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Establishment of primary cultures of human brain microvascular endothelial cells: a new and simplified method to obtain cells for an in vitro model of the blood-brain barrier

Michael J Bernas¹, Filipa L Cardoso², Sarah K Daley¹, Martin E Weinand¹, Alexandre R Campos³, António J Gonçalves Ferreira³, James B Hoying⁴, Marlys H Witte¹, Dora Brites², Yuri Persidsky⁵, Servio H Ramirez⁵, and Maria A Brito²

Michael J Bernas: michaelb@u.arizona.edu; Filipa L Cardoso: filipacardoso@ff.ul.pt; Sarah K Daley: skdaley@email.arizona.edu; Martin E Weinand: mweinand@u.arizona.edu; Alexandre R Campos: alexandrera@scampos.org; António J Gonçalves Ferreira: ajgonfer@fm.ul.pt; James B Hoying: jay.hoying@louisville.edu; Marlys H Witte: lymph@u.arizona.edu; Dora Brites: dbrites@ff.ul.pt; Yuri Persidsky: Yuri.Persidsky@tuhs.temple.edu; Servio H Ramirez: servio.ramirez@temple.edu; Maria A Brito: abrito@ff.ul.pt

¹ Department of Surgery, University of Arizona, Tucson, USA

² Centro de Patogénese Molecular-UBMBE/iMed.UL, Faculdade de Farmácia, University of Lisbon, Lisbon, Portugal

³ Serviço de Neurocirurgia, Hospital de Santa Maria, Lisbon, Portugal

⁴ Cardiovascular Innovation Institute, Louisville, Kentucky, USA

⁵ Department of Pathology and Laboratory Medicine, Temple University School of Medicine, Philadelphia, Pennsylvania, USA

Abstract

We describe a method for generating primary cultures of human brain microvascular endothelial cells (HBMVEC). HBMVEC are derived from microvessels isolated from temporal tissue removed during operative treatment of epilepsy. The tissue is mechanically fragmented and size-filtered using polyester meshes. The resulting microvessel fragments are placed onto type-I collagen-coated flasks to allow HBMVEC to migrate and proliferate. The overall process takes under 3 h and does not require specialized equipment or enzymatic processes. HBMVEC are typically cultured for approximately 1 month until confluence. Cultures are highly pure (~97% endothelial cells; ~3% pericytes), reproducible, and display characteristic brain endothelial markers (von Willebrand factor, glucose transporter-1), robust expression of tight and adherens

Corresponding authors: Maria Alexandra Brito, PhD, Centro de Patogénese Molecular, Faculdade de Farmácia da Universidade, de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal, Tel: +351 217946400, Fax: +351 217946491, abrito@ff.ul.pt, Servio H. Ramirez, PhD, Department of Pathology and Laboratory Medicine, Temple University School of Medicine, 3550 N. Broad Street, MERB 1059, Philadelphia, PA 19140, USA, Tel: 1 2157072741, Fax: servio.ramirez@temple.edu.

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There are no competing financial interests by any of the authors.

Author contributions audit

MJB, developed the protocol; FLC, implemented the protocol in Lisbon laboratory and performed cell culture characterization; SKD performed and validated the protocol in Arizona; MW contributed to the developmental inception of the project, maintains IRB approval, and provided the brain tissue in Arizona; ARC and AJGF were responsible for IRB approval as well as selected and provided human brain tissue for culture in Lisbon; JBH provided key technical improvements to the protocol; MHW inspired and conceived the project, and established and maintains the United States collaborations; DB, provided assistance in the Lisbon protocol as well as helpful discussion of the work and of the manuscript; YP helped develop and provided funding and support for the United States collaboration; SHR contributed experience in the use and characterization of these cells; MAB worked on the implementation of the protocol in Lisbon laboratory and on the cell culture characterization, provided financial support for this work, established the international collaboration, and assembled the manuscript for publication.

junction proteins, caveolin-1, and efflux protein P-glycoprotein. Monolayers of HBMVEC display characteristic high transendothelial electric resistance and have proven useful in multiple functional studies for *in-vitro* modeling of the human blood-brain barrier.

