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An immortalized human blood-nerve barrier endothelial cell line for *in vitro* permeability studies

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Abstract

Solute and macromolecular transport studies may elucidate nutritional requirements and drug effects in healthy and diseased peripheral nerves. Endoneurial endothelial cells are specialized microvascular cells that form the restrictive blood-nerve barrier (BNB). Primary human endoneurial endothelial cells (pHEndECs) are difficult to isolate, limiting their widespread availability for biomedical research. We developed a simian virus-40 large T-antigen (SV40-LTA) immortalized human BNB cell line via stable transfection of low passage pHEndECs and observed continuous growth in culture for >45 population doublings. As observed with pHEndECs, the immortalized BNB endothelial cells were Ulex Europaeus agglutinin-1 (UEA-1)-positive and endocytosed low density lipoprotein, but lost von Willebrand factor (vWF) expression. Glucose transporter-1 (GLUT-1), P-glycoprotein (P-gp), γ -glutamyl transpeptidase (γ -GT), large neutral amino acid transporter-1 (LAT-1), creatine transporter (CRT) and monocarboxylate transporter-1 (MCT-1) mRNA expression were retained at all passages with loss of alkaline phosphatase (AP) expression after passages 16-20. Compared with an SV40-LTA immortalized human blood-brain barrier (BBB) endothelial cell line, there was increased γ -GT protein expression, equivalent expression of organic anion transporting polypeptide-C (OATP-C), organic anion transporter 3 (OAT-3), MCT-1 and LAT-1, and reduced expression of AP, CRT and P-gp by the BNB cell line at passage 20. Further studies demonstrated lower transendothelial electrical resistance (TEER: $\sim 181 \Omega \cdot \text{cm}^2$ vs. $191 \Omega \cdot \text{cm}^2$), equivalent permeability to fluoresceinated sodium (4.84% vs. 4.39%) and lower permeability to fluoresceinated high molecular weight (70 kDa) dextran (0.39% vs. 0.52%) by the BNB cell line. This cell line retained essential molecular and biophysical properties suitable for *in vitro* peripheral nerve permeability studies.

Keywords

Blood-brain barrier; blood-nerve barrier; endoneurial endothelial cell; *in vitro* permeability; transendothelial electrical resistance; transporters

INTRODUCTION

Endoneurial microvascular endothelial cells restrict the passive diffusion of solutes, macromolecules, leukocytes and microbes from the blood circulation into the endoneurium, and can be considered the true blood-nerve barrier (BNB), in contrast to the perineurium that

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CONFLICT OF INTEREST

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restricts diffusion from the freely permeable epineurium into the endoneurium (Olsson 1990; Reina et al. 2000; Reina et al. 2003). These blood-nerve interfaces serve to regulate the influx and efflux of water, nutrients and xenobiotics, needed to maintain the peripheral nerve internal microenvironment. The BNB is second only to the blood-brain barrier (BBB) in terms of restrictive capabilities in mammals (Malmgren and Olsson 1980; Poduslo et al. 1994; Allt and Lawrenson 2000). BNB compromise has been implicated in the pathogenesis of several peripheral neuropathies (Olsson 1990; Mizisin and Weerasuriya 2011).

Expression of specialized transporters by endoneurial microvessels provides insights relevant to understanding peripheral nerve barrier function. Peripheral nerve permeability and solute uptake studies in animals further evaluate BNB function in the intact peripheral nervous system. Certain transporters such as glucose transporter-1 (GLUT-1) and P-glycoprotein (P-gp) have been detected on endoneurial microvessels or isolated BNB-forming endothelial cells across several species (Allt and Lawrenson 2000; Froehner et al. 1988; Kanda et al. 1997; Saito et al. 2001; Sano et al. 2007; Yosef et al. 2010). Interspecies differences in specialized transporter expression and function have also been described (e.g. AP, γ -GT, MCT-1), suggesting that variations in BNB function may occur, implying differential nutrient utilization or susceptibility to drugs and toxins in peripheral nerves (Véga et al. 1998; Latker et al. 1987; Bell and Weddell 1984; Orte et al. 1999; Yosef et al. 2010). Although the BBB and BNB are restrictive neural microvascular barriers, differences in specialized transporter expression have been described in rodents (Sano et al. 2007). Significant advances have been made in understanding mammalian BBB function due to the widespread availability of primary and immortalized cerebral microvascular endothelial cell lines. In contrast, very few peripheral nerve endoneurial endothelial cell lines exist (Kanda et al. 1997; Sano et al. 2007; Yosef et al. 2010; Shimizu et al. 2011; Argall et al. 1994). The compartmentalized structure of peripheral nerves and low endoneurial microvessel density are possible factors that have limited widespread endoneurial endothelial cell line development.

We describe and characterize a novel immortalized human BNB endothelial cell line developed via stable transfection of primary human endoneurial endothelial cells (pHEndECs), demonstrating retained expression of specialized transporters and restrictive barrier function similar to a commonly studied immortalized human BBB cell line.

