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Limitations of the hCMEC/D3 Cell Line as a Model for Aβ Clearance by the Human Blood-brain Barrier

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Abstract: Alzheimer's disease and cerebral amyloid angiopathy are characterized by accumulation of amyloid-β (Aβ) at the cerebrovasculature due to decreased clearance at the blood-brain barrier (BBB). However, the exact mechanism of Aβ clearance across this barrier has not been fully elucidated. The hCMEC/D3 cell line has been characterized as a valid model for the BBB. In this study we evaluated the use of this model to study Aβ clearance across the BBB, with an emphasis on brain-to-blood directional permeability. Barrier integrity of hCMEC/D3 monolayers was confirmed for large molecules in both the apical to basolateral and the reverse direction. However, permeability for smaller molecules was substantially higher, especially in basolateral to apical direction, and barrier formation for Aβ was completely absent in this direction. In addition, hCMEC/D3 cells failed to develop a high TEER, possibly caused by incomplete formation of tight junctions. We conclude that the hCMEC/D3 model has several limitations to study the cerebral clearance of Aβ. Therefore, the model needs further characterization before this cell system can be generally applied as a model to study cerebral Aβ clearance.

Key words: amyloid-β; blood-brain barrier; clearance; hCMEC/D3; *in vitro* model

INTRODUCTION

Alzheimer's disease (AD) is the most common neuropathological disease among elderly. Pathologically, AD is characterized by accumulation of the amyloid-beta (Aβ) protein and Aβ-associated proteins in extracellular plaques, hyperphosphorylated tau protein in the form of intracellular neurofibrillary tangles and wide-spread neuronal loss (LaFerla and Oddo, 2005; Selkoe, 1991; Timmer et al., 2010a). In addition, in approximately 80 percent of AD patients, accumulation of Aβ is also seen in the cerebral blood vessels (Kumar-Singh, 2008; Rensink et al., 2003). This cerebral amyloid angiopathy (CAA) of the Aβ type can severely affect the integrity of blood vessel walls, which often results in small or larger intracerebral bleedings and eventually may lead to hemorrhagic stroke.

Brain levels of Aβ are determined by the balance between local cerebral production, possibly in combination with influx from the peripheral circulation, and clearance of the protein from the brain. Whereas in familial AD production levels of Aβ are clearly increased due to mutations in genes involved in Aβ production, this is not the case for patients with the sporadic form of AD (Bali et al., 2012). It is conceivable that a disturbance of the balance between production and clearance of the Aβ protein towards decreased clearance, is the cause of development of sporadic AD (Mawuenyega et al., 2010).

Clearance of Aβ from the brain can take place via multiple pathways (reviewed by (Miners et al., 2011; Sagare et al., 2012)). One of these pathways is receptor mediated transport of Aβ across the blood-brain barrier

SIGNIFICANCE

Faulty amyloid-β (Aβ) clearance across the blood-brain barrier (BBB) attributes to the pathogenesis of Alzheimer's disease and cerebral amyloid angiopathy. For further elucidation of these clearance mechanisms, proper *in vitro* models are necessary. These models should especially be suitable to measure Aβ transport in the brain-to-blood direction. The hCMEC/D3 cell line has been used as a valid BBB model, but has hardly been characterized for transport in the brain-to-blood direction. By doing so, we observed certain limitations of the hCMEC/D3 cell line for this purpose and we further strengthen the need for the development of new *in vitro* BBB models.

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(BBB) into the systemic circulation. The accumulation of Aβ in CAA is likely a result of impaired clearance across the BBB, emphasizing the role of receptor mediated clearance of Aβ. At the capillary level the BBB is composed of highly specialized endothelial cells supported by pericytes and astrocytes (Zlokovic, 2011). The specialized endothelial cells form tight junctions with neighboring endothelial cells. By forming these tight junctions, passive transcytosis, as occurs in systemic blood vessels, is almost absent at the BBB. With the exception of small lipid-soluble compounds which can passively cross the BBB, other compounds can only pass the intact BBB by active transport. Several receptors on the BBB have been implicated in Aβ clearance, the best known are low-density lipoprotein receptor related protein-1 (LRP1) for the transport from brain to blood and the receptor for advanced glycation end products (RAGE) for transport from blood to brain (Candela et al., 2010; Deane et al., 2003; Deane et al., 2004; Wilhelmus et al., 2007). Several other receptors, such as megalin, P-glycoprotein (P-gp) and other members of the ATP-binding cassette (ABC) transporter family may also be involved in this bidirectional transport of Aβ (Cirrito et al., 2005; Elali and Rivest, 2013; Zlokovic et al., 1996).

We aimed to validate an *in vitro* transport model for the human BBB to study the transport mechanisms of Aβ across the BBB. The hCMEC/D3 cell line has previously been developed to serve as a model for the human BBB (Weksler et al., 2005). This model is most frequently used for transport studies in the apical to basolateral direction (blood-to-brain) and has been applied to Aβ transport as well (Andras et al., 2010; Andras et al., 2008; Tai et al., 2009). However, to study cerebral Aβ clearance, the basolateral to apical (or brain-to-blood) transport is more relevant. Therefore, we evaluated the use of this hCMEC/D3 cell line as a model to characterize the transport of Aβ across the BBB in the brain-to-blood direction.

