Neonatal stroke occurs in ≈1 in 4000 live births and is associated with significant morbidity and mortality.1 Neonates with perinatal stroke often develop long-term disabilities, including motor deficits, cognitive dysfunction, and epilepsy.2 Currently, there are no accepted treatment options for this vulnerable group of infants. Therefore, development of new treatment strategies is urgently needed.

During the past decades, several studies have evaluated potential therapies to prevent progression of injury via pharmacological neuroprotection. More recently, means to enhance repair of the damaged immature brain are being investigated. Several studies, using different types of brain injury, including adult stroke models of middle cerebral artery occlusion (MCAO) and neonatal hypoxic-ischemic (HI) brain injury, have shown that administration of mesenchymal stem cells (MSC) promotes functional neurological recovery.3,4 This beneficial effect of MSC transplantation might involve replacement of damaged cells by the transplanted cells. However, data in the literature suggest that it is more likely that transplanted MSC induce repair by stimulating secretion of growth and differentiation factors, thereby providing an environment that stimulates repair processes like neurogenesis and angiogenesis.5 We and others have shown that in response to the growth factor environment in the damaged brain, MSC secrete several factors that have the potential to stimulate repair processes in the brain.5–7

In this study, we investigated whether MSC transplantation has beneficial effects on functional outcome and lesion volume in a rat model of neonatal stroke. Furthermore, we determined whether the therapeutic potential of MSC could be enhanced by genetically modifying the MSC to secrete more brain-derived neurotrophic factor (BDNF).

Background and Purpose—Brain injury caused by stroke is a frequent cause of perinatal morbidity and mortality with limited therapeutic options. Mesenchymal stem cells (MSC) have been shown to improve outcome after neonatal hypoxic-ischemic brain injury mainly by secretion of growth factors stimulating repair processes. We investigated whether MSC treatment improves recovery after neonatal stroke and whether MSC overexpressing brain-derived neurotrophic factor (MSC-BDNF) further enhances recovery.

Methods—We performed 1.5-hour transient middle cerebral artery occlusion in 10-day-old rats. Three days after reperfusion, pups with evidence of injury by diffusion-weighted MRI were treated intranasally with MSC, MSC-BDNF, or vehicle. To determine the effect of MSC treatment, brain damage, sensorimotor function, and cerebral cell proliferation were analyzed.

Results—Intranasal delivery of MSC- and MSC-BDNF significantly reduced infarct size and gray matter loss in comparison with vehicle-treated rats without any significant difference between MSC- and MSC-BDNF-treatment. Treatment with MSC-BDNF significantly reduced white matter loss with no significant difference between MSC- and MSC-BDNF-treatment. Motor deficits were also improved by MSC treatment when compared with vehicle-treated rats. MSC-BDNF–treatment resulted in an additional significant improvement of motor deficits 14 days after middle cerebral artery occlusion, but there was no significant difference between MSC or MSC-BDNF 28 days after middle cerebral artery occlusion. Furthermore, treatment with either MSC or MSC-BDNF induced long-lasting cell proliferation in the ischemic hemisphere.

Conclusions—Intranasal administration of MSC after neonatal stroke is a promising therapy for treatment of neonatal stroke. In this experimental paradigm, MSC- and BDNF-hypersecreting MSC are equally effective in reducing ischemic brain damage. (Stroke. 2013;44:1426-1432.)

Key Words: cell transplantation ■ cerebral ischemia ■ mesenchymal stem cells ■ neonatal stroke ■ postnatal
Methods

Adenoviral Vector

Adenoviral vector (pAd-HM41-K7; Alphagen, Yokohama, Japan) carrying the gene for polylysine-mutated fibronectin was constructed, as described previously. Mouse BDNF cDNA was cloned using real-time–polymerase chain reaction with total RNA isolated from brain as template. BDNF sequence was confirmed by sequencing and compared with GenBank sequence NM_007540. Mouse BDNF-primer sequences were Forward 5′-TCTAGACACCCACCACATGACCATCTTTTCTCTT-3′, Reverse 5′-TCTTCCCCCTTTTATGGTCAGT-3′. BDNF cDNA was cotransfected into pShuttle2 vector between the Xhol and Ap/II sites, resulting in the pShuttle2-BDNF-IE plasmid. pAd-HM41-K7-BDNF-IE was constructed by ligation of I-Cell/PI-Scl-digested pShuttle2-BDNF-IE with I-CeuI/PI-Scl-digested pAd-HM41-K7. An empty vector control was generated and consisted only of the IRES-eGFP sequence insert (pAd-HM410K7-IE).

