One-Year Follow-Up After Bone Marrow Stromal Cell Treatment in Middle-Aged Female Rats With Stroke

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Background and Purpose—We sought to evaluate the long-term effects of bone marrow stromal cell (BMSC) treatment on retired breeder rats with stroke.

Methods—Female retired breeder rats were subjected to 2-hour middle cerebral artery occlusion (MCAO) followed by an injection of $2 \times 10^6$ male BMSCs ($n=8$) or phosphate-buffered saline ($n=11$) into the ipsilateral internal carotid artery at 1 day after stroke. The rats were humanely killed 1 year later. Functional tests, in situ hybridization, and histochemical and immunohistochemical staining were performed.

Results—Significant recovery of neurological deficits was found in BMSC-treated rats beginning 2 weeks after cell injection compared with control animals. The beneficial effects of cell transplantation persisted for at least 1 year ($P<0.01$). In situ hybridization for the Y chromosome showed that donor cells survived in the brains of recipient rats, among which 22.3 ± 1.95% of cells expressed the astrocyte marker glial fibrillary acidic protein, 16.8 ± 2.13% expressed the neuronal marker microtubule-associated protein 2, and 5.5 ± 0.42% and 1% of cells colocalized with the microglial marker IB4 and the endothelial cell marker von Willebrand factor, respectively. Only very few BMSCs, however, were found in peripheral organs such as the heart, lung, liver, spleen, and kidney in recipient rats. BMSCs significantly reduced axonal loss ($P<0.01$), the thickness of the lesion scar wall ($P<0.01$), and the number of Nogo-A–positive cells ($P<0.05$) along the scar border; meanwhile, synaptophysin expression ($P<0.05$) was significantly increased in BMSC-treated ischemic brains compared with control untreated brains.

Conclusions—The beneficial effects of BMSCs on ischemic brain tissue persisted for at least 1 year. Most surviving BMSCs were present in the ischemic brain, but very few were found in other organs. The long-term improvement in functional outcome may be related to the structural and molecular changes induced by BMSCs. (Stroke. 2007; 38:000-000.)

Key Words: bone marrow stromal cells ■ middle cerebral artery occlusion ■ follow-up studies ■ Y chromosome ■ axonal restoration

Bone marrow stromal cells (BMSCs), a heterogeneous population of plastic-adherent cells, have been successfully used for the treatment of experimental stroke. In those studies, BMSCs selectively targeted injured brain tissue and promoted functional recovery via various cell delivery routes. Therapeutic benefit has been shown for cells injected from 1 day to 1 month after stroke onset, and the longest time of follow-up to date is 4 months after the ischemic attack. In the present study, we measured the effects of BMSCs for a 1-year period on retired breeder rats subjected to middle cerebral artery occlusion (MCAO). Our efforts focused on tracking donor cells and their eventual fate in the brain, the impact of BMSCs on axonal regeneration in ipsilateral brain tissue, and functional outcome.

Materials and Methods

All experimental procedures were conducted in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the institutional animal care and use committee of Henry Ford Hospital.

Animal Model

Female retired breeder Wistar rats ($N=27$, 10 to 12 months old) weighing 300 to 450 g were used in our experiment. Transient MCAO was induced with a method of intraluminal vascular occlusion modified in our laboratory. In brief, rats were initially anesthetized with 3.5% halothane, and anesthesia was maintained with 1.0% to 2.0% halothane in 70% N2O and 30% O2 via facemask. Rectal temperature was controlled at 37°C with a feedback-regulated water heating system. The right common carotid artery, external carotid artery (ECA), and internal carotid artery were exposed. A
3.0 monofilament nylon suture (~19.5 mm, determined by animal weight), with its tip rounded by heating near a flame, was advanced from the ECA into the lumen of the internal carotid artery until it blocked the origin of the MCA. Two hours after MCAO, animals were reanesthetized with halothane, and reperfusion was performed by withdrawal of the suture until the tip cleared the lumen of the ECA.

Cell Transplantation Procedures
Primary cultures of BMSCs were obtained from donor young adult male rats (Theradigm, Inc, Baltimore, Md) as previously described. At 1 day after ischemia, randomly selected animals underwent cell transplantation. In brief, the same anesthesia protocol was reinstated, and a modified polyethylene catheter (PE-50, Becton Dickinson, Sparks, Md) was advanced through the same small puncture (identical to the procedure used for the nylon suture as described earlier) in the ECA into the lumen of the internal carotid artery for a distance of ~15.0 mm, lodging ~2 mm proximal to the origin of the MCA. Approximately 2 × 10^6 male BMSCs in 200 μL phosphate-buffered saline (PBS) (n = 8) or control fluid (200 μL PBS, n = 11) were slowly injected over a 5-minute period into each rat. Immunosuppressant was not used in any animal in this study.