MATERIALS AND METHODS

Antibody and Reagents

1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate-labeled acetylated low density lipoprotein (DiI-Ac-LDL) was purchased from Biomedical Technologies (Stockton, Massachusetts, U.S.A). Sodium fluorescein (uranine), fluorescein isothiocyanate (FITC)-dextran 70 kDa, FITC-conjugated UEA-1 and monoclonal mouse anti-human P-gp IgG1 (clone F4) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Mouse monoclonal anti-human vWF IgG1 (clone 2F2-A9) was purchased from BD Biosciences (San Jose, California, U.S.A.). Mouse anti-SV40-LTA IgG2a (clone PAb416) was purchased from EMD Biosciences (La Jolla, California, USA). Mouse monoclonal anti-human tissue non-specific AP IgG1 (clone D-3), anti-human OATP-C IgG1 (clone A3), anti-human γ -GT (clone 3E6) and anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) IgG1 (clone 0411); polyclonal rabbit anti-human OAT-3 IgG and anti-human CRT IgG; and polyclonal goat anti-human MCT-1 IgG and anti-human LAT-1 IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Fluorescent secondary antibodies (polyclonal goat anti-rabbit IgG FITC and R-phycoerythrin conjugates, and monoclonal goat anti-mouse FITC and R-phycoerythrin) were obtained from SouthernBiotech (Birmingham, Alabama, USA), while horseradish peroxidase-conjugated

polyclonal goat anti-mouse IgG antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania, USA), polyclonal goat anti-rabbit IgG antibodies (Fc fragment specific) from Thermo Fisher Scientific (Rockford, Illinois, USA) and polyclonal donkey anti-goat IgG antibodies from Santa Cruz.

Primary human endoneurial endothelial cell (pHEndEC) isolation and culture

The work described was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki), conforming to the Uniform Requirements for manuscripts submitted to Biomedical journals. An exemption from the Baylor College of Medicine Institutional Review Board (protocol number H-22347) was obtained to harvest sciatic nerves from recently decedent individuals undergoing routine medical autopsy. pHEndECs were isolated, purified and characterized as previously described (Yosef et al. 2010) and expanded by culture on rat tail collagen (RTC)-coated Corning CellBIND® tissue culture plates in regular growth medium [RPMI-1640 media (Invitrogen) containing 10% NuSerum (BD Biosciences, Franklin Lakes, New Jersey, USA), 10% fetal bovine serum (PAA Laboratories, Dartmouth, Massachusetts, USA), 1X penicillin-streptomycin (Mediatech, Herndon, Virginia, USA), 1X vitamin solution, 1X non-essential amino acid solution, 2 mM L-glutamine, 1 nM sodium pyruvate, 10 mM HEPES buffer (all from Invitrogen), 1 ng/mL recombinant human bFGF (Roche Applied Sciences), 10 U/mL heparin (Sigma-Aldrich) and 50 µg/mL endothelial cell growth supplements (BD Biosciences), pH 7.4]. The cells were cultured at 37°C in a humidified atmosphere with 95% air/ 5% CO₂ in an incubator, as previously described (Yosef et al. 2010), and passaged at 80-90% confluence.

Immortalization of pHEndECs by stable transfection

A commercially available plasmid vector containing simian virus-40 large T antigen (SV40-LTA) and a neomycin resistance cassette (known as pSV3-neo) was transformed and expanded within *Escherichia coli* strain HB101 (both from American Type Culture Collection, Manassas, Virginia, USA) in ampicillin-containing Luria Broth Base for 16 hours, followed by purification using the Qiagen QIAfilter™ Plasmid Maxi kit, according to the manufacturer's instructions. DNA purity and concentration were determined by ultraviolet spectrophotometry at 260 nm. Ethidium bromide-stained agarose gel electrophoresis was performed to verify plasmid size.

The purified pSV3-neo plasmid was transferred into the nuclei of proliferating pHEndEC cultures (passage 4, 70-80% confluent) by non-viral technology using an Amaxa Nucleofector™ device (program M-03), according to the manufacturer's instructions. Briefly, aliquots of 5×10^5 pHEndECs were resuspended in Basic Nucleofector™ solution for primary endothelial cells containing 7 µg of pSV3-neo plasmid, electroporated and plated in 80% regular growth media + 20% pHEndEC-conditioned growth media for 24 hours. Surviving adherent cells were cultured for 10 days in neomycin analog G418 sulfate-containing growth medium (as above), changed every 48 hours. SV40-LTA transfected human endoneurial endothelial cells (THEndECs) were pooled and continuously expanded in regular growth medium on CellBIND® tissue culture plates for >80 days *in vitro*. THEndECs were passaged when >80% confluent as previously described with pHEndECs (Yosef et al. 2010). Endothelial cell aliquots were cryopreserved by a controlled rate cooling process from passage 7 onwards for use in subsequent experiments.

