Advances in Brain Vascular Research

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Edited by

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CHAPTER ONE

ENDOTHELIAL CYTOARCHITECTURE AND HETEROGENEITY IN THE BRAIN

MARIA ALEXANDRA BRITO

The vascular endothelium

The term endothelium was first used by Swiss anatomist Wilhelm His in 1865 to differentiate the epithelium from the inner lining of the body cavities, which included blood vessels, lymphatics, and mesothelial-lined cavities, a definition that was later narrowed down to include only the inner cell layer of blood vessels and lymphatics (Aird 2007). Within the developing vertebrate embryo, a rudimentary vascular meshwork of endothelial cells is lumenized into epithelial tubes that are differentiated into specialized subtypes, including arteries, veins, and capillaries, which become stabilized by mural cells (Fish and Wythe 2015). Arteries give rise to the microvasculature, which sequentially comprises arterioles (10-100 μm), capillaries (4-10 μm), and venules (10-100 μm) (Ge, Song, and Pachter 2005), which in turn end up in veins. Arterial and venous blood vessels are formed by an inner endothelial layer (tunica intima), a middle smooth muscle layer (tunica media) and an outer connective tissue layer (tunica adventitia). In contrast, capillaries lack a tunica media, so endothelial cells are ensheathed by cells with contractile properties, known as pericytes (Sá-Pereira, Brites, and Brito 2012).

Brain endothelial cells

The central nervous system (CNS) is protected by a complex interface, known as the blood-brain barrier (BBB). The BBB prevents a wide range of molecules present in the plasma from entering that otherwise could enter the brain and induce cellular activation and neural tissue damage; moreover, it strictly regulates the concentration of ions in the brain

parenchyma, which is essential for normal brain function (Cardoso, Brites, and Brito 2010). The anatomic basis of the BBB is formed by brain microvascular endothelial cells (BMEC), which possess unique features that limit paracellular and transcellular permeability, rendering the brain endothelium a selective barrier. Despite the fact that important interactions are established with the remaining components of the neurovascular unit, particularly the basement membrane, pericytes and astrocytes (Sá-Pereira, Brites, and Brito 2012), this chapter is focused on the main cellular and molecular characteristics of BMEC. As it is currently accepted that endothelial cells display genotypic and phenotypic heterogeneity, BMEC's variable properties are also addressed.

Endothelial cell cytoarchitecture

BMEC are characterized by elaborate junctional complexes that restrict permeability across the endothelium, by cell adhesion molecules that are involved in the crosstalk between the endothelium and immune cells, and also by specific transport systems that ensure the supply of necessary molecules to the brain and the elimination of potentially harmful molecules, the main features of which are presented below.

Junctional complexes and cell adhesion molecules

The connection between barrier integrity and function and the presence of endothelial intercellular junctions was an important hallmark in studying the BBB. Junctional complexes are formed by tight junctions (TJ), adherens junctions (AJ) and gap junctions (GJ). While in epithelial cells the distribution of TJ and AJ is more organized, with apical TJ and basolateral AJ, in endothelial cells the junctional architecture is less defined, and AJ are intermingled with TJ (Bazzoni and Dejana 2004). GJ are involved in intercellular communication (Stamatovic et al. 2016), though this type of junctions at the BBB has been far less described than TJ and AJ, or than GJ in epithelia.

Tight junctions

TJ proteins form strains along the intercellular junction region, establishing a connection with the opposing membrane and obliterating the intercellular space. TJ are formed by a set of transmembrane proteins that are linked to cytosolic proteins, which in turn establish the connection with the cytoskeleton. There are two major types of transmembrane proteins,

classified according to the number of membrane-spanning domains they contain: four-pass transmembrane proteins such as claudins, occludin, and tricellulin, and single-span proteins, including junctional adhesion molecules (JAM) (Mariano et al. 2011). Claudins form dimers and bind homotypically to claudins on adjacent cells to constitute the primary seal of the TJ. The claudin family has 27 elements that have been identified until now and are differentially expressed in epithelia and endothelia (Mineta et al. 2011). However, claudin-5 has been the family member most researched in BBB studies so far, both in vitro and in vivo (Cardoso et al. 2012, Liu et al. 2018). Interestingly, the expression of several family members has recently been demonstrated, namely claudins-1, -3, -4, -5, -11, -20 and -25, in BBB microvessels, and claudin-5 is the dominant protein in vitro (Berndt et al. 2019), explaining why it has been the most extensively studied.

