Therapeutic Benefit of Intravenous Administration of Bone Marrow Stromal Cells After Cerebral Ischemia in Rats

Jieli Chen, MD; Yi Li, MD; Lei Wang, MD; Zhenggang Zhang, MD, PhD; Dunyue Lu, MD; Mei Lu, PhD; Michael Chopp, PhD

Background and Purpose—We tested the hypothesis that intravenous infusion of bone marrow derived–marrow stromal cells (MSCs) enter the brain and reduce neurological functional deficits after stroke in rats.

Methods—Rats (n=32) were subjected to 2 hours of middle cerebral artery occlusion (MCAO). Test groups consisted of MCAO alone (group 1, n=6); intravenous infusion of 1×10⁶ MSCs at 24 hours after MCAO (group 2, n=6); or infusion of 3×10⁶ MSCs (group 3, n=7). Rats in groups 1 to 3 were euthanized at 14 days after MCAO. Group 4 consisted of MCAO alone (n=6) and group 5, intravenous infusion of 3×10⁶ MSCs at 7 days after MCAO (n=7). Rats in groups 4 and 5 were euthanized at 35 days after MCAO. For cellular identification, MSCs were prelabeled with bromodeoxyuridine. Behavioral tests (rotarod, adhesive-removal, and modified Neurological Severity Score [NSS]) were performed before and at 1, 7, 14, 21, 28, and 35 days after MCAO. Immunohistochemistry was used to identify MSCs or cells derived from MSCs in brain and other organs.

Results—Significant recovery of somatosensory behavior and Neurological Severity Score (P<0.05) were found in animals infused with 3×10⁶ MSCs at 1 day or 7 days compared with control animals. MSCs survive and are localized to the ipsilateral ischemic hemisphere, and a few cells express protein marker phenotypic neural cells.

Conclusions—MSCs delivered to ischemic brain tissue through an intravenous route provide therapeutic benefit after stroke. MSCs may provide a powerful autologous therapy for stroke. (Stroke. 2001;32:1005-1011.)

Key Words: bone marrow transplantation ■ middle cerebral artery occlusion ■ neuronal plasticity ■ stroke, experimental ■ stromal cells ■ rats

Bone marrow (BM) contains cells that meet the criteria for stem cells of nonhematopoietic tissues. The precursors of nonhematopoietic tissues are referred to as mesenchymal stem cells or marrow stromal cells (MSCs). These cells have attracted interest because of their capacity for self-renewal in a number of nonhematopoietic tissues, their multipotentiality for differentiation, and their possible use for renewal in a number of nonhematopoietic tissues, the precur-

Successful stromal chimerism has been achieved in murine systems with this approach. Successful stromal chimerism has been achieved in murine systems with this approach. Koc et al have demonstrated the feasibility and safety of infusing culture-expanded autologous MSCs in patients with advanced breast cancer undergoing peripheral blood stem cell transplantation. In light of the utility of MSCs to treat neural injury and the potential vascular route of administration, in the present study, we test the hypothesis that intravenous infusion of MSCs from marrow reduces functional deficits after stroke in rats.

Materials and Methods

Animal Middle Cerebral Artery Occlusion Model
Adult male Wistar rats (n=32) weighing 270 to 300 g were used in our experiments. Briefly, rats were initially anesthetized with 3.5%...
halothane and maintained with 1.0 to 2.0% halothane in 70% N₂O and 30% O₂ by a face mask. Rectal temperature was maintained at 37°C throughout the surgical procedure by means of a feedback-regulated water heating system. We induced transient (2 hours) middle cerebral artery occlusion (MCAO) by using a previously described method of intraluminal vascular occlusion.16–18

