Isolated Rat Brain Capillaries Possess Intact, Structurally Complex, Interendothelial Tight Junctions; Freeze-Fracture Verification of Tight Junction Integrity

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(Accepted April 17th, 1984)

Key words: isolated brain capillaries — freeze-fracture — blood–brain barrier — tight junction — rat

INTRODUCTION

Investigations of the functional characteristics of brain capillaries, including the phenomenon of the blood–brain barrier, have been facilitated by the use of populations of isolated brain capillaries. Brain capillaries, pelleted from brain tissue from a variety of large mammals, have been intensively studied and shown to exhibit in vitro, many of the physiological and biochemical (enzymatic) properties they display in vivo.

Recently, studies on the pathology of brain vasculature and blood–brain barrier have employed in vitro preparations of capillaries as models of in vivo vascular elements. The blood–brain barrier, the anatomical basis of which is the system of occluding interendothelial junctions, is presumed present and functional during these investigations of isolated brain capillaries. However, the presumed lability of endothelial zonulae occludentes as suggested by numerous studies purporting to have experimentally 'opened' endothelial tight junctions (see Rapoport21 for review) casts uncertainty on the wisdom of an a priori assumption of barrier integrity in the in vitro models of brain capillaries. Thorough assessment of the structural nature of endothelial zonulae occludentes of isolated brain capillaries, therefore, is mandatory before experimentation on the permeability of isolated capillaries, based on presumed impermeability of endothelial junctions, can proceed.

Perhaps the best tool for assessing brain endothelial tight junction morphology, and integrity, is freeze-fracture. This technique has been convincingly used to characterize zonulae occludentes in brain...
capillary endothelium of reptiles and rats, and has proven essential for verification of junction integrity in protocols that insult the brain neuropil vasculature. The present study was initiated to address the question, do the endothelial zonulae occludentes of isolated brain capillaries retain their structural integrity (and seal) during the rigors of the isolation procedures? If so, then isolated brain capillaries can be used with confidence as models of brain neuropil microvessel systems.

MATERIALS AND METHODS

Capillaries used in this study were isolated from Sprague−Dawley rats according to the procedure of Betz et al. Pellets of brain capillaries in HEPES buffer were lightly fixed at room temperature for 1 h by resuspension in 3% glutaraldehyde buffered to pH 7.35 with 0.1 M sodium cacodylate. Following aldehyde fixation, the capillary fragments were pelleted at 400 g for 5 min and resuspended 3 times in several changes of buffer (0.1 M sodium cacodylate) and finally dispersed in 30% glycerol in 0.1 M sodium cacodylate (pH 7.35) overnight in the refrigerator. The following day, the glycerol−capillary solution was spun in a clinical centrifuge at 400 g for 15 min until the capillary fragments formed a soft pellet at the bottom of a 15-ml plastic centrifuge tube. This pellet was then resuspended in 1 or 2 drops of fresh 30% glycerol in buffer and single drops of the highly concentrated suspension of capillaries were placed on each of several gold specimen discs and frozen in a slurry of liquid nitrogen-cooled Freon-22. The samples, which had been stored in liquid nitrogen until fracturing, were fractured and replicated with platinum and a thin layer of stabilizing carbon, in a Balzers BAF 301 Freeze-Etch Unit (Balzers, Liechtenstein) according to the method of Shivers and Brightman. Platinum replicas of the isolated brain capillaries, mounted on bare 200-mesh copper grids, were examined in a Philips 201 electron microscope operating at an accelerating voltage of 60 kV.

RESULTS

Pellets of isolated rat brain capillaries are seen in platinum replicas of freeze-fractured samples, to consist primarily of capillary segments of variable length (Fig. 1). The fracture plane often passes along the surface of some of the vessel segments to yield profiles of the intact pieces of capillary that are enwrapped by the PF fracture face of either the pericyte plasma membrane or the PF fracture face of the endothelial plasmalemma (Fig. 1). Alternatively, the path of the fracture may cleave a capillary longitudinally, or cross-fracture it (Fig. 2), to reveal the internal features of the vessel lumen and endothelial cytoplasm. Electron microscopic examination of the platinum replicas of fractured capillary pellets at low magnification reveals several conspicuous features including the general purity of the pellet, with little extraneous neuropil or cellular debris (Fig. 1) and, more importantly, the remarkable preservation of structural integrity and organization displayed by most pieces of capillary (Figs. 1, 2).