INTRODUCTION

The Blood-Brain Barrier

The blood-brain barrier (BBB) is a dynamic and complex interface between the blood and the central nervous system (CNS). The BBB strictly controls the exchanges between the blood and brain compartments which protects and maintains the delicate interstitial environment that is optimal for neuronal communication. The anatomic structure of the BBB is comprised of a tightly sealed monolayer of brain microvascular endothelial cells (BMVEC) which are characterized by the absence of fenestrations, a low level of pinocytotic vesicles, and an elaborate junctional complex formed by both tight junctions and adherens junctions¹. Due to the paracellular impermeability of the brain endothelium, the bidirectional movement of hydrophilic and lipophilic molecules occurs through BBB transport systems. For instance, the glucose transporter-1 (GLUT-1) transports glucose and other hexoses into the brain. Whereas the members of the ATP-Binding Cassette (ABC) family, P-glycoprotein (P-gp) and the multidrug resistance associated proteins (MRP), transport lipophilic molecules, in particular xenobiotics, out of the brain^{2,3}.

With the discovery of the BBB in 1885 by Paul Ehrlich⁴ and the use of either endogenous or exogenous tracers, came an era of exploration of the unique permeability properties of the cerebral endothelial interface *in-vivo*. However, great advances in our understanding of the molecular mechanisms driving BBB function did not begin to occur until Joó and Karnushina⁵ successfully isolated viable microvessels and generated the *in-vitro* BBB model system from rat brain in 1973. In the following decades, the *in-vitro* BBB model coupled with various technological platforms and co-culture configurations has become a powerful mainstay tool for studying CNS drug pharmacokinetics, CNS therapeutic targeting, neuroinflammation, neurodegeneration, neuroprotection, and neurotoxicity⁶. Isolation of cells from human brain tissue continues to be focused on obtaining microglia, astrocytes and more recently neural progenitor cells, while only a small effort has been devoted to endothelial cell isolation.

Development of the Procedure

The Lymphology Laboratories at the University of Arizona (M. Witte and M. Bernas) has had a long standing focus on endothelial cells and endothelial cell cultures from both blood and lymphatic vessel sources^{7,8}. Because of a unique collaboration between this laboratory and a neurosurgical colleague (M. Weinand) with access to human brain samples, efforts were undertaken to combine our expertise to isolate and culture brain microvascular endothelial cells. It was found that most published methods for isolation of BMVEC are time-consuming and involve multi-step procedures including multiple enzymatic digestions with gradient density centrifugations^{9,10}. Some use more complex methodologies including glass bead and magnetic separation¹¹. Although some methodologies have been performed with human brain tissue^{12,13,14}, these techniques have commonly been applied using tissue from other species with most of these studies performed in rat^{9,15,16}, mouse¹⁷, porcine¹⁸ or bovine^{10,19}. This may be due to the difficulty in obtaining an adequate amount of human brain tissue for these procedures. As a result, the literature regarding brain endothelial function is largely representative of non-human brain endothelium, which prompts caution in the interpretation of data due to species differences and highlighting the need for human cells. In addition, in all these methods, the presence of supporting cell types such as

pericytes^{20,21} remains a common problem. There have been some recently produced brain microvascular endothelial cell lines which have demonstrated some similar characteristics to non-transformed cells²². Despite their promising usefulness in research, molecular cytogenetic characterization has recently highlighted complex karyotype changes, which renders genetic testing of cell lines advisable prior to their application in *in vitro* studies²³.

