Intravenous Administration of Human Bone Marrow Stromal Cells Induces Angiogenesis in the Ischemic Boundary Zone After Stroke in Rats

Jieli Chen, Zheng Gang Zhang, Yi Li, Lei Wang, Yong Xian Xu, Subhash C. Gautam, Mei Lu, Zhenping Zhu, Michael Chopp

Abstract—We tested the hypothesis that intravenous infusion of human bone marrow stromal cells (hMSCs) promotes vascular endothelial growth factor (VEGF) secretion, VEGF receptor 2 (VEGFR2) expression and angiogenesis in the ischemic boundary zone (IBZ) after stroke. hMSCs (1×10^6) were intravenously injected into rats 24 hours after middle cerebral artery occlusion (MCAo). Laser scanning confocal microscopy (LSCM), immunohistochemistry and ELISA were performed to assay angiogenesis and levels of human and rat VEGF in the host brain, respectively. In addition, capillary-like tube formation was measured using mouse brain-derived endothelial cells (MBDECs). Morphological and three dimensional image analyses revealed significant (P<0.05) increases in numbers of enlarged and thin walled blood vessels and numbers of newly formed capillaries at the boundary of the ischemic lesion in rats (n=12) treated with hMSCs compared with numbers in rats (n=12) treated with PBS. ELISA measurements showed that treatment with hMSCs significantly (P<0.05) raised endogenous rat VEGF levels in the IBZ from 10.5±1.7 ng/mL in the control group to 17.5±1.6 ng/mL in the hMSC-treated group. In addition, treatment with hMSCs increased endogenous VEGFR2 immunoreactivity. In vitro, when MBDECs were incubated with the supernatant obtained from cultured hMSCs, capillary-like tube formation was significantly (P<0.01) induced. However, hMSC-induced capillary-like tube formation was significantly (P<0.01) inhibited when the endothelial cells were incubated with the supernatant from hMSCs in the presence of a neutralizing anti-VEGFR2. These data suggest that treatment of stroke with hMSCs enhances angiogenesis in the host brain and hMSC-enhanced angiogenesis is mediated by increases in levels of endogenous rat VEGF and VEGFR2. (Circ Res. 2003;92:692-699.)

Key Words: middle cerebral artery occlusion ■ bone marrow stromal cell ■ angiogenesis ■ vascular endothelial growth factor ■ rats

In rats subjected to stroke and trauma, intravenously injected bone marrow stromal cells (MSCs) pass through blood brain barrier, migrate selectively and target damaged brain, and improve functional recovery.1–3 However, the mechanisms by which MSCs promote functional recovery are not clear. Stroke induces angiogenesis, and angiogenesis is associated with improved neurological recovery.4,5 Under normal circumstances after stroke, the contribution of angiogenesis to the brain capillary network is insufficient to support the brain plasticity required for functional recovery. MSCs secrete several growth factors including VEGF.6,7 Vascular endothelial growth factor (VEGF) has a prominent role in vascular formation.8 We have previously demonstrated that administration of recombinant human VEGF 165 (rh-VEGF165) to rats 48 hours after stroke significantly increased angiogenesis in the penumbra and improved functional recovery.4 Thus, it is reasonable to propose that functional benefit after treatment of stroke with human bone marrow stromal cells (hMSCs) may derive from cerebral angiogenesis. In the present study, we therefore tested the hypothesis that treatment of stroke with hMSCs enhances VEGF and thereby promotes angiogenesis in the host brain.

Materials and Methods

Animal Model
Adult male Wistar rats weighing 270 to 300 g were used in all our experiments. Rats were purchased from Charles River Breeding Company (Wilmington, Mass). All experimental procedures have been approved by the Care of Experimental Animals Committee of Henry Ford Hospital. Transient middle cerebral artery occlusion (MCAo) was induced using a method of intraluminal vascular occlusion modified in our laboratory.9 Briefly, the right common
hMSCs were obtained from 20 mL aspirates from the iliac crest of one normal human donor. Each 20 mL of aspirate was diluted 1:1 with Hanks' balanced salt solution (HBSS; Gibco) and layered over 10 mL of Ficoll (Ficoll-Paque; Pharmacia). After centrifugation at 2500g for 30 minutes, the mononuclear cell layer was removed from the interface and suspended in HBSS. Cells were centrifuged at 1000g for 10 minutes and resuspended in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FBS. The cells were incubated at 37 °C in 5% CO2 in flasks for 3 days and nonadherent cells were removed by replacing the medium. After the cultures reached confluence, usually 2 to 3 weeks, the cells were harvested with 0.05% trypsin and 0.53 mmol/L EDTA (GIBCO) for 5 minutes at 37 °C, replated in a flask, again cultured for about 2 weeks, and harvested. They were then frozen for later use. Cells used in these experiments were harvested from 3 to 5 passages.

