Expression of Blood-Brain Barrier Antigens in Non-Brain Derived Endothelial Cells Despite Lack of Astrocyte Interactions

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Authors’ contributions

This work was carried out in collaboration between all authors. Author AM performed the experiments, interpreted the data, and drafted the manuscript. Author ZK was involved in the conceptual design of the study, interpretation of the data, and writing the final paper. All authors read and approved the final manuscript.

ABSTRACT

Endothelial cells of different vascular beds display diverse functional and morphological characteristics. Specifically, brain-derived endothelial cells exhibit specialized properties that form the blood-brain barrier, an important structure that ensures homeostasis within the neural environment. Classic proteins associated with the blood-brain barrier include neurothelin (also known as CD147), neuroblast differentiation-associated protein, glucose transporter-1 and gamma-glutamyltranspeptidase. Astrocytes are believed to be responsible for inducing the expression of blood-brain barrier proteins in brain endothelial cells. We evaluated the induction property of astrocytes in this study. Human umbilical vein endothelial cells and human dermal microvascular endothelial cells, both non-brain derived, were grown as co-cultures with human astrocytes or as monocultures for various periods. Blood-brain barrier marker mRNA levels were quantified using real-time reverse transcriptase polymerase chain reaction and protein localization was determined through immunofluorescence staining. Our results demonstrate expression of neurothelin, glucose transporter-1, and gamma-glutamyltranspeptidase mRNA in endothelial cells even in the absence of astrocytes. Co-culturing endothelial cells with astrocytes induced the expression of glucose transporter-1 and gamma-

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glutamyltranspeptidase but not neurothelin. Immunofluorescence staining revealed expression and localization of neuroblast differentiation-associated protein at cell membranes of non-brain derived endothelial cells. Non-brain endothelial cells also showed glucose transporter-1, gamma-glutamyltranspeptidase and neurothelin immunoreactivity in expected subcellular compartments. These findings indicate that endothelial cells in culture express markers of blood-brain barrier and that astrocytes have differential inductive capacity depending of the endothelial cell type.

Keywords: Blood-brain barrier; endothelial cell; astrocytes; co-culture; induction; CD147; AHNAK; glut.

ABBREVIATIONS

AHNAK : neuroblast associated protein; desmoyokin
BBB : blood-brain barrier
BSA : bovine serum albumin
CD147 : cluster of differentiation 147; neurothelin; basigin
DMEM : dulbecco’s modified eagle medium
EBM-2 : endothelial basal media-2
EC : endothelial cell
FBS : fetal bovine serum
FN : fibronectin
γ-GTP : gamma-glutamyltranspeptidase
Glut-1 : glucose transporter-1
HA : human astrocyte
HDMEC : human dermal microvascular endothelial cell
HUVEC : human umbilical vein endothelial cell
PSF : penicillin-streptomycin-fungizone
RT-PCR : reverse transcriptase polymerase chain reaction

1. INTRODUCTION

Endothelial cells (ECs) exhibit various morphological and functional properties among different vascular beds within the body [1, 2]. Heterogeneity occurs at the level of cell size and organization [3], protein and antigen expression [4], and cell function. Brain capillary ECs have specialized properties that form the blood-brain barrier (BBB), an important interface between the bloodstream and central nervous system that maintains homeostasis within the brain [5, 6]. Because small changes in neural environment compromise synaptic signalling and can lead to cell death, the BBB regulates the movement of solutes between blood and neural tissue. Regulation occurs at three levels: 1) a physical barrier, where tight junctions restrict solute movement (diffusion) through the gaps between ECs, 2) an enzymatic barrier, where enzyme systems metabolize solutes passing through ECs, and 3) a transport barrier, where specialized transport systems regulate which materials are able to travel through ECs via endocytosis, facilitated diffusion and active transport [7].

