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Review

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In vitro models of the blood-brain barrier

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The blood-brain barrier (BBB) is an active interface between the circulation and the central nervous system (CNS) with a dual function: the barrier function restricts the transport from the blood to the brain of potentially toxic or harmful substances; the carrier function is responsible for the transport of nutrients to the brain and removal of metabolites. The BBB plays a crucial role in the clinical practice as well. On the one side there is a large number of neurological disorders including cerebral ischemia, brain trauma and tumors, neurodegenerative disorders, in which the permeability of the BBB is increased. On the other hand due to the relative impermeability of the barrier many drugs are unable to reach the CNS in therapeutically relevant concentration, making the BBB one of the major impediments in the treatment of CNS disorders. The significant scientific and industrial interest in the physiology and pathology of the BBB led to the development of several *in vitro* models of the BBB. These models are mainly based on the culture of cerebral endothelial cells. The best *in vitro* models which mimic the best way the *in vivo* anatomical conditions are the co-culture models in which brain endothelial cells are co-cultured with astrocytes and/or pericytes. Our *in vitro* BBB model is characterized by high transendothelial electrical resistance (TEER regularily above 200 Ohm×cm^2), low permeability and expression of several transporters. Our experiments have proven that the model is suitable for basic research and for testing the interaction between the BBB and potential drug candidates (toxicity, permeability, interaction with efflux transporters) as well.

Key words: cerebral endothelial cells, blood-brain barrier, *in vitro* models, permeability, tight junction

INTRODUCTION

The blood-brain barrier (BBB) is an active interface between the circulation and the central nervous system (CNS) which restricts the free movement of different substances between the two compartments and plays a crucial role in the maintenance of the homeostasis of the CNS. The BBB has a dual, a barrier and a carrier function. The barrier function means that the BBB restricts the transport of potentially toxic or harmful substances from the blood to the brain which is achieved through a fourfold defense line:

1) The paracellular barrier formed by interendothelial junctions restricts the free movement of water soluble compounds between two adjacent cells.

2) The transcellular barrier is made possible by the low level of endocytosis and transcytosis characteristic for brain endothelial cells and inhibits transport of substances through the cytoplasm.

3) The enzymatic barrier is provided by a complex set of enzymes, including acetylcholinesterase, alkaline phosphatase, gamma-glutamyl transpeptidase, monoamine oxidases, and other drug metabolizing enzymes capable to degrade different chemical compounds.

4) In addition to these, the cerebral endothelium expresses a large number of efflux transporters (ABC, ATP-binding cassette transporters) like ABCB1 (P-glycoprotein), ABCC1, ABCC4 and ABCG2 (BCRP).

Besides the barrier function the BBB possesses an important carrier function which is responsible for the transport of nutrients to the brain and removal of metabolites. Small lipid-soluble molecules and blood gases like oxygen and carbon dioxide diffuse passively the BBB, while essential polar nutrients like glucose and amino acids require specific transport proteins (solute carriers, SLC transporters) in order to reach the brain.

The BBB plays a crucial role in the clinical practice as well. On the one side there is a large number of neurological disorders including cerebral ischemia, brain

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trauma, tumors, neurodegenerative disorders in which the permeability of the BBB is increased (Weiss et al. 2009). On the other hand, due to the relative impermeability of the barrier many drugs are unable to reach the CNS in therapeutically relevant concentration, making the BBB one of the major impediments in the treatment of CNS disorders (Jeffrey and Summerfield 2010).

CELLULAR COMPONENTS OF THE BBB

The principal components of the BBB are the endothelial cells, astrocytes, pericytes (Fig. 1). Some other cellular elements like neurons or microglia may also play a significant role in the function of BBB.

Endothelial cells

From the point of view of the permeability the most important cell types of the BBB are cerebral endothelial cells (CECs) which form a continuous sheet covering the inner surface of the capillaries. Endothelial cells are interconnected by tight junctions which form a belt-like structure at the apical region of the cells. Brain endothelial cells have both endothelial-like features (i.e., expression of von Willebrand factor, uptake of acetylated LDL, high activity of alkaline phosphatase and gamma-glutamyl transpeptidase) and epithelial-like features like continuous line of tight junctions, low level of pinocytosis, high transendothelial electrical resistance (TEER).

Pericytes

Endothelial cells are sitting on the basal membrane which consists mainly of collagen IV, fibronectin, laminin and proteoglycans. Engulfed in the basal membrane are the pericytes which cover approximately 22-32% of the endothelium. Pericytes play an important role in the regulation of endothelial proliferation, angiogenesis and inflammatory processes (for review see: Dore-Duffy 2008). In the absence of pericytes an abnormal vasculogenesis, endothelial hyperplasia and increased permeability occurs in the brain (Armulik et al. 2010).

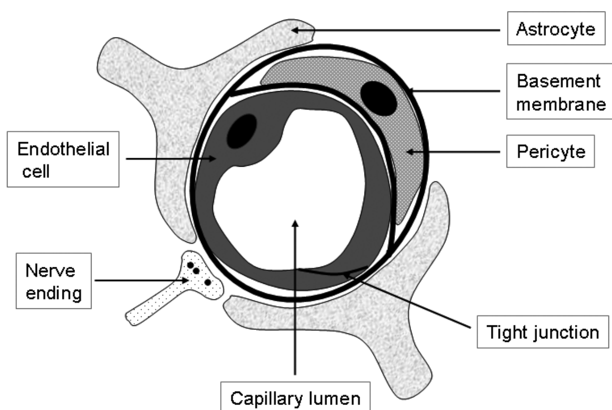

Fig. 1. Cellular structure of the BBB.

