Proteomic Analysis of a Rat Cerebral Ischemic Injury Model after Human Cerebral Endothelial Cell Transplantation

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Objective: Cerebral endothelial cells have unique biological features and are fascinating candidate cells for stroke therapy. Methods: In order to understand the molecular mechanisms of human cerebral endothelial cell (hCMEC/D3) transplantation in a rat stroke model, we performed proteomic analysis using 2-dimensional electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Protein expression was confirmed by quantitative real-time PCR and Western blot.

Results: Several protein spots were identified by gel electrophoresis in the sham, cerebral ischemia (CI), and CI with hCMEC/D3 treatment cerebral ischemia with cell transplantation (CT) groups, and we identified 14 differentially expressed proteins in the CT group. Proteins involved in mitochondrial dysfunction (paraplegin matrix AAA peptidase subunit, SPG7), neuroinflammation (peroxiredoxin 6, PRDX6), and neuronal death (zinc finger protein 90, ZFP90) were markedly reduced in the CT group compared with the CI group. The expression of chloride intracellular channel 4 proteins involved in post-ischemic vasculogenesis was significantly decreased in the CI group but comparable to sham in the CT group. Conclusion: These results contribute to our understanding of the early phase processes that follow cerebral endothelial cell treatment in CI. Moreover, some of the identified proteins may present promising new targets for stroke therapy.

Key Words: Brain · Ischemia · Cell therapy · Proteomics · 2-dimensional electrophoresis.

INTRODUCTION

Stroke is the third most common cause of death in Korea. 1 Stroke is a serious and growing public health problem. It is also a major cause of long-term disability due to its associated mortality, morbidity, and economic burden. Thus, it is important to improve functional recovery after stroke through various therapeutic modalities, including exogenous cell therapy.

Exogenous cell therapy has shown promise as a means for augmenting brain repair, as evidenced by enhanced endogenous angiogenesis, neurogenesis, and immunomodulation. 2-4 Neural stem cells and mesenchymal stem cells, which originate from a variety of sources, have been used for treatment in a focal cerebral ischemic animal model 5-7 and in human stroke patients 8-10. Cerebral endothelial cells have unique features compared to human umbilical vein endothelial cells (HUVEC) or endothelial precursor cells 11 and play an important role in maintaining the function of neurovascular units and promoting endogenous neural stem cell proliferation. 12 The potential therapeutic benefits of human cerebral endothelial cells (HEN6) applied directly to the striatum of experimental ischemic stroke rats have been demonstrated. 13 Recently, we reported that systemically injected human immortalized cerebral endothelial cells (hCMEC/D3) in a focal cerebral ischemia (CI) model successfully migrated into the ischemic hemisphere and had several neuroprotective effects, including reduced infarct size, neuroinflammation, and blood-brain barrier (BBB) breakdown, and enhanced functional recovery. 14-16

Understanding the molecular alterations in the cell therapy model for animal stroke is critical for optimal translational strategy development. Very few studies have reported global protein expression changes in the human or rat ischemic brain. 17,18 Monitoring various kinds of cell therapy uses two-dimensional (2-D) electrophoresis-based protein expression analy-
sis. After the 2-D electrophoresis, distinct messenger ribonucleic acid (mRNA)-protein expression have evaluated by quantitative methods. Although neuroprotective events by transplanted hCMEC/D3 are usually observed around 3 days following cell therapy, the potential mechanisms of action are not completely understood.

The aim of the present study was to compare the protein expression pattern in focal CI model rats treated with human cerebral endothelial cells in order to find new and robust potential therapeutic targets for stroke.

MATERIALS AND METHODS

Cells and reagents

The human cerebral microvascular endothelial cell line (hCMEC/D3) was obtained from Merck Millipore (SCC066, Temecula, CA, USA) and cultured in an Endothelial Growth Medium-2 BulletKit (Lonza, Walkersville, MD, USA) as in our previous report. Primary antibodies against chloride intracellular channel 4 (CLC4, Abcam, Cambridge, UK), paraplegin matrix AAA peptidase subunit (SPG7, OriGene, Rockville, MD, USA), peroxiredoxin 6 (PRDX6, Abcam, Cambridge, UK), zinc finger protein 90 (ZFP90, NovoPro, Shanghai, China), Eph receptor B1 (EPHB1, R&D Systems, Minneapolis, MN, USA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Cell Signaling, Donvers, MA, USA) were used. Horseradish peroxidase (HRP)-conjugated secondary antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animal model and cell transplantation