Occludin was the first transmembrane TJ protein to be identified, but its requirement for TJ formation, regulation or both is still controversial. On the one hand, occludin-deficient mice did not show alterations in the barrier properties of the intestinal epithelium (Saitou et al. 2000). On the other hand, occludin's role in TJ sealing has been demonstrated (Lacaz-Vieira et al. 1999), with a direct influence on the permeability of epithelial and endothelial barriers, including the BBB (Argaw et al. 2009, Jiao et al. 2011). The recently identified tricellulin belongs to the same family as occludin and is concentrated at the intersection between three epithelial cells. Further studies on tricellulin have demonstrated that this TJ protein plays an essential role in the regulation of permeability to macromolecules (Krug et al. 2009). Tricellulin was later identified in the brain microvasculature in situ, as well as in cultured BMEC (Mariano et al. 2013), suggesting a role in BBB integrity and properties. Although the potential interactions of tricellulin with other TJ proteins and the mechanisms that regulate the expression and function of this protein are still unclear, they have recently started to be unraveled (Mariano et al. 2011).

JAM are other transmembrane proteins that mediate protein interactions in the TJ region. The JAM family is divided into two subgroups based on different binding-domain motives. Class I members interact with TJ accessory proteins and have been identified in endothelial and epithelial cells, and they have an active role in controlling endothelial permeability and leukocyte transmigration (Martin-Padura et al. 1998). Members of class II include the endothelial cell-selective adhesion molecule, a JAM protein found exclusively in endothelial cells (Nasdala et

al. 2002), loss of which was shown to enhance vascular endothelial growth factor (VEGF)-initiated permeability (Wegmann et al. 2006).

The cytoplasmic proteins of the TJ are responsible for the connection of junction complexes to the actin cytoskeleton. The most studied TJ accessory components are zonula occludens (ZO) proteins. In contrast to ZO-3, ZO-1 and -2 are both present in endothelial cells (Balda and Anderson 1993, Jesaitis and Goodenough 1994). Alterations in ZO-1 levels in BMEC have been associated with TJ disruption and loss of BBB integrity in pathological conditions (Zhong et al. 2012, Jiao et al. 2011, Palmela et al. 2012). All ZO proteins share common domains that are essential for signal transduction and enable the anchoring of transmembrane TJ proteins and actin filaments (Furuse et al. 1994, Van Itallie et al. 2009). Actin filaments serve both structural and dynamic roles in the cell, and alterations of the structural organization of this cytoskeleton protein have a direct influence on TJ integrity, as suggested in hypoxia conditions (Brown and Davis 2005).

Adherens junctions

AJ not only provide a second site for cell adhesion but are also involved in cell polarization and tissue morphogenesis (Nishimura and Takeichi 2009), as well as in receptor signaling and transendothelial migration of leukocytes (Sweeney et al. 2019). Similarly to TJ, different classes of proteins are required for AJ assembly: transmembrane and the cytoplasmic proteins, which serve as a link between the transmembrane components and the cytoskeleton (Niessen and Gottardi 2008). The most widely characterized transmembrane and cytosolic proteins in endothelial cells are vascular endothelial (VE)-cadherin and catenins, respectively.

Classical cadherins are single-pass transmembrane glycoproteins that function as homophilic receptors to mediate Ca^{2+}-dependent intercellular adhesion. Cadherin clustering is crucial for maintaining AJ integrity and is sustained by interactions between cadherin molecules of the same cell and on the opposite cell (Gumbiner 2005). The role of VE-cadherin in vascular integrity has been shown through experiments targeting the extracellular domain of this transmembrane protein, with a consequent increase in endothelial permeability (Corada et al. 2001). Studies with BMEC reinforced the role of VE-cadherin in BBB function since protein loss or redistribution by the action of cytokines led to an impairment in barrier integrity (Shen et al. 2011).