Immunocytochemistry

Nuclear expression of SV40-LTA by THEndEC cultures was evaluated to verify stable transfection. THEndECs were cultured to confluence on autoclaved RTC-coated borosilicate glass coverslips placed in 12-well Corning costar tissue culture plates, seeding at 20,000

cells/cm². The coverslips were fixed with 1% paraformaldehyde in 1X phosphate buffered saline (PBS) for 10 minutes at room temperature (RT), washed with 1X PBS and permeabilized with cold acetone (-20°C) for 1 minute at RT. Non-specific blocking was performed with 10% normal goat serum (NGS) for 30 minutes followed by incubation with 5 µg/mL of a specific mouse anti-SV40 LTA IgG2a antibody in 2% NGS in 1X PBS for 1 hour at RT. Omission of this antibody served as a negative control. Coverslips were washed with 2% NGS in 1X PBS, and treated with 1 µg/mL goat anti-mouse IgG2a-FITC for 1 hour at RT in the dark. Following another wash, coverslips were treated with 0.45 µM 4',6-diamidino-2-phenylindole (DAPI) for 5 minutes to detect nuclei, washed and mounted in Vectashield (Vector Laboratories, Burlingame, California, USA). UEA-1 binding and vWF expression, as well as endocytosis of DiI-Ac-LDL, were evaluated on confluent THEndECs, as previously published (Yosef et al. 2010). Images were collected with a Zeiss Axioskop Epifluorescent microscope equipped with an Axiocam MRc 5 digital camera using the 10X to 40X objective lenses. Images were initially processed with the Zeiss Axiovision software and merged using Adobe Photoshop CS2 software.

THEndEC proliferation

THEndEC were expanded in culture, plating 1×10^6 cells per 75 cm² Cell BIND® flask in regular growth medium as described above. Endothelial cells were harvested at >80% confluence using 0.05% trypsin-0.53 mM ethylenediamine tetraacetic acid (EDTA) in Hank's Balanced Salt Solution (Mediatech, Herndon, Virginia, USA) and quantified in duplicate using a hemacytometer. The number of population doublings (p.d.) per passage was estimated based on the following formula: $p.d. = \log_2(\text{THEndEC}_c / \text{THEndEC}_p)$; where THEndEC_c indicates number of cells at confluence, and THEndEC_p indicates number of cells initially plated at each passage. Cumulative p.d. was determined by adding consecutive p.d. values, and this was plotted against time in continuous culture (in days).

Polymerase chain reaction (PCR)

All reagents, reaction kits and cartridge purified primers were purchased from Invitrogen (Carlsbad, California, USA), unless otherwise stated. Total RNA was obtained from confluent THEndECs cultured on Corning CellBIND® 6-well tissue culture plates using TRIzol® reagent according to the manufacturer's instructions. THEndECs at passage 8, 12, 16, 20, 24 and 27 were used for these experiments. Total RNA was spectrophotometrically quantified using a nanodrop® ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) as previously published (Yosef et al. 2010). Previously verified primer sequences for AP, CRT, γ-GT, GAPDH, GLUT-1, LAT-1, MCT-1, P-gp and vWF were used (Yosef et al. 2010), as shown on Table 1. Reverse transcription and PCR amplification were carried out using a TC-412 Thermal Cycler (Techne, Burlington, New Jersey, USA) as previously published (Yosef et al. 2010). Omission of specific primers served as negative controls while GAPDH amplification served as an internal control. PCR products were resolved and visualized on 1% ultra-pure agarose gels stained with ethidium bromide. The 1-kb plus DNA ladder kit was used to confirm band size. Digital images were generated using an AlphaImager HP image documentation system (Cell Biosciences, Santa Clara, California, USA) attached to a Sony ICX267AL 1.39 Megapixel CCD camera and processed using the AlphaView software (version 3.2.2.0) and Adobe Photoshop CS2 (Adobe Systems, San Jose, California, USA) software programs. At least three independent experiments were performed.

Western blot analysis

Total cellular (cytoplasmic and membrane) proteins were extracted from THEndEC and SV40-LTA immortalized human brain microvascular endothelial cell (THBMEC: kind gift from Dr. Monique Stins) cultures grown to confluence in 75 cm² Corning CellBIND® tissue

culture flasks at passage 20. THBMEC were seeded at 1×10^6 cells per flask, and expanded as previously published. Protein extraction was performed by gentle scraping in cold lysis buffer (10 mM potassium chloride + 10 mM HEPES + 0.1 mM ethylene diamine tetraacetic acid + 0.1 mM ethylene glycol tetraacetic acid + 1 mM dithiothreitol + 0.5 mM phenylmethanesulfonyl fluoride + 0.05% tergitol® nonyl phenoxy-polyethoxyethanol-40 in deionized distilled water) containing protease inhibitor cocktail (1:300 dilution; Sigma-Aldrich) on ice, following by brief sonication and centrifugation at 18,000g for 20 minutes at 4°C. Protein extracts were quantified using the Bradford Coomassie assay and stored at -80°C. Equal amounts of protein (15 µg) were denatured, reduced and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% slab gel) using the Bio-Rad MiniProtein III Apparatus (Hercules, California, USA), then electrotransferred to polyvinylidene fluoride membranes in transfer buffer using the Bio-Rad Trans-Blot Apparatus, according to manufacturer instructions.