Viruses particles were generated by transfection of PacI-digested pAd-HM41-K7-BDNF-IE into 293 cells with Lipofectamine2000 (Invitrogen). Before being used, virus titer was determined, and stocks were examined for potential contamination with replication-competent viruses.

Mesenchymal Stem Cells

Rat Sprague-Dawley MSC (GIBCO) were cultured according to manufacturer’s instructions. Cells were negative for myeloid and hematopoietic cell lineage–specific antigens and positive for CD29, CD44, CD90, and CD106. MSC were seeded at a density of 3×10⁵ cells per 25-cm² flask, exposed to pAd-HM41-K7-BDNF-IE or pAd-HM41-K7-IE virus particles in 7.5-mL Dulbecco’s modified eagle medium and recultured with normal medium for 6 hours, after which cultures were washed 3 times with Dulbecco’s modified eagle medium and recultured with normal medium. After infection, MSC were cultured for an additional 24 hours after which transplantation was performed.

Animals

All animal research was approved by the University of California San Francisco Institutional Animal Care and Use Committee and performed in accordance with the Guide for Care and Use of Laboratory Animals (US Department of Health and Human Services, Publication Number 85-23, 1985).

Transient 1.5-hour right MCAO was performed in 10-day-old Sprague-Dawley rats, as described previously. Briefly, surgery was performed on spontaneously breathing pups anesthetized with 1.75% isoﬂurane in a mixture of 70% N₂O and 30% O₂. The internal carotid artery was dissected, and a temporary ligature was applied at its origin using a 6-0 silk suture. A second suture was looped around the internal carotid artery, just above the pterygopalatine artery, and retracted laterally to prevent retrograde blood flow. A small arteriotomy was made in the proximal isolated internal carotid artery segment. A coated 6-0 Dermalon filament was inserted and advanced 7.5 to 8.5 mm depending on the weight of the animal and secured with a temporary suture. The filament and both sutures into the internal carotid artery were removed 1.5 hours later, re-establishing blood flow. Spin-echo planar diffusion-weighted MRI was performed using a T1 magnet 3 days after MCAO to identify injured animals; only animals with injury extending throughout the middle cerebral artery territory on diffusion-weighted MRI were used. Injury volume was determined in 6 consecutive 2-mm-thick coronal sections. A total of 36 pups met inclusion criteria based on diffusion-weighted MRI after MCAO (85%). Overall survival of injured animals was 87%.

At 3 days post-MCAO, MSC, BDNF-secreting MSC (MSC-BDNF), or vehicle was delivered intrasurally to awake rats. Thirty minutes before MSC or vehicle administration, 2 doses of 5-µL hyaluronidase (Sigma-Aldrich) in PBS were applied to each nostril and spontaneously inhaled. Subsequently, a total of 1×10⁶ MSC in 20 µL PBS or vehicle were administered as 2 doses of 5-µL were applied to each nostril.

To evaluate cell proliferation/survival, rats received ethynyl-2-deoxyuridine (EdU; 50 mg/kg, IP; Invitrogen, Carlsbad, CA) at days 3 to 5 post-MCAO and bromodeoxyuridine (BrdU; 50 mg/kg, IP; Sigma-Aldrich) at days 21 to 23 post-MCAO. Animals were euthanized at 28 days post-MCAO and perfused with 4% paraformaldehyde in PBS.

Functional Outcome

The cylinder rearing test was used to assess forelimb use asymmetry. The weight-bearing forepaw(s) to contact the wall during a full rear was recorded as left (impaired), right (nonimpaired), or both. Paw preference was calculated as (nonimpaired−impaired)/nonimpaired forepaw−impaired forepaw×100. Adhesive removal test was performed at 28 days after MCAO. Stickers (tough-spots, Diversified Biotech, Boston, MA) were placed on left and right forepaw, and the latency to removal was recorded. The mean time until complete removal of 3 stickers per forepaw was recorded. Sticker placement on left and right forepaw was alternated between and within animals.

Histology and Immunohistochemistry

Coronal paraffin sections (10 µm) were incubated with mouse-anti-myelin basic protein (MBP) (Sternerberger Monoclonals, Lutherville, MD) or mouse-anti-microtubule-associated protein 2 (Sigma-Aldrich, Steinheim, Germany), and binding was visualized with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Volumetric injury analysis was performed on a series of 6 microtubule-associated protein 2, MBP, and cresyl violet stained sections (ImageJ, NIH, Bethesda, MD). Injury volume was expressed as ratio of ipsilateral/contralateral hemisphere volume.

