In Vitro Blood–Brain Barrier Modeling adapted for Peripheral Blood Mononuclear Cell Transmigration from HIV-Positive Patients for Clinical Research on Therapeutic Drug Intervention

Robert Oda, BS*; Bruce Shiramizu, MD†; Melissa Agsalda-Garcia, PhD†;
Joanna Kettlewell, BS†; Valerie Wojna, MD‡

Objective: HIV-associated cognitive impairment (HACI) continues to persist for HIV-seropositive individuals who are on antiretroviral therapy (ART). HACI develops in part when HIV-infected monocytes (MOs) transmigrate through the blood–brain barrier (BBB) and secrete pro-inflammatory cytokines and chemokines, which leads to neuronal damage. *In vitro* BBB models are important tools that can elucidate mechanisms of MO transmigration. Previously described *in vitro* BBB models relied on pathology specimens, resulting in potentially variable and inconsistent results. This project reports on a reliable and consistent alternative *in vitro* BBB model that has the potential to be used in clinical research intervention studies analyzing the effects of ART on the BBB and on MO transmigration.

Methods: A bilayer BBB model was established with commercially available astrocytes and endothelial cells on a 3µm PET membrane insert to allow the contact of astrocytic foot processes with endothelial cells. Inserts were cultured in growth medium for 7 days before exposure to HIV- or HIV+ peripheral blood mononuclear cells (PBMCs). PBMCs were allowed to transmigrate across the BBB for 24 hours.

Results: Confluency and integrity measurements by trans-endothelial electrical resistance (TEER) (136.7 ± 18.3Ω/cm^2) and permeability (5.64 ± 2.20%) verified the integrity of the *in vitro* BBB model. Transmigrated MOs and non-MOs were collected and counted (6.0x10^4 MOs; 1.1x10^5 non-MOs). Markers indicative of glial fibrillary acidic protein (GFAP), von Willebrand factor (vWF), and p-glycoprotein (Pgp) were revealed in immunofluorescence staining (IF), indicating BBB phenotype and functionality.

Conclusion: Potential applications for this model include assessing the HIV DNA copy numbers of transmigrated cells (pre- and post-targeted ART) and understanding the role of oxidative stress related to HIV DNA and HACI. [*P R Health Sci J 2018;37:155-159*]

Key words: HIV, Cognition, Blood–brain barrier, Neurocognition, Brain

The persistence of human immunodeficiency virus type-1 (HIV)–associated cognitive impairment (HACI) remains a major morbidity-related concern for patients on antiretroviral therapy (ART) (1). Previous data have suggested that residual chronic central nervous system (CNS) inflammation is a factor in HACI pathogenesis; however, some patients experience cognitive improvement while on ART, implying that CNS impairment might be reversible (2).

High HIV DNA copy numbers in monocytes (MOs) were found in patients with HACI compared to patients with normal cognition (3, 4). Increased HIV DNA levels in CD14+ cells from cerebral spinal fluid (CSF) correlated with greater HACI (5). To identify strategies to study MOs as targets for novel treatment paradigms, both mono- and bilayer *in vitro* BBB models have been described (6, 7). As a monolayer, brain microvascular endothelial cells are crucial to BBB models because they form tight junctions in vascular homeostasis (8). However, endothelial-only BBB models are limited since the BBB is multi-layered (9). The addition of astrocytes regulates tight junctions and helps facilitate cellular trafficking (10). Evidence of the increased integrity and function of brain microvascular endothelial cells through their interaction with astrocytes would contribute to the benefits of a bi-layer BBB model.

*Department of Molecular Biosciences & Bioengineering, College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa, Honolulu, Hawaii; †Department of Tropical Medicine, Medical Microbiology & Pharmacology, John A. Burns School of Medicine, University of Hawaii, Honolulu, Hawaii; ‡Department of Medicine, Neurology Division, University of Puerto Rico Medical Sciences Campus, San Juan, PR

The author/s has/have no conflicts of interest to disclose.

