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Review article

Monocultures of primary porcine brain capillary endothelial cells: Still a functional in vitro model for the blood-brain-barrier

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ABSTRACT

The main obstacle for the treatment of brain diseases is the restriction of the passage of pharmaceuticals across the blood-brain barrier. Endothelial cells line up the cerebral micro vessels and prevent the uncontrolled transfer of polar substances by intercellular tight junctions. In addition to this physical barrier, active transporters of the multi-drug-resistance prevent the passage of hydrophobic substances by refluxing them back to the blood stream. This paper reviews the development and selected applications of an in vitro porcine brain derived primary cell culture system established in the authors lab that closely resembles the BBB in vivo and could thus be used to study beyond other applications drug delivery to the brain. An essential technique to control the intactness or destruction of the barrier, the impedance spectroscopy, will be introduced. It will be shown that nanoparticles can cross the blood brain barrier by two mechanisms: opening the tight junctions and thus allowing parallel import of substances into the brain as well as receptor mediated endocytosis using brain specific target molecules. However cytotoxic effects have to be considered as well which beside standard cytotoxicity assays could be also determined by impedance technology. Moreover it will be shown that enzymes e.g. for enzyme replacement therapy could be transferred across the barrier by proper tuning or chemical modification of the enzyme. Since this review is based on a conference presentation it will mainly focus on applications of the monoculture system developed in the authors lab which under given culture conditions is useful due to its easy availability, robustness, good reproducibility and also due to its simplicity. Improvements of this model made by other groups will be acknowledged but not discussed here in detail.

1. Introduction

The small capillaries of the brain are lined by specialized endothelial cells and form a complex barrier system, the blood-brain barrier (BBB). This cellular layer is special with respect to the low number of fenestrations, reduced pinocytosis and almost impermeable cell-cell-contacts, so-called tight junctions. Tight junction proteins close the intercellular cleft preventing the uncontrolled passage of most polar substances from the blood stream to the brain. Adherens junctions support the intercellular attachment; gap junctions are present to allow intercellular communication. Astrocytes as well as pericytes are known to regulate the barrier phenotype of this specialized endothelium forming the so-called neurovascular unit (NVU) [1–6]. Although the interaction within this network is not quite clear it is well accepted now that the extracellular matrix (basement membrane) produced by all the cells of the NVU plays an important role for the maintenance of the barrier integrity [7,8,9].

Since passive diffusion into the brain is only possible for small and lipophilic molecules but almost impossible for hydrophilic substances, specialized transport systems are needed to maintain the brain homeostasis and its supply with nutrients. These membrane integral transporter proteins are responsible for passive (along a concentration gradient) and active (against a concentration gradient) transport. The latter one are using ATP as energy source and thus are called ABC-Transporters (ATP-binding cassette transporters), responsible for the efflux of substrate from the brain to the blood. A subfamily of these transporter are the exporters of the multi-drug resistance forming beside the physical barrier an active defense line that prevents the invasion of substrates into the endothelial cells and thus possibly into the brain [10–13]. However although important enough their structure and

Abbreviations: ABC, ATP binding casette; ASA, Arylsulfatase A; ATP, Adenosintriphosphate; Apo-E, Apolipoprotein E; AuNP, Gold nanoparticle; PBCA-NP, poly-(butylcyano) acrylate; BBB, Blood-brain barrier; ERT, Enzyme replacement therapy; LSD, Lysosomal storage disease; MDCK, Madin-Darby canine kidney cells; NP, Nanoparticle; NVU, Neurovascular unit; PBCA-NP, Poly-(butyl-cyanoacrylate); PEG, Polyethyleneglycol; TEER, Transendothelial (epithelial) electrical resistance

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function is not the topic of this review.

1.1. Cell culture models

Due to the complex interconnected cellular system of the NVU, model systems have been developed to investigate the molecular components of the cellular barrier, their interaction and the regulation of the barrier tightness as well as the transport processes in vitro. Although the cell culture models are simplified and miss the regulation caused by the cellular surrounding in full organisms, their advantage is the simplicity, which allows a clear molecular interpretation of observed effects. In any case results from model systems must be accompanied by corresponding in vivo experiments to validate their importance. Stepwise combination of the different cell types in multicellular in vitro systems using transwell filters even in 3-D cultures have been engineered even leading to a lab-on-the chip tool [14]. The role of shear stress to mimic a close in vivo situation is coming more and more into the focus of present research [15–17]. In addition 3D-multicellular spheroidal systems are now becoming very useful to model the blood – brain barrier close to the in vivo situation [18].

Many cell culture systems have been established to study structure and function at the blood brain barrier originating from different animals. A comprehensive overview on these commonly used brain endothelial cell culture models written by the experts in the field has been published recently [19]. In the present paper some aspects of the original porcine model from the authors lab are summarized. However it should be mentioned that also epithelial barriers like the blood-cerebrospinal fluid barrier or the blood-retina barrier and others are also blood brain barriers [20–22].