At 1 day after ischemia, randomly selected animals received either hMSCs (n = 12) or phosphate buffered saline (PBS, n = 12). Animals were anesthetized with 3.5% halothane and then maintained with 1.0% to 2.0% halothane in 70% N2O and 30% O2 using a face mask. Approximately, 1 × 10^6 hMSCs in 1 mL total fluid volume PBS were injected into a tail vein at 24 hours after stroke. We have previously shown that treatment of stroke in rat with dead MSCs or with rat liver fibroblasts provides no functional benefit compared with PBS-treated rats. Therefore, the present study uses PBS as the control treatment. Immunosuppressants were not used in any animals. All animals were euthanized at 14 days after MCAo.

To measure VEGFR1 (flt-1) and VEGFR2 (flk-1) expression in the ischemic brain after hMSC treatment, 6 rats received either 1 × 10^6 hMSCs (n = 3) or PBS (n = 3) at 1 day after stroke. These animals were euthanized at 3 days after treatment, and frozen sections were analyzed for VEGFR1 and VEGFR2 immunoreactivity.

Bromodeoxyuridine Labeling
Bromodeoxyuridine (BrdU, Sigma Chemical), the thymidine analog that is incorporated into the DNA of dividing cells during S-phase, was used for mitotic labeling. BrdU (50 mg/kg) was intraperitoneally injected daily for 14 consecutive days into ischemic rats starting 1 day after MCAo.

Three-Dimensional Image Acquisition and Analysis
To examine neovascularization in ischemic brain, fluorescein isothiocyanate (FITC) dextran (2 × 10^6 molecular weight, Sigma; 1 mL of 50 mg/mL) was administered intravenously to the ischemic rats subjected to 14 days of MCAo. FITC-dextran remains dissolved and free in plasma. The brains were rapidly removed from the severed subject and subjected to 14 days of MCAo. FITC-dextran remains dissolved and free in plasma.4 The brains were rapidly removed from the severed subject and subjected to 14 days of MCAo. FITC-dextran remains dissolved and free in plasma.4

To test whether hMSC treatment induces VEGFR1 and VEGFR2 expression in cerebral endothelial cells, flt-1 and flk-1 immunohistochemistry staining was performed on 20-μm thick frozen sections. A mouse monoclonal anti-VEGF1 (flt-1, Santa Cruz Biotechnology) and anti-VEGF2 (flk-1, Santa Cruz Biotechnology) were used at a titer of 1:100, respectively. To quantify cell types of VEGF immunoreactivity and the cellular phenotype of grafted hMSCs, double immunofluorescence labeling for von Willebrand factor (vWF), a marker for endothelial cells, or glial fibrillary acidic protein (GFAP), a marker for astrocytes, were performed. A monoclonal antibody against vWF (DAKO) and a polyclonal antibody against GFAP (DAKO) were used at a titer of 1:400 and 1:1000, respectively. Coronal sections were incubated with the antibody against vWF, GFAP at 4 °C, and sections were then incubated with the anti-mouse or anti-rabbit immunoglobulin antibody conjugated to Cy5 (Vector). These sections were incubated with the antibody against VEGF, human cell nuclei (MAB1281), or BrdU at 4 °C, and then with the anti-goat or anti-mouse immunoglobulin antibody conjugated to FITC. For BrdU immunostaining, DNA was first denatured by incubating brain sections (6 μm) in 50% formamide 2 × SSC at 65 °C for 2 hours and then in 2N HCl at 37 °C for 30 minutes. Sections were then rinsed with Tris buffer and treated with 1% of H2O2 to block endogenous peroxidase. Sections were incubated with a mouse monoclonal antibody (mAb) against BrdU (1:1000, Boehringer Mannheim) overnight and incubated with biotinylated secondary antibody (1:200, Vector) for 1 hour. BrdU (Cy5) with MAB1281 (FITC) double staining was performed. Control experiments consisted of staining brain coronal tissue sections as outlined above, but omitted the primary antibodies.
Neutralizing antibody to VEGF receptor 2 (VEGFR2, DC101, 10 μg/mL, Imclone System). All assays were performed in triplicate. For quantitative measurements of capillary tube formation, Matrigel dishes were digitized under a 2.5× objective (Olympus BX40) for measurement of total tube length of capillary tube formation using a video camera (Sony DVC-970 MD) interfaced with MCID image analysis system (Imaging Research). Tracks of endothelial cells organized into networks of cellular cords (tubes) were counted and averaged in randomly selected 3 microscopic fields.13

Statistical Analysis
Cerebral microvessel angiogenesis was analyzed by calculating the total surface area of microvessels under a field of view (M. Lu, G.Z. Zhang, H. Soltanian-Zadeh, N. Oja-Tebbe, M.M. Khalighi, D. Morris, M. Chopp, unpublished data, 2002). Due to a lack of normality, ranked data were employed for the analysis microvessel surface area. Data in the two groups were first analyzed by a one-way ANOVA. All data are presented as mean±SD. A value of P<0.05 was taken as significant.