Address correspondence to: Bruce Shiramizu, MD, College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa, Honolulu, Hawaii. Email: bshirami@hawaii.edu

BBB models typically utilize fresh fetal brain tissue in the isolation of astrocytes (11, 12). Isolating astrocytes can be challenging: The cellular quality-control mechanisms must be preserved, and the tissue itself must be not only reliably available but also kept viable during the isolation process (13). Thus, an *in vitro* BBB model using astrocytes from more reliable sources would be valuable. While previous studies used a time-intensive procedure for isolating primary astrocyte/pericyte cells (14), buying commercially available cells would eliminate the need for such a process and would—it is hoped—prove to be a more reliable method of providing the needed material.

An *in vitro* bi-layer BBB model was adapted for use in our clinical research study to assess changes in MO transmigration. The integrity of the model was assessed by trans-endothelial electrical resistance (TEER) (to test tight junctions) and Evans blue dye conjugated to bovine serum albumin (EBA) (to test BBB permeability) and was further validated by immunofluorescence. The resulting data described a reproducible in vitro BBB model that could potentially be a useful tool in future clinical–translational research.

Materials and Methods

A previously described *in vitro* BBB model utilized primary fetal human astrocytes and primary human endothelial cells (11). This report describes an adaptation of that model that is intended to improve consistency and reproducibility by using commercially available cells.

Astrocytes and Endothelial cells

Primary adult human astrocytes and endothelial cells (Angio-Proteomie, Boston, MA) were established on plates that were initially coated with 0.2% porcine skin gelatin at room temperature. The cells were seeded in astrocyte (AGM) or endothelial (EGM) growth medium (Angio-Proteomie). The media were changed every 2 to 3 days until confluency was reached. The cells were used from 5 to 10 passages.

Blood–Brain Barrier model

Cell culture inserts (Falcon, BD Biosciences, Billerica, MA), each with 3μm pores and a surface area of 0.3cm^2, were first coated with a 0.2% gelatin solution on both sides. Astrocytes (1.2x10^5 cells) were placed on the underside of the trans-well insert and incubated at 37°C. The inserts were flipped and placed into a 24-well trans-well plate with AGM. Endothelial cells (1.2x10^5 cells) were then seeded on the inside of the insert. The media were changed every 2 to 3 days, until confluency was reached at 7 days, as assessed by TEER and EBA (Figure 1A).

Peripheral blood mononuclear cells

Whole blood was obtained from volunteers (comprising both HIV-seronegative and HIV-seropositive individuals), per the guidelines established by the University of Hawaii Institutional Review Board. PBMCs were isolated by Ficoll-Paque (GE Healthcare, Chicago, IL) gradient centrifugation.

In Vitro transmigration

The media were removed from the inserts, and the inserts were transferred to a new 24-well plate containing FBS/basal growth medium (BGM) with SDF1-α (R&D Systems, Minneapolis, MN). The inserts were layered with 1x106 PBMCs per insert for 24 hours.

Blood–Brain Barrier integrity assessment

At day 7, inserts were selected at random to assess the permeability of the BBB by EBA and BBB integrity by TEER (11, 15). The medium was aspirated from the upper chamber of each insert and transferred to a new well and washed with phenol red–free Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, St. Louis, MO).

The insert was transferred to a new well containing FBS/DMEM. To test permeability, EBA was added to the upper chamber and incubated at 37°C. Flow-through was analyzed at 620nm using a NanoDrop Spectrophotometer (Thermo Fisher, Waltham, MA). EBA permeability was evaluated before and 24 hours after PBMC introduction. TEER was analyzed using an epithelial voltmeter (World Precision Instruments, Sarasota, FL).

Immunofluorescence

On day 7, inserts were selected at random and moved to a new plate, with paraformaldehyde/PBS added to the astrocyte and endothelial sides of the insert and fixed. After fixation, the membrane of the inserts was cut out of the barrier and placed in optimal cutting temperature compound (Sakura Finetek, Torrance, CA) in a mounting block and cross-sectioned and mounted on slides (Leica Biosystems, Nussloch, Germany). The cross-sections were permeabilized using Triton-X100 (Sigma-Aldrich, St. Louis, MO) and then blocked (EDTA, fish gelatin, BSA, horse serum). Primary antibodies for glial fibrillary acidic protein (GFAP), von Willebrand factor (vWF), and p-glycoprotein (pGP) were added, followed by secondary antibodies, and visualized using a Zeiss Axioskop 2 Plus fluorescent microscope.