Materials and Methods

Aβ Solutions. Aβ42 labeled with HiLyte-488 (Anaspec) was dissolved in DMSO at 410 μM and stored at -80 °C. Non-labeled Aβ42 (21st Century Biochemicals) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Sigma-Aldrich Chemie BV), which was evaporated overnight. Subsequently, peptide films were dissolved in DMSO to five mM stock solutions and stored at -80 °C. Further dilutions in assay culture medium were made directly before use.

hCMEC/D3 Cell Culture. The hCMEC/D3 cell line was obtained under license from INSERM. Culture flasks were first coated with collagen-I (150 μg/ml in PBS, for a minimum of one hour). Cells (passage 28 to 35) were cultured to confluence in EBM2 basal medium (Lonza Clonetics, Basel, Switzerland) supplemented with FBS (5 percent), hydrocortisone (1.4 μM), ascorbic acid (5 μg/ml), chemically defined lipid concentrate (1 percent), human bFGF (1 ng/ml), HEPES (10 μM) and antibiotics. Medium was changed every 2-3 days. Alternatively, hCMEC/D3 cells were cultured in EBM2 basal medium supplemented with EGM-2 MV SingleQuots (Lonza) according to guidelines by the manufacturer.

Dextran Permeability of hCMEC/D3 Monolayers. For permeability experiments hCMEC/D3 cells were cultured on transwell inserts (24-well, 0.4 μm pores, PET, Corning) in assay medium (EBM2 basal medium supplemented only with 2 percent FBS, 10 μM HEPES, 1.4 μM hydrocortisone, 0.5 ng/ml bFGF). Cells were grown to confluence for six days and medium was changed after three days. Under these culture conditions, cells formed a monolayer. Fluorescently labeled substrates were added to the cells in phenol red-free EBM2 basal medium (without supplements) to eliminate interference with the fluorescence detection method. For barrier integrity measurements in the apical to basolateral direction fluorescently labeled 70 kDa dextran (Sigma-Aldrich) or 4 kDa dextran (Sigma-Aldrich) at a final concentration of 1 μM, was added to the apical compartment (total volume of 100 μl). Samples (50 μl) were taken at given time points (30 min – six hours) from the basolateral chamber (total volume of 600 μl) and volume of phenol red-free medium was added to compensate for reduction in volume due to sampling. For barrier integrity measurements in the basolateral to apical direction, fluorescently labeled dextran (70 kDa or 4 kDa), at 1 μM final concentration, was added to the basolateral chamber and samples (10 μl) were taken from the apical chamber with addition of phenol red-free medium to compensate for reduction in volume due to sampling.

To assess the effect of Aβ toxicity on barrier integrity, 1 μM fluorescently labeled 70 kDa dextran was added in combination with 1 μM non-labeled Aβ42 in the basolateral chamber, followed by quantification of dextran permeability in the basolateral to apical direction.

All experiments were performed in duplicate and transwell inserts without cells were included as control for membrane interference. Fluorescence of the samples was measured using a Fluorstar reader with excitation at 492 nm and emission at 520 nm (BMG Labtech). Repeated measures two-way ANOVA with Bonferroni's *post hoc* test was used to compare the mean fluorescent signal in the receiving chamber using Prism 5 for windows.

The apparent permeability of the endothelial monolayer (Pe) was calculated as described (Weksler et al., 2005). In brief, Pe was determined as the permeability surface area product (Ps) per surface area (cm^2) (Pe (cm/min) = Ps/s), with: $1/Ps = 1/me - 1/mf$, and me is the slope of the cleared volume plotted against time for filters containing endothelial cells; mf is the slope of the cleared volume plotted against time for filters without endothelial cells.

To study dextran internalization by the hCMEC/D3 cells, cells were lysed in RIPA-buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1.0 percent Nonidet P40, 0.5 percent sodium deoxycholate, 0.1 percent SDS and protease inhibitors) and fluorescence was measured as described above.

TABLE I. Primary Antibodies Used

Antigen	Description of Immunogen	Source, Host Species, Cat. #, Clone or Lot#, RRID	Concentration Used
ZO-1	Human recombinant ZO-1 fusion protein encompassing amino acids 334-634	Source: ThermoFisher Scientific Host Organism: Mouse Clonality: Monoclonal Cat.# : 339100 Lot#: 1100420A RRID: AB_2533147	10 μg/ml
Claudin-5	Synthetic peptide (the amino acid sequence of the immunogen is considered to be commercially sensitive by the manufacturer)	Source: Abcam Host Organism: Rabbit Clonality: Polyclonal Cat. #: Ab15106 Lot#: GR234078-2 RRID: AB_301652	5 μg/ml
Mouse IgG	Mouse IgG gamma 1	Source: ThermoFischer Scientific Host: Goat Clonality: Polyclonal Conjugate: Alexa Fluor 488 Cat.#: A21121 Lot#: 1704461 RRID: AB_2535764	10 μg/ml
Rabbit IgG	Gamma Immunoglobins (Heavy and Light chains)	Source: ThermoFischer Scientific Host: Goat Clonality: Polyclonal Conjugate: Alexa Fluor 488 Cat:#: A11034 Lot#: 1737902 RRID: AB_2576217	10 μg/ml

Aβ Permeability of hCMEC/D3 Monolayers. Aβ permeability experiments were performed as described for dextran permeability experiments in both directions. Instead of dextran, fluorescently labeled Aβ42 (1 μM) or diluted DMSO as a vehicle control, was added to either the apical or basolateral chamber. Repeated measures two-way ANOVA with Bonferroni's *post hoc* test was used to compare the mean fluorescent signal in the receiving chamber using Prism 5 for windows.

Transendothelial Electrical Resistance (TEER). Transwell inserts (Corning) were first coated with collagen-I. hCMEC/D3 cells were seeded onto transwell inserts at 25,000 cells/cm^2 and grown to confluence (approximately five to six days). Transendothelial electrical resistance (TEER) was measured in duplicate inserts using the STX01 electrode of the Millicell-ERS Volt-Ohm meter. TEER was measured on day three – six of culture. Repeated measures ANOVA with Bonferroni's *post hoc* test was used to compare the mean TEER values using Prism 5 for Windows.