All surgical procedures and postoperative care in this study were performed in accordance with the guidelines of the Chonnam National University Animal Care and Use Committee. A total of 33 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 reports. Rats were randomly assigned to three groups: phosphate-buffered saline (PBS)-treated (sham), focal CI, and cell transplantation after focal CI (CT). A single cell suspension of 1×10^6 hCMEC/D3 cells in 10 μL of saline was injected into the left common carotid artery with a custom Hamilton syringe with a 33-gauge needle as in our previous report. Successful lesion making was confirmed by sharply departed cortical lesion in the motor cortex after open craniectomy. All brain samples for mRNA and protein expression analysis were obtained 3 days after focal CI induction.

Protein extraction

Following sacrifice (n=3, each group), tissue samples were isolated, weighed, mixed in three volumes of protein extraction buffer (8 M urea, 2 M thiourea), and homogenized. After centrifugation (8000 rpm, 30 min), the supernatant was collected and mixed with the same volume of 20% TCA/acetone to precipitate the proteins (-20°C, 1 h). The TCA was washed away with cold acetone (3 times), and the proteins were collected by centrifugation (10000 rpm, 15 min, 4°C). The precipitate was dried, dissolved in 200 μL protein extraction buffer, and stored at -70°C. Protein quantification was conducted using the BCA method with bovine serum albumin and confirmed with Western blotting against GAPDH.

Two-dimensional gel electrophoresis (2-DE)

Proteins were separated using 2D-PAGE and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as described previously. The extracted proteins were solubilized with 200 μL of rehydration buffer containing 8 M urea, 2% CHAPS, 2% IPG buffer, 100 mM dithiothreitol (DTT), and 0.001% bromophenol blue. Then, 250 μL of rehydration buffer containing 1 mg of protein was applied to an immobilized pH 4–7 (NL) gradient (IPG) dry strip (13 cm; GE Healthcare, Amersham, UK), and the IPG strip was rehydrated for 12 h at 20°C. After rehydration, proteins were focused by isoelectric focusing (IEF). The voltage used was 300 V for 1 h, 3500 V for 90 min, and 3500 V for 4 h. The current was limited to 2 mA per strip. Following IEF, the resulting strip was equilibrated for reduction of proteins with equilibration buffer (50 mM Tris-Cl pH 8.8, 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), 0.001% bromophenol blue, and 1% DTT) for 20 min at room temperature and then incubated for protein alkylation with equilibration buffer containing 2.5% iodoacetamide but not DTT in the dark for 20 min at room temperature. The strips were separated on a 12% gel by the second dimension of electrophoresis and run in 45 mA at 20°C. To ensure reproducibility in the gel pattern, we repeated 2-DE against the same sample three times.

Gel staining and detection of proteins

The resulting gel was fixed in 12% trichloroacetic acid for 1 h and stained with Coomassie Blue G250 according to the colloidal Coomassie staining method. To identify the spots of interest, gels were excised and transferred into a microcentrifuge tube and then rinsed with distilled water for 15 min. Fifty percent acetonitrile (ACN) was added to the tube and incubated for 15 min. On discarding the ACN, 100% ACN was added to the tube and allowed to stand for 15 min until gel pieces became white and had shrunk. After removal of ACN, the gel was incubated with 100 mM ammonium bicarbonate (ambic) for 5 min. One hundred percent ACN was added to the tube and incubated for 15 min. This procedure was repeated until dye was no longer visible. Destained gel pieces were dried using a Speed Vac. Dried gel pieces can be stored at -70°C prior to use.