Astrocytes

Astrocytes are also important components of the BBB and are capable to induce BBB properties in endothelial cells (for review see: Abbott et al. 2006). Endfeet of astrocytes cover a significant part of the endothelial surface (Kacem et al. 1998). Astrocytes are sources of important regulatory factors like TGF-beta, GDNF, bFGF, IL-6. Astrocytes deficient in GFAP with incomplete functionality are not able to induce BBB properties (Pekny et al. 1998).

Other cellular components associated with the BBB

Although neurons are not directly involved structurally in the formation of the BBB, cerebral capillaries are innervated by different noradrenergic, serotonergic, cholinergic or GABA-ergic neurons (Hawkins and Davis 2005). Neurons can regulate important aspects of BBB function and can induce the expression of BBB-related enzymes in cultured cerebral endothelial cells (Tontsch and Bauer 1991). Microglial cells are also found in the perivascular space, playing a very important immunological role, however, their contribution to the BBB properties is not well characterized.

DETERMINING ELEMENTS OF BBB PERMEABILITY

Intercellular junctions

From the point of view of paracellular permeability the interendothelial junctions including tight junctions (TJs) and adherens junctions (AJs) play a crucial role.

Tight junctions

Tight junctions of CECs act as a physical barrier forcing most molecular traffic to take a transcellular

route across the BBB, rather than moving paracellularly through the junctions, as in most endothelia. Presence of a continuous line of tight junctions at the cell-cell borders is one of the most important elements of the BBB phenotype of CECs. In this respect brain endothelial cells resemble epithelial cells. TJs are responsible for the separation of the apical and the basolateral membrane domain leading to the polarization of the cell ('fence function'), and for the restriction of the paracellular pathway ('gate function') (for review see: Gonzalez-Mariscal et al. 2003).

The molecular components of the TJs can be separated into transmembrane and cytoplasmic plaque proteins. Transmembrane proteins of endothelial TJs include occludin (Furuse et al. 1993), junctional adhesion molecules (Martin-Padura et al. 1998) and members of the claudin family (Furuse et al. 1998). Brain endothelial cells express claudin-5 (Morita et al. 1999) and to a smaller extent claudin-3, -10, -12, and possibly other subtypes (Ohtsuki et al. 2008). Plaque proteins link transmembrane proteins to the actin cytoskeleton and include PDZ-containing proteins, like zonula occludens (ZO)-1 (Stevenson et al. 1986), ZO-2 (Gumbiner et al. 1991) and non-PDZ proteins like cingulin (Citi et al. 1988, 1989) or JACOP (junction-associated coiled-coil protein)/paracingulin (Ohnishi et al. 2004).

Adherens junctions

AJs are ubiquitous in the vasculature and mediate adhesion of endothelial cells to each other, contact inhibition during vascular growth and remodeling, initiation of cell polarity, and – in part – regulation of paracellular permeability. The transmembrane proteins of the adherens junctions are the cadherins, in the case of vascular endothelial cells mainly VE-cadherin (Breier et al. 1996), which is linked through the catenins (alpha, beta and gamma) to the cytoskeleton. A proper function of the adherens junction is needed for tight junction formation (Schulze and Firth 1993). In addition, TJs and AJs may be even structurally interconnected, since it has been shown that ZO-1 and ZO-2 can interact with alpha-catenin (Itoh et al. 1997, 1999).

Paracellular permeability has been reported to be increased in several neurological disorders including cerebral ischemia, brain tumors, and neurodegenerative disorders. These functional changes are usually associated with changes in the expression and localization of principal TJ proteins (for review see: Weiss et al. 2009).

Efflux transporters

From the point of view of drug permeability efflux transporters are of special importance. A large number of drugs are substrates of ABC transporters and therefore cannot reach their CNS target in adequate concentrations. Brain endothelial cells are able to express a whole set of ABC transporters. One of the most important is P-glycoprotein (ABCB1) which is able to transport a large variety of lipophilic drugs out of CECs (for review see: Demeule et al. 2002, Begley 2004). In addition, other members of the ABC transporters may also have a significant contribution. CECs have been shown to express members of the multidrug resistance proteins (MRPs, or according to the new nomenclature the ABCC family) like MRP1, MRP4, MRP5, MRP6 and expression of BCRP (ABCG2) is also well documented (for review see: Loscher and Potschka 2005, Shen and Zhang 2010). Expression profile of these transporters largely determines permeability properties and their functionality is an important requirement for the quality of the *in vitro* models.

SLC transporters

The SLC family of membrane transport proteins includes over 300 members organized in 48 subfamilies. Brain endothelial cells express high amounts of glucose transporter 1 (GLUT1, SLC2 family) and a large number of other transporters as well. These include transporters of the organic anion/cation transporter family (OAT or SLC22 family), organic anion transporter family (OATP or SLC21 family), cationic amino acid transporters (SLC7 family members), monocarboxilate transporters (MCT or SLC16 family members) and members of the proton/oligopeptyde transporters like PEPT1 and 2, PHT1 and 2 (SLC15) (Carl et al. 2010). The importance of these transporters is not yet fully understood, however, it is evident that drugs which are substrates of these transporters can reach high concentrations in the CNS.

Other mechanisms

The negatively charged endothelial glycocalyx is also involved in maintaining low vascular permeability and the asymmetrically distributed enzymes (enzy-

matic barrier) contribute to inactivation of their specific substrates. Under physiological conditions the vesicular transcytosis in CECs is considerably limited, however, in some pathological conditions the number of pinocytotic vesicles may rise leading to an increased permeability (Cipolla et al. 2004).