Aβ Toxicity Towards hCMEC/D3 Cells. Aβ toxicity was determined using an MTT assay. In short, hCMEC/D3 cells were grown to confluence in collagen-I coated 96-wells plates, as described above. Duplicate wells were incubated with serum-free medium for a minimum of four hours. After incubation of the cells with 1 μM of Aβ42 or a vehicle control for 20 hours, MTT (Sigma-Aldrich) was added at a final concentration of 0.8 mg/ml and incubated for another four hours. After removal of the culture medium, MTT precipitates were dissolved in isopropanol containing 0.1 percent NP-40 and 3 mM HCl, followed by measurement of the absorbance at 560 nm using a 96-well absorbance reader (Tecan, Männedorf). Results were expressed as percentage of vehicle control. Statistical analysis for cellular toxicity comparing vehicle control and Aβ42 was performed using the student's t-test with Prism 5 for windows.

Immunocytochemistry of hCMEC/D3 Cells. Cells were grown to confluence as described above for permeability experiments. Cells were grown either on transwell inserts or on collagen-coated 8-well glass chamberslides (Lab-Tek, ThermoFisher Scientific). Cells were stained for the tight junction (associated) proteins zona occludens 1 (ZO-1) and Claudin-5 (see Table I for used antibodies). Cells were washed twice with warm PBS and then fixed for 10 minutes using two percent paraformaldehyde followed by permeabilization with 0.5 percent Tween for 20 minutes. Primary antibody was added for overnight incubation at 4 °C. Subsequently, cells were incubated with fluorescently labeled goat anti-mouse or goat anti-rabbit antibody (see Table I for used antibodies) for one hour at room temperature. All antibodies were diluted in PBS/0.1 percent BSA, which also served as the negative control. After each incubation, inserts or wells were extensively washed with PBS. Subsequently, the transwell membranes were cut out of the insert and placed on object slides. Slides were mounted in Fluoromount G containing DAPI (Southern Biotech) for nuclear staining,

TABLE II. Permeability Coefficients of the hCMEC/D3 Cell Line

	Permeability Coefficients ($\times 10^{-3}$ cm/min) (±SD)		
	A –> B	B –> A	*p-value*⋆
70 kDa dextran	0.13 (± 0.01)	0.29 (± 0.06)	
4 kDa dextran	0.37 (± 0.21)	1.71 (± 0.07)	
70 kDa dextran + Aβ42	n.d.	0.18 (± 0.002)	n.s. ($p = 0.13$)

Values are expressed as mean (± standard deviation).
⋆Mean permeability coefficients of 70 kDa dextran and 70 kDa dextran + Aβ were compared using a t-test.
Abbreviations: A→B, apical to basolateral direction. B→A, basolateral to apical direction. n.d., not determined; n.s., not significant.

followed by visualization using a fluorescent microscope (Leica, Wetzlar).

Results

Paracellular Dextran Leakage. The transwell insert system was used to separate a culture well into an apical and basolateral compartment representing the blood and brain compartment respectively. Since endothelial cells do not actively transport dextran across compartments, dextran permeability is often used to measure paracellular leakage of a BBB model. To confirm hCMEC/D3 cells do not internalize dextran, fluorescence was measured in hCMEC/D3 cell lysates after incubation with fluorescently-labeled dextran. Indeed, no fluorescent signal was detectable in these lysates for up to 24 hours of incubation with dextran (data not shown).

To study the barrier integrity of hCMEC/D3 cells we used both 70 kDa dextran and 4 kDa dextran. Permeability of dextran was determined in the apical to basolateral direction (A→B) for both 70 kDa and 4 kDa dextran. The paracellular leakage for 4 kDa dextran was substantially larger than for 70 kDa dextran. This difference in efficacy of the hCMEC/D3 barrier towards the two dextran preparations is reflected in the Pe as shown in Table II ($Pe_{70kDa\ A\rightarrow B}$: 0.13×10^{-3} cm/min compared with $Pe_{4kDa\ A\rightarrow B}$: 0.37×10^{-3} cm/min).

However, since the brain-to-blood transport is more relevant concerning Aβ clearance we measured paracellular leakage of dextran in the basolateral to apical direction (B→A). We also observed an intact barrier in the basolateral to apical direction when applying high-molecular weight dextran (70 kDa) for at least six hours (Figure 1A). Only a minor increase in fluorescent signal in the apical chamber was observed for the inserts containing hCMEC/D3 cells compared to empty inserts indicating a tight barrier formation under these conditions ($p = 0.0019$). Even though barrier formation appeared to be successful, permeability was higher than the apical to basolateral paracellular leakage, as indicated by the Pe values shown in Table II ($Pe_{70kDa\ A\rightarrow B} = 0.13$ vs. $Pe_{70kDa\ B\rightarrow A} = 0.29 \times 10^{-3}$ cm/min).

