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Regulation of human brain vascular pericytes and human astrocytes in a blood–brain barrier model using human brain microvascular endothelial cells: Expression of TGF-β1, VEGF, MMP-9 and P-gp

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ABSTRACT

An *in vitro* human blood–brain barrier (BBB) model was developed using human brain microvascular endothelial cells (HBMECs) co-cultured with human brain vascular pericytes (HBVPs) and human astrocytes (HAs) at various ratios (HBVPs:HAs = 1:1, 1:2, 1:6), associated with pericyte-conditioned medium (PCM) and astrocyte-conditioned medium (ACM). After 7 days of co-culturing of HBMECs with HBVPs and HAs at a ratio of 1:2, and a 1:1 ratio of PCM and ACM, the transendothelial electrical resistance was enhanced to $319 \pm 16.67\ \Omega \times cm^2$ (225% increase) and the permeability coefficient of propidium iodide was reduced to $2.09 \pm 0.5 \times 10^{-6}$ cm/s (61% decrease). Analysis of three major barrier integrity modulators: transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF), and matrix metalloproteinases (MMPs), revealed a higher TGF-β1 expression and lower VEGF and MMP-9 expressions in a co-culture of HBMECs with HBVPs and HAs at a ratio of 1:2. Calcein-AM analysis showed higher P-glycoprotein (P-gp) activity in the model using PCM and ACM with HBVPs:HAs = 1:2 (100%) than that with HBVPs:HAs = 1:1 (55%) and HBVPs:HAs = 1:6 (49%). The effect of TGF-β1 receptor inhibitor SB431542, VEGF inhibitor asteric acid, and MMP-9 inhibitor CTT (H-Cys-Thr-Thr-His-Trp-Gly-Phe-Thr-Leu-Cys-OH) on the BBB model suggested that TGF-β1 up-regulates P-gp activity and VEGF down-regulates P-gp activity. In the presence of PCM and ACM, co-culturing of HBMECs with HBVPs and HAs at a ratio of 1:2 could approach the *in vivo* BBB in a well representative manner.

1. Introduction

The central nervous system (CNS) is immensely protected by the blood–brain barrier (BBB) and the blood–cerebrospinal fluid barrier. The BBB acts as a strict shield that regulates the selective transport system for the entrance of endogenous and exogenous molecules [1]. The nature of the BBB consists of maintaining the dynamic homeostasis in the CNS, defending the microenvironment of the brain, providing nutrients stably from special pathways, and changing the state of a reaction to inflammatory cells [2,3]. The communications among the various cell types that exist between the blood and the brain give the BBB a structure with distinctive biochemical properties [4,5]. The components of the BBB include the basement membrane, brain microvascular endothelial cells (BMECs), astrocytes, pericytes, and blood cells. BMECs have unique biological traits, such as the presence of Factor VIII antigen, particular enzymes, limited vesicles, adenosine triphosphate (ATP)-binding cassette transporters, membrane polarity, high transendothelial electrical resistance (TEER), and low permeability [2]. Hence, the delivery of substances to traverse the BBB into the human brain is considered to be energy-dependent.

Brain microvascular astrocytes play a significant role in the synthesis of proteoglycans, thereby enhancing charge selectivity and modulation of BMECs. They also take part in carrying nutrients to nerve cells with many receptors and specific channels, and connect with the cells closely related to each other in the BBB to produce the neurovascular units (NVU) [6,7]. Astrocytes can secrete soluble factors, including transforming growth factor-β (TGF-β), glial-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), and angiopoietin 1 (Ang 1). Astrocytes assist in improving the tightness of BMECs and inducing proteins in cell membrane, and can be used to establish an *in vitro* BBB model and replicate human brain internality [8,9]. Pericytes regulate angiogenesis and blood vessel function, and are responsible for the production of Ang 1. Ang 1 has restored vascular stabilization in platelet-derived growth factor-β (PDGF-β)-deficient mice. Moreover, TGF-β and TGF-β type II receptor knockout mice revealed a defective vascular wall, suggesting that pericytes are involved in TGF-β-mediated vascular stability [10,11]. Pericytes and BMECs

