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Original Article

Neuroprotective effects of systemic cerebral endothelial cell transplantation in a rat model of cerebral ischemia

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Abstract: Human cerebral microvascular endothelial cell line (hCMEC)/D3 cells, which are from a stable clonal cell line of human immortalized cerebral endothelial cells, were intra-arterially transplanted through the common carotid artery in a rat model of photochemical-induced cerebral ischemia. Their therapeutic effects on infarct size, blood-brain barrier (BBB) breakdown, and outcome were examined. The hCMEC/D3 cells were genetically modified with the firefly luciferase gene for in vivo imaging post-transplantation. Transplanted hCMEC/D3 cells were identified in the infarcted brain by bioluminescence imaging at 1 day after transplantation. Compared with the control group, the hCMEC/D3-transplanted group showed reduced infarct size on day 3, reduced Evans blue dye leakage on day 1 indicating decreased BBB breakdown, and early recovery from Rotarod test neurological deficits. The hCMEC/D3-transplanted group also showed decreased levels of matrix metalloproteinase (MMP)-9, which were inversely correlated with TIMP-1 levels on post-transplantation days 1 and 3. The expression of tumor necrosis factor-α and interleukin-1β were markedly diminished in the hCMEC/D3-transplanted group compared with controls. The systemically transplanted cells selectively migrated and integrated into the ischemically lesioned area, which accelerated neurological recovery. This new cerebral endothelial cell-based therapy may hold promise for clinical trials in patients with ischemic stroke.

Keywords: Stroke, cell transplantation, cerebral endothelial cell, experimental neurorestoration

Introduction

Cerebrovascular disease is the leading cause of death and disability in most countries, including Korea. Clinicians and researchers are very interested in exploring more effective and safer treatments for patients with stroke [1]. In the last two decades, various studies in cell transplantation have provided strong evidence for the therapeutic benefits of cell transplantation in the treatment of stroke [2-6]. Several kinds of cell transplantation therapy eventually decrease brain infarct size, reduce blood-brain barrier (BBB) injury, and promote neurogenesis and neurological recovery [6-9]. The therapeutic potential of cell therapy in patients with stroke initially appears to be independent of cell differentiation and relies on multiple bystander mechanisms exerted by the transplanted cells to boost endogenous restorative mechanisms and modulate the injured microenvironment [10, 11].

Although the neuroprotective effects of endothelial precursor cells have been widely studied in cerebral ischemia models [12-14], endothelial cells, whether differentiated from embryonic stem cells [15] or cultured from cerebral microendothelial cells [16, 17], have not been similarly examined. Endothelial cells, a major component of the neurovascular unit, help maintain cerebral homeostasis [18]. Comparison of the brain vasculome to that of other organs has shown that endothelial gene expression patterns are highly organ-dependent [19, 20]. Human cerebral endothelial cells exhibit large

physiological and pharmacological differences from the stem-cell-like endothelial cells, such as human umbilical vein endothelial cells [21], and they stimulate the self-renewal of neural stem cells [22]. Moreover, the stereotaxic transplantation of human cerebral endothelial cells attenuates the behavioral and histological deficits that occur with vasculogenesis and neurogenesis at 7 days after middle cerebral occlusion in an animal model of stroke [17]. However, the role of transplanted cerebral endothelial cells in the attenuated effects of focal cerebral ischemia are not fully understood. Numerous mechanisms could be involved in the neuroprotective effects exhibited by cerebral endothelial cells following focal cerebral ischemia in rats. However, little is known about the role of transplanted cerebral endothelial cells in the ischemic brain apart from their induction of angiogenesis and neurogenesis [17].

In this study, we used immortalized cells from the human cerebral microvascular endothelial cell (hCMEC)/D3 line. This cell line, derived from microvessels of the human temporal lobe, has unique physiological and pharmacological properties that have been well established by various in vitro disease models [23, 24]. Here, we describe for the first time the diverse capabilities of systemically transplanted hCMEC/D3 cells in the enhancement of endogenous responses after focal cerebral ischemia.

Materials and methods

The human cerebral microendothelial cell line and culture

The hCMEC/D3 cells provided by Dr. Couraud [23] were cultured in an Endothelial Growth Medium-2 Bullet kit (Lonza Walkersville, Inc., Walkersville, MD, USA). The medium was changed the following and every other day thereafter until the cells were 80-90% confluent. All of the experiments were performed between passages 29 to 35.