Results
Administration of hMSCs Induces Angiogenesis
Measurements of BrdU immunoreactive endothelial cells revealed few BrdU immunoreactive endothelial cells in the contralateral hemisphere (Figures 2A and 2D), which is consistent with fact that proliferation of the cerebral endothelial cells essentially ceases in the adult brain.16 In the ipsilateral hemisphere, however, there was a significant (P<0.05) increase in the number of BrdU immunoreactive endothelial cells in rats treated with PBS compared with the contralateral hemisphere (Figures 2B, arrow, and 2D). Treatment with hMSCs significantly (P<0.05) enhanced the number of BrdU immunoreactive endothelial cells in the ipsilateral hemisphere compared with the numbers in rats treated with PBS (Figures 2C, arrows, and 2D). BrdU immunoreactive endothelial cells primarily localized to enlarged cerebral vessels (Figures 2B and 2C). Enlarged thin-walled vessels are termed “mother” vessels and are markers of angiogenesis after cerebral ischemia.4 16 Treatment with hMSCs significantly (P<0.05) increased vascular perimeters in the ipsilateral hemisphere compared with perimeters in rats treated with PBS (Figure 2E).

To further examine angiogenesis, three-dimensional analysis was performed using software developed in our labora-
tory, which measures numbers of branch points, segment lengths, and diameters of vessels. Segments of capillaries were significantly (P<0.05) shorter in the hMSC-treated and MCAo-PBS groups (Figure 3 and Table) than in the homologous tissue in the contralateral hemisphere (Figure 3 and Table). Treatment with hMSCs (Figure 3 and Table) significantly (P<0.05) increased numbers of branch points in the boundary regions of ischemia compared with numbers in the homologous tissue in the contralateral hemisphere (Figure 3 and Table) and the ipsilateral hemisphere of rats treated with PBS (Figure 3 and Table). To avoid errors in statistical analysis for unpaired correlated data, three-dimensional cerebral vessels were further analyzed by calculating the total surface area of microvessels under a field of view. Vessel surface area in the ipsilateral hemisphere significantly (P<0.05) increased in rats treated with hMSCs compared with animals treated with PBS (Table).

**Administration of hMSCs Increases Endogenous Brain Levels of VEGF and VEGFR2**

Rats treated with PBS did not show any MAB1281 (a marker for human cells) immunoreactivity in brain tissue. In contrast, MAB1281 immunoreactive cells were detected in multiple brain regions of the ipsilateral hemisphere including cortex and striatum in rats treated with hMSCs, indicating specificity of this antibody for human cells (Figure 4A). The vast majority of hMSCs were localized to the ischemic boundary zone, with some cells within vessels (Figure 4B). Few hMSCs were detected in the contralateral hemisphere of brain. Scattered hMSCs (1% to 2%) showed vWF immunoreactivity. Likewise, less than 1% of BrdU immunoreactive cells were MAB1281-positive, indicating that the newly formed cells do not originate from the injected hMSCs.

To examine whether administration of hMSCs increases brain levels of VEGF, ELISAs for endogenous rat VEGF and exogenous human VEGF were performed. Treatment with hMSCs significantly (P<0.05) increased endogenous rat VEGF levels in the ischemic boundary regions from 10.5 ± 1.7 ng/mL in the control group to 17.5 ± 2.1 ng/mL in the hMSC-treated group. Human VEGF level was not detected in ischemic brain extracts. Parallel to ELISA results, immunostaining for endogenous rat VEGF showed increased in VEGF immunoreactivity within the ischemic boundary in rats treated with hMSCs (Figures 4C and 4D). Many rat VEGF immunoreactive cells exhibited GFAP immunoreactivity (Figures 4E through 4G). Semiquantitative analysis revealed that rat VEGF immunoreactivity was significantly (P<0.05) increased in rats treated with hMSCs compared with the control.

