Understanding the Physiology of the Blood-Brain Barrier: In Vitro Models

Gerald A. Grant, N. Joan Abbott, and Damir Janigro

Endothelial cells exposed to inductive central nervous system factors differentiate into a blood-brain barrier phenotype. The blood-brain barrier frequently obstructs the passage of chemotherapeutics into the brain. Tissue culture systems have been developed to reproduce key properties of the intact blood-brain barrier and to allow for testing of mechanisms of transendothelial drug permeation.

The modern view of the mammalian blood-brain barrier (BBB) has shifted from a purely anatomic concept to a more physiological and dynamic definition. Morphologically, the BBB is formed by specialized endothelial cells (ECs) lining the intraluminal portion of brain capillaries. These ECs are characterized by specialized regions of intercellular contact (tight junctions) that prevent leakage of blood-borne substances into the brain parenchyma. The kinetic aspects of the passage of ions and molecules from the blood into the brain and vice versa better approximate the physiological function of the BBB. It is clear, however, that the distinct morphological properties of the BBB, as seen at both the light microscopic and ultrastructural levels, account for the "restraining" nature of brain capillary ECs. In contrast, the often-neglected fact that the BBB does not act as an absolute barrier, together with the asymmetry of its permeation properties, is less evident from a simple morphological investigation of brain microvessels.

The failure of BBB structural integrity and function plays a pivotal role in the pathogenesis of many diseases of the central nervous system (CNS) (Table 1). Thus, during ischemia, inflammation, trauma, neoplasia, hypertension, and epilepsy, altered BBB permeability is commonly observed. In barrier pathology, it is useful to take into account not only the endothelial dysfunction but also damage to the basal lamina, pericytes, astrocytes, vascular innervation, and components of the immune system. The extravasation of plasma proteins associated with BBB dysfunction may occur through a number of different transcellular or paracellular routes, including altered tight junctions, induction of fluid-phase or nonspecific pinocytosis and transcytosis, formation of transendothelial channels, or disruption of the endothelial cell membrane. Of course, these pathways may open in combination and are not mutually exclusive. Irrespective of whether the BBB disruption is the main pathogenic factor, or an inevitable consequence of the disease itself, our understanding of the cellular mechanisms that lead to the disruption of the BBB is limited. This may be due, in part, to the lack of available models of BBB. Any such in vitro models must reproduce important features of brain EC (Table 2) while allowing for manipulations aimed at mimicking the disease process itself. For example, the vascular permeability changes associated with neoplasia and inflammation are clearly manifest and of practical importance with regard to the clinical application of diagnostic and therapeutic measures. However, a suitable model to study tumor (or pathogen)-BBB interactions has yet to be developed.

Several approaches have been attempted to investigate the unique characteristics of the BBB endothelium in both the normal and disease state. Experimental observations first made by Paul Ehrlich in 1885 and Edwin Goldman in 1901 (that the CNS is not stained by intravascular water-soluble dyes) provided the first demonstration of a BBB to polar compounds. Pioneering studies of the BBB were performed in vivo using intracarotid injection single-pass techniques (11). Further characterization of the BBB at the cellular level has led more recently to the development of in vitro experimental approaches. Isolated brain capillary preparations as well as tissue culture systems using brain ECs have proven to be a promising methodology to define the characteristics of the brain capillary endothelium at the molecular and cellular level. The purpose of this review is to describe the virtues and pitfalls of cell culture-based models of the BBB. In our opinion, none of these models yet fully expresses the unique features of the BBB in situ; it is thus important for physiological, pharmacological, and preclinical studies to compare, whenever possi-

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ble, results obtained in vitro with data collected from intact animals. However, it should be emphasized that, in addition to enormous technical difficulties associated with in vivo studies of 2-µm-thick ECs lining the brain parenchymal vessels, in vivo models have other technical disadvantages. These include the impossibility of simultaneously sampling brain and plasma compartments with minimal damage to the BBB itself. Finally, the development of in vitro models holds significant promise for the identification of therapeutic strategies for the treatment of neurological diseases. In this review, we will first describe the BBB in vivo and in isolated vessels and then discuss several cell culture-based in vitro models of the BBB and their application to the study of neuropathological diseases.

