Immortalized Human Brain Endothelial Cell Line HCMEC/D3 as a Model of the Blood-Brain Barrier Facilitates In Vitro Studies of Central Nervous System Infection by Cryptococcus neoformans

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Cryptococcus neoformans cells must cross the blood-brain barrier prior to invading the central nervous system. Here we demonstrate that the immortalized human brain endothelial cell line HCMEC/D3 is a useful alternative to primary brain endothelial cells as a model of the blood-brain barrier for studies of central nervous system infection.

Cryptococcus neoformans is a fungal human pathogen that causes meningitis in a predominantly immunocompromised population (26). To invade the central nervous system (CNS), cryptococcal cells must cross the blood-brain barrier (BBB) (2). Despite efforts to understand the propensity of this pathogen for the CNS, little progress has been made in the past couple of decades (9, 18-20). The major reasons for this have been the inability to recapitulate the properties of the BBB in vitro and the many challenges posed by BBB studies using live animals (13, 21, 22, 24, 32, 35). Although commercially generated primary human brain microvascular endothelial cells (HBMEC) are now available for BBB studies, there are several disadvantages to developing models of the BBB using these cells. Mainly these primary cells are unstable after a limited number of passages, and they can be very expensive. The alternative, obtaining primary HBMEC from discarded brain tissues, is also undesirable since the process is labor-intensive and introduces variability from batch to batch (4, 8). To facilitate the study of the BBB in vitro, researchers have tried to develop human brain endothelial cell lines that retain critical features of primary cells, such as the expression of endothelial cell markers, transporters, and tight junctional proteins (1, 15, 23, 25, 27, 29, 30, 33, 34, 36). The recent development of one particular line of immortalized human brain endothelial cells (HCMEC/D3) that recapitulates many of the key characteristics of primary brain endothelial cells without the need to coculture with glial cells is proving to be a promising cell line for in vitro studies of the BBB (36). Indeed, the HCMEC/D3 cell line has already been successfully used as a BBB model in several studies, further attesting to its high quality and its potential to replace primary cells for in vitro BBB studies (10, 11, 12, 14-16, 28, 31, 37).

Here we show that the HCMEC/D3 cell line can serve as a useful in vitro model of the BBB to study the mechanisms used by C. neoformans to breach the brain endothelium and enter the CNS. In order to test the feasibility of this cell line as a BBB model to study the migration of C. neoformans across the BBB, a transcytosis assay was used. This assay consisted of a transwell apparatus with endothelial cells growing in rich endothelial growth medium (EGM-2; Lonza) on a collagen-coated porous membrane (8 μm; Bioscience). The medium was changed from 10% to 2.5% human serum. The number of cryptococcal cells that crossed the endothelial cell barrier (36). Since cell growth in 0.25-strength was very slow, this medium was not used. Also, the HCMEC/D3 barrier (36). Since cell division appeared to impair the permeability of the HCMEC/D3 barrier (36) and 35. HCMEC/D3 cells were seeded here between passages 25 and 35. HCMEC/D3 cells were seeded based on the growth area ratio. A confluent monolayer in a culture flask of 25 cm² was trypsinized and resuspended in 12 ml of medium. The ratio 12 ml/25 cm² (0.5 ml/1 cm²) was used to determine how much volume was needed to seed the insert. Seeding 500 μl was essentially the equivalent of adding 1 cm² of confluent monolayer to the transwell apparatus. A 50% seeding would mean using 250 μl of trypsinized suspension from a fully confluent monolayer to seed the transwell apparatus. Once added to the transwell apparatus, the HCMEC/D3 cells were cultivated for approximately 6 to 7 days at 37°C and 5% CO₂. Prior to starting transcytosis, the medium of both transwell chambers was changed to include 2.5% human serum. The medium was changed 1 day after seeding from 1× strength to 0.5× strength 24 h before the assay. The use of the lower-strength medium was required to reduce the growth factors in the medium as the cells reached confluence so that the monolayer could differentiate, rather than allowing individual cells to continually divide, since cell division appeared to impair the permeability of the HCMEC/D3 barrier (36). Since cell growth in 0.25×-strength medium is very slow, this medium was not used. Also, the multiplication rate of fungal cells in the lower chamber was not significant when reduced-strength medium (0.5×) was used and when CFU counts were taken prior to 24 h.

Cells of a wild-type strain of C. neoformans (H99; 1 × 10⁶ cells per well) were added to the top chamber, and the number of cryptococcal cells that crossed the endothelial cell barrier was monitored over time by taking aliquots (100 μl) from the

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crossed the endothelial cell monolayer significantly better than the acapsular strain. Cells to BBB crossing was examined using a wild-type encapsulated strain B3501 and its isogenic acapsular counterpart. The encapsulated strain significantly better than the negative control, that cryptococcal cells crossed the HCMEC/D3 monolayer significantly better than the capsular strain. *, results determined by t test \( (P < 0.05) \). Data are expressed as means ± standard deviations. The contribution of the capsule of \( C. neoformans \) cells to BBB crossing was examined using a wild-type encapsulated strain B3501 and its isogenic acapsular counterpart. The encapsulated strain crossed the endothelial cell monolayer significantly better than the acapsular strain. *, results determined by t test \( (P < 0.05) \). Data are expressed as means ± standard deviations. The interaction between CD44 and hyaluronic acid appears to be crucial for the adherence of \( C. neoformans \) to the brain endothelium, since either eliminating hyaluronic acid or knocking down CD44 drastically reduced the adherence of cryptococcal cells to the endothelium (3, 17, 19). Consistent with these studies, we also found that a strain of \( C. neoformans \) that lacked hyaluronic acid was not able to effectively cross the HBMEC/D3 cell barrier (data not shown).