Plasmid and virus production for in vivo imaging

For the bioluminescent imaging (BLI), the hCMEC/D3 cells were transduced with a pFU-FGW vector provided by Dr. Gambhir and containing a firefly luciferase (Fluc) gene. The pFU-FGW lentivirus was produced by the triple transfection of pFU-GFW, psPAX2, and pMD2.G in 293T cells with polyethylenimine (Polysciences, Inc., Warrington, PA, USA). Three hours after transfection, the medium was changed to Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and a penicillin/streptomycin solution. The virus-containing medium was harvested 48 h after transfection. The hCMEC/D3 cells were infected by overnight incubation in medium containing the pFU-FGW lentivirus and 8 μg/mL of Polybrene (Sigma-Aldrich Co. LLC, St. Louis, MO, USA). Stable cells were selected after treatment with 200 μg/mL of zeocin for 7 days.

The subjects and the photochemical-induced focal cerebral ischemia model

All of the surgical procedures and postoperative care were performed in accordance with the guidelines of the Chonnam National University Animal Care and Use Committee. A total of 120 male Sprague-Dawley rats (8 weeks old) weighing 250-300 g were maintained on a 12 h light/dark cycle. Focal cortical ischemia was induced by photothrombosis of the cortical microvessels as described in our previous report [25].

Cell transplantation

Rats were randomly assigned to two groups: phosphate-buffered saline (PBS)-treated and cell transplanted. The left common carotid artery was exposed and a single cell suspension of 1×10^6 hCMEC/D3 cells in 10 μL of saline was injected into the artery with a custom Hamilton syringe with a 33-gauge needle; a previously described method with some modifications [26]. Bleeding was controlled by using Gelfoam (Pfizer, New York, NY, USA) at the injection site until it ceased.

BLI

The BLI was performed with the In Vivo Imaging System 100 (PerkinElmer Inc., Waltham, MA, USA), a modified version of a previously described method [27]. Before imaging, D-luciferin (Promega Corporation, Madison, WI, USA) was dissolved in PBS and injected intraperitoneally at a dose of 100 mg/kg by body weight. BLI was performed at 12 h and 1, 3, and 7 days after transplantation. Living Image software (version 3.0, PerkinElmer Inc.) was used to quantify the maximum number of photons within the brain regions of interest.

Measurement of infarct volume

Triphenyltetrazolium chloride (TTC) (Sigma-Aldrich Co. LLC) staining was performed as previously described [28]. At 12 h and 1, 3, and 7 days after transplantation, brains (n = 5 per time point) were removed under deep anesthesia. Two-mm-thick coronal slices were incubated in PBS with 2% TTC at room temperature for 1 h and were then transferred to a 4% buffered paraformaldehyde solution for fixation. The area of brain damage over the entire ipsilateral hemisphere was measured with image processing software (ImageJ, http://imagej.nih.gov/ij/) and the total volume of infarct (mm^3) was calculated from the sum of the damaged volume (i.e., damaged area × thickness) of the brain slices.

Rotarod test

The rotarod test was performed by two investigators blind to group assignment for 8 weeks (n = 5 per group). Rats were placed on the rotarod cylinder and the length of time that each animal remained on the rotarod was measured, as previously described [28]. The speed of the rotarod was slowly increased from 4 to 40 rpm over a period of 5 min.

BBB permeability

Vascular permeability was quantitatively evaluated by the fluorescent detection of extravasated Evans blue dye, as previously described [29]. Briefly, disruption of the BBB was assessed at 6, 12, 24, and 72 h after transplantation (n = 5 rats per time point). Evans blue dye (2%, 4 mL/kg, Sigma-Aldrich Co. LLC) was injected for over 2 min into the left femoral vein. One hour after the injection, the brains were removed under deep anesthesia and coronal sections were divided by hemisphere. The brains were weighed and homogenized in a 10-fold volume of 50% trichloroacetic acid solution. The supernatant was obtained by centrifugation and diluted 4-fold with ethanol. The Evans blue extravasation was quantified in the resultant supernatant using spectrophotometry.

Gelatin zymography

Gelatinase activity was determined using a previously described protocol [29]. Briefly, 100 μg protein samples were prepared and mixed with an equal volume of sample buffer. Samples from the PBS-treated and hCMEC/D3-transplanted groups at 12 h and 1, 3, and 7 days post-transplantation (n = 4 per time point) were separated using Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 0.1% gelatin at 4°C. To visualize enzymatic activity, the gels were stained with 0.5% Coomassie Brilliant Blue R-250 for 30 min and then de-stained in methanol/acetic acid/water. An equal amount of protein from an HT1080 human fibrosarcoma was loaded as a positive control. The gelatinase activity was measured using a densitometric analysis of the gels.