DETERMINATION OF PARACELLULAR BARRIER CHARACTERISTICS *IN VITRO*

Measurement of TEER

There are several methods to determine the barrier properties of *in vitro* models. The paracellular permeability can be relatively monitored using measurement of transendothelial electrical resistance (TEER). The most widely used "classical" method to determine TEER is based on the culture of endothelial cells on semipermeable filters which define two compartments: the apical, upper compartment which can be considered as "blood-side" and the basolateral, lower compartment which is the "brain side". For the measurement of TEER two electrodes are used, one being placed in the upper and the other in the lower chamber and the electrodes are separated by the endothelial layer. Since the surface of the filter should be taken into consideration the results are expressed in Ohm×cm^2. Good models have values in the order of magnitude of hundreds. The other method is the use of ECIS method (electrical cell substrate impedance sensing). Here, endothelial cells are cultured on special electrode surfaces and the resistance is determined based on the voltage drop between these electrodes and a larger counter electrode placed in the medium. A monolayer with low paracellular permeability has resistance valued in the order of magnitude of 10000 Ohm (Hartmann et al. 2007). This method was not designed to use with co-culture models. Resistance values referred in the present manuscript were obtained with the "classical" method.

Measurement of permeability

Besides "electrical" methods permeability of tracer substances with known molecular weight can also be used. Sodium fluorescein (MW = 376 Da) and FITC-dextran are the most widely used tracers. The apparent permeability is determined using the following formula: $P_{app} = dQ/(dT \times A \times C_0)$, where dQ is the transported amount, dT the incubation time, A the surface of filter and C_0 the initial concentration. Permeability values in the order of magnitude of 10^{-6} cm/s for sodium fluorescein are considered good values.

IN VITRO MODELS USED FOR THE STUDY OF THE BBB

The significant scientific and industrial interest in the physiology and pathology of the BBB led to the development of several *in vitro* models of the BBB.

Models based on cells of non-cerebral origin

There is a significant number of publications using different types of epithelial cells or endothelial cells of non-cerebral origin for the study of different aspects of BBB function. Especially MDCK (Madin-Darby canine kidney) cells are relatively widely used in this respect. MDCK cells have good paracellular permeability characteristics; however, there are major differences between MDCK and brain endothelial cells. Although epithelial and endothelial TJ structures show considerable similarities, in MDCK cells claudin-1 is the principal claudin whereas in CECs claudin-5 is the most important, moreover, ZO-3 is not expressed in CECs. Further differences are in transporter expression which may determine brain penetration of drugs. Despite these disadvantages studies on MDCK cells can be used with limitations in the study of paracellular permeability. Furthermore, since they are easy to culture and to transfect, modified MDCK cells (overexpressing P-glycoprotein or LRP) have been used for permeability screens (Wang et al. 2005, Nazer et al. 2008). Human umbilical endothelial cells (HUVECs) have also been used as BBB models (Langford et al. 2005). The advantage of HUVECs is that they are a human cell line, however, not of cerebral origin.

Taken together, these models might be suitable to address some specific questions of BBB function; however, they cannot be considered real BBB models.

In vitro BBB models based on the culture of cerebral endothelial cells

An important step towards an *in vitro* BBB model was the succesful isolation of brain capillaries (Joó and Karnushina 1973) which was followed a few years later by the first cerebral endothelial cultures (Panula

et al. 1978, DeBault et al. 1979). Soon the first cultures on semipermeable supports appeared (Bowman et al. 1983) which after recognition of the importance of astrocytic factors opened the way to the co-culture models (Tao-Cheng et al. 1987, Laterra et al. 1990, Rubin et al. 1991).

Primary cultures

Primary cultures of endothelial cells isolated from a relatively broad spectrum of mammals are in use nowadays. The most widely used are brain endothelial cells of rat, mouse, pig and bovine origin (for review see: Deli et al. 2005). A great advantage of the rodent models is the availability of experimental animals and the possibility to use transgenic animals in the case of mouse models. Furthermore, these animals are the best characterized ones, and the availability of antibodies, cloned genes also favors their use. However, due to their small size, relatively low amounts of endothelial cells can be obtained from them. The advantage of using pig or bovine endothelial cells relies on the large quantity of cells obtainable, good permeability properties, however, the access to these animals is restricted and they are not so well characterized from biochemical or molecular point of view. The use of human primary cells is also restricted by the unavailability of experimental material. Brain tissue for isolation usually originates from surgical material (Persidsky et al. 1997, Bernas et al. 2010) which often cannot be considered as a "healthy" tissue.

Cell lines

The relatively high costs and special skills required for the isolation of brain endothelial cells led to the development of several cell lines. Brain endothelial cells are able to form spontaneus cell lines without transformation. Such cell lines originating from mice and pig were used up to 35-40 passages (DeBault et al. 1981, Tontsch and Bauer 1989).

One of the best characterized brain endothelial cell lines is RBE4 obtained by the transfection of rat brain microvessel endothelial cells with a plasmid containing the E1A adenovirus gene (Roux et al. 1994). RBE4 cells have been shown to retain many BBB characteristics like high alkaline phosphatese and gamma-glutamyl transpeptidase activity (Roux et al. 1994) and expression of P-glycoprotein (Régina et al. 1998). This cell line has been used in the study of signalling characteristics of brain endothelial cells (Krizbai et al. 1995, Fabian et al. 1998, Smith and Drewes 2006, Zhang et al. 2009), regulation of P-glycoprotein (Pilorget et al. 2007, Yu et al. 2007), cell migration (Barakat et al. 2008) and permeability studies (Pan et al. 2005).