Results

TEER resistance and EBA permeability

TEER resistance increased steadily on consecutive days, with the maximum resistance measured on day 7. The resistance steadily decreased after day 7, with no BGM change (Figure 1B).

After switching to BGM, the resistance and permeability values were stable over time. The values of TEER resistance and of EBA permeability were both measured after the introduction of BGM (Figure 2A). Statistical analysis by ANOVA showed no significant differences in TEER or EBA, which maintained the values of approximately 100 Ω/cm^2 ($p = 0.065$) and lower than 15% ($p = 0.075$), respectively, over 5 days.

Initial tests of the BBB model showed the stabilization of permeability after the addition of BGM; a value of 7.73 (± 0.87%) was maintained. Control wells showed permeability

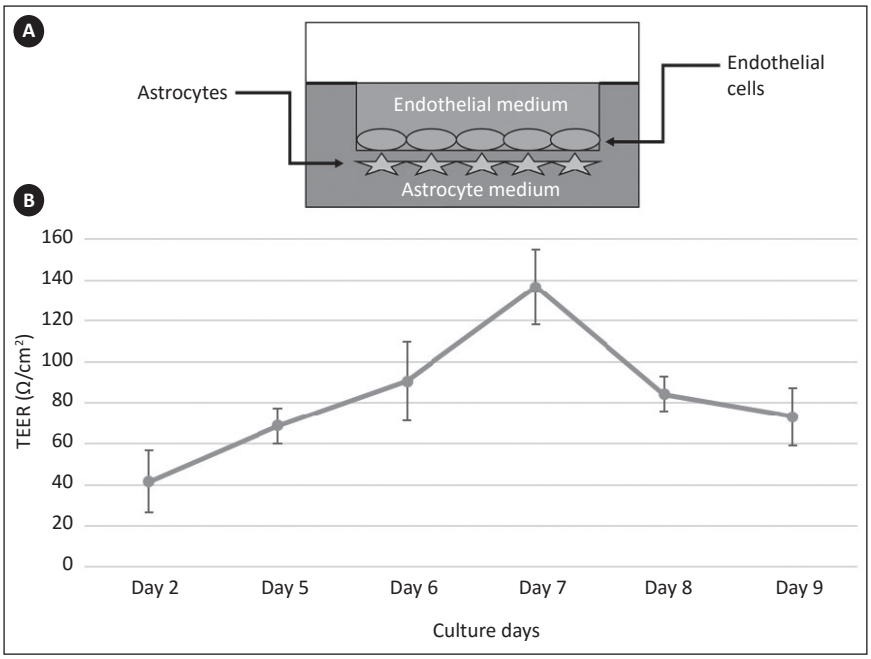
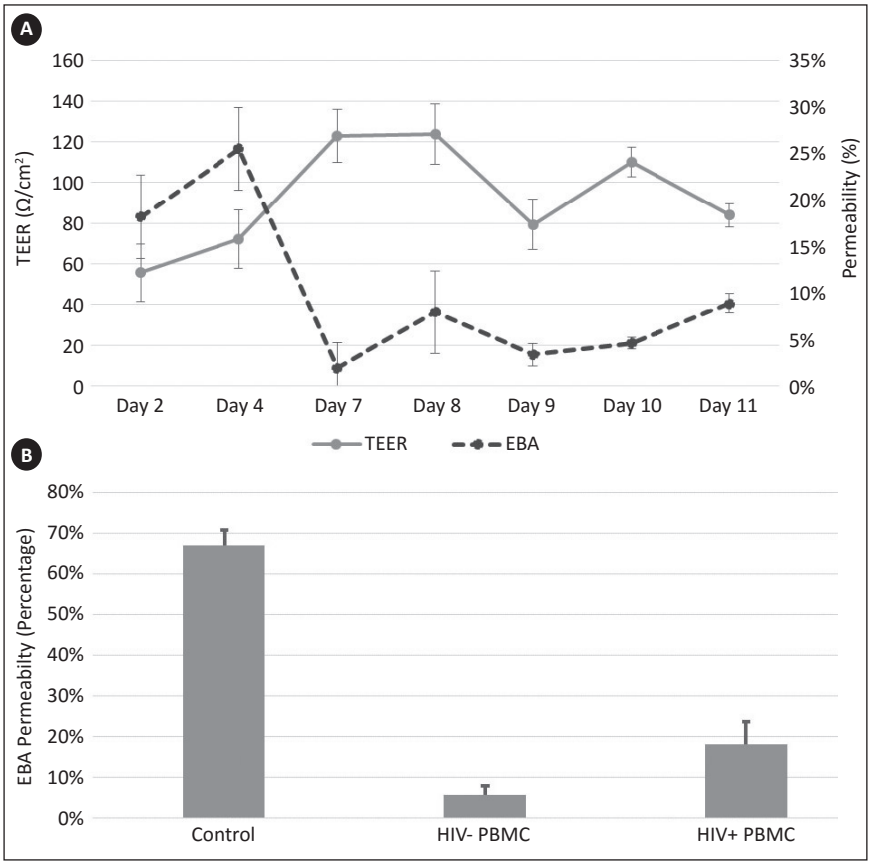