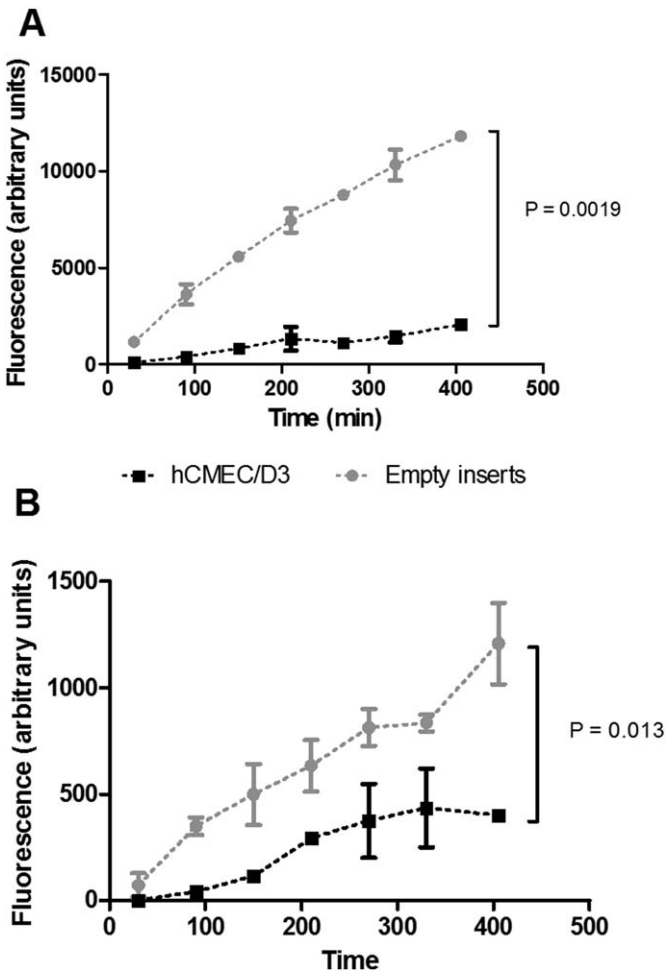

Fig. 1. Dextran Permeability of the hCMEC/D3 Cell Line in Basolateral to Apical Direction. hCMEC/D3 cells were cultured in the presence of 70 kDa (A) or 4 kDa (B) fluorescently labeled dextran (1 μM) in the basolateral chamber. Fluorescence was measured in the apical chamber at given time points. Barrier formation was observed for 70 kDa dextran (A) when comparing empty inserts with inserts containing hCMEC/D3 cells ($p = 0.0019$). For dextran with a size of 4 kDa (B) this barrier formation is substantially lower when comparing empty inserts with inserts containing hCMEC/D3 cells ($p = 0.013$). Bars indicate standard deviation. Repeated measures Two-way ANOVA with Bonferroni's *post hoc* test was used to compare the mean fluorescent signal in the receiving chamber using Prism 5 for windows. Experiments were performed in duplicate wells, representative figure of > 10 experiments.

Since Aβ is a small protein we also tested the paracellular leakage for small molecules in the basolateral to apical direction. Hereto, we used the 4 kDa fluorescently labeled dextran, similar in size to Aβ. Even though there was a significant difference in fluorescent signal between inserts containing cells and those that did not ($p = 0.013$) (Figure 1B), leakage was considerably larger for 4 kDa dextran compared to 70 kDa. As shown in Table II, paracellular leakage in the basolateral to apical direction was approximately 4.5-fold higher than the apical to basolateral leakage ($Pe_{4\ kDa\ A\rightarrow B}$: 0.37×10^{-3} cm/min

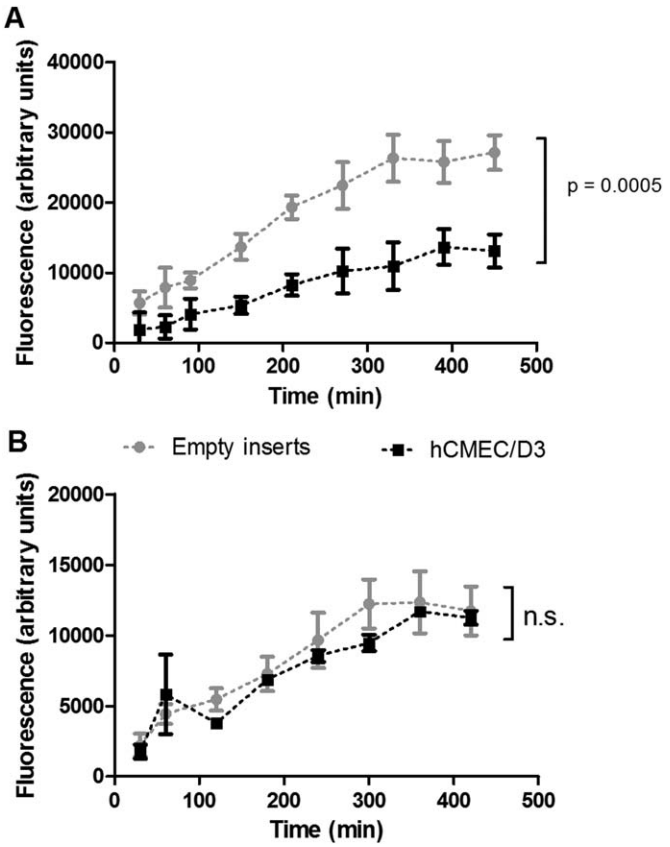

Fig. 2. Permeability of Aβ42 Across the hCMEC/D3 Barrier. Passage of Aβ42 across the hCMEC/D3 cell barrier was measured in apical to basolateral (A) and basolateral to apical (B) direction. While barrier formation was present in the apical to basolateral direction ($p = 0.0005$), no functional barrier formation of hCMEC/D3 cells for Aβ42 was observed in basolateral to apical direction when comparing empty inserts (grey) with hCMEC/D3 monolayer containing inserts (black). Bars indicate standard deviation. Repeated measures Two-way ANOVA with Bonferroni's *post hoc* test was used to compare the mean fluorescent signal in the receiving chamber using Prism 5 for windows. Experiments were performed in duplicate wells, representative figure of > 10 experiments.