The BBB involves numerous cellular associations between ECs, astrocytes, pericytes, neurons, and microglia [8, 9]. ECs form a capillary tube with tight junctions forming between the edges of cells. Pericytes, which inhibit EC proliferation, make contacts with ECs [6]. As well, astrocytes make many contacts with the EC abluminal membrane through endfoot...
projections [2, 7] (Fig. 1a). This astrocyte contact assists in the formation of tight junctions between ECs [2, 7] and the expression of BBB markers in ECs [6, 10, 11]. Classic BBB markers include gamma-glutamyltranspeptidase (γ-GTP), neurothelin (CD147), neuroblast differentiation-associated protein (AHNAK), and glucose transporter-1 (Glut1) [6, 12, 13]. These BBB proteins regulate various cellular activities in brain ECs. For example, γ-GTP catalyzes the shift of the gamma-glutamyl functional groups from glutathione to amino acids or other compounds in ECs [14]. Neurothelin is a membrane glycoprotein found mainly in brain capillaries and is important for cell recognition [6, 15]. AHNAK, also known as desmoyokin, coordinates with other tight junction proteins to help ECs maintain low levels of blood-tissue exchange [13]. Finally, Glut1 transports glucose in an insulin-independent manner [7].

This study aims to determine whether non-brain derived ECs express BBB markers in culture. We also examined whether co-culturing non-brain derived ECs with astrocytes induces BBB markers. We hypothesize that ECs from non-neural tissues will express BBB markers and that the level of BBB marker expression will increase with astrocyte co-culture. To test this hypothesis, we have measured and compared mRNA levels and protein expression between EC monocultures and co-cultures, as well as cultures from different periods.

2. MATERIALS AND METHODS

2.1 Cell Culture

Human dermal microvascular ECs (HDMECs; Cat# CC-2516), human umbilical vein ECs (HUVECs; Cat# CC-2517), and normal human astrocytes (HAs; Cat# CC-2565) were obtained from Lonza Inc. (Walkersville, Maryland, USA). All cell types were cultured on human plasma fibronectin-coated (FN, 1 g/cm², Millipore, Temecula, California, USA) chambered slides (Lab-Tek; Thermo Scientific, Ottawa, Ontario, Canada) or multiwall culture plates (BD Biosciences, Mississauga, Ontario, Canada). For EC culture, we used Endothelial Basal Media-2 (EBM-2; Lonza Inc.) containing SingleQuots®, 20% fetal bovine serum (FBS; Lonza Inc.) and 1X penicillin-streptomycin-fungizone (PSF; Invitrogen, Grand Island, New York, USA). Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen) supplemented with 20% FBS and 1X PSF was used for HA. Monocultures were established using respective EC and HA media described above and a cell density of 20,000 cells/cm². Cell numbers were determined using Scepter™ handheld automated cell counter (Millipore). For co-culture experiments, we cultured HAs for 5 days, and then seeded ECs on top to create a direct co-culture system (Fig. 1b). For these experiments, we used the cells at passage 2-6.
Fig. 1. Schematic illustration of the blood-brain barrier and the experimental design
(a) Schematic showing the cellular interactions comprising the blood-brain barrier. Endothelial cells form a barrier between the blood and the brain tissue. Tight junctions between endothelial cells restrict solute movement. Astrocyte end-feet (shown in orange) and pericytes (shown in green) make contacts with the endothelial cells. Modified from Abbott et al., 2010 and Schlosshauer, 1993.
(b) Schematic showing the experimental layout. Chamber slides (cell staining) or multiwell culture plates (gene expression studies) were used to perform monoculture of endothelial cells and astrocytes or direct co-culture. Cells were cultured for one and seven days after seeding and media was changed every other day.

2.2 Real Time Reverse Transcriptase-Polymerase Chain Reaction (Real Time RT-PCR)

Total RNA was isolated from cell cultures using QiagenRNeasyPlus Mini Kit (QIAGEN, Mississauga, Ontario, Canada). Five hundred ng of total RNA was used to prepare cDNA with iScriptcDNA Synthesis Kit (BioRad, Hercules, California, USA). All primer sets were
obtained from Qiagen (CD147: QT00074564, Glut1: QT00068957, and γ-GTP: QT01668373). ANHAK expression was determined by immunocytochemistry (described below). First Choice human brain reference RNA (Invitrogen) was used as a positive control for real time RT-PCR experiments. mRNA levels were quantified using Lightcycler™ (Roche Diagnostic Canada, Laval, Quebec, Canada). For each sample, 2 L of prepared cDNA, 1 L of both forward and reverse primers (2 L total), 10 L of SYBR Green Master Mix (Clonetech, Mountain View, California, USA), and 6 L of dH₂O were added to Roche glass capillaries. The RT-PCR program consisted of one denaturation step (95°C for 3 minutes, 20°C/sec), and then 40 PCR cycles (95°C for 0 sec, 55°C for 10 sec, 72°C for 15 sec; all at 20°C/sec). Melting curve analysis was used to confirm the specificity of the amplification. BBB marker mRNA expression was calculated by the standard curve method. mRNA levels were normalized to 18S rRNA housekeeping gene and presented relative to the reference brain RNA positive control.

