Anesthesiology 2010; 113:1109–17

Intrathecal Injection of Hepatocyte Growth Factor Gene-modified Marrow Stromal Cells Attenuates Neurologic Injury Induced by Transient Spinal Cord Ischemia in Rabbits

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ABSTRACT

Background: Our previous studies showed that transfer of hepatocyte growth factor (HGF) gene or transplantation of marrow stromal cells (MSCs) remarkably attenuated neurologic injuries after spinal cord ischemia. We sought to investigate a novel neuroprotective strategy of transplantation of human HGF gene-modified MSCs on ischemic spinal cords.

Methods: Human HGF gene was transferred into MSCs *in vitro*. The HGF gene-modified MSCs were transplanted by means of intrathecal injection. Two days later, spinal cord ischemia was induced by occlusion of the infrarenal aorta with a balloon catheter for 40 or 50 min. Hind-limb motor function was assessed during a 14-day recovery period with Tarlov criteria, and then histologic examination was performed.

Results: Human HGF was detected in the cerebrospinal fluid from 2 to 16 days after transplantation of HGF gene-modified MSCs. Compared with the controls, transplantation of HGF gene-modified MSCs or MSCs alone significantly improved the Tarlov scores 1, 2, 7, and 14 days after spinal cord ischemia of 40 or 50 min ($P < 0.01$, respectively) and increased the number of intact motor neurons in the lumbar spinal cord ($P < 0.01$, respectively). When the ischemic period was extended to 50 min, the Tarlov scores and the number of intact motor neurons of rabbits transplanted with HGF gene-modified MSCs were markedly higher than those of the rabbits transplanted with MSCs only ($P < 0.05$, respectively).

Conclusions: Transplantation of HGF gene-modified MSCs induces powerful neuroprotection on spinal cords against ischemia-reperfusion injury and is more therapeutically efficient than transplantation of MSCs only.

What We Already Know about This Topic

- Both growth factors and transplantation of marrow stromal cells (MSCs) have the potential to attenuate neurologic injuries after spinal cord ischemia.

What This Article Tells Us That Is New

- Transplantation of human growth factor gene-modified MSCs can provide spinal cord neuroprotection in the setting of ischemia-reperfusion injury.

PARAPLEGIA remains a major devastating and unpredictable complication after surgical repair of descending and thoracoabdominal aortic aneurysms. This complication is attributed primarily to temporary or permanent intervention of the blood supply of spinal cords. Regardless of the progress with surgical techniques and pharmacologic interventions, the incidence of paraplegia is still high at 6.6–8.3% in patients with extent II thoracoabdominal aortic aneurysms.[1,2]

Hepatocyte growth factor (HGF) was originally found from plasma and serum as a molecule that could stimulate DNA synthesis in rat and human hepatocytes.[3] HGF has been identified as a potential angiogenetic factor.[4] Moreover, HGF also plays a role as a neurotrophic and survival factor to maintain the structure and function of the nervous system.[5] HGF has been reported to induce versatile neuroprotective effects both *in vitro* and *in vivo*.[6] In our previous report, transfer of human HGF gene has been shown to attenuate the neurologic injury caused by spinal cord ischemia, as evidenced by the improvement of hind-limb motor function and the protection of the motor neurons.[7]

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Received from the Department of Cardiac Surgery and Department of Anesthesiology, First Affiliated Hospital, China Medical University, Shenyang, China. Submitted for publication May 14, 2010. Accepted for publication August 3, 2010. Supported by Grant no. 30801130 from the National Natural Science Foundation of China (Beijing, P. R. China) and Research Grant no. 05239 for Japan Society for the Promotion of Science Postdoctoral Fellows (Tokyo, Japan).

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Bone marrow contains the precursors of nonhematopoietic tissues that are referred to as mesenchymal stem cells or marrow stromal cells (MSCs). In different microenvironments, MSCs can differentiate into multiple mesodermal lineage cells as well as neuroectodermal cells.[8,9] MSCs have been considered to be a source for autoplastic therapies to central nervous system injuries. Several research studies have reported that transplantation of MSCs improved functional recovery after stroke.[10,11] We reported that, in a rabbit model of spinal cord ischemia, prophylactic transplantation of MSCs markedly protected spinal cords against ischemia-reperfusion injury.[12]

Recent experiments indicated that the existing therapeutic potential of MSCs could be enhanced by gene transduction. Brain-derived neurotrophic factor gene-modified MSCs were shown to promote functional recovery and reduce infarct size in the rat cerebral artery occlusion model.[13] Transplantation of angiopoietin-1 gene-modified human MSCs showed enhanced regions of increased angiogenesis at the lesion border and modest additional improvement in functional outcome after cerebral ischemia.[14] Furthermore, transplantation of human MSCs transfected with both angiopoietin-1 gene and the vascular endothelial growth factor gene showed a greater structural-functional recovery after cerebral ischemia than transplantation of human MSCs only.[15] The HGF gene was also used to modify MSCs and further enhanced the neuroprotective effects of MSCs in a stroke model of rat.[16]