A well characterized rat brain endothelial cell line is GP8 as well, which was obtained by immortalization of rat cerebral endothelial cells using SV40 large T antigen (Greenwood et al. 1996). This cell line has also been used successfuly in different signalling studies (Etienne et al. 1998, Vandamme et al. 2004, Lupo et al. 2005) and regulation of P-glycoprotein activity (Régina et al. 1999, Deli et al. 2001). Several studies in which GP8 or its derivatives were used parallelly with RBE4 show similar characteristics for both cell lines (Fabian et al. 1998, Etienne et al. 1998, Vandamme et al. 2004). The GPNT cell line was obtained from GP8 cells retransfected with a selection

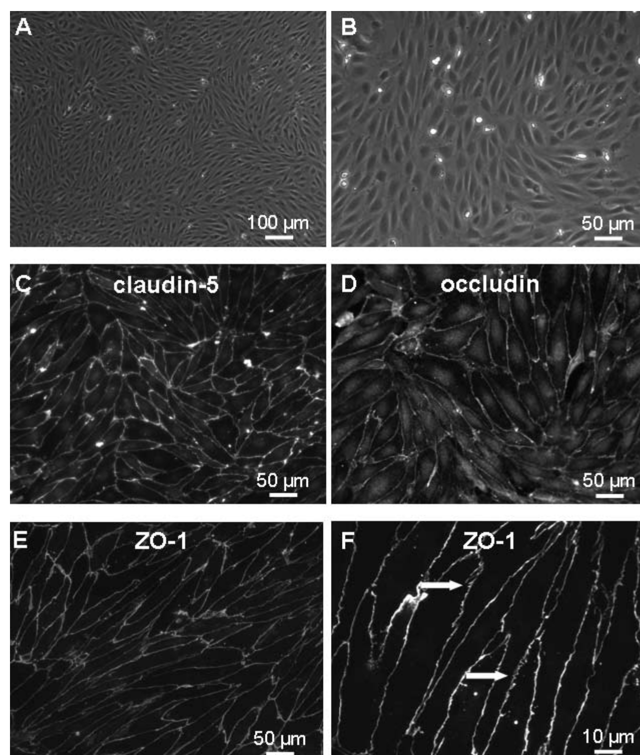

Fig. 2. Expression of junctional proteins in CECs. Phase contrast images (A, B) show the spindle shaped morphology of RBECs in culture. Transmembrane tight junction proteins claudin-5 (C), occludin (D) and the plaque protein ZO-1 (E, F) show a continuous membrane staining at the cell-cell borders. Arrows indicate interdigitations at contact sites characteristic for the endothelium (F).

plasmid containing the puromycin resistance gene (Régina et al. 1999).

Another widely used cerebral endothelial cell line is the commercially available b-end 3-5 of murine origin also used mainly in signalling studies (Lee et al. 2010) but permeability studies as well (Tan et al. 2001, Omidi et al. 2003). Porcine (Neuhaus et al. 2006) and bovine (Sobue et al. 1999) cell lines are also available but far less well characterized.

Due to the restricted availability of human material for the isolation of primary human CECs, development of a reliable human brain cerebral endothelial cell line was of primordial importance. The best characterized human cell line is the hCMEC/D3 which has been shown to retain important BBB characteristics, like expression of junctional proteins and efflux transporters (Weksler et al. 2005). Besides widespread signalling studies (Schreibelt et al. 2007, Lim et al. 2008, Wilhelm et al. 2007, 2008, Zhong et al. 2008, Fischer et al. 2009) transporter regulation was also investigated (Dauchy et al. 2009, Zastre et al. 2009, Carl et al. 2010) and has been proposed as a model system for drug transport investigations as well (Poller et al. 2008). Recently, a new conditionally immortalized human microvascular endothelial cell line was established using a temperature sensitive SV40-T antigen. These cells express occludin and claudin-5 at the cell boundaries as well as several influx and efflux transporters (Sano et al. 2010).

Co-cultures

Although cerebral endothelial cells are the principal components of the BBB several other cell types play important regulatory roles in the induction and maintenance of a properly functioning BBB. This led to the inclusion of glial cells, pericytes and even neurons in different BBB models (for review see: Deli et al. 2005).

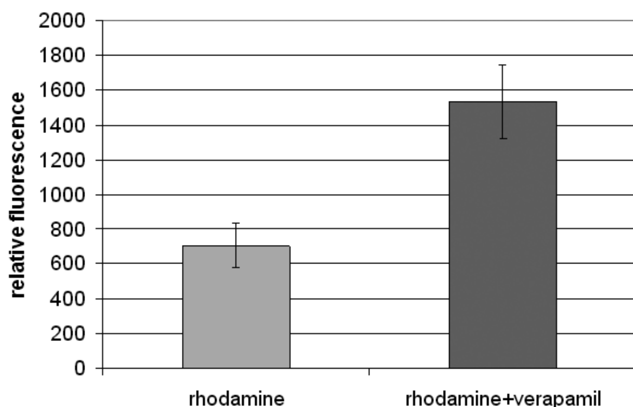

Fig. 3. Rhodamine-123 uptake in primary rat brain endothelial cells.

Co-culture of endothelial cells with glial cells

Most *in vitro* co-culture models use brain endothelial cells and glial cells. A great step towards the understanding of the BBB was the discovery that glial cells are able to induce BBB properties (DeBault and Cancilla 1980). Since the interaction of cerebral endothelial cells with astrocytes has been extensively studied (Abbott et al. 2006) endothelial glial co-culture models have became the most widespread models. Endothelial cells are usually primary cells of rat, bovine or mouse origin; however, even cell lines are used. Glial cells can also be primary cells or the C6 cell line, which has been intensively used for the study of gliomas (Hu et al. 2010). Some models are singeneic, where both brain endothelial cells and glial cells come from mouse (Stamatovic et al. 2005), or rat (Veszelka et al. 2007, Hutamekalin et al. 2008). Other models use cells of different origin: for example bovine endothelial cells and rat astrocytes.

