Brain Research, 324 (1984) 313–322
Elsevier

BRE 10448

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

RICHARD R. SHIVERS[1], A. LORRIS BETZ[2] and GARY W. GOLDSTEIN[2]

[1]*Department of Zoology, University of Western Ontario, London, Ontario (Canada) and* [2]*Department of Pediatrics, University of Michigan, Ann Arbor, MI (U.S.A.)*

(Accepted April 17th, 1984)

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

Populations of isolated brain capillaries have been proposed as useful models for in vitro studies of the blood–brain barrier. Preliminary investigations of barrier properties using such preparations of brain microvessels have suggested that the tight interendothelial junctions (zonulae occludentes) are intact and retain the impermeability to the protein tracer horseradish peroxidase, exhibited by them in vivo. The endothelial junctions of isolated capillaries are therefore assumed to be functionally 'tight' in vitro. In order to determine the precise structural organization of these occluding junctions, including an estimate of their tightness (complexity), and to demonstrate a method for simple but precise assessment of junctional integrity, pellets of isolated rat brain capillaries were freeze-fractured and then replicated with platinum and carbon. The freeze-fracture images of interendothelial zonulae occludentes revealed complex arrays of intramembrane ridges and grooves characteristic of tight junctions. Longitudinal fractures of the cellular lining of capillaries exposed vast expanses of interendothelial plasma membrane interfaces and the junctional complexes situated between the cells. From such arrays, the elaborate and complex architecture of the zonulae occludentes could be readily appreciated. Situated on the PF fracture faces are 6–8 parallel ridges which display a high degree of anastomosing between adjacent strands. The EF fracture face contains grooves complementary to the PF face ridges. The zonulae occludentes of these capillary endothelial cells are similar in complexity to those reported in the literature for reptilian brain capillaries and therefore can be presumed 'very tight'. This study demonstrates that freeze-fracture of pellets of brain capillaries alleviates sampling problems inherent in whole tissue preparations and, in addition, demonstrates the usefulness of freeze-fracture as a tool to monitor junction structure during in vitro investigation of the blood–brain barrier.

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[11,13,20,32,34], have been intensively studied and shown to exhibit in vitro, many of the physiological and biochemical (enzymatic) properties they display in vivo[1,2,4,10,12,13,15,16,17,30,32].

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[7,14,18,23,33]. The blood–brain barrier, the anatomical basis of which is the system of occluding interendothelial junctions[3,26], 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 Rapoport[21] 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

Correspondence: R. R. Shivers, Department of Zoology, University of Western Ontario, London, Ontario, Canada N6A 5B7.

0006-8993/84/$03.00 © 1984 Elsevier Science Publishers B.V.

capillary endothelium of reptiles[26], and rats[9], and has proven essential for verification of junction integrity in protocols that insult the brain neuropil vasculature[27]. 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.[2]. 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[28]. 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).

