Comparison of Bone Marrow Stromal Cells Derived From Stroke and Normal Rats for Stroke Treatment

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Background and Purpose—We compared the effect of treatment of stroke with bone marrow stromal cells from stroke rats (Isch-BMSC) and normal rats (Nor-BMSC) on functional outcome.

Methods—Isch-BMSCs and Nor-BMSCs were intravenously injected into rats 24 hours after middle cerebral artery occlusion. To test the mechanism of Isch-BMSC-enhanced neurorestoration, Isch-BMSC and Nor-BMSC cultures were used.

Results—Isch-BMSC significantly promoted functional outcome and enhanced angiogenesis, arterial density, and axonal regeneration compared with Nor-BMSC treatment animals. Isch-BMSCs exhibited increased Angiopoietin-1, Tie2, basic fibroblast growth factor, glial cell-derived neurotrophic factor, vascular endothelial growth factor, and Flk1 gene expression compared with Nor-BMSC. Using transwell coculture of BMSCs with brain-derived endothelial cells, Isch-BMSCs increased phosphorylated-Tie2 activity in brain-derived endothelial cells and enhanced brain-derived endothelial cells capillary tube formation compared with Nor-BMSCs. Inhibition of Tie2 gene expression in brain-derived endothelial cells using siRNA significantly attenuated BMSC-induced capillary tube formation.

Conclusions—These data suggest that Isch-BMSCs are superior to Nor-BMSCs for the neurorestorative treatment of stroke, which may be mediated by the enhanced trophic factor and angiogenic characteristics of Isch-BMSCs. (Stroke. 2010;41:524-530.)

Key Words: Ang1 ▪ angiogenesis ▪ axonal regeneration ▪ bone marrow stromal cell ▪ cerebral infarct ▪ Tie2

Cell-based treatments of stroke include neural stem and progenitor, cord blood, and bone marrow stromal cells (BMSCs).1-5 BMSCs have potent therapeutic potential for stroke and neurodegenerative disease.4 BMSCs from normal animals when administered intravenously selectively migrate to the ischemic brain, induce angiogenesis, and promote functional recovery after stroke.5 BMSC treatment of stroke increases angiogenesis and vascular stabilization, partially mediated by VEGF/Flk1 and Angiopoietin-1 (Ang1)/Tie2.9 Ang1 belongs to a family of endothelial growth factors and promotes migration, sprouting, and survival of endothelial cells (ECs) and thereby mediates vascular remodeling through activation of signaling pathways triggered by the Tie2 tyrosine kinase receptor.9

A major question involved in the use of BMSCs for the treatment of stroke and neurological disease is whether to use autologous or allogeneic cells. Allogeneic cells can be obtained from young, healthy donors, amplified, and stored for immediate use when needed. However, there is an additional consideration that has not been factored in when weighing the question of allogeneic versus autologous transplantation. In addition to the convenience issues of using autologous cells compared with autologous cells, what has not been considered is whether BMSCs from patients with stroke are altered by the stroke. Do donor cells from a healthy nonstroke patient provide the same beneficial effects as those from a patient with stroke? Are the cells from a patient with stroke changed in some way so as to impact their therapeutic potential?

In the present study, we begin to address this clinically important question. BMSCs extracted from a rat subjected to stroke (Isch-BMSCs) are compared with cells extracted from a normal healthy rat (Nor-BMSCs) and their therapeutic effects are tested and compared in a model of middle cerebral artery occlusion (MCAo) in the rat. Although not truly a model of autologous transplantation, because the cells are not obtained from the animal receiving the treatment, the present study does test the relative benefit of BMSCs from a stroke animal to a normal animal.

Methods

Animal Middle MCAo Model

Adult male Wistar rats weighing 270 to 300 g were used (Charles River Breeding Co, Wilmington, Mass). Transient right MCAo was induced for 2 hours by advancing a 4-0 surgical nylon suture with its
tip rounded by heating near a flame to block the origin of the middle cerebral artery. Reperfusion was performed by withdrawal of the suture 2 hours after MCAo. At 2 days posts ischemia, MCAo rats were euthanized and bone marrow cells were isolated for BMSC culture.