2.3 Immunofluorescence Staining

Cells were analyzed for BBB marker expression by using rabbit anti-human CD147 (Abcam, Cambridge, MA), mouse anti-human AHNAK (Abnova, Walnut, CA), rabbit anti-human Glut1 (Abcam), and mouse anti-human γ-GTP (Abcam). Cells were fixed in cold methanol for five minutes and then incubated with primary antibody (1:200) in 1% bovine serum albumin (BSA; Sigma-Aldrich, Oakville, Ontario, Canada). Anti-rabbit or anti-mouse Alexa-Fluor 488-tagged secondary antibody (1:200; Invitrogen) was used for detection. Slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted using fluoromount mounting media (Sigma-Aldrich). Images were taken with Olympus BX-51 fluorescence microscope (Olympus Canada Inc., Richmond Hill, Ontario, Canada) equipped with a Spot Pursuit™ digital camera (SPOT Imaging Solutions, Sterling Heights, Michigan, USA).

2.4 Statistical Analyses

Data was analyzed by ANOVA and Student’s t-test. Data is presented as mean ± SEM. Differences were considered significant at a p value less than 0.05.

3. RESULTS

3.1 Ecs Express CD147 and Astrocytes Only Cause a Transient Induction

We first wanted to know whether non-brain derived ECs express CD147. CD147 is highly expressed in hepatocarcinoma cells [16] and BBB endothelial cells [6, 17]. Real time RT-PCR showed that CD147 mRNA is present in HDMECs and HUVECs as well as HAs (Fig. 2). In EC-HA co-cultures, only HUVECs increased the expression of CD147 (Fig. 2a). Interestingly, this was only evident at day 1 and by day 7, the difference in CD147 mRNA levels between co-cultured and mono-cultured HUVECs was not significant (Fig. 2b). However, when we examined monocultures at day 1 and 7, we found that the mRNA levels were significantly downregulated in HAs (Fig. 2c). Further, mRNA levels were not different in monocultured HUVECs at day 1 and day 7. This suggested that the reason for no induction seen in CD147 in 7-day co-cultured cells might be the downregulated of the transcripts in HAs.
3.2 Induction of Glut1 in Endothelial Cells upon Astrocyte Co-Culture

We next examined the expression of Glut1 in endothelial cells. Glut1 is one of the best-characterized markers of brain capillary ECs [18] and has been shown to be expressed in cultured brain ECs [19]. Glut1 is also present in astrocytes in vivo [20]. Our results show that both EC types and HAs express Glut1 in culture (Fig. 3). However, in contrast to CD147, expression of Glut1 mRNA revealed a more defined pattern. Glut1 mRNA levels were higher in ECs co-cultured with astrocytes when compared to the corresponding EC monocultures at both day 1 and day 7 (Fig. 3a and 3b). Glut1 mRNA levels in day 1 HDMEC co-culture were significantly higher (2.8x increase) than those of day 1 HDMEC monocultures. This difference was even more drastic at day 7 (14.8x increase). Similar results were observed in HUVEC cultures (2.9x increases at day 1, and 8.25x increases at day 7). We then examined
whether this dramatic (14.8- and 8.25-fold) increase is due to longer times in cultures (7 days vs 1 day). Our results show that both HDMECs and HUVECs significantly downregulated Glut1 mRNA at day 7 in the absence of astrocytes (Fig. 3c). A slight increase (1.16x) was noted in HAs cultured alone for 7 days. Therefore, increased Glut1 mRNA levels in co-cultured HDMECs and HUVECs reflect the inductive effect of astrocytes and not the cell density or time in culture.