In vivo studies and isolated vessels

Unlike peripheral endothelia, brain microvessel ECs are characterized by the presence of a high transendothelial electrical resistance (TER), intercellular tight junctions, minimal pinocytotic activity, and the virtual absence of fenestrations (6). In vivo, the endothelial BBB actually consists of a luminal plasma membrane, the cytosol, and the abluminal membrane of the endothelial cell. The capillary endothelium is closely invested by endfeet of glia, which develop in proximity to the brain ECs (15). Studies both in vivo and in vitro provide evidence that astrocytes contribute at least in part to the induction and maintenance of BBB characteristics in the brain endothelium (3, 7, 13); thus it appears that the cerebral endothelium is under direct influence from neighboring (or “perivascular” glia) astrocytes and that chemical signals released by or present on the membrane of these cells are in part responsible for the unique characteristics of the mammalian BBB.

Table 1. Central nervous system disorders involving BBB dysfunction

<table>
<thead>
<tr>
<th>Neoplasia</th>
<th>Vascular</th>
<th>Trauma</th>
<th>Metabolic</th>
<th>Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain tumors (histamine, tissue necrosis factor, interferons, interleukins, permeable tumor vessels)</td>
<td>Ischemia, hypoxia (glutamate, free radicals, vasodilatation, lactic acidosis, prostaglandins, glial dysfunction)</td>
<td>Open and closed head injury (intracranial hypertension, endothelial disruption, vascular spasm and loss of cerebral autoregulation)</td>
<td>Diabetes (hyperglycemia, ischemia)</td>
<td>Multiple sclerosis/experimental allergic encephalomyelitis</td>
</tr>
<tr>
<td>Meningiomas (vascular endothelial growth factor)</td>
<td>Hypertension (mechanical damage to endothelium, free radicals, vasopressin, angiotensin)</td>
<td>Vasogenic (EC damage, intracranial hypertension, arachidonic acid metabolites, histamine, oxygen free radicals, polyamines)</td>
<td>Toxins: lead, aluminum, mercury, dimethyl sulfoxide (endothelial damage)</td>
<td>Meningitis: bacterial, viral, fungal (bradykinin, ATP, histamine, serotonin, interleukins)</td>
</tr>
<tr>
<td>Subarachnoid hemorrhage (complement system-C3a, EC damage, vasospasm)</td>
<td>Arteriovenous malformations (endothelial damage due to ischemia and high flow state)</td>
<td>Cytotoxic</td>
<td>Epilepsy</td>
<td>Dementia: AIDS demential complex, Alzheimer’s (B-amyloid)</td>
</tr>
<tr>
<td>X-irradiation (endothelial damage)</td>
<td>Migraines (serotonin)</td>
<td>Metabolic</td>
<td>Seizures (glutamate, glial dysfunction following neuronal activation, hypertension)</td>
<td></td>
</tr>
</tbody>
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BBB, blood-brain barrier; EC, endothelial cell.
signals while controlling the rate of transport of metabolites and ions (e.g., K+, Na+, and H+).

The concept of the polarity of the brain endothelium emerged from functional transport studies (2). For example, the Na+-dependent amino acid transporter (type A) is found only on the abluminal side and transports neutral amino acids from the brain to the blood against a concentration gradient. In contrast, the Na+-independent L system carries neutral amino acids and is expressed on both the luminal and abluminal membranes (6). Carriers mediating specific efflux from the brain have also been described, including P-glycoprotein, which actively exports lipophylic molecules out of cells and confers insensitivity to drugs used for cancer chemotherapy (multidrug resistance).

Classically, in vivo experiments were used to determine the permeability of compounds across the brain endothelium (6). Such approaches offered valuable information about the behavior of different classes of compounds and helped identify specific transport systems. A primary advantage of in vivo preparations is the preservation of the normal anatomic arrangement of cells at the blood-brain interface. In addition, regional differences within the brain can be studied.