**BMSC Culture**
Normal and MCAo rats (n=6/group) bone marrow was isolated and cultured in alpha Dulbecco modified Eagle medium (DMEM) with 20% fetal bovine serum and 1% penicillin streptomycin. Cells were maintained at 37°C in 5% CO2 and nonadherent cells were removed. BMSCs were used within Passage 4. The conditioned media (media from Passages 3 and 4) was collected from Isch-BMSC and Nor-BMSC culture for in vitro study. The conditioned media is the BMSC culture media (alpha DMEM with 20% fetal bovine serum and 1% antibiotic/antimitotic) after BMSCs have been cultured in it for 2 to 3 days. Because we have found that there is no significant difference in cell survival and selected gene expression between BMSCs derived from surgical shunt control rats and Nor-BMSCs (data not shown), the Nor-BMSCs were used as controls in this study.

**Functional Tests**
A modified Neurological Severity Score evaluation and foot fault tests were performed before MCAo and at 1, 7, and 14 days after MCAo.

**BMSC Administration**
Rats were subjected to MCAo and treated with: (1) phosphate-buffered saline for control; (2) Isch-BMSCs (1×10^6); and (3) Nor-BMSCs (1×10^6; n=8/group) into a tail vein at 24 hours after MCAo. Previous studies have shown that 1×10^6 Nor-BMSCs is a subtherapeutic dose in stroke rats. The animals were treated with nor-BMSC-conditioned media; (4) 50% Isch-BMSC-conditioned media; (5) 50% BMSC-conditioned media; or (6) 50% BMSC-conditioned media for 24 hours.

**Histological and Immunohistochemical Assessment**
At 14 days after MCAO, rats were euthanized and brains were fixed by 4% paraformaldehyde before being embedded in paraffin. Seven coronal sections of tissue were processed and stained with hematoxylin and eosin for calculation of volume of cerebral infarction. Lesion volume is presented as percentage of the ipsilateral hemisphere lesion volume to the contralateral hemisphere.

**Immunohistochemical Staining**
Six-micron thick sections were cut from the center of the lesion block. Every tenth coronal section for a total 5 sections was used for immunohistochemical staining. Von Willebrand Factor (vWF, dilution 1:400; Dako), α-smooth muscle actin (mouse monoclonal IgG, 1:800; Daco), Ang1 (rabbit polyclonal IgG, 1:2000; Abecam), and Tie2 (rabbit polyclonal IgG antibody, 1:80 dilution; Santa Cruz) were used, respectively. Control experiments consisted of staining brain coronal tissue sections as outlined previously, but the primary antibodies were omitted. Bielschowsky silver immunostaining was used to demonstrate axons.

**Quantification**
For measurement of vascular density, vWF and α-smooth muscle actin immunostaining was digitized. Five sections and 8 brain regions within the ischemic border zone (IBZ) adjacent to the ischemic lesion in each section were acquired and the total number of vWF-positive vessels and α-smooth muscle actin-positive arterial (diameter size ≥10 μm) in the IBZ was counted using the MCID computer imaging analysis system (Imaging Research, St Catharines, Canada). The total number of vessels and arteries/mm² area are presented.

ECs are a thin layer of cells that line the interior surface of blood vessels. To quantify Tie2-immunoreactive ECs, numbers of total ECs and Tie2-immunoreactive ECs in 10 enlarged thin walls vessels and 10 arteries were counted separately. Data are presented as percentage of Tie2-immunoreactive ECs to total ECs in 10 thin wall vessels or arteries from each rat.

**Lactate Dehydrogenase Assay**
Using the Cytotoxicity Detection kit (Roche), following the standard protocol, rat brain ECs (rBECs) were treated with (n=6/group): (1) Nor-BMSC-conditioned media; or (2) Isch-BMSC-conditioned media for 24 hours. Secreted and total (media and cells) lactate dehydrogenase levels were measured. Data are presented as percentage of lactate dehydrogenase level in the media to total lactate dehydrogenase.

**rBEC Migration**
A confluent rBEC monolayer was wounded by scraping the cells and then treating with 50% DMEM with: (1) 50% BMSC culture media for control; (2) 50% Nor-BMSC-conditioned media; or (3) 50% Isch-BMSC-conditioned media (n=6/group). Fifty percent rBEC culture media and 50% BMSC culture media were used. RNA/protein was isolated from rBECs with TRIzol Reagent for real-time polymerase chain reaction and Western blot assays.