High TEER (500-600 Ohm×cm^2) and low permeability values have been obtained with bovine endothelial cells co-cultured with astrocytes (Dehouck et al. 1990, Zysk et al. 2001), C6 glioma cells (Raub 1996) or cultured in the presence of astrocyte-conditioned medium (Rubin et al. 1991). Similarily, good results were obtained with models based on rat or mouse endothelial cells and astrocytes (Kis et al. 2001, Deli et al. 2003, Hutamekalin et al. 2008). There is a considerable effort to establish human models but success is limited by the difficulty to access human brain tissue. A further difficulty is that the available tissue is usually of surgical origin, which can be considered intact only with serious limitations. These difficulties are reflected by the high variability of the permeability of these models; however, TEER values of 200-300 Ohm×cm^2 have been measured (Megard et al. 2002). Porcine models proved to be also useful (Franke et al. 2000), co-culture of porcine brain endothelial cells with C6 astroglioma showed TEER values up to 900 Ohm×cm^2 (Smith et al. 2007).

Using porcine brain endothelial cells and astrocytes in contact and without contact Cohen-Kashi Malina

and coworkers (2009) have shown that a direct contact between endothelial cells and astrocytes is necessary to obtain good TEER values. Other systems using no direct contact between endothelial cells and astrocytes showed low pemeability values. Based on the method of Dehouck and others (1990) a modified BBB model was introduced suitable for high throughput screening in which a "BBB-inducing medium" is used. This BBB-inducing medium contains 1% conditioned medium from the glial-endothelial co-culture, harvested 48 h after refreshing the co-culture system medium and frozen until further use (Culot et al. 2007).

Endothelial cell lines in co-culture with glial cells have also been used as *in vitro* BBB models. The highest controversy is linked to the use of ECV304 cells. This cell line although shows endothelial characteristics (Kiessling et al. 1999, Suda et al. 2001) and in co-culture with glial cells can reach relatively high TEER values, proved to be a non-endothelial human cell line (Brown et al. 2000). Despite this controversy this model is still used as an *in vitro* BBB model (Kuhlmann et al. 2006, Wang et al. 2010). The mouse cerebral endothelial cell lines b-end 3 and 5 in co-culture with C6 glioma cells exhibit TEER values well below 100 Ohm×cm^2. Similarly RBE4 cells and rat astrocytes or C6 cells also show high permeability values (in the range of 10^{-5} cm/s for sucrose). A triple culture model using RBE4.B cells has demonstrated that neurons and astrocytes are able to induce a significant decrease in the permeability for sucrose (Schiera et al. 2005).

Co-culture of endothelial cells with pericytes

Pericytes are in close contact with endothelial cells therefore a co-culture of endothelial cells with pericytes in *in vitro* models is plausible. Co-culture of brain endothelial cells with pericytes was shown to increase TEER in a rat model (Hayashi et al. 2004), and pericytes have been shown to induce MRP6 expression (Berezowski et al. 2004) in endothelial cells. Surprisingly, other data indicate that pericytes induce MMP secretion in endothelial cells (Zozulya et al. 2008). In the interaction between pericytes and endothelial cells endothelin-1 (Dehouck et al. 1994), TGF-beta (Dohgu et al. 2005) and angiopoetin-1 (Hori et al. 2004) may play a significant role. More recently, a triple co-culture model including endothelial cells, astrocytes and pericytes was characterized and shown to have high TEER and low permeability (Nakagawa et al. 2007, 2009).

Co-culture of endothelial cells with other cells

Neurons have been shown to induce blood-brain barrier related enzymes in cultured cerebral endothelial cells (Tontsch and Bauer 1991). A co-culture model of RBE4.B cells and cortical neurons was established by Cestelli and colleagues (2001) and it has been demonstrated that it is not necessary a direct contact among endothelial cells and neurons for the induction of occludin expression. Moreover, not only mature neurons but differentiating embryonic neural progenitor cells are also able to induce BBB properties in cerebral endothelial cells (Weidenfeller et al. 2007). Interestingly, Zenker and coauthors (2003) found that the TEER of brain capillary endothelial cells was increased by 50% in a non-contact co-culture with macrophages.

Dynamic models

There is increasing evidence that shear stress is able to affect endothelial barrier function (Tarbell 2010). This led to the development of dynamic *in vitro* models. For this purpose usually hollow fibers are used which mimic capillaries and allow co-culture of other cell types as well. The first models were based on the co-culture of bovine aortic endothelial cells and glial cells (Stanness et al. 1997, Cucullo et al. 2002). Recently a dynamic model allowing pulsatile flow and using the human cerebral endothelial cell line hCMEC/D3 and astrocytes was developed. The model showed TEER around 1000 Ohm×cm^2. Interestingly, the same cells used in a static model provided only TEER values around 70 Ohm×cm^2 (Cucullo et al. 2008). A further modification of the dynamic BBB model was described in which hollow fibers were used with transmural microholes (2 to 4

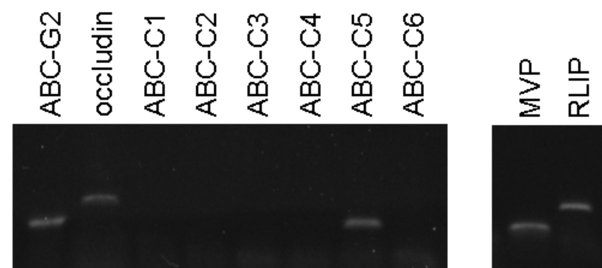

Fig. 4. Expression of transporters in CECs. RT-PCR was performed using cDNA of RBECs and specific primer pairs for rat transporters.

micrometers) making the model suitable for immune cell transmigration studies. The model retained high TEER and low permeability properties (Cucullo et al. 2010).