Western blot

Western blot analyses of the matrix metalloproteinase (MMP)-2 and MMP-9 protein and tissue inhibitor of metalloproteinases (TIMP)-1 and TIMP-2 levels were performed as previously described, with some modifications [30]. The protein extracts were prepared from the ischemic hemisphere with the PRO-PREP™ Protein Extraction Solution (iNtRON Biotechnology, Gyeonggi-do, Korea). Samples containing 30 μg of protein were loaded into a 10% sodium dodecyl sulfate-polyacrylamide gel. Following electrophoresis, the gel was blotted and membranes were incubated for 1 h at room temperature in blocking buffer. The gel was then probed overnight at 4°C with one of the following primary antibodies: MMP-2 (1:1,000; Abcam plc, Cambridge, MA, USA), MMP-9 (1:1,000, Abcam plc), TIMP-1 (1:500; Cell Signaling Technology, Inc., Danvers, MA, USA), TIMP-2 (1:500; Cell Signaling Technology, Inc.), or β-actin (1:15,000; Sigma-Aldrich Co. LLC). The western blots were visualized using an enhanced chemiluminescence kit (ECL; GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) and the optical densities were measured using a Fujifilm LAS-3000 luminescent image analyzer (FUJIFILM Corporation, Tokyo, Japan).

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

RNA was obtained from the ischemic hemisphere using an RNeasy kit (QIAGEN Inc., Valencia, CA, USA) and the complementary DNA (cDNA) was synthesized with a GoScript™ Reverse transcriptase kit (Promega Corporation) in accordance with the manufacturer's instructions. Real-time qRT-PCR was conducted for tumor necrosis factor (TNF)-α, interleu-

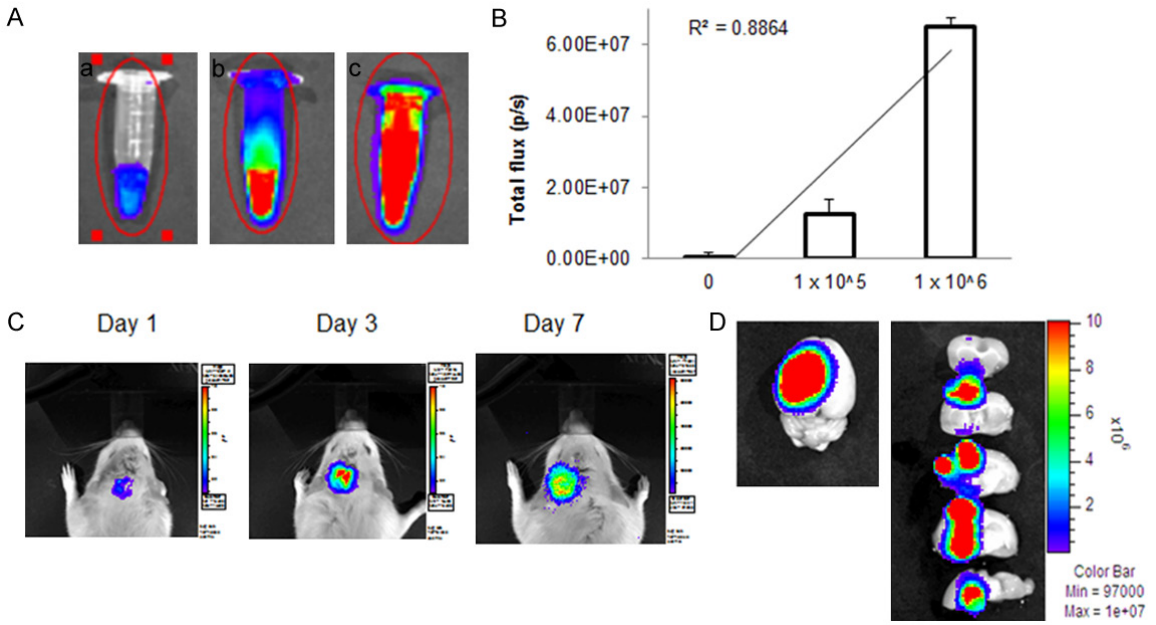

Figure 1. Generation of firefly luciferase (Fluc)-transduced human cerebral microvascular endothelial cell (hCMEC)/D3 cells and in vivo bioluminescent imaging (BLI). A. hCMEC/D3 human cerebral endothelial cells are transduced using a reporter gene encoding a Fluc gene. Successfully transduced cells are selected with zeocin. The selected cells exhibit increased luciferase activity (tubes b and c) compared to the nontransduced hCMEC/D3 cells (tube a). B. The luciferase activity gradually increases with the number of cells. C, D. BLI signals are observed in experimental animals at 1 day after transplantation while increased photon flux is observed on days 3 and 7. n = 5 in each group.

