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Cell-Culture Models of the Blood–Brain Barrier

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Keywords

astrocytes; blood-brain barrier; endothelial cells; in vitro; pericytes

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With the increase in average life span, the percentage of patients with neurological diseases is getting higher and higher.[1] Thus, developing effective neuropharmaceuticals is critically important and urgent. Sadly, many promising candidates fail to show expected effects because of their inability to cross the blood–brain barrier (BBB), a dynamic interface that separates the central nervous system (CNS) from the circulation system.[1] BBB maintains the homeostasis of CNS microenvironment and proper neurological functions by regulating the exchange of substances between the 2 systems.[2] Perturbation of BBB has been found in many neurological disorders, including neurodegenerative diseases,[3–6] trauma,[7,8] brain tumors,[9,10] and stroke.[11–13] Restoring BBB integrity in pathological conditions to maintain brain homeostasis and opening BBB temporarily to allow efficient delivery of drugs to the CNS are potential therapeutic options for patients with these disorders. For these purposes, a lot of research has focused on the regulation of BBB permeability. Because of the complexity of the in vivo BBB, many simplified in vitro BBB models have been developed and studied, including the monolayer models, coculture models, dynamic models, and microfluidic BBB models. Because no in vitro BBB models can fully replicate the in vivo conditions, there is no perfect in vitro BBB model. Understanding the limitations of these in vitro BBB models would be critical to the design of experiments and interpretation of data.

There have been a large number of excellent reviews on in vitro BBB models in the literature. For example, Gumbleton and Audus[14] reviewed immortalized cell lines and primary cells used in in vitro BBB models and suggested that an ideal model should have low permeability, possess endothelial-like morphology, express functional transporters, and be easy to construct. Deli et al[15] summarized permeability data on in vitro BBB models in both normal and pathological conditions. They also reviewed the effects of various biological factors and pharmaceutical molecules on signaling transduction and BBB

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permeability.[15] Additionally, Abbott et al[16] recently published an in-depth review on in vitro culture models of the CNS barriers, including stem cell–based approaches and techniques used to characterize the BBB properties. Here in this review, we summarize the most widely used in vitro BBB models, including the newly developed nonhollow fiber-based microfluidic models, compare their strengths and weaknesses, and provide suggestions on model selection in BBB research and new-drug research and development (R&D).

Blood–Brain Barrier

The existence of a barrier between the CNS and the systemic circulation was first described by Paul Ehrlich in 1885[17] and Edwin Goldmann in 1913.[18] The term BBB was first used by Stern and Gaultier in 1922.[19] The BBB shields the brain from harmful substances in the blood and prevents the entrance of blood cells, but it allows the uptake of nutrients and hormones from blood (see below). The major BBB components include brain microvascular endothelial cells (BMECs), astrocytes, and pericytes.[20] To discuss in vitro BBB models, we first briefly introduce the biological properties and functions of individual BBB components. A more detailed illustration of the BBB can be found in other references.[1,21]

Brain Microvascular Endothelial Cells

BMECs are a specialized type of endothelial cells. Structurally, BMECs have more mitochondria and less pinocytotic vesicles/fenestrations compared with peripheral endothelial cells.[22–25] Functionally, BMECs form much tighter capillary endothelium than peripheral endothelial cells.[26] The brain is basically not permeable to polar molecules, although it is estimated that capillaries in human brain have a length of ≈650 km and a surface area of ≈10 to 20 m.[2,27–29] This tight barrier property can be attributed to the unique paracellular and intracellular transportation properties of BMECs.

In the interendothelial space, tight junctions (TJs) seal gaps between BMECs and limit paracellular permeability through the expression of tight junction proteins (TJPs), such as occludin, claudins, and zonula occludens (ZO-1, ZO-2, and ZO-3).[25,30–33] Accumulating evidence shows that the levels of TJPs negatively correlate with paracellular permeability, and loss of TJP expression leads to paracellular leakage,[25,31,33–36] suggesting that TJPs play a crucial role in the regulation of paracellular permeability. Another way to regulate BBB permeability is via vesicular transport.[4,37–39] Two major mechanisms are used by BMECs to regulate intracellular transportation. First, small lipophilic molecules, such as oxygen and carbon dioxide, diffuse across BMECs freely.[40] Second, some hydrophilic molecules are transported across BMECs via specific transporters and receptors. Depending on the subcellular distribution, 3 major types of transporters and receptors are found: (1) Bidirectional transporters and receptors expressed on both the luminal and abluminal sides of BMECs. These transporters and receptors usually function to facilitate nutrient transportation. For example, glucose transporter 1, mono-carboxylate transporter 1, L1 amino acid transporters, and y+cationic amino acid transporter transport glucose, lactate, and large neutral and cationic essential amino acids in and out of BMECs, respectively.[41,42] (2) Unidirectional transporters and receptors expressed on both the luminal and abluminal sides of BMECs. This group of transporters and receptors shifts molecules either in or out of

