Video Article

Improved Method for the Preparation of a Human Cell-based, Contact Model of the Blood-Brain Barrier

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Abstract

The blood-brain barrier (BBB) comprises impermeable but adaptable brain capillaries which tightly control the brain environment. Failure of the BBB has been implied in the etiology of many brain pathologies, creating a need for development of human *in vitro* BBB models to assist in clinically-relevant research. Among the numerous BBB models thus far described, a static (without flow), contact BBB model, where astrocytes and brain endothelial cells (BECs) are cocultured on the opposite sides of a porous membrane, emerged as a simplified yet authentic system to simulate the BBB with high throughput screening capacity. Nevertheless the generation of such model presents few technical challenges. Here, we describe a protocol for preparation of a contact human BBB model utilizing a novel combination of primary human BECs and immortalized human astrocytes. Specifically, we detail an innovative method for cell-seeding on inverted inserts as well as specify insert staining techniques and exemplify how we use our model for BBB-related research.

Video Link

The video component of this article can be found at http://www.jove.com/video/50934/

Introduction

The BBB is a specialized interface between the peripheral blood circulation and the central nervous system, crucially responsible for the maintenance of brain hemostasis. It comprises distinct brain microvascular endothelial cells (BECs) which are functionally influenced by few cellular and acellular components (below) to form a tight and dynamic gateway into the brain. Under physiological conditions the BBB restricts the passage of blood cells, plasma components and harmful substances, all potentially neurotoxic, into the brain. In parallel, the BBB selectively exchanges key ions and nutrients (glucose and amino-acids) and metabolic waste products between the brain and the circulation to precisely maintain the brain environment[1,2]. In recent years it is becoming evident that failure of the BBB occurs in a variety of chronic brain pathologies, such as neurodegenerative or inflammatory-related diseases (*e.g.* Alzheimer's disease and multiple sclerosis, respectively)[3], as well as in acute conditions like ischemic stroke[4].

The unique BBB properties of brain endothelial cells (BECs) are largely induced by their cerebral environment[5], and in particular by astrocytes[6,7]. There is a growing understanding that other cell-types, such as pericytes[8], neurons and microglia[1,3], as well as the basement membrane[9], support BECs and form together a functional unit termed the "neurovascular unit" (NVU) which simultaneously couples neuronal metabolic demands to their supplying capillaries[10].

The involvement of the BBB in pathological situations underlies numerous attempts to develop *in vitro* BBB models to assist in BBB-related research[11,12]. These models aim to mimic as close as possible *in vivo* BBB characteristics according to the NVU principle. *In vitro* BBB models generally rely on a monolayer of tight-junction-forming BECs (mainly from bovine[13], human[14], rat[15], mouse[16], and porcine[17,18] origins), cultured on a porous membrane together with supporting astrocytes (extensively reviewed by Deli *et al.* 2005[11]).

Astrocytes can be grown in non-contact conditions on the bottom of a tissue culture well, separated from BECs (cultivated on the upper surface of the membrane) by the culture medium yet communicating with BECs via soluble factors[16]. In more advanced models which better resemble the anatomical structure of the BBB *in vivo*, astrocytes are maintained in contact conditions and cultured directly on the opposite side of the membrane in close proximity to BECs[13,15,17] (**Figure 1**). This configuration enables physical contact between BECs and astrocytes, established when astrocytes project their processes through the porous membrane. Importantly, for a true contact to occur the pores should be ≥1µm in diameter, since astrocytic end-feet cannot pass through smaller pore sizes (*i.e.* 0.4 µm)[14,15]. Notably, contact BBB systems are demonstrated in some studies to be superior to their non-contact counterparts regarding their trans-endothelial electrical resistance (TEER) and endothelial permeability values of various tracers[13,17,18]. An additional dimension of media flow was recently added in a number of *in vitro* BBB models to apply shear forces to the endothelium for closer simulation of the brain vasculature[12,19].

One technical obstacle to overcome when generating a contact BBB model is the seeding of astrocytes against gravity on the abluminal surface of the porous membrane. Previous protocols[13,20], where astrocytes were simply seeded in a drop of media on top of an inverted insert, allowed only short seeding times (*i.e.* 10 min[13] or 2 hr[20]) which were found in our hands to be insufficient for proper cell attachment. Using this

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basic method, a longer astrocyte attachment period requires constant monitoring of the inserts by frequent opening of the incubator (causing fluctuations in temperature, pH and humidity) and is also prone to uneven cell seeding due to leakage of media through the pores, especially if pores larger than 1 µm are employed.

Here, we describe a general protocol for preparation of a contact BBB model. Our procedure includes an alternative method for cell-seeding on inverted inserts, which addresses the above mentioned limitations. The method permits undisturbed adherence of astrocytes onto the abluminal membrane surface, in an equilibrated incubator, for an extended period of time. As a result, a uniform seeding of astrocytes is achieved which increases barrier quality and minimizes basal permeability variations between inserts.