**Experimental Groups**

Experimental groups consist of group 1 (control): rats given MCAO alone without donor cell administration (n = 6); group 2: rats given low-dose MSCs (1×10⁶) injected intravenously at 24 hours after MCAO (n = 6); and group 3: rats given high-dose MSCs (3×10⁶) injected intravenously at 24 hours after MCAO (n = 7). The animals of groups 1, 2, and 3 were killed at 14 days after MCAO. To test the effects of delayed (7-day) treatment, we included 2 additional groups: Group 4 rats (control) were given MCAO alone without donor cell administration (n = 6) and were killed at 14 days after MCAO. To test the effects of delayed (7-day) treatment, we included 2 additional groups: Group 4 rats (control) were given MCAO alone without donor cell administration (n = 6) and were killed at 14 days after MCAO; group 5 rats were given high-dose MSCs (3×10⁶) injected intravenously at 7 days after MCAO and were killed at 35 days (n = 7) after MCAO. The selection of an extended survival time (35 days) was based on the supposition that late treatment, 7 days after MCAO, provides a delayed functional benefit.

**Transplantation Procedures**

Primary cultures of BM cells were obtained 48 hours after treating donor rats with 5-fluorouracil (150 mg/kg); MSCs were separated, as previously described.3,11 All transplantation procedures were performed under aseptic conditions. At 1 or 7 days after ischemia, randomly selected animals received transplantation. Animals were anesthetized with 3.5% halothane and then maintained with 1.0% to 2.0% halothane in 70% N₂O and 30% O₂ by a face mask mounted in a Kopf stereotaxic frame (model 51603, Stoelting Co). Approximately 1×10⁶ or 3×10⁶ MSCs in 1 mL total fluid volume were injected into a tail vein. Immunosuppressants were not used in any animal.

**Behavioral Testing**

In all animals, a battery of behavioral tests was performed before MCAO and at 1, 7, 14, 21, 28, and 35 days after MCAO by an investigator who was blinded to the experimental groups. For the rotarod test,18, 19 rats were placed on an accelerating rotarod cylinder, and the time the animals remained on the rotarod was measured. The speed was slowly increased from 4 to 40 rpm within 5 minutes. A trial ended if the animal fell off the rings or gripped the device and spun around for 2 consecutive revolutions without attempting to

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### TABLE 1. Neurological Severity Scores (NSS)

<table>
<thead>
<tr>
<th>Points</th>
<th>Motor tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Raising rat by the tail</td>
</tr>
<tr>
<td>1</td>
<td>1 Flexion of forelimb</td>
</tr>
<tr>
<td>1</td>
<td>1 Flexion of hindlimb</td>
</tr>
<tr>
<td>3</td>
<td>1 Head moved &gt;10° to vertical axis within 30 s</td>
</tr>
<tr>
<td>3</td>
<td>Placing rat on the floor (normal=0; maximum=3)</td>
</tr>
<tr>
<td>0</td>
<td>Normal walk</td>
</tr>
<tr>
<td>1</td>
<td>Inability to walk straight</td>
</tr>
<tr>
<td>2</td>
<td>Circling toward the paretic side</td>
</tr>
<tr>
<td>3</td>
<td>Fall down to the paretic side</td>
</tr>
<tr>
<td>2</td>
<td>Sensory tests</td>
</tr>
<tr>
<td>2</td>
<td>1 Placing test (visual and tactile test)</td>
</tr>
<tr>
<td>2</td>
<td>2 Proprioceptive test (deep sensation, pushing the paw against the table edge to stimulate limb muscles)</td>
</tr>
<tr>
<td>6</td>
<td>Beam balance tests (normal=0; maximum=6)</td>
</tr>
<tr>
<td>0</td>
<td>Balances with steady posture</td>
</tr>
<tr>
<td>1</td>
<td>Grasps side of beam</td>
</tr>
<tr>
<td>2</td>
<td>Hugs the beam and one limb falls down from the beam</td>
</tr>
<tr>
<td>3</td>
<td>Hugs the beam and two limbs fall down from the beam, or spins on beam (≥60 s)</td>
</tr>
<tr>
<td>4</td>
<td>Attempts to balance on the beam but falls off (&gt;40 s)</td>
</tr>
<tr>
<td>5</td>
<td>Attempts to balance on the beam but falls off (≥20 s)</td>
</tr>
<tr>
<td>6</td>
<td>Falls off: No attempt to balance or hang on to the beam (&lt;20 s)</td>
</tr>
<tr>
<td>4</td>
<td>Reflexes absent and abnormal movements</td>
</tr>
<tr>
<td>1</td>
<td>1 Pinna reflex (head shake when touching the auditory meatus)</td>
</tr>
<tr>
<td>1</td>
<td>1 Corneal reflex (eye blink when lightly touching the cornea with cotton)</td>
</tr>
<tr>
<td>1</td>
<td>1 Startle reflex (motor response to a brief noise from snapping a clipboard paper)</td>
</tr>
<tr>
<td>1</td>
<td>1 Seizures, myoclonus, myodystony</td>
</tr>
<tr>
<td>18</td>
<td>Maximum points</td>
</tr>
</tbody>
</table>

