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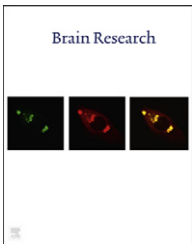

Research Report

Establishment of a new conditionally immortalized cell line from human brain microvascular endothelial cells: A promising tool for human blood–brain barrier studies

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ABSTRACT

The blood–brain barrier (BBB) is formed by brain microvascular endothelial cells (BMEC) working together with astrocytes and pericytes, in which tight junctions and various transporters strictly regulate the penetration of diverse compounds into the brain. Clarification of the molecular machinery that provides such regulation using *in vitro* BBB models has provided important insights into the roles of the BBB in central nervous system (CNS) disorders and CNS drug development. In this study, we succeeded in establishing a new cell line, hereinafter referred to as human BMEC/conditionally immortalized, clone β (HBMEC/ciβ), as part of our ongoing efforts to develop an *in vitro* human BBB model. Our results showed that HBMEC/ciβ proliferated well. Furthermore, we found that HBMEC/ciβ exhibited the barrier property of restricting small molecule intercellular penetration and possessed effective efflux transporter functions, both of which are essential to a functioning BBB. Because higher temperatures are known to terminate immortalization signals, we specifically examined the effects of higher temperatures on the HBMEC/ciβ differentiation status. The results showed that higher temperatures stimulated HBMEC/ciβ differentiation, marked by morphological alteration and increases in several mRNA levels. To summarize, our data indicates that the newly established HBMEC/ciβ offers a promising tool for use in the development of a practical *in vitro* human BBB model that could make significant contributions toward understanding the molecular biology of CNS disorders, as well as to CNS drug development. It is also believed that the development of a specific culture method for HBMEC/ciβ will add significant value to the HBMEC/ciβ-based BBB model.

1. Introduction

The blood–brain barrier (BBB), which is formed by brain microvascular endothelial cells (BMEC) working together with astrocytes and pericytes, strictly regulates the entrance of various compounds into the brain (Eyal et al., 2009; Persidsky et al., 2006). The key components providing such regulation are tight junctions along with the specialized transport

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system existing in BMEC. Tight cell junctions between BMEC are established by multiple components including claudin-5 and occludin, which produce a physical barrier that seals the paracellular permeable route. The transport system is equipped with efflux transporters that actively export diverse compounds back into systemic circulation, as well as influx transporters and physiological receptors that allow the uptake of essential substances, such as glucose, into the brain. These sophisticated BMEC functions play a vital role in maintaining the homeostasis of the central nervous system (CNS).

One of the important aspects related to the BBB is that its functional impairment is associated with several CNS disorders. For example, abnormal BBB permeability of gadolinium-diethylenetriamine pentaacetic acid has been reported in patients diagnosed with subcortical ischemic vascular disease (Taheri et al., 2010) (other examples are also found in Rosenberg, 2012). Both the BBB disruption and reconstitution patterns are likely to play important roles in the overall clinical courses of the CNS disorders, even though their precise roles are not yet fully understood. Another important consideration is that the BBB has been a critical obstacle to pharmacotherapy for CNS diseases, as well as an impediment to the development of CNS drugs (Ohtsuki and Terasaki, 2007; Pardridge, 2007). For example, it has been shown that P-glycoprotein (Pgp) and breast cancer resistant protein (BCRP), which are representative BBB efflux transporters, severely restrict the distribution of anti-cancer drugs into mouse and monkey brains (Lagas et al., 2010; Yamasaki et al., 2011). In addition, recent successes in antibody-based therapy have been limited to peripheral diseases due to the inability of antibodies to access the brain parenchyma.

Considering the above-mentioned circumstances, it is likely that clarification of the nature of the BBB using *in vitro* BBB models could provide fundamental insights into understanding CNS diseases, as well as assist in CNS drug developments (Naik and Cucullo, 2012; Wilhelm et al., 2011). Furthermore, noting the qualitative and quantitative species-differences observed at the BBB (Uchida et al., 2011; Warren et al., 2009), it was considered preferable to use primary human BMEC (prHBMEC) for human BBB investigations (Lacombe et al., 2011). However, prHBMEC proliferate at a low rate and are prone to rapid senescence, even upon limited passages, which critically hampers their use in routine experiments. Therefore, alternative human cells possessing high proliferation ability together with stable cellular functionality are considered necessary for the development of useful *in vitro* human BBB models.