**Transwell Coculture Model**
rBECs were plated in 6-well plates. Isch-BMSCs or Nor-BMSCs were added into the upper chambers of Falcon 0.4-mm cell culture inserts for incubation overnight (n=6/group). Fifty percent rBEC culture media and 50% BMSC culture media were used. RNA/protein was isolated from rBECs with TRIzol Reagent for real-time polymerase chain reaction and Western blot assays.

**Real-Time Polymerase Chain Reaction**
Total RNA was isolated from cultured cells with TRIzol (Invitrogen), and quantitative polymerase chain reaction was performed using the SYBR Green real-time polymerase chain reaction method as previously described. Ang1, Tie2, BFGF, GDNF, VEGF, Flk1, tumor necrosis factor-α (TNFα), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression were measured. Relative gene expression data were analyzed using the 2-ΔΔCT method.

**Western Blot**
Protein was isolated from cultured cells with TRIzol (Invitrogen). Protein concentrations were determined by a DC protein assay kit (Bio-Rad, Hercules, CA), and Westerns for β-actin, Ang1, Tie-2, and antiphospho-Tie2 were measured in BMSCs and rBECs.

**Tie2 Small Interfering RNA for rBECs**
Tie2 small interfering RNA (siRNA; Santa Cruz Biotechnology) was transfected using Lipofectamine 2000 (Invitrogen) following standard protocol. Briefly, rBECs were transfected with 8 μg Tie2 siRNA or scramble siRNA in serum-free media for 6 hours. DMEM+20% fetal bovine serum was added and cells were incubated overnight. Tie2 gene expression was measured 2 days after transfection.

**Capillary Tube Formation Assay**
rBECs were treated with (n=6/group): (1) serum-free DMEM for control, 50% DMEM with: (2) 50% BMSC culture media; (3) 50% Nor-BMSC-conditioned media; or (4) 50% Isch-BMSC-conditioned media.
media; (5) Condition 2 in scramble BECs; (6) Condition 3 in scramble BECs; (7) Condition 4 in scramble BECs; (8) Condition 2 in Tie2 siRNA BECs; (9) Condition 3 in Tie2 siRNA BECs; and (10) Condition 4 in Tie2 siRNA BECs and incubated for 5 hours. Matrigel wells were digitized under a 10× objective, and measurement of total tube length was performed using a MCID image analysis system. Tracks of ECs were counted and averaged in 6 randomly selected microscopic fields.16

Statistical Analysis
All measurements were performed blinded. The global test using the Generalized Estimating Equation was implemented to test the group difference on functional recovery. Pairwise treatment comparison was conducted if the overall treatment effect was detected at the 0.05 level. If a global test was significant, the group difference was evaluated on each functional test at the 0.05 level. Otherwise, the pairwise treatment comparison or the treatment comparison on each functional outcome was considered as exploratory. The correlation between 2 outcomes was estimated using Generalized Estimating Equation. One-way analysis of variance was used to test the treatment effect on histological evaluation. Spearman or Pearson correlation coefficients were calculated.

Results

Neurological Outcome and Lesion Volume
Animals with mild lesions, that is, with a modified Neurological Severity Score test score <5, were excluded. The mortality rates after stroke are 11% (one of 9) in the MCAo control group; 11% (one of 9) in the Nor-BMSC treatment group, and 0% in the Isch-BMSC treatment group. The total animal number remaining in this study was 8/group. The 3 groups were balanced at the baseline (P=0.74). The global test showed significant group difference on functional recovery at Day 14 (P<0.001). Isch-BMSC significantly and Nor-BMSC marginally improved the functional recovery compared with the phosphate-buffered saline-treated MCAo control (P=0.05). Isch-BMSC improved recovery compared with the Nor-BMSC-treated group (P=0.038). Rats treated with Isch-BMSCs improved functional recovery compared with the phosphate-buffered saline–MCAo control (P=0.05). Isch-BMSC improved recovery compared with the phosphate-buffered saline–MCAo control (P=0.05).

