Blood-brain barrier-specific properties of a human adult brain endothelial cell line

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SPECIFIC AIMS

Endothelial cells that line the microvasculature of the central nervous system (CNS) differ fundamentally from other vascular endothelia in their capacity to regulate exchanges between blood and the neural parenchyma and constitute the blood-brain barrier (BBB). Establishment of a human model of the BBB has been difficult. We report here the production and extensive characterization of an immortalized human brain endothelial cell line which recapitulates most of the unique properties of brain endothelium and may thus constitute a unique in vitro model of the human BBB.

PRINCIPAL FINDINGS

1. Immortalization of human brain endothelial cells

After lentiviral infection of preconfluent primary brain endothelial cell cultures in passage 0, several hundred parent cell lines of immortalized human brain microvessel endothelial cells were obtained by limiting dilution cloning, from which one clonal population, named hCMEC/D3, was selected. The hCMEC/D3 cell line showed a morphology similar to primary cultures of brain endothelial cells, with confluent monolayers of tightly packed elongated cells that exhibited contact inhibition. The hCMEC/D3 cell line maintained a nontransformed phenotype over more than 100 population doublings (33 passages), without any sign of senescence or de-differentiation, notably showing a normal diploid karyotype and the capacity to form a branched network of capillary-like cords on reconstituted extracellular matrix (Matrigel).

2. Expression of endothelial and BBB-specific markers

The hCMEC/D3 cell line constitutively expresses many endothelial and/or BBB markers with the appropriate subcellular localization pattern, including the junctional proteins PECAM-1, VE-cadherin, β- and γ-catenins, ZO-1, JAM-A, and claudin-5 (**Fig. 1**). These results strongly suggest that hCMEC/D3 cells maintain the overall tight junction organization known to be present in brain endothelium. No change was detected with passage in the junctional expression of VE-cadherin, β-catenin, or any other endothelial marker tested (not shown).

Moreover, the membrane expression of a number of adhesion molecules and chemokine receptors was detected by flow cytometric analysis in basal conditions or after treatment by the inflammatory cytokines IFN-γ and TNFα. Indeed, hCMEC/D3 cells constitutively express ICAM-1, ICAM-2, PECAM-1, and CD40; upon cytokine activation, ICAM-1 and CD40 are up-regulated while expression of VCAM-1 is induced. These observations further establish that hCMEC/D3 cells stably maintain in culture the expression of a large number of membrane proteins known to be involved in leukocyte migration into the CNS in inflammatory conditions.

3. Drug permeability: correlation with in situ brain perfusion

Permeability assays using fluorescent dextrans of increasing molecular size (4–70 kDa) revealed that hCMEC/D3 monolayers, grown to confluence on semipermeable filters, exert a better restriction than primary cultures of bovine brain endothelial cells and a much more stringent restriction than the immortalized GPNT rat brain endothelial cell line. Compared with permeability values reported using the reference BBB in vitro model constituted by cocultures of bovine brain

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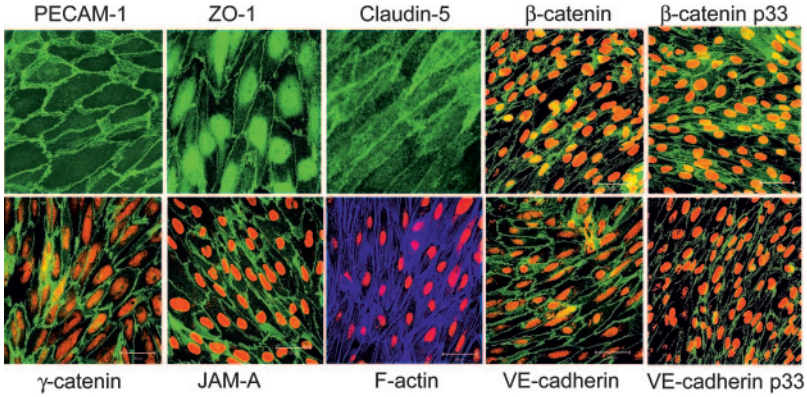