Cytosolic catenins include α-catenin and β-catenin. The majority of β-catenin in the cell is associated with AJ, where it interacts with the

cytoplasmic region of cadherin (Aberle et al. 1994). There is also a small and dynamic pool of β-catenin in the cytoplasm and nucleus that is responsible for the transduction of Wnt signals. The reduction of β-catenin levels has been shown to cause AJ fragility and increased BMEC permeability in injurious situations (Wylezinski and Hawiger 2016). Besides binding to cadherins, β-catenin contains binding information in a different domain for α-catenin (Pokutta and Weis 2000), involved in the connection of the AJ complex to actin. Despite the shortage of studies on the function of α-catenin, interference with its binding to β-catenin has shown to affect endothelial integrity and impair leukocyte transmigration (van Buul, van Alphen, and Hordijk 2009). Although alterations in TJ proteins have been associated with compromised barrier function, a growing number of studies have highlighted the crucial role of accurate AJ assembly in such endothelial function. In particular, both VE-cadherin and β-catenin have been shown to mediate the expression of BMEC claudins (Taddei et al. 2008, Liebner et al. 2008), validating their role in BBB integrity.

Platelet endothelial cell adhesion molecule-1 (PECAM-1), also known as cluster of differentiation 31 (CD31), is a transmembrane protein highly enriched at interendothelial junctions of vascular endothelial cells, suggested to contribute to the regulation of vascular integrity and remodeling. Although it has been considered by some authors to be an AJ protein (Sweeney et al. 2019), others consider it to be a transmembrane protein located outside junctional complexes (Wimmer et al. 2019). Considering that its role as a cell adhesion molecule (CAM) is widely accepted, in this chapter it is described in further detail in the section about such molecules.

Gap junctions

GJ are formed by members of the connexin (Cx) family (Eugenin et al. 2012), which is composed of several transmembrane isomers with tissue-specific expression. Canonically, connexins function as homo- or hetero-hexamers at the plasma membrane and can exist as hemichannels (HC) or GJ, the latter being characterized by the alignment of two neighboring cell surface hexamers to oppose each other. GJ mediate intercellular communication by connecting the cytoplasm of adjacent cells and allowing the passage of ions and small molecules, thus leading to signal transduction between neighboring cells (Stamatovic et al. 2016). In the brain, GJ complexes mediate the important signaling between BMEC and both astrocytes and pericytes (De Bock et al. 2014). Interestingly, in

BMEC, Cx have been shown to contribute to BBB permeability control and suggested to be associated with occludin and claudin-5, as well as to stabilize brain endothelial junctions, while nonspecific Cx channel blockers inhibited the barrier function of the TJ. The brain microvascular endothelium expresses Cx37 and Cx40, whereas Cx43 was observed in freshly isolated capillary and cultured BMEC, but not in brain slices (De Bock et al. 2014). These apparently inconsistent findings may be explained by the fact that Cx43 is expressed in the growing microvessels of the 18 week-old human telencephalon but disappears as their differentiation progresses (Virgintino et al. 2001). Cx43 redistribution in endothelial cells with increased BBB permeability has been observed with ageing, suggesting that a loss of endothelial cell communication may underpin ageing-associated barrier defects (De Bock et al. 2011).

Cell adhesion molecules

CAM, which are involved in immune responses, have been classified into different families of proteins according to their structure: integrins, selectins and proteins from the immunoglobulin superfamily. Integrins are cell-surface transmembrane αβ heterodimers that recognize specific extracellular matrix (ECM) ligands. The expression of integrin subunits α1 and α6 is associated with the presence of β1-ECM ligands, like laminin, collagen IV and fibronectin (Paulus et al. 1993). Importantly, the diversity of roles attributed to integrins appears to be dependent on the specific combination of proteins. For instance, the function of integrin αvβ3 seems to include the induction of endothelial migration and angiogenesis, being greater after the onset of focal ischemia and influencing vascular hyperpermeability (Abumiya et al. 1999). On the other hand, loss of β1 integrin in ischemic injury correlates with maximal neuronal injury and loss of microvessel integrity (Tagaya et al. 2001). Moreover, β1 integrin block has been linked to a reduction in claudin-5 expression and an increase in microvessel permeability (Osada et al. 2011). These observations point to a novel regulatory mechanism with β1 integrin/ECM adhesion and BMEC TJ, promoting BBB integrity.

