Human Umbilical Vein Endothelial Cells Protect Against Hypoxic-Ischemic Damage in Neonatal Brain via Stromal Cell-derived Factor 1/C-X-C Chemokine Receptor Type 4

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Background and Purpose—Agents that protect against neurovascular damage provide a powerful neuroprotective strategy. Human umbilical vein endothelial cells (HUVECs) may be used to treat neonates with hypoxic-ischemia (HI) because of its autologous capability. We hypothesized that peripherally injected HUVECs entered the brain after HI, protected against neurovascular damage, and provided protection via stromal cell–derived factor 1/C-X-C chemokine receptor type 4 pathway in neonatal brain.

Methods—Postpartum day 7 rat pups received intraperitoneal injections of low-passage HUVEC-P4, high-passage HUVEC-P8, or conditioned medium before and immediately after HI. HUVECs were transfected with adenovirus-green fluorescent protein for cell tracing. Oxygen–glucose deprivation was established by coculturing HUVEC-P4 with mouse neuroblastoma neuronal cells (Neuro-2a) and with mouse immortalized cerebral vascular endothelial cells (b.End3).

Results—HUVEC-P4–treated group had more blood levels of green fluorescent protein–positive cells than HUVEC-P8–treated group 3 hours postinjection. Intraperitoneally injected HUVEC-P4, but not HUVEC-P8, entered the cortex after HI and positioned closed to the neurons and microvessels. Compared with the condition medium–treated group, the HUVEC-P4–treated but not the HUVEC-P8–treated group showed significantly less neuronal apoptosis and blood–brain barrier damage and more preservation of microvessels in the cortex 24 hours after HI. On postpartum day 14, the HUVEC-P4–treated group showed significant neuroprotection compared with the condition medium–treated group. Stromal cell–derived factor 1 was upregulated in the ipsilateral cortex 3 hours after HI, and inhibiting the stromal cell–derived factor 1/C-X-C chemokine receptor type 4 reduced the protective effect of HUVEC-P4. In vitro transwell coculturing of HUVEC-P4 also significantly protected against oxygen–glucose deprivation cell death in neurons and endothelial cells.

Conclusions—Cell therapy using HUVECs may provide a powerful therapeutic strategy in treating neonates with HI. (Stroke. 2013;44:00-00.)

Key Words: human umbilical vein endothelial cell ■ neonatal brain ■ neurovascular unit ■ SDF-1/CXCR4

Hypoxic-ischemia (HI) is a major cause of neonatal mortality and neurological morbidity among survivors.1 Neurovascular unit has been shown to be a major target of injury.2–5 Dysfunction of the neurovascular unit may disrupt microcirculation and, hence, promote progression of neurological diseases. Neurovascular damage may be present early in neurological diseases, even before the onset of neuronal death.2,5 Evidence has indicated that neurons and vascular cells are closely related developmentally, structurally, and functionally.2 Communication between the nervous and vascular system is required for maintaining the blood–brain barrier (BBB) integrity and promoting neural function and regeneration.6 Therefore, for effective therapies in newborns with HI, it is necessary to target early the protective pathway that simultaneously acts on neurons and vessels.1,3–5

Stem cells have been used in the experimental studies of neonatal HI.7 Human umbilical cord blood stem cells (HUCSCs) contain many stem cell types that can differentiate into mature cell types. Many studies have evaluated the effect of HUCSCs in perinatal brain damage,4,5 however, few studies have focused on the protective effect of human umbilical vein endothelial cells (HUVECs). Both HUVECs and HUCSCs have autologous potentials and low immunogenicity. However, compared with HUCSCs, the advantages of HUVECs include cell type homogeneity, well-characterized surface markers, late endothelial progenitor cell characters, and obtainable

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huge cell numbers via rapid expansion property. Autologous treatment using HUVECs for newborns, especially preterm infants, is particularly appropriate because an umbilical cord contains a sufficient number of vascular endothelial cells. Through its paracrine effect, cell therapy using HUVECs may not only produce protective signals for cerebral vessels but also promote neuronal survival after HI in the neonatal brain.