**Three-Dimensional Quantitative Measurements of Vascular Structure**

<table>
<thead>
<tr>
<th></th>
<th>Contralateral</th>
<th>MCAo</th>
<th>MCAo + hMSCs</th>
</tr>
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<tbody>
<tr>
<td>Diameter, μm</td>
<td>4.2±0.1</td>
<td>4.3±0.1</td>
<td>5.2±0.1†</td>
</tr>
<tr>
<td>Length, μm</td>
<td>32.8±1.6</td>
<td>25.5±1.2*</td>
<td>28.1±1.8*</td>
</tr>
<tr>
<td>No. of branch points (density)/0.002 mm³</td>
<td>134.3±7.6</td>
<td>131.6±11</td>
<td>177.7±11.4†</td>
</tr>
<tr>
<td>Total vessel surface area (μm²)/0.002 mm³</td>
<td>66 958±4626</td>
<td>44 558.5±9979</td>
<td>76 847.2±4601†</td>
</tr>
</tbody>
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*P<0.05 compared with contralateral; †P<0.05 compared with MCAo. n=3 per group.
with the immunoreactivity in the control rats (Figure 4H). However, immunostaining with the anti-human VEGF antibody exhibited a few VEGF-positive cells in the ischemic brain. Furthermore, treatment with hMSCs significantly \((P<0.05)\) increased the number of flk-1 immunoreactive endothelial cells in the ipsilateral hemisphere compared with the number in control rats (Figures 4I through 4K), whereas the number of flt-1-positive cells did not significantly increase \((14.8\pm2.1\% \text{ in hMSCs versus } 11.4\pm1.6\% \text{ in control})\). This suggests hMSC treatment after stroke induces endogenous cells to express VEGF and VEGFR2.

**hMSCs Upregulate VEGF and VEGFR2 Expression in Brain Endothelial Cells**

To further examine the effect of hMSCs on expression of VEGF and its receptors in endothelial cells, mouse-brain endothelial cells were treated with or without 50% hMSC supernatant for 24 hours. Consistent with in vivo findings, incubation with hMSC supernatant significantly \((P<0.05)\) increased the number of VEGF (Figures 5A through 5C) and flk-1 (Figures 5D through 5F) immunoreactive cells compared with the number in control group.

**hMSCs Induce VEGF, Which Mediates Angiogenesis In Vitro**

To examine whether hMSCs induce angiogenesis, a capillary-like tube formation assay was performed.\(^{17,18}\) A significant \((P<0.05)\) increase in capillary-like tube formation was detected when the endothelial cells were incubated with 100% hMSC supernatant (Figures 6B and 6D) compared with the endothelial cells incubated with regular cell culture medium (Figures 6A and 6D). However, when the endothelial cells were incubated with 100% supernatant from hMSCs in the presence of a rat anti-mouse neutralizing antibody to VEGFR2, capillary-like tube formation was significantly \((P<0.05)\) inhibited compared with the supernatant alone (Figures 6C and 6D). The blockade of the VEGFR2 with anti-VEGFR2 antibody did not completely abolish hMSC-induced capillary-like tube formation (Figure 6D).

**Discussion**

Our data demonstrate that treatment of stroke with hMSCs significantly enhances angiogenesis in ischemic brain, and hMSC-enhanced angiogenesis is likely mediated by increases in endogenous rat VEGF and VEGFR2 expression.

The present investigation was undertaken to identify potential mechanisms underlying the promotion of neurological functional recovery induced by hMSC treatment of stroke.\(^1\) Angiogenesis is associated with improved neurological recovery from stroke.\(^4\) Stroke patients with a higher cerebral blood vessel density make better progress and survive longer than patients with lower vascular density. Newly formed vessels improve tissue perfusion around the ischemic boundary zone and enhancement of angiogenesis promotes functional recovery in rats after stroke.\(^4,5\) hMSC-enhanced angiogenesis in ischemic brain, therefore, likely contributes to improvement of neurological functional recovery.

Although proliferation of cerebral endothelial cells ceases in the adult brain, stroke induces neovascularization within the ischemic boundary zone.\(^5\) Newly formed vessels are derived from enlarged thin wall vessels by sprouting and by invagination.\(^4\) Our findings that treatment with hMSCs significantly increased vascular perimeter and numbers of capillaries are consistent with previous reports on dynamic formation of new vessels in ischemic brain.\(^4\) In addition, our observations that treatment with hMSCs increased numbers of proliferated endothelial cells and a few hMSCs \((\approx 1\%)\) exhibit endothelial phenotype support the concept that formation of new vessels arises from the proliferation and migration of endothelial cells from the adjacent tissue and from circulating endothelial progenitor cells.\(^5,16\) To our knowledge, this is the first study using
three-dimensional image analysis to demonstrate that hMSCs promote angiogenesis in adult brain after stroke.