![Graph showing Glut1 expression in endothelial cells](image)

**Fig. 3. Glut1 expression in endothelial cells**

Glut1 mRNA expression in monocultures and co-cultures after 1 (a) and 7 days (b) [mRNA is expressed as a ratio of target to 18S rRNA and shown relative to human brain reference RNA; *p<0.05 compared to monocultures]. (c) Effect of culture time on Glut1 expression in monocultures of ECs and HAs [ *p<0.05 compared to day 1].

### 3.3 Differential Effect of Astrocytes on γ-GTP Expression

γ-GTP is one of the most widely used markers of BBB [6]. In vivo, γ-GTP marks brain ECs and pericytes [21, 22]. In fact, the major source of brain γ-GTP is the perivascular cells [22]. We analyzed mRNA expression of γ-GTP in our cultured cells and show that ECs as well as HAs express this BBB-specific enzyme (Fig. 4a). mRNA levels of γ-GTP were lower in HUVECs as compared to HDMECs (Fig. 4a). However, co-culture of HUVECs with HAs significantly increased the expression of γ-GTP within 24 hours and this induction was retained at day 7 (Fig. 4b). In contrast, γ-GTP expression was significantly lower in HDMEC-astrocyte co-culture at 24 hours but the levels were comparable to monocultures at day 7 (Fig. 4a and 4b). We next determined the effect of long-term culture. Only HUVECs cultured for 7 days increased mRNA expression of γ-GTP in the absence of astrocytes (Fig. 4c).
except for B in the absence of astrocytes. We also analyzed protein expression and localization in ECs by immunocytochemistry and observed expression of CD147, Glut1, and γ-GTP in monocultures (Fig. 5). There was also no change in the subcellular localization of γ-GTP expression in endothelial cells

\[ \text{γ-GTP expression in endothelial cells} \]

γ-GTP mRNA expression in monocultures and co-cultures after 1 (a) and 7 days (b) [mRNA is expressed as a ratio of target to 18S rRNA and shown relative to human brain reference RNA; *p<0.05 compared to monocultures]. (c) Effect of culture time on γ-GTP expression in monocultures of ECs and HAs [*p<0.05 compared to day 1].

3.4 Expression of BBB Marker Proteins in Endothelial Cells

Our gene expressions studies have shown a varied response of astrocytes on cultured non-brain ECs. More importantly, both HDMECs and HUVECs expressed three commonly used markers of BBB in the absence of astrocytes. Except for Glut1, the mRNA levels were comparable to the reference human brain mRNA. We also analyzed protein expression and localization in ECs by immunocytochemistry and observed expression of CD147, Glut1, and γ-GTP in monocultures (Fig. 5). There was also no change in the subcellular localization of these proteins with varying time in culture (data not shown). In addition to these three markers of BBB, we also examined the expression pattern of AHNAK. AHNAK (also known as neuroblast differentiation-associated protein, and/or desmoyokin) is an important signalling molecule involved in neuronal cell differentiation and is selectively expressed in ECs exhibiting barrier properties [13]. Within BBB ECs, AHNAK associates with tight junction...
Induction of BBB marker expression in ECs using astrocytes has been heavily studied. AHNAK reactivity localized to cell membranes, which is consistent with its function at the tight junctions. Similar results were obtained for HUVECs (data not shown).

![Fig. 5. Expression of BBB protein in endothelial cells](image)

Expression of AHNAK/desmoyokin, CD147 (neurothelin), γ-GTP, and Glut1 in HDMEC monocultures as detected by AlexaFluor 488-tagged secondary antibodies (green; right panel; 20x magnification). Slides were counterstained with DAPI (blue). Left panel shows high power images (40 x magnification). AHNAK and Glut1 were predominantly localized to the cell membrane. CD147 localized to the cytoplasm and cell membrane. γ-GTP immunoreactivity was seen in the cytoplasm.