Stromal cell–derived factor 1 (SDF-1) is a chemokine secreted by tissues after hypoxia. C-X-C chemokine receptor type 4 (CXCR4), a SDF-1 receptor, is widely expressed in tissues, including neurons and vascular endothelial cells. The SDF-1 and CXCR4 are key regulators of endothelial progenitor cells mobilization and recruitment to tissue injury areas in many organs. SDF-1 expression increases after HI in the neonatal mouse brain. Whether peripherally injected HUVECs migrate to the neonatal brain and protect against HI through SDF-1/CXCR4 axis remains unknown.

We used an in vivo model of neonatal HI and established in vitro coculture models of HUVEC/neurons and HUVEC/vascular endothelial cells against oxygen–glucose deprivation (OGD) to test the following hypotheses: (1) intraperitoneally injected low-passage HUVECs entered the brain after HI, protected neurovascular unit, and provided neuroprotection via SDF-1/CXCR4 pathway; and (2) transwell coculturing with HUVECs protected against OGD cell death in neurons and endothelial cells via paracrine effect.

### Materials and Methods

This study was approved by the Institutional Review Board of National Cheng Kung University Hospital and the Animal Care Committee of National Cheng Kung University.

#### Primary Culture of HUVECs

Human umbilical cords were rinsed and washed. Endothelial cell suspensions were harvested by collagease perfusion and centrifuged, and the pellets were resuspended in the complete medium (online-only Data Supplement) before being plated onto culture dishes. Low-passage HUVECs (HUVEC-P4) and high-passage HUVECs (HUVEC-P8) from the same umbilical cords were used (online-only Data Supplement Methods). Recombinant adenosines were produced using AdEasy (Stratagene). The adenosine-green fluorescent protein (GFP) construct was transfected into HUVECs for 24 hours.

#### HI Injury and HUVECs Treatment

Postpartum day 7 (P7) male Sprague-Dawley rat pups were anesthetized, and the right common carotid artery was permanently ligated. After surgery, the pups were returned to their dams for a 1-hour recovery before hypoxia. The pups were then placed in air-tight 500-mL containers partially submerged in a 37°C water bath through which humidified 8% oxygen was maintained at a flow rate of 3 L/min for 2 hours. Pups received intraperitoneal injections of HUVECs (1 × 10⁶ per injection), conditioned medium, or saline solution before and immediately after HI. The experiments were performed by laboratory technicians, whereas the quantitative measurements were performed by the investigators who were blinded to the grouping during the assessment. The brain was sectioned (20-μm thick) from the genu of the corpus callosum to the end of the dorsal hippocampus post-HI.

#### Brain Damage Measurement

Brain damage was determined by Nissl staining at P14. Corresponding to the plates 15, 18, 27, 31, and 39 in a rat brain atlas, the cross-sectional area of the cortex (plates 15, 18, 27, 31, and 39), hippocampus (plates 27, 31, and 39), and striatum (plates 15, 18, 27, and 31) were calculated, and the percentage of area loss in the lesion versus the nonlesion hemisphere was determined.

#### Immunofluorescence

The sections were incubated with a mixture of 2 of the following primary antibodies: anti-neuronal nuclear antigen (NeuN; 1:100; Millipore), anticleaved caspase-3 (1:100; Cell Signaling), anti-rat endothelial cell antigen-1 (RECA-1; 1:100; Abcam), and anti-GFP (1:100; Santa Cruz). The secondary antibodies included the following: Alexa Fluor 594–conjugated antimouse IgG (1:400; Invitrogen) or Alexa Fluor 488-conjugated antirabbit IgG (1:400; Invitrogen). The fluorescence signals were recorded at excitation–emission wavelengths of 596 to 615 nm (Alexa Fluor 594, red) and 470 to 505 nm (Alexa Fluor 488, green).