the brain/blood system. For example, transferrin receptor and insulin receptor mediate endocytosis of transferrin and insulin, respectively, leading to accumulation of these ligands in BMECs.[43–45] (3) Transporters and receptors expressed on either the luminal or abluminal side of BMECs. These unevenly distributed transporters and receptors contribute to the polarity of BMECs and are involved in unidirectional transportation of substances. For instance, multidrug resistance-related protein 1 (also called ATP-binding cassette subfamily C member 1),[46–50] P-glycoprotein (also called ATP-binding cassette subfamily B member 1),[51,52] and breast cancer resistance protein (also called ATP-binding cassette subfamily G member 2)[53–58] are predominantly expressed in the luminal side of BMECs. These transporters recognize a large spectrum of lipophilic substrates and pump them into capillary lumen to prevent their penetration across the BBB.[59] Beside BMECs, these transporters have also been found in many types of tumor cells, including glioma,[60] leukemia,[61] esophageal carcinoma,[62] and colon/lung/kidney cancer.[63,64] Because many anticancer drugs are substrates of these transporters,[59,65] the access of these drugs to CNS tissue or tumors is limited, making patients resistant to anticancer drug treatment, a phenomenon known as multidrug resistance.[65] In contrast to tumor cells, lower expression of these transporters was found in blood vessels supplying CNS metastases.[66] For example, P-glycoprotein levels in blood vessels around CNS metastases of melanoma and lung cancer were ≈5% and 40% of that in normal brain tissue, respectively.[67] These data suggest that systematically delivered chemotherapy may reach CNS tumor cells, whereas it is hard to penetrate and kill them. Various inhibitors for these transporters, including verapamil, probenecid, and fumitremorgin C, have been used to increase drug delivery to brain tumors and improve the therapeutic efficacy.[66] On the other hand, excitatory amino acid transporters 1 to 3 are found solely on the abluminal side of BMECs to efficiently remove the excitatory neurotransmitter glutamate from the brain.[68] Low-density lipoprotein receptor-related protein 1, which is predominately expressed on the abluminal side of BMECs, facilitates the elimination of amyloid-β from the brain,[69–71] although there is also evidence suggesting that lipoprotein receptor-related protein 1 may not contribute to the efflux of amyloid-β across the BBB.[72,73] Expressed only on the abluminal side of BMECs, (Na^+-K^+)ATPase regulates ion homeostasis in the brain and thus proper neuronal and synaptic functions.[42,58] The expression of these transporters and receptors is summarized in Figure 1. For a more complete list of transporters in BMECs, please see the other excellent reviews.[57,58] Additionally, other mechanisms may also modulate substance transport in and out of BMECs. It has been shown that BMECs express high levels of γ-glutamyl transpeptidase and alkaline phosphatase, which modify many molecules to prevent their entrance into the brain.[74,75]

Astrocytes

Using chimeric grafting experiments, Stewart and Wiley[76] in 1981 elegantly showed that CNS tissue induces the unique properties of BMECs mentioned above. It has been suggested that astrocytes, the most abundant glial cells in the brain, whose endfeet cover 99% of the vascular surface,[26,77–83] contribute to the unique properties of BMECs and thus integrity of the BBB. In agreement with this hypothesis, adding astrocytes to BMECs significantly increased the transendothelial electric resistance (TEER) and decreased their permeability to various molecules in vitro.[34,84–87] Additionally, astrocyte numbers have been positively

correlated with the BBB integrity in vivo,[88] suggesting that astrocytes do contribute to the integrity of the BBB.

Pericytes

A group of perivascular cells, first described by Rouget in 1873,[89] was named pericytes by Zimmermann in 1923.[90] Sandwiched in between BMECs and astrocytic endfeet,[91] pericytes are embedded in the endothelial basement membrane.[21,92] Pericytes cover capillaries in the brain and the degree of coverage negatively correlates with capillary permeability.[92] Pericytes have been shown to enhance TEER in the BMEC–astrocyte coculture BBB model.[93,94] Additionally, pericytes upregulate P-glycoprotein functional activity and regulate TJ permeability in endothelial cells.[95–97] Recently, 3 groups concomitantly reported a key role of pericytes in BBB maturation/regulation in vivo. Using mice lacking pericytes, Daneman et al[98] elegantly demonstrated that pericyte numbers positively correlate with BBB tightness. Consistent with this report, Armulik et al[99] and Bell et al[4] found that loss of pericytes leads to BBB breakdown in vivo. These data strongly suggest that pericytes actively regulate BBB integrity and should be included in in vitro BBB models to better mimic the in vivo conditions. It should be noted that pericyte differentiation status affects their function in BBB permeability. Smooth muscle actin-α-high pericytes have been shown to decrease BBB integrity, whereas smooth muscle actin-α-low pericytes stabilize BBB integrity.[100] Therefore, the differentiation status of pericytes should be taken into consideration when they are used in in vitro BBB models.

Other Cellular Components

Besides the cells mentioned above, other components, such as neurons and microglia, may also contribute to the integrity of the BBB. For example, neurons may regulate BBB permeability indirectly by modulating BMECs and astrocytes.[2,101–107] It has been shown that neurons decrease sucrose leakage across BBB in vitro,[108] probably via regulating the localization of occludin.[109,110] Neurons are also able to induce BBB properties on BMECs.[111,112] Like neurons, microglia are speculated to modulate BBB integrity because of their close relations with the neurovascular unit.[113] How microglia modulates BBB permeability, however, remains controversial. There is evidence showing that microglial activation promotes the restoration of a disrupted BBB.[114] Microglia-released tumor necrosis factor-α, however, has been reported to impair BBB integrity, probably via tumor necrosis factor-α receptor-mediated cytotoxicity.[115] These controversial results could be explained by the complexity of microglial biology. In physiological conditions, microglia exist in the brain in the resting state with a ramified morphology.[116] On injury, they quickly change their morphology/gene expression profiles and become activated.[117] Early after injury, microglia take the M1 proinflammatory phenotype, characterized by the secretion of tumor necrosis factor-α and nitric oxide, to prevent the spread of injury.[118,119] With the progress of disease, microglia transit to the M2 phenotype, characterized by the secretion of interleukin-10 and transforming growth factor-β, to clean up cell debris and promote recovery.[118,119] Because the functions of microglia depend on their phenotypes and the transition between these phenotypes is differentially regulated in different types of insults,[117–119] the roles of microglia in BBB permeability may vary depending on injury types and time after injuries.