Similar results were obtained by other investigators as well. Human fetal astrocytes and especially astrocyte-conditioned medium reduced significantly the permeability of an *in vitro* BBB model based on the culture of primary human brain endothelial cells. Under static conditions TEER values of about 500 Ohm×cm^2 could be reached. Fluid shear stress of 1-2 dyne/cm^2 reduced the permeability to approximately 50% (Siddharthan et al. 2007).

The large number of different *in vitro* models suggests that there is no „perfect" model so far and – depending on the studied question – one or the other model can be more advantageous. Studies on intracellular signalling have been performed in the majority of cases on monocultures cultured in Petri dishes. This method has the advantage that endothelial cells can be used in large quantities for biochemical studies, physiological investigations (migration, proliferation) and the optical characteristics of the plastic Petri dishes are superior to different filters. Besides this, the experimental costs are also lower. The disadvantage of these approaches lies in that cellular interactions within the BBB cannot be taken into consideration. Primary cultures from different species have been successfully used including human, bovine, porcine; rat or mouse origin. The use of cell lines is also widespread, however they cannot be recommended when investigations on tight junction structure and function are in focus.

The culture on different filter systems is usually the method of choice when cellular interactions are important and these can be regarded as true BBB models. These models are mainly used when barrier properties (TEER, permeability) are investigated or in drug transport studies. TEER and permeability are two important parameters in the determination of the quality of the *in vitro* model. Unfortunately, under culture conditions brain endothelial cells do not show the same characteristics as *in vivo*, especially regarding the barrier properties (Wolburg et al. 1994). However, under carefully chosen experimental conditions *in vitro* models may have good barrier characteristics and are useful tools in drug permeability and transport experiments. It has been demonstrated that above TEER values of 120-130 Ohm×cm^2 the permeability for sodium fluorescein and FITC-labeled dextrane (4 kDa) does not decrease with the increase of TEER (Gaillard and deBoer 2000) indicating that barriers with at least 120-130 Ohm×cm^2 can be used for transport experiments. Best results are obtained with models based on primary cerebral endothelial cells; the major limitation of the models using endothelial cell lines is their relatively high paracellular permeability (Roux and Couraud 2005).

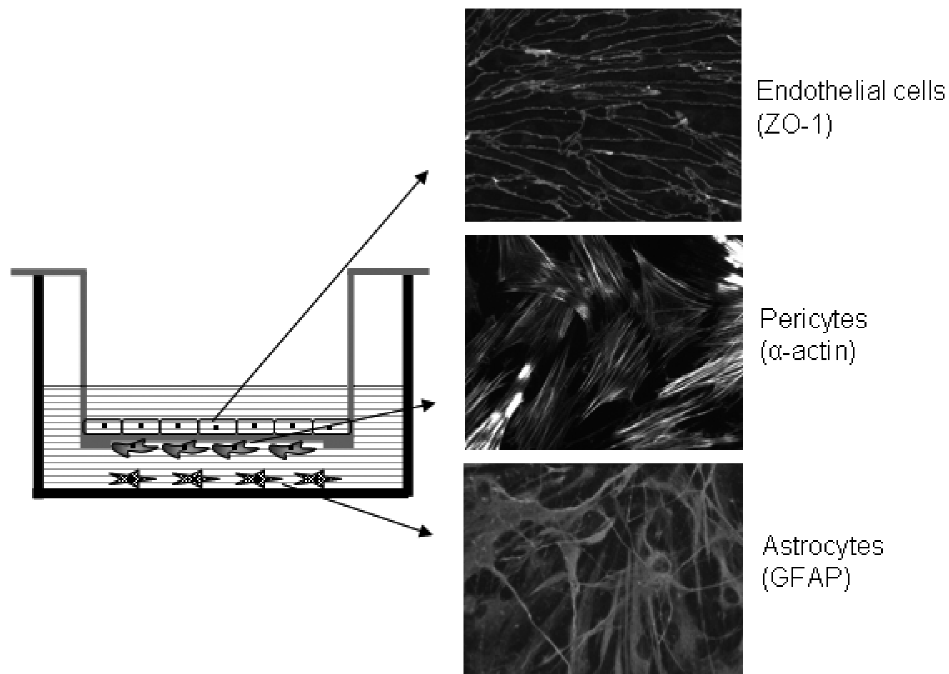

Fig. 5. *In vitro* model of the BBB.

CHARACTERIZATION OF A RAT *IN VITRO* BBB MODEL

Culture of primary cerebral endothelial cells

Our *in vitro* BBB model is based on the culture of primary rat brain endothelial cells (RBECs). Endothelial cells are isolated from cortices of 2-3-week old Wistar rats, using a two step enzymatic digestion and centrifugation on Percoll gradient (Wilhelm et al. 2007, Hutamekalin et al. 2008, Nagyoszi et al. 2010). Following plating on collagenIV/fibronectin-coated Petri dishes cells reach confluency within 5 days. Cells show a spindle shaped morphology (Fig. 2A, B) and form a continuous line of tight junctions at the cell borders as revealed by staining with antibodies against the TJ proteins claudin-5, occludin and ZO-1 (Fig. 2C-F).