Figure 1. (A) Schematic representation of the blood–brain barrier model. Astrocytes are cultured on the underside of the trans-well insert and flipped into a well with AGM. Endothelial cells are then cultured on the inside of the barrier and fed with EGM. PBMCs are introduced from the endothelial side and allowed to transmigrate to the astrocyte layer after 24 hours. (B) Trans-endothelial electrical resistance (TEER) over time. TEER values (Ω/cm2) of the bilayer BBB model over the course of 9 days (n = 6). Data showed peak point at day 7, where inserts reached maximum confluency, when media were changed to BGM for transmigration experiments.



values of approximately 65%. HIV-negative PBMC transmigration resulted in no compromise of BBB integrity. The transmigration of HIV+ PBMC resulted in a significant increase in EBA permeability, as assessed by an independent 2-sample t-test ($p = 0.004$) (Figure 2B).

Immunofluorescence

Our model exhibited presence of functional proteins; vWF and GFAP are both shown on a cross section of our barrier (Figure 3). GFAP was imaged on both sides of the barrier. Staining for pGP revealed the presence of an efflux transporter on our endothelial cell cultures (Figure 3).

Transmigration

Cell-count data showed the transmigration of 10% of the initial cell population (1x106 cells) per insert. PBMCs were collected after transmigration and pooled from 20 wells and retrieved. Cells were collected and re-suspended in PBS and filtered (CellTrics, 30μm, Sysmex, Kobe, Japan). Cells were separated by EasySep™ Human Monocyte Enrichment Kit

Figure 2. (A) Trans-endothelial electronic resistance (TEER) pre- and post-media switch. All measurements were made after media had been changed for that time point. TEER values (n = 24) before and after basal growth media (BGM) switch (media change at day 7). TEER remained elevated and was validated with low EBA permeability. Permeability of these barriers (n = 24) initially was high (>15%); the permeability of the barriers decreased and maintained at optimal values after BGM switch (<15%). Analysis by ANOVA showed that there were no significant differences between TEER and EBA values between days 7 through 11 ($p = 0.065$ and $= 0.075$, respectively). (B) Evans blue albumin (EBA) permeability. EBA permeability values (n = 3) of a control insert (no cells) and the bilayer model after transmigration of HIV-negative and HIV-positive PBMCs. The bi-layer model demonstrated a high level of membrane integrity and low EBA permeability after HIV- transmigration and showed significant integrity loss after HIV+ PBMC transmigration ($p = 0.00405$) (B).