kin (IL)-1β, and IL-6 in triplicate on 25 ng equivalents with the Applied Biosystems 7300 Sequence Detection System (Thermo Fisher Scientific Inc., Waltham, MA, USA) and SYBR Green Assays (Thermo Fisher Scientific Inc.). The threshold amplification cycle-number data from multiple plates were combined with Applied Biosystems Relative Quantitation software (SDS1.2) and the delta-delta cycle threshold method (ref). All primers were synthesized by Macrogen Inc. (Seoul, Korea) and the primer sequences used in this study for qRT-PCR were as follows: rat TNF-α: sense, 5'-AAATGGGCTCCCTCTATCAGTTC-3', antisense, 5'-TCTGCTTGGTGGTTTGCTACGAC-3'; rat IL-1β: sense, 5'-CACCTCTCAAGCAGAGCACAG-3', antisense, 5'-GGGTTCCATGGTGAAGTCAAC-3'; rat IL-6: sense, 5'-TCCTACCCCAACTTCCAATGCTC-3', antisense, 5'-TTGGATGGTCTTGGTCCTTAGCC-3'; and rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH): sense, 5'-GGGCATCCTGGGCTACACTGA-3', antisense, 5'-CCTTGCTGGGCTGGGTGGT-3'.

Statistical analyses

The statistical analyses were performed with PRISM 5.0 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). The summary data are presented as mean ± standard deviation. Student's *t*-tests were used for comparisons between two groups, and analyses of variance and Bonferroni posthoc tests were used for comparisons among multiple groups. The criterion for statistical significance was a *p*-value of less than 0.05. All measurements were analyzed by observers blind to group assignment.

Results

Evaluation of the reporter cell line and detection of injected hCMEC/D3 cells in the ischemic hemisphere

First, we evaluated the grossly transduced population of hCMEC/D3 cells. Both of the grossly transduced populations (Tube B: 5×10^5 cells, Tube C: 1×10^6 cells) of the Fluc-labeled hCMEC/D3 cells exhibited an increased in vitro luciferase activity that was cell count-dependent ($R^2 = 0.8865$) compared to that of the wild-type parental cell line (Tube A, 5×10^5 cells).

We then injected Fluc-labeled hCMEC/D3 cells at 1 h after cerebral ischemia through the left