4. DISCUSSION

In this study, we have demonstrated that non-brain derived ECs express markers selective to the BBB. Presence of astrocytes induced the expression of Glut1 and γ-GTP but not CD147. Induction of BBB marker expression in ECs using astrocytes has been heavily investigated since DeBault and Cancilla first discovered that rat glial cells are able to induce the expression of γ-GTP in mouse vascular ECs [23]. In a study by Hayashi and colleagues [2], culturing HUVECs and rat fetal brain astrocytes on opposite sides of a porous membrane revealed that γ-GTP and Glut1 are upregulated in HUVECs making physical contact with astrocyte end-feet. This upregulation is not as significant in HUVECs without physical interaction, suggesting astrocyte end-foot contact is responsible for the expression of BBB proteins in non-brain derived ECs [10]. In the same study, γ-GTP expression decreased in HUVECs following the removal of the astrocyte layer [2]. In the present study, we show that HUVECs and HDMECs express all BBB markers in culture. Except for Glut1, mRNA levels of these markers were comparable to normal brain RNA sample. We also show that the
inductive effect of astrocytes may be time-dependent especially for Glut1. The reason for the discrepancy between our study and the previous Hayashi and colleagues work may be the cell source and some experimental conditions. For example, fetal rat brain astrocytes were used in the previous study alongside human ECs, whereas we have used human astrocytes and human ECs. In addition, we performed all of our experiments in passage 2-6 cells as compared to 8-14 in the previous study.

Astrocytes assist in the formation of tight junctions between ECs in vitro [24, 25]. Tight junctions are essential to the impermeability of the BBB. As well, increased electrical resistance across EC co-cultured with astrocytes indicate impermeability of these ECs to ions [2, 26]. In our study, non-brain derived ECs expressed AHNAK, which is associated with tight junctions in brain ECs exhibiting barrier properties. We analyzed the expression in both monocultures (absence of astrocytes) and in co-cultures. The co-culture system consisted of a layer of ECs cultured on top of an established layer of human astrocytes. Because astrocytes and ECs were not separated, AHNAK expression in HA could not be differentiated from that in ECs in co-culture systems. However, monocultures revealed robust expression in ECs. This was in accordance with our RT-PCR data, which shows that HUVECs and HDMECs express all BBB markers.

Further research is required to understand the effects of astrocyte co-culturing on ECs. A potential limitation of our study, which needs to be addressed, is the quantitative assessment of BBB marker protein levels. Although, we have determined the localization by immunocytochemistry, western blot analysis may be necessary to show that all BBB marker proteins are expressed in non-brain derived ECs and the potential induction of these proteins during long-term culture as well as astrocyte co-culture. These studies can then be expanded to include human brain-derived ECs. We have compared our results to human brain tissue and show that the BBB marker level in culture is similar to human brain tissue and it would be critical to determine whether these levels correspond to levels seen in brain derived ECs in culture. These studies may suggest a ‘cut-off’ level and may illustrate whether the astrocyte mediated induction level we see in our study is of significance. Rodent astrocytes and human ECs could also be used to explore cross-species induction between animal and human cells as shown by Hayashi and colleagues [2]. Studies have shown that rat-derived astrocytes induce the expression of BBB properties in porcine ECs [11], human ECs [25], and bovine ECs [26], suggesting the induction signal is conserved [2]. Such cultures would allow the use of species-specific primers to amplify mRNA derived for only ECs, as well as species-specific BBB protein primary antibodies to differentiate EC and astrocyte signals; however, antibody cross-reactivity may limit this objective. Additionally, functional tests would allow for the comparison of ECs before and after co-culturing. For example, quantifying EC permeability may show changes in ion transport (measured by electrical resistance) and organization (i.e. tight junction formation), and suggest a possible correlation with the upregulation of BBB marker proteins.

5. CONCLUSION

We have demonstrated that BBB marker proteins are expressed in non-brain derived ECs. We have also shown that astrocytes are capable of upregulating the expression of Glut1 and γ-GTP in HUVECs but only Glut1 in HDMECs. Further studies are needed to understand the barrier properties of ECs. Our studies have shown that astrocytes alone do not provide sufficient inductive signals. Other sources of these signals regulating EC phenotype may include the basement membrane and the pericytes.
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COMPETING FINANCIAL INTERESTS

All authors confirm that there is no competing financial interest.

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