Many capillary fragments possess a pericyte sheath which had apparently withstood the rigors of the initial isolation procedure (Figs. 1, 2, 3). Careful examination of capillary segments ensheathed with pericyte cytoplasmic processes revealed substantive morphological differences between the endothelial cell plasma membrane fracture face and that of the pericyte (Figs. 1, 3, 5) which serve as useful structural criteria for discrimination between the two. The pericyte plasmalemma possesses a striking population of EF fracture face protrusions — the pinocytotic vesicles (Figs. 1, 5), whereas the EF fracture face of the capillary endothelial plasma membrane (both

Fig. 1. Intact segment of isolated brain capillary. The outer surface of the vessel is covered by the PF fracture face of the pericyte plasmalemma which exhibits numerous small depressions (arrows) characteristic of pinocytotic vesicles. The area around this vessel (asterisk) is conspicuously free of debris and elements of CNS neuropil. × 19,600.

Fig. 2. Cross-fracture of capillary and its pericyte (P) investment. No evidence of structural damage is visible. The endothelial cytoplasm (E) exhibits few pinocytotic vesicles (arrows) and in this region of the vessel, the pericyte (P) is generally devoid of vesicular elements. F, marginal fold; BL, basal lamina of capillary; L, capillary lumen. × 32,250.

Note: arrow in lower right corner of each figure denotes direction of platinum shadowing on the replica.
luminal and abluminal) was generally free of structural protrusions except, near sites of apposition (junctional) of endothelial cytoplasmic processes (Fig. 2).

Cross-fractures of capillary endothelial cytoplasm (Figs. 2–4) show it to be well-preserved and free of apparent structural damage as a result of isolation stress. For example, pinocytotic vesicles exhibit a typically uniform flask-shaped profile (Figs. 2, 3) and the plasma membrane of the endothelial cells is intact with absolutely no evidence of disruptions, perforations, or clumping of intramembrane particles. Examination of the replicas in the immediate vicinity of the capillary fragments (Figs. 1, 2) demonstrates the cleanliness of the pellets and absence of fragmented vessels and debris. The cytoplasm of endothelial cells is also free of debris, vacuoles and other features symptomatic of poor preservation and freezing damage.

Zonulae occludentes situated between apposed endothelial cytoplasmic processes of the capillary segments appear in platinum replicas either as cross-fractured focal tight junctions (Fig. 2, inset Fig. 7) or as a highly complex network of parallel rows of anastomosing ridges on exposed PF fracture face surface (Figs. 6–9). Intramembrane EF fracture face grooves complementary to the ridges are often seen as well (Fig. 8). The exposed intramembrane elements of endothelial zonulae occludentes are remarkably regular in construction and integrity (Fig. 7) with no evidence of damage from either the isolation procedure or routine preparation of them for freeze-fracture. The parallel ridges are generally complete (Fig. 9) and in instances where intervals in their profiles are apparent, the gaps can clearly be accounted for by the particles situated in the grooves on the complementary intramembrane EF fracture face (Fig. 8). Furthermore, the extensive anastomoses between parallel intramembrane ridges (Figs. 6, 7) and grooves (Fig. 8) exhibited by these isolated brain capillary endothelial tight junctions correspond precisely to the profiles obtained from brain capillaries sampled in vivo.

Samples of rat brain capillaries that have been isolated, purified, and concentrated into generally homogeneous pellets contain vessels lined with endothelial cells which, when freeze-fractured, are not only structurally indistinguishable from those sampled from intact neuropil, but also, they more frequently yield extensive views of the junctional membrane interface between endothelial processes (Fig. 6). In these instances, the areas of intramembrane fracture face that possess the ridge and groove components of the tight junctions are unusually large and present a much more complete blueprint of interendothelial zonula occludens architecture (Figs. 6–8). For example, the extent of junction-containing membrane exposed in the platinum replica in Fig. 6, is much greater than any that has been published in the blood–brain barrier literature to date, except for interendothelial junctions of brain capillaries of the American chameleon, Anolis carolinensis⁴. The frequency of capillaries which display large areas of junction-containing membrane is greater in replicas of capillary pellets than in replicas of intact brain tissue simply because the pellets contain mostly capillary segments and little, if any, brain tissue, thus the frequency of finding fractured capillaries is greater.

DISCUSSION

Populations of isolated, purified rat brain capillaries have constituted an invaluable in vitro model for studies of the blood–brain barrier. Both physiological as well as biochemical investigations of preparations of brain capillaries have unquestionably con-

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Fig. 3. Cross-fracture through a longitudinally oriented segment of capillary endothelial cell. The cytoplasm (C) is filled with pinocytotic vesicles and similar vesicles are attached to both luminal (white arrowheads) and abluminal (black arrows) surfaces of the cell. L, capillary lumen; BL, basal lamina; P, pericyte. × 40,000.

Fig. 4. High magnification of a portion of the EF (EF) fracture face of the luminal plasma membrane of an endothelial cell. Arrows denote sites where pinocytotic vesicles appear to arise from this surface of the vessel. No evidence of structural damage can be seen. L, capillary lumen. × 51,000.