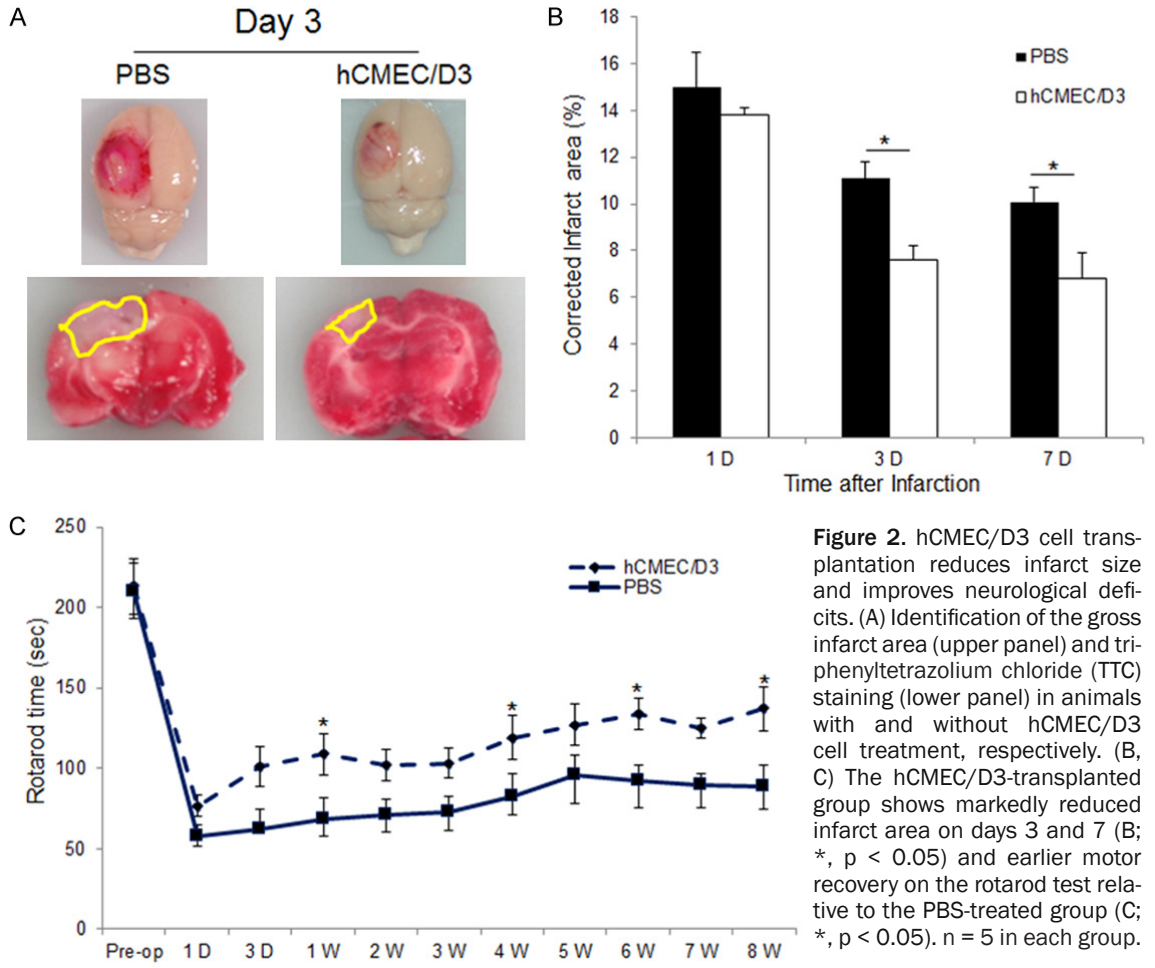

Figure 2. hCMEC/D3 cell transplantation reduces infarct size and improves neurological deficits. (A) Identification of the gross infarct area (upper panel) and triphenyltetrazolium chloride (TTC) staining (lower panel) in animals with and without hCMEC/D3 cell treatment, respectively. (B, C) The hCMEC/D3-transplanted group shows markedly reduced infarct area on days 3 and 7 (B; *, $p < 0.05$) and earlier motor recovery on the rotarod test relative to the PBS-treated group (C; *, $p < 0.05$). n = 5 in each group.

common carotid artery. BLI signals were measured 12 h and 1, 3, and 7 days after these intra-arterial (IA) injections (**Figure 1C** and **Figure 1D**) in each transplant group. Twenty-four hours later, all animals showed luciferase activity in the lesioned hemisphere (**Figure 1A**). The BLI signal peaked on day 3 after the IA injections. On day 7 after the IA injections, in vivo BLI signals gradually decreased to less than those observed on day 3, but ex vivo imaging still showed strong luciferase activity in the lesioned hemisphere (**Figure 1B**). These findings indicated that hCMEC/D3 cells successfully migrated to the brain infarct after IA injection.

hCMEC/D3 cell transplantation attenuated brain infarct size, BBB disruption, and promoted neurological recovery

TTC staining on days 3 and 7 after the IA injection of the hCMEC/D3-transplanted group showed markedly reduced ischemic lesions compared to those in the PBS-treated group (**Figure 2A** and **Figure 2B**, $p < 0.05$ for both days). Compared to the PBS-treated group, the hCMEC/D3-transplanted group showed an earlier recovery of their neurological functional impairments on day 7 that was sustained in the 4 weeks after hCMEC/D3 IA transplantation on the rotarod task (**Figure 2C**, $p < 0.05$).

As described in our previous study [31], Evans blue dye is a good marker of BBB leakage in photothrombotic stroke models. The effects of hCMEC/D3 cell transplantation on BBB integrity were assessed by Evans blue extravasation and gelatin zymography. As shown in **Figure 3A**, the photothrombotic focal cerebral ischemia in the PBS-treated group resulted in significantly higher levels of Evans blue extravasation. However, the hCMEC/D3-transplanted group showed lower dye extravasation relative to the PBS-treated group on days 1 and 3 ($p < 0.05$