Behavioral Tests
Two behavioral tests, the modified neurological severity scores (mNSS) test and the adhesive-removal somatosensory test, were performed before MCAO (baseline) and at 1 day, 2 weeks, 4 weeks, and then monthly after cell administration by an investigator who was blinded to the experimental groups.

mNSS Test
The mNSS test is a composite of motor, sensory, balance, and reflex tests. Neurological function was graded on a scale of 0 to 18 (normal score = 0, maximal deficit score = 18). In the severity scores of injury, 1 point is awarded for a specific abnormal behavior or for the lack of a tested reflex; thus, the higher the score, the more severe the injury.

Adhesive-Removal Somatosensory Test
For the adhesive-removal somatosensory test, 2 small pieces of adhesive-backed paper dots (113.1 mm^2 at first and 56.6 mm^2 at 2 months after treatment and thereafter to increase the sensitivity of the test) were used as bilateral tactile stimuli occupying the distal-radial region on the wrist of each forelimb. The rat was then returned to its home cage. The time to remove each stimulus was recorded during 3 trials per day. Individual trials were separated by at least 5 minutes. Before surgery, the animals were trained for 3 days. Once the rats were able to remove the dots within 10 seconds, they were subjected to MCAO.

Histological and Immunohistochemical Assessment
Under deep ketamine anesthesia, all rats were humanely killed 1 year after MCAO. Rats were fixed by transcardial perfusion with saline, followed by perfusion with 4% paraformaldehyde. The brain, heart, lung, liver, spleen, and kidney were collected and embedded in paraffin. The brain tissue was sectioned into 7 equally spaced (2 mm) paraffin sections. The brain sections were analyzed by light and fluorescence microscopy (Olympus BH-2, Tokyo, Japan). After deparaffinization, brain sections were processed for either Bielshowsky silver–Luxol fast blue staining or immunostaining. Double staining for Bielschowsky silver and Luxol fast blue was used to demonstrate axons and myelin, respectively. In brief, for Bielschowsky silver staining, slides were placed in 20% AgNO3 in the dark, and then NaOH and sodium thiosulfate were added to the slides in turn. Slides were then stained in Luxol fast blue solution, washed in 95% alcohol, and placed in Li2CO3. For immunohistochemical staining, brain sections were placed in boiled citrate buffer (pH 6) in a microwave oven (650 to 720 W). After being blocked in normal serum, sections were incubated with antibodies against glial fibrillary acidic protein (GFAP; dilution 1:5000, Dako, Carpenteria, Calif), Nogo-A (dilution 1:250, Santa Cruz Biotech Inc, Santa Cruz, Calif), or synaptophysin (monoclonal antibody; dilution 1:1000, Chemicon, Temecula, Calif). For the first 2 antibodies, the sections were incubated with avidin-biotin horseradish peroxidase complex and developed in 3′,3′-diaminobenzidine tetrahydrochloride; for synaptophysin, the sections were visualized with CY3-conjugated secondary antibody. Negative control sections from each animal received identical preparations for immunohistochemical staining, except that the primary antibodies were omitted.

In Situ Hybridization
Fluorescein isothiocyanate–conjugated in situ hybridization for the Y chromosome was performed as described previously to track donor male BMSCs in female recipients. In brief, the brain or organ slides were digested with proteinase K (50 μg/mL) for 20 minutes at 37°C. The prepared cDNA probe for the Y chromosome was labeled with use of a random primer DIG labeling and detection kit (Boehringer Mannheim, Indianapolis, Ind). Hybridizations were performed in a hybridization mixture (consisting of 50% deionized formamide, 10% salmon test DNA, 5% dextran sulfate, 1× Denhardt’s solution, 2× standard saline citrate, 250 μg/mL dithiothreitol, 250 μg/mL poly A, and 500 ng digoxigenin-labeled probe) at 50°C for 40 hours. The digoxigenin-labeled, Y chromosome–positive BMSCs were visualized with the fluorescent antibody enhancer set (Boehringer Mannheim). The slides were then counterstained with 10 ng/mL propidium iodide for nuclear staining and mounted with antifade solution and coverslips. Negative control slides from each animal received identical staining preparation, except that the probe or the anti-digoxigenin antibodies were omitted.