One point is awarded for the inability to perform the tasks or for the lack of a tested reflex; 13 to 18 indicates severe injury; 7 to 12, moderate injury; 1 to 6, mild injury.
walk on the rungs. The animals were trained 3 days before MCAO. The mean duration (in seconds) on the device was recorded with 3 rotarod measurements 1 day before surgery. Motor test data are presented as percentage of mean duration (3 trials) on the rotarod compared with the internal baseline control (before surgery).

For the adhesive-removal somatosensory test,18,20,21 somatosensory deficit was measured both before and after surgery. All rats were familiarized with the testing environment. In the initial test, 2 small pieces of adhesive-backed paper dots (of equal size, 113.1 mm²) were used as bilateral tactile stimuli occupying the distal-radial region on the wrist of each forelimb. The rat was then returned to its cage. The time to remove each stimulus from forelimbs was recorded on 5 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

Animals were allowed to survive for 14 or 35 days after MCAO, and at that time animals were reanaesthetized with ketamine (44 mg/kg) and xylazine (13 mg/kg). Rat brains were fixed by transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde, and the brain, heart, liver, spleen, lung, kidney, and muscle were embedded in paraffin. The cerebral tissues were cut into 7 equally spaced (2 mm) coronal blocks. A series of adjacent 6-μm-thick sections were cut from each block in the coronal plane and were stained with hematoxylin and eosin. The 7 brain sections were traced by the Global Laboratory Image analysis system (Data Translation). The indirect lesion area, in which the intact area of the ipsilateral hemisphere was subtracted from the area of the contralateral hemisphere, was calculated.27 The lesion volume is presented as a volume percentage of the lesion compared with the contralateral hemisphere.

Single and double immunohistochemical staining28 was used to identify cells derived from MSCs. Briefly, a standard paraffin block was obtained from the center of the lesion, corresponding to coronal coordinates for bregma ~1~1 mm. A series of 6-μm-thick sections at various levels (100-μm interval) were cut from this block and were analyzed by light and fluorescent microscopy (Olympus, BH-2). To detect the distribution of transplanted MSCs in other organs (ie, heart, liver, lung, spleen, kidney, muscle, and bone marrow), 3 sections (6 μm thick, 100-μm interval) from each organ were obtained and numbers of bromodeoxyuridine (BrdU)-reactive cells measured. Measurements of BrdU-reactive cells in organs other than brain were performed in all rats subjected to 1-day or 7-day treatment with 3×10^6 MSCs. After deparaffinization, sections were placed in boiled citrate buffer (pH 6.0) within a microwave oven (650 to 720 W). After blocking in normal serum, sections were treated with the monoclonal antibody against BrdU (Calbiochem) diluted at 1:100 in PBS. After sequential incubation with peroxidase-conjugated rabbit anti-mouse IgG (dilution 1:100; Dakopatts), the secondary antibody was bound to the first antibody against BrdU. Diaminobenzidine (DAB) was then used as a chromogen for light microscopy. Counterstaining of sections by hematoxylin was also performed. Cells derived from MSCs were identified by morphological criteria and by immunohistochemical staining with BrdU (the tracer) present in the nuclei of donor cells. BrdU found in the parenchymal cells or in the cytoplasm of macrophage-like cells was not counted. Analysis of BrdU-positive cells is based on the evaluation of an average of 10 histology slides of brain. All BrdU-reactive cells, with BrdU clearly localized to the nucleus, were counted throughout all 10 coronal sections. For information on the relative presence of MSCs within other organs, 3 slides from each
organ were obtained from each experimental animal, and an estimate was made of the percentage of BrdU-positive cells to endogenous organ-specific cells.