Membranes were blocked with 5% non-fat milk or 5% donkey serum in Tris buffered saline + 0.05% Tween-20 (TBS-T) for 1 hour at RT, followed by incubation with primary antibodies diluted in TBS-T containing 1-5% non-fat milk or 1% donkey serum overnight at 4°C (final concentration 0.2 µg/mL for OAT-3 and CRT, 0.5 µg/mL for γ-GT, 1 µg/mL for AP, GAPDH, LAT-1, MCT-1 and OATP-C and 5 µg/mL for P-gp). Following several washes, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies diluted in TBS-T/ 1-5% non-fat milk or TBS-T/ 1% donkey serum (1:10,000 dilution) for 1 hour at RT, washed as above, treated with Pierce Enhanced Chemiluminescence western blotting substrate plus detection reagents (Thermo Fisher Scientific, Rockford, Illinois, U.S.A.) and visualized by generating autoradiographs following film exposure from 1 minute to 1 hour using an SRX-101A film processor (Konica Minolta, Tokyo, Japan).

Digital autoradiograph images were taken using an AlphaImager HP image documentation system (Cell Biosciences) attached to a Sony ICX267AL 1.39 Megapixel CCD camera. Semi-quantitative protein expression (relative to GAPDH) was determined by spot densitometry (based on integrated density values with background correction) from concurrently developed films imaged at the same time point using the AlphaView software program (version 3.2.2.0). Data from three independent protein isolates were averaged to determine differences in transporter protein expression between the BNB and BBB cell lines. Molecular weight markers confirmed protein size.

Transendothelial electrical resistance (TEER) assessment

TEER was directly measured on confluent passage 20 THEndECs and THBMECs initially seeded at 10,000 to 80,000 cells per insert on Corning polyester membrane transwell inserts (3.0 µm pore size, 6.5 mm diameter, growth surface area 0.33 cm²) in 24-well tissue culture plates on day 7 using an epithelial volttohmmeter with STX2 electrodes (World Precision Instruments, Sarasota, Florida, USA) as previously described (Yosef et al. 2010). The effect of the extracellular matrix on TEER in each cell line was tested by coating inserts with rat-tail collagen (RTC) gel, glutaraldehyde (GTA)-crosslinked RTC and GTA-crosslinked RTC followed by RTC gel coating prior to seeding. Transwell inserts were gel-coated with RTC in ammonia vapor as previously described (Yosef et al. 2010). Other inserts were treated with 1:1 mix of 0.2% GTA in 1X PBS and 50 µg/mL RTC in 0.02N acetic acid for 1 hour at RT, washed twice with 1X PBS and treated with 2% glycine for 5 minutes to neutralize residual GTA and washed again. Finally, several inserts were initially coated with GTA-crosslinked RTC, then gel-coated with RTC as above. These experiments were performed to determine the optimum conditions for THEndEC barrier formation and determine if significant differences in TEER existed between THEndECs and THBMECs *in vitro*.

Solute Permeability assessments

The permeability of optimally cultured, confluent passage 20 THEndEC and THBMEC layers to sodium fluorescein (molecular weight 376 D) and FITC-dextran 70 kDa was determined on transwell inserts on day 7, using previously published methods (Yosef et al. 2010). Briefly, 100 μ L of a 1 mg /mL solution of either sodium fluorescein or FITC-dextran-70 kDa (warmed to 37°C) was added into each transwell insert placed in a 24-well tissue culture plate containing 600 μ L of basal medium at 37°C for 15 minutes in a humidified incubator containing 95% air/ 5% CO₂. The concentration of sodium fluorescein or FITC-dextran-70 kDa in the receiving well was determined fluorometrically against a standard curve of known concentrations using a FLUOstar Optima microplate reader (BMG Labtech, Durham, North Carolina, USA). The permeability coefficient is directly proportional to the ratio of solute that permeates the transwell membrane to the input, expressed as a percentage of the input (Yosef et al. 2010). Comparisons between THEndECs and THBMECs were performed to determine if differences in solute permeability to low and high molecular weight molecules existed between BNB and BBB-forming endothelial cell lines *in vitro*.