Figure 1. Expression of endothelial and BBB markers by hCMEC/D3 cells. Confluent monolayers of hCMEC/D3 cells (passage 22) were stained for the endothelial junctional markers PECAM-1 and VE-cadherin and for the junction-associated proteins β-catenin, γ-catenin, ZO-1, JAM-A, and claudin-5 as indicated. Cy2-phalloidin staining of F-actin (blue) is essentially localized at the cell cortex (nuclei counterstained nuclei in red). No change was detected in the junctional expression of VE-cadherin or β-catenin at passage 33 (p33).

microvessel endothelial cells and rat glial cells, inulin permeability through hCMEC/D3 cells was similar (0.36 vs. 0.37×10^{-3} cm/min) and sucrose permeability was higher (1.65 vs. 0.75×10^{-3} cm/min). These permeability values are much lower than those reported for the rat brain endothelial cell lines RBE4 and GPNT ($4–7x10^{-3}$ cm/min for sucrose and 1.3×10^{-3} cm/min for inulin). Transendothelial electric resistance (TEER) was found to remain at constant low levels (<40 $\Omega.cm^2$), reflecting a high ionic permeability.

Permeability of hCMEC/D3 monolayers grown to confluence on semipermeable polycarbonate filters was measured for [^{14}C]-sucrose, -diazepam, -morphine-6-glucuronide (M6G) or [^{3}H]-inulin, -prazosin, -colchicine, -vincristine. For comparison, similar experiments were performed with human umbilical vein endothelial cells (HUVECs), which are not expected to form a tight diffusion barrier, and with the rat brain endothelial cell line GPNT. We observed that hCMEC/D3 permeability coefficients (Pe in cm/min) were well correlated (R=0.938) with in vivo permeability data, expressed as Kin transport coefficients and assessed by the brain perfusion technique. Our results reveal a good correlation between hCMEC/D3 and GPNT permeability data (R=0.990), but no correlation between hCMEC/D3 and HUVEC permeability data (R=0.628). Although this study will need to be extended to a larger number of compounds, these observations clearly point to the hCMEC/D3 cell line as a potential in vitro model of the human BBB.

4. Expression and functionality of multidrug resistance proteins

In conjunction with the presence of inter-endothelial tight junctions, BBB endothelia are characterized by a highly restrictive permeability to hydrophobic molecules resulting from the expression of several multidrug resistance proteins of the ATP binding cassette (ABC) transporter family. By RT-PCR and Western blot analysis, we observed that hCMEC/D3 cells, like primary human brain endothelial cells, express MDR1 (or P-glycoprotein), MRP1, and BCRP, ABC transporters expressed by brain endothelium.

The functionality of these pumps was then evaluated by assessing the uptake by hCMEC/D3 monolayers of ABC transporter substrates: calcein-AM, a P-gp, and MRP1 substrate; rhodamine 123, a P-gp and BCRP substrate; [^{3}H] daunorubicin, a shared substrate of P-gp, MRP1, and BCRP. Drug uptake was measured in the absence or presence of specific transporter inhibitors: PSC-833, MK-571 or KO-143 for P-gp, MRP1, and BCRP, respectively. We observed that PSC-833 markedly increased calcein-AM, rhodamine 123 and daunorubicin uptake by hCMEC/D3 cells, indicating that P-gp was highly active in these cells. MRP1 and BCRP were also found to be functional in hCMEC/D3 cells.

CONCLUSIONS

The present results constitute an extensive phenotypic characterization of an immortalized human brain microvascular endothelial cell line that maintains in culture a stable and physiologically normal endothelial phenotype and most of the unique structural and biochemical properties of brain endothelium in vivo (**Fig. 2**).

The tight junctions of the BBB are the major contributor to the extremely low permeability to both solutes and cells. Junctional expression of claudins and occludin, the integral membrane components of tight junctions, as well as continuous junctional expression of JAM-A and ZO-1, altogether correlate in situ with functional tight junctions. Characterization of occludin- and claudin-5 genetically deficient mice revealed that, whereas occludin unexpectedly appeared to be dispensable, loosening of the BBB was reported in the former animals, highlighting the key contribution of claudin-5 to BBB integrity. We present evidence here that hCMEC/D3 cells display a junctional expression of JAM-A, claudin-5, and ZO-1, while occludin was not consistently detected at cell-cell contacts. These results support the conclusion that hCMEC/D3 cells maintain the overall tight junction organization known to be present in brain endothelium.