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interconnect with each other through several elements, such as soluble factors, tight junctions (TJs), gap junctions, and adhesion plaques [2,12]. TJs are one of the essential anatomical tissue sites controlling the performance of the BBB. The dense alignment of TJs located in the luminal region between BMECs has a sealing capability to limit penetration and adjusts the formation of apical and basolateral plasma membrane [4,13]. In terms of molecular arrangement, TJ proteins adhered to BMECs are interlocked with transmembrane proteins and linked to the basement membrane to generate a firm blockade [3].

A practical *in vitro* BBB model is vital in understanding the complicated surroundings that arise in the *in vivo* BBB, and further facilitates treatment methods associated with CNS-based disorders. Therefore, biomedical researchers and practitioners are keen to pursue the use of *in vitro* BBB models that are simple and highly reproducible, and as much as possible, that can offer mechanistic simulation of standard and pathological conditions on BBB-related issues [14]. TJs can be characterized by TEER and penetration of foreign matter to determine the compactness of BMECs [15]. P-glycoprotein (P-gp), an ATP-dependent efflux transporter in the apical membrane of BMECs, is considered to have an ability of multidrug resistance, leading to the poor quality of numerous pharmaceuticals to infiltrate the brain. Thus, P-gp should be highly expressed in an effective BBB model [16-18]. Apart from that, BBB integrity can be also affected by secreted physiological factors, including TGF-β, Ang 1, GDNF, bFGF, vascular endothelial growth factor (VEGF), and matrix metalloproteinases (MMPs), through interactions with BMECs [8-11].

In 1973, for the first time, an *in vitro* BBB model was achieved using a primary culture method and viable microvessels isolated from the rat brain [19]. This undeniable landmark inspired the subsequent development of *in vitro* BBB models established with BMECs using various animal species, including pig and steer [19,20]. The primary culture retained some of the phenotypic feature of BMECs. But, the presence of peripheral cells in the basement membrane incurred difficulty purifying BMECs [21]. Then, a more complex co-culture was introduced as a BBB model to obviate the problems associated with the primary culture and to enhance the function of the model [7]. A co-culture can access the effects of various cell types on BMECs, for example astrocytes, glial cells [22], pericytes [23], and neurons [24], and form adequate NVU [25]. In addition, a double or triple co-culture method may reproduce the state of the *in vivo* BBB and is beneficial in BBB-related studies, including those on neuroinflammation, drug abuse, and active drug transport in the CNS [26-28]. This work involves the development of an *in vitro* BBB model using a co-culture of human BMECs (HBMECs) with human brain vascular pericytes (HBVPs) and human astrocytes (HAs) (Fig. S1). In addition, we wanted to find a better co-culture scheme to imitate the *in vivo* system by altering the ratio of HBVPs and HAs with those of pericyte-conditioned medium (PCM) and astrocyte-conditioned medium (ACM).