As the use of human cells is important for human-relevant research[21], we additionally demonstrate in this article the specific utilization of a novel combination of primary human BECs and immortalized human astrocytes for establishment of a contact human BBB model with a high throughput screening capacity. Since viewing of cells on porous membranes can be difficult, we also detail staining techniques which can assist in determination of confluence and cell morphology on the porous membranes. Finally, we exemplify how our human BBB model can be utilized to examine the effect of tissue-type plasminogen activator (t-PA) - a clot busting enzyme which serves as a sole treatment option for acute ischemic stroke - on the BBB.

Protocol

1. Cell Culture (3-7 Days Prior to BBB Assembly)

1.1. Primary Human Brain Microvascular Endothelial Cells (BECs)

BECs were commercially obtained. The cells were produced by dispase dissociation of normal human brain cortex tissue and provided frozen at passage 3 (<12 population doublings).

1. *Substratum*: Coat tissue-culture vessels with "Attachment Factor" as per the manufacturer instructions.
2. *Cell maintenance*: Maintain BECs in serum-containing complete medium. For experimentation, culture BECs in complete serum-free medium. Maintain the cells in a humidified 5% CO_2, 21% O_2 incubator at 37 °C

   Note: As an alternative to specialized reagents, 0.1% (w/v) gelatine solution can serve as a coating reagent while DMEM/F-12 supplemented with 15 mM HEPES, 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 50 µg/ml gentamycin, 20 µg/ml heparin and 20 µg/ml endothelial cells growth supplement can be used for BEC maintenance.
3. *Sub-culturing for experimentation*: Split confluent BECs at a ratio of 1:3 to 1:6, depending on the intended time for next use (3-7 days). Change the maintenance medium every 3 days. Use BECs up to 15 passages.

1.2. SVG Human Fetal Astroglial Cell-line

SVG human fetal astroglial cells[22] were originally derived from primary cultures of human fetal brain transformed with replication-deficient simian virus 40.

1. *Maintenance*: Culture SVG cells in minimum essential medium with Earle's balanced salt solution supplemented with 20% (v/v) FCS (same serum percentage used to maintain their parental primary cells [22]), 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. Maintain the cells in a humidified 5% CO_2, 21% O_2 incubator at 37 °C.
2. *Sub-culturing for experimentation*: Split confluent SVGs at a ratio of 1:5 to 1:10, depending on the intended time for next use (3-7 days). Removal of trypsin is not required due to the high content of serum in the medium. Change the medium every 3 days. Use SVGs up to 20 passages.

2. Assembly and Coculturing of the Human *In vitro* BBB Model (Day 0)

Comment: The human *in vitro* contact BBB model is prepared according to published protocols [13,23], with modifications.

2.1. Coating of Inserts

1. Place tissue-culture inserts (6.5 mm in diameter with polyester porous membrane, 3 µm pore-size) in the wells of the supplied 24-well plate. Coat the luminal surface of the inserts overnight in a humidified 37 °C incubator with rat collagen I (20 µg/cm^2; 50 µl of 132 µg/ml collagen I in 0.02% acetic acid in Milli-Q water (MQH_2O)). Wash the inserts once (both from the abluminal and luminal side) with MQH_2O to remove the residual acid.

   Note: Basement membrane of brain capillaries contains no collagen I but mainly laminins, collagen type IV isoforms, nidogens, and heparan sulfate proteoglycans[9]. For this reason, some researchers utilize coating agents such as collagen IV, fibronectin[13], or Matrigel (BD biosciences)[16] for a more authentic BBB modeling.

2.2. Seeding SVGs on the Underside (Abluminal) Membrane Surface

1. Invert the insert and gently fit around the rim of the porous membrane a short piece of elastic silicone tubing ("external tubing"; ~10 mm long, 8 mm internal diameter, 1.6 mm wall), creating essentially a new well above the abluminal surface (**Figure 2A**).
2. The underside of the insert needs to be sealed to avoid media leakage. First, prepare a plug (**Figure 2A** and **2B**) made of a silicone tubing (termed "internal tubing"; ~8 mm long, 3.2 mm internal diameter, 1.6 mm wall; **Figure 2B**), sealed at one end with a plastic sealing cone (~3mm long; prepared by cutting the bottom of a 0.2 ml polymerase chain reaction (PCR) tube; **Figure 2B**). This cone not only seals the

internal tubing lumen, but also widens its edge for tighter fitting into the insert. Next, using a sterile forceps, insert the silicone plug into the luminal cavity and advance it until it reaches up-to ~1-2 mm from the membrane (**Figure 2A**).