To visualize the cellular colocalization of the Y chromosome and certain markers in the same cell in the brain, the fluorescein isothiocyanate–conjugated antibody and 3′,3′-diaminobenzidine hydrochloride were used for double labeling. Each coronal brain section was first treated with cell type–specific antibodies: microtubule–associated protein 2 (MAP2; dilution 1:200, Chemicon) for neurons; GFAP for astrocytes; Griffonia simplicifolia agglutinin B4 (GSA IB4; dilution 1:10, Sigma, St Louis, Mo) for microglia; or von Willebrand factor (vWF; dilution 1:400, Dako) for endothelial cells, and was visualized with 3′,3′-diaminobenzidine hydrochloride staining. Subsequently, fluorescein isothiocyanate–conjugated in situ hybridization staining was used for identification of male BMSCs. Negative control sections from each animal received identical preparations for immunohistochemical staining, except that the primary antibody was omitted.

Quantification
Immunoreactive cells and in situ hybridization–positive cells were analyzed with National Institutes of Health Image software (Image J) based on evaluation of an average of 3 histology slides (6 μm thick, 54-μm interval, every 10th slide) from the standard block of each animal. The number of Y chromosome–positive cells was counted in the ipsilateral hemisphere zone. In each animal, 200 Y chromosome cells were measured to obtain the percentage of Y chromosome cells colocalized with the cell type–specific markers (MAP2, GFAP, GSA IB4, or vWF) by double staining.

For measurements along the scar border, 8 fields of view were digitized with the use of either a ×20 objective or a ×40 objective and the MCID computer imaging analysis system (Figure 1). The number of Nogo-A–positive cells was counted, and the synaptophysin–positive area was measured in each field of view. For
measurement of scar wall thickness and axon density, 4 fields of view along the scar border within the ipsilateral striatum (fields 5 to 8 in Figure 1) were digitized and evaluated.

Statistical Analysis
For each functional test, ANOVA was used to test the group effect by the repeated time of assessments, excluding baseline (day 1). The analysis began testing for the group×time interaction, followed by testing the group effect at each time point when a significant group×time interaction or a group effect was detected at the critical value of 0.05. Immunohistochemistry data were analyzed by Student’s t test. Data are presented as mean±SE.

Results
Nineteen of 27 stroke rats survived up to 1 year. Among the 8 animals that died prematurely, 3 were in the BMSC group and 5 in the PBS control group. Six rats (2 BMSCs, 4 PBS) died within 2 weeks after surgery. One rat in each group was humanely killed at ≈2 months after surgery because of wasting.

Neurological Outcome
Functional status was balanced between the BMSC-treated (n=8) and the control retired breeder rats (n=11) at baseline, and no significant difference on each behavioral score (mNSS and adhesive-removal test) was detected before cell administration. Figures 2A and 2B show the temporal profile of functional recovery in control and BMSC-treated rats. Significant functional improvement was detected in the BMSC

Figure 1. A representative coronal brain section with hematoxylin and eosin staining shows 8 fields selected for quantitative measurement of cells and structure along the cavity.

Figure 2. Line graphs showing the temporal profile of functional recovery in control (n=11) and BMSC-treated rats (n=9). A and B show the average scores; C and D show the median score in the mNSS test and the adhesive-removal test. Significant BMSC effects were detected at each time point starting 2 weeks after treatment on the mNSS (A, C) and the adhesive-removal test (B, D). **P<0.01 vs control group.
group at each time point starting 2 weeks after treatment on both tests (P<0.01) compared with controls.

**Histology**

**Lesion Volume**

By hematoxylin and eosin staining, reproducible neuronal damage was observed in the ischemic core of the striatum and cortex in all rats subjected to MCAO. Ventricles were enlarged in all MCAO rats (Figure 1), indicating brain atrophy at 1 year after MCAO. Ischemic core tissues were transformed into cysts of various size in all stroke rats. The damaged tissue consisted of a central cavity surrounded by a scar wall (densely arranged GFAP-positive fibrils) and a scar border. The lesion volume decreased from 36.4±1.7% (n=11) to 29.5±3.9% (n=8) after BMSC treatment. This change, however, was not statistically significant (P=0.091).