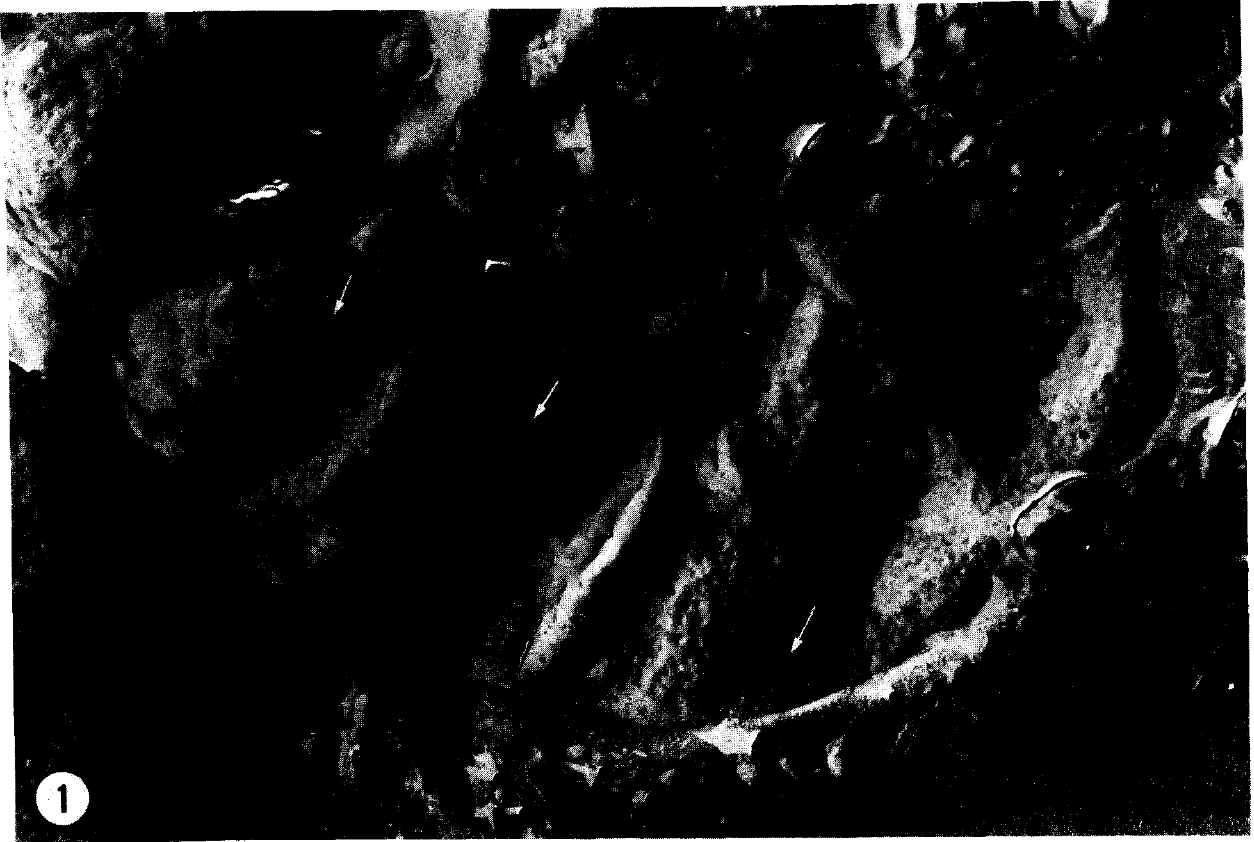
Many capillary fragments possess a pericyte sheath which had apparently withstood the rigors of the initial isolation procedure (Figs. 1, 2, 3). Careful examination of capillary segments ensheathed with pericyte cytoplasmic processes revealed substantive morphological differences between the endothelial 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

→

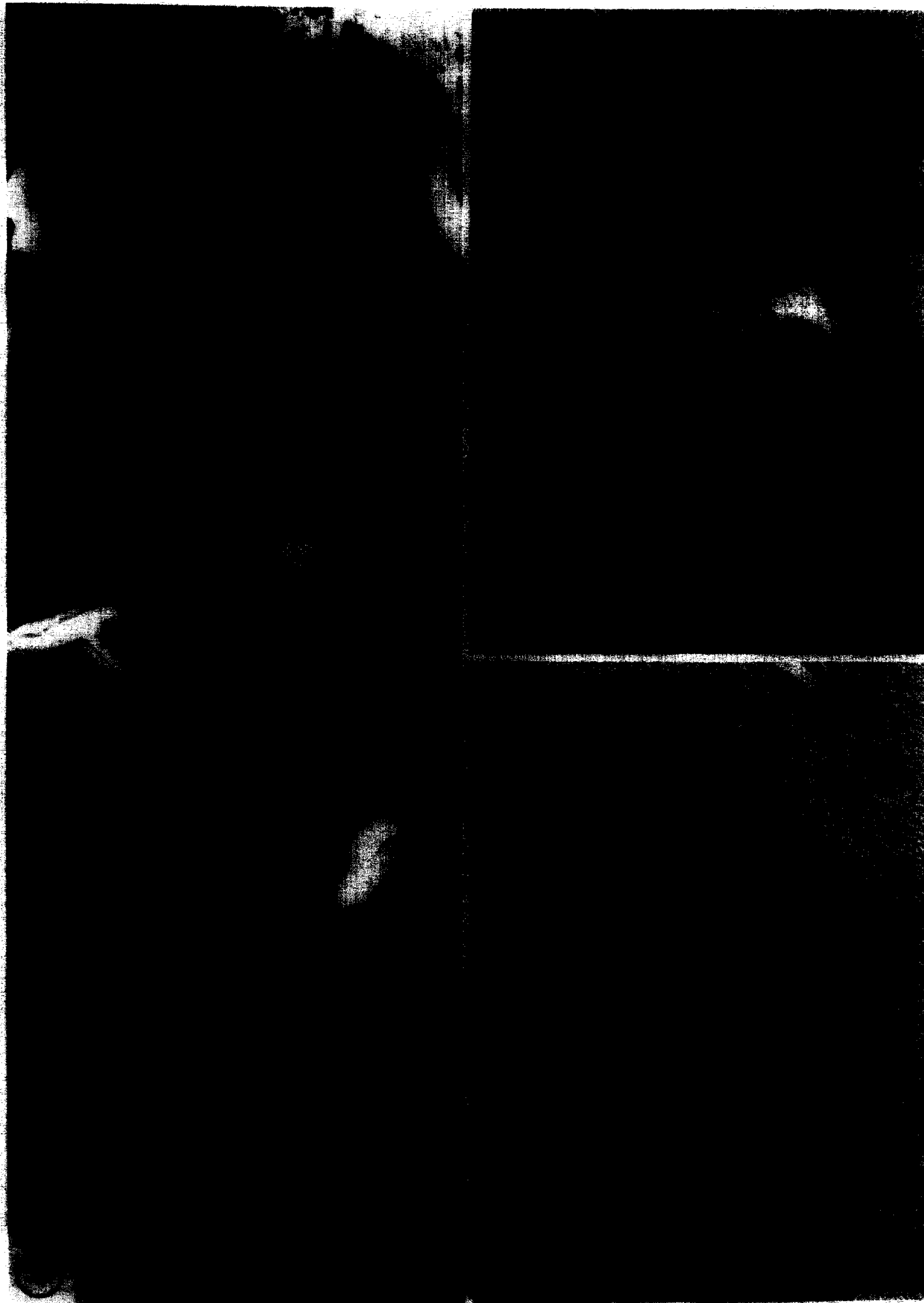
Fig. 1. Intact segment of isolated brain capillary. The outer surface of the vessel is covered by the PF fracture face of the pericyte plasmalemma which exhibits numerous small depressions (arrows) characteristic of pinocytotic vesicles. The area around this vessel (asterisk) is conspicuously free of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