To visualize the cellular colocalization of BrdU-specific and cell-type–specific markers in the same cells, double staining was used. Brain sections were treated with cell-type–specific antibodies, a neuronal nuclear antigen (NeuN for neurons, dilution 1:200; Chemicon), microtubule-associated protein 2 (MAP-2 for neurons, dilution 1:200; Boehringer Mannheim), and glial fibrillary acidic protein (GFAP for astrocytes, dilution 1:100; Dako). Each coronal section was first treated with the primary BrdU monoclonal antibody, as described above. FITC-conjugated antibody (Calbiochem) was used for double-label immunoreactivity identification. Negative control sections from each animal received identical preparations for immunohistochemical staining, except that primary antibodies were omitted. A total of 500 BrdU-positive cells per animal from multiple adjacent (100-μm interval) sections were counted to obtain the percentage of BrdU cells colocalized with cell-type–specific markers (NeuN, MAP-2, and GFAP) by double staining.

**Laser Scanning Confocal Microscopy**

Colocalization of BrdU with neuronal marker was conducted by laser scanning confocal microscopy (LSCM) with the use of a Bio-Rad MRC 1024 (argon and krypton) laser-scanning confocal imaging system mounted onto a Zeiss microscope (Bio-Rad).19 For immunofluorescence double-labeled coronal sections, green (FITC for BrdU) and red cyanine-5.18 (Cy5 for MAP-2 or NeuN) fluorochromes on fluorescence double-labeled coronal sections, green (FITC for BrdU) and red cyanine-5.18 (Cy5 for MAP-2 or NeuN) fluorochromes on the sections were excited by a laser beam at 488 nm and 647 nm; emissions were sequentially acquired with 2 separate photomultiplier tubes through 522 nm and 680 nm emission filters, respectively. Areas of interest were scanned with a ×40 oil immersion objective lens in 260.6×260.6-μm format in the x-y direction and 0.5 μm in the z direction.

**Statistical Analysis**

The behavior scores (rotarod test, adhesive-removal test, and NSS), were evaluated for normality. Repeated-measures analysis was conducted to test the treatment effect on the behavior score. The analysis began with testing for the treatment-time interaction at the significance level 0.1, then testing for the overall treatment effect if there was no interaction detected at the 0.05 level. A subgroup analysis began with testing for the treatment-time interaction at the 0.05 level. A subgroup analysis was conducted to test the treatment effect on the behavior score. The analysis was then conducted for the control group, the control group had complete behavioral scoring up to 14 days after ischemia before they were euthanized for histological analysis; the other control group had complete behavioral scores up to 35 days after ischemia. The control animals were shared for testing the treatment effect with different doses and treatment given at different times after ischemia.

