Development, Characterization and Potential Applications of a Multicellular Spheroidal Human Blood–Brain Barrier Model Integrating Three Conditionally Immortalized Cell Lines

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Received March 11, 2021; accepted April 21, 2021; advance publication released online April 24, 2021

In vitro blood–brain barrier (BBB) models are essential research tools for use in developing brain-targeted drugs and understanding the physiological and pathophysiological functions of the BBB. To develop BBB models with better functionalities, three-dimensional (3D) culture methods have gained significant attention as a promising approach. In this study, we report on the development of a human conditionally immortalized cell-based multicellular spheroidal BBB (hiMCS-BBB) model. After being seeded into non-attachment culture wells, HASTR/ci35 (astrocytes) and HBPC/ci37 cells (brain pericytes) self-assemble to form a spheroid core that is then covered with an outer monolayer of HBMEC/ci18 cells (brain microvascular endothelial cells). The results of immunocytochemistry showed the protein expression of several cellular junction and BBB-enriched transporter genes in HBMEC/ci18 cells of the spheroid model. The permeability assays showed that the hiMCS-BBB model exhibited barrier functions against the penetration of dextran (5 and 70 kDa) and rhodamine123 (a P-glycoprotein substrate) into the core. On the other hand, facilitation of 2-([N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino]-2-deoxyglucose (2-NBDG; a fluorescent glucose analog) uptake was observed in the hiMCS-BBB model. Furthermore, tumor necrosis factor-alpha treatment elicited an inflammatory response in HBMEC/ci18 cells, thereby suggesting that BBB inflammation can be recapitulated in the hiMCS-BBB model. To summarize, we have developed an hiMCS-BBB model that possesses fundamental BBB properties, which can be expected to provide a useful and highly accessible experimental platform for accelerating various BBB studies.

Key words blood–brain barrier; in vitro model; immortalized cell; spheroid; drug development; central nervous system disease

INTRODUCTION

The central nervous system (CNS) is physiologically isolated from the rest of the body by the blood–brain barrier (BBB). The BBB is characterized by tight intercellular junctions (tight and adherens junctions) and efflux transporters (e.g., P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP)) that strictly regulate the movement of endogenous as well as xenobiotic compounds, including drugs, from the blood into the brain. Therefore, while the BBB intrinsically serves as a protective barrier against blood-derived substances that could otherwise be involved in CNS diseases, the same property has been a major obstacle to creating effective drugs for CNS diseases.

The BBB also restricts the migration of immune cells into the CNS, thereby enforcing a low level of immune surveillance. However, in cases involving certain diseases, such as multiple sclerosis and stroke, immune cell invasion into the CNS has been observed in association with BBB impairment, which can be related to the disease progression.

Collectively, the BBB has been a critical research focus for identifying therapeutic approaches and innovative drugs that can fight against various CNS diseases. These are areas where in vitro BBB models can be powerful experimental tools.

Since the BBB is formed in vivo by brain microvascular endothelial cells (BMEC) that are ensheathed by pericytes and astrocytes, these three cell types are often integrated into in vitro BBB models. Conventional, in vitro BBB model setups utilize Transwell culture systems. They provide many experimental advantages, including easy preparation, and have enabled researchers to readily monitor BBB integrity and drug permeability.

However, cells in a Transwell culture system survive in a two-dimensional (2D) manner, and the three cell types cannot communicate directly with each other. This lack of in vivo microenvironments significantly limits their functions to prematurity, eventually posing increased false-negative/positive risks in certain experiments.

Meanwhile, three-dimensional (3D) culture methods have gained significant attention as a promising approach to developing BBB models with better functionalities. These new systems, which include BBB-on-a-chip, organoid, and spheroid models, are designed to mimic in vivo BBB conditions more closely and are thus expected to achieve higher BBB func-
tional levels than ever.\textsuperscript{20–22} However, the systems are still in the formative stages, and there are several points where they need to be improved or must await alternative developmental approaches. For example, the use of primary cells is always hampered by their limited availability, and several BBB-on-a-chips are difficult to setup. Therefore, in order to ensure 3D BBB models are both practical and widely accessible, several improvements are necessary to increase their availability and applicability.