We approached the isolation from an endothelial standpoint and endeavored to resist the relatively harsh and time-consuming digestions (in our hands, we have found that enzyme digestions can negatively impact endothelial cell viability) while also eliminating density centrifugations. Goldstein et al.²⁴ did not use enzyme digestion (retaining the density centrifugations) for the isolation of microvascular capillaries for metabolic studies, and we incorporated their methods into our procedure. Beginning in 1990, we undertook studies to isolate microvascular capillaries from human temporal lobe brain samples removed during operation to control intractable epilepsy without enzymatic digestion. Using simple unit gravity separation in tissue culture medium, we could easily and quickly complete the isolation and initiation of cell cultures in the tissue culture hood. These early efforts were successful and the cells were used for a variety of multi-institutional collaborative experiments focusing on cocaine and HIV-1 effects on the BBB^{25–29}. Although the method was successful, contaminating cells and difficulty in reproducibility requiring a large number of samples were troublesome. We therefore reinstituted some of the digestion methods and centrifugations in an effort to improve the procedure. These refinements were successful³⁰ and are still in use by other investigators³¹. However, we were still troubled by the amount of time, effort, and the quantity of brain tissue that the procedure required. We also had observed that the enzymatic steps could harm endothelial cells if they are used for too long or are too harsh in the procedures. Thus, we reworked the procedure by eliminating the enzymatic treatment and density centrifugations. We added an improved method to isolate the microvascular fragments by adding a simplified tissue disruption technique, using size exclusion and inclusion with polyester membranes from another endothelial cell isolation procedure in place at the University of Arizona (J. Hoying)³².

New Procedure and Characterization of BMVEC Cells

We have developed a protocol for a reproducible, consistent, economical and simplified isolation/culture of human BMVEC (HBMVEC) (Fig. 1) that can be implemented in any laboratory with minimal equipment and accessories. Human brain samples can be obtained, after informed consent and Institutional Review Board (IRB) ethical approval, from discarded temporal lobe tissues removed from patients with intractable epilepsy undergoing neurosurgical operative procedures (removal of hippocampal epileptogenic foci). The procedure can be completed in approximately 2 1/2 h with readily visible endothelial cell clusters after 8–10 days. As can be seen by phase contrast microscopy (Fig. 2), it is necessary to allow culture expansion to occur for nearly 1 month in order to achieve confluency. After confluence is reached, characterization to evaluate the purity of the cell population can be undertaken. To confirm the endothelial phenotype, several endothelial cell markers can be examined using immunohistochemistry with cells grown on coverslips using standard methods (Figures 3 and 4), provided as supplementary method. Markers such as von Willebrand factor (vWF), and specially the GLUT-1 are distinctively expressed in these BMVEC cells. To determine the degree of non-endothelial cell contamination, the widely used pericyte marker, α -smooth muscle actin (α -SMA) can be employed (Fig. 3). The formation of the “physical barrier” by the HBMVEC which acquires the attributes required for modeling of the BBB *in-vitro* relies on the expression of the proteins present in the tight junction complex. Proteins found at tight junctions, such as the cytosolic zonula occludens (ZO)-1 and ZO-2, and the transmembrane occludin, claudin-5 and junction adhesion molecule (JAM)-2, as well as at adherent junction proteins such as β -catenin are

prominently expressed (Fig. 4 and 5). In addition, caveolin-1, a marker of the cell vesicular transport machinery, and the efflux protein P-gp are also expressed (Fig. 4). Confluent monolayers of HBMVEC present a high transendothelial electric resistance (TEER) that characterize these brain-specific endothelial cells (Fig. 6). TEER enables direct evaluation of an experimental condition that may induce barrier “tightness” or “leakiness”. TEER measurements can be acquired with various technologies, including the ECIS system from Applied Biophysics (Fig. 6A). The system works by using the free ions in the culture media, which are then used to generate an AC current flow between an electrode and counter-electrode located in specialized tissue culture arrays. The instrument measures continuously the complex impedance, providing readouts for impedance, resistance and capacitance. Typical TEER readings from a low passage culture of HBMVEC plated at 1×10^4 on collagen type I-coated electrode arrays (8W10E + cultureware array, Applied Biophysics) and monitored (at 1h intervals using 1000Hz AC signal settings) until monolayer confluence and barrier formation occurs are displayed (Fig. 6B). Once the steady state TEER is reached, the barrier can be evaluated in its response to a particular stimulus and the change from normalized resistance values of HBMVEC can be recorded (Fig. 6C).