3. Next, seed 4 x 10^4 SVG cells in 200 µl SVG maintenance medium (section 1.2.2 above) directly into the silicone well above the abluminal membrane surface (**Figure 2A**). Allow SVGs to adhere for at least 4 hr in the incubator.

   Note: To maintain sterility transport the assembled inserts in between two 6-well plates (one plate inverted over the other; **Figure 2C**) to minimize exposure to unfiltered air during the procedure. Silicone tubes and plugs are washed in ethanol and autoclaved for later use.

   Note: Use plugs (section 2.2.2) only for inserts with membrane pore sizes larger than 1 µm. Membranes with pore diameter ≤1 µm rarely leak and require only the fitting of the external silicone tubing (section 2.2.1). In addition, some coating agents other than collagen I (see note to section 2.1) may prevent leakiness even through 3 µm pores. Determine this empirically.
4. In order to monitor the adherence state of your astrocytes, seed in parallel into a standard 96-well an identical volume of the astrocyte cell-suspension. This well has the same surface area as the 6.5 mm insert (0.33 cm^2) and permits easier visualization of cells by phase-contrast microscopy than the insert membrane. When you observe sufficient adherence of the astrocyte in the control 96-well, the astrocytes have adequately adhered also to the insert membrane.
5. When astrocytes have sufficiently adhered in the control 96-well, transfer the insert assemblies to the tissue culture hood (see note above) and gently remove the external silicone tubing and plugs. Return inserts to the normal (*i.e.* upright) orientation into the supplied wells containing 800 µl/well of BEC maintenance medium (section 1.1.2 above).

2.3. Seeding of BECs into the Luminal Chamber above the Astrocytes

Seed 2x10^4 BECs in 200 µl onto the collagen-coated luminal surface and return the inserts into the incubator.

2.4. Coculturing

Coculture the SVG/BEC-seeded inserts for 3 days in BEC medium without media change before experimentation.

3. Experimentation with the Human BBB Model (Day 3)

1. To stimulate the *in vitro* BBB first wash cells by replacing the abluminal and luminal media with 600 µl and 100 µl of serum-free BECs medium, respectively.
2. Aspirate the luminal medium again and replace with 100 µl of serum-free medium with stimulating agents.

   Note: If stimulation of the abluminal chamber is required (to activate the *in vitro* BBB from the astrocytic compartment), aspirate the abluminal medium and replace with 600 ul of serum free medium with stimulating agents.
3. Test each experimental group in triplicate (hence, if luminal stimulation is performed, which requires 3 x 100 µl, we recommend diluting the reagents to their final concentrations in 350 µl of serum-free medium per group to minimize pipetting errors between wells).
4. Perform standard paracellular and/or transcellular permeability assays of labeled tracers [16,24] (for the latter we use fluorescein isothiocyanate (FITC)-conjugated albumin) and/or measurement of TEER [13,16,25] as previously described.

To account for fluctuations in baseline permeability between experiments, analyze changes in permeability relative to a coated insert without cells (which serves as a reference for maximal permeability) and to the vehicle-treated group using the formula: permeability (% of max) = (permeability value of experimental insert - average permeability value of the vehicle group) / (permeability value of the blank insert - average permeability value of the vehicle group) X 100.

4. Visualization of Cells on Porous Membranes

Comment: Unstained cells on insert membranes are difficult to observe by phase contrast or differential interference contrast (DIC) microscopy since the membrane is quite opaque and interferes with light transmission. To deal with this issue we developed various staining procedures of cells on inserts, which greatly enhance cell appearance on the membrane. Generally, stain the insert membranes while still attached to the insert, in the wells. The membranes should be removed only at the end of the staining procedure for mounting purposes.

4.1. Hematoxylin Staining

1. Fix inserts with ice-cold 4% (w/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS; 130 mM NaCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, pH 7.2) for 20 min. Wash once in PBS.
2. Submerge the insert in 0.1% Mayer's hematoxylin solution for 2 min.
3. Gently wash the insert with tap water (by submerging the insert in a beaker containing tap water and gently pipetting the excess water out). Transfer the insert to Scott's tap water solution (2 g/L $NaHCO_3$, 20 g/L $MgSO_4$) for ~20 sec until the desired blue color develops. Wash the insert again with tap water (section 4.1.3). An optional eosin stain can be introduced here by dipping the insert in 1% eosin solution for 10 sec followed by a tap water wash.
4. Air-dry the insert membrane. Using a scalpel (cutting around the membrane edge) remove the membrane and mount it on a glass slide for bright-field imaging

   Note: If di-N-butylphthalateinxylene (DPX) mounting medium is utilized wash the insert with 100% ethanol followed by xylene before removing and mounting the membrane.