Trypsin digestion and mass spectrometry

Destained gel pieces were treated with 0.1% (w/v) RapiGest SF in 50 mM amnic buffer for 10 min at 37°C and then dried
using a Speed Vac. Gel pieces were treated with 30 μL of trypsin solution (10 μg/mL) and incubated for 45 min on ice. After discarding excess trypsin solution, 30 μL of hydrolysis buffer (50 mM ampic/1 mM CaCl₂) was added to the tube containing the gel piece. Finally, the gel pieces were incubated overnight at 37°C. Tryptic peptides were transferred into a new microcentrifuge tube and 20 μL of 50 mM ampic buffer was added to the tube containing the gel piece for 15 min. Then, 20 μL of ACN was added to the tube containing the gel piece and incubated for 15 min, and 40 μL of the eluted peptides was collected into the new tube containing tryptic peptides. Gel pieces were also incubated with 20 μL of 5% formic acid for 15 min, and 20 μL of ACN was added to the gel tube. The 5% formic acid/ACN (ratio 1 : 1) step was repeated to elute tryptic peptides. After adding 100 mM DTT to the tryptic peptides to a final concentration of 1 mM, the peptides were dried by using a vacuum centrifuge and stored at -70°C for further analysis. The dried peptides were dissolved with 3 μL of buffer [50% ACN/0.1% trifluoroacetic acid (TFA)]. One microliter of peptide solution was mixed with 1 μL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution (10 mg/mL of CHCA, 50% ACN, 49.9% EtOH, and 0.1% TFA), and then 1 μL of the sample/matrix mixture was directly deposited onto the plate and dried. Protein identification by peptide mass fingerprinting (PMF) was carried out using the Mascot database (matrixscience.com). The parameters for PMF were as follows: mass error tolerance, 100 ppm; variable modification, oxidation of methionine and carbamidomethylation of cysteine; and the number of missed cleavages, 1. The mass data were calibrated with Angiotensin I (m/z 1296.6853) as an external standard and a peptide derived from trypsin (m/z of 2211.1046) as an internal standard.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed as described previously. The ribonucleic acid (RNA) of the ischemic hemisphere (n=5, each group) was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and converted into complementary deoxyribonucleic acid (cDNA) by use of the GoScript™ cDNA Synthesis Kit (Promega Corporation, Madison, WI, USA) according to the manufacturer’s instructions. The qRT-PCR was run on a QuantStudio 3 (ThermoFisher Scientific Inc., Waltham, MA, USA) in the presence of a fluorescent dye (SYBR® Green master mix, ThermoFisher Scientific Inc., Waltham, MA, USA). The relative level of mRNA was calculated with the delta-delta cycle threshold method and presented after normalization to GAPDH. All primers were designed for Rattus norvegicus-derived sequences, and synthesized by Macrogen Inc. (Seoul, Korea), and the primer sequences used in this study for qRT-PCR were as follows: rat Cltc4: sense, 5’-ACATCTGCAGAACTTGGCTCCT-3’; antisense, 5’-GTAAAGAGAAGAACCTTGCTTAT-3’; rat Zfp90: sense, 5’-AGGGTTTCTCTCCGCTATGTGTGA-3’; antisense, 5’-GGTCCTGGGAGCATGATGAGTGATG-3’; rat Ephb1: sense, 5’-GGTCCTCCAGCGATGTTCTAC-3’; antisense, 5’-CCTGTCGCTGGCTGGTGT-3’.

Western blot analysis

Western blot analysis was performed as previously described with proper modification. Briefly, ischemic hemispheres (n=4, each group) were lysed by the PRO-PREP™ Protein Extraction Kit (iNTRON Biotechnology, Seoul, Korea). After separation by 8–10% SDS-PAGE, proteins were transferred to polyvinylidene difluoride membrane and then incubated with primary antibody at 4°C overnight. Each primary antibody described in the reagents section was used at 1:1000 dilution, except SPG7 (1:2000 dilution). GAPDH was used as the control. The membranes were washed with Phosphate Buffered Saline with Tween® 20 three times and further incubated with host-matched HRP-labeled secondary antibodies for 1 h at room temperature (1:1000; Santa Cruz). 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).

Statistical analysis

The statistical analyses were performed with PRISM 5.0 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). Student’s t-tests were used for comparisons between two groups, and analyses of variance and Bonferroni post hoc tests were used for comparisons among multiple groups. p-value of less than 0.05 was considered statistically significant.