#### TissueFAXS Fluorescence Quantification Analysis

TissueFAXS, Zeiss AxioImager Z1 Microscope System (Tissue-Gnostics) was used to quantify the GFP-positive HUVECs, NeuN-positive neurons, apoptotic neuronal cells, and the vascular and extravascular location of GFP-positive HUVECs in the cortex post-HI. Three visual fields (0.145 mm²/field) per section (magnification: 20×) (Figure I in the online-only Data Supplement), and 3 brain sections (plates 27, 31, and 39) per rat were counted.

#### GFP-positive HUVECs

Cells were identified by their nuclei (4',6-diamidino-2-phenylindole [DAPI] staining) and applied to determine gray values in the corresponding channels, fluorescein isothiocyanate (GFP). The percentages of GFP and DAPI double-positive cells among DAPI-positive cells were determined.

#### Neuronal Apoptosis

Cells were identified by their nuclei (DAPI staining) and applied to determine gray values in the 2 corresponding channels, fluorescein isothiocyanate (cleaved caspase-3) and Cy3 (NeuN). The percentages of cleaved caspase-3+, NeuN−, and DAPI-triple–positive cells among DAPI-positive cells were determined.

#### Location GFP-positive HUVECs

Cells were identified by their nuclei (DAPI staining) and then applied to determine gray values in the 2 corresponding channels, fluorescein isothiocyanate (GFP) and Cy3 (RECA-1). The percentages of RECA-1 double–positive cells were determined.

#### Quantitative Analysis

#### Cerebral Vessel Length and Density

Assessment of RECA-1–positive vessel density at 24 hours post-HI was performed using the length density stereological parameter. The region containing the highest vessel density was chosen. Three visual fields were acquired (20× magnification) to calculate the length density.

#### BBB Damage, Microglia, Astrogliosis, and SDF-1

IgG extravasation (an indicator of BBB permeability) and microglia activation (ED-1) were assessed 24 hours post-HI and astroglosis (an indicator of BBB permeability) and microglia activation (ED-1) were assessed 24 hours post-HI and astroglosis (antiglial fibrillary acidic protein) at 7 days post-HI (online-only Data Supplement). The IOD signals were analyzed (200× magnification) per visual field (0.145 mm²). Three visual fields per section and 3 sections (plates 27, 31, and 39) per brain were analyzed.

#### Quantifying Blood Levels of GFP-Positive HUVECs

To quantify the GFP-labeled HUVECs after intraperitoneal injection, peripheral blood (100 μL) samples were put into 1-mL syringes containing 100:1 heparin. The cells were fixed, and the GFP-positive HUVECs and nucleated blood cells were detected by flow cytometry.
HUVEC Migration Assays In Vitro

Boyden Chamber Assay

The specimens were loaded into the lower parts of a 48-hole Boyden chamber (BD Biosciences). The cell suspension was loaded into the upper chamber with a loading volume of 50 μL with a total cell number of 5x10⁴ for each hole. After 12-hour incubation, cells on the bottom of the filter were stained with Giemsa and counted (144 fields of each quadruplicate filter) with tissue cytometry (online-only Data Supplement).¹⁸

OGD

Neurons and Endothelial Cells

Mouse neuroblastoma cells (Neuro-2a; Bioresource Collection and Research Center) and mouse immortalized cerebral endothelial cells (b.End3; American Type Culture Collection) were cultured, plated (4x10⁴ cells per dish), and grown for 24 hours in a normoxia under complete medium. For OGD, the Neuro-2a and b.End3 cells were washed, switched to OGD medium, and placed in a hypoxia chamber (NexBiOxy). The chambers were sealed and maintained under a gas mixture of 95% N₂/5% CO₂ for 6, 12, 18, and 24 hours. OGD was terminated by returning the cell culture to a normoxic condition under the complete medium for 24 hours.³

HUVEC Coculturing With Neurons or With Endothelial Cells

The Neuro-2a cells and b.End3 cells were cocultured with HUVEC-P4. After the cells had been washed and placed in OGD medium, the HUVECs were added to the Neuro-2a and b.End3 cells, respectively, using transwells before OGD. OGD was terminated by returning to a normoxic condition and replacing the medium with complete medium.