1

2



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 carolinensis*[26]. 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-

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[1,4,10,11,12,13,17,20,32]. 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[2,7,14,15,16,18,23].

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[1,2,13,17,18,20]. Yet, the anatomical basis of the barrier, interendothelial zonulae occludentes of the capillary endothelium[3,21,22,26], has been neglected.

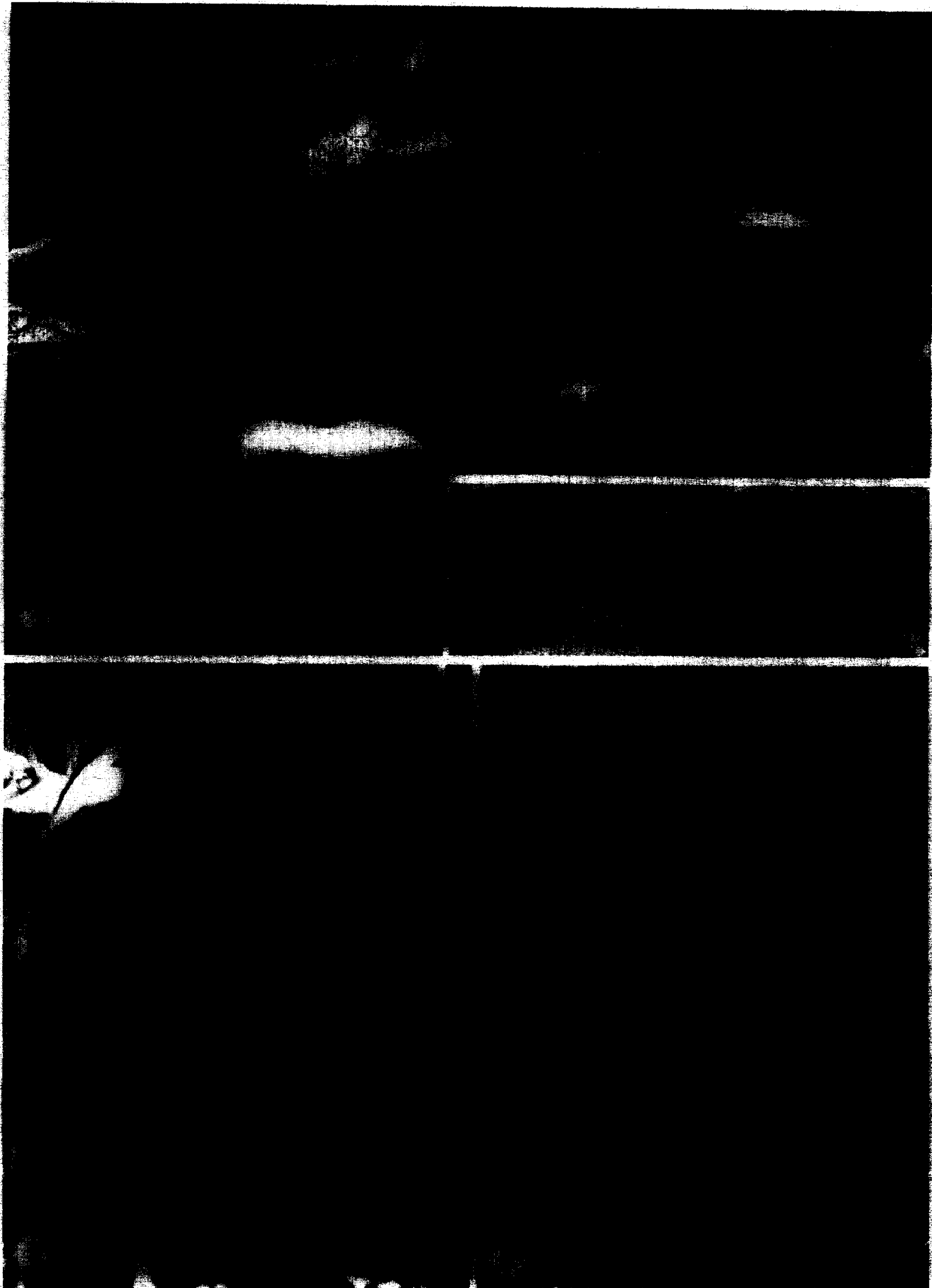
The structural integrity of the interendothelial zonulae occludentes of the endothelial lining of isolated brain capillaries is reported here for the first time. The freeze-fractured capillary pellets have revealed capillaries with tight endothelial junctions that are more structurally complex than those reported for other brain capillaries studied to date[6,8,31], with the exception of those in the lizard brain neuropil which are more elaborate[26]. 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'[5,19,24,25,26,27]. 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[21]. 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[21], 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[9,26,27,29].