RESULTS

2-D electrophoresis spot screening and proteomic analysis

The spot distribution patterns for the sham group, phototherm-
Table 1. List of identified differentially expressed proteins among three groups

| Spot number | Gene ID | Protein name                                      | MW (Da)/PI | Matched score (%) |
|-------------|---------|---------------------------------------------------|------------|-------------------|
| A. Down-regulated protein in hCMEC/D3 transplantation group compared to injury group |
| 652         | 79126   | Complement factor I                                | 67234/7.55 | 48                |
| 1106        | 361003  | Dual specificity phosphatase and pro isomerase domain containing 1 | 24058/6.04 | 48                |
| 1107        | 171051  | Calcium-binding protein 1                          | 32996/6.05 | 46                |
| 867         | 307940  | Disrupted in schizophrenia 1                       | 92505/5.8  | 51                |
| 874         | 26363   | Acyl-CoA dehydrogenase, very long chain            | 70705/9.01 | 48                |
| 887         | 24338   | Eph receptor B1                                    | 109812/6.03| 50                |
| 1104        | 498945  | Zinc finger protein 90                             | 72392/8.84 | 51                |
| 1133        | 24235   | Complement component 4 binding protein, alpha      | 62225/7.06 | 58                |
| 1266        | 94167   | Peroxiredoxin 6                                   | 24803/5.64 | 55                |
| 1498        | 353231  | SPG7, paraplegin matrix AAA peptidase subunit      | 82071/9.15 | 52                |
| 1589        | 314906  | Kinesin family member 5A                           | 116843/5.56| 45                |
| 1938        | 302538  | Synaptotagmin-like 5                               | 83593/8.91 | 46                |
| B. Rescued to normalized level after hCMEC/D3 transplantation |
| 1154        | 305482  | Myotubularin related protein 3                     | 133415/5.71| 54                |
| 1487        | 83718   | Chloride intracellular channel 4                   | 28615/5.92 | 52                |

DISCUSSION

In this study, we used 2-DE-based proteomics to elucidate the early phase neuroprotective mechanisms of human cerebral endothelial cell transplantation in focal CI. In the spot analysis, we identified 14 sequence-matched proteins based on the protein database, and using qRT-PCR and immunoblot analyses, we confirmed several protein expression changes after hCMEC/D3 transplantation in the early period of rat focal CI.

During CI, various detrimental processes occur, including overproduction of oxidants, inactivation of detoxification systems, and consumption of antioxidants. Mitochondria are known to play a key role in ischemic/reperfusion injury in the brain[16]. Increased synthesis of TNF-α aggravates neuronal death by promoting mitochondrial dysfunction, calcium dysregulation, and oxidative stress[20]. In our previous report, the hCMEC/D3 transplantation model decreased infarct size and attenuated TNF-α expression at day 3 after focal CI induction[20]. In the present study, SPG7 expression is increased in the CI group at day 3 compared with sham expression, but in the CT group, expression is similar to sham level (Fig. 3). SPG7 has recently become well known and is required for Ca²⁺ overload- and ROS-induced permeability transition pore (PTP) opening, mitochondrial dysfunction, and cell death[20]. In fact, abrupt SPG7 overexpression induces PTP opening after ischemic injury, suggesting that reduction of SPG7 expression could protect mitochondria from Ca²⁺- and ROS-induced PTP-dependent necrosis[20]. Our findings suggest that hCMEC/D3 application in the ischemic rat model may be neuroprotective by reducing TNF-α and SPG7 expression following CI.

Brain ischemia induces PRDX6 and toll-like receptor 4 (TLR4) expression, which may contribute to the sequential neuroinflamm-
mation associated with CI\(^{20}\). Interestingly, ligustilide may provide early and direct neuroprotection by inhibiting TLR4/PRDX6 signaling, suggesting that TLR4/PRDX6 blocking agents are potent neuroprotective tools\(^{32}\). In the photothrombotic focal ischemia model, Prdx6 mRNA expression was markedly increased at day 3 and significantly reduced in the hCMEC/D3 treated group (Fig. 2D), but the change in protein expression did not reach statistical significance (Fig. 3B). These findings are consistent with our previous study that showed hCMEC/D3 transplantation can ameliorate production of neuroinflammatory cytokines, such as TNF-α and IL-1β, in the early phase of CI.