Fig. 5. High magnification of a segment of EF (EF) fracture face of pericyte plasmalemma which illustrates the numerous protrusions (P) associated with pinocytotic vesicles. Compare this figure with that of the endothelial plasmalemma (Fig. 4). × 51,000.
Fig. 6. Freeze-fracture frequently exposes broad expanses of tight junction-containing intramembrane surface (area between brackets) of the capillary endothelium (E). The complexity and beauty of this zonula occludens is clearly appreciated and seen to consist of numerous parallel ridges (R) which are highly anastomosed (arrows). This view of a brain vessel endothelial tight junction is the most extensive that has been demonstrated to date. L. lumen; BL. basal lamina; PF, PF fracture face  × 40,000
firmed the functional identity of the model system to in vivo systems. In addition, these studies, and others, have elegantly demonstrated the use of the model of isolated brain capillaries for numerous physiological and biochemical probes of brain endothelial function and of course, exploration of the function of the blood–brain barrier. 

In spite of the remarkable similarity of populations of isolated brain capillaries to those examined in vivo, and the increasing use of them for a broad spectrum of functional and experimentally-oriented investigations, relatively little is known about the morphological features of these vessels. This is especially surprising since many studies of isolated brain capillaries have been directed toward those features associated with their participation in the blood–brain barrier. Yet, the anatomical basis of the barrier, interendothelial zonulae occludentes of the capillary endothelium, has been neglected.

The structural integrity of the interendothelial zonulae occludentes of the endothelial lining of isolated brain capillaries is reported here for the first time. The freeze-fractured capillary pellets have revealed capillaries with tight endothelial junctions that are more structurally complex than those reported for other brain capillaries studied to date, with the exception of those in the lizard brain neuropil which are more elaborate. In fact, there is striking similarity between the architecture of tight junctions of isolated brain capillaries and tight junctions of other tissues which have been considered 'very tight'. On the basis of the observations on the anatomy of interendothelial zonulae occludentes of isolated brain capillaries made in this study, it can be concluded that the tight junctions are, in the first instance, intact, and secondly, probably very tight and impermeable to most of the same molecules as their in vivo counterparts. With respect to tight junction integrity, the model of isolated brain capillaries is acceptable and desirable for blood–brain barrier studies.

A very significant aspect of this study has been to demonstrate the high degree of stability of tight junction structure exhibited following the physically stressful procedure of isolation and purification of them from intact neuropil. If the intramembrane components of the zonulae occludentes are as labile as proposed by some investigators of the blood–brain barrier, then certainly, isolation of them should provide sufficient force to tear them apart. This is clearly not so! The junctions have remained stable, highly complex units with normal functional properties. Thus, results of this study which have shown intact zonulae occludentes, provide circumstantial support for the notion that these junctions in intact neuropil are sufficiently stable that they may not readily disorganize following physical stress. Moreover, this type of preparation may serve as a useful model for further studies of tight junctional stability.

Future studies of the functional activities of isolated brain capillaries under normal physiological parameters, and experiments designed to explore the effects of pathologic agents on capillary activity and blood–brain barrier integrity, should benefit from monitoring the capillary and interendothelial junction morphology with freeze-fracture techniques. This tool is clearly the only method of preparing the capillary tight junctions whereby vast areas of junction-containing intramembrane surface can be consistently exposed for detailed analyses of junction integrity.

ACKNOWLEDGEMENTS

Supported by the Natural Science and Engineering Council of Canada (R.R.S.), The National Foundation — March of Dimes (G.W.G.) and Grants HL-25492 (A.L.B.) and ES-02380 (G.W.G.) from the National Institutes of Health. A.L.B. is the recipient of an Established Investigatorship from the American Heart Association.
REFERENCES


Fig. 7. High magnification of capillary endothelial plasmalemma (PF) fracture face showing details of the anastomosed (arrows), parallel ridges (R) that constitute the zonula occludentes. These intramembrane junctional elements are intact and the complexity of their interrelationships is obvious. Comparison of this view of the tight junction with that seen in cross-fracture (arrows, inset) exemplifies the usefulness of freeze-fracture in revealing the true character of brain endothelial tight junctions. L, lumen. × 47,000. Inset: × 54,000.

Fig. 8. The EF fracture face (EF) of capillary endothelial plasma membrane reveals the complex system of intramembrane grooves (G) complementary to ridges seen on PF fracture faces (see Figs. 6, 7). The grooves possess some intramembrane particles (arrows) that have been lost from the ridges during cleavage. The high degree of interlock between the junctional elements is particularly conspicuous in this micrograph. × 54,000.

Fig. 9. Many intramembrane ridges of the brain capillary endothelium zonulae occludentes appear double (black arrows) when viewed at very high magnification. The branches between parallel ridges are of narrower width (white arrows). L, lumen; BL, basal lamina: × 102,000.