4.2. Scanning Electron Microscope (SEM)

1. Fix inserts with ice-cold 4% (w/v) PFA in PBS for 20min. Wash three times in PBS.

2. Post-fix the membranes for 30 min in 1% (w/v) osmium tetroxide in MQH_2O.
3. Wash inserts in water (section 4.1.3) and dehydrate in an escalating ethanol gradient (50%, 70%, 90% for 5 min each, then 3x with 100% ethanol for 10 min per interval).
4. Treat dehydrated inserts with a mixture of hexamethyldisilazane (HMDS):ethanol (1:2 then 2:1 for 5 min).
5. Treat inserts with 100% HMDS for 5 min, air-dry and store under desiccated conditions.
6. Remove the membrane using a scalpel (cutting around the membrane edge). Examine membranes by scanning electron microscopy.

4.3. Immunofluorescence

1. Fix inserts with ice-cold 4% (w/v) PFA in PBS for 20 min, wash once in Tris-buffered saline (TBS; 50 mM Tris-HCl pH 7.6, 154 mM NaCl, 0.22 µm filtered).
2. Perform immunostaining of the cells on the insert membrane as previously described[26].
3. Remove the membrane using a scalpel (cutting around the membrane edge) and mount it on a glass slide with fluorescence mounting medium. Examine membrane by fluorescent microscopy.

Representative Results

In order to establish a human, contact BBB model we had to cultivate SVGs and BECs on porous membranes with a 3 µm pore-size, shown to permit passage of astrocyte end-feet for contact with endothelial cells[14,15,27,28]. A schematic representation of the complete contact model is illustrated in **Figure 1** (left illustration). The main technical challenge presented by a contact system is the need to seed astrocytes against gravity on the abluminal surface of the membrane. We have succeeded in this task by principally creating a removable silicone well on top of the abluminal membrane surface while preventing leakage of media with an additional silicone plug inserted into the luminal cavity (**Figure 2**). The method allowed an optimal, uninterrupted seeding period of SVGs (at least 4 hr), which in turn became strongly attached to the porous surface with minimal cell loss. Indeed, 3 days post-seeding both SVGs and BECs appeared confluent and covered the membrane uniformly, as judged from images of hematoxylin-stained inserts (see section 4.1; **Figure 3A**, left panels). Additionally, both cell types maintained their cell-specific markers on porous membranes (a diffuse staining pattern of glial fibrillary acidic protein in SVGs[22] and expression of von Willebrand factor in Wiebel-Palade bodies and cytoplasmic vesicles in BECs; **Figure 3A**, right panels), observed by immunofluorescence (section 4.3). Furthermore, from SEM imaging (section 4.2) it became apparent that SVGs and BECs were capable of growing directly over the membrane pores (**Figure 3A**, middle panels, arrow heads), a phenomenon which artificially contributes to the physical barrier function of *in vitro* BBB contact models1[3,28]. Functionally, in the presence of SVGs the endothelial permeability value (*Pe*)[16,24] of fluorescent albumin[28] improved ~4-fold relative to BECs alone ($P<0.01$; n=4), from 0.574±0.199 $x10^{-3}$ to 0.126±0.028 $x10^{-3}$ cm/min without or with astrocytes (SVGs), respectively. These albumin *Pe* results are comparable to another study using mouse BECs with rat C6 glioma cells[29]. Our seeding protocol was successfully applied also to freshly-isolated primary mouse BECs and primary mouse astrocytes grown on 1 µm pores. In this mouse contact model we obtained a reasonable physical barrier for the small hydrophilic tracer sodium fluorescein (*Pe*=0.83±0.22 $x10^{-3}$ cm/min; n=3). Hence, our seeding method can be extended for preparation of BBB models from other species.

The most fundamental feature of a contact BBB model is the ability of astrocytes to project their end-feet through the membrane pores to physically contact endothelial cells. A few studies made use of scanning electron microscopy to identify astrocytes processes which pass from the abluminal membrane surface (where astrocytes are seeded) to the luminal surface, as a proof of principle that contact between astrocytes and EC is plausible[14,15,27]. Following these studies we imaged by SEM the luminal membrane after seeding SVGs on the abluminal surface. As shown in **Figure 3B**, astrocytic (SVG) end-feet could be observed passing through 3 µm pores into the luminal compartment, confirming that contact can potentially exist between SVGs and BECs seeded on pores of this dimension. Collectively, our seeding method was simple, efficient, and yielded an authentic contact BBB model.