For cell proliferation analysis, sections were incubated with biotinylated sheep-anti-BrdU and rabbit-anti-Ki67 (Abcam). Visualization was done with AlexaFluor-594 conjugated streptavidin and donkey-anti-rabbit AlexaFluor488 (Molecular Probes). EdU incorporation was detected by incubating sections in 100 mmol/L Tris containing 0.5 mmol/L CuSO₄, 50 mmol/L ascorbic acid, and 10 µmol/L AlexaFluor-594-azide (Molecular Probes). EdU- and BrdU-positive cells were counted in 3 high magnification fields in the striatum of the damaged hemisphere in 3 sections per brain. Ki67-positive cells were counted in subventricular zone (SVZ) in 3 sections per brain.

Statistical Analysis

All data are expressed as mean±SEM. Functional outcome measured with cylinder rearing test and adhesive removal test were analyzed using 2-way ANOVA with Fisher Least Significant Difference post-tests. Histological measures were analyzed using 1-way ANOVA with Bonferroni post-tests. P<0.05 was considered statistically significant.

Results

MSC Treatment After Neonatal Stroke Reduces Lesion Volume

Rats underwent MCAO at p10 and were treated with MSC, MSC-BDNF, or vehicle at 3 days after the insult. Animals that underwent MCAO had significant brain tissue loss in the ipsilateral hemisphere at 28 days post-MCAO (Figure 1A). After treatment with MSC, there was a significant reduction in ipsilateral tissue loss in comparison with vehicle-treated rats (P<0.05). Treatment with MSC-BDNF resulted in a reduction of tissue loss when compared with vehicle (P<0.01 versus vehicle), but not significantly different from MSC-treated rats.

We also analyzed the effect of MCAO and subsequent MSC treatment on gray and white matter loss. At 28 days post-MCAO, microtubule-associated protein 2–positive area loss, as a measure of gray matter injury, was significantly lower
after treatment with MSC or MSC-BDNF in comparison with vehicle-treated rats ($P<0.05$ and $P<0.01$, respectively). Treatment with MSC-BDNF did not result significantly in lower MAP-2 loss than treatment with MSC (Figure 1B).

Injury to white matter was analyzed by measuring MBP-positive area. MCAO resulted in significant loss of MBP-positive tissue in vehicle-treated rats (Figure 1C). Treatment with MSC did not significantly decrease MBP area loss when compared with vehicle-treated rats. Treatment with MSC-BDNF, however, did cause a significant reduction in MBP-positive area loss ($P<0.05$) with no significant difference between MSC and MSC-BDNF treatment.

**MSC Transplantation After Neonatal Stroke Reduces Motor Deficits**

To determine the extent of lateralizing motor deficits caused by our stroke model, the cylinder rearing test was performed at 14, 21, and 28 days after MCAO. In this test, sham-operated rats did not show any paw preference (Figure 2A). MCAO caused lateralization shown by a $\approx 30\%$ preference to use the right unimpaired forepaw in vehicle-treated rats. After treatment with MSC, performance in the cylinder rearing test significantly improved at all 3 time points measured in comparison with vehicle-treated rats. At 14 days post-MCAO, forepaw impairment in MSC-BDNF treated rats was significantly lower than in MSC-treated rats ($P<0.05$). However, the effect of MSC-BDNF-treatment was only temporary because there was no difference between MSC-BDNF and MSC-treated rats at 28 days post-MCAO.

At 28 days post-MCAO, all rats were subjected to the adhesive removal test. This test evaluates sensory and motor deficits related to the paw. Sham-operated controls did not show a difference in adhesive removal latency between left and right forepaw (Figure 2B). After MCAO, all rats were impaired in the adhesive removal test, indicating that the latency to remove the sticker from the impaired forepaw was significantly higher than removing the sticker from the unimpaired forepaw. More importantly, MSC- and MSC-BDNF–treated rats showed a reduced latency to remove the adhesive from the impaired forepaw when compared with vehicle-treated rats. There was

![Figure 1. Effect of mesenchymal stem cells (MSC) transplantation on lesion size. Quantification of damage using Nissl staining (A), microtubule-associated protein 2 (MAP2)-positive area loss (B), myelin basic protein (MBP)-positive area loss (C), and representative sections. Data represent mean±SEM. Sham controls n=5, vehicle (VEH) n=8, MSC n=9, MSC–brain-derived neurotrophic factor (BDNF) n=8. *$P<0.05$ vs VEH, **$P<0.01$ vs VEH.](http://stroke.ahajournals.org/)}
MSC Treatment After Neonatal Stroke Induces Long-Lasting Cell Proliferation in the Ipsilesional Striatum and SVZ

To determine the effect of MSC transplantation and modified-MSC transplantation on cell proliferation in the brain after neonatal stroke, rats were injected with cell proliferation markers EdU on days 3 to 5 post-MCAO and, to check for late proliferation, with BrdU on days 21 to 23 post-MCAO. At 28 days post-MCAO, the number of EdU- and BrdU-labeled cells and expression of the cell proliferation marker Ki67 were analyzed.

