Treatment of stroke in rat with intracarotid administration of marrow stromal cells

Y. Li, MD; J. Chen, MD; L. Wang, MD; M. Lu, PhD; and M. Chopp, PhD

Article abstract—*Objective:* To measure the therapeutic efficacy for the treatment of stroke with intra-arterial administration of bone marrow stromal cells (MSC). *Background:* MSC have characteristics of stem and progenitor cells. The hypothesis that MSC injected into the internal carotid artery after stroke enter into ischemic brain and improve neurologic recovery was tested. *Methods:* Twenty-five adult Wistar rats were subjected to transient (2-hour) middle cerebral artery occlusion alone (n = 9), or treated with intracarotid arterial injection of 200 μL phosphate-buffered saline (n = 8) or 2×10^6 MSC in 200 μL phosphate-buffered saline (n = 8) 1 day after ischemia. MSC were harvested and isolated from additional adult rats and then cultured and labeled with bromodeoxyuridine. Rats were subjected to neurologic functional tests (adhesive-removal, modified neurologic severity scores) before and at 1, 7, and 14 days after middle cerebral artery occlusion. Immunohistochemistry was used to identify cell-specific proteins of bromodeoxyuridine-reactive MSC. *Results:* Bromodeoxyuridine-reactive cells (~21% of 2×10^6 injected MSC) distributed throughout the territory of the middle cerebral artery by 14 days after ischemia. Some bromodeoxyuridine-reactive cells expressed proteins characteristic of astrocytes and neurons. Rats with intra-arterial transplantation of MSC exhibited improvement on the adhesive-removal test ($p < 0.05$) and the modified neurologic severity scores ($p < 0.05$) at 14 days compared with controls. *Conclusions:* MSC injected intra-arterially are localized and directed to the territory of the middle cerebral artery, and these cells foster functional improvement after cerebral ischemia.

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Stem cells are capable of extended self-renewal and can generate multilineage cell types.[1] The fate of stem cells in vivo is greatly influenced by contextual cues, and stem cells respond to the microenvironment into which they are seeded.[2-4] Neural stem cells have been transplanted into adult brain for treatment of neurodegenerative disorders such as PD.[5] However, fetal tissue transplants are plagued by ethical and logistical problems. Intracranial transplantation of stem cells other than from fetal brain may be a suitable graft in the CNS. Bone marrow is a composite of cells and soluble stroma. One cellular component of bone marrow is hematopoietic stem and progenitor cells, which replenish the blood system. A second cellular component of bone marrow is marrow stromal cells (MSC; also referred to as mesenchymal stem and progenitor cells), which give rise to cells of mesenchymal lineage, such as osteoblasts and cartilage.[6] MSC secrete growth factors and cytokines into the soluble stroma,[6-9] which promote the growth and differentiation of the hematopoietic stem cells.[8,9]

MSC transplantation has been employed as a source of progenitor cells for both cell therapy and gene therapy in animals and in patients with severe osteogenesis imperfecta and with cancer, opening the door to MSC therapy for a number of diseases.[10-14] MSC secrete neurochemicals[7,15] and become glia when transplanted intracerebrally into normal rat brain.[16] Bone marrow cells from male mice (y chromosome cells) injected intravenously into irradiated female adult mice contribute to both microglia and macroglia in the brain of adult mice.[17] In addition, bone marrow cells injected intravenously into rats subjected to middle cerebral artery occlusion (MCAo) migrate across the blood–brain barrier into damaged ischemic tissue and provide a small number of astrocytes to the brain.[18] Whole bone marrow cells and cultured MSC intracerebrally transplanted into the ischemic boundary zone of adult rats and mice 1 day after MCAo express neural phenotype and promote functional recovery.[19,20]

In an effort to identify a more effective mode of MSC transplantation and stroke treatment, we injected MSC into the carotid artery of rats subjected to MCAo. Our data indicate that intra-arterial MSC transplantation provides an efficient route for the treatment of stroke.

From the Departments of Neurology (Drs. Li, Chen, Wang, and Chopp) and Biostatistics and Research Epidemiology (Dr. Lu), Henry Ford Health Sciences Center, Detroit; and the Department of Physics (Dr. Chopp), Oakland University, Rochester, MI.