The selectin family consists of three Ca^{2+}-dependent CAM: leukocyte (L-), endothelial (E-), and platelet (P-) selectins, which vary depending on the number of extracellular consensus repeats with homology to complement regulatory proteins: two, six or nine, respectively (Barthel et al. 2007). In endothelial cells, both P- and E-selectins are expressed (Yoshida et al. 1996), and their levels can be increased by several stimuli, like cytokines and pathogens (Huang, Gonzalez, and Eniola-Adefeso

2013, Wong and Dorovini-Zis 1996). The selectin family plays a key role in mediation of early neutrophil rolling and adherence to BMEC, through interaction with L-selectin on the leukocyte surface (Engelhardt 2008). Although the upregulation of selectins is particularly important to recruit immune cells to resolve the injury, it may also play a detrimental role if the aberrant homing of leukocytes aggravates the symptoms. In fact, in brain ischemic injury, selectins appear to play an important role in exacerbating immune cell recruitment and consequent injury (Zhang et al. 1998), and blocking these adhesion molecules has been shown to constitute a promising therapeutic approach (Yilmaz et al. 2011). In contrast, studies in an animal model of multiple sclerosis have demonstrated that the expression of endothelial selectins is not involved in immune cell recruitment through the BBB (Doring et al. 2007). Therefore, the role of selectins in the recruitment of immune cells in pathological conditions appears to be complex and might depend on the expression of other molecules.

Proteins from the immunoglobulin superfamily share the presence of several extracellular immunoglobulin domains (Wang and Springer 1998). The most studied proteins from this family in endothelial cells are intercellular CAM-1 (ICAM-1), PECAM-1 and vascular CAM-1 (VCAM-1), which typically have 5, 6 and 7 immunoglobulin domains, respectively. In BMEC, these three CAM are crucial for the recruitment of immune cells to the injured brain area, especially in pathological conditions such as multiple sclerosis (Engelhardt et al. 1997, Washington et al. 1994).

Transport across the BBB

Due to elaborate junctional complexes, the brain endothelium can be seen as a continuous cell membrane across which lipid molecules can diffuse and enter the brain passively, whereas polar and high molecular weight molecules are not able to freely cross the endothelium (Abbott et al. 2010). These properties allow the movement of blood gases oxygen and carbon dioxide across the BBB but prevent many essential molecules from entering the brain, including nutrients, hormones and vitamins, and restrict the elimination of unwanted compounds (Abbott et al. 2010, Sweeney et al. 2019). To ensure the metabolic demands of the brain and its homeostasis, endothelial cells of brain capillaries are equipped with a number of transport systems that can be categorized as efflux transporters, solute carrier-mediated transporters, and transcytosis.

Efflux transporters

Some molecules that have a high lipid solubility have a lower brain penetrance than might be expected based on their physicochemical characteristics. This fact results from the existence of efflux transporters in BMEC that promote the efflux from endothelial cells into the blood and/or extrusion out of the brain of both endogenous molecules and xenobiotics, thus preventing their accumulation in the brain. Despite the relevance of efflux transporters for CNS homeostasis, they represent an enormous obstacle for the treatment of brain pathologies.

The most studied efflux transporters are the ATP-binding cassette (ABC) family, which mediates the exporting of substrates from cells coupled with the hydrolysis of ATP (Shen and Zhang 2010). There are 49 genes encoding the transporters in this family, arranged into seven subfamilies (termed A–G), among which the subfamilies B, C and G, which encode for P-glycoprotein (ABCB1), MRP (or multidrug resistance-associated protein) (ABCC1-9) and BCRP (or breast cancer resistance protein) (ABCG2) (Videira, Reis, and Brito 2014). P-glycoprotein and BCRP are expressed at BBB level (Gomez-Zepeda et al. 2019) in the same order of magnitude (Morris, Rodriguez-Cruz, and Felmlee 2017). Both BCRP and P-glycoprotein are expressed at the luminal surface (Roberts et al. 2008), although a fraction of P-glycoprotein in the abluminal membrane has also been detected (Tai et al. 2009). A clear expression of MRP4 has been demonstrated in the luminal membrane of rat BBB endothelial cells (Roberts et al. 2008). As far as other MRP are concerned, and particularly for MRP1, conflicting results can be found in the literature. In fact, some researchers have described a weak expression at BBB capillaries (Roberts et al. 2008), while others have stated that it is present in choroid plexus capillaries but not in those of the BBB (Gazzin et al. 2011), which overall indicate a low expression of this efflux transporter at the BBB. Differences among species in the expression of the efflux transporters have been described, with a higher expression of BCRP and a lower expression of P-glycoprotein and of MRP4 in humans than in rodents (Morris, Rodriguez-Cruz, and Felmlee 2017).