Cell Viability

At 24 hours post-OGD, cell viability was assessed using a cell proliferation kit (XTT; Roche). The Neuro-2a cells (1x10⁵) and b.End3 cells (1x10⁵) were incubated with XTT labeling reagent for 2 hours. The quantity of formazan was measured by the amount of 492 nm absorbance, and cell viability was expressed as the percentage of optical density in the OGD group over that of the normoxia control.³

Statistics

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software). Numeric continuous data were presented as mean±SEM and analyzed using 1-way ANOVA, and Tukey test was used for post hoc comparisons. For comparisons of brain area reduction between groups, we used Kruskal-Wallis test and Tukey test for post hoc comparisons. P<0.05 was considered statistically significant, and all probabilities were 2-tailed.

Results

Low-Passage HUVECs Entered the Brain and Positioned Close to the Neurons and Microvessels After HI

Boyden chamber (Figure 1A) revealed that HUVEC-P4 had significantly higher migratory properties than HUVEC-P8. Flow cytometry showed similar transfection efficiency between HUVEC-P4 and HUVEC-P8 at 24 hours after adenovirus-GFP transfection (Figure 1B). After injection, HUVEC-P4–treated pups had significantly more HUVEC-positive cells (GFP-positive cells/all nucleated cells) in the blood at 3 hours post-HI than HUVEC-P8–treated pups (Figure 1C). The circulating

Figure 1. Boyden chamber assay revealed human umbilical vein endothelial cell (HUVEC)-P4 had higher migratory properties (A, left) and significantly more Giemsa-stained cells migrated from the bottom of the filter 12 hours postincubation (A, right, tissue cytometry) than HUVEC-P8 (144 fields of each quadruplicate filter); n=4 in each group. B, HUVEC-P4 and HUVEC-P8 had comparable green fluorescent protein (GFP) transfection efficiency. C, HUVEC-P4 pups had significantly more circulatory GFP-positive cells 3 hours posthypoxia than HUVEC-P8 pups; n=6 to 10 in each group. Scale bar in B =200 μm. Data are mean±SEM. *P<0.05, **P<0.01, #P<0.001.
HUVEC-positive cells in the HUVEC-P4 group decreased significantly from 3 hours to 12 hours and 24 hours post-HI. Immunofluorescence revealed many GFP-positive cells in the ipsilateral cortex of the HUVEC-P4–treated pups but not of the HUVEC-P8–treated pups 24 hours post-HI (Figure 2A). Tissue cytometry showed that the HUVEC-P4–treated group had higher percentages of HUVECs (GFP-positive cells/DAPI-positive cells; Figure 2B) in the cortex 24 hours post-HI than the HUVEC-P8–treated group. The GFP and DAPI double-positive cells significantly increased from 3 hours to 24 hours post-HI in HUVEC-P4 pups. HUVEC-P4–treated group had significantly more GFP and DAPI double-positive cells than the HUVEC-P8–treated group at 24 hours and 7 days post-HI (Figure 2C). Immunofluorescence in HUVEC-P4–treated pups by guest on April 5, 2017 http://stroke.ahajournals.org/ Downloaded from