Accordingly, as part of our ongoing efforts to address the growing interest of 3D BBB models, we herein report on the development of a human immortalized cell-based multicellular spheroidal BBB model (hiMCS-BBB). Our immortalized cells have shown to be used in the development of Transwell-type BBB models capable of discriminating a variety of high and low BBB permeable drugs.\textsuperscript{23–25} In addition, spheroid models are often easier to set up compared to transwell- or chip-type models. The spheroidal culture method employed here further enhances their properties and has allowed the creation of a hiMCS-BBB model that can be a useful research tool in future BBB studies.

**MATERIALS AND METHODS**

**Cells and Culture Methods** Human BMEC/conditionally immortalized clone 18 (HBMEC/ci18), human astrocytes/conditionally immortalized clone 35 (HASTR/ci35), and human brain pericytes/conditionally immortalized clone 37 (HBPC/ci37) were developed in our previous studies.\textsuperscript{23–25} These immortalized cells carry temperature-sensitive simian virus large T antigen (tsSV40T) and human telomerase catalytic subunit (hTERT) as immortalization genes. These immortalized cells were processed using Cell Visualizer software (SCREEN) to eliminate noise derived from the medium surrounding spheroids.

**Development of BBB Spheroid Models** The hiMCS-BBB models were developed by sequentially combining the three human immortalized BBB cell lines in the wells of PrimeSurface 96V plates (Sumitomo Bakelite, Tokyo, Japan). The number of each cell type for model development was designated to 1.0 × 10^3 (namely, the ratio is 1 : 1 : 1) based on the previous report.\textsuperscript{23} Further preparations are similar but have some differences from those reported previously,\textsuperscript{26–28} as described below (see also Fig. S1).

On the first day of the process, HASTR/ci35 and HBPC/ci37 cells were concomitantly seeded into each well of the PrimeSurface 96V plates along with the Spheroid medium (100 µL, which consists of Endothelial Cell Basal Medium-2 (Lonza, Basel, Switzerland) containing SingleQuots Endothelial Cell Growth Medium-2 (Lonza), penicillin-streptomycin, and 0.48 mg/mL methylcellulose-400 (Wako)). Methylcellulose was added to the medium to increase its viscosity to prevent cell adhesion, as described in a previous report.\textsuperscript{26} The cells were then cultured for 48 h, allowing them to form a spheroidal core structure.

On the third day, HBMEC/ci18 cells suspended in the Spheroid medium (50 µL) were added to the wells containing the spheroid core, after which the cells were cultured for another 48 h. The culture temperature for the hiMCS-BBB model development was usually done at 37°C, but 33°C temperature was also used only in an initial trial.

A Transwell-type BBB model was also developed for comparison purposes using the same process and setup procedure described in our previous report.\textsuperscript{23}

**3D Imaging if the hiMCS-BBB Model** Label-free 3D images of the hiMCS-BBB model were captured by optical coherence tomography (OCT) using a Cell\textsuperscript{\textregistered} iMager Estier (SCREEN, Kyoto, Japan). The original OCT images were processed using Cell Visualizer software (SCREEN) to eliminate noise derived from the medium surrounding spheroids.

**Cell Localization Analysis** The cells were initially pre-labeled with CellTracker Green or Orange CMFDA Dye (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and with red fluorescent cytoplasm membrane staining kits (Promocell, Heidelberg, Germany). The hiMCS-BBB models (approximately 10–20 spheroids) with the pre-labeled cells were collected and fixed by 4% paraformaldehyde (PFA) for 15 min at room temperature (r.t.), after which they were mounted on glass slides with Fluoro-KEEPER Antifade Reagent (Nacalai Tesque, Kyoto, Japan).

Fluorescence was then detected using the FLUOVIEW FV1000 (Olympus, Tokyo, Japan) confocal microscope, and confocal z-stack images were captured in 2.0 μm intervals up to through to a 50 μm depth.