At 28 days after HI, only some EdU- and BrdU-positive cells could be detected in the SVZ. However, in the injured striatum and near the ischemic boundary zone, a significant number of both EdU- and BrdU-positive cells were visible. No increase in EdU-positive cell number in the striatum was detected at 28 days post-MCAO in vehicle-treated rats in comparison with sham-operated controls. Treatment with either MSC or MSC-BDNF significantly increased the number of EdU-positive cells in the ipsilateral striatum. There was no difference in the number of EdU-positive cells between MSC- and MSC-BDNF–treated rats (Figure 3A and 3D), indicating that there was no difference in cell proliferation induced by either treatment with MSC or MSC-BDNF.

Measurement of late cell proliferation by analysis of the number of BrdU-positive cell showed that MCAO with vehicle-treatment had no effect on cell proliferation at 21 to 23 days post-MCAO. After treatment with MSC and MSC-BDNF, the number of BrdU-positive cells increased when compared with vehicle-treated rats. However, there was no additional effect of MSC-BDNF when compared with MSC treatment (Figure 3B and 3D).

At 28 days post-MCAO cell proliferation in the MSC-treated rats, proliferation was still increased as compared with vehicle-treated MCAO rats as witnessed by increased Ki67-positive cell number in the SVZ. Treatment with MSC-BDNF did significantly increase the number of Ki67-positive cells in the SVZ when compared with vehicle-treated rats but was not different from treatment with MSC alone (Figure 3C and 3D).

Discussion

This study, for the first time, shows that intranasal application of MSC or BDNF-hypersecreting MSC after neonatal stroke in rats effectively reduces long-term functional impairment and infarct volume and increases cell proliferation in the ischemic hemisphere.

Improved outcome after transplantation of MSC has been attributed to inhibition of inflammation, neuroprotection, direct replacement of lost tissue by MSC, and stimulation of endogenous repair processes. MSCs secrete a variety of factors, including neurotrophins like BDNF and nerve growth factor, growth factors like vascular endothelial growth factor and insulin-like growth factor, and interleukins. Recently Kim et al showed that intraventricular administration of human umbilical cord blood–derived MSC 6 hours after onset of neonatal stroke in a nonreperfusion model reduced ipsilesional tissue loss. This was accompanied by a dampened inflammatory response and decreased cell death. It has been suggested that the beneficial effects of MSC are mediated by the immunomodulatory effect of MSC, as well as by delivery of trophic factors. We observed a decrease in lesion volume after MSC treatment. However, in the adult MCAO model, treatment with MSC has been shown to improve neurological function without reducing lesion volume. All of these results could either be interpreted as MSC-induced endogenous repair or MSC-induced neuroprotection. In some studies, in juvenile animals, there is an evidence for significant late neuronal loss. However, we did not observe a deterioration of neuronal function between day 3 and 10 post-HI when using the HI model in p9 mice. Therefore, we proposed that MSC transplantation in the neonatal HI model may well promote endogenous neuronal repair. In the model of neonatal stroke currently described, we do not know at present whether late neuronal loss develops, and we cannot...
conclude whether MSC mainly function by protecting the brain against late neurodegeneration or by endogenous repair mechanisms. The fact that treatment with MSC increase cell proliferation in the SVZ lasting for at least 28 days post-MCAO may indicate that endogenous repair processes are contributing to the therapeutic effects of MSC in our neonatal stroke model.

Recent results from our group showed that MSC transplantation 10 days after induction of neonatal HI induces various changes in the cytokine and growth factor environment.\(^5\)\(^,\)\(^7\)
After transplantation of MSC after neonatal HI, the gene expression profile in the brain shifts toward a growth promoting environment. In vivo, this is visible as increased cell proliferation, formation of new neurons, and decreased gliosis, suggesting decreased inflammation after MSC treatment of ischemic brain injury. In the present study, transplantation of both MSC and MSC-BDNF resulted in long-lasting cell proliferation in the SVZ. The SVZ contains neural stem cells, which under normal circumstances differentiate into neurons and migrate toward the olfactory bulb. Local injury can stimulate cell proliferation in the SVZ on which cells migrate toward the injured area and differentiate into neurons, oligodendrocytes, and astrocytes. Our results in this study show that MSC treatment increases cell proliferation for at least 28 days post-MCAO. Cell proliferation markers, EdU and BrdU, were injected directly after administration of the MSC and 2 weeks later, respectively, to determine cell proliferation during the course of the experiment. At 28 days after HI, only some EdU- and BrdU-positive cells could be detected in the SVZ. However, in the injured striatum and near the ischemic boundary zone, a significant number of both EdU- and BrdU-positive cells was visible. These EdU- or BrdU-labeled cells probably originate from the SVZ and migrated toward the injured area, where they potentially contribute to repair of the injured tissue and decreased lesion volume that was observed after MSC transplantation.