Statistical Analyses

Statistical analyses (unpaired two-tailed Student's t-test or analysis of variance) were performed using JMP® version 8 software (SAS Institute), with statistical significance set at $p < 0.05$. Data sets were determined to be normally distributed based on tests for skewness and kurtosis. Data in the text represent mean values. Variations of the mean are expressed as standard errors of mean, demonstrated by error bars in representative graphs.

RESULTS

Establishment and characterization of the THEndEC line

We transfected early passage pHEndECs using a purified pSV3-neo plasmid, establishing a stable THEndEC line. Endothelial cell survival was ~80% 24 hours following non-viral nuclear transfer by electroporation. All THEndECs selected by G418 resistance expressed SV40-LTA in their nuclei, indicative of successful stable transfection (Figures 1A-C). THEndECs were spindle-shaped, bound UEA-1 lectin (indicative of expression of α -L-fucose residues) and intensely endocytosed DiI-Ac-LDL (Figures 1D-E), as previously described with pHEndECs (Yosef et al. 2010). THEndECs did not express vWF by immunocytochemistry, in contrast to pHEndECs (data not shown). Loss of Weibel-Palade bodies or vWF expression may occur with immortalized mammalian endothelial cell lines (Takahashi et al. 1990; Schwartz et al. 1991; van Leeuwen et al. 2000).

THEndECs continuously grew in culture for >80 days (45 population doublings) without senescence (Figure 2). There was loss of contact inhibition, with a mean population doubling rate of 27.8 hours from passages 8-12, 43.8 hours from passages 13-15, 59.2 hours from passages 16-21, 38.8 hours from passage 22-26 and 53.3 hours from passage 27-28 (Figure 2A). This contrasts with pHEndECs (passage 3-8) that consistently double in number approximately over 48 hours during the logarithmic phase of growth (first 2-4 days depending on seeding density), followed by contact inhibition (Yosef et al. 2010). Despite differences in mean population doubling rate, there was a near linear relationship between passage number and cumulative population doublings (B) suggesting stable proliferation characteristics *in vitro*.

THEndEC specialized transporter expression

THEndECs expressed mRNA for γ -GT, LAT-1, CRT, MCT-1, and GLUT-1 at all passages tested (P8-P27), as previously demonstrated on pHEndECs (Figure 3). THEndECs did not

express P-gp at passage 8. Expression of splice-variants or multimers of the expected cDNA product (based on data derived from pHEndECs) was observed at passages 12 to 27. The expected cDNA product became clearly apparent at passage 20 and persisted until passage 27. An additional band was observed at passages 24 and 27, as shown in Figure 3. AP expression was routinely observed from passages 8 to 16, with variable loss of cDNA expression after passage 16 or 20 (Figure 3). Consistent with the immunocytochemistry data, THEndECs did not express vWF cDNA at any of the passages tested (data not shown).

Comparative expression of specialized transporters by the BNB and BBB cell lines

We also compared the expression of specialized endothelium transporters on confluent THEndECs and THBMECs by western blot at passage 20. We determined that THBMECs expressed more AP, CRT and the efflux transporter, P-gp. THEndECs expressed more γ -GT, while both cell lines equivalently expressed LAT-1, MCT-1, OAT-3 and OATP-C, as shown in Figure 4. These data suggest differential uptake and release of solutes and macromolecules across the *in vitro* human BNB and BBB, suggesting similarities as well as important differences in peripheral nerve endoneurium and cerebral cortex nutrient utilization and susceptibility to drug intoxication.

Comparative BNB and BBB biophysical characteristics *in vitro*

We determined that GTA-crosslinked RTC provided the best extracellular matrix for THEndEC proliferation and barrier formation on transwell inserts *in vitro* based on TEER measurements, with initial seeding at 80,000 cells per insert (Figure 5A). Maximum mean TEER was $180.7 (\pm 0.7) \Omega \cdot \text{cm}^2$ with GTA-crosslinked RTC, compared to $173.3 (\pm 0.9) \Omega \cdot \text{cm}^2$ with RTC gel and $175.0 (\pm 1.5) \Omega \cdot \text{cm}^2$ with GTA-crosslinked RTC followed by RTC gel ($p < 0.05$). This is in contrast to THBMECs, as RTC-gel coating provided optimal TEER ($191.3 \pm 0.3 \Omega \cdot \text{cm}^2$, vs. $168.7 \pm 0.3 \Omega \cdot \text{cm}^2$ with GTA-crosslinked RTC and $171 \pm 1.5 \Omega \cdot \text{cm}^2$ with GTA-crosslinked RTC followed by RTC gel; $p < 0.05$), although seeding at 80,000 cells per insert also maximized TEER (Figure 5B). At optimal seeding densities and preferred extracellular matrix coating, confluent THEndECs demonstrated lower TEER than THBMECs ($p < 0.001$), implying the human BNB may be less restrictive than the BBB *in vitro* (Figure 5C).