**Figure 2.** A. Human umbilical vein endothelial cell (HUVEC)-P4 pups had many green fluorescent protein (GFP)-positive cells in the cortex 24 hours after hypoxic-ischemia (HI) than HUVEC-P8 pups. Tissue cytometry revealed that HUVEC-P4 pups had (B) higher percentages of HUVECs at 24 hours and (C) significantly more GFP and 4',6-diamidino-2-phenylindole (DAPI) double-positive cells per visual field than HUVEC-P8 pups at 24 hours and 7 days post-HI; n= 4 to 6 per group at each time point. Data are means±SEM. Immunofluorescence (D) of the HUVEC-P4 pups showed the HUVEC (arrows) locations were 70.7% extravascular (top left) and 29.3% vascular (top middle). The GFP-positive cells did not express neuronal nuclear antigen (NeuN; **top right**). Confocal microscopy (**D, bottom**) showed the GFP-positive HUVECs were located close to endothelial cells. Scale bar in A =100 μm (inset =10 μm); in **D, top**=50 μm, **D, bottom** =10 μm. *P<0.05, **P<0.01.

**Figure 3.** At 24 hours after hypoxic-ischemia (HI) (A) immunofluorescence revealed the human umbilical vein endothelial cell (HUVEC)-P4 group had fewer neuronal nuclear antigen (NeuN)-positive neurons that coexpressed cleaved caspase-3 than the HUVEC-P8 or condition medium group. The HUVEC-P4 group had lower percentage of apoptotic neurons (cleaved caspase-3- and NeuN double-positive cells/4',6-diamidino-2-phenylindole [DAPI]-positive cells) (B) and significantly less triple-positive (cleaved caspase-3, NeuN, and DAPI) cells per visual field (C) than the other 2 groups; n= 4 to 6 in each group. Data were mean±SEM. *P<0.05, **P<0.01. Scale bar, 100 μm.
pups (Figure 2D, top) showed that the percentages of the GFP-positive HUVEC location were 70.7% ± 1.1% extravascular and 29.3% ± 1.1% vascular. The GFP-positive cells coexpressed DAPI, but not NeuN; they remained endothelial character. Z-section confocal microscopy confirmed that the HUVEC-P4 were positioned close to endothelial cells (Figure 2D, bottom).

**HUVEC-P4 Protected Against Neurovascular Injury and Provided Neuroprotection**

At 24 hours post-HI, immunofluorescence in the ipsilateral cortex showed the HUVEC-P4–treated group had more NeuN-positive neurons than the HUVEC-P8– or condition medium–treated group (Figure II in the online-only Data Supplement). The HUVEC-P4–treated group also had fewer NeuN-positive neurons that coexpressed cleaved caspase-3 than the HUVEC-P8– and condition medium–treated groups (Figure 3A). Tissue cytometry revealed that the HUVEC-P4–treated group had lower percentages of apoptotic neurons (Figure 3B) and significantly less cleaved caspase-3, NeuN and DAPI triple-positive cells (Figure 3C) than the other 2 groups. RECA-1 immunofluorescence showed the HUVEC-P4–treated group had more preservation of microvessels than the HUVEC-P8– or condition medium–treated group (Figure 4A). The HUVEC-P4–treated group had significantly more vascular area (Figure 4B) and vascular length density (Figure 4C) than the HUVEC-P8–treated or condition medium–treated group.

**HUVEC-P4 Provided Neuroprotection via SDF-1/CXCR4**

Neuropathological examination on P14 showed that HUVEC-P4–treated group, but not HUVEC-P8–treated group, had significantly less brain area reduction than condition medium–treated or normal saline–treated groups (Figure 5A). Immunohistochemistry showed that compared with control, the SDF-1 level was significantly upregulated in the cortex 3 hours post-HI (Figure 5B). The pups received intra-peritoneal injections of AMD3100 (7.5 mg/kg per dose), a CXCR4 antagonist, or normal saline immediately before and after HI. Neuropathology showed that the AMD3100- and normal saline–treated group had similar brain area reduction, suggesting that inhibiting the SDF-1/CXCR4 during HI did not worsen damage (Figure 5C). The HUVEC-P4–treated pups were then administered with AMD3100 (7.5 mg/kg per dose) immediately before and after HI. Neuropathology showed that the HUVEC-P4 AMD3100 group had significantly more brain damage (Figure 5D) than the HUVEC-P4 normal saline group.
HUCV Coculturing Provided Neuronal and Vascular Protection Against OGD