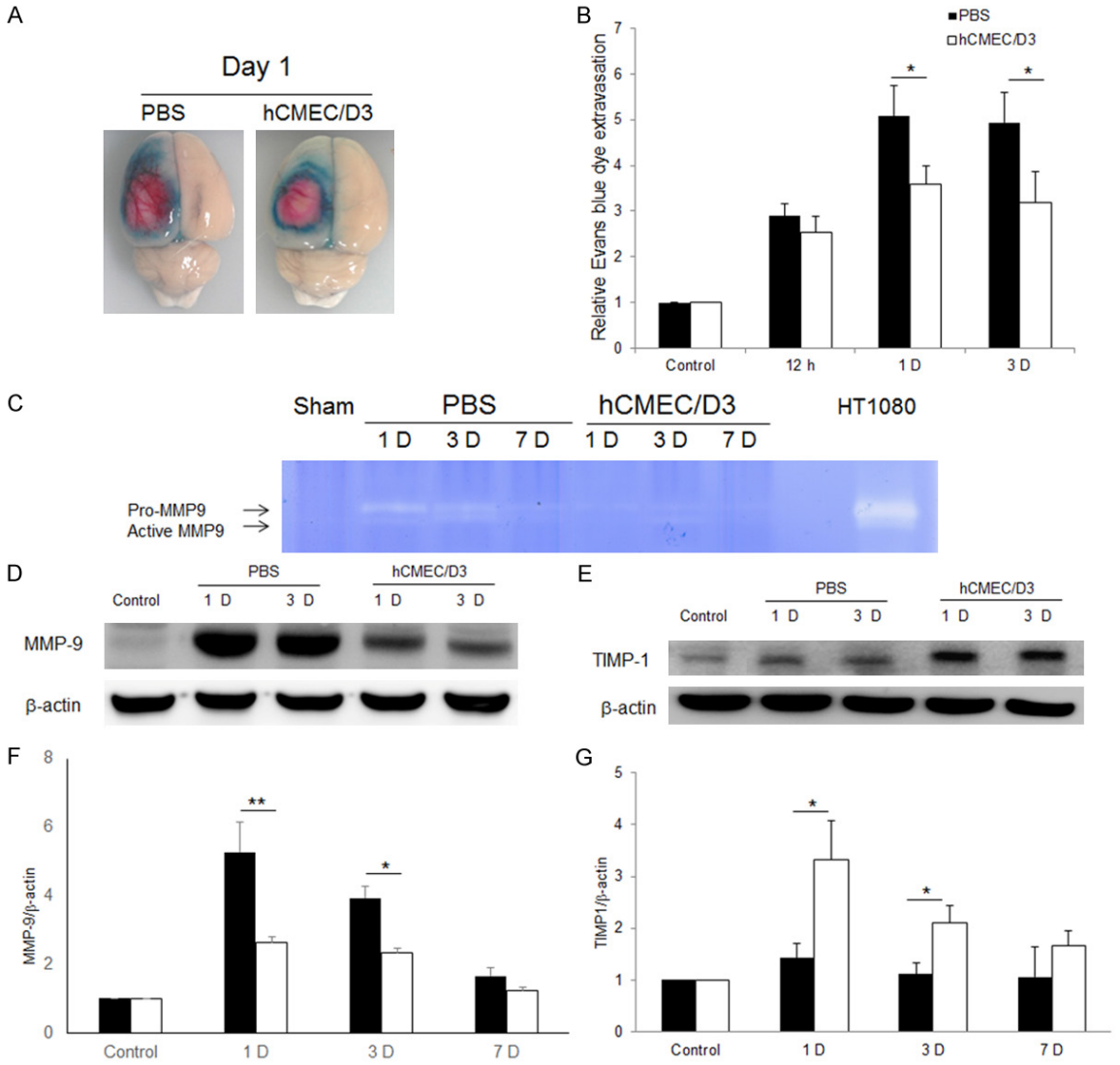

Figure 3. hCMEC/D3 cell transplant decreases blood-brain barrier (BBB) leakage after ischemia. (A) Representative gross brains images show Evans blue extravasation (blue-colored area). (B) BBB permeability by quantification of Evans blue staining was compared between groups (*; $p < 0.05$). Gelatin zymogram (C) and western blot (D-G) show reduced MMP-9 and increased TIMP-1 expression in the hCMEC/D3-transplantatrf group relative to the PBS-treated group. n = 4 in each group; *$p < 0.05$, **$p < 0.01$.

for both). Zymography of brain homogenate showed markedly increased levels of pro-MMP-9 and activated MMP-9 in the 24 h after ischemic onset in the PBS-treated group. In the hCMEC/D3-transplanted group, the pro-MMP-9 and activated MMP-9 levels were markedly reduced compared with those in the PBS-treated group on days 1 and 3. However, the MMP-2 levels in the gelatin zymography and western blot tests (data not shown) were not significantly different between the PBS-treated and hCMEC/D3-transplanted groups. In the western blot, the MMP-9 levels were higher in the PBS-treated group than in the hCMEC/D3-transplanted group on days 1 and 3, and the levels of TIMP-1 were inversely correlated with MMP-9 at the same time points.