To examine whether the integrity of the HCMEC/D3 barrier was compromised by \( C. neoformans \), we monitored transendothelial electrical resistance (TEER) of the HCMEC/D3 monolayer using an endometer (World Precision Instruments, Sarasota, FL) throughout the duration of the in vitro transcytosis assay in the presence or absence of cryptococcal cells (Fig. 1D). TEER values are routinely used to assess cell barrier integrity; however, these values are indicative only of a tight and functional barrier and are not conclusive (4, 8, 36). The final TEER value was obtained by subtracting the resistance of collagen-coated inserts from the resistance obtained in the presence of the endothelial cells. We found that the TEER values for the HCMEC/D3 monolayer in the presence of cryptococcal cells did not change over time and were not significantly different from those of control wells (Fig. 1D). Constant TEER values,
which are indicative of a restrictive barrier, suggested that the integrity of the HCMEC/D3 barrier was maintained, and they supported the prevailing hypothesis that C. neoformans can cross the brain endothelium transcellularly without causing significant damage to the endothelial cells, since a major loss of barrier integrity would result in a reduction of TEER values over time (4, 8). Although the HCMEC/D3 monolayer had lower TEER values (~60 Ω) than those reported for primary brain endothelial cells (~150 Ω), it has been clearly demonstrated that this feature of the immortalized cell line is independent from its ability to form a highly restrictive barrier (36). In addition, the TEER values do decrease when the endothelial barrier is intentionally disrupted, indicating that TEER values of HCMEC/D3 cells (although low) do reflect an intact and functional barrier (36). Extensive characterization of HCMEC/D3 using permeability of low-molecular-weight compounds (including [14C]sucrose, [14C]diazepam, [14C]morphine-6-glucuronide or [3H]inulin, [3H]imipramine, [3H]prazosin, [3H]colchicine, [3H]vincristine, or fluorescein isothiocyanate-labeled dextran [4, 40, and 70 kDa]) has shown that this cell line exerts better restriction than primary cultures of bovine brain endothelial cells and resulted in permeability values similar to those obtained for primary cultures of human brain endothelium (4, 8, 36). This is likely because the HCMEC/D3 cell line retains all major functional features of primary brain endothelial cells, including the expression of tight junctional proteins at the cell borders (36). Accordingly, we confirmed the expression of transcripts in the HCMEC/D3 cell line (in the absence of cryptococcal cells) for two additional key junctional proteins, occludin 1 and claudin 7, and the surface glycoprotein receptor CD44 (Fig. 2). Taken together, these results indicate that the HCMEC/D3 cell line maintains an overall tight junction orga-
nization known to be present in the brain endothelium, which further supports the usefulness of HCMEC/D3 cells as an in vitro model of the BBB for cryptococcal studies.

Ultrastructural examination of brain endothelial cells infected with C. neoformans suggested that cryptococcal cells penetrated the HCMEC/D3 cell barrier, consistent with a transcellular movement of C. neoformans across the BBB (Fig. 3D and F) (4). Scanning electron microscopy (SEM) images revealed that HCMEC/D3 cells had undergone morphological changes that were observed only in the presence of cryptococcal cells and not when glass beads (20 to 50 μm; Sigma) were added to the endothelial cells (Fig. 3). Our results suggested that these changes in HCMEC/D3 cells were not due to a general stress reaction but rather occurred specifically in response to cryptococcal cells. Among the morphological changes that occurred was the production of microvilli on the surface of the endothelium (Fig. 3C to F, arrows). The microvilli could be seen attaching to and surrounding cryptococcal cells; however, microvilli were not observed anywhere near the glass beads (Fig. 3A). Interestingly, cryptococcal cells appeared healthier (more turgid and round) in the absence of the HCMEC/D3 cells (but still bathed in the EGM-2 medium), suggesting that perhaps the endothelium had created a hostile environment for C. neoformans (Fig. 3B). The SEM images also revealed the outline of cryptococcal cells just below the surface of the endothelial barrier, suggesting that these cryptococcal cells had been completely internalized by HCMEC/D3 cells, while others were only partially internalized (Fig. 3D and F, arrows). The morphological changes in the HBMEC/D3 cells reported here are consistent with similar changes observed in primary brain endothelial cells, further underscoring the tight similarity between the immortalized HCMEC/D3 cells used in this study and other primary cells (4, 36). Taken together, our results suggest that the HCMEC/D3 cell line is very well suited as a model of the BBB for resolving molecular mechanisms that determine how C. neoformans cells penetrate the brain endothelium and invade the CNS. This notion is supported by recent studies that have successfully used the HCMEC/D3 cell line as an in vitro BBB model to examine how bacterial and viral pathogens breach the BBB (10, 15).

In summary, we have shown that the recently developed immortalized brain endothelial cell line HCMEC/D3 is a suitable alternative to primary brain endothelial cells for in vitro studies of the BBB that will facilitate studies aimed at elucidating the invasion of C. neoformans into the CNS. Understanding its strong propensity for the CNS and its routes of entry will be essential for the identification of new targets required for future drug development aimed at treating CNS infection.

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