BCRP and P-glycoprotein are important elements of barrier function. These transporters have substrate overlap, as shown by the fact that inhibition of one of the two transporters is not sufficient to deliver anticancer drugs into the brain because of compensation by the other transporter, which led to the paradigm that P-glycoprotein and BCRP work as a “cooperative team of gatekeepers” at the BBB (Agarwal et al. 2011). This does not exclude some substrate specificity, as recently shown for

benzylpenicillin that is transported by BCRP but not by P-glycoprotein (Li et al. 2016).

To overcome the action of efflux transporters in therapeutics, their inhibition has been used in the treatment of brain pathologies like amyotrophic lateral sclerosis (Jablonski et al. 2014) and brain cancer (On and Miller 2014). The importance of P-glycoprotein and BCRP to BBB function and brain homeostasis has been highlighted and their deregulation in neurodegenerative diseases has been demonstrated. In fact, an upregulation of both P-glycoprotein and BCRP has been shown in amyotrophic lateral sclerosis, epilepsy, as well as in stroke and ischemic brain injury, whereas a selective decrease of P-glycoprotein, with no changes in BCRP, has been observed in Alzheimer's disease (AD) and multiple sclerosis (Qosa et al. 2015).

Solute carrier-mediated transport

Endothelial cells forming the BBB express a large number of solute carriers that provide transport of a wide variety of solutes and nutrients, mediating their flux into and out of the brain. These transporters have a specific and polarized distribution in endothelial cells and are expressed in either the luminal or abluminal membrane, or both, depending on the direction of transport into or out of the brain, or can be bidirectional (Abbott et al. 2010). These transporters are grouped based on the type of molecule transported, which include carbohydrates, amino acids, nucleotides, and organic anions and cations (Ohtsuki and Terasaki 2007, Sweeney et al. 2019). Energy transporters are one of the most important systems, and energy sources include glucose, mannose, and creatine, among others. One of the most studied groups of transporters in BMEC is the glucose transporter (GLUT) family with 14 isoforms (GLUT 1-14) (Patching 2017), responsible for mannose and glucose transport. GLUT-1 is highly enriched in BMEC, with higher expression on the luminal side than at the abluminal membrane, as a mechanism to generate a glucose transport gradient (Moura et al. 2019). Due to the relevance of this transporter at the BBB, glucose and other sugar derivatives have been considered as promising shuttles to achieve drug delivery to the brain via GLUT-1 (Patching 2017). Evidence highlights the importance of GLUT-1 expression for proper brain function, as this transporter is reduced in disorders that affect the brain, like AD (Hooijmans et al. 2007).

Transcytosis

Transcytotic mechanisms allow the transporting of a variety of large molecules and complexes across the BBB that otherwise would be physically prevented from entering the brain parenchyma (Abbott et al. 2010). This type of transport involves endocytic mechanisms, in which extracellular molecules are internalized at one pole of the plasma membrane and subsequently are transported via caveolae or clathrin-coated vesicles to the opposite side, where they are exocytosed. Caveolae are vesicles rich in sphingolipids and cholesterol, with caveolins as the main protein component (Stan 2005), and particularly caveolin-1 (Cardoso, Brites, and Brito 2010). The expression of caveolin-2 alone does not lead to caveolae formation and requires caveolin-1 for transportation (Parolini et al. 1999), whereas experiments in mice lacking caveolin-1 highlighted its importance in the formation of caveolae (Park et al. 2002). In the brain, caveolin-1 has been identified in BMEC (Ikezu et al. 1998, Bernas et al. 2010) and suggested to play an active role in the regulation of BBB vesicular permeability (Predescu, Predescu, and Malik 2007), partially because of its influence on junction proteins (Nag, Venugopalan, and Stewart 2007, Zhong et al. 2008, Kronstein et al. 2012). Clathrin-coated vesicles are negatively charged and, thus, repel anionic molecules. So, only a small fraction of the plasma proteins, characterized by a positive charge, can be transcytosed by clathrin-coated vesicles (Herve, Ghinea, and Scherrmann 2008).