**Immunocytochemistry (ICC)** The hiMCS-BBB models (approximately 10-20 spheroids) were cultured in a 1.5 mL protein lobind tube and washed with phosphate-buffered saline (PBS)(–). The sample preparation methods used for a Transwell-type BBB model are described in our previous report.\textsuperscript{23}

ICC was then performed as described previously.\textsuperscript{23–25,29} The primary and secondary antibodies used are listed in Table S1. All the antibodies were diluted to the indicated concentrations with CanGetSignal Immunostain Solution A (TOYOBO, Osaka, Japan). For nuclear counter-staining, 4′,6-diamidino-2-phenylindole (DAPI) was used. Fluorescence was detected using a FLUOVIEW FV1000 confocal microscope (Olympus).

**Dextran Permeability Assay** In addition to the BBB spheroids, those without HBMEC/ci18 cells (ABMEC-hiMCS) were prepared for comparison purposes. Up to 90% of the culture medium was removed, and the Spheroid medium containing fluorescein isothiocyanate (FITC)-labeled dextran (5 or 70 kDa, 75 μg/mL, Sigma, St. Louis, MO, U.S.A.) was added to the spheroids. The incubation was performed for 12 h at 37°C.

The hiMCS-BBB models (approximately 10–20 spheroids) were collected and put into a 1.5 mL tube. The medium was then gently aspirated, after which the BBB spheroids were washed with PBS(–) twice. The hiMCS-BBB models were then fixed with 4% PFA, and then mounted on slides. The intraspheroidal fluorescence was detected using a FLUOVIEW FV1000 confocal microscope (at 35–40 μm depth, Olympus), and the signal intensity was quantified using FV10-ASW software (Olympus).

**Functional Assays for BBB Transporters** The functions of glucose transporter type 1 (GLUT1) and P-gp in the BBB spheroids were determined by permeability assays using 2-(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino)-2-deoxyglucose (2-NBDG, Wako) and rhodamine123 (R123,
Wako), respectively. The medium replacement processes for those containing 2-NBDG (100 µM) or R123 (1.0 µM) were performed as described above, and the hiMCS-BBB and the ΔBMEC-hiMCS models were then incubated for 90 min. The R123 permeability assay was also performed in the presence of elacridar (10 nM, Wako). The signal intensity of the intraspheroidal fluorescence was quantified as described above.

**Total RNA Extraction, cDNA Synthesis and Quantitative (q)PCR**

Using the hiMCS-BBB model, total RNA extraction and cDNA synthesis were prepared using the methods described previously.23–25,29) qPCR was conducted using the cDNA and the primers listed in Table S2, and the amplification efficiency of each qPCR result was confirmed to be close to one. Data were obtained using the delta-delta-CT method, where the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA level was used as a normalization control.

**Tumor Necrosis Factor-α (TNF-α) Treatment Assay**

Two days after HBMEC/ci18 cell seeding, a switch was made to a serum-free spheroid medium containing TNF-α (20 ng/mL, Peprotech, Rocky HILL, NJ, U.S.A.). After culturing for 24 h, the hiMCS-BBB spheroids were collected and then subjected to RNA extraction.

TNF-α treatment assays were also performed in the presence or absence of THP-1 cells. Briefly, the THP-1 cells (1.0 × 10^5) were pre-labeled with CellTracker Orange and then suspended in the Spheroid medium (with or without TNF-α). The cell suspension was added to the BBB spheroid one day after the HBMEC/ci18 cell seeding process, followed by co-culturing for 48 h. The spheroids were then collected, washed with PBS(−) and fixed with 4% PFA, after which their fluorescence level was detected using the FLUOVIEW FV1000 confocal microscope during which confocal z-stack images were captured in 2.0 µm intervals up to a depth of 50 µm.

**Statistical Analysis**

Statistical analyses were performed using a statistical software package (Statcel, OMS, Saitama, Japan) to determine whether the differences between the multiple values were significant. The values obtained from qPCR and permeability analyses were first analyzed by one-way ANOVA and permeability analyses were first analyzed by the Student’s t-test.