Various cell culture models have been developed in the last 30 years [for details see 19]. Most of them are based on either primary or immortalized brain capillary endothelial cells. Within the group of primary endothelial cells most cultures are of non-human origin, e.g. murine, bovine and/or porcine and others. The porcine model developed by the group of Galla et al. is especially useful since the barrier properties are best developed in this model [23,24]. This became even more improved by the application of a special culture condition exchanging the serum after reaching cellular confluence by serum free medium and by the addition of hydrocortisone [25–27]. With this special recipe transendothelial electrical resistances (TEER) of up to 1800 Ωcm^2 have been reached corresponding to sucrose permeabilities down to 10 cm/s although the range between different preparations is pretty large sometimes reaching values below 1000 but also above 2000 Ωcm^2 [personal communication]. Co-culture systems with astrocytes and/or pericytes are often used seeding them on the opposite site of the filter (so-called contact co-cultures) or at the bottom of the well (non-contact co-cultures) [19,28,29]. Also triple cultures are used [30,31] and even conditioned media are reported to have an increasing effect on the TEER [25,32]. Moreover the extracellular matrix has been reported to be of major importance for the development of the tight junctions [8,9]. The porcine model has been successfully used through the last 30 years and has been considerably improved by others in modified forms using monocultures as well as co-cultures with astrocytes and pericytes [33–37]. Importantly enough Chen-Kashi Malina et al. [38] reported in agreement with our findings [39], that only co-culture in close proximity to glial cells (so-called contact mode) mimic the in vivo BBB with an TEER up to 1650 Ωcm^2.

The culture of primary cells ist cost and labour intensive. Thus different groups established immortalized cell lines [19]. Some are even commercially available (bEND 3 and 5) but most of the immortalized cell lines generally develop low electrical resistances of less then 50 Ωcm^2 which corresponds to highly leaky endothelial cells [19]. Förster et al. [40–42] were able to create a cell line called cEND with resistances up to 800 Ωcm^2 and correspondingly strong tight junction protein occluding and claudin 5 expression. These cells, like the primary porcine cells, responded to glucocorticoids with a barrier increase up to 1000 Ωcm^2. For further cell culture systems the reader is referred to the paper by Helms et al. [19]. But it is important to note, that the development of the endothelial cell lines also opened the door to get immortalized human endothelial cell lines. Very new and promising BBB models however are endothelial cells based on stem cells [43] Recently, Katt et al. [44] reported the importance of the applied extracellular matrix to obtain cultures that exhibit the key features of a reliable BBB model, namely expression of tight junction proteins, high barrier properties with high electrical resistances of several thousand Ωcm^2 and corresponding low permeabilites. These cultures reach the values of the primary porcine cells but have the advantage of almost unlimited accessibility and the human origin.

1.2. Impedance spectroscopy: a technique to automatically monitor the barrier properties

As mentioned before, epithelial and endothelial cells form semipermeable interfaces between compartments of different chemical composition. They control the diffusive permeation of solutes between the compartments of adjacent cells by forming a tight barrier via tight junction proteins. Moreover they are essential to maintain chemical gradients formed across the cell layers, which are needed for example to drive an active transport along the transcellular pathways. Any experiment that is focused on transport processes needs an access to the apical as well as basolateral side of the cells. Thus in such an experimental design cells are grown on permeable filter supports. For a reliable transport experiment it is inevitable to control the maintenance of the barrier continuously throughout the experiment. Thus techniques are needed to allow a continuous and long-term monitoring of the cellular barrier properties without disturbing the barrier integrity. Very often markers like fluorescent or radioactive probes are used to quantify the permeability and thus the tightness of such a cell layer. However in these cases sample have to be drawn and analyzed separately. Impedance analysis uses the direct correlation between permeability and electrical resistance of the epithelial or endothelial layer, so-called TEER (trans endothelial/epithelial electrical resistance) [45] Cells with low permeability exhibit a high, cells with high permeability a low TEER value. Thus the TEER value is a quantitative measure of the tightness of the barrier. In impedance spectroscopy a small AC-voltage (alternating current) is applied across the cell layer grown on porous membranes of commercially available filter inserts. By placing two electrodes, one in the upper and one in the lower compartment, a small AC voltage can be applied which does not disturb the cellular properties, and the electrical impedance is measured typically in a frequency range from 1 Hz to 100 kHz yielding the impedance spectrum. Two quantities determine the impedance Z of a cell layer, the resistance TEER and in addition the electric capacitance c_{cl} (Fig. 1).

Both contribute to the measured total impedance leading to a non-linear frequency dependence. The TEER is a measure for the tightness or leakiness of the cell layer; the capacitance gives additional information about geometric cell properties like the formation of microvilli or protrusions. It might also be used as a parameter to quantify cell death in in vitro assays. Electric equivalent circuits are used to model mathematically the barrier properties thus yielding the corresponding parameters where mainly the ohmic resistance (TEER) is extracted as a measure for the tightness of the cellular junctions. A commercially automated cell monitoring system is available (see www.nanoanalytics.com for further information).

1.3. Nanoparticles as tools to overcome the blood brain barrier

The transfer of drugs to treat neurological diseases is often limited by the low permeability of drugs into the brain. Due to the selective barriers separating the blood from the brain parenchyma. Although transport systems are present at the BBB, the brain import of drugs and therapeutics are hampered due to the strict specificity and the fine