hMSC treatment of ischemic stroke activates the endogenous cell expression of both VEGF and its receptor VEGFR2. VEGF exerts biological function via at least two related receptor tyrosine kinases, VEGFR1 and VEGFR2. The binding of VEGF to VEGFR2 elicits activation of signaling cascades that trigger the biological effects of VEGF. VEGFR2 has been shown to be essential for endothelial progenitor cell proliferation and differentiation. Blocking VEGFR2 inhibits angiogenesis in adult mice. Data in the present study demonstrate that treatment with hMSCs significantly increases rat brain levels of VEGF and VEGFR2 but not human VEGF levels. Distribution of increased VEGF corresponds with the localization of hMSCs in the host brain. Therefore, these data suggest that hMSCs increase endogenous levels of VEGF and VEGFR2. Our in vivo findings were confirmed.

**Figure 5.** VEGF and VEGFR2 expression in cultured brain endothelial cell. VEGF immunoreactive cells were increased in hMSC supernatant-treated group (B, arrows) compared with control medium (A, arrow). flk-1-reactive cells were increased in hMSC supernatant-treated group (E, arrows) compared with control (D, arrow). C and F, Quantitative data of percent of VEGF (C) and flk-1 (F) immunoreactive cells in cultured mouse brain endothelial cell. Scale bars in B = 25 μm.

**Figure 6.** Capillary-like tube formation. When mouse brain endothelial cells were incubated with a regular medium, capillary-like tube formation was not detected (A, n = 3). However, incubation with hMSC supernatant strongly induced capillary-like tube formation (B, n = 3). A rat anti-mouse neutralizing antibody to VEGFR2 significantly inhibited hMSC-induced capillary-like tube formation (C, n = 3). D, Quantitative analysis of capillary-like tube formation. Note: the blockade the VEGFR2 with anti-VEGFR2 antibody did not completely inhibit hMSC-induced capillary-like tube formation (D). Scale bar in A = 25 μm.
by in vitro data showing that incubation of endothelial cells with hMSC supernatant increases endothelial cell expression of VEGF and VEGFR2. Furthermore, hMSC-induced capillary-like tube formation is inhibited by a neutralized antibody against VEGFR2. Collectively, we propose that administration of hMSCs enhance expression of VEGF in astrocytes and endothelial cells and VEGFR2 in endothelial cells, and that enhanced VEGF interacts with increased VEGFR2 in endothelial cells, which consequently promotes angiogenesis in adult ischemic brain.

Although the present study was not designed to investigate mechanisms of how hMSCs upregulate endogenous VEGF and VEGFR2, a possible tie between the exogenous hMSC and parenchymal cell local production of growth factors may be related to the ability of MSCs to produce a wide variety of trophic factors and cytokines, some of which activate the production of VEGF and VEGFR2. For example, hMSCs secrete bFGF,20,22 and bFGF promotes expression of VEGF and VEGFR2.23,24 We have also demonstrated that when hMSCs are cocultured with injured or rat ischemic brain, an array of trophic factors are produced, including bFGF.22 Thus, we speculate that hMSCs, by producing cytokines and trophic factors, indirectly upregulate the production of endogenous VEGF and VEGFR2, which promote angiogenesis in the ischemic brain.

The mechanisms of action of bone marrow stromal cells to treat neural injury and stroke stand in sharp contrast to those of stem and progenitor cells. Stem and progenitor cells, being totipotent cells, when placed in injured brain, are designed to replace dead and injured tissue.5,25,26 Embryonic stem cells placed in brain have been shown to differentiate into parenchymal cells and to migrate to sites of injury. Bone marrow stromal cells also likely contain a subpopulation of stem-like cells, which can differentiate into brain cells.27 However, these cells are a minor subpopulation of the hMSCs we use and do not contribute to the restoration of function, and only a very small percentage of the hMSCs assume parenchymal cell phenotype.10 We use MSCs not to replace tissue, but to augment function and to enhance plasticity of remaining tissue, particularly tissue in the boundary zone of the ischemic lesion. Thus, hMSCs when injected intravenously enter brain and evoke the expression of growth and trophic factors from the endogenous cells, primarily astrocytes and endothelial cells. The hMSCs are essentially catalysts for the production angiogenic remodeling of injured brain by means of VEGF and its receptor, VEGFR2.

The present study shows that treatment with MSCs enhances angiogenesis by increasing endogenous levels of VEGF and VEGFR2, suggesting that administration of MSCs provides a microenvironment to activate endogenous restorative mechanisms of ischemic brain. Therefore, treatment with MSCs may offer an additional avenue for stroke therapy.

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References


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