We have utilized our human contact BBB model to explore the effect of t-PA, a fibrinolytic agent, on the BBB. A number of articles have suggested that t-PA may be able to induce opening of the BBB during its intravenous administration for the treatment of acute ischemic stroke[30-33]. This interaction between t-PA and the BBB may contribute to bleeding complications associated with t-PA-induced thrombolysis[4,34,35] and is thus a subject for current research efforts. Our studies *in vitro* confirmed that t-PA indeed increased the permeability of the intact BBB in a concentration- (**Figure 4A**) and time-dependent manner (**Figure 4B**), which were both within a pharmacologically-relevant range[36,37]. Furthermore, dramatic morphological changes of SVG astrocytes could be observed on the porous membranes post exposure to t-PA (**Figure 4C**), suggesting that astrocytic responses to t-PA underlie t-PA-induced BBB opening. Subsequent investigation identified that t-PA stimulates astrocytes via activation of plasminogen - its natural substrate - into the potent and broad-spectrum protease plasmin. Furthermore, t-PA and plasmin were found to activate Rho-kinase (ROCK) signaling in astrocytes, and blockade of the ROCK pathway prevented t-PA-induced BBB opening[28]. This is a good example of how utilization of BBB models can lead to identification of novel biological processes and therapeutic opportunities.

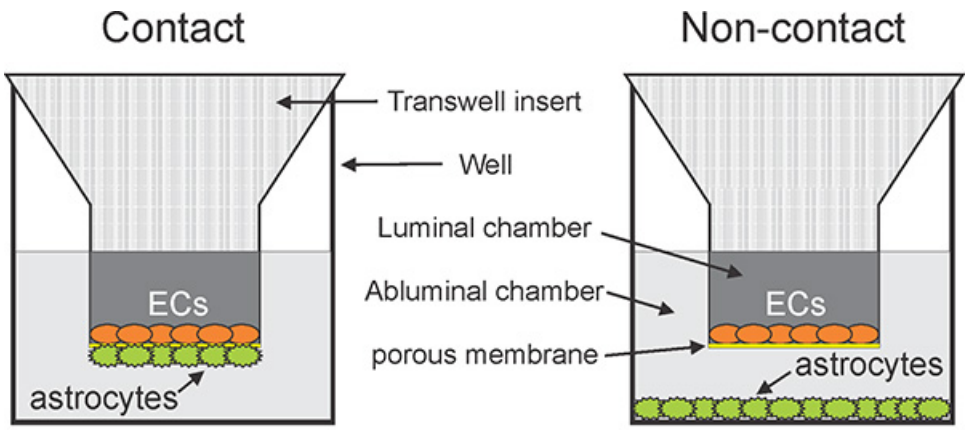

Figure 1. Insert-based configurations of *in vitro* BBB models. Schematic representations of static (without flow) *in vitro* BBB models. In basic models brain endothelial cells (ECs; orange) are cultured alone on a porous membrane (yellow). In more advanced approaches ECs are cocultured with astrocytes (green). Astrocytes can be grown either in non-contact conditions, on the bottom of the well (right), or on the abluminal surface of the porous membrane in contact conditions (left). Click here to view larger image.

