Bone Marrow Stromal Cells That Enhanced Fibroblast Growth Factor-2 Secretion by Herpes Simplex Virus Vector Improve Neurological Outcome After Transient Focal Cerebral Ischemia in Rats

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Background and Purpose—Fibroblast growth factor-2 (FGF-2) administration and bone marrow stromal cell (MSC) transplantation could improve neurological deficits after occlusive cerebrovascular disease. In the present study, we examined the effects of neurological improvement after transient middle cerebral artery occlusion (MCAO) in rats by a novel therapeutic strategy with FGF-2 gene–transferred MSCs by the herpes simplex virus type 1 (HSV-1) vector.

Methods—Adult Wistar rats were anesthetized. Nonmodified MSCs, FGF-2–modified MSCs with HSV-1 1764/-4/pR19/ssIL2-FGF-2, or PBS was administered intracerebrally 24 hours after transient right MCAO. All animals underwent behavioral tests for 21 days, and the infarction volume with 2,3,5-triphenylterazolium was detected 3 days and 14 days after the MCAO. Three days and 7 days after the MCAO, the FGF-2 production in the ipsilateral hemisphere of the MCAO was measured with ELISA. Seven and 14 days after the MCAO, immunohistochemical staining for FGF-2 was applied.

Results—The stroke animals receiving FGF-2–modified MSCs demonstrated significant functional recovery compared with the other groups. Fourteen days after the MCAO, there was a significant reduction in infarction volume only in FGF-2–modified MSC-treated group. FGF-2 production in the FGF-2–modified MSC-treated brain was significantly higher compared with the other groups at 3 and 7 days after MCAO. Administered FGF-2–modified MSCs strongly expressed the FGF-2 protein, which was proven by ELISA.

Conclusions—Our data suggest that the FGF-2 gene–modified MSCs with the HSV-1 vector can contribute to remarkable functional recovery after stroke compared with MSCs transplantation alone. (Stroke. 2005;36:2725-2730.)

Key Words: bone marrow cell ■ cerebral infarct ■ FGF-2 ■ HSV-1 vector

Recent animal experiments demonstrated that basic fibroblast growth factor-2 (FGF-2) can improve neurological function after stroke by the neuroprotective effect or vasodilating effect.1,2 Whereas bone marrow stromal cell (MSC) transplantation improved neurological deficits after occlusive cerebrovascular disease. MSCs can differentiate into some cell types not derived from a mesenchymal origin such as glia and neurons.3,4 MSCs also have the potential to secrete some growth factors. This production was considered key to the benefits provided by transplanted MSCs in the ischemic brain.5,6 Therefore, we expect that enhancement of growth factor secretion including FGF-2 by MSCs can achieve functional recovery after central nervous system occlusive disorders more effectively. In the present study, we examined the effects of neurological improvement after transient middle cerebral artery occlusion (MCAO) in rats by a novel therapeutic strategy with FGF-2 gene–transferred MSCs by the herpes simplex virus type 1 (HSV-1) vector.

Materials and Methods

HSV-1 Vector
We prepared a replication-incompetent HSV-1 vector expressing FGF-2 with an artificially fused interleukin-2 (IL-2) secretory signal sequence (HSV-1 1764/-4/pR19/ssIL2-FGF-2). This vector originated from HSV-1 strain 17 and was made replication incompetent by the deletion of the ICP4, ICP34.5, and VP16 genes.7 HSV-1
Preparation of Bone MSCs

We prepared MSCs from 7- to 9-week-old Wistar rats (Japan SLC, Inc; Hamamatsu, Japan) as described previously. The bilateral femurs and tibias were aseptically dissected and the bones were cut off. The marrow was extruded with the culture medium (minimum essential medium with 2 mMol/L L-glutamate and 10% FBS) to flask and cultured in 5% CO₂ at 37°C. After 48 hours, the nonadherent cells were removed by changing the medium. When the cells were grown to confluence, they were lifted by 0.25% trypsin and 0.02% EDTA in PBS. The cells were passed 4 to 6 times and were used in all experiments.