**Results**

**Effect of MSCs Given 1 Day After Ischemia**

For rats treated with low-dose MSCs at day 1 after stroke, no treatment-by-time interaction was detected on each behavioral score; no significant difference in functional recovery (rotarod, adhesive-removal, and NSS tests) was found in animals treated with 1×10^6 MSCs compared with MCAO alone animals (Figure 1). For high-dose MSCs given at day 1 after ischemia, treatment-by-time interaction was detected on adhesive-removal and NSS scores but not on the rotarod score. Rats treated with the high-dose MSCs had significantly lower adhesive-removal times and NSS scores at day 14 compared with the control group (P<0.01) (Figure 1). For high-dose MSCs given at day 7 after ischemia, the treatment effects over the time were marginal for NSS scores (P=0.06). The treatment effects over time were significant for the rotarod and adhesive-removal tests (P<0.05). The treated rats had a lower NSS mean score at day 21 (P=0.046) and 28 (P=0.02) and had a significant (P<0.05) recovery of adhesive-removal time and rotarod score at day 35 (Figure 2).

**Histology**

The blood gasses were within normal ranges for all animals and did not differ among groups (data not shown). Within the 6-μm-thick coronal sections stained with hematoxylin and eosin, dark red and dark neurons were observed in the ischemic core of all rats subjected to MCAO with and without donor transplantation at 14 and 35 days after MCAO. No significant reduction of volume of ischemic damage was detected in rats with donor treatment compared with control rats subjected to MCAO alone (Table 2).

Within the brain tissue, cells derived from MSCs were characterized by round-to-oval dark brown nuclei with irregularly shaped and thin cytoplasm by BrdU staining. Only cells with this morphology and with BrdU localized solely to nucleus were counted as MSCs (Figure 3a). MSCs identified by BrdU immunoreactivity survived and were distributed throughout the damaged brain of recipient rats. Some cells, the vast majority within the lesion, contained BrdU within the cytoplasm (Figure 3b). These cells are considered as macrophages and are not counted as MSCs. The number of BrdU-reactive cells detected from an average of 10 histology slides per MSC transplantation animal is given in Table 2. Higher levels BrdU-reactive cells were seen in the brain at high-dose (3×10^6) MSC transplantation group than in the low-dose (1×10^6) MSC group at 24 hours after MCAO (P<0.01) (Table 2). BrdU-reactive cells were observed in multiple areas of the ipsilateral hemisphere, including cortex, striatum of the ipsilateral hemisphere. The vast majority of BrdU-labeled MSCs were located in the ischemic core and its boundary zone. Few cells were observed in the contralateral hemisphere.

**In organs other than brain, as an approximate percentage of endogenous organ-specific cells, scattered BrdU-positive cells were detected in bone marrow (≈2% to 4%) and in muscle, spleen, kidney, lung, and liver (≈0.01% to 0.5%) (Figure 3c and d). Most BrdU-positive cells encircle vessels in these organs, with few cells located in parenchyma.
Double-staining immunohistochemistry of brain sections revealed that some BrdU-positive cells were reactive for the neuronal markers NeuN (Figure 3, e and f) and MAP-2 and for the astrocyte marker GFAP (Figure 3, g and h). The percentage of BrdU that labeled expressed NeuN, MAP-2, and GFAP proteins was ≈1%, ≈2%, and ≈5%, respectively.

Figure 4 shows LSCM images from the coronal sections immunofluorescently stained with antibodies against MAP2, BrdU, and NeuN. Colocalization of immunofluorescent labels for MAP2 and BrdU (Figure 4, A to G) and for NeuN and BrdU (Figure 4, H to J) were present.

Discussion
We have demonstrated that intravenous injection of $3 \times 10^6$ MSCs at 1 or 7 days after stroke significantly improves functional outcome compared with nontreated rats. Morphological analysis of the tissue indicates that BrdU-labeled MSCs are more likely to enter into damaged brain than into contralateral nonischemic brain. Many MSCs survive, and a few cells express protein markers for parenchymal cells.