Soluble plasma molecules can be randomly taken up by caveolae with a bulk of blood plasma and then be transported across brain endothelial cells. This process, known as bulk-phase or fluid-phase transcytosis (FMT), is independent of any interaction between the transported molecules and the caveolar vesicle membrane. Due to the negative charge of clathrin-coated vesicles, only a very small portion of the plasma proteins can be transcytosed randomly within the fluid phase of transport by these vesicles (Herve, Ghinea, and Scherrmann 2008). In contrast to the FMT, transcytosis can involve the interaction of a ligand with moieties expressed at the luminal surface of endothelial cells. This type of transcytosis may occur via specific binding to a receptor (receptor-mediated transcytosis, RMT) or by a nonspecific process (adsorptive-mediated transcytosis, AMT). Both in RMT and AMT, the lysosomal compartment needs to be avoided to prevent degradation of the protein or peptide and achieve transcytosis (Abbott et al. 2010).

RMT is characterized by the binding of a macromolecular ligand to the corresponding receptor in the endothelial cell surface that triggers an endocytic process with formation of a caveola. The caveola is then

released and the ligand-receptor cluster is internalized into the endothelial cell and routed across the cytoplasm towards the opposite pole of the cell where the vesicle content is exocytosed, with dissociation of the complex occurring during cellular transit or the exocytotic event (Abbott et al. 2010). Among the several receptors involved in RMT at the BBB are the insulin receptor, the transferrin receptor, the lipoprotein receptor-related protein receptor-1 (LRP-1), and the receptor for advanced glycation end products (RAGE) (Abbott et al. 2010, Sweeney et al. 2019). Insulin and transferrin receptors allow insulin and iron-loaded transferrin to enter the brain to supply it with the hormone and iron respectively (Paterson and Webster 2016, Lajoie and Shusta 2015). Importantly, these receptors have been exploited as BBB shuttles for the delivery of drugs to the brain, in a strategy known as Trojan horses (Pardridge 2006). LRP-1 is mainly localized in the abluminal membrane of endothelial cells, while RAGE is mainly expressed at the luminal membrane (Sweeney et al. 2019). LRP-1 and RAGE transport amyloid-β (Aβ) peptide, one of the hallmarks of AD across the BBB. LRP-1 transports Aβ out of the brain by transcytosis, mediating its efflux from the brain and representing the major efflux transporter for Aβ across the microvasculature (Shibata et al. 2000). LRP-1 is decreased in the BBB of AD patients (Silverberg, Messier, et al. 2010), which contributes to Aβ accumulation in the brain parenchyma. In contrast to LRP-1, RAGE constitutes an entrance gate for peripheral Aβ to enter the brain parenchyma and is upregulated in AD (Silverberg, Miller, et al. 2010), thus promoting Aβ accumulation in the brain. Targeting of these transport systems to promote Aβ elimination and/or to prevent peripheral Aβ entrance into the brain parenchyma has emerged as a promising strategy for AD treatment (Deane et al. 2012, Kook et al. 2012, Sagare et al. 2013).

In AMT, a compound is internalized through a direct electrostatic interaction with the endothelial cell surface, which triggers the endocytic process (Moura et al. 2019). Thus, positively charged molecules like albumin interact with the negatively charged cell luminal membrane of BBB endothelial cells to be internalized (Pulgar 2018). Interestingly, AMT was recently suggested to be the transcytosis mechanism by which α-synuclein-containing erythrocyte-derived extracellular vesicles cross the BBB towards the brain and further points to AMT as a mechanism involved in the communication between the periphery and the brain, as well as in α-synuclein-related pathology initiation and spread (Matsumoto et al. 2017). The AMT mechanism has been used for drug delivery to the brain of large therapeutic molecules such as neuropeptides and proteins or even drug-encapsulated vectors like liposomes and nanoparticles using

cationic proteins and basic oligopeptides, such as cell-penetrating peptides, as targets (Lu 2012).