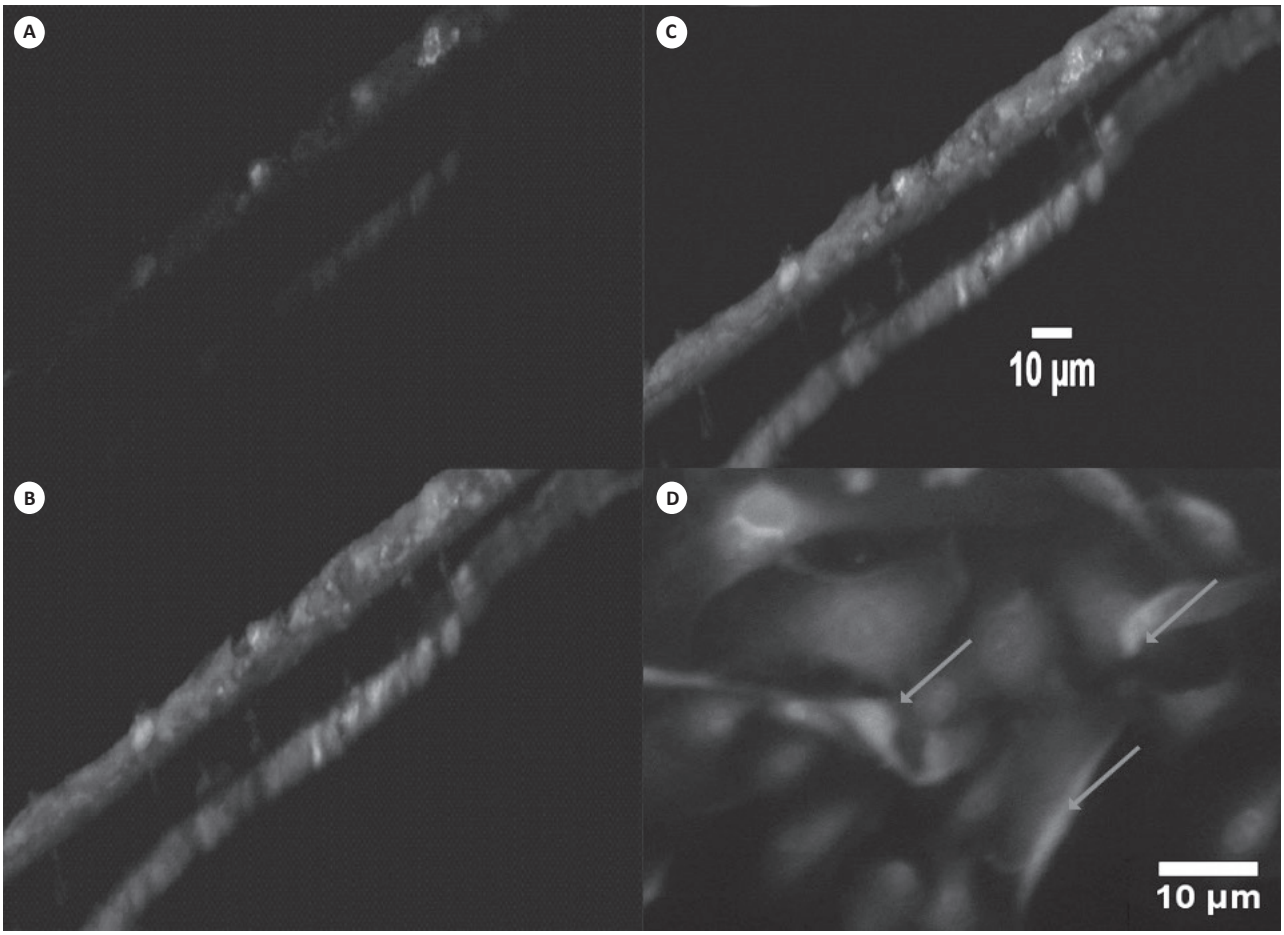


Figure 3. (A) Cross-sectional and top-view staining of the BBB model for quality assessment of the presence of functional proteins. (A) vWF staining is shown in green, and (B) GFAP staining is shown in red. (C) The merged view of the image showing the colocalization of the astrocytes and endothelial cells, with nuclei of cells in blue. (D) Top view of endothelial cells showing the nucleus in blue and the Pgp transporters in green. Arrows point to the transporters (D).

(STEMCELL Technologies, Vancouver, Canada) on a RoboSep (STEMCELL) and counted. After cell separation, 6.0x104 MOs were found to transmigrate (per insert) in the BBB model.