What are the mechanisms or factors that promote reduced deficits with MSC transplantation after stroke? One possibility is that the MSCs integrate into the tissue, replace damaged cells, and reconstruct neural circuitry. However, we have no clear evidence that the MSCs function in this way, and although some cells express neural cell phenotype, we have no evidence that these cells develop contacts with other neurons. Reconstruction of neural circuitry is not always a prerequisite for functional recovery. A more reasonable
hypothesis is that interaction of MSCs with the host brain may lead to production of trophic factors,\textsuperscript{31} which may contribute to recovery of function lost as a result of lesions, the mechanisms of which are unidentified.\textsuperscript{32} MSCs constitutively secrete interleukins (IL)-6, IL-7, IL-8, IL-11, IL-12, IL-14, IL-15, macrophage colony-stimulating factor, Flt-3 ligand, and stem-cell factor.\textsuperscript{33,34} These cytokines are survival, growth, and/or differentiation factors for murine hippocampal neuronal progenitor cells.\textsuperscript{35–39} MSCs also contain catecholamines and may release specific neurotransmitters.\textsuperscript{40} Thus, reduction of ischemic-induced deficits by MSC transplantation may be due to the production of trophic factors by MSCs.

Nonhematopoietic BM stroma is composed of mesenchymal cells, including fibroblasts, osteoblasts, and adipocytes, in addition to endothelial cells.\textsuperscript{1} Most nonhematopoietic stromal progenitor cells appear to be more consistent with an endothelial rather than a fibroblast cell origin.\textsuperscript{41,42} In response to severe ischemia or cytokine stimuli, stromal progenitors may expand and be recruited along with endothelium progenitor cells (EPCs) and consequently contribute to neovascularization and/or wound-healing processes.\textsuperscript{43} In the present study, BrdU-labeled cells encircled vessels of organs at 14 days after injection. We speculate that transplanted MSCs may function as EPC. EPC mobilization may ultimately represent a potential strategy for clinical therapy of ischemic vascular disease.

More MSCs were found in the lesioned hemisphere than in the intact hemisphere. These data are consistent with reports that male BM cells systemically infused into female ischemic rats migrate preferentially to ischemic cortex.\textsuperscript{13} The mechanisms responsible for intravenously infused BM migration into brain and its intraparenchymal distribution are not clear. Disruption of the blood-brain barrier may facilitate selective entry of MSCs into ischemic brain compared with nonspecific contralateral cerebral tissue. In addition, there are other mechanisms that may promote migration of MSCs into brain. Approximately 20\% of microglia are thought to originate from the marrow.\textsuperscript{44,45} MSCs intravenously injected into irradiated mice continue to replicate in vivo, and over a period of weeks they populate several connective tissues including bone, cartilage, lung, and brain.\textsuperscript{5} By systemic administration, BM-derived myogenic progenitors migrate into a degenerating muscle and participate in the regeneration process.\textsuperscript{46} These cells appear to be recruited by long-range, possibly inflammatory, signals originating from the degenerating tissue, and they probably access the damaged muscle from the circulation, together with granulocytes and macrophages.\textsuperscript{46} Natural migration of BM cells from one hematopoietic microenvironment to the other may occur.\textsuperscript{47} This movement is genetically controlled in part through changes in expression of cell surface adhesion molecules, such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and neural cell adhesion molecule.\textsuperscript{48,49}

In our previous study, we transplanted BM or MSCs cells into ischemic brain,\textsuperscript{10,11,18} and these cells migrate, differentiate, and reduce functional deficits after stroke.\textsuperscript{11,18} Injection of devitalized BrdU-labeled MSCs directly into brain resulted in no improvement in functional outcome, and outcome was no different from that detected in rats with intracerebral PBS injection. Local intracerebral injection induces local brain damage,\textsuperscript{50} and particularly, multiple injections may not be clinically acceptable. The most important finding of this study is that BM-derived MSCs delivered to ischemic tissue through an intravenous route provide therapeutic benefit. This simple approach for cell therapy, which does not necessitate invasive stereotaxic operations, could potentially target pathological sites in a number of brain disorders. Logistical and ethical concerns about the use of fetal cells for transplantation therapy can be eliminated by exploiting MSCs as an alternative autologous graft source.

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References


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