Validation, Replication, and Utilization

As a demonstration of the validation, simplicity, and transportability, this protocol which was initially developed (over 50 samples) at the University of Arizona, Tucson, AZ, USA, has recently allowed for a rapid implementation of HBMVEC cultures in a Portuguese laboratory. Moreover, confluent monolayers resulting from this procedure can be sent from the lab where the cells were isolated to other labs, rendering the application of the *in-vitro* model of the BBB feasible worldwide. Although we have not analyzed each isolation using all the methods for comparison, this improved technique has been successful in obtaining microvascular endothelial cells from every sample and demonstrates low inter-sample variations in terms of the profile and expression of junctional proteins. For example, cultures obtained from three different resections showed a similar expression profile for transmembrane (occludin and claudin5) and cytoplasmic (ZO-1 and ZO-2) tight junction proteins (Fig. 5A).

An expanded gene expression analysis of proteins involved in junctional complexes (ZO-1, ZO-2, occludin, claudin-1, -3, and -5, and JAM-2) confirms the presence and also a similar degree of expression between various HBMVEC isolates (Fig. 5B). The HBMVEC generated from this procedure have been used for *in-vitro* modeling of the BBB for studies of neuroinflammation and disease processes (including HIV-1), substance abuse research (alcohol, cocaine and methamphetamine), and CNS drug delivery^{33–39}.

MATERIALS REAGENTS

- Human brain tissue (see REAGENT SETUP) ! CAUTION Consult IRB regulations and obtain informed consent from all subjects before collecting and processing human brain tissue and all human tissue must be treated with Universal Precautions.
- Type I Collagen I, rat tail tendon (100 mg; BD Biosciences, cat. no. 354236)
- 0.02 N Acetic Acid solution
- DMEM/Ham's F12 (500 ml; Biochrom, cat. no. F4815)
- Fetal Bovine Serum (FBS) (500 ml; Biochrom, cat. no. S0615)
- Antibiotic-Antimycotic, stabilized (100x; SIGMA, cat. no. A5955)
- Glutamax (100x; Invitrogen, cat. no. 35050–038)

- Endothelial Cell Growth Supplement (ECGS) (15mg; BD Biosciences, cat. no. 354006). Critical! The ECGS reagent is preferred since other growth supplement cocktails may adversely affect the initial growth of the culture.
- Heparin (5000 U/ml; Biochrom, cat. no. L6510)

EQUIPMENT

- Biosafety cabinet (hood) suitable for cell culture and equipped with UV light for decontamination
- Water-bath with temperature control
- Centrifuge suitable for 50-ml tubes
- Cell culture incubator with both temperature and gas composition controls (Heraeus, model Function line)
- Stereomicroscope (Zeiss, model Stemi DV4)
- Inverted microscope with phase-contrast (Olympus, model CK2-TR) and digital Camera (Nikon, model L1)
- Upright microscope fitted with widefield epi-fluorescence (Zeiss, model Scope. A1) and integrated digital camera (Leica, model DFC490)
- Electrical Cell-Substrate Impedance Sensing (ECIS) system (Applied Biophysics, model 1600R with electrode arrays 8W10E+)
- 50 ml tubes
- Filtration units for solutions (0.2 μ m)
- 100 mm tissue culture dishes
- Graefe forceps, 0.8 mm tips straight (Fine Science Tools, cat. no. 11050–10)
- Dumont forceps, standard tip, 0.10 mm $\times$ 0.06 mm, Dumoxel, 11 cm (Fine Science Tools, cat. no. 11251–30)
- 25 ml serological pipettes
- 10 ml serological pipettes (wide and normal bore)
- 5 ml serological pipettes
- 500 μ m polyester mesh (Small Parts, Miramar, FL, USA)
- 30 μ m polyester mesh (Small Parts, Miramar, FL, USA)
- A wire frame for holding the polyester mesh. Alternatively a metallic cell strainer can also be used
- T-25 flasks
- 24-well plates