Collectively, these results led to the hypothesis that additional neuroprotection against ischemia-reperfusion injury of spinal cords can be induced by transplantation of HGF gene-modified MSCs. In the current study, the human HGF gene was transferred into MSCs by using the hemagglutinating virus of Japan (HVJ)–envelope vector. We investigated whether intrathecal injection of HGF gene-modified MSCs could improve the neuroprotective effects in a well-characterized rabbit model of spinal cord ischemia.

Materials and Methods

Animal Care and Surgical Procedure

Japanese white rabbits weighing 1.8–2.5 kg were used in the study. The animal protocol was approved by the Ethics Review Committee for Animal Experimentation of Hamamatsu University School of Medicine (Hamamatsu, Japan) and was in accordance with the National Institutes of Health *Guide for the Use and Care of Laboratory Animals* (National Institutes of Health, Bethesda, MD).

Surgical preparation was conducted according to the method described previously.[17] The rabbits were anesthetized with intravenous sodium pentobarbital (25 mg/kg). Core body temperature was continuously monitored with a rectal probe and was maintained at 38.5 ± 0.5°C with the aid of a heating lamp. A 4-French balloon-tipped catheter (Goodtec, Inc., Huntington Beach, CA) was inserted through an arteriotomy in the left femoral artery and advanced 15 cm forward into the abdominal aorta. Preliminary investigations confirmed that the balloon should be positioned 0.5–1.2 cm distal to the left renal artery.[18] After systemic heparinization (200 mg/kg), spinal cord ischemia was induced by inflation of the balloon. Complete aortic occlusion was confirmed by reduction in distal aortic blood pressure to less than 20 mmHg, which was measured through the side hole of the balloon catheter. At the end of the operation, the catheter was removed and the femoral artery was reconstructed.

Preparation of HVJ-envelope Vector

Human HGF complementary DNA (2.2 kilobases) was inserted between the EcoRI and NotI sites of the pUC-Sr expression vector plasmid to produce an HGF expression plasmid.[19] HVJ-envelope vector was prepared according to the procedures recommended by the manufacturer (Ishihara Sangyo Kaisha, LTD., Osaka, Japan). In brief, HVJ-envelope (20 Assay Units) was mixed with 200 μg plasmid DNA and 0.3% Triton-X. After centrifugation, the mixture was washed with 1 ml balanced salt solution to remove the detergent and unincorporated DNA. Then the envelope vector was suspended in phosphate-buffered saline, and the suspension was stored at 4°C until use.

MSCs Culture

MSCs were isolated and cultured as described previously.[12] Bromodeoxyuridine (BrdU; 3 μg/ml [Sigma, St. Louis, MO]) was added into the medium 72 h before transplantation.

Gene Delivery to MSCs In Vitro

The cultured MSCs from the fifth to seventh passages were infected with the HVJ-envelope vector suspension by incubation for 1 h at a multiplicity of infection of 10. After infection, the envelope suspension was changed to normal culture medium for MSCs and the infected MSCs were continuously cultured for the subsequent 24 h before transplantation.

Intrathecal Injection

After anesthesia with pentobarbital, the intervertebral space between L5 and L6 was punctured with a 16-gauge needle, and polyethylene-10 tubing was inserted through it into the subarachnoid space. The desired position of the catheter was confirmed by cautious aspiration of cerebrospinal fluid (CSF). After intrathecal injection of either the MSCs or the vehicle, the catheter was removed. Then, the animals were placed head up for 60 min. The animals were included in the study only if they had a normal hind-limb motor function 2 days after intrathecal injection.[12]

Measurement of Human HGF Concentration

In vitro, approximately 2×10^5 MSCs were cultured in each well of a six-well dish. The MSCs were transferred with the human HGF gene by incubation with the HVJ-envelope vector at multiplicity of infection of 0, 1, 5, or 10 for 1 h. Then, the MSCs were cultured with normal medium for another 24 h. The culture supernatant was collected to measure the concentration of human HGF.