In vitro study for different OGD durations (6 hours, 12 hours, 18 hours, and 24 hours) revealed that there were progressive decreases in cell survival rate, with 26.8% and 52.8% survival, respectively, in the Neuro-2a cells (Figure 6A) and b.End3 endothelial cells (Figure 6B) after 24-hour OGD. Transwell coculture of HUVECs increased survival ≈20% in the Neuro-2a cells (Figure 6C), and increased survival ≈40% in the b.End3 cells (Figure 6D) against OGD, suggesting HUVECs protect against neuronal and vascular injury via paracrine effect.

Discussion

Neurovascular unit is a major target of neuroprotection in newborns with HI. We found that peripheral injections of low-passage HUVECs entered the neonatal brain post-HI, positioned close to the neurons and vessels, protected against neuronal and vascular endothelial cell damage, and provided neuroprotection. Inhibiting the SDF-1/CXCR4 pathway did not affect HI brain damage; however, the neuroprotection provided by peritoneal injection of HUVEC-P4 was attenuated by inhibiting the SDF-1/CXCR4 pathway. In addition, transwell coculturing with HUVECs protected against OGD cell death in neurons and endothelial cells. Taken together, our findings suggest that cell therapy using HUVECs may be a powerful therapeutic strategy against HI in neonatal brain potentially via its paracrine effect on neurons and endothelial cells.

The neurovascular unit provides a framework for the development of new treatment that targets both neurons and vessels of neurological diseases. Significant neurovascular protection post-HI is related to long-term neuroprotection in the neonatal brain. It has been well-known that HUVECs alter their biochemical and cellular behaviors according to passages. Higher passage of HUVECs may result in lost cell functions by downregulation of its migration molecules. We showed that the transmigration ability was decreased in the high-passage HUVECs. Peripheral injection of low-passage HUVECs positioned close to the neurons and microvessels after entering the brain and protected against neuronal and vascular injury via paracrine effect.
vascular damage after HI. The correlation of HUVECs presented in the lesioned brain area and the beneficial effects observed is strengthened by the relevance of spatial proximity for neurovascular protective effects observed in the in vitro HUVEC coculture system, which showed that HUVECs protected neurons and endothelial cells against OGD.

Most studies of cell therapy in neonatal HI injury use intracerebral transplantation. Cell delivery via intravenous or intraperitoneal route is a less invasive method that allows widespread distribution of transplanted cells to cover the injured area. In addition, this peripheral procedure enables exposure of cells to the chemotactic signals at the injury site, which selectively attracts cells to migrate to the target tissue. Our study shows that the HUVECs delivered via peritoneal injection were able to reach the systemic circulation, migrate to the vulnerable area, and, more importantly, position close to the neurons and vessels after HI.

The intraperitoneal route has been used for cell transplantation in neonatal HI brain injury, demonstrating cell migration from the peritoneal cavity into the injured areas. These studies suggest the importance of precise chemotactic signals and BBB opening in the injured area, so that the cells injected can migrate over such long distances. In adult rats, bone marrow–derived stem cells homed to the ischemic brain via the interaction of SDF-1 and CXCR4. In neonatal brain injury, the increased expression of SDF-1 in the vulnerable area was observed post-HI and the intraperitoneally transplanted HUCSCs migrated to the lesion site within 1 day after injection. Although studies have shown that SDF-1/CXCR4 was related to the homing effect of the HUCSC after neonatal HI, significant neuroprotection was not observed at pathological levels when treatment started 24 hours after injury. We demonstrated that SDF-1 level was upregulated in the cortex 3 hours post-HI, and peripherally injected HUVECs before and immediately after HI were observed in the cortex 24 hours post-HI and provided significant neuroprotection. AMD3100 is a bicyclam molecule that antagonizes the binding of SDF-1 to its cognate receptor CXCR4 and inhibits SDF-1–induced calcium flux and chemotaxis. AMD3100 is highly specific for CXCR4 and has no detectable agonist activity in calcium flux and chemotaxis assays. Inhibiting the SDF-1/CXCR4 axis using AMD3100 significantly attenuated the neuroprotection afforded by HUVECs providing evidence that HUVEC-mediated neuroprotection in neonatal brain required SDF-1/CXCR4 axis.