**RESULTS**

**Development of a Multicellular Spheroid Using the Three Types of Immortalized BBB Cells**

First, a hiMCS-BBB model was developed in 96-well V-bottom plates under 33 °C conditions, as shown in Fig. 1. Upon seeding, the HBPC/ci37 and HASTR/ci35 cells were found to have self-assembled into a core spheroid within 24 h. The HBMEC/ci18 cells were then added to the core to form a multicellular spheroid (Fig. 1A). However, under the 33 °C condition, its size was somewhat large (Fig. S2A) and the HBMEC/ci18 cells did not uniformly cover the spheroid (Figs. 1B, C). These results indicated a need for methodological manipulation that would improve spheroid formation.

**Effects of Immortalization Signal Elimination on De-
To solve the issue mentioned above, we took advantage of the conditionally immortalized properties of the BBB cells. Previously, we showed that the conditionally immortalized BBB cells undergo growth arrest and simultaneous differentiation upon elimination of their immortalization signal, which was achieved by changing the culture temperature from 33 to 37 °C (which causes tsSV40T protein degeneration). Therefore, we tested the effects of 37 °C culture temperature on spheroid formation. The results of 3D imaging analysis and hematoxylin and eosin staining showed that 37 °C culture temperature suppressed spheroid growth (Figs. 1B, S2A), and that the spheroidal core did not undergo necrosis during culturing (Fig. S2B). We also observed that the spheroid structure remained intact at least for three days in this condition (data not shown).

We then analyzed the intraspheroidal localization of each cell type in the model obtained at 37 °C using fluorescent dyes. The results showed that HASTR/ci35 cells (red) accumulated in the central part of the spheroid and that most HBPC/ci37 cells (blue) were located on the outer side of the core, along which a continuous surface monolayer of HBMEC/ci18 cells (green) had formed (Fig. 1D).

Taken together, our results showed that 37 °C culture conditions played a crucial role in the formation of an integrated multicellular BBB spheroid.

Paracellular Barrier Properties in the hiMCS-BBB Model

Next, we analyzed expression and function of the intercellular junctions in the hiMCS-BBB model. A Transwell BBB model was also prepared for comparison purposes (Fig. 2A). The results of ICC for the BBB-enriched barrier genes showed that HBMEC/ci18 cells in the hiMCS-BBB model clearly expressed tight junction proteins (claudin-5 (CLDN-5) and zonula occludens 1 (ZO-1)), adherens junction proteins (vascular endothelial (VE)-cadherin and β-catenin). The expression levels of these proteins were significantly higher than those found in the Transwell model.

To examine the barrier functions, permeability assays were performed using 70 and 5 kDa FITC-dextran (Fig. 2B). The hiMCS-BBB without HBMEC/ci18 model (ΔBMEC-hiMCS) was also used for comparison purposes. The results showed that the hiMCS-BBB model exhibited significantly lower permeabilities than the ΔBMEC-hiMCS model (0.58 ± 0.05-fold [p < 0.001] and 0.70 ± 0.05-fold [p < 0.001] for 70 and 5 kDa FITC-dextran, respectively). The clear restriction of dextran penetration into the hiMCS-BBB model indicates its physical barrier property achieved by the intercellular junction formation.

BBB-Enriched Transporter Expression and Functions in the hiMCS-BBB Model

We then moved on to examination of gene expression and functions of the BBB enriched influx/efflux transporters. The results of ICC showed that the expression levels of GLUT1, P-gp and BCRP were significantly higher in the hiMCS-BBB models than those in the Transwell model (Fig. 3A). Furthermore, in addition to the non-polarized...
localization of GLUT1, the polarized localization of the efflux transporters on the apical membrane of HBMEC/ci18 cells was observed. This proper transporter distribution is expected to contribute to their functional achievements.