Besides junctional proteins brain endothelial cells express a whole set of transporters as well. One of the most important efflux transporters with a decisive impact on drug delivery is P-glycoprotein (P-gp/mdr1/Abcb1). The presence of a functional P-glycoprotein was tested using rhodamine-123 uptake. Verapamil, a specific inhibitor of P-gp was able to induce a more than twofold increase in the rhodamine-123 uptake indicating that this transporter is present and active in RBECs (Fig. 3). RT-PCR experiments have demonstrated the expression of other transporters as well, like ABCC5, ABCG2, MVP (major vault protein) and RLIP (non-ABC transporter RalA binding protein) as well (Fig. 4).

Co-culture systems

For the co-culture system pericytes are obtained from cerebral microvessels plated onto non-coated dishes. Within 10 days an almost pure pericytic culture is obtained as assessed by staining with alpha-actin antibody (Fig. 5).

Glial cultures, consisting mainly of astrocytes (Fig. 5) are prepared from newborn rats and are cultured on poly-L-lysine coated surfaces.

As *in vitro* BBB models both double and triple co-culture systems can be used, based on the culture of endothelial cells with astrocytes and/or pericytes. The triple co-culture system, which is the closest to the *in vivo* situation, is prepared as follows: rat brain pericytes are plated onto the backside of 12-well Transwell filters (pore size: 0.4 μm; 1.5×10^4 cells/filter). On the next day endothelial cells are plated onto the upper surface of the filters. After reaching confluency, the endothelial monolayer is supplied with 550 nM hydrocortisone, 250 μM CPT-cAMP and 17.5 μM RO-201724 and placed into dishes containing glial cultures for 24 h. In the lower compartment astrocyte-conditioned medium can also be used with similar barrier characteristics of the model. We have obtained TEER values of 264 ± 67 Ohm×cm^2 when astrocytes were used vs. 252 ± 32 Ohm×cm^2 when astrocyte-conditioned medium was used, calculated from four independent experiments.

Measurement of TEER

We can measure TEER using chopstick electrodes and an EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, Florida, USA). The disadvantage of this method is that the plates containing the cultures need to be removed from the thermostate for measurements, which can be made only at certain time points.

The cellZscope system (NanoAnalytics, Münster, Germany) allows for continuous monitoring of the transendothelial electric resistance of up to 24 filters. After reaching confluency on the filter inserts endothelial cells already develop TEER values above 100 Ohm×cm^2. At this point, the endothelial monolayer is supplied with 550 nM hydrocortisone, 250 μM CPT-

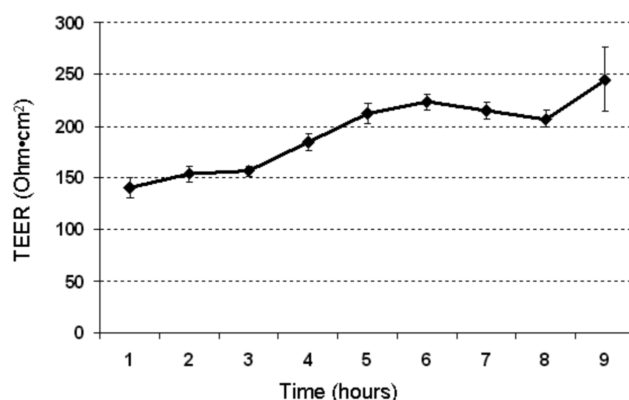

Fig. 6. Induction of TEER by hydrocortisone, cAMP and astrocyte-conditioned medium. Confluent RBECs grown on semipermeable filter inserts were supplied with 550 nM hydrocortisone, 250 μM CPT-cAMP and 17.5 μM RO-201724 from the apical side and astrocyte-conditioned medium from the basolateral side. TEER was monitored using the CellZscope system.

cAMP and 17.5 μM RO-201724 and placed into the wells of the instrument containing astrocyte-conditioned medium. TEER values increase gradually reaching values above 200 Ohm×cm^2 (Fig. 6).

Permeability measurements

The barrier function can also be evaluated by measuring the permeability of the cells to sodium-fluorescein (SF, MW = 376 Da) and Evans blue labeled albumin (EBA, MW = 67 kDa). Transwell filters containing endothelial cells and pericytes are washed with Ringer-HEPES solution (pH 7.4). Ringer-HEPES is added to the abluminal side of the filter. The luminal side is loaded with Ringer-HEPES containing 10 μg/ml SF, 170 μg/ml Evans blue and 10 mg/ml BSA. The cells are then incubated at 37ºC for 1 h with gentle shaking, and samples are collected from the abluminal side. Concentration of SF and EBA can be measured using a fluorescent microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 520 nm for SF, and 584/680 nm excitation/emission wavelengths for EBA. Apparent permeability (P_{app}) is calculated as described previously. We have achieved permeability values of 2.48×10^{-6} ($\pm$ 1.87×10^{-6}) cm/s for SF and 8.32×10^{-7} ($\pm$ 2.26×10^{-7}) for EBA (8 independent