2. Materials and methods

2.1. Preparation of conditioned mediums

The T75 cell culture plate (Corning, Cambridge, MA, USA) was pretreated with 4 mL of 2% gelatin (Sigma-Aldrich, St. Louis, MO, USA) solution and placed in a humidified CO_2 incubator (US NU-4750, autoflow water-jacketed automatic CO_2 incubator; NuAire, Plymouth, MN, USA) at 37 °C for 1 day. To carry out the cell subculture pretreatment, the first 3 generations of HBMECs (Sciencell Research Laboratories, Corte Del Cedro Carlsbad, CA, USA) or HBVPs (Sciencell Research Laboratories) or HAs (Sciencell Research Laboratories) were added to a T75 cell culture pretreated with 4 mL of fibronectin (Sigma-Aldrich) solution (150 µL fibronectin in 10 mL Dulbecco's phosphate buffered saline (DPBS; Sigma-Aldrich)) and placed in a humidified CO_2 incubator at 37 °C for 1 day. The Transwell culture inserts (polyethylene terephthalate (PET) membrane; BD Biosciences, San Jose, CA, USA) were placed in a 24-well cell culture plate (Midwest Scientific, St. Louis, MO, USA) with sterilized stainless steel tweezers. 500 µm fibronectin or gelatin solution was added to the inside and outside of each well to evenly distribute the PET film in the Transwell culture dish, and each well was placed in a humidified CO_2 incubator at 37 °C for 1 day. Endothelial cell medium (Sciencell Research Laboratories) and endothelial cell growth supplement (Sciencell Research Laboratories) for HBMECs cell culture, pericytes medium (Sciencell Research Laboratories) and pericytes growth supplement (Sciencell Research Laboratories) for HBVPs cell culture, and astrocytes medium (Sciencell Research Laboratories) and astrocyte growth supplement (Sciencell Research Laboratories) for HAs cell growth were mixed at 37 °C in an aseptic operation station. HBVPs or HAs at a density of 4×10^5 cells/cm^2 were seeded in a pretreated T75 cell culture plate and placed in a humidified CO_2 incubator at 37 °C; the medium was changed every day. The old medium was collected after cell growth for 8 h (PCM_1 or ACM_1) and 1 day (PCM_2 or ACM_2), respectively. PCM or ACM was collected through filtration using a sterile filter with a pore size of 0.2 µm, and stored in a refrigerator at −80 °C.

2.2. Development of in vitro BBB models

2.2.1. Co-culture of HBMECs and HAs

HAs were seeded at a density of 4×10^5 cells/cm^2 in a base of the pretreated Transwell petri dish and placed in a humidified CO_2 incubator at 37 °C for 1 h. Then, the upper layer was injected with HBMECs at a density of 4×10^5 cells/cm^2, and placed in a humidified CO_2 incubator at 37 °C. The medium was changed every day, and HBMECs and HAs were co-cultured for 7 days for further experiments.

2.2.2. Co-culture of HBMECs and HAs with PCM and ACM

HAs were seeded in the base of the pretreated Transwell petri dish, and placed in a humidified CO_2 incubator at 37 °C for 1 h. Then, the upper layer was injected with HBMECs and placed in a humidified CO_2 incubator at 37 °C. The seeding density was 4×10^5 cells/cm^2. After 80% of HBMECs were in the HAs, the medium in the Transwell culture dish was changed to ACM_1, ACM_2, PCM_1, PCM_2 or PCM_2:ACM_2 (1:1), and the culture dish was placed in a humidified CO_2 incubator at 37 °C. The medium was changed every day and co-cultured for 7 days.

2.2.3. Co-culture of HBMECs, HBVPs and HAs

HBVPs and HAs at various ratios (1:1, 1:2, 1:6) were seeded in the base of the pretreated Transwell petri dish and placed in a humidified CO_2 incubator at 37 °C for 1 h. Then, the upper layer was injected with HBMECs and placed in a humidified CO_2 incubator at 37 °C. The seeding density was 4×10^5 cells/cm^2. The medium was changed every day, and HBMECs, HBVPs and HAs were co-cultured for 7 days.

2.2.4. Co-culture of HBMECs, HBVPs and HAs with PCM and ACM

HBVPs and HAs at various ratios (1:1, 1:2, 1:6) were seeded in the base of the pretreated Transwell petri dish and placed in a humidified CO_2 incubator at 37 °C for 1 h. Then, the upper layer was injected with HBMECs and placed in a humidified CO_2 incubator at 37 °C. The seeding density was 4×10^5 cells/cm^2. After 80% of HBMECs were in the HBVPs and HAs, the medium in the Transwell culture dish was changed to ACM_1, ACM_2, PCM_1, PCM_2 or PCM_2:ACM_2 (1:1), and the culture dish was placed in a humidified

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