Consistently, the results of the functional assays using 2-NBDG (a GLUT1 substrate) showed that the hiMCS-BBB model exhibited higher 2-NBDG permeabilities compared with those of the ΔBMEC-hiMCS model (1.42 ± 0.20, \( p < 0.01 \)) (Fig. 3B), thereby indicating that 2-NBDG uptake is facilitated in the hiMCS-BBB model. On the other hand, the hiMCS-BBB model showed significantly lower R123 (a P-gp substrate) permeabilities than the ΔBMEC-hiMCS model (0.60 ± 0.02, \( p < 0.001 \)) (Fig. 3C). Furthermore, treatment with elacridar (a P-gp inhibitor) resulted in an increase of R123 influx into the hiMCS-BBB model (1.41 ± 0.07, \( p < 0.001 \)) (Fig. 3C), highlighting the P-gp-mediated restriction of R123 entrance into the spheroid core. Therefore, these results suggest that the hiMCS-BBB model possesses functional expression of GLUT1 and P-gp.

Response to TNF-α Treatment in the hiMCS-BBB Model Since the BBB participates in the formation of brain inflammation (e.g., inflammatory cytokine secretion and lymphocyte recruitments), it was intriguing to determine how the hiMCS-BBB model responds to an inflammatory stimulus. To accomplish this, the hiMCS-BBB models with or without TNF-α treatment were collected, after which their mRNA expression profiles of typical endothelial inflammatory mediators (C–X–C motif ligand 10 (CXCL10), CXCL8, interleukin-6 (IL-6), and CC chemokine ligand 2 (CCL2)) and endothelial adhesion molecules (E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1)) were compared (Fig. 4A). The qPCR results showed that all of the mRNA levels tested were significantly higher in the TNF-α-treated hiMCS-BBB models than in the non-treated ones. The TNF-α-induced E-selectin and ICAM-1 protein expression were confirmed by ICC (Fig. 4B).

To ask whether the inflammatory responses help lymphocytes recruitment to activated HBMEC/ci18 cells in the hiMCS-BBB model, the TNF-α treatment assays were performed in the presence or absence of THP-1 cells (a cell line that is often used as a monocyte model in inflammation studies). The results showed that TNF-α stimuli prompted THP-1 cells to adhere to the surface of the hiMCS-BBB model (Fig. 4C).

Therefore, our results indicate that the hiMCS-BBB model responds well to the TNF-α challenge, and thus can be used to create a BBB inflammation model.

DISCUSSION

In this study, we newly developed a conditionally immortalized cell-based MCS-BBB model, which we have named the hiMCS-BBB model. Since the elimination of the immortalization signal is a prerequisite for appropriate spheroid formation, the use of “conditionally immortalized” (not just “immortalized”) cells are essential to successful model development. To demonstrate the potential applicability of our model to BBB studies, we will first explain its basic BBB characteristics.

From a structural viewpoint, the model exhibits a three-layered structure of BMECs, pericytes, and astrocytes (in order from the exterior to the interior), in which the HBMEC/ci18 cells appear to uniformly cover the spheroid core surface. The severe restriction of paracellular marker (dextran) penetration into the spheroid clearly indicates that HBMEC/ci18 cells form...
the intercellular junctions necessary to create a monolayer without crevices. While in the reverse order of the structure found in vivo, the layered structure can provide a suitable platform for cell-to-cell communication. Moreover, spheroid culture allows the cells to exist in a 3D manner, which is also similar to what they are in vivo. It can thus be assumed that these in vivo-mimetic cultural conditions bring out BBB characteristics in HBMEC/ci18 cells. Consistently, we found that the protein expression levels of BBB-enriched genes, including those related to cellular junctions, are significantly higher than those found in a Transwell model. Therefore, we can conclude that appropriate cell disposition along with 3D culture are a critical necessity if HBMEC/ci18 cells are to exhibit BBB structural properties in the hiMCS-BBB model.

It is also important to note that the hiMCS-BBB models possess functional influx/efflux transporters. The proper transporter distribution is likely to contribute to their functional achievements. More specifically, polarized localization of P-gp (and BCRP) on the apical membrane of HBMEC/ci18 cells makes it easy for them to pump out the substrate used (R123 in this study), and the non-polarized localization of GLUT1 facilitates transcellular transport of its substrate (2-NBDG). Therefore, it is considered likely that at a minimum, the hiMCS-BBB models functionally express BBB-enriched transporters through which the traffic of compounds between the interior and exterior of the spheroid are facilitated or restricted, as can be seen in in vivo BBB.