By interacting with their receptors, cells may release growth factors and cytokines, thus inhibiting apoptosis, increasing angiogenesis, and stimulating the differentiation of endogenous precursor cells. Endothelial-produced brain-derived neurotrophic factor can protect neurons against OGD and oxidative damage. Vascular endothelial growth factor also has neurovascular protective effects in the neonatal brain.
We showed the spatial proximity HUVECs have with neurons and endothelial cells is associated with neurovascular protection after HI. Transwell coculturing with HUVECs significantly increased neuronal and endothelial cell survival against OGD, suggesting that HUVECs may provide the neurovascular protection by releasing trophic factors.

Further study is important to examine the neuroprotective effect and the therapeutic time window of HUVECs when administered after HI insult. Cell therapy using HUVECs has the potential for protective intervention in the neonatal brain because of its homogeneity, well-characterized surface markers, regenerative potential, and autologous capability. A proposed diagram (Figure III in the online-only Data Supplement) is provided to show how the intraperitoneally injected HUVEC enters the brain post-HI to provide neuroprotection. Elucidating the shared neuronal and vascular protective molecules provided by HUVECs may yield neuroprotective drugs that mimic the beneficial effects of HUVECs for treating high-risk newborns with asphyxia.

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Disclosures
The authors have no conflicts of interests to report.

References
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SUPPLEMENTAL MATERIAL

Method

Human umbilical cords were rinsed and washed. Endothelial cell suspensions were harvested by collagenase perfusion, centrifuged, and the pellets re-suspended in the complete medium [M199 supplemented with 1% penicillin/streptomycin, 20% fetal bovine serum (FBS) and endothelial growth medium (EGM)], before being plated onto culture dishes. HUVECs can be propagated for 30-70 population. After about 5-7 passages, cells gradually start to increase in size, to grow more slowly and to lose specific functions. It has been well known that HUVECs alter their bio-chemical and cellular behaviors according to passages. Soluble intracellular adhesion molecule-1 (sICAM-1), an endothelial cell marker, is different dependent on passages. Biological processes categories are most significant with HUVEC population, including downregulation of cell cycle and proliferation, and up-expression of programmed cell death suggesting that higher passage of HUVEC may lose cell functions by downregulating some transmigration molecules.

The percentage of circulatory GFP-positive cells was derived from the % of circulatory GFP-(+) cells/all circulatory nucleated cells. The rat peripheral bloods were first lysed with RBC lysis buffer, and then analyzed the rest of the cells using flow cytometry.

AMD3100 (Plerixafor, Genzyme Corporation) is a bicyclam molecule that antagonizes the binding of the chemokine stromal cell-derived factor-1 (SDF-1) to its cognate receptor CXCR4 and inhibits SDF-1-induced calcium flux and chemotaxis. AMD3100 is highly specific for CXCR4 and has no detectable agonist activity in calcium flux, GTP-binding and chemotaxis Assays. AMD3100 results in the rapid and reversible mobilization of hematopoietic stem cells into the peripheral circulation.