REAGENT SETUP

- **Tissues:** Human samples of either temporal lobe or hippocampus can be obtained from discarded tissue during operative treatment of epilepsy (outside of epileptogenic foci). Tissue should be collected without saline and processing should initiate as soon as possible. Delay of more than a few hours should be avoided. Tissue should remain at room temperature (20–24°C). This protocol is optimized

for use with human tissue samples, but it may be applied to tissues from other species, as rat⁴⁰, as well. Operative samples vary in size with successful isolations obtained from tissues ranging in size from 5–10 mm³ to as large as 4 cm³.

- **Isolation medium (IM):** For 100 ml combine 10 ml FBS, 1ml Glutamax, 1ml Antibiotic-Antimycotic, 88 ml DMEM-F12, and filter through a 0.2 µm filter. Store at 4 °C for up to 1 week.
- **Isolation supplemented medium (ISM):** For 100 ml combine 10 ml FBS, 1ml Glutamax, 1ml Antibiotic-Antimycotic, 2 ml ECGS (50µg/ml), 3.4 ml Heparin (1 mg/ml) and 82.6 ml DMEM-F12. Filter through a 0.2 µm filter and store at 4 °C for up to 1 week.
- **0.02 N acetic acid:** Dissolve 1.14 ml acetic acid in sterile water. **CRITICAL** This reagent should be freshly made.
- **Collagen solution:** For coating of T-25 flask, prepare a 50 µg/ml solution of collagen in 0.02 N acetic acid. **CRITICAL** This reagent should be freshly made.

PROCEDURE

Prior to HBMVEC isolation

- 1) Coat a T-25 flask using an incubation of 2.5 ml of collagen solution for 1 hour. Wash three times with HBSS. Note that coated cultureware can be stored in aluminium foil at 4 °C for up to one week.
- 2) Prepare both IM and ISM.

HBMVEC isolation and culture

- 3) Maintain IM and ISM at 37°C in water-bath.
- 4) Collect the brain sample in a 100 mm tissue culture dish containing ~5 ml of IM (Fig. 1A). **CRITICAL STEP** To avoid contamination with microorganisms, processing of the tissue should be performed in a class II biosafety cabinet using sterile reagents.
- 5) Carefully, remove meninges and visibly large vessels, using sterilized surgical forceps and a dissecting stereomicroscope to facilitate the visualization (Fig. 1B).
- 6) Fragment tissue using repeated titration with sterile pipettes of 25, 10 and 5 ml, until the sample can be passed easily back and forth through the 5 ml pipette (Fig. 1 C, D).
- 7) Place a 500 µm polyester screen (large enough to overlap) over a 100 mm non-coated Petri dish. The membrane is strong enough to not require a wire backing. Wet the screen with ~5 ml IM.

CRITICAL STEP Always practice proper sterile cell culture technique to avoid contamination. SEE TROUBLESHOOTING.

- 8) Pipet the sample onto the polyester screen by starting in the center and working your way out without pushing the sample through the screen (Fig. 1E). Wash the screen with ~10 ml IM. This step eliminates larger non-dissociated tissues which will not pass through the membrane. These tissues may contain some microvascular fragments, but their inclusion in the culture initiation greatly reduces the establishment and purity of the endothelial cultures.

CRITICAL STEP Perform this step quickly to avoid drying out the polyester screen.

- 9) Discard the polyester screen and large chunks and preserve the flow through fluid.
- 10) Place a sterile wire frame on a sterile non-coated 100 mm Petri dish and then place a sterile 30 μ m polyester screen on top. Wet the screen with ~5 ml IM. SEE TROUBLESHOOTING.
- 11) Pipet the collected pass-through fluid (from step 9) onto the 30 μ m nylon screen. Start in the center and work in a circle and do not try to push sample through the membrane (Fig. 1F). Wash with ~10 ml IM.

CRITICAL STEP Perform this step quickly to avoid drying out the nylon screen.