Endothelial cell heterogeneity

Endothelial cells have the ability to sense and respond to their local environment and meet the physiological requirements of the underlying tissue (Aird 2007). Therefore, in contrast to the long naively considered layer of functionally invariant cells, endothelial heterogeneity is now acknowledged at several levels, namely between different organs, between large and small vessels within the same organ, and between adjacent vascular segments, which reflect functional diversity (Ge, Song, and Pachter 2005). Accordingly, gene expression analysis has revealed significant variation in gene expression patterns between endothelial cells from microvessels of different tissues, from large vessels and microvessels, and from arteries and veins, as well as in pathological conditions, as reviewed by Ge et al. (Ge, Song, and Pachter 2005). These authors have also pointed out the differences between arterioles, capillaries and venules in the organization of junctional complexes, rate of transcytosis, and the expression of some enzymes. Furthermore, segmental characterization has revealed that as the vessels' size increases, there is a progressive decrease in the expression of transporters enriched at the BBB, P-glycoprotein and GLUT-1, together with a progressive increase in the expression of α-smooth muscle actin. It has also been demonstrated that P-glycoprotein expression is homogeneous in the whole cerebral capillary bed, reflecting the well-known protective role of the efflux transporter (Saubamea et al. 2012). Expression of the key marker of rat BBB, endothelial barrier antigen (EBA), is not uniform in the cerebral vasculature, being undetectable in arterioles and strongly expressed in venules, while an uneven expression has been observed in capillaries, with some vascular segments strongly labelled and others only faintly stained. This unexpected pattern for a protein associated with the integrity of the BBB suggests that it may be dynamically regulated at single-cell level in healthy brains (Saubamea et al. 2012). Variability in the endothelial surface charge has also been shown by the less intense anionic sites in venules than in capillaries or arterioles (Vorbrodt 1987), in line with the negative surface charge of the BBB mentioned above. Leukocyte extravasation occurs solely at the level of post-capillary venules (Engelhardt and Ransohoff 2005), which have leaky junctions and are loosely covered by a layer of pericytes; moreover, local changes in hemodynamics result in greatly reduced blood flow rates that increase the

chances of leukocyte contact with the vessel's endothelial lining (Muller 2013).

Along the wall of pre-capillary arterioles, capillaries, and post-capillary venules, there are variations in the abundance of pericytes, which rise at post-capillary venules and tend to disappear and be replaced by smooth muscle cells as the vein caliber increases (Sá-Pereira, Brites, and Brito 2012). Moreover, the degree of vascular coverage by pericytes varies with tissue type, appearing to be correlated with the degree of tightness of the interendothelial junctions. Although it is recognized that CNS pericytes are numerous surrounding brain capillaries, the precise extent of the vascular surface covered by pericytes is still unclear. In fact, Frank et al. (Frank, Dutta, and Mancini 1987) mention that pericytes cover 22–30% of the cerebral capillary surface, whereas Dalkara and colleagues (Dalkara, Gursoy-Ozdemir, and Yemisci 2011) state 30–70%, and Engelhardt and Sorokin (Engelhardt and Sorokin 2009) claim that they cover 99% of the abluminal surface of the capillary basement membrane in the brain. In any case, the pericyte-to-endothelia ratio in the brain is higher than in other organs (1:3 compared with 1:100 in striated muscles) (Dalkara, Gursoy-Ozdemir, and Yemisci 2011), and pericyte coverage in retina is even higher than in brain capillaries (Frank, Dutta, and Mancini 1987), in accordance with the suggested role of pericytes in maintaining the BBB and the blood-retinal barrier. There are also variations among species, as the average ratio of pericytes to ECs in the rat capillary is 1:5, whereas it is 1:4 in the mouse, and 1:3-4 in humans (Dore-Duffy and Cleary 2011).