AMD3100, a now well-documented specific antagonist of CXCR4, is widely applied
as SDF-1/CXCR4 blocker clinically and experimentally. AMD3100 rapidly mobilizes hematopoietic progenitor cells in both humans and mice by reversibly blocking the SDF-1–CXCR4 interaction. AMD3100 injection after surgical ligation of the coronary artery increases the mobilization of bone marrow progenitor cells, enhances greater bone marrow progenitor cells accumulation in infarcted tissue and improves myocardial performance. AMD3100 also prolongs bone marrow progenitor mobilization and improves recovery from ischemia/reperfusion cardiac injury through eNOS-dependent mechanism.

BBB damage, microglia, astrogliosis and SDF-1. Sections (20-μm thick) were incubated with 0.3% H2O2 /methanol. IgG extravasation (1:200; Chemicon) and microglia activation (ED-1, 1:100; Millipore) were assessed at 24 hours, and astrogliosis (anti-glial fibrillary acidic protein [GFAP], 1: 800; Millipore) at 7 days post-HI. Different time points were assessed for SDF-1 antibody (1:100; Cell signaling). Biotin-peroxidase signals were detected and images acquired. The integrated optical density (IOD) was analyzed (200× magnification) per visual field (0.145 mm²).

Wound closure assay. HUVEC-P4 and HUVEC-P8 monolayers were carefully wounded by scratching with a sterile plastic pipette tip along the diameter of the well. After injury, the cells were incubated with the complete medium. HUVEC migration was quantified by measuring the distance between the wound edges at the time of injury and after incubation.

GFP-positive HUVECs number. Cells were identified by their nuclei (DAPI staining), and applied to determine gray values in the corresponding channels FITC (GFP). The percentages of GFP- and DAPI-double positive cells among DAPI-positive cells were determined.

Neuronal cells. Cells were identified by their nuclei (DAPI staining), and applied to
determine gray values in the corresponding channels Cy3 (NeuN). The percentages of NeuN- and DAPI-double positive cells among DAPI-positive cells were determined.
Results

The postpartum day 7 rat pups have nucleated cell counts of about 5000/uL in the blood circulation and a total blood volume of 400 uL. We intraperitoneally injected $2 \times 10^5$ GFP-positive HUVECs and detected 0.75% of GFP-positive cells/all nucleated blood cells at 3 hours after hypoia. Therefore, the calculated % of injected HUVEC that could be detected in systemic circulation at 3 hours post-hypoxia is about 7.5%.
Figure 1. The representative region for the measurement of GFP-positive HUVEC cells, NeuN-positive neurons, apoptotic neuronal cells (NeuN colocalized with cleaved caspase-3), and the vascular and extravascular location of GFP-positive HUVEC cells in the cortex post-HI using TissueFAXS®, Zeiss AxioImager Z1 Microscope System (Tissue-Gnostics, Vienna, Austria).
Supplemental Figure 2.

**Figure 2.** At 24 hours post-HI, (A) immunofluorescence of the ipsilateral cortex showed that the HUVEC-P4 group had more NeuN-positive neurons than the HUVEC-P8 or condition-medium group. (B) Tissue Cytometry showed that the HUVEC-P4 group (59.04%) had higher percentages of NeuN-positive neurons (NeuN-positive cells/DAPI-positive cells) than the HUVEC-P8 (42.42%) or condition medium (41.56%) group 24 hours post-HI. (C) The HUVEC-P4 group also had significantly more NeuN- and DAPI-double positive cell number per visual field than the HUVEC-P8 or condition-medium group. n= 4-5 in each group. Data were mean ± SEM. *P<0.05; **P<0.01. A: Scale bar = 100 μm, and =10 μm in inset.
Figure 3. A proposed diagram shows how peripherally-injected low-passage HUVEC HUVECs migrate into systemic circulation, enter the brain after hypoxic-ischemia via SDF-1/CXCR4 migratory pathway, and position in proximity to the neurons and vessels to provide neurovascular protection in the neonatal brain. The HUVEC-mediated neurovascular protection may work via its paracrine mechanisms on the neurons and vascular endothelial cells.