In Vitro BBB Models

To facilitate cerebrovascular research and expedite the R&D of novel drugs for various neurological diseases, many in vitro BBB models have been developed. However, because none of these in vitro models fully replicates the in vivo conditions, there is no perfect in vitro BBB model. Thus, cautions should be taken when one chooses in vitro BBB models and interprets the data. Here we summarize the most widely used in vitro BBB models, including the recently developed microfluidic BBB models, and analyze their advantages and disadvantages. Based on whether shear stress is replicated, these in vitro BBB models are divided into 2 categories: static and dynamic models.

Static Models

Static models do not replicate the shear stress generated by the flow of blood in in vivo conditions. According to the number of cell types involved, static BBB models are further categorized into monolayer and coculture models.

Monolayer Models—A monolayer of endothelial cells grown in the Transwell insert (Figure 2A) is used as a simple in vitro BBB model. The insert mimics the blood (luminal) side, whereas the well in which the insert sits mimics the parenchymal (abluminal) side. The microporous membrane support (0.4 μm) allows the exchange of small molecules and cell-secreted growth factors but prevents the migration of cells between the 2 compartments. To mimic the unique properties of BMECs (see Brain Microvascular Endothelial Cell section of this article), primary or low passage BMECs are used. Accumulating evidence shows that primary or low passage BMECs retain many of the biochemical and morphological properties that characterize the BBB in vivo, such as the presence of TJs and polarized expression of transporters, receptors, and enzymes.[1] Two big challenges, however, exist in isolating and culturing primary BMECs: the low yield and contamination by mural cells. Because the brain vasculature accounts only for 0.1% (v/v) of the brain, a large number of rodents are usually needed to generate enough BMECs.[120] Therefore, larger species, including bovine, porcine, and nonhuman primate, are used to increase the yield of BMECs. The problems with larger species, however, are that these animals are usually nontransgenic and there are no or few respective antibodies available for these species. For these reasons, rodents are still the most widely used animals in BBB research. Recently, temporary application of puromycin in the culture medium has been shown to dramatically improve the purity of primary BMECs.[34,121,122] This methodological breakthrough enables the use of BMECs at high purity and significantly advances BBB research.

Human cells may be needed when human-specific transporters/receptors or immunologic issues are involved. Primary human cells, however, are not usually available because of ethical reasons. To circumvent this issue, many immortalized human cell lines, such as human cerebral microvascular endothelial cell line (hCMEC/D3)[123] and immortalized human cerebral endothelial cells,[124] have been generated. One big benefit of the immortalized cell lines is their significantly enhanced ability to proliferate in culture. Because of this advantage, immortalized brain endothelial cell lines from rodents, such as rat brain endothelial cell line, mouse brain endothelial cell line, and mouse cerebral endothelial

cell line, have also been generated. These immortalized cell lines, however, unlike primary or low passage BMECs, lose some of the unique properties that characterize the in vivo BBB.[125–129] It has been shown that some of the BBB-specific transporters and enzymes are expressed at much lower levels in the immortalized cell lines. For example, the expression of TJPs, including occludin and claudin-5, in hCMEC/ D3 cells is lower than that in primary cerebral endothelial cells.[130] RBE4 and hCMEC/D3 cells have been found to have less glucose transporter 1 compared with brain endothelium in situ.[130–132] Additionally, γ-glutamyl transpeptidase and alkaline phosphatase activity is dramatically reduced in RBE4 cells and other rat brain endothelial cell lines, compared with isolated rat brain capillaries.[133,134] Because of the above-mentioned limitations, these immortalized cell lines are not able to generate a tight monolayer and thus have inadequate barrier function. To overcome this problem, reagents that modulate BBB properties, such as cAMP and glucocorticoids, have been used to improve the tightness of the endothelial monolayer.[135–140]

An alternative way to generate human BMECs is through human pluripotent stem cells (hPSC). Although previous studies showed that hPSC-generated endothelial cells do not have organ-specific properties,[141–143] Lippmann et al[144] successfully differentiated hPSC into endothelial cells with unique BBB properties. These hPSC-derived endothelial cells expressed TJPs, various nutrient receptors, amino acid and peptide transporters, and efflux transporters.[144]

It should be noted that the monolayer model only has 1 major cell type (BMECs) and lacks the communication among different cell types. Accumulating evidence shows that the BBB properties are dictated by the microenvironment in the brain rather than intrinsic to BMECs, and cell–cell communications play a critical role in the maintenance of these properties.[76,145–147] Thus, this oversimplified monolayer model is not ideal for the study of BBB integrity. Because of the intrinsic simplicity,[148] however, monolayer models have been widely used in signaling pathway and transporter kinetic studies, binding affinity measurement, and high-throughput screening.