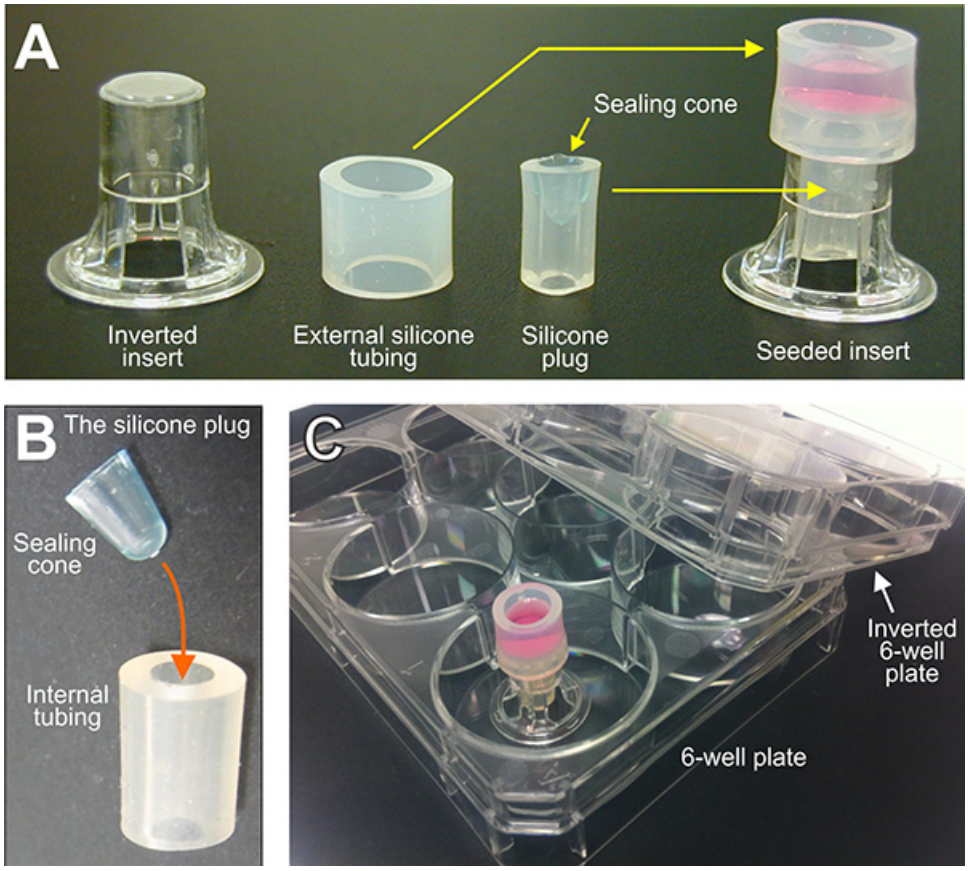

Figure 2. An improved method for astrocyte seeding on the abluminal membrane surface for preparation of a contact BBB model. (A) The insert is inverted, a short piece of silicone tubing ("external tubing") is assembled around its perimeter and a silicone plug fitted into the luminal cavity. This assembly creates a well on top of the abluminal surface and prevents media from leaking through the pores. Cells can be seeded in the well and allowed to adhere undisturbed for extended periods of time (right) **(B)** The silicone plug is prepared from another piece of silicone tubing ("internal tubing"), sealed at one end with a sealing cone which is inserted into its cavity. **(C)** Once seeded, the assembled inserts are transported in between two 6-well plates (one plate inverted over the other) to minimize the risk of contamination. Click here to view larger image.