Comparative permeability studies demonstrated equivalent permeability between confluent THEndECs and THBMECs to sodium fluorescein (mean $4.84 \pm 0.19\%$ vs. $4.39 \pm 0.18\%$; $p = 0.12$), with a statistically significant (but probably physiological insignificant) lower permeability to fluoresceinated high molecular weight (70 kDa) dextran ($0.39 \pm 0.01\%$ vs. $0.52 \pm 0.01\%$; $p < 0.00001$) by THEndECs (Figures 6A-B). It is important to recognize that BBB endothelial function is directly enhanced by the glia limitans *in vivo* (formed by astrocyte and microglial foot processes); a structure that does not exist in peripheral nerves. These data suggest differences exist in the restrictive barrier function between the human BNB and BBB *in vitro*, despite both being restrictive barrier-forming microvascular endothelial cells of human neural origin.

DISCUSSION

Compared to other microvascular barriers, relatively little is known about the mammalian BNB. *In vitro* endothelial and epithelial barrier studies have significantly increased our knowledge of tissue nutritional requirements and drug susceptibility, as well as leukocyte and microbial interactions with the microvasculature. Phenotypic and functional differences exist between endothelial cells from different tissues and species and between macrovascular and microvascular endothelial cells from the same tissue (Aird 2007a, b; Yano et al. 2007; Orte et al. 1999; Murphy et al. 1998; Man et al. 2008; Bell and Weddell

1984). This implies that there are limitations in extrapolating observations from microvascular endothelial cells derived from other tissues or species to others. This provides the impetus to study human BNB function using a cell line derived from peripheral nerve endoneurial endothelial cells.

Despite stable transfection and immortalization with SV40LTA, THEndECs retained their vascular endothelial cell nature supported by specific binding to UEA-1 lectin and uptake of DiI-Ac-LDL (implying expression of α -fucose residues and retention of membrane scavenger receptors, respectively). We observed continued growth for 45 population doublings *in vitro*. However, THEndECs do not express vWF. Loss of vWF has been described in some immortalized endothelial cell lines (Takahashi et al. 1990; van Leeuwen et al. 2000). Loss of Weibel-Palade body synthesis may be responsible for the absence in vWF expression, implying limitations of this cell line in studying platelet adhesion/aggregation and blood-nerve barrier wound healing (Rondaij et al. 2006; van Mourik et al. 2002). Based on our published pHEndEC studies, loss of vWF expression during senescence mirrored loss of constitutive P-selectin expression (Yosef et al. 2010). Therefore, it is possible that other proteins expressed in Weibel-Palade bodies may be lost in THEndECs as well.

We tested the retention of BNB transporter expression by THEndECs following serial passaging (passages 8-27) based on our previously published observations with pHEndECs (Yosef et al. 2010). Immortalization and serial passaging did not affect CRT, γ -GT, GLUT-1, LAT-1 and MCT-1 mRNA expression, suggesting that this cell line may be useful for facilitated receptor-mediated *in vitro* BNB permeability/kinetic studies. Such studies have the potential to increase our understanding of how peripheral nerves utilize essential amino acids and glucose during normal nutritional states, or creatine and lactate at times of starvation. The potential role of MCT-1 as an efflux transporter (facilitating the removal of lactate from the endoneurium of injured nerves) can be tested with this cell line.

P-gp is an important efflux transporter at microvascular barriers, and is a substrate for many xenobiotics such as vincristine (Sharom 2011). There was an initial loss in P-gp mRNA expression following immortalization, followed by the expression of multimers or splice variants in addition to the expected cDNA product based on our data derived from pHEndECs (Yosef et al. 2010). Alternative splicing of the P-gp gene has been described at a higher frequency in ovarian tumors (He et al. 2004). Although we clearly observed the expected P-gp cDNA product from passage 20 onwards, further analysis on the effect of immortalization of P-gp function is needed with the THEndEC cell line. AP is a known marker of human BNB capillary endothelium *in vivo* and *in vitro*, and may function as an adhesion molecule, enzyme and membrane pump (Bell and Weddell 1984; Gallo et al. 1997; Yosef et al. 2010). We observed variable loss of expression after passages 16 or 20, similar to the loss of expression in pHEndECs during senescence (Yosef et al. 2010), implying a role for serial passaging in enzyme loss. Early THEndEC passages could be studied in order to understand the role of this enzyme in human BNB function *in vitro*.

We sought to determine whether several transporters previously demonstrated at the mammalian BNB or BBB were expressed by the THEndEC cell line, and if differences exist between these cell lines. We demonstrated increased THEndEC γ -GT expression compared with THBMECs. THBMECs retain γ -GT expression demonstrated by primary human brain microvascular endothelial cells (Stins et al. 2001). γ -GT transfers amino acids across restrictive barrier-forming endothelium, as well as catalyzes the transfer of the gamma-glutamyl moiety of glutathione to an acceptor [amino acid, peptide or water] (Courtay et al. 1992; Hawkins et al. 2006). This provides a pathway for drug and xenobiotic detoxification that may be preferentially utilized by the human BNB compared to the BBB. OATP-C and

OAT-3 were also expressed by our human BNB cell line, in contrast to an immortalized rat BNB cell line (Sano et al. 2007).