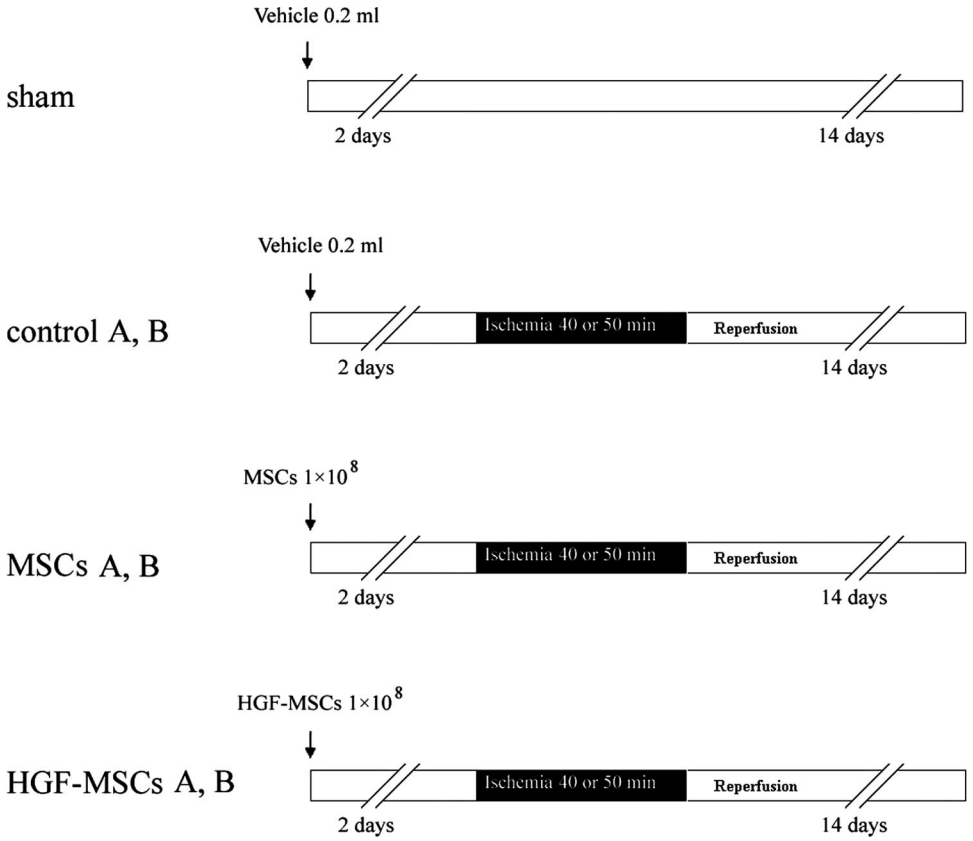

Fig. 1. Experimental groups and protocol. A = ischemia for 40 min; B = ischemia for 50 min; HGF = hepatocyte growth factor; HGF-MSCs = HGF gene-modified marrow stromal cells; MSCs = marrow stromal cells.

CSF (100 μl) was collected by means of intrathecal puncture before transplantation of MSCs and 2, 9, and 16 days after transplantation to measure the concentration of human HGF. The concentration of HGF was determined by means of enzyme immunoassay with an antihuman HGF antibody (Institute of Immunology, Tokyo, Japan) that reacts only with human HGF and not with rabbit HGF.[7,20]

Experimental Protocol

Rabbits were assigned to seven groups randomly by means of the random number table (n = 4 for the sham group; n = 8 for each of the other six groups), as shown in figure 1. Sham rabbits received the same surgical procedure without spinal cord ischemia. Two days before spinal cord ischemia, approximately 10^8 MSCs or human HGF gene-modified MSCs (HGF-MSCs) in a total fluid volume of 0.2 ml were intrathecally injected into each rabbit of groups of MSCs A, MSCs B, HGF-MSCs A, or HGF-MSCs B, whereas the vehicle alone of the same volume was injected into the two corresponding control groups. Groups of control A, MSCs A, and HGF-MSCs A were subjected to a 40-min spinal cord ischemia, and the ischemic duration was extended to 50 min in groups of control B, MSCs B, and HGF-MSCs B.

Neurologic Assessment

During a 14-day recovery after ischemia, hind-limb motor function was assessed by two blinded observers using the modified Tarlov scale: 0, no movement; 1, slight movement; 2, sit with assistance; 3, sit alone; 4, weak hop; and 5, normal hop.[21]

Histologic and Immunohistochemical Study

All animals were killed 14 days after the transient ischemia. Paraffin-embedded sections (4 μm) of lumbar spinal cords (L4–L6) were stained with hematoxylin and eosin. In cases in which the cytoplasm was diffusely eosinophilic, the large motor neuron cells were considered to be necrotic or dead. When the cells demonstrated basophilic stippling (containing Nissl substance), the motor-neuron cells were considered to be viable or alive.[22] The intact motor neurons in the ventral gray matter were counted by a blinded investigator in three sections for each rabbit, and the results were then averaged.