Transplantation of hCMEC/D3 cells attenuated acute inflammatory cytokine induction in focal cerebral ischemia

On days 1 and 3 following hCMEC/D3 cell transplantation, IL-1β, IL-6, and TNF-α mRNA expression, indices of acute inflammation, were measured using qRT-PCR. IL-1β and TNF-α levels

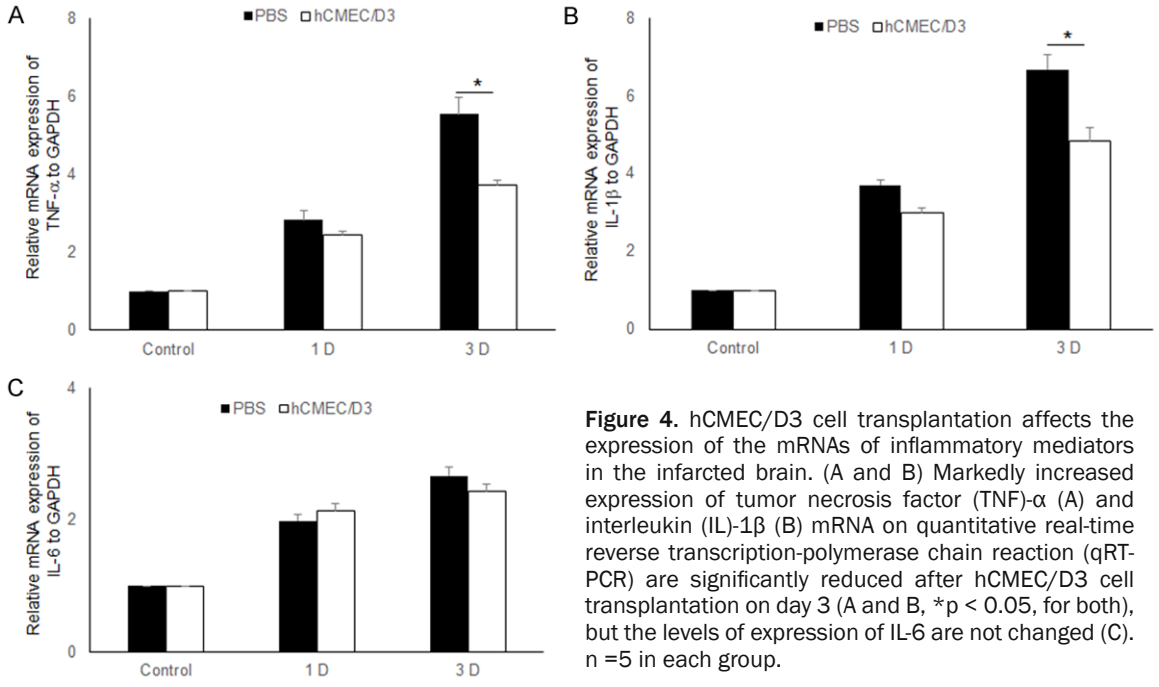

Figure 4. hCMEC/D3 cell transplantation affects the expression of the mRNAs of inflammatory mediators in the infarcted brain. (A and B) Markedly increased expression of tumor necrosis factor (TNF)-α (A) and interleukin (IL)-1β (B) mRNA on quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) are significantly reduced after hCMEC/D3 cell transplantation on day 3 (A and B, *$p < 0.05$, for both), but the levels of expression of IL-6 are not changed (C). n =5 in each group.

were markedly reduced in the hCMEC/D3-transplanted group relative to the PBS-treated group on day 3 after cerebral ischemia (**Figure 4**, $p < 0.05$ for both). However, there were no significant differences in IL-6 levels between the groups. These findings suggest that hCMEC/D3 cell therapy attenuated the acute inflammatory response following focal cerebral ischemia.

Discussion

In this study, we demonstrated that human cerebral endothelial cells successfully migrated to the area of infarct and exhibited various neuroprotective mechanisms, including infarct size reduction, attenuation of BBB disruption, and the restoration of early neurological deficits.

Several therapy candidates have been widely investigated in the treatment of stroke, including embryonic stem cells, adult neural stem cell/progenitor cells, and bone marrow-derived mesenchymal stem cells [32]. The neurovascular unit, of which the cerebral endothelial cell is a major cell type, is an important compartment that regulates brain homeostasis under healthy and pathological conditions [18]. The present study found that hCMEC/D3 cell-transplantation therapy reduced infarct size, BBB breakdown, and eventually promoted neurological recovery following focal cerebral ischemia. These results suggest that hCMEC/D3 cell transplantation is a promising therapy for patients with stroke.