OATP-C is a member of a family of solute carriers that are selectively expressed in the liver and function as Na⁺ and ATP-independent transporters for amphipathic organic compounds such as steroids, thyroid hormones, anionic peptides, drugs (such as 3-Hydroxy-3-Methyl-Glutaryl -coenzyme A reductase inhibitors) and xenobiotics (Hsiang et al. 1999; Konig et al. 2000). OAT-3 is selectively expressed on the basolateral membranes of renal proximal tubules and is involved in the transport and excretion of drugs such as benzyl penicillin, methotrexate, ciprofloxacin, indomethacin and cimetidine, and toxic organic ions. OAT-3 also functions as an organic anion exchanger, coupling the uptake of one organic anion molecule with the efflux of one endogenous dicarboxylic acid molecule (Burckhardt and Burckhardt 2011; VanWert et al. 2010). These transporters were also expressed at similar levels by the human BBB cell line, implying possible important homeostatic function within human neural tissues.

THEndECs expressed less AP and CRT than THBMECs *in vitro*. The reduction in AP expression in the human BNB cell line may reflect alterations induced by immortalization or serial passaging. CRT transports creatine (required for the storage of high energy phosphate groups) across vascular endothelium (Ohtsuki et al. 2002). Since AP also acts as a phosphotransferase, one may speculate that the reduced expression of these transporters by the human BNB cell line occurs due to less demanding metabolic demands within the peripheral nerve endoneurium compared to the cerebral cortex. The reduced expression of P-gp by the BNB cell line may reflect post-immortalization alternative splicing events at the mRNA level (as suggested by the PCR data) that could alter functional protein synthesis. It is also plausible that the BBB is more efficient at removing P-gp substrates from the cerebral cortex than the BNB is for peripheral nerve endoneurium. The equivalent expression of LAT-1 and MCT-1 suggest that these neural barriers may similarly transport neutral and aromatic amino acids and monocarboxylates (such as lactate), respectively.

Further studies are needed to determine the full repertoire of transporters expressed by the human BNB. Solute and drug permeability assays are also needed to further deduce influx and efflux transport mechanisms at the human BNB *in vitro*. These studies may provide important insights into the regulation and maintenance of peripheral nerve endoneurial homeostasis, as well as mechanisms of specific drug uptake and removal following systemic administration in normal and pathologic states.

Based on *in vivo* animal studies, the BNB is second only to the BBB in terms of restrictive barrier function. Unlike the BBB, the BNB lacks a glial limitans. Furthermore, the endoneurial microenvironment is also regulated by the perineurium (Olsson 1990; Mizisin and Weerasuriya 2011). These observations suggest the human BNB and BBB, although similar in function, demonstrate important differences despite being of neural origin. We determined the optimal conditions (seeding density and matrix coating) for THEndECs and compared TEER and solute permeability to a well-established immortalized BBB cell line (THBMECs). Consistent with *in vivo* observations, the BNB had a lower TEER than the BBB. These cell lines had similar permeability to sodium fluorescein, suggesting similar function in regulating sodium influx into the endoneurial and cerebral microenvironments. Interestingly, confluent THEndECs demonstrated a statistically significantly lower permeability to high molecular weight dextran compared to THBMECs. These values were of the same order of magnitude, so differences may or may not have physiological significance. However this difference could reflect the effect of immortalization on these human neural-derived microvascular cells (i.e. induced autocrine expression of mitogens

that may enhance or impair barrier function), or reflect the importance of the glial limitans in enhancing human BBB function to large macromolecules *in vivo*.

CONCLUSIONS

THEndECs retain essential characteristics of microvascular endothelial cells derived from peripheral nerves and demonstrate expression of selected influx and efflux transporters expected of a restrictive barrier-forming endothelial cell line. Confluent THEndECs form resistant barriers *in vitro*, with slightly less TEER, equivalent solute permeability to sodium fluorescein and slightly reduced permeability to high molecular weight dextran compared to a well-established human BBB cell line. Differences in solute and macromolecular transport proteins exist between the human BNB and BBB cell lines, suggesting differences in microvascular endothelial barrier function between peripheral nerves and the brain. THEndECs provide an important tool required for detailed *in vitro* solute, macromolecular and drug transport studies that may provide further insights into human BNB function in health and disease.

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Figure 1. Characterization of the THEndEC line

Representative indirect fluorescent immunocytochemistry photomicrographs demonstrate SV40 LTA expression (green; **A**) within THEndEC nuclei detected with DAPI (blue; **B**). The merged photograph shows SV40 LTA expression in the nuclei of ~100% of THEndECs (**C**), indicative of stable transfection. THEndECs (bluish green nuclei) retain expression of α -L-fucose residues, detected by UEA-1 lectin binding (green) as shown in a representative merged photomicrograph (**D**) and intensively endocytose DiI-Ac-LDL (orange; **E**). Original magnification 200X for A-C, 300X for D-E.