Most studies of BBB permeability in vivo use one of two different methodological approaches. In the first approach, a solute is injected as a bolus into the carotid artery; brain uptake or extraction is determined from a single pass of the bolus through the brain capillaries. The brain uptake index technique was later introduced by Oldendorf in 1970 as an intracarotid injection single-pass method to measure cerebrovascular transport and permeability. In this method, the brain uptake of a test tracer is normalized by the use of a permeant reference tracer of known extraction. In vitro modeling of the BBB

Isolated brain microvessels. A better understanding of the physiology and pathophysiology of the BBB was gained with the development of methods pioneered by the late Ferenc Joó to obtain functionally and morphologically intact cerebral microvessels dissociated from surrounding neurons and glia. Several methods are now available for the isolation and purification of brain microvessels, which involve separation of brain capillaries from the brain by combinations of mechanical homogenization, enzymatic dissociation, filtration, density gradient centrifugation, and glass bead column filtration. Capillaries, venules, and arterioles of 5- to 25-µm diameter are typically isolated by these procedures. Cortical microvessels isolated from adult mammalian brain are enriched in the putative BBB markers, alkaline phosphatase and γ-glutamyl transpeptidase (GGTP), but are deficient in choline

**Table 2. Description of the main cellular and functional properties of the in situ BBB**

<table>
<thead>
<tr>
<th>BBB-specific markers</th>
<th>Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inductive influence from glia</td>
<td>Mandatory</td>
</tr>
<tr>
<td>Tight junctions</td>
<td>Present</td>
</tr>
<tr>
<td>TER</td>
<td>$&gt;1,500 \ \Omega \cdot \text{cm}^2$</td>
</tr>
<tr>
<td>$P_{\text{acq}}$</td>
<td>Low ($&lt;10^{-7}$ cm/s)</td>
</tr>
<tr>
<td>$K^+$ permeability</td>
<td>Low</td>
</tr>
<tr>
<td>Exposure to flow membrane</td>
<td>Luminal</td>
</tr>
<tr>
<td>Polarized transporters</td>
<td>Ubiquitous (e.g., $K^+$, amino acids)</td>
</tr>
<tr>
<td>Stereoselective transport</td>
<td>Glucose, amino acids</td>
</tr>
</tbody>
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BBB, blood-brain barrier; TER, transendothelial resistance; $P$, permeability.

"Carriers mediating specific efflux from the brain...."
acetyltransferase, a protein exclusively expressed at the BBB (10). The expression of BBB markers can be used to assess the purity of the microvessel preparations but do not guarantee the integrity or preservation of the normal BBB function. Isolated capillaries can be successfully used for physiological, biochemical, and developmental studies of the BBB as well as transport studies. Such preparations have also been used for the identification of membrane receptors and transporters. The advantages of this isolated capillary system include the preservation of its three-dimensional structure, differentiation, availability, and ease of use. The disadvantages include the difficulties associated with the isolation procedure, limited viability of the endothelium, possible metabolic deficiencies induced by the isolation procedures, and the inability to study transendothelial flux.

Given the unique interactions between EC and glia, and owing to the difficulties associated with the use of the capillary microvasculature in vitro, it is not surprising that among the most successful models of the BBB are actually isolated pial brain arterioles. Surface pial microvessels appear to share many of the molecular, physiological, and morphological properties of cortical parenchymal vessels, despite a lack of the perivascular astrocytic ensheathment. Although the ultrastructural features, permeability to tracers, electrical resistance, and molecular properties are quite similar between pial and cortical vessels, the distribution of the expression of BBB-specific antigens (e.g., endothelial barrier antigen) as well as the structure of tight junctions visualized by electron microscopy may differ (4). Because technical limitations have precluded the in vitro study of cortical parenchymal vessels, investigators have pursued cell culture-based in vitro models of the BBB.