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

1 Betz, A. L. and Goldstein, G. W., Polarity of the blood–brain barrier: neutral amino acid transport into isolated brain capillaries, *Science*, 202 (1978) 225–227.
2 Betz, A. L., Csejtey, J. and Goldstein, G. W., Hexose transport and phosphorylation by capillaries isolated from rat brain, *Amer. J. Physiol.*, 236 (1979) C96–C102.
3 Brightman, M. W. and Reese, T. S., Junctions between intimately apposed cell membranes in the vertebrate brain, *J. Cell Biol.*, 40 (1969) 648–677.
4 Cardelli-Cangiano, P., Cangiano, C., James, J. H., Jeppsson, B., Brenner, W. and Fischer, J. E., Uptake of amino acids by brain microvessels isolated from rats after portacaval anastomosis, *J. Neurochem.*, 36 (1981) 627–632.
5 Claude, P. and Goodenough, D. A., Fracture faces of zonulae occludentes from 'tight' and 'leaky' epithelia, *J. Cell Biol.*, 58 (1973) 390–400.
6 Connell, C. J. and Mercer, K. L., Freeze-fracture appearance of the capillary endothelium in the cerebral cortex of mouse brain, *Amer. J. Anat.*, 140 (1974) 595–599.
7 DeBault, L. E. and Cancilla, P. A., γ-Glutamyl transpeptidase in isolated brain endothelial cells: induction by glial cells in vitro, *Science*, 207 (1980) 653–655.
8 Dermietzel, R., Junctions in the central nervous system of the cat. IV. Interendothelial junctions of cerebral blood vessels from selected areas of the brain, *Cell Tiss. Res.*, 164 (1975) 309–329.
9 Farrell, C. L. and Shivers, R. R., Capillary junctions of the rat are not affected by osmotic opening of the blood–brain barrier, *Acta Neuropath.*, 63 (1984) 179–189.
10 Goldstein, G. W., Relaton of potassium transport to oxidative metabolism in isolated brain capillaries, *J. Physiol. (Lond.)*, 286 (1979) 185–195.
11 Goldstein, G. W., Wolinsky, J. S., Csejtey, J. and Diamond, I., Isolation of metabolically active capillaries from rat brain, *J. Neurochem.*, 25 (1975) 715–717.
12 Head, R. J., Hjelle, J. T., Jarrott, B., Berkowitz, B., Cardinale, G. and Spector, S., Isolated brain microvessels: preparation, morphology, histamine and catecholamine contents, *Blood Vessels*, 17 (1980) 173–186.
13 Hjelle, J. T., Baird-Lambert, J., Cardinale, G., Spector, S. and Udenfriend, S., Isolated microvessels: the blood–brain barrier in vitro, *Proc. nat. Acad. Sci. U.S.A.*, 75 (1978) 4544–4548.
14 Hwang, S. M., Weiss, S. and Segal, S., Uptake of L-[^{35}S]cystine by isolated rat brain capillaries, *J. Neurochem.*, 35 (1980) 417–424.
15 Hwang, S. M., Miller, M. and Segal, S., Uptake of L-[^{14}C]proline by isolated rat brain capillaries, *J. Neurochem.*, 40 (1983) 317–323.
16 Karnushina, I. L., Palacios, J. M., Barbin, G., Dux, E., Joó, F. and Schwartz, J. C., Studies on a capillary-rich fraction isolated from brain: histaminic components and characterization of the histamine receptors linked to adenylate cyclase, *J. Neurochem.*, 34 (1980) 1201–1208.
17 Kolber, A. R., Bagnell, C. R., Krigman, M. R., Hayward, J. and Morell, P., Transport of sugars into microvessels isolated from rat brain: a model for the blood–brain barrier, *J. Neurochem.*, 33 (1979) 419–432.
18 Micic, D., Swink, M., Micic, J., Klatzo, I. and Spatz, M., The ischemic and postischemic effect on the uptake of neutral amino acids in isolated cerebral capillaries, *Experientia*, 35 (1979) 625–626.
19 Peek, W. D., Shivers, R. R. and McMillan, D. B., Freeze-fracture analysis of junctional complexes in the nephron of the garter snake, *Thamnophis sirtalis*, *Cell Tiss. Res.*, 179 (1977) 441–451.
20 Phillips, P., Kumar, P., Kumar, S. and Waghe, M., Isolation and characterization of endothelial cells from rat and cow brain white matter, *J. Anat.*, 129 (1979) 261–272.
21 Rapoport, S. I., *The Blood–Brain Barrier in Physiology and Medicine*, Raven Press, New York, 1976.
22 Reese, T. S. and Karnovsky, M. J., Fine structural localization of a blood–brain barrier to exogenous peroxidase, *J. Cell Biol.*, 34 (1967) 207–217.
23 Silbergeld, E. K., Wolinsky, J. S. and Goldstein, G. W., Electron probe microanalysis of isolated brain capillaries poisoned with lead, *Brain Research*, 189 (1980) 369–376.
24 Simionescu, M., Simionescu, N. and Palade, G. E., Segmental differentiations of cell junctions in the vascular endothelium. The microvasculature, *J. Cell Biol.*, 67 (1975) 863–886.
25 Simionescu, M., Simionescu, N. and Palade, G. E., Segmental differentiations of cell junctions in the vascular endothelium. Arteries and veins, *J. Cell Biol.*, 68 (1976) 705–723.
26 Shivers, R. R., The blood–brain barrier in the lizard, *Anolis carolinensis;* a freeze-fracture study, *Brain Research*, 169 (1979) 221–230.