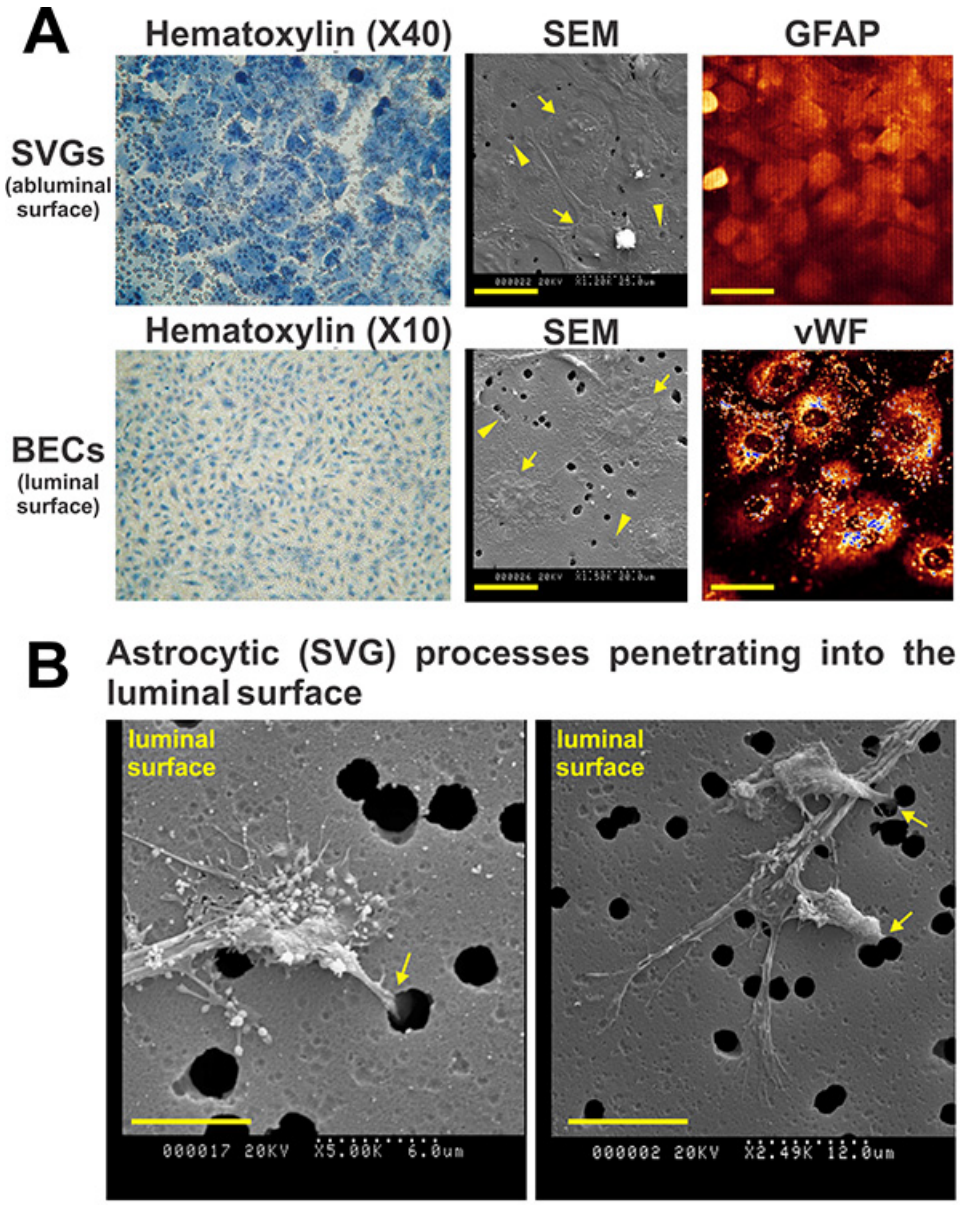

Figure 3. Visualisation of brain endothelial cells (BECs) and astrocytes on a 3 µm porous membrane. A) Hematoxylin (left panels), scanning electron microscope (SEM; middle panels) and immunofluorescence images (right panels) of SVGs (on the abluminal surface, 40,000 per 6.5 mm insert, top panels) and BECs (20,000 per 6.5 mm insert on the collagen I-coated, luminal surface, bottom panels) grown on a 3 µm porous membrane for 3 days (without coculturing). Both cell types reach confluence within this time frame and maintain their cell-specific markers (glial fibrillary acidic protein (GFAP) for SVG and von Willebrand factor (vWF) in Wiebel-Palade bodies for BECs, right panels) on the membrane. Arrow heads in the SEM panels further show the ability of both cell types to directly grow over and cover the pores, while arrows represent cell nuclei. Scale bars on SEM images represent 25 µm (top) or 20 µm (bottom). Scale bars on immunofluorescence images represent 40 µm. **(B)** SEM images of SVG end-feet passing through 3 µm pores into the luminal (BEC) surface 3d post seeding of SVGs (alone) on the abluminal membrane surface. These images validate the potential for development of true contact between SVGs and BECs in our human BBB model. Scale bars represent 6 µm on the left panel and 12 µm on the right panel. Click here to view larger image.

* For further clarification of the cells position please refer to **Figure 1**, left panel.

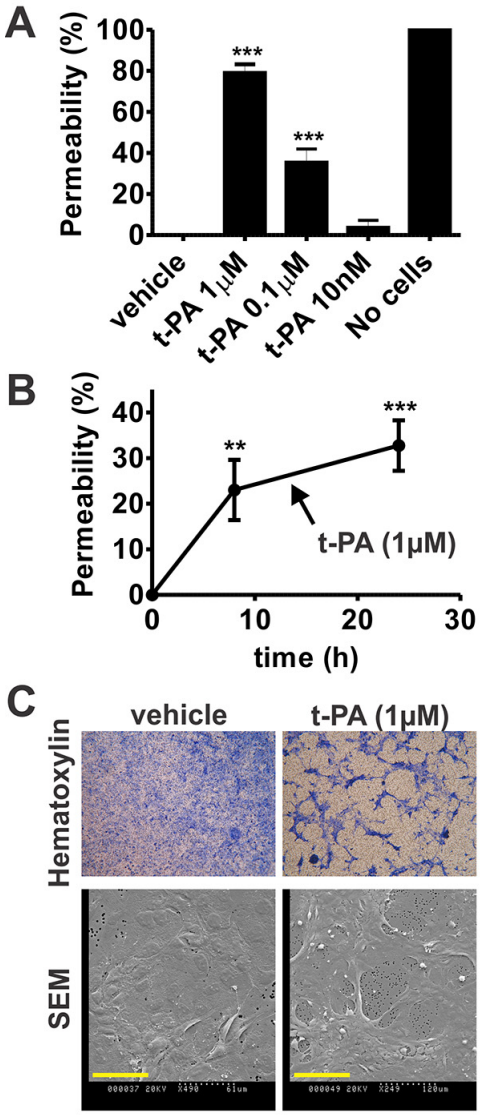

Figure 4. tissue-type plasminogen activator (t-PA)-mediated increase in BBB permeability is driven by morphological changes of astrocytes. (A) Increasing t-PA concentrations were added to the luminal chamber of the human *in vitro* BBB and permeability was assessed 24 hr later by passage of fluorescent albumin into the abluminal chamber. t-PA increased BBB permeability in a concentration-dependent manner. n=4-6 for all groups but t-PA 10nM, where n=2. ***P<0.001 compared to all other groups by one way ANOVA with Newman-Keuls post hoc. **(B)** t-PA (1 μM) was added to the luminal chamber of the human *in vitro* BBB for 8 hr and 24 hr before permeability assessment. As shown, t-PA-mediated BBB opening was already substantial 8 hr post stimulation (70.26% of the maximum at 24 hr) but a further increase was observed at 24 hr. n=6, ***P<0.001, **P<0.01compared to zero by one way ANOVA with Newman-Keuls post hoc. In both (**A**) and (**B**), bars/data points represent mean±SEM. **(C)** Representative bright-field images of Haematoxylin-stained SVGs (top panels) or scanning electron micrographs of SVGs (bottom panels) on polyester insert membranes (3 μm pore size) 24 hr post treatment with vehicle or t-PA (1 μM). Pronounced t-PA-induced morphological changes and disruption of the SVG monolayer integrity can be observed, suggesting that astrocytic responses to t-PA underlie the effect of t-PA on BBB permeability. Images are representative of two independent experiments. Scale bars on SEM images represent 61 μm (left) or 120 μm (right). Click here to view larger image.