Discussion

The BBB model that was adapted was reproducible and demonstrated integrity and reliability. TEER was used to measure the functionality of the tight junctions; the model was consistent with such models as used by other studies (16). TEER values decreased after reaching maximum confluence, possibly due to cell overgrowth, as previously reported (17). Measures of EBA permeability were consistent with those of other studies, in that EBA is typically impermeable to an intact BBB (11, 18). The combination of low EBA permeability and high TEER values indicated that the model had a barrier with integrity and junctions.

The model also showed human BBB characteristics. vWF and GFAP are markers of endothelial cells and astrocytes, respectively. GFAP presence on both sides of the barrier suggested that astrocytes can come into physical contact with the endothelial cells, which is desirable, as astrocytic foot processes are regulators of endothelial cells. pGP is a "gatekeeper" in the BBB and has a major influence on drug trafficking, as it is broadly complementary to many substrates and acts as an efflux transporter to move these substances out of the brain and into the blood stream.

The transmigration of MOs through the BBB was also of interest, as we wanted to adapt the model for clinical research studies. CD14+ cells isolated from the transmigration of PBMCs could be further analyzed for HIV DNA content. Previously, CD14+ cells were shown to directly correlate with increased brain injury and CSF immune activation (19). Circulating HIV-infected MOs were hypothesized to contribute to HACI by increasing pro-inflammatory cytokine levels and oxidative stress (14). A review on HACI and oxidative stress by Valcour & Shiramizu proposed mitochondrial DNA (mtDNA) as an additional factor leading to impaired cognitive performance (20). An exploration of mtDNA in CD14+ cells after transmigration and their role in oxidative stress could provide avenues for more therapeutic options for HACI.

This adapted in vitro BBB model was reliable and consistent in terms of PBMC transmigration and should be suitable for use in

future clinical research intervention trials. The stability of this BBB model could provide a window of opportunity in a clinical trial setting for research visits relying on clinical specimens in clinical–translational studies. To further understand the pathogenesis of HACI, the BBB model could be a tool for studying oxidative stress mechanisms and the impact of HIV-infected transmigrated cells in the setting of intervention clinical trials.

Resumen

Objetivo: Deterioro cognitivo asociado al VIH (HACI, por sus siglas en inglés) continua prevalente en individuos seropositivos en tratamiento antirretroviral (TAR). HACI se desarrolla en parte por el daño neuronal que ocurre como consecuencia de la transmigración de monocitos (MOs) infectados por la barrera hematoencefálica (BHE) y la secreción de citoquinas inflamatorias y quimioquinas. Modelos in-vitro de BHE son herramientas importantes para elucidar los mecanismos de la transmigración de MOs. En modelos in-vitro previamente descritos de BHE usan tejidos patológicos con resultados potencialmente variables e inconsistentes. Este estudio reporta un modelo in-vitro de BHE alterno de confianza y consistente que tiene el potencial de uso en estudios de ensayos clínicos analizando los efectos de TAR en la BHE y la transmigración de MOs. Métodos: La BHE de dos capas se estableció usando un encarte con células astrocíticas y endoteliales. Los encartes se cultivaron en medio de crecimiento por siete días antes de ser expuestos a células mononucleares de sangre periférica (CMSP), se permitió la transmigración de las CMSP a través de la BHE por 24 horas. Resultados: La integridad del modelo in-vitro de BHE se verificó determinando las medidas de confluencia e integridad usando la resistencia eléctrica trans-endotelial (RETE) (136.7 ± 18.3Ω/cm^2) y permeabilidad (5.64 ± 2.20%). MOs se recogieron, incluyendo los que transmigraron y los que no, y se confirmó el número esperado para cada población (6.0x104 MOs; 1.1x105 non-MOs). El fenotipo y la funcionalidad de BHE fue verificado determinando los marcadores de la proteína acídica fibrilar glial (PAFG, Factor de von Willebrand (WvF y la p-glicoproteína (Pgp) usando tinciones de inmunofluorescencia (IF). Conclusión: Posibles usos de este modelo incluyen determinar la cantidad de copias de ARN del VIH (HIV DNA, por sus siglas en inglés) en las células que transmigran (antes y posterior al TAR) y entender el rol de daño oxidativo relacionado a ARN VIH y HACI.

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