Coculture Models—To better mimic the anatomic structure of the in vivo BBB, BMECs are cocultured with other cells that directly contribute to the barrier properties of BBB.[4,26,77–83,98,99] Based on the evidence that interaction between BMECs and astrocytes increases the expression of transporters and TJs in BMECs, induces the formation of cell polarity in BMECs, and promotes a phenotype more closely mimicking the in vivo BBB,[26,149–158] BMECs are cocultured with astrocytes to improve their barrier properties. In this coculture model, BMECs are seeded in the Transwell insert and astrocytes are grown either on the underside of the Transwell insert or at the bottom of the well in which the insert sits (Figure 2A). Consistent with previous reports, higher TEER and lower permeability to molecular tracers are observed in this BMEC–astrocyte coculture model.[135,159–161] Cells of different origins have been used in this model. For example, mouse BMECs cocultured with astrocytes generated a TEER of 200 to 300 $\Omega \cdot cm^2$,[162] whereas bovine BMECs cocultured with astrocytes produced a higher TEER around 500 to 600 $\Omega \cdot cm^2$.[159–161] Additionally, a TEER of 900 $\Omega \cdot cm^2$ was achieved when porcine BMECs were used.[163] When hPSC-derived endothelial cells were cocultured with rat astrocytes, the

TEER reached 860 $\Omega \cdot cm^2$.[144] The different TEER values and thus the barrier properties of BMECs are probably because of more efficient BMEC isolation/purification methods rather than species difference, given that equally high TEER values (600–1000 $\Omega \cdot cm^2$) were observed in the BMEC–astrocyte coculture model independent of species.[16,164–166] However, to answer the question clearly whether there is a species difference in BMEC tightness, comparative studies using the same methods and conditions within the same laboratory are needed. It should be noted that the TEER values obtained from the BMEC–astrocyte coculture model are still lower than that in in vivo conditions, which is estimated to be more than 1000 $\Omega \cdot cm^2$.[14,167] suggesting that other players also contribute to the integrity of BBB.

The BMEC–astrocyte coculture model has been widely used to study stroke, the leading cause of disability and the third most common cause of death in the United States.[168] Hypoxia and hypoglycemia, 2 pathological changes in stroke, can be easily mimicked in vitro by lowering oxygen and glucose levels.[169] This in vitro stroke model has been applied to investigate endothelial pathophysiology,[30,170] endothelial barrier permeability,[171,172] cell–cell communication,[173] and free radicals[174] during stroke. Additionally, it has also been used in large-scale screening for compounds that have protective function in hypoxia-reoxygenation induced injury.[175] The advantages of using this model to study stroke include[169] (1) the severity of stroke can be easily controlled by regulating oxygen and glucose levels, (2) reperfusion-induced injury can be replicated by reoxygenation, (3) the contribution of hypoxia/hypoglycemia alone or together can be investigated, (4) the contribution of each cell type can be easily identified, (5) the molecular mechanism underlying stroke can be dissected out because of the intrinsic simplicity of this system, and (6) it allows large-scale screening of compounds that have therapeutic potentials for stroke. One major disadvantage of this in vitro model is that it lacks shear stress (see Dynamic Models section of this article for details).

Because of the key role of pericytes in BBB regulation,[4,98,99] a BMEC–pericyte coculture model has also been developed. The cells are grown in Transwell insert as described above. Pericytes dramatically enhanced the TEER in this coculture model, compared with the monolayer model.[176] In addition, multidrug resistance-related protein 6 expression and matrix metalloproteinase secretion in BMECs were increased in this model.[177,178] This model is particularly useful in the study of BEMC–pericyte interaction/communication. Although pericytes enhance BMEC tightness, astrocytes are absent in this BMEC–pericyte coculture model, suggesting that this model may be further improved by incorporating astrocytes.

To replicate the anatomic structure of the in vivo BBB fully, a BMEC–pericyte–astrocyte triple coculture system was built and characterized. In this model, BMECs are plated in the Transwell insert with pericytes on the underside of the insert and astrocytes at the bottom of the well (Figure 2A). The direct contact of BMECs and pericytes mimics the anatomic structure of neurovascular unit in vivo. Although direct cell–cell communication between astrocytes and BMECs/ pericytes is missing in this triple-culture model, indirect effect of astrocytes in BBB regulation via secreted soluble factors is included. As expected, this triple coculture model has significantly higher TEER and lower permeability,[94,179] indicating that

both pericytes and astrocytes contribute to the integrity of BBB. These data suggest that the BMEC–pericyte–astrocyte triple coculture model is a more reliable in vitro BBB model. Therefore, this model has been widely used in many BBB permeability studies.[180–185] Like the BMEC–astrocyte coculture model, this triple-culture model can also be used as an ischemia-reperfusion model for pathophysiological studies or large-scale screenings.

In addition to the above-mentioned cell types, neurons and other cells have also been cultured with BMECs. It has been shown that neurons induce the expression of BBB-related enzymes and occludin in BMECs.[111,112] Neurons also significantly decreased the leakage of sucrose in the BMEC–astrocyte coculture model.[108–110] Additionally, macrophages when cocultured with BMECs significantly increased the TEER.[186] Recently, stem cells and stem cell-derived cells have been used in in vitro BBB models. For example, embryonic neural progenitor cells have been shown to induce BBB properties in BMECs.[187–189] Neural progenitor cell-derived cell mixtures, including neurons, astrocytes, oligodendrocytes, and proliferating neural progenitors, have also been shown to induce the BBB phenotype in rat BMECs.[190]

The coculture models incorporate important players other than BMECs and thus better mirror the anatomic structure of the in vivo BBB. These coculture models generate a tighter barrier and are ideal for permeability studies, cell–cell interaction, and lead compound identification/optimization in new-drug R&D. The most significant disadvantage of these coculture models is the lack of shear stress, which plays a critical role in the induction and maintenance of the BBB phenotype.[86,191] Therefore, it is advisable to validate the results generated from the static models in dynamic models and in vivo.

Dynamic Models

Shear stress, generated by the flow of blood in physiological conditions, affects transporter and TJ expression as well as endothelial barrier function.[191] It has been shown that incorporation of flow significantly increases the expression of ZO-1 and decreases the permeability of human BMEC monolayer.[86] Thus, dynamic BBB models, which include shear stress, have been developed. There are 3 major types of dynamic BBB models: the cone-plate apparatus, dynamic in vitro BBB model, and microfluidic in vitro BBB models.