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Address correspondence and reprint requests to Dr. Michael Chopp, Department of Neurology (E&R3056), Henry Ford Health Sciences Center, 2799 West Grand Boulevard, Detroit, MI 48202; e-mail: chopp@neuro.hfh.edu

Materials and methods. Experimental procedures were approved by the Care of Experimental Animals Committee of Henry Ford Hospital.

Donor cells. Cultured bone marrow cells were obtained from adult Wistar rats (8 to 12 weeks old, weighing 270 to 290 g) given 5-fluorouracil, 150 mg/kg intraperitoneally 2 days before harvesting[21] to ablate mature blood cells and thereby induce stem and progenitor cells into cycle. Using a syringe (1 mL) with phosphate-buffered saline (PBS; in all cases 0.1 M phosphate/140 mM NaCl; pH 7.6), fresh complete bone marrow was harvested aseptically from tibias and femurs. Bone marrow was then mechanically dissociated until a milky, homogenous single-cell suspension was obtained. Erythrocytes were removed from bone marrow using 0.84% NH_4Cl. Numbers of nucleated marrow cells were measured by a cytometer and 2×10^6 nucleated cells were seeded into each 25 cm^2 tissue culture flask in medium (ie, 5 mL Iscove modified Dulbecco medium [IMDM] supplemented with 10% fetal bovine serum). After 72 hours of incubation, MSC tightly adhered to plastic and were resuspended to fresh IMDM in new flasks. MSC were grown at 37 °C in a 5% CO_2 water-jacketed incubator for three passages. To identify cells derived from MSC, 3 μg/mL bromodeoxyuridine (BrdU)(Sigma Chemical Corp., St. Louis, MO), a thymidine analog and marker of newly synthesized DNA,[22] was added to the medium at 72 hours before transplantation. Upon harvest, cells were isolated by treatment with 0.25% trypsin and 0.5 mM ethylenediaminetetraacetic acid (EDTA) at 37 °C for 10 minutes at room temperature. The digestion was stopped by adding 8 mL IMDM. Cells were then washed five times with PBS. Nucleated MSC were counted using a cytometer to ensure adequate cell number for transplantation. For immunostaining, MSC were subcultured in chambered slides and more than 90% of MSC were BrdU reactive.

Host rats with intra-arterial transplantation. Experiments were performed in which MSC from donor adult Wistar rats were administered to an immunologically identical strain of male Wistar rats (n = 25, 8 to 12 weeks old, weighing 270 to 290 g). In all surgical procedures, anesthesia was induced in rats with 3.5% halothane, and maintained with 1.0% halothane in 70% N_2O and 30% O_2 using a face mask. The rectal temperature was controlled at 37 °C with a feedback-regulated water heating system. Transient MCAo was induced using a method of intraluminal vascular occlusion.[23] Rats subjected to transient MCAo for 2 hours produce a consistent and reproducible ischemic lesion in the unilateral striatum and cortex.[24] Briefly, a 2-cm incision was made at the center of the neck, and the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed under an operating microscope (Carl Zeiss, Thornwood, NY). The CCA and ICA were temporarily clamped using microsurgical clips (Codman & Shurtleff, Randolf, MA). A 5-0 silk suture was tied loosely at the origin of the ECA and ligated at the distal end of the ECA. A length (18.5 to 19.0 mm) of 4-0 surgical nylon suture, determined according to the animal's weight, with its tip rounded by heating near a flame was introduced into the ECA lumen through a small puncture. The silk suture around the ECA origin was tightened around the intraluminal nylon suture to prevent bleeding, and the microsurgical clips were removed. The nylon suture was gently advanced from the ECA into the lumen of the ICA until it blocked the origin of the MCA. After 2 hours of MCAo, reperfusion was achieved by withdrawal of the suture until the tip cleared the ICA lumen and reached the origin of ECA. Intracarotid injection of MSC was carried out 24 hours after MCAo. Anesthesia was reinstituted and a modified polyethylene (PE-50, Becton Dickinson, Sparks, MD) catheter was advanced through the same small puncture (identical to the procedure used for the nylon suture above) in the ECA into the lumen of the ICA for a distance of ~15.0 mm, lodging ~2 mm proximal to the origin of the MCA. Approximately 2×10^6 MSC in 200 μL PBS (n = 8) or control fluid (200 μL PBS; n = 8) was slowly injected over a 5-minute period into each rat. Immunosuppressants were not used in any animal in this study.[25] All MCAo rats with (n = 16) or without (n = 9) donor transplantation were killed 14 days after MCAo.