Isch-BMSCs and Nor-BMSC Treatment of Stroke Regulates Vascular Density and Axonal Regeneration
Isch-BMSCs and Nor-BMSCs treatment of stroke increased vascular density (vWF-positive vessels) compared with the control animals (Figure 2A). Treatment with Isch-BMSCs enhanced arterial density in the ipsilateral hemisphere compared with the control rats and Nor-BMSC-treated animals (Figure 2B). The vWF-positive vessel number was correlated with arterial density (r=0.60, P=0.01). Isch-BMSC treatment also increased Bielschowsky silver expression in the striatal bundles compared with control MCAo and Nor-BMSC treatment animals (Figure 2C).

Ang1 and Tie2 Expression in the Ischemic Brain After Isch-BMSC and Nor-BMSC Treatment
Isch-BMSC and Nor-BMSC treatment of stroke both increased Ang1 expression in the IBZ (Figure 2D) and increased Tie2 expression in the ECs in the IBZ of thin wall vessels (Figure 2E) and arterial vessels (Figure 2F) compared with the MCAo control. In addition, Isch-BMSC treatment enhanced arterial EC Tie2 expression in the IBZ compared with Nor-BMSC treatment animals (Figure 2F).

Trophic Factor Expression in the Isch-BMSC and Nor-BMSC
Trophic factors regulate brain plasticity after stroke. To test trophic factor expression in the Isch-BMSC and Nor-BMSC, real-time polymerase chain reaction and Western blot were performed. Isch-BMSCs increased of Ang1, Tie2, VEGF, Flk1, bFGF, and GDNF but not TNFα gene expression compared with Nor-BMSC (N=6/group; Figure 3A). Isch-BMSCs also increased Ang1 protein expression compared with Nor-BMSC (Figure 3B–C).

Coculture rBEC With Isch-BMSCs Enhances rBEC Trophic Factor Expression Compared With Nor-BMSCs
Coculture Isch-BMSC with rBEC did not increase total Tie2 expression but increased p-Tie2

Figure 1. Isch-BMSC and Nor-BMSC treatment of stroke improves functional outcome after stroke in rats. A, Modified Neurological Severity Score test; (B) foot fault test; (C) lesion volume (percentage of lesion compared with contralateral hemisphere). n=8/group.
Figure 2. Isch-BMSC and Nor-BMSC treatment of stroke increases angiogenesis, arteriogenesis, and axonal regeneration in the ischemic brain. A, vWF immunostaining and vascular density quantitative data in MCAo control, Nor-BMSC treatment rats, and Isch-BMSC treatment rats. B, α-smooth muscle actin immunostaining and arterial density quantitative data. C, Bielschowsky immunostaining and quantitative data. D–F, Ang1 (D), Tie2 expression in thin wall vessels (E) and artery (F) immunostaining and quantitative data. *P<0.05 compared with MCAo control (n=8/group). Scale bar in A–F = 100 μm.
expression compared with coculture of rBECs with Nor-BMSCs (Figure 4B–C).

**rBEC Migration, Cell Death, and Tube Formation**

rBEC migration was measured at 0 hour and 24 hours after culture (Figure 5A–D). Isch-BMSCs-conditioned media increases rBECs migration measured by scratch assay (Figure 5E) and decreases cell death measured by lactate dehydrogenase assay (Figure 5F) compared with Nor-BMSC-conditioned media. Conditioned media derived from Nor-BMSCs (Figure 5I) and Isch-BMSCs (Figure 5J) both increased capillary tube formation compared with control groups (Figure 5G–H). Isch-BMSCs media enhanced capillary tube formation compared with Nor-BMSCs media (Figure 5K, $P < 0.05$). Tie2 siRNA, but not scramble siRNA, decreased Tie2 gene expression in rBECs (Figure 5L). Knockdown of Tie2 expression using Tie2 siRNA did not completely inhibit Isch-BMSC-induced tube formation.