Cone-Plate Apparatus—The cone and plate viscometer was first used to build shear force.[192] In this model, a rotating cone generates shear force, which is transmitted to the endothelial monolayer via the medium (Figure 2B). The angular velocity and the cone angle determine the shear stress it generates. Because the shear stress is not evenly distributed along the radius of the plates, the endothelial monolayer receives different shear stress depending on its location in the plates. In addition, other cell components of the BBB, such as astrocytes and pericytes, are not included in this model, which limits its application in BBB research and diminishes the reliability and significance of data it generates.

Dynamic In Vitro BBB Model—To incorporate both shear stress and other cell types, microporous hollow fibers are used (Figure 2C).[193–196] In this model, BMECs and astrocytes are plated in the inner (luminal) and outer (abluminal) sides of the porous hollow fibers, respectively (Figure 2D). The culture medium is then pumped into the system via a

variable-speed pump to generate shear stress comparable with that seen in physiological conditions in vivo (5–23 dynes/cm^2).[197,198] To maintain the stable microenvironment, a gas-permeable tubing system is used for the exchange of O_2 and CO_2. Using bovine aortic endothelial cells and C6 glioma cell line, a TEER value of ≈ 600 $\Omega \cdot cm^2$ was achieved in this dynamic model.[199] Further side-by-side comparative studies showed that this dynamic model generated a TEER >10× higher than that in the Transwell coculture model and that its permeability to sucrose and phenytoin was, respectively, 10× and 5× less than that in the coculture system,[200] suggesting a tighter barrier function of this model. Cucullo et al[196] also compared the barrier function of this model using different endothelial cells and found that brain endothelial cells (human BMECs and hCMEC/D3) generated a much higher TEER (1200 $\Omega \cdot cm^2$) and less permeability to sucrose or phenytoin than endothelial cells of peripheral origin (human umbilical vein endothelial cells), rationalizing the use of brain endothelial cells to construct in vitro BBB models. Additionally, the expression of transporters, ion channels, and efflux proteins was dramatically induced in BMECs in this model.[199,201,202] Altogether, these data suggest that this dynamic model better mimics the in vivo BBB by replicating its anatomic and physiological properties.[86,167,194,196,199,200]

This dynamic in vitro BBB model has been used to study the pathophysiology of various CNS diseases, including ischemia-reperfusion-induced injury and epilepsy.[167,196,202] The molecular events during ischemia include flow disturbance (loss of shear stress) and induction of inflammatory response (release of reactive oxygen species and cytokines by white blood cells).[203] These changes have been replicated in the dynamic BBB model by flow cessation and reperfusion in the presence of white blood cells.[204] It has been shown that flow cessation for 1 hour in this system induces a biphasic opening of the BBB[167,196] and ibuprofen pretreatment partially prevents BBB failure and decreases the duration and degree of BBB breakdown,[196] suggesting that the inflammation immediately after ischemic stroke is one of the major causes of BBB failure. In another study, endothelial cells and astrocytes from normal or drug-resistant epileptic human brain tissue were cultured in this dynamic system.[202] The permeability to phenytoin, a substrate for P-glycoprotein, was significantly reduced when endothelial cells from epileptic brain were used, although TEER and permeability to sucrose were not affected.[202] This effect was independent of the origin of astrocytes and could be reversed by P-glycoprotein blocker XR9576,[202] suggesting that the drug-resistant BBB phenotype in epileptic patients can be replicated in this dynamic BBB model.

Recently, a revised model with hollow fibers with trans-mural microholes of 2 to 4 μm has been developed to allow transmigration/trafficking studies.[167] Extravasation of white blood cells was observed when the BBB is breached by flow cessation and reperfusion.[167] It should be noted that the space between BMECs and astrocytes (the thickness of the hollow fiber) is 150 μm[167] compared with the immediate contact of these cell types in vivo. This extremely thick layer may affect the extravasation of immune cells from the luminal side to the abluminal side. Thus, cautions should be taken when interpreting data generated from this dynamic in vitro BBB model. In addition, this dynamic in vitro BBB model also has three other disadvantages. First, it does not allow direct visualization of the endothelial morphology in the luminal compartment. Although cells can be characterized after trypsin treatment, the harvesting process may change the morphology and physiological

characteristics of the cells. Second, the cell number ($>1\times10^6$) and technical skills required to build this model are relatively high. Third, the time required to reach steady-state TEER is longer (usually 9–12 days)[167,199] compared with that in coculture models (usually 3–4 days). These shortcomings prevent the use of this dynamic in vitro BBB model in large-scale screens. This model, however, may be useful in lead compound validation/optimization in new-drug R&D.