Evaluation of the Effectiveness of FGF-2 Gene Transfer to MSCs With HSV-1 Vector

Experimental groups (n=4 each group) consisted of group 1: only culture medium (without MSCs); group 2: MSCs without HSV-1 vector infection were incubated in culture medium; and groups 3, 4, 5, and 6: MSCs were infected with HSV-1 1764/4/pr19/ssl2-L2- FGF-2 at a multiplicity of infection (MOI) of 0.1, 1, 5, and 10 for 1 hour, and then incubated in the culture medium. Twenty-four hours after HSV-1 vector infection, we determined the FGF-2 concentration in the culture supernatant with an ELISA (Wako Pure Chemical). Absorbance was measured with a microplate reader (450 nm; NALGEN-NUNC Immunoreader NJ-2001).

In Vivo Study

Stroke Model

Procedures and virus inoculates were approved by the Committee of Recombinant DNA and Animal Experiments, Osaka Medical College. Briefly, adult male Wistar rats weighting 250 to 300 g (Japan SLC, Inc; Hamamatsu, Japan) were anesthetized initially 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. We induced transient occlusion with minor modifications. Two hours after MCAO, the rats were treated with PBS or fluorescent marked blood (Japan SLC, Inc; Hamamatsu, Japan) as described previously. The bilateral femurs and tibias were aseptically dissected and the bones were cut off. The marrows were extruded with the culture medium (minimum essential medium with 2 mMol/L L-glutamate and 10% FBS) to flask and cultured in 5% CO₂ at 37°C. After 48 hours, the nonadherent cells were removed by changing the medium. When the cells were grown to confluence, they were lifted by 0.25% trypsin and 0.02% EDTA in PBS. The cells were passed 4 to 6 times and were used in all experiments.

Experimental Groups: Stereotactic MSC Transplantation Procedure

Twenty-four hours after MCAO, the animals were initially reanesthetized with halothane and maintained with intraperitoneal pentobarbital (10 μg/g body weight) administration, and then fixed with a stereotactic frame (David Kopf; model 900). The experimental groups consisted of group 1 (sham-operated group), composed of rats given MCAO without cell administration but with 10 μL PBS; group 2 (nonmodified MSC-treated group), consisting of rats given MSCs without gene transfer (1×10⁴ cells) stereotactically to the ipsilateral striatum of MCAO; and group 3 (FGF-2-modified MSC-treated group), consisting of rats given MSCs (1×10⁴ cells) that had been transfected the FGF-2 gene with HSV-1 1764/4/pr19/ssl2-L2- FGF-2 to the ipsilateral striatum of MCAO. Twenty-four hours before the stereotactic injection, the FGF-2 gene was transferred to MSCs with HSV-1 1764/4/pr19/ssl2-L2- FGF-2. The MSCs were incubated with the virus vector (gene transfer to MSCs at MOI of 5). One hour after, the virus solution was removed, and the MSCs infected with the virus vector were cultured for another 24 hours. Nonmodified MSCs and FGF-2-modified MSCs were lifted as above just before transplantation and harvested. The harvested cells were diluted to 1×10⁶ cells/10 μL PBS.

Behavioral Testing

All animals (n=6 for each group) underwent behavioral tests before MCAO and 1, 3, 7, 14, and 21 days after MCAO. A modified neurological severity score (mNSS; supplemental Table I, available online at http://stroke.ahajournals.org) was used to grade the aspects of neurological function. Each test in mNSS has been used to grade various aspects of neurological function. mNSS is composite of the motor (muscle status, abnormal movement), sensory (visual, tactile, and proprioceptive), and reflex tests. The higher score, the more severe the injury.

Measurement of Infarct Volume

Three and 14 days after the MCAO, all group rats (n=5 for each group) were deeply anesthetized with halothane. Then transcardiac perfusion was performed with saline. The brain was immediately removed and sectioned into 7 equally spaced (2 mm) coronal blocks using a rodent brain matrix. These sections were stained with 2% 3,3'-3,5'-triphenyltetrazolium (TTC) with normal saline for 30 minutes at 37°C. With TTC staining, the area without staining was determined to be the infarct area. The lesion volume is presented as a volume percentage of the lesion compared with the contralateral hemisphere.