**Donor Cells in Recipients**

Donor-derived male BMSCs were identified by Y chromosome in situ hybridization in recipient brain and other organ tissues. No Y chromosome–positive cells were found without BMSC treatment. BMSCs were distributed throughout the damaged brain, with the majority located close to the injured tissue. The number of donor-derived Y chromosome–positive cells in the ipsilateral hemisphere was 38±4.7 per section. Double-staining immunohistochemistry of brain sections revealed that some Y chromosome–positive cells were reactive for the neuronal marker MAP2 (Figures 3A through 3C), astrocyte marker GFAP (Figures 3D through 3F), the microglial marker GSA IB4 (Figures 3G through 3I), or the endothelial cell marker vWF (Figures 3J through 3L). The percentage of Y chromosome–positive cells that expressed MAP2 and GFAP protein was 22.3±1.95% and 16.8±2.13%, respectively. Only a small fraction (5.5±0.42%) of Y chromosome–positive cells colocalized with the microglial marker GSA IB4, and <1% of Y chromosome–positive cells expressed the endothelial marker vWF. In organs other than the brain, very few Y chromosome–positive cells were detected in the heart, lung, liver, spleen, or kidney (supplemental Figure I, available online at http://stroke.ahajournals.org).

**Morphological Changes Along the Scar Border**

At 1 year after MCAO, high-density GFAP-positive fibrils were interwoven into a scar wall around the cystic core, especially along the striatal scar border (Figures 4A and 4B). After BMSC treatment, a significantly (P<0.01) thinner scar wall (Figures 4B and 4C) was observed compared with that of the control MCAO rats (Figures 4A and 4C).
Nogo-A, a potent neurite growth inhibitor, restricts axonal regeneration after central nervous system (CNS) injury.\textsuperscript{2,10,11}

In the adult brain, Nogo-A is selectively expressed in oligodendrocytes.\textsuperscript{1,12} Figures 4D through 4F show that BMSC treatment markedly reduced the number of Nogo-A–positive cells in the area along the cavity ($P<0.05$).

Double staining for Bielshowsky silver and Luxol fast blue identifies axons and myelin, respectively, in the white matter in the brain. The ischemic attack destroys white matter in the core lesion area and leaves axon-myelin bundles in a partially damaged and disorganized state in the scar border area (Figures 4G and 4H). Axonal density along the cavity in the striatum was significantly increased in BMSC-treated rats compared with control animals (Figures 4G through 4I, $P<0.01$), which may serve as one of the mechanisms underlying the improvement of neurological function induced by BMSC treatment. Synaptophysin is an indicator of presynaptic plasticity and synaptogenesis.\textsuperscript{13} As shown in Figures 4J through 4L, BMSC treatment significantly increased synaptophysin expression ($P<0.05$) compared with control rats.

**Discussion**

Our data demonstrate that transplantation of BMSCs via the carotid artery at 1 day after stroke has long-lasting beneficial
effects on neurological functional recovery in retired breeder female rats. Most surviving BMSCs were located in the ischemic brain tissue, with very few cells scattered in other organs. Along with the marked reduction of axonal loss, BMSC-treated rats had a significantly reduced scar wall and fewer Nogo-A–positive cells in the scar border area than did control rats. In addition, a significant increase of synaptophysin expression was detected after BMSC administration compared with control animals. To our knowledge, this is the first 1-year follow-up report of BMSC therapy in MCAO rats.

Stroke is principally a disease of the elderly. There are now ≈4 million stroke survivors with neurological deficits in the United States, most of them over the age of 65. Therefore, recently established guidelines on how to improve the predictive validity of animal models of stroke have stressed the importance of using older animals. In this regard, in the present study we chose retired breeder rats, which are 10 to 12 months old instead of the 3- to 4-month-old young adult animals that are normally used for the focal ischemia model.

BMSCs have been extensively studied as a candidate for stroke therapy. Among various cell injection routes adopted in experimental stroke studies, intravenous and intracarotid approaches are considered more clinically feasible. Although most BMSCs administered intravenously target the brain, these cells also spread to other organs; hence, intracarotid injection was used in this study to minimize out-of-brain distribution of donor cells. As anticipated, only very few BMSCs were found in the heart, lung, liver, spleen, and kidney 1 year after MCAO.