Fig. 7. High magnification of capillary endothelial plasmalemma (PF) fracture face showing details of the anastomosed (arrows), parallel ridges (R) that constitute the zonula occludens. 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.

27 Shivers, R. R., The effect of hyperglycemia on brain capillary permeability in the lizard, *Anaolis carolinensis*. A freeze-fracture analysis of blood–brain barrier pathology, *Brain Research,* 170 (1979) 509–522.

28 Shivers, R. R. and Brightman, M. W., Trans-glial channels of crayfish ventral nerve roots in freeze-fracture, *J. comp. Neurol.,* 167 (1976) 1–26.

29 Shivers, R. R. and Harris, R. J., Opening of the blood–brain barrier in *Anolis carolinensis:* a high voltage electron microscope–protein tracer study, *Neuropath. appl. Neurobiol.,* in press.

30 Shivers, R. R., Edmonds, C. L. and Del Maestro, R. F., Microvascular permeability in induced astrocytomas and peritumor neuropil of rat brain: a high voltage electron microscope–protein tracer study, *Acta neuropath.,* in press.

31 Tani, E., Yamagata, S. and Ito, Y., Freeze-fracture of capillary endothelium in rat brain, *Cell Tiss. Res.,* 176 (1977) 157–165.

32 Williams, S. K., Gillis, J. F., Matthews, M. A., Wagner, R. C. and Bitenskey, M. W., Isolation and characterization of brain endothelial cells: morphology and enzyme activity, *J. Neurochem.,* 35 (1980) 374–381.

33 White, F. P., Differences in protein synthesized in vivo and in vitro by cells associated with the cerebral microvasculature. A protein synthesized in response to trauma? *Neuroscience,* 5 (1980) 1793–1799.

34 White, F. P., Dutton, G. R. and Norenberg, M. D., Microvessels isolated from rat brain: localization of astrocyte processes by immunohistochemical techniques, *J. Neurochem.,* 36 (1981) 328–332.