Behavioral tests. Each rat was subjected to a series of behavioral tests to evaluate neurologic function before MCAo and 1, 7 and 14 days after MCAo.

Adhesive-removal somatosensory test. Somatosensory deficit was measured[26] both before and after MCAo. Two small pieces of adhesive-backed paper dots (113.1 mm^2) 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 the limb was recorded during five trials per day for 3 days. Individual trials were separated by at least 5 minutes. Once the rats were able to remove the dots within 10 seconds, they were subjected to MCAo.

Modified neurologic severity scores. The table shows a set of modified neurologic severity scores used to grade neurologic function.[26-32] The neurologic severity score is composite of motor (muscle status, abnormal movement),[23,26,30] sensory (visual, tactile, and proprioceptive),[28] and reflex tests.[29,26,32] These tests are similar to contralateral neglect testing in humans.[33] In the severity scores of injury, one point is awarded for the inability to correctly perform the tasks or for the lack of a tested reflex. The higher the score, the more severe the injury.

Histologic and immunohistochemical assessment. The rat brains were fixed by transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde, and brain blocks were embedded in paraffin. The brain was sectioned into seven equally spaced (2-mm) coronal blocks using a rodent brain matrix. A series of adjacent 6-μm-thick sections were cut from each block and stained with hematoxylin and eosin. The sections were traced using the Global Lab Image analysis system (Data Translation, Marlboro, MA). The indirect lesion area, in which the intact area of the ipsilateral hemisphere was subtracted from the area of the contralateral hemisphere, was calculated.[34] The lesion volume is presented as a volume percentage of the lesion compared with the contralateral hemisphere.

A standard paraffin embedded block (within the center of the lesion of MCAo), corresponding to coronal coordinates bregma −1.0~1.0 mm[35] was obtained from which a series of adjacent 6-μm-thick sections were analyzed using light and fluorescent microscopy (Olympus BH-2, Tokyo, Japan). After deparaffinizing, brain sections were placed in a boiled citrate buffer (pH 6) in a microwave oven (650 to 720 W). Following blocking in normal serum, sections

Table *Modified neurological severity score*

Motor tests[23,26,30]	Points
Muscle status: hemiplegia	
Raising the rat by the tail	
Flexion of forelimb	1
Flexion of hindlimb	1
Head moving more than 10° (vertical axis)	1
Placing the rat on the floor	
Inability to walk straight	1
Circling toward the paretic side	1
Falling down to the paretic side	1
Abnormal movements	
Immobility and staring	1
Tremor (wet-dog shakes)	1
Myodystony, irritability, seizures	1
Sensory tests[27,28]	
Visual and tactile placing	1
Proprioceptive test (deep sensory)	1
Reflexes (blunt or sharp stimulation)[26,29,32] absent of:	
Pinna reflex (a head shake when touching the auditory meatus)	1
Corneal reflex (an eye blink when lightly touching the cornea with cotton)	1
Startle reflex (a motor response to a brief loud paper noise)	1
Maximum points	14

One point is awarded for the inability to perform the tasks or for the lack of a tested reflex. Score: 10–14 severe; 5–9 moderate; 1–4 mild injury.