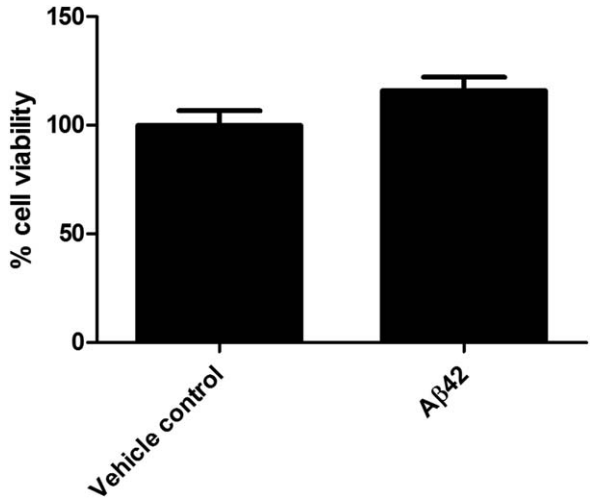

Fig. 3. Toxicity of Aβ Towards hCMEC/D3 Cells. Toxicity of 1 μM of Aβ42 to hCMEC/D3 cells was measured using an MTT assay. Cells were incubated with Aβ for 20 hours. When compared to the vehicle control, no toxic effect of 1 μM Aβ42 was observed. To compare the toxicity between conditions a t-test was performed using Prism 5 for Windows. Experiments were performed in duplicate wells, representative figure of 4 experiments.

compared with $Pe_{4\ kDa\ B\rightarrow A}$: 1.71 x$10^{-3}$ cm/min for 4 kDa dextran). Culturing the cells in EGM2-MV SingleQuots supplemented medium did not improve barrier formation for 4 kDa dextran (data not shown).

Aβ Permeability. Permeability of the hCMEC/D3 barrier was also determined for Aβ. Barrier formation for Aβ42 in the apical to basolateral direction was observed, indicating the cells are indeed capable of regulating passage of Aβ to some extent in this direction (Figure 2A, $p = 0.0005$). However, in contrast, and also in contrast to the limited barrier formation seen for 4 kDa dextran in the basolateral to apical direction, we did not observe a functional barrier for Aβ42 in the basolateral to apical direction; i.e., a monolayer of cells cultured on the membrane did not alter the amount of Aβ appearing on the apical side compared to the empty membrane (Figure 2B, $p =$ n.s.). There was no indication that the displacement of Aβ to the apical side was caused by transport of Aβ since no intracellular fluorescent signal could be detected using fluorescent microscopy (data not shown). Finally, western blotting analysis confirmed that the Aβ peptide preparations added to cell cultures were in monomeric form (data not shown), which excludes aggregation of Aβ as a cause for this non-functional barrier for Aβ42.

Aβ Toxicity. Since it is known that Aβ is toxic to cerebrovascular cells we hypothesized that Aβ may disrupt the barrier in our model, resulting in the limited *in vitro* barrier formation. Therefore, we added non-labeled Aβ42 to the basolateral compartment in combination with 70 kDa fluorescently labeled dextran. As shown in Table II, Aβ42 did not increase the paracellular passage of 70 kDa dextran when compared to passage of 70 kDa dextran alone ($p = 0.13$), indicating that Aβ did not disrupt barrier function at the concentration used for transport experiments. In addition, we performed an MTT toxicity assay to determine the possible toxic effect of 1 μM Aβ42 on the hCMEC/D3 cells, but cell viability was not decreased upon treatment with Aβ compared to vehicle control (Figure 3, $p > 0.05$.). These results indicate that lack of barrier formation for Aβ peptides is not caused by toxicity of Aβ towards cerebrovascular cells.

Barrier Integrity. In addition to dextran permeability we measured TEER as an indicator of barrier integrity. TEER was expected to develop over time when the hCMEC/D3 cells become more confluent and form monolayers with tight junctions between neighboring cells. However, TEER of the hCMEC/D3 monolayer was only slightly higher compared to transwell inserts without cells and reached 6.8 $\Omega \cdot cm^2$ on day 5

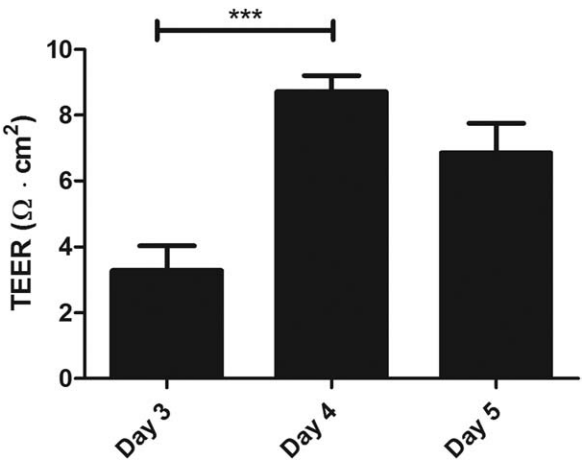

Fig. 4. Development of TEER by hCMEC/D3 Cells. TEER measurement in hCMEC/D3 cells (cultured at 25,000 cells/cm^2, reaching confluence on day 5). hCMEC/D3 cells developed a higher TEER when cultured up to 5 days in transwell inserts (TEER = 6.8 Ω·cm^2, corrected for values of empty inserts). In addition, no increase in TEER was observed when cells grow from non-confluent monolayers (day 3: 3.3 Ω·cm^2) to confluent monolayers (day 5: 6.8 Ω·cm^2). While the increase in TEER from day 3 to day 4 did reach statistical significance (day 4: 8.7 Ω·cm^2), an increase of approximately 5 Ω·cm^2 was not considered as a relevant increase in TEER. Repeated measures one-way ANOVA with Bonferroni's *post hoc* test was used to compare the mean TEER values: ***, $p < 0.001$. Experiments were performed in duplicate wells, representative figure of 4 experiments.

(Figure 4). In addition, TEER was also not substantially increased with increasing confluence from day 3 (3.3 Ω·cm^2) and day 4 (8.7 Ω·cm^2) till day 5 (confluent monolayers, 6.8 Ω·cm^2). Longer culture of the cells up to 10 days did not improve TEER and the formation of multiple cell-layers was often observed after more than 5 days of culture in complete culture medium (data not shown).