Collectively, based on their physical barrier properties along with their transporting systems, it is considered likely that hiMCS-BBB models possess the fundamental characteristics required for BBB models. Although we cannot state so categorically, it can be speculated that direct cell-to-cell interaction in 3D structure plays a critical role for HBMEC/ci18 cells in exhibiting the above-described BBB properties in the hiMCS-BBB model.

In view of pathophysiological aspects, it has long been known that pro-inflammatory cytokines play an important role in the development and progression of neuroinflammation, as exemplified by immune cell recruitment when augmenting inflammatory responses. Accordingly, we show that the hiMCS-BBB model can respond to TNF-α, thereby inducing heightened expression levels of cytokines/chemokines along with leukocyte adhesion molecules, in HBMEC/ci18 cells. This is presumably the guide for THP-1 cells to be recruited and migrated into the spheroid. This indicates that our hiMCS-BBB models can, at least in part, recapitulate an immune reaction at the BBB, and therefore have the potential to be valuable tools for investigating the molecular mechanisms behind BBB inflammation and thus provide an enhanced method for exploring new druggable targets.

In addition to their functional features, we would like to underscore unique advantages of our newly developed model that contribute to their profound application potentials in various BBB studies. The hiMCS-BBB models have a substantial

![Fig. 4. An Inflammatory Response of the hiMCS-BBB Model to TNF-α Treatment](image-url)
level of practicability because the excellent proliferation abilities of the immortalized cells confer a high level of availability on the model (to the best of our knowledge, ours is the first spheroidal BBB model composed entirely of human conditionally immortalized cells). In addition, the model development method is simple, does not require complicated handling and specific culture devices, which reduces the time-consuming labor and economic burden imposed on researchers. These strengths will allow the performance of large-scale experiments and/or trial-and-error experimental processes in future BBB studies.

Nevertheless, it should also be borne in mind that further studies aimed at enhancing the value of hiMCS-BBB-based models will be necessary. One is that the current protocol needs further refinement aimed at improving its functions and endurance. Another is that the model’s BBB functions should be further characterized using a variety of BBB-permeable or non-permeable small and large molecules, where a non-destructive method for assessing drug permeability may also be worthwhile to create to obtain its kinetic parameters.

Finally, it should also be briefly mentioned that we do not claim that the hiMCS-BBB model is, or will always be, superior to the Transwell-BBB model. Rather, we note that since each model has unique advantages (such as the fact that the Transwell model is currently more suitable for determining the BBB permeability coefficients of drugs (i.e., $P_e$ or $P_{app}$ values), both model types can be regarded as providing important experimental options that can accelerate future BBB studies.

To summarize, in this paper, we introduce a new human immortalized cell-based BBB model, hiMCS-BBB. The model delivers fundamental BBB characteristics while also providing excellent scalability and handling simplicity. We expect that the hiMCS-BBB models have the potential to be a unique platform for various human BBB studies, including screenings for BBB-penetrating drugs/carriers or anti-CNS inflammatory drugs, as well as elucidation of molecular mechanisms behind the physiology and pathophysiology of the human BBB.

Acknowledgments This work was supported by Grants from JSPS KAKENHI (19K07214), AMED under Grant No. JP17be0304322h0001, the Mochida Memorial Foundation for Medical and Pharmaceutical Research (Tokyo, Japan), Eisai (Tokyo, Japan), and Ono Pharmaceuticals (Osaka, Japan). Otherwise, we have no financial relationship to disclose for this manuscript.

Conflict of Interest Conflict of interest statements related to research funds are provided in the acknowledgement section, and the model development method herein has been applied for a patent (No. 2020-007041). There is another related patent application (No. 2020-065670). The authors declare that they do not have any other conflicts of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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