were incubated with a monoclonal antibody against BrdU (diluted at 1:100 in PBS; Calbiochem (CN Biosciences), San Diego, CA), and then incubated with biotinylated secondary antibody (dilution 1:100, Dakopatts, Carpinteria, CA). Subsequently, sections were washed in PBS and incubated with avidin-biotin-horseradish peroxidase complex. Diaminobenzidine was then used as a chromogen for light microscopy. Counterstaining of some sections by hematoxylin was also performed. Double immunohistochemistry was employed to determine the potential of MSC to differentiate along multiple lineages after MCAo. Cell type–specific antibodies were used to identify the phenotypic fate of these cells.[36,37] Briefly, each coronal section was treated by the first primary BrdU monoclonal antibody, as described above. Subsequently, the second primary antibodies against cell type–specific markers of microtubule-associated protein 2 (MAP-2) for neurons, dilution 1:200 (CHEMICON International, Temecula, CA), and glial fibrillary acidic protein (GFAP) for astrocytes, dilution 1:1000 (Dakopatts), were applied on the same sections. Fluorescein isothiocyanate–conjugated secondary antibody, dilution 1:20 (Calbiochem), was then added for double-label immunoreactivity identification. Negative control sections from each animal received identical preparations for immunohistochemical staining, except those primary antibodies were omitted. Cells derived from bone marrow cells were identified using morphologic criteria[38-40] and by immunohistochemical staining with antibody against BrdU. We measured the total number of BrdU-reactive cells derived from bone marrow based on the evaluation of an average of 10 histology slides (50-μm interval) per experimental animal. Only the cells with clearly labeled nuclei by BrdU immunoreactivity were counted (×132 magnification). A total of 500 BrdU-reactive cells per animal were counted to obtain the percentage of BrdU-reactive cells colocalized with cell type–specific markers (MAP-2, GFAP) by double staining.

Statistical analysis. The lesion volume was subjected to Student's *t*-tests to evaluate differences between control rats (MCAo alone or MCAo with injection of PBS) and treated rats (MCAo with MSC transplantation). The behavioral scores (time to adhesive removal and neurologic severity score) were evaluated for normality. Repeated-measure analysis of variance was conducted to test the treatment effect on each of the behavior scores including the test for treatment and time interaction. A subgroup analysis of treatment effect at each time point was performed, if there was a treatment by time interaction or treatment effect at the 0.05 level. If no interaction was detected at the 0.05 level or there was no overall treatment effect, the subgroup analysis was considered as exploratory. Mean values (standard division of the mean, STD) and *p* value for testing the difference between treated and control groups are presented.

Results. By hematoxylin-eosin staining, reproducible neuronal damage was observed in the ischemic core of the striatum and cortex in all rats subjected to MCAo. No significant difference of lesion volume was observed in ischemic rats with and without MSC transplantation. Ischemic lesion volume 14 days after the onset of ischemia was 36.6% ± 10.1% in rats subjected to MCAo alone, 35.3% ± 11.9% in ischemic rats injected with PBS, and 31.2% ± 7.3% in ischemic rats treated with MSC transplantation. A nearly complete loss of MAP-2 and GFAP immunoreactivity in parenchymal cells in the ischemic core was observed after MCAo.

No BrdU reactivity was observed in rats without MSC treatment. Cells derived from MSC were distributed throughout the ischemic regions in the MCA territory (figure 1, A). These cells were identified and counted as derived from MSC only if BrdU reactivity was localized in the intact nuclei (see figure 1, B). BrdU-reactive MSC were observed in multiple areas of the ipsilateral hemisphere, including all six layers of the cortices, and the rostral-caudal axis of the striatum of the ipsilateral hemisphere. Many more (~90%) BrdU-reactive MSC localized in the ischemic core and boundary zone than in the relatively intact regions of the ipsilateral hemisphere. Numbers of BrdU-reactive MSC measured from an average of 10 histology slides (50-μm interval) per animal, were 433,700 ± 55,100 (~21% of 2×10^6 transplanted cells) 14 days after MCAo. Double immunostaining (see figure 1, D through G) demonstrated that, from a total of 500 BrdU-reactive cells per animal, approximately 10% of BrdU-reactive cells expressed GFAP (astrocytes) and 1% expressed MAP-2 (neurons). Scattered BrdU-reactive cells (~1% of 2×10^6 transplanted cells) were also observed in the contralateral hemisphere: approximately 3% of BrdU-positive cells ex-