The suitability of the hCMEC/D3 model for transendothelial permeability studies is supported by our

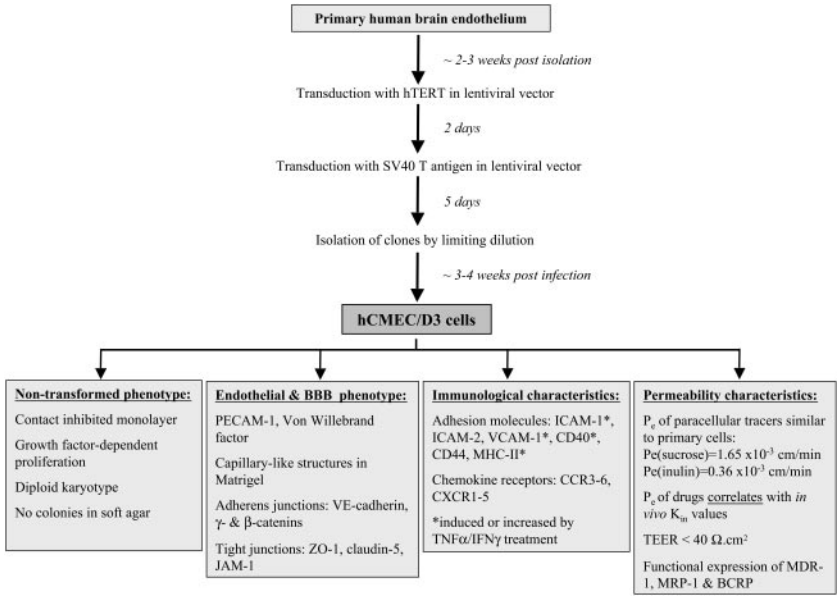

Figure 2. Schematic diagram summarizing development of cell line and its properties.

results: we observed that hCMEC/D3 cell monolayers display a highly restricted permeability to standard drugs over a wide range of hydrophobicity, in good correlation with in vivo permeability values from brain perfusion studies, which was not the case for HUVECs. These data clearly indicate that the hCMEC/D3 cell monolayers display highly restrictive permeability properties, quite similar to the reference bovine coculture model, although sucrose permeability was slightly higher and TEER values were constantly low. Altogether, our results demonstrate that hCMEC/D3 cell monolayers may constitute a valuable in vitro model of the human BBB that mimics brain endothelial-restricted permeability for a wide variety of compounds, except for small hydrophilic molecules (<300 Da) and ions.

The trans-endothelial permeability of many hydrophobic compounds at the BBB is known to be highly limited by the expression of multiple ABC transporters, including P-gp (ABCB1), the recently identified BCRP (ABCG2), and several members of the MRP family. In the present study, we show that P-gp, MRP1, and BCRP are expressed and functional in hCMEC/D3 cells. Coculture of brain endothelial cells with glial cells was reported to increase the functional expression of P-gp and other ABC efflux transporters, in parallel with the above-mentioned decrease in monolayer permeability. Accordingly, future optimization of the hCMEC/D3 model might include coculture with human glial cells.

Although the brain has often been described as an "immune-privileged site" due to the presence of the BBB, it is now well established that, during inflammatory situations such as multiple sclerosis, massive infiltration of leukocytes can be observed in the brain tissue. This leukocyte recruitment coincides with the up-regulated expression of a number of adhesion molecules on the surface of cerebral vascular endothelium. We report here the constitutive or cytokine-inducible expression of multiple adhesion molecules by hCMEC/D3 cells, together with the expression of a large number of chemokine receptors. A couple of immortalized human brain endothelial cell lines were reported earlier, but their phenotype was described only in terms of expression of a small number of endothelial markers, without any strong evidence of stability, limiting any conclusion regarding their potential use as a BBB in vitro model.

The hCMEC/D3 cell line constitutes to our knowledge the first example of an extensively characterized human brain endothelial cell line that recapitulates most of the unique properties of the BBB, even without coculture with glial cells. We believe this cell line can be a useful model for studying the biology of human brain endothelium in the context of neuroinflammatory or infectious diseases and for large-scale screening of CNS drug candidates. FJ