Determination of Human FGF-2 in the Brain Tissue With ELISA

Three and 7 days after MCAO, the rats (n=5 for each group) were deeply anesthetized with halothane, and brains were removed and dissected on ice immediately. The ipsilateral cerebral hemisphere of the MCAO was dissected from the whole brain.

The wet weight of each sample was rapidly measured. The samples were then stored at −80°C. Subsequently, each sample was homogenized in cell lysis buffer. The homogenate was centrifuged for 45 minutes at 10 000g at 4°C, and the supernatant was collected for ELISA as stated above.

Identification of the Transplanted MSCs and Immunohistochemical Assessment for FGF-2

Before transplantation, the MSCs were incubated with culture medium containing 1 μg/mL bis-benzimide (Hoechst 33258) over 24 hours to label the nuclei fluorescently. Twenty-four hours after MCAO, the rats were treated with PBS or fluorescent marked nonmodified or FGF-2-modified MSCs as described above.

Fourteen days after MCAO, the rats were deeply anesthetized with halothane and their brains were transcardially perfused with normal saline, followed by 4% paraformaldehyde. Then their brains were removed and incubated in 20% sucrose at 4°C overnight. The samples were immediately frozen by liquid nitrogen, and 6-μm sections were prepared with a cryostat for immunohistochemical staining. After permeabilization with 0.02% Triton X and blocking in 3% BSA, these sections were treated with the mouse monoclonal antibody against FGF-2 for 120 minutes at 4°C. Subsequently, the sections were washed with PBS, and fluorescent rhodamin–conjugated donkey secondary antibody against mouse IgG was added and incubated for 20 minutes at 4°C. The prepared sections were observed with a fluorescent microscope (Olympus AX70). FGF-2- or Hoechst-positive cells were counted in the specimen-contained implantation center using a ×100 objective (n=4 for each group).

Statistical Analysis

Data were expressed as mean±SD. The 1-way ANOVA test followed by Bonferroni’s post hoc analysis was used, and values of P<0.05 were considered statistically significant.
Results

In Vitro Study: Gene Transfer to MSCs With HSV-1 Vector and FGF-2 Secretion

The supernatant of all groups of MSCs transferred the FGF-2 gene with our HSV-1 vector contained FGF-2. FGF-2 production by FGF-2–modified MSCs increased with the proportion to MOI (Figure 1).

Neurological Function Test

At 7 days after MCAO, the rats treated with FGF-2–modified MSCs showed significant improvement in mNSS compared with the sham-operated rats (P<0.05). At 14 and 21 days after MCAO, significant functional recovery was found in the nonmodified MSC-treated group compared with the sham-operated rats (P<0.05). In addition, that was also found in the rats treated with FGF-2–modified MSCs compared with not only the sham-operated group but also the nonmodified MSC group at 14 and 21 days after MCAO (Figure 2).

Measurement of Infarction Volume

Three days after MCAO, infarct volume tended to decrease in FGF-2–modified MSC-treated rats. However, there was no significant difference in the total infarct volume among each group (data not shown). Whereas 14 days after MCAO, there were significant difference in the total infarction volume between the sham-operated group or the nonmodified MSC-treated group and FGF-2–modified MSC group (Figure 3).

FGF-2 Measurements With ELISA in Brain Extracts

At 3 and 7 days after MCAO, the level of FGF-2 in the brain tissue of the FGF-2–modified MSC-treated group was significantly higher than that of the sham-operated or nonmodified MSC-treated group. The mean level of FGF-2 in the brain tissue of the nonmodified MSC-treated group was higher than that of the sham-operated group but statistically not significant (Figure 4). In the sham-operated group, FGF-2 level at 7 days after the MCAO was significantly decreased compared with that at 3 days after MCAO. It is noteworthy that in the FGF-2–modified MSC-treated group, the mean level of FGF-2 at 7 days after MCAO was higher than that at 3 days after MCAO (statistically not significant).