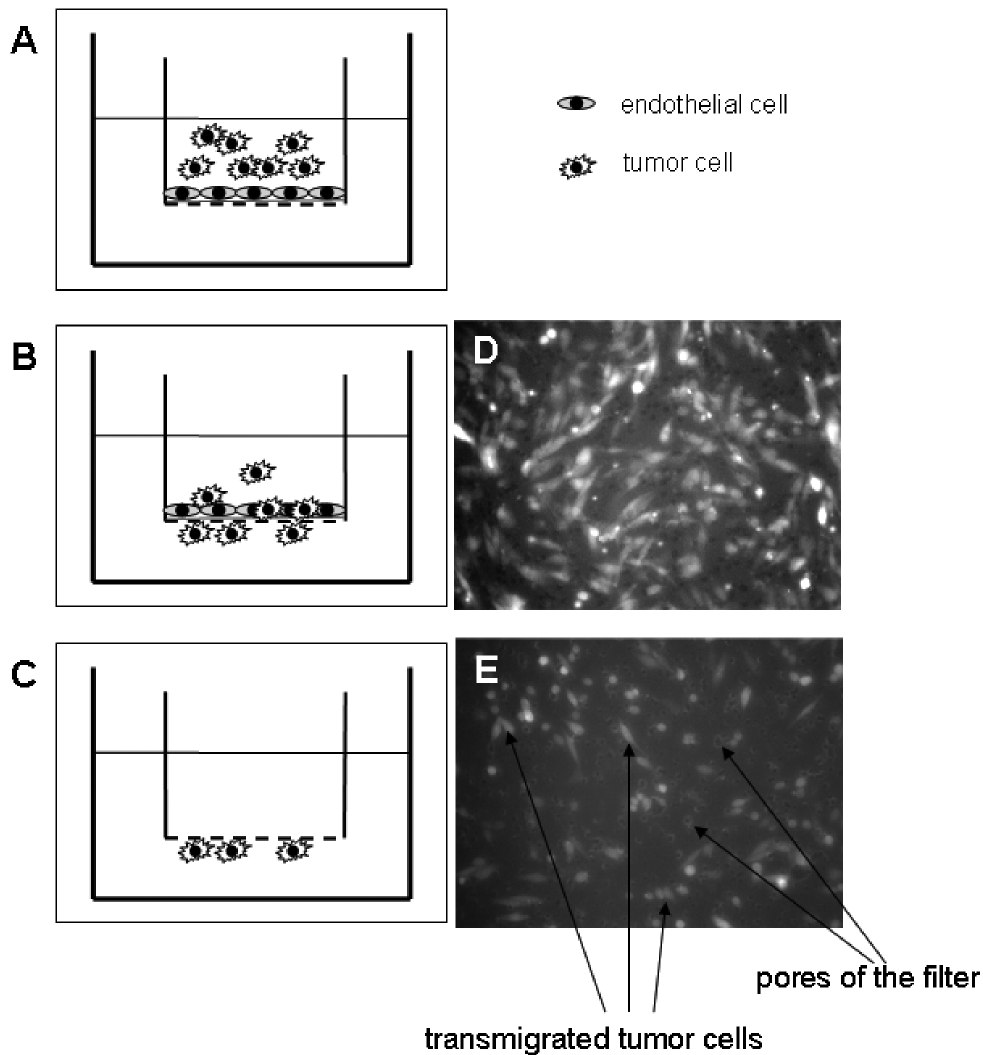

Fig. 7. Use of the *in vitro* model for brain metastasis investigations. Fluorescently labeled cancer cells are plated on confluent endothelial cultures (A). At the end of co-culture (B) cells from the upper compartment are removed (C). Fluorescence microscopy images of the tumor cells (A2058 melanoma cells) at the end of co-culture (D) and after wiping off the cells from the apical side of the filter (transmigrated cells, E).

experiments, TEER values of 278 ± 58 Ohm×cm^2). Our results are in line with previous literature data (Gaillard and deBoer 2000) showing that a monolayer with a TEER above 120-130 Ohm×cm^2 is accompanied by a low paracellular permeability.

Using the same experimental setup, the apparent permeability of test drugs can also be evaluated. In this case the test drug is added to the luminal side and its concentration is measured in the abluminal buffer. Calculations are performed similarly.

This model is also suitable for testing substrates of efflux transporters. The test chemical is applied to either the apical (A) or the basolateral (B) side of the endothelial monolayer in the presence or absence of known inhibitors of specific transporters. Samples are taken from the opposite chamber. $P_{app\ (A\text{-}to\text{-}B)}$ and $P_{app\ (B\text{-}to\text{-}A)}$ is calculated. If the ratio between them is >1 when the test compound is given alone, and close to 1 when the test compound is given together with the specific efflux transporter inhibitor, the test drug is probably a substrate of the transporter and *in vivo* will reach lower concentrations in the brain.

Metastasis model

In order to study the routes and mechanisms of transmigration of tumor cells through the BBB we have constructed a transmigration experimental setup consisting of brain endothelial cells cultured on large pore size filter inserts (Fig. 7).

Primary RBECs are gently trypsinized and passed onto fibronectin/collagen-coated filter inserts (8 µm pore size, 1.13 cm^2, Millipore) which are placed into 12-well plates. After reaching confluency, endothelial cells are supplemented with hydrocortisone and cAMP from the apical side and astrocyte-conditioned medium from the basolateral side for 24 h in order to tighten the junctions. The following day 10^5 fluorescently labeled tumor cells (A2058 melanoma cells) are plated into the upper compartment onto the endothelial monolayer (Fig. 7A). Tumor cells are able to migrate through the endothelial cell layer and the pores of the filter and accumulate on the other side of the filter (Fig. 7B). After 5-24 h cells are fixed. Cells from the upper compartment are removed with a cotton swab (Fig. 7C) and tumor cells migrated through the endothelial monolayer and the pores of the filter can be counted using a fluorescence microscope (Fig. 7E).

CONCLUSION

In summary, our *in vitro* BBB model is characterized by high transendothelial electrical resistance (TEER regularily over 200 Ohm×cm^2), low permeability and expression of different transporters. Our experiments have proven that the model is suitable for the study of different aspects of BBB function in basic research and for testing the interaction between the BBB and potential drug candidates (toxicity, permeability, interaction with efflux transporters) as well.

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