Immunohistochemical staining with the monoclonal antibody against BrdU (Lab Vision, Fremont, CA) was used to identify cells derived from MSCs as described previously.[23] The BrdU-positive cells in the ventral gray matter of the lumbar spinal cords were counted and averaged in three slides.

Statistical Analysis

Values were expressed as mean ± SD. Statistical analysis of the neurologic scores and the number of intact large motor neurons were performed with Kruskal-Wallis test. For parametric values, unpaired *t* test, a one- or a two-way repeated-measures (time and group) analysis of variance, as appropri-

Table 1. Physiologic Parameters

Parameter	Sham	A			B		
		Control	MSCs	HGF-MSCs	Control	MSCs	HGF-MSCs
Body weight, kg	2.2 ± 0.1	2.2 ± 0.2	2.1 ± 0.2	2.1 ± 0.1	2.2 ± 0.2	2.1 ± 0.2	2.2 ± 0.2
Proximal MAP, mmHg							
Baseline	89.5 ± 3.9	91.1 ± 2.5	89.3 ± 3.3	89.4 ± 3.5	92.0 ± 2.5	87.1 ± 1.2	92.5 ± 2.6
Ischemia 10 min	—	91.1 ± 2.0	89.3 ± 3.9	90.8 ± 2.3	91.8 ± 2.8	88.4 ± 2.5	91.5 ± 2.9
Ischemia 20 min	—	89.8 ± 1.3	91.8 ± 2.4	90.9 ± 2.9	91.0 ± 2.3	90.4 ± 1.8	91.5 ± 1.9
Ischemia 30 min	—	91.0 ± 1.7	91.5 ± 1.6	92.1 ± 2.0	91.4 ± 1.9	91.6 ± 1.6	91.0 ± 1.5
Ischemia 40 min	—	89.3 ± 1.3	91.3 ± 1.2	90.6 ± 1.9	90.0 ± 1.5	91.3 ± 1.5	90.8 ± 2.4
Ischemia 50 min	—	—	—	—	89.9 ± 1.1	90.6 ± 1.5	88.5 ± 2.3
Reperfusion 20 min	—	88.6 ± 3.1	89.6 ± 3.6	88.0 ± 2.0	89.9 ± 2.9	88.8 ± 2.1	87.9 ± 2.4
Distal MAP, mmHg							
Baseline	90.0 ± 2.9	90.1 ± 5.3	89.5 ± 1.9	89.9 ± 2.2	92.4 ± 2.7	90.4 ± 1.9	89.6 ± 2.6
Ischemia 10 min	—	14.8 ± 1.8*	15.0 ± 1.3*	15.3 ± 1.7*	15.4 ± 1.8*	15.3 ± 1.8*	15.8 ± 1.5*
Ischemia 20 min	—	15.5 ± 1.6*	14.8 ± 1.5*	15.9 ± 1.2*	15.1 ± 1.5*	15.4 ± 1.3*	15.0 ± 1.3*
Ischemia 30 min	—	15.3 ± 1.0*	15.5 ± 1.5*	15.0 ± 1.6*	15.1 ± 1.1*	15.3 ± 1.0*	14.8 ± 1.3*
Ischemia 40 min	—	14.8 ± 1.0*	15.0 ± 1.3*	15.3 ± 1.3*	15.8 ± 1.3*	14.8 ± 1.3*	15.1 ± 1.2*
Ischemia 50 min	—	—	—	—	14.4 ± 1.1*	15.4 ± 1.1*	15.1 ± 1.1*
Reperfusion 20 min	—	87.9 ± 2.2	88.8 ± 3.9	87.0 ± 1.3	89.5 ± 3.7	86.6 ± 2.1	86.9 ± 1.7
Heart rate, beats/min							
Baseline	246.3 ± 5.6	248.9 ± 8.7	242.9 ± 7.9	246.8 ± 4.7	247.6 ± 13.8	243.3 ± 9.7	243.4 ± 9.1
Ischemia 10 min	—	249.8 ± 9.8	240.9 ± 5.1	248.5 ± 11.6	247.0 ± 8.0	238.8 ± 5.2	247.4 ± 6.1
Ischemia 20 min	—	243.6 ± 10.3	249.4 ± 7.7	251.3 ± 12.3	251.0 ± 15.0	252.4 ± 8.7	248.1 ± 10.6
Ischemia 30 min	—	246.3 ± 8.6	249.8 ± 12.7	247.6 ± 12.9	247.9 ± 6.6	245.1 ± 6.7	241.8 ± 11.1
Ischemia 40 min	—	246.4 ± 12.4	253.6 ± 6.7	242.9 ± 6.0	245.9 ± 10.0	240.6 ± 8.7	250.0 ± 15.1
Ischemia 50 min	—	—	—	—	244.5 ± 10.3	241.4 ± 6.3	249.8 ± 11.0
Reperfusion 20 min	—	246.8 ± 8.8	243.5 ± 9.8	249.3 ± 8.3	253.8 ± 8.9	243.8 ± 5.4	252.5 ± 8.2
Rectal temperature, °C							
Baseline	38.4 ± 0.3	38.4 ± 0.2	38.6 ± 0.5	38.6 ± 0.3	38.7 ± 0.1	38.7 ± 0.2	38.4 ± 0.5
Ischemia 10 min	—	38.8 ± 0.3	38.5 ± 0.4	38.2 ± 0.4	38.7 ± 0.3	38.5 ± 0.3	38.5 ± 0.5
Ischemia 20 min	—	38.6 ± 0.2	38.5 ± 0.2	38.7 ± 0.2	38.7 ± 0.3	38.6 ± 0.2	38.5 ± 0.2
Ischemia 30 min	—	38.6 ± 0.4	38.5 ± 0.3	38.6 ± 0.2	38.4 ± 0.1	38.5 ± 0.3	38.3 ± 0.2
Ischemia 40 min	—	38.6 ± 0.4	38.3 ± 0.3	38.6 ± 0.2	38.4 ± 0.4	38.2 ± 0.4	38.4 ± 0.3
Ischemia 50 min	—	—	—	—	38.4 ± 0.2	38.6 ± 0.3	38.6 ± 0.2
Reperfusion 20 min	—	38.8 ± 0.3	38.7 ± 0.2	38.4 ± 0.3	38.5 ± 0.4	38.6 ± 0.5	38.7 ± 0.2