Discussion

Medical research into brain pathologies suffers much translational difficulty. In the area of acute ischemic stroke, for example, many drugs which showed great promise in animal models failed at the clinic[38,39]. The reasons for these disappointing results are diverse and include infidelity of the preclinical test systems to the human stroke scenario and overstatement of results obtained from animal studies[21]. One strategy to improve the predictive value of preclinical findings to the clinical phase is additional development of **human** cell-based *in vitro* models; these may provide potent and cost-effective tools for screening novel drugs and mechanisms and allow better focusing on clinically-relevant avenues [21]. Within this context, we described here in detail the process undertaken in our laboratory for the establishment of a human contact BBB model utilizing a novel combination of human immortalized astrocytes (SVGs) and human primary BECs for examination of stroke-related phenomena.

Under static conditions (without flow), contact BBB models (**Figure 1**) resemble best the *in vivo* anatomical structure and are shown to be superior in their barrier properties[13,17,18]. Importantly, true contact between BECs and astrocytes occurs only on membranes with larger pore diameter (≥1 µm), which permit passage of astrocytic end-feet and subsequent induction of tighter (less permeable) BEC monolayers[14,15,27]. However, the seeding of astrocytes on inverted inserts with higher pore size - the core technical element in the preparation of a contact BBB model - presents a double technical challenge, since in addition to the limited medium volume which can be added to the inverted 6.5 mm inserts (~50 ul), the membranes are also prone to leakage of medium due to their large pore diameter. These limitations can make the astrocytic seeding awkward and inconsistent.

Our innovative technique for seeding astrocytes on inverted inserts with 3 µm pores offers a simple yet effective solution for this procedure. By creating a removable, impermeable silicone well on top of the abluminal surface we greatly improved the astrocytes' ability to successfully adhere to the porous membrane. This enables uniform growth of astrocytes on the abluminal surface and allows reproducible preparation of a large number of inserts for simultaneous screening of many experimental parameters (for example, our recently performed screening of the effect of five different plasminogen-activators on the BBB[28]) . Hence, despite its simplicity, we found our method to dramatically improve the throughput and quality of our research using this contact BBB model. One limitation of our astrocyte seeding method is the requirement for extra manual handling of the inserts, which harbor thin (10 µm thick) membranes. Excessive or rough handling can cause micro-tears and damage to the membrane. Hence, the use of soft and flexible silicone tubing is essential as the assembly/disassembly has to be performed with minimal force.

Regarding its overall concept, our human BBB model was established based on a previously-described protocol[14,23] which originally utilized primary human cells. Despite their superiority for BBB modeling, one drawback in employment of primary human brain cells is the inability to routinely access live human brain tissue for regular production of primary BECs and astrocytes and/or the cost involved in commercially obtaining them. Since many BBB models successfully substitute primary astrocytes with immortalized astrocytes (for example with a rat C6 glioma cells[29]), it was reasonable to employ SVG astrocytes[22] as a novel, abundant and cost-effective option for human BBB modeling. The ability of SVGs to positively influence the BEC barrier function validated in principle their suitability for BBB studies. In contrast, we chose to keep primary BECs in our BBB model for their critical role in the BBB *in vivo*[1,2,9]. Importantly, prolonged culturing of primary BECs can result in de-differentiation and deterioration of their BBB-specific phenotype. This indeed may be evident in our system by a rather high endothelial permeability values for albumin. For this reason, we have used our model mainly in a relative fashion[28] (**Figure 4**), taking into account its elevated basal permeability. If a very tight human BEC monolayer is desired the barrier function can be further improved by addition of chemicals such as the glucocorticoid hydrocortisone[17] or cyclic AMP with phosphodiesterase inhibitors[13]. Immortalized human BECs, such as the hCMEC/D3 cell line[40], could also be employed for their ability to better maintain tight junctions over time. Yet, BEC cell-lines often display reduced barrier qualities compared to low-passage primary BECs[41,42] and needs to be carefully considered because of other potential changes (*i.e.* altered expression of cell-surface receptors).

Collectively, our modification of the astrocytic cell type (*i.e.* the employment of SVGs) and the substantial improvement to the astrocyte seeding protocol resulted in generation of a reproducible, easy to assemble and authentic human contact BBB model. Bearing in mind its limitations, this system can serve as a useful tool for *in vitro* investigation not only of acute ischemic stroke, as we exemplified here[28], but also of several other brain pathologies involving modulation of the BBB.

Disclosures

Authors have nothing to disclose.

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