Figure 2. THEndEC growth characteristics

The graphs depict THEndEC proliferation as a measure of population doublings over time (A) and the relationship between passage number and population doublings (B). Proliferation rate (slope of curve) was highest during the first 10 days of culture, slowed down over the next 40 days, increased over the following 25 days and slowed down over the last week following continuous culture (A). Despite differences in proliferation rate, a near linear relationship is observed between passage number and cumulative population doublings, indicative of steady THEndEC proliferation over time (B).

Figure 3. Expression of specialized transporters by THEndECs following serial passaging
Representative digital photographs of ethidium bromide-stained agarose gels visually depict stable PCR product (cDNA) expression for γ -GT, LAT-1, CRT, MCT-1 and GLUT-1 at all passages tested. P-gp is not detected at passage 8, with the expression of splice-variants or multimers from passages 12 to 27. The expected cDNA product (*) is clearly evident from passages 20 to 27. AP expression is lost by passage 20 in this representative experiment. Numerals indicate passage numbers.

Figure 4. Comparative THEndEC and THBMEC transporter protein expression

Representative composite digital autoradiographs following Western blot to detect and quantify specialized transporters expressed by confluent THEndEC (N; nerve-derived) and THBMEC (B, brain-derived) is shown in **A**. Protein extracts from three isolates are displayed on the same autoradiograph to demonstrate similarity in transporter protein expression by these cell lines. Comparative semi-quantitative transporter protein expression (relative to GAPDH expression) is shown by bar histograms (**B**). THEndECs significantly express more γ -GT, while THBMEC express more AP, CRT and P-gp. No statistically significant differences are observed with OATP-C, OAT-3, MCT-1 and LAT-1 expression. * indicates p-value <0.05, N.S. indicates not significant. Error bars indicate standard errors of the mean. N=3.

Figure 5. Effect of seeding density and extracellular matrix composition on TEER
 Seeding density influences THEndEC (A) and THBMEC (B) TEER, with maximal TEER observed at a seeding density of 80,000 cells per insert irrespective of the extracellular matrix coating used. Maximal THEndEC TEER is observed with glutaraldehyde-crosslinked rat tail collagen [GTA-RTC] (black arrow, A), while maximal THBMEC TEER is observed with rat tail collagen [RTC] alone (black arrow, B). At optimal seeding density and most favorable extracellular matrix, THBMEC demonstrates a statistically significantly higher TEER than THEndEC (C). * indicates p-value <0.05. Error bars indicate standard errors of the mean. TEER values from at least 6 transwell inserts were averaged in these series of experiments, measured 7 days after plating.

Figure 6. Comparative THEndEC and THBMEC solute permeability

No significant difference in permeability to sodium fluorescein is observed between THEndEC and THBMEC grown to confluence at optimal seeding and extracellular matrix conditions (A). In contrast, a statistically significant higher permeability to dextran-70-FITC is observed with confluent THBMEC compared to THEndEC (B). N.S. indicates not significant, * indicates p-value < 0.05. Error bars indicate standard errors of the mean. Solute permeability values from at least 10 transwell inserts were averaged in these series of experiments.

Table 1

Oligonucleotide primers used for reverse transcription-polymerase chain reaction to detect THEndEC transporter expression with serial passaging.

cDNA	Forward Primer (5'-3')	Reverse Primer (5'-3')	Annealing Temp (°C)	Product length (bp)
AP	ACG TGG CTA AGA ATG TCA TC	CTG GTA GGC GAT GTC CTT A	52	475
CRT	GCC GGC AGC ATC AAT GTC	GGT GTT GCA GTA GAA GAC GAT CAC	56	86
γ -GT	AAT GGA CGA CTT CAG CTC TCC	AGC CGA ACC AGA GGT TGT AGA	56	218
GAPDH	GGA AGG TGA AGG TCG GAG TC	CAG TAG AGG CAG GGA TGA TG	56	605
GLUT-1	CAT GTC CTT CCA GTA TGT GG	GTC AGG TTT GGA AGT CTC AT	50	313
LAT-1	AGG AGC CTT CCT TTC TCC TG	CTG CAA ACC CTA AGG CAG AG	56	181
MCT-1	ACC AGC AGT TGG AGG TCC AGT TGG ATA	AAC TGA TTA ATT GTT TGG AAG ACT GAT	56	750
P-gp	GCC TGG CAG CTG GAA GAC AAA TAC ACA AAA TT	CAG ACA GCA GCT GAC AGT CCA AGA ACA GGA CT	56	283
vWF	CAA CAA AGG AAC GTC TCC	TAC ACA ACA GAG CCA TTG G	50	758