The RE1-Silencing Transcription factor (REST), also known as Neuron-Restrictive Silencer Factor (NRSF), acts as a transcriptional repressor\(^{27}\). ZFP90 is a novel NRSF-binding protein that inhibits the repressor activity of NRSF by preventing its binding to deoxyribonucleic acid\(^{13,21}\). ZFP90 is highly expressed in pathological conditions such as congestive heart failure and aggravates cardiac dysfunction via increased expression of NRSF target genes, such as brain natriuretic peptide, present during adverse cardiac remodeling. However, the role of REST and

**Fig. 2.** qRT-PCR for selected target genes in the CT group compared with sham and CI groups. The mRNA levels of Ephb1, Zfp90, Prdx6, Spg7, and Clic4 were measured by qRT-PCR and calculated by the ΔΔCt methodology as described in methods. The assays were run in triplicate, and the standard error of the mean (SEM) was determined. Relative expression of Zfp90, Prdx6, and Spg7 was markedly decreased in the CT group compared with the CI group, but changes in Ephb1 did not reach statistical significance (A-D). mRNA for Clic4 was markedly decreased in the CI group but was rescued in the CT group (E; n=5 per group). Data are presented as mean±SEM. *p<0.05, **p<0.01 vs. CT group. qRT-PCR : quantitative real-time PCR, CI : cerebral ischemia, mRNA : messenger ribonucleic acid, EPHB1 : eph receptor B1, ZFP90 : zinc finger protein 90, PRDX6 : peroxiredoxin 6, SPG7 : paraplegin matrix AAA peptidase subunit, CLIC4 : chloride intracellular channel 4, GAPDH : glyceraldehyde 3-phosphate dehydrogenase.
ZFP90 in CI is only partially understood. In an in vitro model of ischemia, acute knockdown of REST in hippocampal slices subjected to oxygen glucose deprivation prevented GluA2 downregulation and neuronal death. In a stroke model, RNAi-mediated depletion of REST or administration of dominant-negative REST delivered directly into the hippocampus in vivo prevented epigenetic modifications, restored gene expression, and rescued hippocampal neurons.

During CI/reperfusion injury, cerebrovascular maintenance functions are disrupted, and the goal of CI treatment is to restore vasculogenesis and neurogenesis, focusing on the ischemic penumbra. As our previous results and other studies have shown, cerebral endothelial cell transplantation plays a potent neurorestorative role, which involves protection against BBB breakdown through increased tight junction proteins and promotion of vasculogenesis via vascular endothelial growth factor (VEGF) signaling. In the present study, we identified changes in CLIC4 expression using the 2-DE method; while CLIC4 is significantly decreased in the CI group, it is normalized relative to the sham group level in the hCMEC/D3 transplantation group. CLIC4 is normally expressed in endothelial cells and vascular smooth muscle cells and is essential for normal vasculogenesis through endothelial tubulogenesis during embryogenesis and ischemia. CLIC4 gene deficiency results in reduced expression of Vegfr2, Vegfr1, Vegfc, and mural cell markers associated with a reduction in ischemia-induced expression of hypoxia inducible factor-1α (HIF-1α) and hypoxic cell death. Recent work showed that overexpression of VEGF-A in Clic4 (-/-) mice rescued defects in collateral maturation by preventing mural cell loss and collateral pruning, thus restoring collateral number and diameter and reducing stroke severity in adults. In fact, hCMEC/D3 showed high Clic4 mRNA and protein expression, whereas HUVEC did not (data not shown). Considering previous reports and the present study, transplanted Clic4-expressing hCMEC/D3 cells could migrate to the ischemic lesion and contribute to microenvironmental improvement by restoring the HIF-1α/VEGF axis.

In this study, focal CI induced various protein expression changes associated with promoting apoptosis and neuronal cell death and repressing vasculogenesis. Transplanted cerebral endothelial cells (hCMEC/D3) may partly play a role in controlling neuroinflammation via SPG7 and PRDX6 suppression, reducing fetal gene re-expression via ZFP90, and modulating the damaged vasculature via CLIC4 upregulation compared with the CI group. And we could assume that these effects are strongly corroborated by the paracrine mediated neuroprotection in the cell transplantation group was recovered to sham expression level. These results contribute to our understanding of the early phase processes that follow cerebral endothelial cell transplantation in CI. Moreover, some of the identified proteins may present promising new targets for stroke therapy.

**CONCLUSION**

Human cerebral endothelial cell transplantation following focal CI induced several protein expression changes. Aberrantly increased expression of proteins associated with neuroinflammation or apoptosis, such as ZFP90, PRDX6, and SPG7, was decreased following cell transplantation. CLIC4 expression in the cell transplantation group was recovered to sham expression level. These results contribute to our understanding of the early phase processes that follow cerebral endothelial cell treatment in CI. Moreover, some of the identified proteins may present promising new targets for stroke therapy.

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- **Conflict of Interest**
  The authors certify that there is no authorship dispute between them.
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