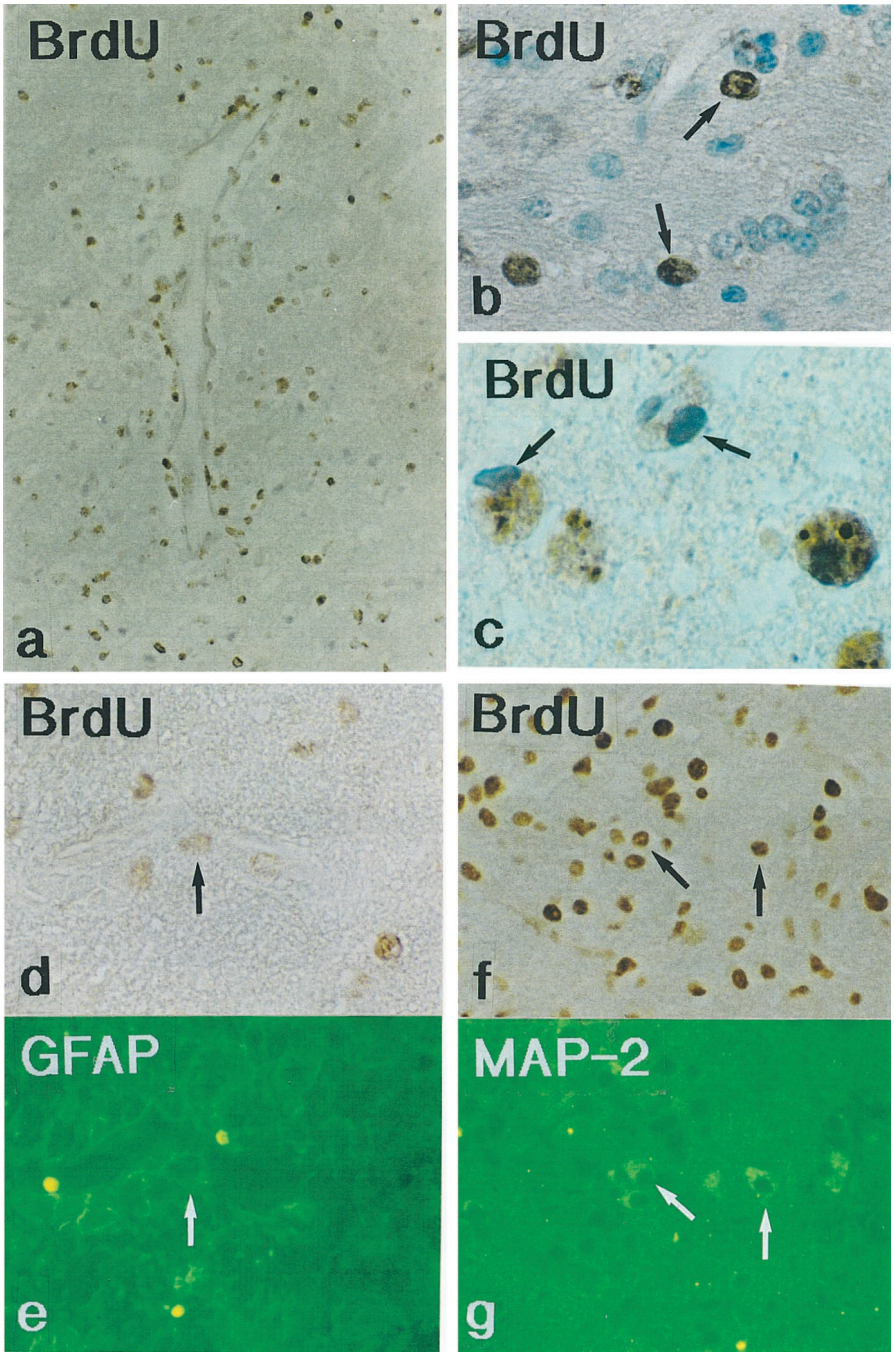

Figure 1. (A) Fourteen days after middle cerebral artery occlusion (MCAo) (13 days after marrow stromal cell [MSC] intra-arterial transplantation), cells derived from MSC identified by bromodeoxyuridine (BrdU) immunoperoxidase with 3,3-diaminobenzidine (DAB; brown) distribute in the territory of the MCA. (B) BrdU reactivity is localized in the nuclei of cells derived from MSC. (C) Some endogenous macrophage uptake of BrdU from BrdU-reactive MSC is observed. However, in macrophages BrdU is present in cytoplasm but not nucleus. In contrast, in cells derived from exogenous MSC (B), BrdU reactivity is localized within the nucleus. Double staining of immunoperoxidase-DAB (brown) and fluorescein isothiocyanate (green) shows that BrdU-reactive cells (D and F) are colocalized (arrows) with glial fibrillary acidic protein (GFAP; E) and microtubule-associated protein 2 (MAP-2; G) in the same cells. (Original magnifications: A, ×90; B through E, ×360; F through G, ×260.)

pressed GFAP, but no MAP-2 immunoreactivity was detected.

In order to avoid counting BrdU-positive cells other than those derived from exogenous MSC, which might have skewed the results, we employed morphologic criteria to identify endogenous macrophages (large, amoeboid, phagocytic cells; see figure 1, C) and neutrophils (cells with highly lobulated nucleus) by hematoxylin-eosin and BrdU staining. These inflammatory cells are morphologically distinct from MSC (small cells with a single nucleus; see figure 1, B). Very few neutrophils were found 14 days after treatment in all experimental rats, which is consistent with our previous study.[41] Some macrophage uptake of BrdU was observed. However in macrophages, BrdU was present in the cytoplasm but not the nucleus (see figure 1, C), In contrast, cells derived from exogenous MSC (see figure 1, B) show BrdU reactivity localized within the nucleus. No apparent difference of numbers of macrophages and neutrophils was detected in MSC-treated compared with nontreated rats.[37] There was no evidence of increased inflammatory response in the ischemic brain after MSC transplantation compared with MCAo alone.

No significant differences in results of the behavioral tests were detected among groups before MCAo. Severe behavioral deficits were evident in all animals 1 day after the ischemic insult. All rats subjected to MCAo alone or treated with PBS or MSC showed a progressive decrease in behavioral deficit over time to 14 days after MCAo, with the neurologic severity score treatment effect depending on time ($p = 0.008$) but not on time for the adhesive-removal test ($p = 0.47$ for the interaction). There was no overall treatment effect on time for the adhesive-removal test ($p =$