The IA transplantation of any cell type has been successfully targeted to the damaged hemisphere through the injured BBB at least three times compared to intravenous injections by bypassing lung entrapment [33]. Bioluminescence has been used to track the biodistribution [33], migration [34], and survival [35] of several types of grafted cells. In order to trace the distribution of the transplanted hCMEC/D3 cells in the ischemic hemisphere, we generated Fluc-labeled hCMEC/D3 cells. As shown in **Figure 1**, the observed photon flux correlated with approximately 3.0×10^5 cells at 3 days after transplantation and more than 20% of the initial cell signal was still present at 7 days after transplantation. Our results indicated that the IA-injected hCMEC/D3 cells successfully migrated to the damaged hemisphere and were retained during the remodeling period that follows cerebral ischemia. This cell line might be a useful tool for in vivo cell viability assessments, biodistribution, and for the investigation of the long-term effects of cerebral endothelial cell transplantation as a therapy following ischemia.

MMPs have been involved in the pathogenesis of ischemic stroke in both animal and human studies [36]. Abnormally increased levels of MMP-9 expression following ischemic stroke increases BBB permeability and infarct volume; hence, its attenuation is considered a potential therapeutic target for the treatment of ischemia [37]. In this study, we showed that Evans blue dye extravasation gradually increased after photothrombotic cerebral ischemia. Following hCMEC/D3 cell transplantation, this extravasation decreased on days 1 and 3 (**Figure 3B**). Simultaneously, levels of MMP-9, an important catalyst of BBB breakdown, was markedly decreased (**Figure 3C**, **3D**). TIMP-1, but not TIMP-2, was increased on days 1 and 3 as evidenced by the western blots of the same animals (data not shown). In vivo MMP activity is tightly controlled by endogenous TIMPs [38], and therefore its overexpression may play a neuroprotective role in global cerebral ischemia in mice [39] and attenuate BBB permeability in a model of acute liver failure [40]. These previous reports, together with our findings, suggest that the upregulation of TIMP-1 reverses the activation of MMP-9, BBB breakdown, and eventually contributes to neurorestoration. The molecular mechanisms underlying these phenomena should be studied further, perhaps with a focus on changes in associated tight junction proteins and signaling pathways, such as the p38 mitogen-activated protein kinase pathway.

The expression of pro-inflammatory cytokine mRNA after cerebral ischemia have been examined and these molecules are closely related to functional recovery after injury [41]. In the present study, the expression of TNF-α, IL-1β, and IL-6 were increased markedly 1 day after photothrombotic cerebral ischemia. Interestingly, expression of TNF-α and IL-1β, but not IL-6, was significantly decreased after hCMEC/D3 cell transplantation (**Figure 4A**, **4B**). TNF-α and IL-1β are among the most widely studied cytokines in neuroinflammation following acute ischemic stroke and their expression is associated with the extent of stroke damage. The direct application of exogenous TNF-α and IL-1β enhances brain edema formation and worsened ischemic injury in a model of stroke [42]. Although TNF-α modulation has demonstrated both deleterious and beneficial effects on cerebral ischemia [43], recent reports have suggested that TNF-α interference therapy might successfully prevent post-ischemic neuronal injury [44, 45]. Compared to previous reports, suppression of the increased early expression of post-ischemic inflammatory cytokines TNF-α and IL-1β might be accomplished by cerebral endothelial cell transplantation. However, further studies examining profiles of gene expression after transplantation are needed to better understand the underlying mechanisms of this therapeutic modality.

Remodeling of the microenvironment by vasculogenesis and neurogenesis starts immediately after cerebral ischemia. The hypoxia-inducible factor-1β axis is a canonical pathway involved in the early stages of recovery following cerebral ischemia. Several downstream genes, including the vascular endothelial growth factor (VEGF), are dynamically activated in this early period of ischemia [46]. Increased levels of endogenous VEGF could phenotypically affect the transplanted cerebral endothelial cells. Moreover, VEGF-stimulated cerebral microendothelial cells show various changes in cytokine, chemokine, and extracellular matrix protein release in vitro [21] and these gene profile changes play an important role in the protection of the brain against cerebral ischemia, referred to as the bystander effect [32]. During the late period of cerebral ischemia, another study reported that another immortalized human cerebral endothelial cell line, the HEN6, is actively involved in the promotion of vasculogenesis and neurogenesis following transplantation [17].

The present study focused on the effects of cerebral endothelial cell transplantation on the early progression of cerebral ischemia. The systemically transplanted cerebral endothelial cells migrated to the ischemic hemisphere and were involved in mediating recovery through the attenuation of BBB breakdown and anti-inflammatory effects after focal cerebral ischemia in rats.

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