Administration of MSC after neonatal stroke significantly reduces lateralizing motor deficits. Besides the effect of MSC transplantation after neonatal stroke on motor and somatosensory function, it is likely that cognitive function also is improved in rats after treatment with MSC. Recent studies have shown that neurorestorative treatments like MSC transplantation or erythropoietin can indeed improve cognitive function after ischemic brain damage. A study on cognitive effects may be of importance in view of repair of white matter structures induced by treatment with MSC or MSC-BDNF (Figure 2).

Neurotrophic factors play an important role in repair of ischemic brain damage. BDNF, in particular, is an important neurotrophic factor promoting neurogenesis and angiogenesis. It offers neuroprotection, modulates inflammation, and can improve synaptic plasticity after ischemic brain injury. Furthermore, it has been shown that intracranial infusion of BDNF using an osmotic pump can reduce infarct volume after stroke.

Our present study shows that the effect of MSC-BDNF administration seems to be temporary. Treatment with MSC-BDNF causes less lateralizing motor deficits measured at 1 week after administration of the stem cells, after which motor deficits get more pronounced and at 4 weeks reach the level of rats treated with MSC alone. However, at 4 weeks, post-stroke BDNF-MSC are not more effective in reducing gross infarct size, gray and white matter loss, and induction of cell proliferation than treatment with MSC alone. It is possible that the additive effect of BDNF on transplantation of MSC transplantation of MSC observed in the early measurement in the cylinder rearing test (day 14) is a direct effect of BDNF on neuronal activity. BDNF plays a role in several stages of the development of neural circuits, including neural stem cell survival and differentiation, axonal growth, and refinement of developing circuits. The effect of BDNF-MSC might be targeted toward the refinement of neural circuits that are developing. Addition of BDNF in this period of development could lead to selective stabilization of some connections and elimination of others more effectively than MSC alone does.

In this study, we used a nonintegrating adenoviral construct to overexpress BDNF in MSC, indicating that BDNF expression subsides over time as the vector is removed from the cell. The temporary expression of BDNF might have been too short to induce long-lasting effects. Therefore additional administrations of BDNF-secreting MSC at later time points may be necessary to produce a more sustained effect. However, there is a time window of administration for MSC to be effective.

We have recently shown, using the murine HI model, that a second dose of MSC at 17 days post-HI has no additive effect on top of a first dose at 10 days post-HI, whereas 2 doses at 3 and 10 days post-HI effectively reduce HI brain damage. Apart from the fact that efficacy of (modified) MSC transplantation is of great importance, safety is a key issue in light of future clinical application of transplantation of (modified) MSC to babies with brain damage. Allogeneic MSC have been used for many years now in the treatment of hematopoietic diseases, which indicates that protocols regarding isolation, administration, and safety are already established. The use of an adenovirus to modify MSC instead of lenti- or retrovirus reduces the risk of developing neoplasms because adenoviruses do not integrate into the host genome. However, fate of transplanted cells and possible adverse behavioral effects must be monitored closely in a phase II trial applying (modified) MSC transplantation in newborn babies with brain damage.

In summary, this study shows that intranasal application of BDNF-secreting MSC is equally effective in reducing gray and white matter loss, and motor deficits, and inducing cell proliferation after neonatal MCAO as use of MSC. Thus, intranasal treatment with MSC may be a promising therapy for neonatal ischemic brain damage.

The signals responsible for orchestrating repair processes, such as neurogenesis, gliogenesis, and angiogenesis, are complex and depend on an intricate balance of various intra- and extracellular molecules. It may well be possible that MSC are equipped well enough to react to the needs of the ischemic environment to stimulate repair processes in the neonatal ischemic brain.

Sources of Funding
This study was funded by the European Union (HEALTH-F2-2009–241778, NEUROBID); Zon-MW Project (no 116002003), and National Institutes of Health (grant NS35902).

Disclosures
None.

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Mesenchymal Stem Cell Transplantation Attenuates Brain Injury After Neonatal Stroke

Stroke. 2013;44:1426-1432; originally published online March 28, 2013;
doi: 10.1161/STROKEAHA.111.000326
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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