Values are given as mean ± SD.

* $P < 0.01$ compared with baseline.

A = ischemia for 40 min; B = ischemia for 50 min; HGF = hepatocyte growth factor; HGF-MSCs = HGF gene-modified marrow stromal cells; MAP = mean arterial pressure; MSCs = marrow stromal cells.

ate, followed by unpaired Student *t* tests with the Bonferroni correction were used. All statistical analysis was performed using SPSS version 11.5 (SPSS, Inc., Chicago, IL). A two-sided *P* value of less than 0.05 was considered statistically significant.

Results

Physiologic Parameters

Table 1 indicates the physiologic parameters from all the groups. The mean blood pressure of distal aorta of all the groups except the sham group was significantly decreased during the aortic occlusion ($P < 0.01$ *vs.* baseline, respectively). There were no significant differences in body weight ($F = 0.552$, $P = 0.766$), blood pressure, or heart rate or rectal temperature among the groups at any time point ($P > 0.05$, respectively).

Concentration of Human HGF

After MSCs were infected with HVJ-envelope containing human HGF plasmid, human HGF was detected in the culture supernatant. The concentration of human HGF in-

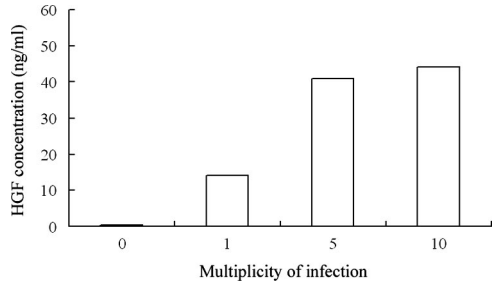

Fig. 2. Human HGF concentration in the supernatant of cultured MSCs 24 h after infection with HVJ-envelope containing human HGF plasmid. HGF = hepatocyte growth factor; HVJ = hemagglutinating virus of Japan; MSCs = marrow stromal cells.

creased with the increase of multiplicity of infection, as shown in figure 2.

Human HGF protein was readily detected in CSF from 2 to 16 days after transplantation of HGF gene-modified MSCs, but not before transplantation, as shown in figure 3. No significant difference of the concentration of human HGF was found between groups of HGF-MSCs A and HGF-MSCs B at any observation time point ($t = 0.155$, $P = 0.879$; $t = 0.576$, $P = 0.574$; $t = 0.882$, $P = 0.393$; respectively). However, no such expression could be detected in control animals or animals that received transplantation of MSCs only at any defined time point.

Migration of Transplanted Cells

In vitro, more than 90% of the cultured MSCs or HGF-MSCs showed BrdU reactivity. Within the spinal cord, transplanted MSCs or HGF-MSCs were identified by BrdU immunoreactivity and characterized by round-to-oval dark brown nuclei. Immunohistochemical staining at 14 days after transient ischemia revealed that many transplanted MSCs or HGF-MSCs still survived and integrated into the host gray matter in groups of MSCs A, MSCs B, HGF-MSCs A, and HGF-MSCs B. No BrdU-positive cells were detected in spinal cords of the rabbits in the two control groups. Representative photographs of sections of immunohistochemical staining are shown in figure 4A. There was no significant difference in the BrdU-positive cell count among the groups of MSCs A, HGF-MSCs A, MSCs B, and HGF-MSCs B (chi-square = 1.335, $P = 0.721$), as shown in figure 4B.