- 12) Open another Petri dish and using an additional 10 ml IM in a serological pipet, hold and fold the screen from step 11 and wash the isolated fragments off the membrane into the new non-coated dish (Fig. 1G). Repeat with ~5 ml IM.

CRITICAL STEP Use caution, this step could potentially introduce microbial contaminants to the preparation. Do not use a coated or tissue culture prepared dish to prevent adherence of fragments. This filtration step washes through red blood cells which are known to inhibit endothelial cell cultures as well as any other single or groups of cells less than 30 microns which are not microvascular vessel isolates.

- 13) Transfer the medium containing all microvascular fragments washed off the membrane in Step 12 into a 50 ml conical tube and centrifuge at 400 rpm (~30 g) for 10 min (Fig. 1H).
- 14) Discard the supernatant and resuspend the pellet in 1 ml ISM.
- 15) Transfer to a collagen-I coated T-25 flask. Gently shake to ensure coverage of the flask. Allow for attachment of microvessel fragments by placing in the incubator for ~1 hour.
- 16) Carefully introduce an additional 3 ml ISM to the T-25 flask (Fig. 1I) and without shaking, move to the incubator.

Expansion of the HBMVEC culture

- 17) In 2 to 4 days, change the medium of the culture for the first time with 4 ml ISM. Under phase-contrast microscopy, endothelial cells should begin migrating out from vessels and proliferating. Also monitor for any bacterial or fungal contaminants in the culture - SEE TROUBLESHOOTING.
- 18) Change the medium once a week. Typically, small endothelial cell clusters are visible after 8-10 days and cell confluency is achieved in 30 days, although this is dependent on the density of seeding. HBMVEC will present as tightly packed triangular shaped cells under phase-contrast microscopy. SEE TROUBLESHOOTING.

TIMING

Day 1, steps 1 and 2, preparation of flask will take about 1 h 15 min, and preparation of mediums will take approximately 15 min.

Day 2, steps 3 – 16, processing of the sample takes ~ 2 h 30 min.

Day 3 – 30, steps 17 and 18, replacing medium and monitoring cell growth takes about 10 minutes, including phase-contrast microscopy and image capture.

TROUBLESHOOTING

Filters (Steps 7 and 10)

Polyester screens used in steps 7 and 10 are discarded after use to avoid clogging and possible contamination in future cultures.

Contamination (Step 17)

Bacteria and fungi can be identified by phase contrast microscopy. Pericytes, which can be present in HBMVEC cultures, can be identified by phase contrast microscopy and by labelling with markers, as α -smooth muscle actin (α -SMA). Although contamination by astrocytes or microglia has not been observed, these cells can be identified by immunohistochemical labelling with markers such as glial fibrillary acidic protein and CD11b, respectively. These cells do not exhibit the cobblestone appearance of endothelial cells and can be phenotypically identified by phase contrast microscopy.

Cell growth (Step 18)

A starting tissue volume of 5–10 mm³ will generally yield one T-25 (0.5–1×10⁶ cells) flask while each successive increase of 5–10 mm³ in starting tissue will yield an additional flask. Since the fragments are not amenable to counting, the technician must become familiar with the needed density of microvessel fragments to initiate culture. In general this will be approximately 1 vessel fragment per 10X field examined, but individual laboratories will vary. Depending on the density of seeding, cells may not yet be visible after 2 weeks and confluence may be achieved latter than 30 days in vitro. To circumvent this time lapse, cultures starting with even smaller amounts of brain tissue can be plated in cell cultureware of smaller surface area (Petri dishes or in a well of a culture plate).