Cultured brain microvascular ECs. Highly purified populations of cultured microvascular cells for the study of the developmental and pathophysiological processes of the BBB became available in the early 1980s. With this technique, a viable and homogeneous population of brain capillary ECs can be isolated for the establishment of a tissue culture system. The first endothelial monolayers were established using cerebral microvessel ECs grown on culture dishes, microcarriers (e.g., dextran beads), and various kinds of filters, including nylon mesh and polycarbonate. The sucrose permeability (P_{sucrose}) of these monolayers ranged from 10^{-4} to 10^{-5} cm/s compared with 10^{-6} to 10^{-8} cm/s in vivo. Despite the differences in P_{sucrose}, the rank order for penetration of test compounds was well maintained. TER is a measure of the ionic conductance of the monolayer and is a useful measure of the “tightness” of the monolayer, which is largely determined by its impenetrability due to tight junctions. The TER in vivo measured ~1,500 Ω·cm², whereas the measurements in endothelial monolayers cultured in vitro have ranged from 20 to 1,400 Ω·cm². Cyclic nucleotides such as adenosine 3',5'-cyclic monophosphate and cyclic guanosine monophosphate have been reported to modulate TER and permeability across endothelial monolayers, but their mechanisms of action are poorly understood (9, 13).

Although primary cultures of brain endothelium alone may form tight intercellular junctions, coculture with astrocytes resulted in the increased formation and complexity of endothelial tight junctions and induced the expression of specific BBB markers, including GGTP, the glucose transporter isoform (GLUT-1), OX-26 (mouse antibody against human and rat transferrin receptor), and P-glycoprotein (1). In contrast, the ubiquitous occurrence of ZO-1 (antibody against zonula occludens-associated protein) in the CNS makes the application of this protein unreliable as a quantitative means to estimate tight junctional permeability but can be used as a tool for the direct detection of de novo tight junction assembly (8, 10). The advantages of cultured endothelium include the potential for using pure cell populations as well as their relative viability compared with isolated arterioles ex situ. The isolation procedure for primary cultures, however, is labor intensive and expensive, and it is difficult to avoid contamination by other cell types, chiefly pericytes, leptomeningeal cells, and smooth muscle. Therefore, developing immortalized cell lines that preserve a stable BBB phenotype is of great interest. Several cell lines have been established (e.g., RBE4). Interestingly, even nonbrain ECs such as bovine aortic ECs can be induced by glia to form complex tight junctions and express a barrier phenotype (7, 14). Sophisticated systems have been developed to preserve the barrier-specific functional polarity of the endothelium in culture, since the cells may rapidly dedifferentiate in the absence of astrocytes with serial cell passage. The addition of flow to the culture system has been shown to cause physiological shear stress and play a critical role in the differentiation of ECs (12).

Comparison of in vitro models. Several in vitro models of the BBB are currently used to explore the influence of diseases on the dynamic barrier on cellular, biochemical, and molecular levels. Each model attempts to mimic the complexity of the mammalian BBB, but each is characterized by different selective permeability to different compounds and may manifest a range of TER values. The term “blood-brain barrier” suggests that brain capillaries are impermeable, but it is obvi-
ous that, although they are indeed impermeable to some plasma solutes, they must be freely permeable to others. A critical feature in any model system is the ability to discriminate between a compound of high permeability through the lipid bilayer (transcellular) and a compound such as sucrose or mannitol that traverses (albeit poorly) via a paracellular pathway. A transcellular vesicular route may also need to be considered.