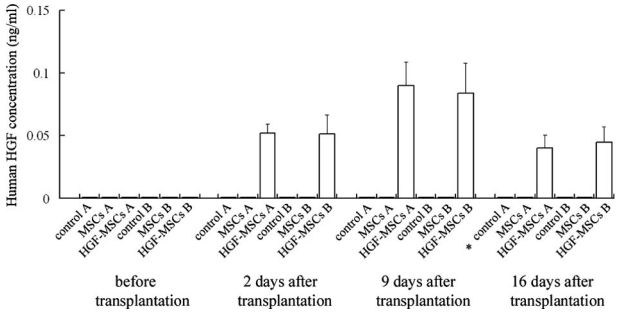

Fig. 3. Concentration of human HGF protein in cerebrospinal fluid before and after transplantation of MSCs or HGF gene-modified MSCs. No significant difference of the concentration of human HGF was found between groups of HGF-MSCs A and HGF-MSCs B at any observation time point ($P = 0.879$, $P = 0.574$, and $P = 0.393$, respectively). A = ischemia for 40 min; B = ischemia for 50 min; HGF = hepatocyte growth factor; HGF-MSCs = HGF gene-modified marrow stromal cells; MSCs = marrow stromal cells.

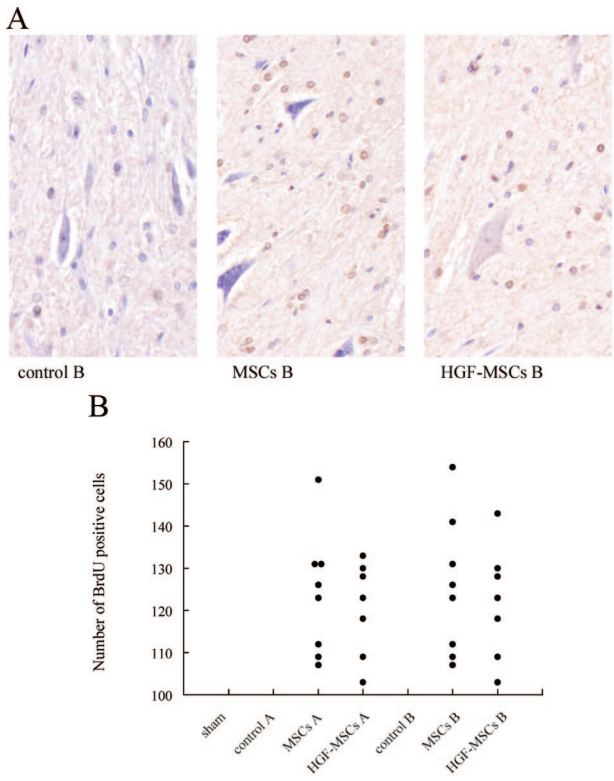

Fig. 4. Micrographs of lumbar spinal cords 14 days after spinal cord ischemia show morphologic characteristics of transplanted MSCs or HGF gene-modified MSCs. (*A*) With immunoperoxidase staining with diaminobenzidine and counterstaining with hematoxylin, bromodeoxyuridine (BrdU) reactivity is present in the nuclei of donor cells (brown, original magnification, 200×). (*B*) Number of BrdU-positive cells in the ventral gray matter. No BrdU-positive cells can be detected in the spinal cords of sham and control animals. There was no significant difference in the BrdU-positive cells count among the groups of MSCs A, HGF-MSCs A, MSCs B, and HGF-MSCs B (chi-square = 1.335, $P = 0.721$). A = ischemia for 40 min; B = ischemia for 50 min; HGF = hepatocyte growth factor; HGF-MSCs = HGF gene-modified marrow stromal cells; MSCs = marrow stromal cells.