ANTICIPATED RESULTS

The yield of a culture largely depends on the amount of brain tissue available. Typically, from 5–10 mm³ of fresh tissue, 1×10⁶ HBMVEC can be harvested after 1 month (Fig. 2). Evaluation of the culture purity by analysis of 50 randomly selected fields reveals the presence of 97% HBMVEC and only 3% contaminating pericytes (Fig. 3). Using this cell culture protocol, specific markers of tight and adherens junctional proteins can be detected (Fig. 4 and 5), with no need of barrier tighter inducers as dexamethasone or hydrocortisone. The cells also express the caveolae marker, caveolin-1, as well as the ABC family protein, P-gp (Fig. 4). Cell culture properties are reproducible, as can be readily assessed by immunofluorescence analysis of GLUT-1, vWF, ZO-1 and β -catenin. Donor to donor variability in tight junction profiles can also be evaluated by analysis of protein expression levels by Western blot of subcellular fractions (Fig. 5A; note β -actin and Na⁺/K⁺ ATPase α 1 as loading controls for cytosol and membrane/organelle fractions respectively), or by evaluation of mRNA expression levels by quantitative real time PCR (Fig. 5B). The CD-31/PECAM-1 endothelial surface marker is present and can be used for characterization especially in flow cytometry related applications. However we would caution that the basal surface expression of this marker is low in resting HBMVEC and that there is a small donor to donor variability (unpublished observations). Cultures can be passaged ~3–4 times before phenotypic, cell culture purity, and barrier properties are affected, as inferred from analysis of markers of endothelial cells (GLUT-1, vWF), pericytes (α -SMA), and junction proteins (ZO-1 and β -catenin) by immunofluorescence, as well as of TEER measurement by ECIS

system or STX2 electrode (World Precision Instruments). Cells have been successfully frozen and recovered; however this does not extend the useful number of passages.

HBMVEC isolated by the present protocol form a barrier, as indicated by the TEER increase over time until the resistance reaches a steady state with the characteristic high TEER values and no longer fluctuates beyond ± 0.1 normalized resistance (subsequent values divided by initial values), as measured by the ECIS 1600R system (Fig. 6A).

Absolute TEER values for HBMVEC monolayers can be expected above >1000 ohms when using the 8W10E+ cultureware array with an AC frequency setting of 1000Hz. Although curve trends and relative TEER should be comparable across the various types of arrays available (i.e 8W1E, 8W10E and 8W10E+), it is important to note that between arrays broad differences in absolute TEER will be observed. For example, the 8W10E+ contains 8-wells with each well featuring 40 circular microelectrodes (each electrode 10^{-4} cm²) whereas the 8W1E contains a single microelectrode per well which can result in absolute TEER values that are 10 times higher than those observed for the 8W10E+. Steady state may be achieved in 8 days as demonstrated in figure 6B, however this greatly depends on HBMEC density at the time of cell plating. Once the HBMEC has reached steady state, cells can then be treated accordingly. Figure 6C shows the response of the endothelial barrier following exposure to a high concentration of Lysophosphatidic acid (LPA), the precipitous decrease in TEER (Fig. 6C) indicates a disruption or “leakiness” to the barrier. Conversely, the introduction of dexamethasone will generate a “tightening” of the barrier and increase the resistance gradually over time (data not shown).

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Figure 1.

Representative steps of the procedure to isolate and culture human brain microvascular endothelial cells. (A) Brain sample placed in a 100 mm dish containing isolation medium (IM); (B) removal of meninges and large vessels using sterilized surgical forceps and a stereomicroscope to facilitate the visualization; (C) fragmentation of the tissue by repeatedly sterile pipettes of 25 and 10 ml until (D) the sample can be passed effortlessly back and forth through a 5 ml pipette; (E) passage of the sample through a 500 µm polyester screen for removal of large fragments; (F) filtration of the collected fluid through a 30 µm polyester screen placed over a wire frame for support; (G) collection of the fragments retained on the screen by washing into a new dish; (H) pellet of fragments following centrifugation (note this picture depicts the collection of several membranes and the total volume is usually less); (I) resuspended microvessels and introduction to a collagen coated T-25 flask.

Fig. 2.

Phase contrast microscopy of human brain microvascular endothelial cells. Isolated brain microvascular fragments are obtained as a result of the procedure (A). Cells emerge from the end of these fragments over the first few days and form islands of cells with varying phenotypes as the density increases (B). As the confluent monolayers are formed in approximately one month, cell density increases and the cells display the typical cobblestone appearance (C-F). Scale bar = 100 μm .