Cell immortalization techniques offer promising possibilities related to the development of such cells because, generally speaking, immortalized cells are known to proliferate well and are capable of retaining the important morphological and functional characteristics of their parental cells (Ide, 2006). In cell immortalization, simian virus 40 large tumor antigen (SV40T) and its temperature-sensitive mutant (tsSV40T) are often used in order to take advantage of their ability to bind with p53 and pRB (as well as proteins that are currently unknown), which facilitates cell proliferation. Unlike wild-type SV40T, tsSV40T gradually degrades at non-permissive temperatures (>37 °C) even though it is stably expressed at 33 °C. Accordingly, a unique characteristic of a conditionally (=temperature-sensitive) immortalized cell is its differentiation tendency in response to the temperature-induced elimination of the tsSV40T expression (Saleem et al., 2002; Tabuchi et al., 2002). In addition to SV40T, human telomerase reverse transcriptase subunit (hTERT) plays a key role in cell immortalization. hTERT is often used in combination with SV40T to prevent the telomere shortening associated with cell division, which consequently reduces crisis risk (proliferation stoppage), genome instability and senescence (Kowolik et al., 2004; O'Hare et al., 2001; Sharma et al., 2003).

Previously, no human BMEC-derived cell line immortalized by both tsSV40T and hTERT has been established. Therefore, in the present study, we sought to establish a new conditionally immortalized cell line derived from human BMEC using both tsSV40T and hTERT. Our results were successful and, based on the results reported here; it is believed that our cells will provide a promising tool for use in the development of a functional *in vitro* human BBB model.

2. Results

2.1. *HBMEC/ciβ establishment*

Once cell immortalization and individualization were complete, a new clonal cell line was obtained, which we named human BMEC/conditionally immortalized, clone β (HBMEC/ciβ). The cells of this line showed high proliferation ability at 33 °C (doubling time=38.7 h) (Fig. 1A). Furthermore, they were found to retain their proliferation ability for up to at least 37 continuous passages, while prHBMEC proliferation stopped at continuous passage 12 (data not shown). Consistent with this high proliferation ability, HBMEC/ciβ expressed tsSV40T and hTERT functionally, as can be seen in the results of western blot analysis (Fig. 1B) and telomerase activity determination (Fig. 1C), respectively. Microscopic observation showed that HBMEC/ciβ exhibited a spindle-shaped morphology, and formed a monolayer at confluence, which was maintained for up to at least 21 days. This morphological feature was similar to that of prHBMEC (Fig. S1).

2.2. *BMEC-enriched gene expression in HBMEC/ciβ*

The gene expression profile in HBMEC/ciβ was examined by reverse transcription-polymerase chain reaction (RT-PCR) analysis (Fig. 2). We found that HBMEC/ciβ clearly expressed endothelial marker gene mRNAs (von Willebrand factor (vWF), platelate endothelial cell adhesion molecule-1 (PECAM-1) and vascular endothelial-cadherin (VE-cad)), thus confirming that HBMEC/ciβ retained endothelial cell characteristics (Engelhardt, 2003). We then analyzed BMEC-enriched gene mRNA expression and found that HBMEC/ciβ expressed various junction molecule gene mRNAs (occludin, claudin-3, -5 and -12, zonula occludens-1 (ZO-1), junctional adhesion molecule-A (JAM-A) and β-catenin), along with various transporter gene mRNAs such as Pgp, BCRP glucose transporter 1 (GLUT1), L-type amino acid transporter 1 (LAT1), organic anion transporting polypeptide 1A2 (OATP1A2), OATP1C1 and OATP2B1. In addition, HBMEC/ciβ expressed transferrin receptor (TfR), lipoprotein