Brain endothelial diversity

In line with the organ-specific heterogeneity in both genotypic and phenotypic characteristics and signature gene regulatory networks, brain endothelial cells present the most prominent upregulation of the "Wnt signaling" and "adherens junction" pathways. Moreover, analysis of regulatory transcription factors that could maintain brain endothelial cell-specific upregulation of the Wnt signaling pathway have revealed the upregulation of lymphoid enhancer-binding factor 1, known to interact with β-catenin and regulate differentiation of the BBB in vivo (Jambusaria et al. 2018).

Besides organ-specific endothelial heterogeneity, there is also variability in endothelial cell populations within the brain. In fact, there are regional differences in BBB function that reflect functional diversity within the CNS' anatomically distinct regions (Wilhelm et al. 2016) and there are certain brain regions devoid of BBB, as at the level of

circumventricular organs (CVO) (Cardoso, Brites, and Brito 2010). CVO include important elements of the neuroendocrine system and secretory organs (e.g. posterior pituitary, pineal gland, median eminence, subcommissural organ and the subfornical organ) where an exchange of circulating substances takes place. In these CVO, capillaries are characterized by thinner endothelial cells that contain fenestrations and discontinuous TJ, together with a lower expression of TJ proteins, which result in enhanced permeability (Wilhelm et al. 2016). It should also be highlighted that there are even differences among CVO microvessels, which display higher permeability in central regions, where TJ proteins like occludin, claudin-5 and ZO-1 are undetectable, whereas a low level of staining was detectable in distal subdivisions of secretory CVO (Morita et al. 2016).

It has been shown that BBB permeability for specific molecules is heterogeneous throughout the brain. For example, brain uptake of insulin in rats is higher in the hippocampus than in the cortex, changes that could not be attributed to differences in insulin receptor expression levels (Banks and Kastin 1998). On the other hand, the chemokine C-C motif ligand 11 (CCL11) crosses the BBB with transport rates that vary between brain regions, with the fastest transport occurring in the striatum (Erickson et al. 2014). Regarding interleukin (IL)-1 transport in SAMP8 mice, the fastest transport was observed in the pons-medulla (Moinuddin, Morley, and Banks 2000). Studies by Villasenor and colleagues (Villasenor et al. 2017) have shown that transcytosis significantly increased in a region-specific manner, and suggested that the regional variations in BBB permeability could be determined by different molecular pathways regulating transcellular permeability in BMEC.

Interestingly, even in the same brain region and tissue section, endothelial variability has been observed in the expression of a widely expanded set of endothelial markers, and it appears that this molecular heterogeneity is a property generalized to many markers (Lee et al. 2017). To explain this observation, the authors propose that the coordinate patterns of expression could define several different endothelial cell types within the brain or that cells exhibit distinct molecular patterns due to environmental cues. Importantly, analysis of such markers may make it possible to target populations for alterations in endothelial cells in specific conditions that could be used in personalized medicines.

In line with regional heterogeneity, pathological processes of specific brain regions are usually associated with BBB impairment in those regions, as exemplified by the fact that after status epilepticus induced in rats, BBB leakage was observed in limbic regions, while cortical brain

regions were not affected (van Vliet et al. 2014). There are also regional differences in BBB permeability associated with ageing as revealed by magnetic resonance imaging (MRI) analysis of humans showing that the hippocampus and the caudate nucleus, but not other brain regions, present BBB hyperpermeability in people with no cognitive impairment, an effect that was accelerated in cognitively impaired individuals (Montagne et al. 2015).

Conclusion

BMEC have the common characteristic of forming a single cell layer that lines all brain blood vessels and regulates exchanges between the bloodstream and the surrounding tissue. The most relevant knowledge about BMEC cytoarchitecture was brought together here, essential for understanding the unique barrier properties of the complex structure that they form, the BBB. Moreover, alterations occurring in BBB endothelial cells in brain disorders were pinpointed, in line with the new paradigm according to which vascular dysfunction is a key player in the onset and progression of brain diseases. Moreover, overcoming the BBB for therapeutic purposes based on the shuttling of drugs relying on BMEC transport systems was addressed. Despite the common and specific properties of brain endothelial cells, heterogeneity within brain microvasculature has increasingly been recognized. Such diversity among different brain regions and even within the same one was also addressed. Importantly, understanding brain microvascular dissimilarities may pave the way to cell-specific targeting approaches in personalized medicine.

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