Figure 3. Characterization of primary cultures of human brain microvascular endothelial cells by immunofluorescence microscopy. Brain endothelial cells were labeled for the glucose transporter-1 (A), and double-labeled for von Willebrand factor (vWF) and α -smooth muscle actin (α SMA) (B and C respectively). In C, a pericyte is visible, identified by the positive immunostaining for α SMA. For quantitative evaluation of the culture purity, the total number of nuclei, as well as the number of cells positive for vWF and for α -smooth muscle actin, were counted in 50 randomly selected fields (D). In A-C, nuclei were stained with Hoechst 33258 dye. Details of the immunocytochemical analysis are provided as supplementary method. Scale bar = 40 μ m.

Figure 4.

Immunofluorescent staining of confluent human brain microvascular endothelial cell monolayers for zonula occludens-1 (A,B; objective magnification 10x and 20x respectively), occludin (C,D; objective magnification 10x and 20x respectively), β -catenin (E), caveolin-1 (F), and P-glycoprotein (G) (E-G; objective magnification 63x). Nuclei were stained with Hoechst 33258 dye. Details of the immunocytochemical analysis are provided as supplementary method. Scale bar = 100 μ m for A, B, C, D and scale bar = 40 μ m for E, F, G.

Figure 5.

Western blot and q RT-PCR analysis of protein and mRNA expression in confluent monolayers of human brain microvascular endothelial cells (HBMVEC) between passages 1–3, from three different donors. A, Western Blot analysis of subcellular fractions were performed as previously described³⁶ using antibodies against occludin, claudin-5, zonula occludens (ZO)-1, and ZO-2 (note: the Na⁺/K⁺ ATPase α 1 and β -actin were used as internal/loading controls). B, Gene expression profile of tight junction proteins from donors in (A). Total RNA was isolated using the RNAqueous-4PCR kit (Ambion, Austin, TX) and RNA purity and concentration was determined with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The conversion to cDNA was performed by reverse transcription using 2 μ g of total RNA with the High-Capacity cDNA Reverse Transcription Kit (ABI, Foster city, CA). The cDNA (diluted 1:20) template was then mixed with both the Taqman universal PCR master mix (ABI) and the corresponding human TaqMan Gene Expression Assay (CLD1: Hs01076359_m1, CLD3: Hs00265816_s1, CLD5: Hs01561351_m1, OCC: Hs00170162_m1, ZO-1: Hs01631876_m1, ZO-2: Hs00910541_m1 and JAM-2: Hs00221894_m1 from ABI) according to the manufacturer's instructions, for internal controls, the human TaqMan Gene Expression Assays for GAPDH and RPLPO (ABI) were also used. The qPCR was performed on an ABI StepOnePlus Real Time PCR system. The raw data was analyzed with the DataAssist software (ABI) using the delta-delta Ct method (Relative Quantification). The results are expressed in relative gene expression levels (fold) compared to a HEK293 control sample. CLD, claudin; OCC, occludin; JAM, junction adhesion molecule.

Figure 6.

Transendothelial electric resistance (TEER) measured with the Electrical Cell-Substrate Impedance Sensing (ECIS) system (model 1600R, Applied Biophysics, Troy, NY). (A) ECIS device with electrode arrays (cultureware 8W10E+, Applied Biophysics) coupled with acquisition software to monitor TEER continuously. (B) TEER values shown as normalized resistance (subsequent values divided by initial values) from the initial plating of HBMVEC to confluence and barrier formation. The resistance, measured at 1 hr intervals, increases over time until a steady state level is reached. (C) Evaluation of the barrier function in response to Lysophosphatidic acid (LPA) addition (arrow), measured at 10 min intervals for 24 h, where the precipitous decrease in TEER by LPA indicates a disruption or “leakiness” to the barrier. The results indicated by the graphs are represented as the average (line) normalized TEER $\pm$ SEM (n=3). Note only the positive SEM is shown for 5C.