The most commonly used tissue culture substrate used for EC culturing consists of cultured brain microvessel endothelial cell (EC) monolayers grown on microporous membranes. Cultured cortical astrocytes are compartmentalized below the endothelial monolayer and release soluble factors, which preserve the BBB properties. This system allows for study of bidirectional transport across the BBB. A: Transwell system. Transwell system consists of cultured brain microvessel endothelial cell (EC) monolayers grown on microporous membranes. Cultured cortical astrocytes are compartmentalized below the endothelial monolayer and release soluble factors, which preserve the BBB properties. This system allows for study of bidirectional transport across the BBB. B: dynamic in vitro BBB model (DIV-BBB). ECs and cortical astrocytes are cocultured on hollow fibers inside a sealed chamber accessible by ports. Left: schematic representation of a cross section of one hollow fiber. ECs are loaded intraluminally, and cortical astrocytes are loaded abluminally. Astrocytic foot processes grow toward ECs to induce and maintain their BBB phenotype. Cartridge-hollow fiber system consists of artificial capillaries connected by gas-permeable tubing to a source of growth medium, thus allowing exchange of O₂ and CO₂ and exposure to pulsatile flow (right). C: isolated vessels. Pial surface and penetrating cortical arterioles have also been used for BBB studies. Left: schematic representation of a cross section of an end arteriole with one layer of endothelium surrounded by a single layer of vascular smooth muscle. Pia mater and its attached penetrating intracerebral arterioles are then separated from a section of cortical parenchyma as shown (right).

FIGURE 1. Schematics of in vitro models of the blood-brain barrier (BBB). A: Transwell system. Transwell system consists of cultured brain microvessel endothelial cell (EC) monolayers grown on microporous membranes. Cultured cortical astrocytes are compartmentalized below the endothelial monolayer and release soluble factors, which preserve the BBB properties. This system allows for study of bidirectional transport across the BBB. B: dynamic in vitro BBB model (DIV-BBB). ECs and cortical astrocytes are cocultured on hollow fibers inside a sealed chamber accessible by ports. Left: schematic representation of a cross section of one hollow fiber. ECs are loaded intraluminally, and cortical astrocytes are loaded abluminally. Astrocytic foot processes grow toward ECs to induce and maintain their BBB phenotype. Cartridge-hollow fiber system consists of artificial capillaries connected by gas-permeable tubing to a source of growth medium, thus allowing exchange of O₂ and CO₂ and exposure to pulsatile flow (right). C: isolated vessels. Pial surface and penetrating cortical arterioles have also been used for BBB studies. Left: schematic representation of a cross section of an end arteriole with one layer of endothelium surrounded by a single layer of vascular smooth muscle. Pia mater and its attached penetrating intracerebral arterioles are then separated from a section of cortical parenchyma as shown (right).
In this system, cerebral or peripheral ECs are cultured intraluminally in the presence of astrocytes cultured abluminally using hollow fiber tubes inside a sealed chamber (Fig. 1B). The hollow fiber cartridge system consists of artificial capillaries that are exposed to luminal pulsatile flow. This system is characterized by an extremely high TER estimated to be >1,000 Ω·cm², resistance that approximates that obtained in vivo. In addition, there is a low permeability to sucrose (10⁻⁶–10⁻⁷ cm/s) and the functional expression of stereoselective transport (e.g., L- vs. D-aspartate) (14). However, because of the compartmentalization of ECs and glia in the hollow fiber system in the presence of pulsatile flow, the study of linear kinetics becomes more complex. Isolated pial arterioles (surface and penetrating) have also been used to study BBB function ex situ (Fig. 1C).

The induction and preservation of selective permeability and transport mechanisms as well as expression of normally occurring ion channels responsible for the maintenance of brain homeostasis are necessary BBB phenotypic features. In vitro models have proven valuable in the rigorous study of biochemical transport at the cellular level in the context of several disease states such as ischemia, neoplasia, and meningitis. Thus far, and in addition to the aforementioned quasi-physiological TER, dynamic BBB models have successfully replicated several morphological and functional characteristics of the intact BBB. These include asymmetric K⁺ transport, stereoselective transport of amino acids, a BBB-like glucose transporter, tight junctions, and negligible permeation by ¹⁴C-sucrose or inulin. Culture models have made it possible to investigate the site and mechanism of action of toxic agents that affect the BBB, including drugs and industrial toxins. The effects of putative toxicants (e.g., lead, aluminum) and other pathogens on BBB viability have been studied using these models. Further studies will be challenged by even more demanding tasks, such as mimicking chronic neurodegenerative processes (i.e., Alzheimer's and AIDS dementia) in long-term endothelial cell cultures.