Severe behavioral deficits were evident in all animals in the acute stage after stroke, and all rats showed a progressive decrease in behavioral deficit over time. In an effort to increase the sensitivity of the adhesive-removal function test, we used half-size (56.6 mm² compared with 113.1 mm²) paper dots in the adhesive-removal test from the second month on. The therapeutic effects of BMSCs on neurological deficits were evident at 2 weeks after cell administration and persisted thereafter. The temporal profile of neurological outcome shows that BMSCs significantly enhanced the initial rate of recovery, which suggests that BMSCs in ischemic brain tissue set the endogenous restorative reactions to stroke to a more efficient level that leads to a persistent improvement in neurological function.

One of the most important questions for a long-term follow-up cell therapy study is the ultimate fate of the cells. In this study, we performed male cell to female recipient transplantation to follow the fate of injected BMSCs by Y chromosome in situ hybridization. Our data show that some donor cells survived in the recipient’s brain, and they localized along the scar border zone. Although it may not be quite comparable with our other studies, in which different cell dosages, injection routes, and injection timing were used, the present 1-year data demonstrate that a higher percentage of donor cells colocalized with neural cell markers than at shorter times. One possible explanation is that more donor cells express brain cell proteins when they are maintained in the brain environment for a longer period of time. Another explanation for the persistence of the Y chromosome, however, is cell fusion. A previous study showed that bone marrow–derived cells fuse spontaneously with neural progenitors in vitro, and they can also fuse with Purkinje neurons in the brain after bone marrow transplantation. Taking into account the report demonstrating cell fusion in the damaged liver in mice, we cannot rule out the possibility of cell fusion in the context of ischemic stroke. Further studies to dissect this issue are warranted.

After focal ischemia, massive neuronal death will result in regions of denervation that provide a stimulus for undamaged neurons to sprout and establish new synaptic connections. Spontaneous axonal reorganization occurs in ischemic brain tissue and may underlie the partial recovery of neurological function over time. Although adult mammalian CNS neurons exhibit a capacity for axonal outgrowth in permissive environments, the injured CNS contains axon-inhibitory factors that limit anatomic and functional recovery. The inhibitory factors include, but are not necessarily limited to, the formation of a glial scar and the presence of axon outgrowth inhibitory proteins.

Stroke induces gliosis in a region of apoptotic cell death that closely borders the infarct, extending several hundred microns into peri-infarct brain tissue in the rat. Over time, the glial scar wall, a narrow region characterized by densely interwoven fibrils, comes into being. The scar wall has been proposed to be a major impediment to regeneration in the adult CNS. The glial scar is a mechanical barrier for outgrowing axons, and various growth-inhibitory molecules are increased within the region of the scar wall, which makes axon penetration difficult. Moreover, some studies have shown that the reduction of scar wall thickness is related to functional improvement after MCAO. The glial scar is actually the remnant of reactive gliosis at the acute and subacute phases of CNS injuries or diseases. However, its cause remains elusive. There is evidence that proinflammatory cytokines, such as interleukin (IL)-1 and IL-6, boost glial scar formation, whereas anti-inflammatory cytokines, such as IL-10 and IL-1 receptor antagonists, can act neuroprotectively. Although BMSCs downregulate IL-1 and upregulate IL-10 expression, the effect of BMSCs on scar formation requires further investigation.

CNS myelin is a primary inhibitor of axonal growth in the adult brain, and Nogo-A is a prominent myelin-derived inhibitor of axonal outgrowth. Previous studies have revealed that Nogo-A is expressed in oligodendrocyte cell bodies and processes and is localized to the innermost axonal and outermost myelin membranes. This localization of Nogo-A fits well with its role as an inhibitor of regenerative fiber growth and structural plasticity. In addition, a recent report demonstrated that interruption of the Nogo-NgR pathway enhanced stroke-induced plasticity of axonal connectivity and promoted stroke recovery. The present study has shown that concomitant with the reduction of axonal loss and the increase of synaptophysin expression, BMSC-treated rats have a thinner glial scar wall and fewer Nogo-A–positive cells along the scar border. Taking into account the aforementioned observations, we suggest that interfering with the inhibitory effects of the scar wall and Nogo-A may underlie the axonal regeneration–improving action of BMSCs.
In summary, we have demonstrated that BMSCs survive and exert therapeutic effects on ischemic brain tissue for at least 1 year. The BMSC effects on glial wall thickness and axonal density in the treated animal may contribute to the observed functional benefits.

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Disclosures
None.

References
Figure I. Few Y-chromosome cells are scattered in heart (A), lung (B), liver (C), spleen (D) and kidney (E). Scale bar = 25 μm in A-I.
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