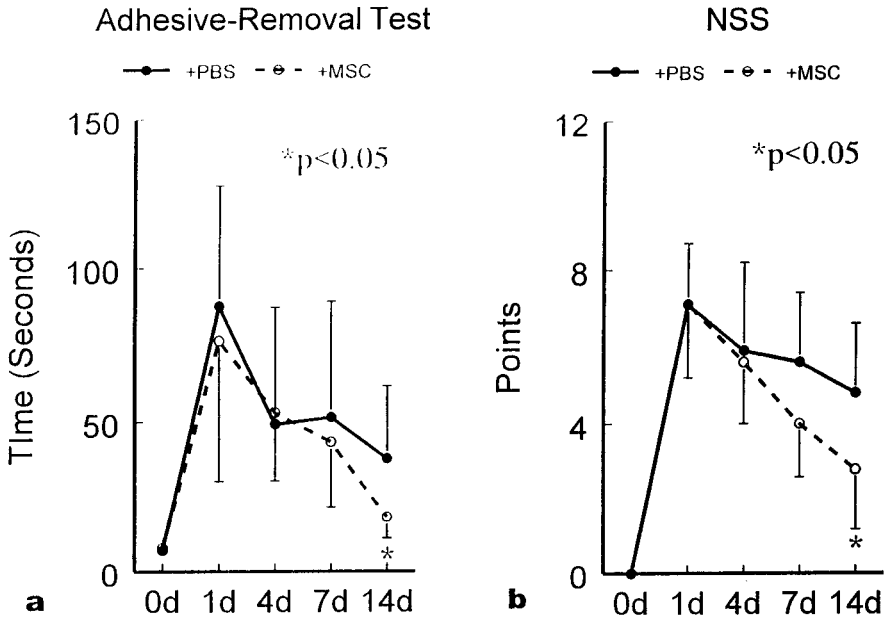

Figure 2. Changes of the adhesive-removal test (A) and modified neurologic severity scores (NSS; B) 1, 7, and 14 days after middle cerebral artery occlusion (MCAo) with or without intra-arterial transplantation of marrow stromal cells (MSC). Partial recovery of neurologic function is present after MCAo followed by phosphate-buffered saline (PBS) injection, but residual deficits persist at 14 days (the endpoint). At 14 days, there is improved recovery in both the adhesive-removal test (A; $p < 0.05$) and NSS (B; $p < 0.05$) in rats treated with MSC intra-arterial transplantation compared with PBS injection. Data are presented as mean values (standard division of mean).

0.38). Subgroup analysis showed a significant treatment effect on both neurologic severity scores ($p = 0.04$) and time of adhesive-removal test ($p = 0.045$) at day 14 after treatment (figure 2).

Discussion. Our data indicate that MSC delivered to the carotid artery enter the ischemic adult rat brain. These cells survive and a small number of MSC express proteins phenotypic of parenchymal-like cells and improve neurologic functional recovery after stroke.

Double immunostaining showed that BrdU-reactive cells expressed GFAP (~10%) and MAP-2 (~1%) in the ischemic ipsilateral hemisphere, but only expressed GFAP (~3%) in the contralateral hemisphere. Thus, within the ischemic tissue, MSC are induced to differentiate into a mixed population of cellular phenotypes, suggesting that the fate of the MSC is dictated by the microenvironment.

Intra-arterial administration of MSC appears superior to intracerebral transplantation. After intracerebral transplantation of BrdU-labeled bone marrow cells, most BrdU-reactive cells localize in the injected regions.[20] In contrast, MSC injected into the carotid artery appear to distribute over a wide area of the ischemic core and penumbra. Intra-arterial injection of MSC is less invasive than intracerebral transplantation, and poses a lower risk to the patient. Larger volumes of MSC in vehicle (200 μL) are permitted by intra-arterial injection than by intracerebral transplantation (10 μL).[19] A larger proportion of BrdU-reactive cells (~21%) derived from intra-arterially administered MSC survive in the ischemic areas than after intracerebral transplantation (~8.6%) 14 days after MCAo in the adult rats (unpublished data). We employed the mechanical "suture" occlusion model of transient stroke, and the MCA is thus patent at the time of MSC injection. However, after embolic stroke, it may be important to ensure that the MCA and its branches are patent prior to MSC injection in order for the intra-arterial treatment to be effective.

The mechanisms responsible for the passage of intra-arterially injected MSC into the brain and their intraparenchymal distribution are unknown. We found little or no evidence of petechial hemorrhage associated with MSC parenchymal localization. At 24 hours after stroke, although the blood–brain barrier permeability may be enhanced, the opening may not allow diffusion of cells into tissue. From the broad distribution of MSC, it appears as if brain were "infected" with inflammatory cells. Thus, we may speculate that similar transport mechanisms responsible for inflammatory cell migration may act on the MSC. After stroke, the local microvasculature expresses adhesion molecules (eg, vascular-cell adhesion molecules) to attract inflammatory cells[42] and, possibly, MSC from the carotid artery. Bone marrow cells migrate into the ipsilateral and contralateral hemispheres after IV administration of bone marrow cells after MCAo in irradiated mice.[17] MSC also have the capacity to pass through the intact blood–brain barrier and migrate throughout neonatal forebrain and cerebellum.[43]