Identification of the Transplanted MSCs and Immunohistochemical Assessment for Human FGF-2

Some Hoechst-positive cells were observed around the implantation truck, but no Hoechst-positive cells migrated to remote site from the implantation point (contralateral brain or ipsilateral...
The FGF-2 secretion by transplanted MSCs was confirmed with immunohistochemical staining 14 days after MCAO at the region around the stereotactically transplanted point. No Hoechst-positive cells were observed in the sham-operated rats. Some Hoechst-marked cells were stained weakly for FGF-2 (3.12 ± 0.76%) in the nonmodified MSC-treated rats. Whereas the Hoechst-marked cells were stained for FGF-2 (39.79 ± 7.32%) in the FGF-2–modified MSC-treated rats (Figure 5). Additionally, we demonstrated that transplanted FGF-2–modified MSCs might enhance the endogenous FGF-2 expression significantly in the ischemic brain. We also demonstrated that transplanted FGF-2–modified MSCs obtained the function to secrete FGF-2 continuously in the ischemic brain even at 14 days after MCAO. These data supported Chen’s data. Furthermore, immunohistochemically, we revealed that the transplanted FGF-2–modified MSCs were alive, and ≈40% of them secreted FGF-2 protein in the ischemic brain even at 14 days after MCAO. These data demonstrated that transplanted FGF-2–modified MSCs obtained the function to secrete FGF-2 continuously in the ischemic brain.

We also demonstrated transplanted FGF-2–modified MSCs could enhance the endogenous FGF-2 expression in the ischemic boundary cortex. This might be the reason that FGF-2 production was significantly higher in the FGF-2–modified MSC-treated group than in the sham-operated group and even the nonmodified MSC-treated group (P < 0.05). In the sham-operated group, FGF-2 level at 7 days after the MCAO was significantly decreased compared with that at 3 days after the MCAO (P < 0.05), whereas in the FGF-2–modified or nonmodified MSC-treated group, the mean level of FGF-2 level was maintained.

**Discussion**

FGF-2 is trophic for neurons, glia, and endothelial cells in the central nervous system. Similarly, FGF-2 prevents downregulation of the antiapoptotic protein Bcl-2 in ischemic brain tissue and limits excitotoxic damage to the brain through an activin-dependent mechanism. Furthermore, FGF-2 appears to be a cerebral vasodilator and, consequently, increases regional cerebral blood flow. In addition, we and other researchers reported that ischemic insult and FGF-2 administration augmented neurogenesis in the rodent brain. These effects of FGF-2 were thought to contribute to infarct volume reduction and neurological improvement after experimental focal cerebral ischemia. We also reported previously that gene therapy for a transient MCAO rat model with adenovirus vector–expressing FGF-2 could improve neurological deficits and reduce infarction volume.

Recent animal experiments demonstrated that bone marrow cell transplantation alone improves neurological deficits in cerebral infarction. Bone marrow cells have the potency to secrete several cytokines, including FGF-2, in the ischemic brain. At least in the acute stage, these cytokines were considered key beneficial substances provided by transplanted MSCs in the ischemic brain. Chen et al reported on cytokine secretion from MSCs in the ischemic brain microenvironment. They found MSCs secreted FGF-2 in the normal and the ischemic brain microenvironment. However, the FGF-2 production levels in the ischemic brain microenvironment decreased over time.

Based on these data about cell therapy with MSCs and FGF-2 administration therapy after stroke, we described how FGF-2 secretion–enhanced MSCs might be more applicable to achieve the functional recovery after ischemia.

