. Aquaporin-4 (AQP4), a member of the water channel family expressed exclusively by astrocytes in the brain, is immunohistochemically distributed on the plasma membrane attaching to the basement membrane of both capillaries and large vessels (Nielsen, 1997\(^26\); Nagelhus, 1998\(^27\); Nico, 2001\(^28\)). Thus, AQP4 immunohistochemistry outlines the entire network of intracerebral blood vessels, demonstrating that astrocytic endfeet almost completely cover the intracerebral vasculature. On the other hand, Cx43, a gap junction protein, is expressed primarily on astrocytes (Giaume and McCarthy, 1996\(^29\)). In the cortex of adult rats, Cx43 immunoreactivity was intensely detected around large and medium-sized vessels. However, whether this difference in immunoreactivity between small and large vessels can indicate some functional difference remains to be determined.

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**References**