Microfluidic-Based BBB Models—To address the limitations of the dynamic in vitro BBB model, microfluidic device-based in vitro BBB models have been developed.[205,206] The microfluidic BBB (µBBB) model is one of the first such models. It contains 2 perpendicularly crossing channels, which allow dynamic flow and generate shear stress; a polycarbonate porous membrane at the intersection of these channels, which enables coculture of BMECs and astrocytes; and multiple built-in Ag/AgCl electrodes for TEER measurement[205,206] (Figure 3A). These channels have a height of 200 μm and widths of 2 mm (luminal) and 5 mm (abluminal). In this model, BMECs and astrocytes are seeded on the luminal and abluminal sides of the membrane, respectively (Figure 3B). A pump and a gas-permeable tubing system are used to generate shear stress and allow O_2–CO_2 exchange, respectively. Under a shear stress of 0.023 dynes/cm^2 (much lower than that in physiological conditions), this µBBB model significantly improved the TEER of bEND.3 cells to 140 $\Omega \cdot cm^2$ after 3 days of culture, in contrast to 15 $\Omega \cdot cm^2$ in the static model.[205,206] When cocultured with astrocytes, the TEER was further increased to 250 $\Omega \cdot cm^2$.[205,206] Additionally, the permeability increased on histamine exposure, followed by recovery, suggesting the stability of this model.[205,206] This µBBB model was further improved by replacing the oxidation sensitive AgCl electrodes with inert platinum ones and decreasing the cross-sectional area.[207] These modifications allow accurate measurement of TEER and reduce the amount of cells needed. Without shear stress, hCMEC/D3 cells reached a TEER of $\approx$40 $\Omega \cdot cm^2$ within 3 days of culture in this improved µBBB model.[207] With only 18 hours of physiological shear stress (5.8 dynes/cm^2), the TEER was increased to 120 $\Omega \cdot cm^2$.[207] Tumor necrosis factor-α, a known inflammatory cytokine, dramatically decreased the TEER to 12 $\Omega \cdot cm^2$.[207] These data suggest that this improved model is able to respond to mechanical and biochemical stimuli and thus can be used in permeability studies. The different TEER values in these studies may be because of different cell types (bEND.3 versus hCMEC/D3 and monolayer versus coculture), shear stress (0.023 versus 5.8 dynes/cm^2), and time (3 days versus 18 hours).

Another microfluidic-based BBB model originally designed to study the BBB permeability of drugs involves a microhole structure.[208] This model is composed of 2 horizontally aligned chambers connected by a microhole structure (Figure 3C).[208] Endothelial cells suspended in medium will be infused in the brain chamber and trapped in the microholes because of pressure gradient. By using human umbilical vein endothelial cells and astrocyte-conditioned medium with a shear stress of 0.28 to 8.91 dynes/cm^2, Yeon et al[208] successfully demonstrated that the permeability of this system to fluorescein isothiocyanate-dextran inversely correlated with its size. Additionally, by comparing the permeability of 5 well-known drugs in this system with their in vivo data, this group further validated the reliability of this model, suggesting its application in predicting the CNS permeability of

new compounds.[208] The TEER measurement in this system, however, has not been reported. Two major concerns of this device are (1) it lacks cell–cell contact, a key feature of the in vivo BBB and (2) it does not replicate the dimensions of microvasculature in vivo. Recently, this microfluidic device has been modified and a new microfluidic-based synthetic microvasculature model of the BBB (SyM-BBB) has been developed.[209] This SyM-BBB model contains 2 microchannels separated by microfabricated pillars with 3-μm gaps (Figure 3D).[209] The pillars separated by 3-μm gaps mimic the porous membrane in the μBBB model. Endothelial cells will be infused to the blood chamber via ports 1 and 2, whereas astrocyte-conditioned medium or astrocytes can be infused to the brain chamber from port 3. The flow speed of medium in these chambers determines the shear stress. This design better mimics the in vivo microcirculatory system by including the diverging and converging bifurcations.[209] It has been reported that astrocyte-conditioned medium significantly increases the expression of TJP in RBE4 cells and decreases their permeability to fluorescein isothiocyanate-dextran in this model.[209] TEER values were not available because of the lack of electrodes in the system at this stage. Additionally, efflux activity assay revealed a functional P-glycoprotein efflux system,[209] suggesting that the Sym-BBB model can be used in drug discovery and BBB permeability studies. Further work should focus on including other cell types (astrocytes or pericytes) and integrating electrodes for TEER measurement.

Compared with the dynamic in vitro BBB model, these microfluidic in vitro BBB models have many advantages. First, because the thickness of the membrane or pillars is <10 μm,[205,206] they allow transmigration/trafficking studies in a condition that better replicates the in vivo BBB structure. Second, nondestructive microscopy is possible because of the transparency of the materials.[205,206] Third, it takes less time (3–4 days) to reach steady-state TEER.[205,206] Fourth, they only require a small amount of cells and are less demanding in terms of technical skills. These microfluidic models, on the other hand, also have some shortcomings. For example, TEER value is not high enough (250–300 $\Omega \cdot cm^2$) in these models. This may be because of the short time in culture (3–4 days). Future work should examine whether the TEER continues to increase at later time points. Additionally, such as the dynamic in vitro BBB model, these microfluidic-based models can only incorporate 2 cell types, given that the membrane and pillars (hollow fibers in the dynamic in vitro BBB model) have only 2 sides. Although there are only limited publications about these microfluidic-based BBB models to date (probably because they are relatively new), they show promising results as dynamic in vitro BBB models.[205,206] With the accumulation of research data, these microfluidic models may be used to aid neurovascular research and new-drug R&D in the future because of its small size, short time to reach steady-state TEER, low-to-moderate technical skill requirement, and low cost. The advantages and disadvantages of these in vitro BBB models are summarized in the Table.

How to Select the Appropriate Models?

Choosing appropriate in vitro models not only saves time and money, but also enables accurate interpretation of the data. One of the most important criteria in model selection is the purpose of study. If the aim is to investigate the permeability of the BBB, it is advisable to use the coculture models and dynamic in vitro BBB model if possible. Some multiculture

models are commercially available now, which significantly reduces the difficulty and time in model preparation, but increases the cost. If cell trafficking/migration is involved, microfluidic BBB model is the first choice because of the thin space between BMECs and astrocytes (<10 μm) and incorporation of shear stress. If the purpose is to explore signaling pathways, study transporter kinetics, or measure binding affinities, the use of monolayer model is very helpful because of its intrinsic simplicity. The results, however, should be validated in more sophisticated dynamic models and in vivo, before a conclusion is drawn. It is also recommended that the results in human cells be validated (if nonhuman cells are used), given that species differences may exist.