**Discussion**

Recovery of neurological function after stroke is mediated by many coupled events, including vascular remodeling and axonal regeneration. The injured brain and its response to stimulation with BMSCs pose an enormous complexity of interweaving pathways responsible for functional recovery. In this study, we are the first to compare the effects of treatment of stroke in rats with Isch-BMSCs and Nor-BMSCs on functional outcome and brain plasticity. We found that a subtherapeutic dose of Nor-BMSC increases angiogenesis but did not promote arteriogenesis and axonal regeneration in the ischemic brain compared with control MCAo animals. Isch-BMSC treatment enhances angiogenesis, promotes arteriogenesis and axonal regeneration in the ischemic brain as well as improves functional outcome after stroke compared with MCAo control. Isch-BMSC treatment also increases arterial density and axonal regeneration as well as enhances functional outcome after stroke compared with Nor-BMSCs. Therefore, Isch-BMSC may be superior to Nor-BMSC in the treatment of stroke.

Isch-BMSCs Express Growth Factors and Increase Endogenous Brain EC Trophic Factor Expression

BMSCs produce many cytokines and trophic factors. Angiogenesis and vascular maturation are regulated by VEGF, its receptors, and the Ang1/Tie2 system. Angiogenesis and vascular maturation are regulated by VEGF, its receptors, and the Ang1/Tie2 system. Hypoxic preconditioning of BMSCs increases trophic factor expression. The Isch-BMSCs used in the present study are not hypoxic and not directly stressed by the MCAo. Our data show that BMSCs from rats with stroke exhibit significant increases of growth factor (Ang1, Tie2, VEGF, Flk1, bFGF, and GDNF) but not TNFα gene expression compared with Nor-BMSC. These data suggest that the BMSCs are altered by a cerebral ischemic insult.

BMSCs also stimulate the local parenchymal cell production of growth factors. Isch-BMSC treatment of stroke increases endogenous brain EC Ang1 and Tie2 expression in the ischemic brain compared with Nor-BMSC treatment and also promotes p-Tie2 activity in in vitro cultured rBECs.
compared with Nor-BMSC-treated rBECs. Isch-BMSCs increase of endogenous rBEC trophic factor expression may augment functional outcome after stroke. BMSCs present in the ischemic brain secrete and stimulate parenchymal cell expression of trophic factors, which may promote functional benefit after stroke. We cannot exclude the possibility that more Isch-BMSCs are present in the ischemic brain than Nor-BMSC. Tracking BMSCs in the ischemic brain warrants investigation.

The Ang1/Tie2 System Contributes to Isch-BMSCs Treatment-Enhanced Angiogenesis

Ang1 and its receptor Tie2 mediate vascular integrity and angiogenesis. Tie2 is highly expressed in ECs and is crucial for angiogenesis and vascular maintenance. Ang1-modified BMSCs increase angiogenesis and arteriogenesis. Autologous secretion of Ang1 by transduced ECs induces Tie-2 activation and promotes arteriogenesis after limb ischemia. Our data show that Isch-BMSC treatment of stroke increases Ang1 and Tie2 expression compared with Nor-BMSCs in the ischemic brain. In addition, coculture of Isch-BMSCs with rBECs also increases rBEC p-Tie2 activity and enhances angiogenesis compared with Nor-BMSC. Inhibition of the Ang1/Tie2 pathway by Tie2 siRNA in rBEC inhibits capillary tube formation and attenuates Isch-BMSC and Nor-BMSC-induced angiogenesis in vitro. Therefore, the Ang1/Tie2 pathway contributes to Isch-BMSC and Nor-BMSC-induced angiogenesis.

In summary, we have found that treatment of stroke with Isch-BMSC increases angiogenesis, arteriogenesis, and axonal regeneration in the ischemic brain as well as improves functional outcome after stroke compared with Nor-BMSC.
Isch-BMSC increases trophic factor expression and the activity of the Ang1/Tie2 pathway, which may promote brain plasticity. Although our data suggest that using BMSCs derived from patients with stroke may be better than BMSCs derived from a normal population, the autologous BMSC treatment requires harvesting bone marrow cells from patients with stroke and subsequent culturing for several days. In addition, patient's age and comorbidities may also influence the BMSC effects. Allogeneic cells can be obtained from young, healthy donors, amplified, and stored for immediate use when needed. Therefore, the relative benefits of using autologous and allogeneic cells need to be weighed. The present article adds to the issues under consideration by indicating that cells from an ischemic animal used for the treatment of stroke may be superior to cells from a normal animal.

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Disclosures
None.

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