Neurologic Assessment

The individual neurologic scores of the 7 groups 1, 2, 7, and 14 days after the transient spinal cord ischemia are shown in figure 5. The sham animals retained normal motor function of lower limbs (Tarlov score = 5) throughout the observation period. A 40-min aortic occlusion resulted in severe lower extremity neurologic deficits in the rabbits of group control A, whereas transplantation of MSCs or HGF-MSCs remarkably enhanced the motor function of the lower limbs

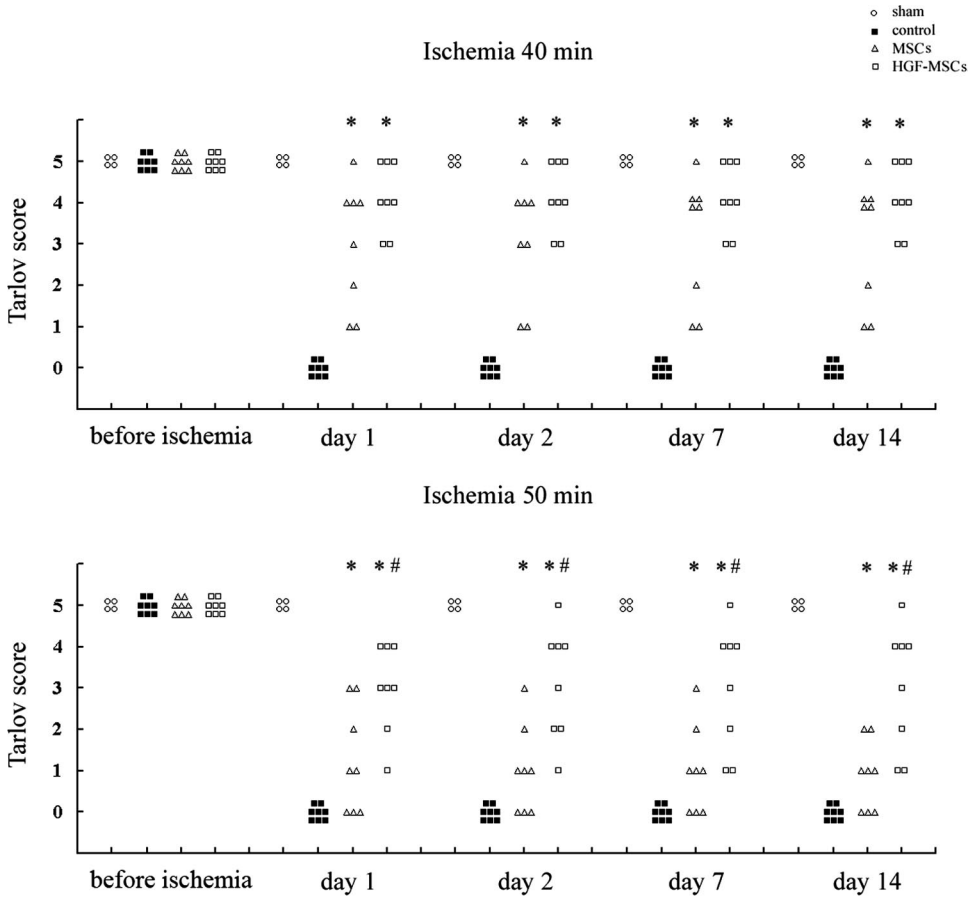

Fig. 5. Neurologic function assessed just before spinal cord ischemia and 1, 2, 7, and 14 days after ischemia. HGF = hepatocyte growth factor; HGF-MSCs = HGF gene-modified marrow stromal cells; MSCs = marrow stromal cells. * $P < 0.01$, compared with control group; # $P < 0.05$, compared with MSCs group.

after spinal cord ischemia, as indicated by the significantly higher Tarlov scores of groups of MSCs A ($P = 0.0003$, 0.0003, 0.0003, and 0.0003, *vs.* group control A, at the four time points, respectively) and HGF-MSCs A ($P = 0.0003$, 0.0003, 0.0003, and 0.0003, *vs.* group control A, at the four time points, respectively). No statistical significance of Tarlov scores was observed between the MSCs A group and HGF-MSCs A group at the four observation time points ($P = 0.127$, 0.154, 0.203, and 0.203, respectively). When the ischemic time was extended to 50 min, significantly higher Tarlov scores were still found in rabbits receiving transplantation of either MSCs ($P = 0.01$, 0.01, 0.01, and 0.01 *vs.* group control B, at the four time points, respectively) or HGF-MSCs ($P = 0.0003$, 0.0003, 0.0003, and 0.0003 *vs.* group control B, at the four time points, respectively). Compared with group MSCs B, much better neurologic function was detected in rabbits of group HGF-MSCs B at any defined time point ($P = 0.015$, 0.008, 0.013, and 0.01, respectively).