In vitro BBB models are extensively used in the first few stages of new-drug R&D processes, including target identification, hit identification, and lead identification/optimization.[1] Once a target (kinases, receptors, etc) is identified, high-throughput screening is used to identify potential drug candidates. At this stage, a very large number of compounds need to be screened, demanding an easy and fast in vitro BBB model. The monolayer models and coculture models, which take 3 to 4 days to reach steady-state TEER and are relatively easy to construct, can be used. It has been shown that, among different immortalized cell lines, the P-glycoprotein transfected Madin–Darby canine kidney cells give the best separation of passively distributed and effluxed compounds and the best correlation between in vitro and in vivo data in permeability assays.[210] Recently, a comparative study showed that the permeability of the P-glycoprotein transfected Madin–Darby canine kidney cells and vinblastine-treated Caco-2 cells alone was similar to that of the BMEC–pericyte–astrocyte triple coculture model.[180] These data suggest that the P-glycoprotein transfected Madin–Darby canine kidney cells and vinblastine-treated Caco-2 cells may be used in high-throughput screening for potential candidate identification. Because these cells are not from the brain vasculature, validation using BMECs in more sophisticated models is usually necessary. In the lead identification/optimization stage, identified compounds are validated, and structure–activity relationships and toxicological profile are investigated. Sensitive in vitro models that replicate the majority of the in vivo conditions are required. Such models include the static coculture system and dynamic models. Because of the high technical skill requirement and long culturing time (9–12 days) of the dynamic in vitro BBB model, the newly developed microfluidic-based BBB models are an alternative choice. It is advisable to use primary cells rather than immortalized cell lines at this stage to better replicate the biological properties of the BMECs in vivo. Validation using human cells is highly recommended to avoid species difference-caused failure in the subsequent R&D stages. The suggestions on the selection of in vitro BBB models at different R&D stages are summarized in Figure 4.

Conclusions

In vitro BBB models are of key importance to our understanding of the BBB functions in physiological and pathological conditions and the R&D of new drugs for various neurological disorders. Although many in vitro BBB models have been developed and used in a variety of studies, none of them can fully replicate the in vivo conditions. Knowing the advantages and disadvantages of these models and choosing the suitable models enable accurate interpretation of the data and significantly facilitate the R&D of new drugs for

many neurological diseases. In this review, we summarize the widely used and newly developed in vitro BBB models, compare their strengths and weaknesses, and provide suggestions for model selection.