**Implications of the BBB in neurological disease**

A group of toxic agents and pathological conditions causes early changes in BBB function, which are mediated via direct effects on the ECs, but is also usually associated with morphological changes in astrocytes. Tumors, for example, disrupt the glial sheath that envelops the ECs and are associated with an increased capillary permeability. The capillary endothelium of tumor vessels is highly abnormal and expresses a varied degree of fenestrated regions, vesicles, open junctions, and fragmented basal lamina to a high degree, depending on tumor type and grade, which lead to a considerable increase in permeability of the tumor vascular bed. Tumors also stimulate the proliferation of abnormal capillaries by releasing angiogenic factors. In more malignant tumors, the capillaries are fenestrated with increased pinocytosis and have an incomplete BBB. Tumors, therefore, permit contrast enhancement on radiographic imaging studies (computerized tomography or magnetic resonance imaging) and may exhibit marked vasogenic edema. More recently, hyperosmolar solutions have been employed to improve the delivery of chemotherapeutic agents to the neoplastic cells. Hyperosmolar agents cause an osmotically induced shrinkage of brain microvessel ECs and a reversible increase to BBB permeability. Future therapeutic strategies depend on an improved understanding of the mechanisms responsible for the induction and maintenance of the barrier under normal and pathological conditions. Furthermore, by coculturing neuronal, glial, and brain ECs with tumor cell lines, we may obtain important information on the efficacy of antineoplastic agents, while simultaneously monitoring passage of these agents across the BBB, and on their potential neurotoxicity.

There is increasing evidence that, in many diseases of the CNS, the barrier dysfunction may be brought about by the release or activation of a cascade of mediator substances from damaged or activated cells. The study of conditions that increase BBB permeability has improved our understanding of the mechanisms that maintain and modulate the barrier and our search for agents that can be used to open the barrier for therapeutic purposes. The role of the BBB in the evolution of viral and bacterial CNS diseases remains incompletely defined and is currently being explored both in vivo and in vitro. Barrier dysfunction secondary to viral or bacterial pathogens may exacerbate the severity of the neurological injury, whereas an intact barrier may hinder recovery from disease by delaying the entry of immune complexes or therapeutic agents into the infected CNS. Seizures, whether induced by electroconvulsive shock or drugs, resulted in increased permeability to intravascular markers. The induction of hypertension also increased BBB permeability; however, the increase was also associated with increased pinocytosis. Hypercapnia had the same effect as hypertension, although vasodilatation and the stretch of tight junctions are critical (1, 6, 10). Classical vasoactive mediators of inflammation (e.g., histamine, serotonin, ATP) cause a rapid and dramatic increase in permeability. Experiments performed on brain microvascular
endothelium showed that tight junction openings caused by agents such as ATP and bradykinin may be mediated via a rise in cytosolic Ca$^{2+}$ and endothelial contraction. Oxygen free radicals released following ischemia or hypoxia caused an even more dramatic opening of the barrier, which was Ca$^{2+}$ independent. The breakdown of the BBB following an ischemic episode has been well documented; however, the extent and duration of the opening largely depends on the duration of the ischemia and degree of reperfusion. The temporal course of the BBB opening following ischemic reperfusion injury and even traumatic brain injury appears biphasic. The early opening of the BBB is thought to contribute to the development of cerebral edema formation both in tumors and following ischemia, although the pathogenesis of the disruption has not been clearly elucidated. Novel dynamic cell culture systems offer valuable tools to study the effect of ischemia and hypoxia on barrier function.

Conclusion

In vitro models of the mammalian BBB are now able to mimic key features of the in situ BBB and, moreover, can provide detailed information about the cellular and molecular mechanisms of dynamic endothelial function. Study of in vitro preparations for application of modern techniques of molecular and cellular biology and physiology at the single cell level is critical for the study of the BBB. Further developments in vitro now will allow sophisticated genetic manipulations to explore novel clinical applications.

References