Tight Junction Formation of hCMEC/D3 Cells. One explanation for the absence of TEER and a barrier by hCMEC/D3 cells for small molecules, in particular Aβ, could be the lack of (functional) tight junction formation by these cells. We therefore stained the cells for expression of ZO-1 and Claudin-5, both tight junction (associated) proteins which are required for the formation of a functional tight junctions. hCMEC/D3 cells grown on glass 8-well chamberslides showed expression of ZO-1, resembling the formation of tight junctions (arrows, Figure 5A); ZO-1 expression was expressed lining the perimeter of the hCMEC/D3 cells. It should be noted though that this staining was not consistent throughout the whole cell culture, and cytoplasmic staining of ZO-1 was also observed. Claudin-5 expression of cells grown on glass chamberslides was mainly present intracellularly under these culture conditions (Figure 5C). Interestingly, when hCMEC/D3 cells were grown on transwell membranes staining of tight junction was even less apparent. While some punctated expression of ZO-1 was observed lining the perimeter of the hCMEC/D3 cells (arrow heads, Figure 5B), cytoplasmic expression was abundantly observed (Figure 5B). Claudin-5 was also found intracellularly when grown on transwell membranes, comparable to the cells grown on glass chamberslides (Figure 5D). It should be noted that the staining result on transwell membranes was independent of incubation with Aβ. Omission of the primary antibodies resulted in no staining indicating staining for either protein was specific (Figure 5, insets). The absence of ZO-1 and Claudin-5 expression on hCMEC/D3 cell membranes could indicate complete or partial failure of tight junction formation when these cells are cultured on transwell inserts.

DISCUSSION

The hCMEC/D3 cell line has become widely used as a model for the human BBB and its barrier formation has been characterized by multiple groups (Alabanza and Bynoe 2012; Markoutsa et al., 2011; Poller et al., 2008; Weksler et al., 2005), however mainly in the apical to basolateral direction. Furthermore, a high permeability to small molecules appears to be a limitation (Boyer-Di Ponio et al., 2014). In this study we therefore evaluated the use of the hCMEC/D3 cell line as a model for studying cerebral Aβ clearance across the human BBB, thus in the basolateral to apical direction. We showed that, while the hCMEC/D3 cells formed a reasonable barrier for molecules with a high molecular weight, this barrier formation was less efficient for smaller molecules, especially for Aβ. We showed that this lack of barrier formation for Aβ was not caused by Aβ-induced cell toxicity, but might be explained by incomplete formation of tight junctions between the hCMEC/D3 cells in this model. Therefore, we concluded that this model is less suitable to study the mechanism of clearance of Aβ from the brain across the BBB.

Restricted permeability in the apical to basolateral direction for compounds with MW > 4 kDa has been described for the hCMEC/D3 cells (Weksler et al., 2013). Permeability to 4 and 70 kDa dextran is usually presented in the form of a permeability coefficient (Pe). Using both a graphical representation and using calculated Pe values for the permeability of 4 and 70 kDa dextran, it can easily be seen that, as expected, barrier formation was more efficient for larger molecules than for smaller molecules. However, the permeability for large molecules in the basolateral to apical direction was substantially higher in our study than previously observed. For comparison, we determined the Pe value (based on passage of 70 kDa dextran) to be 0.13×10^{-3} cm/min, whereas Pe values as low as 0.013×10^{-3} cm/min have been described for the hCMEC/D3 cell line (Poller et al., 2008; Weksler et al., 2005). It should be noted that in these studies higher concentrations of dextran were used. When determining permeability for smaller molecules (4 kDa) we found similar Pe values for the apical to basolateral direction as have been described (Weksler et al., 2013).

To study the cerebral clearance of Aβ using the hCMEC/D3 model, permeability in the opposite