Unilateral MCAo in the rodent induces contralateral hemiplegia and relevant asymmetric neurologic dysfunction. The reasons underlying the mechanisms responsible for neurologic functional recovery are not known. It is unlikely that the generation of few (~1%) BrdU-reactive cells expressing MAP-2 of neuronal phenotype in the ischemic structures was responsible for the behavioral functional recovery observed at 14 days after MCAo. Even if administered MSC reach the right target and survive and express neural proteins, there is little reason to believe that they will readily make proper connections among the denervated targets. Molecules secreted by cells de-

rived from MSC may stimulate activation of parenchymal cell "self-repair," rather than replace missing cells. The salient feature of the CNS is its complexity. There is a fair degree of redundancy within the CNS and it is possible the MSC activate these redundant neurons for functional recovery. However, we have not tested this hypothesis. In addition, in the normal adult brain, the absence of forebrain neuronal production may reflect not a lack of appropriate neuronal stem and precursor cells, but rather a tonic inhibition or a lack of postmitotic trophic and migratory support.[44,45] MSC may provide a source of microenvironmental differentiating factors for the ischemia damaged brain, thereby enhancing the proliferation and migration of cells derived from neural stem and progenitor cells in the adult CNS.[7,8,15,46] The interaction of MSC with the host brain may lead MSC and parenchymal cells to produce trophic factors, which may contribute to recovery of function lost as a result of a lesion.[47,48]

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Aneurysmal SAH

Cognitive outcome and structural damage after clipping or coiling

M. Hadjivassiliou, MD; C.L. Tooth, BSc; C.A.J. Romanowski, FRCP; J. Byrne, MD; R.D.E. Battersby, FRCS; S. Oxbury, MA; C.S. Crewswell, BSc; E. Burkitt, BSc; N.A. Stokes, BSc; C. Paul, BSc; A.R. Mayes, Dphil; and H.J. Sagar, DM

Article abstract—*Background:* Aneurysmal subarachnoid hemorrhage (SAH) and surgical clipping of intracranial aneurysms are associated with substantial morbidity and mortality. *Objective:* To compare cognitive outcome and structural damage in patients with aneurysmal SAH treated with surgical clipping or endovascular coiling. *Methods:* Forty case-matched pairs of patients with aneurysmal SAH treated by surgical clipping or endovascular coiling were prospectively assessed by use of a battery of cognitive tests. Twenty-three case-matched pairs underwent MRI 1 year after the procedure. Matching was based on grade of SAH on admission, location of aneurysm, age, and premorbid IQ. *Results:* Both groups were impaired in all cognitive domains when compared with age-matched healthy control subjects. Comparison of cognitive outcome between the two groups indicated an overall trend toward a poorer cognitive outcome in the surgical group, which achieved significance in four tests. MRI showed focal encephalomalacia exclusively in the surgical group. This group also had a significantly higher incidence of single or multiple small infarcts within the vascular territory of the aneurysm, but both groups had similar incidence of large infarcts and global ischemic damage. *Conclusion:* Endovascular treatment may cause less structural brain damage than surgery and have a more favorable cognitive outcome. However, cognitive outcome appears to be dictated primarily by the complications of SAH.

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For subarachnoid hemorrhage (SAH) patients that reach a neurosurgical unit, the mortality can be as high as 26%.[1] Neurosurgical clipping of the aneurysm has, until recently, been the preferred treatment option to prevent further bleeding. Most patients undergoing craniotomy and surgical clipping of the aneurysm are left with significant cognitive deficits.[2] Neuropsychological assessment has shown that up to 62% of patients with Glasgow Outcome Scale scores of 1 are left with cognitive impairment.[3] The most important factors implicated include grade on admission[4] (according to the World Federation of Neurosurgical Surgeons grading scale[5]), aneurysm location,[6] age,[7] and operative and perioperative factors. Previous attempts to assess the contribution of surgery toward cognitive outcome were hindered by the lack of an appropriate control group. Endovascular embolization[8] using detachable

From the University Department of Clinical Neurology (Drs. Hadjivassiliou, Mayes, Sagar, and C. Tooth) and the Departments of Neuroradiology (Dr. Romanowski) and Neurosurgery (Dr. Battersby), Royal Hallamshire Hospital, Sheffield, UK; and the Departments of Neuroradiology (Dr. Byrne) and Neuropsychology (S. Oxbury, C. Creswell, E. Burkitt, N. Stokes, and C. Paul), The Radcliffe Infirmary, Oxford, UK.

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Address correspondence and reprint requests to Dr. Marios Hadjivassiliou, Department of Neurology, The Royal Hallamshire Hospital, Glossop Road, Sheffield, S102JF, UK.