As we reported previously, our replication-incompetent HSV-1 1764/4+/pR19 vector can safely transfer the gene of interest to MSCs efficiency. In the present study, MSCs were transferred the gene of interest before transplantation with virus vector ex vivo, and these gene-modified MSCs were transplanted into a stroke model. The experimental animals were not exposed to the virus vector directly. Therefore, our therapeutic strategy may be safer than direct gene therapy with virus vectors alone. Kurozumi et al reported the same strategy for the MCAO rat model using a fiber-mutant adenovirus vector.

J. Chen et al demonstrated that in the ischemic brain, transplanted MSCs are able to enhance the FGF-2 expression in the ischemic boundary area. Our in vivo ELISA data demonstrated that FGF-2 secretion was observed even in the brain of the sham-operated rats but decreased over time. The level of FGF-2 protein in the brain of the nonmodified MSC-treated rats was maintained over time. These results supported Chen’s data. Furthermore, immunohistochemically, we revealed that the transplanted FGF-2–modified MSCs were alive, and ≈40% of them secreted FGF-2 protein in the ischemic brain even at 14 days after MCAO. These data demonstrated that transplanted FGF-2–modified MSCs obtained the function to secrete FGF-2 continuously in the ischemic brain. We also demonstrated transplanted FGF-2–modified MSCs could enhance the endogenous FGF-2 expression in the ischemic boundary cortex. This might be the reason that FGF-2 production was significantly higher in the FGF-2–modified MSC-treated hemisphere than in the sham-operated group or nonmodified MSC-treated group, even 7 days after MCAO. The mechanism by which FGF-2–modified MSCs could improve neurological function and induced reduction of the infarction area after transient MCAO has been unclear. However, we felt that a therapeutic benefit was developed with continuous FGF-2 secretion by the transplanted FGF-2–modified MSCs.

The possible mechanisms by which FGF-2 enhances recovery include protection against retrograde cell death and acceleration of new neuronal sprouting and synapse formation. Finklestein and his group demonstrated that this functional recovery by FGF-2 might be attributable to upregulation of the markers of neuronal plasticity, such as synaptophysin and GAP43, and the stimulation of new neuronal sprouting in the uninjured brain. Other mechanisms could include promoting the endogenous neurogenesis and the synaptic plasticity, as we and some authors reported previously.
These mechanisms might have also worked in combination in our study, but this is still unclear, and which mechanism is predominant should be elucidated in the future. Our present data as to reduction of the infarction volume 14 days after the MCAO detected with TTC staining demonstrated that 1 of the main mechanisms on functional recovery by our strategy is an antiapoptotic effect within 14 days after MCAO. Our preliminary study revealed that this strategy reduces the apoptosis of neuronal cells in the ischemic boundary (data not shown).

Some authors stressed that TTC staining is unreliable after 48 hours or so because of uptake in proliferating astroglia. However, Kurozumi et al reported that a correlation between MRI-derived infarction volume and TTC staining results 14 days after MCAO. Furthermore, we proved the number of reactive astroglia in the ischemic border zone was not different among each group at 14 days after the MCAO (data not shown). So we used TTC staining for detection of infarction area at 14 days after the MCAO.

In conclusion, the intracerebral injection of the MSCs transferred FGF-2 gene with our HSV-1 vector resulted in functional recovery after temporally MCAO in a rat model. These data suggested that our fusion therapy with MSCs, which were FGF-2 genes transferred by our HSV-1 vector, was an effective modality for stroke. Our results may be valid for temporally ischemia model. So we would like to examine for permanent ischemia model with our strategy in the future.

Acknowledgments
This work was supported by grants-in-aid for scientific research (B) (14370448) and (C) (12671353) and by a grant-in-aid for exploratory research (14657350) and by a grant-in-aid for young scientists (B) (17790989) from the Japanese Ministry of Education, Science and Culture to S.-I.M. and N.N. Additional support was provided in the form of a grant from the Promotion and Mutual Aid Corporation for Private Schools of Japan and the Science Research Promotion Fund to S.-I.M., and by the High-Tech Research Program of Osaka Medical College.

References


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Stroke. 2005;36:2725-2730; originally published online November 10, 2005;
doi: 10.1161/01.STR.0000190006.88896.d3

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

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