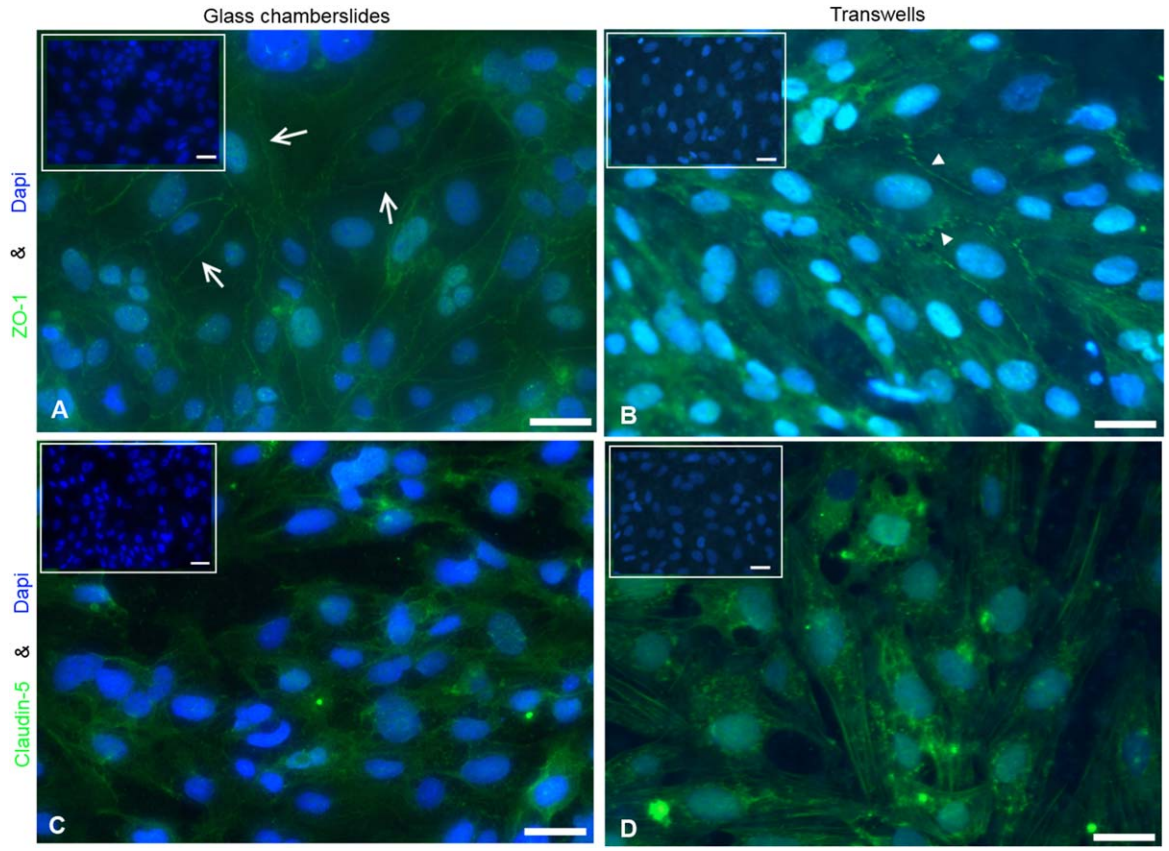

Fig. 5. Expression of ZO-1 and Claudin-5 by hCMEC/D3 Cells. hCMEC/D3 cells grown to confluence on glass chamberslides (A, C) or on transwell membranes (B, D) were stained for ZO-1 (green, A, B) or Claudin-5 (green, C,D). Nuclear counter-staining was performed using Dapi (blue). ZO-1 expression was expressed lining the perimeter of the hCMEC/D3 cells when grown on glass chamberslides (A, arrows). On transwell inserts the ZO-1 staining was mainly cytoplasmic with few tight junction-like patterns (B, arrowheads). Claudin-5 expression was mainly cytoplasmic for both culture conditions and did not clearly show cell membrane expression in a tight junction pattern (C, D). The absence of expression of both markers at the cell membrane indicates failure of tight junction formation when culturing hCMEC/D3 cells on transwell inserts. Omission of the primary antibody resulted in no staining for all conditions (insets). Scale bar = 25 μm. Representative image of four experiments.

direction, basolateral to apical, or 'brain-to-blood', is more relevant. For larger molecules permeability was only slightly (approximately two times) higher in this direction compared to the opposite direction. However, unexpectedly, permeability for small molecules was approximately 4.5-fold higher in the basolateral to apical compared to the apical to basolateral direction. Possibly, a differentiation in pore size in either direction due to the manufacturing process of the inserts is a cause of these discrepant findings. However, since experimental procedures were equal for both dextran preparations, it appeared that barrier formation of the hCMEC/D3 cells is highly direction-specific for small molecules. To our knowledge, permeability of dextran in the basolateral to apical direction has not yet been reported for the hCMEC/D3 cells. However, passage of radio-labeled sucrose across the hCMEC/D3 cell line has been used once as a measure for tightness of the barrier, and permeability has also been reported to be higher in the basolateral to apical direction than in the opposite direction (Poller et al., 2008). Since this non-receptor mediated passage of substances such as dextran and sucrose is a representation of the barrier function of cerebrovascular endothelial cells in the studied direction, we think it is more relevant to determine permeability in the basolateral to apical direction when using the hCMEC/D3 model to study cerebral Aβ clearance. The directional differences in permeability we found for small molecules highlight this importance.

Since clearance of Aβ from the brain to the blood is affected in AD and CAA, a well characterized and functional *in vitro* model for the study of this process is desired. The hCMEC/D3 cells have been characterized to express relevant receptors for Aβ transport, including LRP1 and RAGE (Andras et al., 2010; Andras et al., 2008). However, functional studies on the transport of Aβ are, to our knowledge limited, especially in the 'brain-to-blood' direction. One of the few studies in which the transport of radio-labeled Aβ across the

hCMEC/D3 cells was studied, showed involvement of the P-glycoprotein receptor in apical to basolateral transport of Aβ, but not in the basolateral to apical direction (Tai et al., 2009). Recently, another study demonstrated the ability of mixed Aβ monomers and oligomers to alter Aβ40 transport across hCMEC/D3 cells in the basolateral to apical direction (Qosa et al., 2014). However, it should be noted that in the former study, transport was only measured for a short period of time (i.e., 30 minutes), whereas the latter study only used a single endpoint as readout. In addition, baseline barrier formation in these studies was only determined in the apical to basolateral direction. We now showed that the hCMEC/D3 cell model does not seem suitable for Aβ clearance studies, since baseline permeability for smaller molecules, especially Aβ, was compromised in the basolateral to apical directions. In addition, our results showed that a single time point or a short time course to measure Aβ transport might not be sufficient to characterize the transport.

A complicating factor for measuring Aβ transport by BBB cells is the potential toxicity of Aβ towards cerebrovascular cells. Concentrations of Aβ of 10 μM and higher are toxic for hCMEC/D3 cells, cerebral smooth muscle cells and pericytes (Bruinsma et al., 2010; Carrano et al., 2011; Timmer et al., 2010b; Wilhelmus et al., 2007). In addition, 5 μM of Aβ40 increased the permeability of hCMEC/D3 cells for 70 kDa dextran, in the apical to basolateral direction (Tai et al., 2010). Therefore, we used a concentration of 1 μM of Aβ in our studies, which neither influenced the barrier formation for dextran nor reduced viability of the hCMEC/D3 cells. However, a consequence was that these lower concentrations of Aβ were near the detection limit of the fluorescent detection system, which could have increased the coefficient of variation of our Aβ measurements. Therefore, results may be improved by developing more sensitive detection methods in future studies. In addition, we would like to point out that the porosity of the transwell filters might affect transport of Aβ across the cells. In our current study, we have used transwell filters with 0.4 μm pores while, in contrast, Aβ transport across the BBB was successfully measured previously using transwell filters with 3.0 μm pores (Candela et al., 2010; Merino-Zamorano et al., 2016). However, these studies used primary cells of either murine or bovine origin for the *in vitro* BBB model and did not use hCMEC/D3 cells. Also, in the current study we focused on permeability of the hCMEC/D3 cells for Aβ42, since this is the most prevalent Aβ peptide in the brain. However, it would be interesting to determine if a similar effect is present for permeability of Aβ40.