Acknowledgments

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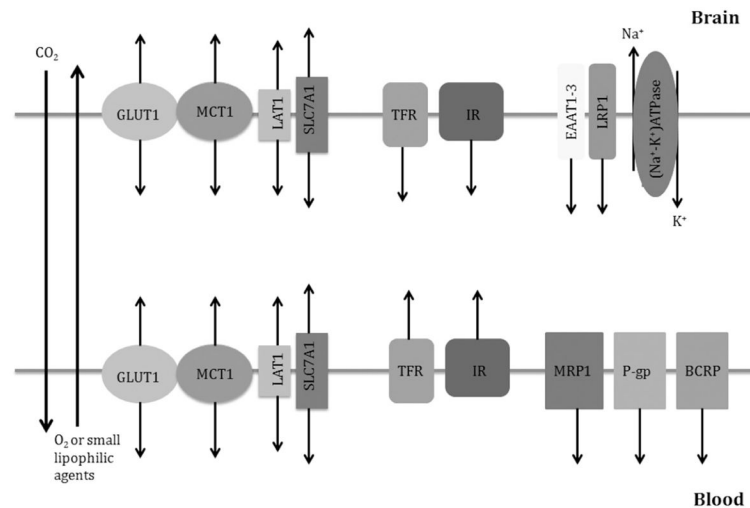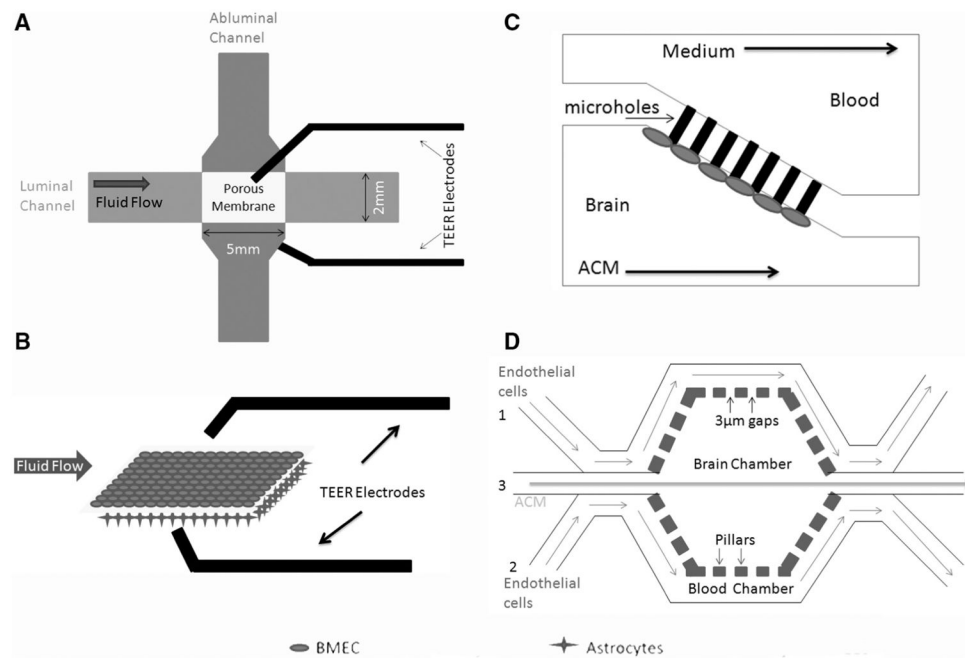
Figure 1.
Major transporters and receptors in brain microvascular endothelial cells (BMECs). Oxygen, carbon dioxide, and mall lipophilic agents diffuse across blood–brain barrier freely. Glucose transporter (GLUT1), lactate transporter (MCT1), essential amino acid transporters (LAT1 and SLC7A1) are expressed on both luminal and abluminal sides of BMECs, and transport nutrients bidirectionally. Transferrin receptor (TFR) and insulin receptor (IR) are also expressed on both sides of BMECs, and mediate endocytosis of transferrin and insulin, respectively, leading to accumulation of these molecules in BMECs. Multidrug resistance-related protein 1 (MRP1), P-glycoprotein (P-gp), and breast cancer resistance protein (BCRP) are predominantly expressed on the luminal side of BMECs and regulate drug efflux. excitatory amino acid transporters 1 to 3 (EAAT1–3) and lipoprotein receptor-related protein 1 (LRP1) are solely expressed on the abluminal side of BMECs and remove glutamate and β-amyloid from the brain, respectively. $(Na^+\text{-}K^+)$ATPase is expressed only on the abluminal side of BMECs, and controls ion homeostasis in the brain.
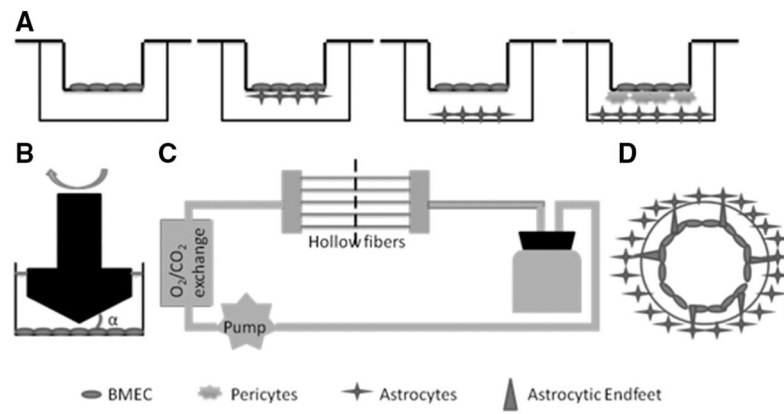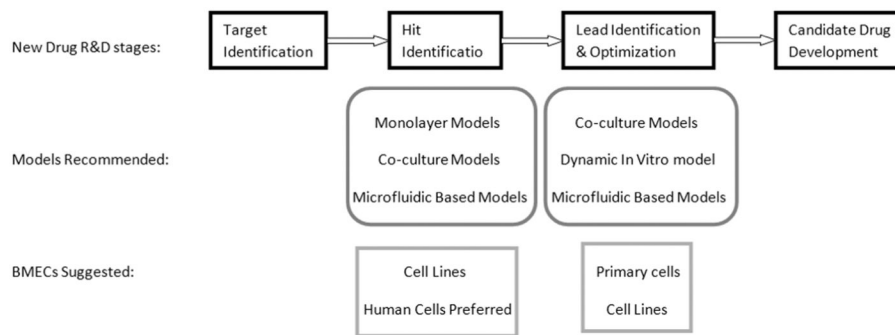
Figure 2.
In vitro blood–brain barrier (BBB) models. **A**, Brain microvascular endothelial cell (BMEC) monolayer model (**left**), BMEC–astrocyte coculture models (**middle** 2), and BMEC–pericyte–astrocyte triple-culture model (**right**). **B**, Cone-plate apparatus. α is the cone angle. **C**, Dynamic in vitro BBB model. **D**, Cross-section of one hollow fiber.

Figure 3.
Microfluidic-based in vitro blood–brain barrier (BBB) models. **A**, Microfluidic BBB
(μBBB) model. **B**, Three-dimensional view of the porous membrane at the intersection of
the flow channels in the μBBB model. **C**, Diagram of the microfluidic device containing
micro-holes. **D**, Structure of the synthetic microvasculature model of the BBB model. ACM
indicates astrocyte-conditioned medium; BMEC, brain microvascular endothelial cell; and
TEER, transendothelial electric resistance.

Table

Comparison Among Different In Vitro BBB Models

	Other Cell Types	Sheer Stress	No. of Animals Needed	Time to Steady TEER	Steady TEER Value	Gap Between Different Cell Types	Suitable for Migration Assays	Technical Requirements	Cost	Nondestructive Microscopy
Monolayer models	No	No	Moderate	3–4 d	Low to moderate	N/A	Yes	Low	Low	Yes
Coculture models	Yes	No	High	3–4 d	Moderate to high	<10 μm	Yes	Moderate	Low to moderate	Yes
Contact apparatus	No	Yes	Moderate	3–4 d	N/A	N/A	No	Low to moderate	Low	No
Dynamic in vitro BBB model	Yes	Yes	Very high	9–12 d	High	>150 μm	No	High	High	No
Microfluidic-based models	Yes	Yes	Low	3–4 d	Moderate to high	<10 μm	Yes	Moderate	Low	Yes

BBB indicates blood–brain barrier; and TEER, transendothelial electric resistance.

Stroke. Author manuscript; available in PMC 2015 December 03.