TEER values are another indication of barrier integrity, and TEER is expected to increase with the formation of functional tight junctions between endothelial cells. In this study we showed that hCMEC/D3 cells develop a low TEER in the culture conditions used. While these results indicated a high ionic permeability for the obtained monolayer of cells, this has been observed previously for endothelial cells (Weksler et al., 2013). Our results confirmed previous findings where low TEER values for the hCMEC/D3 cell line have been found (ranging from 5 – 50 $\Omega \cdot cm^2$ corrected for empty transwell inserts) (Eigenmann et al., 2013; Weksler et al., 2013). Culturing the hCMEC/D3 cells for a longer period after reaching confluence to increase TEER is not possible since this results in overgrowth (Urich et al., 2012). We could confirm that, in our hands, this phenomenon started to appear even earlier (6 days after seeding). It has been reported that TEER of hCMEC/D3 cells can be (slightly) increased in the presence of astrocytes or with addition of extra hydrocortisone (Forster et al., 2008). However, the effect of this latter addition could not be reproduced by us (data not shown). A high increase in TEER (reaching levels of 1000-1200 $\Omega \cdot cm^2$) was found when hCMEC/D3 cells were cultured under shear-stress, indicating this may be an important factor in creation of proper barrier characteristics *in vitro* (Cucullo et al., 2008) and might also improve barrier formation for small molecules like Aβ.

Tight junctions are complex molecular structures that require multiple proteins to interact. Transmembrane proteins, adaptors and regulatory proteins are all needed to form a functional tight junction (Luissint et al., 2012). It has previously been shown that hCMEC/D3 cells express at least three tight junction specific genes, i.e. claudin-5, occludin and JAM2, which are strongly reduced in expression level when compared to primary mouse cerebrovascular endothelial cells (Urich et al., 2012). Another important tight junction associated protein required for functional tight junction formation is ZO-1. We neither observed an expression pattern of ZO-1 nor of Claudin-5 typical for tight junctions in hCMEC/D3 cells grown to confluence on transwell inserts. This is in accordance with a previous study that found low ZO-1 expression by hCMEC/D3 cells without co-culture conditions (Hsuchou et al., 2010). In contrast, culturing of the cells on glass chamberslides resulted in expression of ZO-1 in a tight junction associated pattern, e.g. lining the perimeter of the hCMEC/D3 cells. Other studies indeed report tight junction expression of ZO-1 (Liu et al., 2014; Lopez-Ramirez et al., 2012; Luissint et al., 2012; Sajja et al., 2014; Tai et al., 2010), and most of these studies were not performed on hCMEC/D3 cells grown on transwell membranes but rather on glass cover slips. This further underlines our observation that the hCMEC/D3 cells may show a different behavior on these transwell membranes. These findings might provide an explanation for the negligible barrier formation for small molecules, namely the lack of formation of fully functional tight junctions by hCMEC/D3 cells as indicated by the absence of ZO-1 and Claudin-5 in these tight junctions, but this would require further investigation. Permeability of the hCMEC/D3 cell monolayer was previously reported to decrease in the presence of tight junction-increasing substances, such as simvastatin, a HMG-CoA reductase inhibitor similar to lovastatin (Markoutsa et al., 2011). However, it has also been found that simvastatin increases expression of the Aβ receptors LRP-

1 and RAGE (Andras et al., 2010; Andras et al., 2008). Since these inhibitors are involved in cholesterol function and cholesterol is an important factor in Aβ metabolism, and since LRP-1 and RAGE may affect the transport of our protein of interest (Aβ), including these compounds in our model seemed inappropriate.

Overall, our findings indicate there might be yet unknown subtleties that limit the establishment of the hCMEC/D3 cell line as an *in vitro* BBB model to study cerebral Aβ clearance. While barrier formation of the hCMEC/D3 cell line for larger molecules appears to be appropriate, we showed that this characteristic is substantially less intact for smaller molecules, especially when protein transport is studied in the basolateral to apical direction. In addition, barrier formation did not appear to be functional for Aβ peptides in this direction. While we do not have an explanation for this directional difference in permeability, our study confirms previous observations that the hCMEC/D3 cell line might not be suitable for all studies concerning the BBB due to the relatively high permeability for small molecules (Boyer-Di Ponio et al., 2014) and that these aspects should be taken into account when selecting an *in vitro* model for permeability assessments. In addition, our study highlights the importance to specifically characterize the hCMEC/D3 permeability of Aβ further before applying the cell line as a widespread model of the BBB in AD and CAA research. It should however also be noted that the isolated use of the hCMEC/D3 cells as an *in vitro* BBB model is a limitation. Co-culture of hCMEC/D3 cells with other cells of the BBB, e.g. astrocytes or pericytes, or applying shear stress to the cells, is more reflective of the physiological BBB and might therefore be necessary to improve the barrier formation limitations of hCMEC/D3 cells we describe here.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

E.A.L.M.B designed and performed experiments, analyzed and interpreted the data and prepared the manuscript. L.J. designed and performed immunocytochemistry experiments. H.B.K, R.M.W.d.W and M.M.V co-designed experiments, interpreted the data, and supervised the project. All authors critically revised the manuscript. M.M.V raised funding for the project.

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