Histologic Assessment

Representative sections of lumbar spinal cords stained with hematoxylin-eosin are shown in figure 6A, and the results of counting viable motor neurons are summarized in figure 6B. In the sham-operated animals, the spinal cord was intact, and many large motor neurons were present in the anterior horn. Severe neurologic damage was readily detected in animals of both group control A and group control B 14 days after spinal cord ischemia, as evidenced by vacuolization, frank necrosis, and an almost total loss of motor neurons. There was no significant difference in the intact motor neuron count between the two control groups ($P = 0.161$). In contrast, slighter histologic changes were found in lumbar spinal cords of animals in groups of MSCs A, HGF-MSCs A, MSCs B, and HGF-MSCs B, and the intact motor neurons were preserved to a much greater extent ($P = 0.001$, group MSCs A *vs.* group control A; $P = 0.001$, group HGF-MSCs A *vs.* group control A; $P = 0.001$, group MSCs B *vs.* group control B and $P = 0.001$, group HGF-MSCs B *vs.* group control B). Prolonged spinal cord ischemia of 50 min induced a significant decrease of the intact motor neurons in group MSCs B compared with MSCs A ($P = 0.008$), and a remarkable difference of the intact motor neurons was found between group MSCs B and group HGF-MSCs B ($P = 0.02$).

Discussion

The salient findings of the current study can be summarized as follows: (1) HVJ-envelope vector successfully transferred

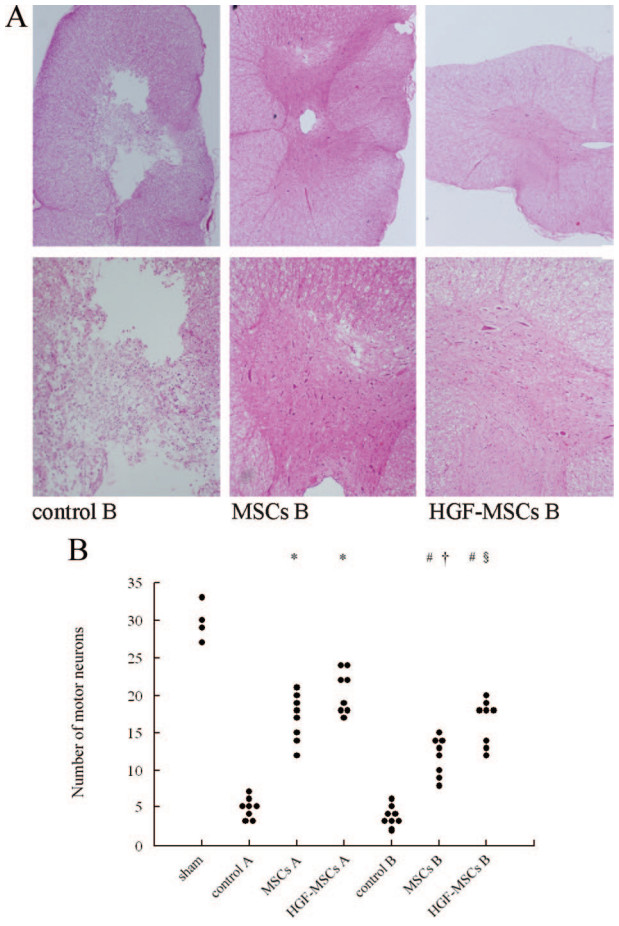

Fig. 6. Histologic assessment of the spinal cord 14 days after transient ischemia. (*A*) Representative sections of lumbar spinal cords stained with hematoxylin and eosin of low magnification (40×, top) and high magnification (100×, bottom). The specimen from a control animal exhibits severe neuronal damage as evidenced by vacuolization and frank necrosis, and almost all motor neurons are lost, whereas animals that received MSCs or HGF-MSCs show minimal evidence of cellular damage. (*B*) Number of large motor neurons in the ventral gray matter. A = ischemia for 40 min; B = ischemia for 50 min; HGF = hepatocyte growth factor; HGF-MSCs = HGF gene-modified marrow stromal cells; MSCs = marrow stromal cells. * $P < 0.01$, compared with control A; # $P < 0.01$, compared with control B; § $P < 0.05$, compared with MSCs B; † $P < 0.01$, compared with MSCs A.

human HGF gene to the cultured MSCs with high efficiency *in vitro*, and the transplanted gene-modified MSCs successfully functioned to express and maintain a high level of human HGF *in vivo*; (2) prophylactic intrathecal injection of HGF gene-modified MSCs significantly attenuated the neurologic injury after transient spinal cord ischemia; and (3) transplantation of HGF gene-modified MSCs induced more efficient neuroprotection than transplantation of MSCs only in a prolonged ischemia model of spinal cords.

An efficient and minimally invasive vector system is the "bottle neck" of gene transfer. Numerous viral and nonviral (synthetic) delivery systems have been developed and improved. HVJ envelope is a purified vesicle prepared from HVJ, the replication activity and infectivity of which have been completely inhibited, whereas the activities of the two envelope proteins (hemagglutinating neuroaminidase and fusion protein) are maintained almost at the same levels as those of live HVJ.[24] HVJ-envelope vector is a novel and unique system that combined the advantages of viral and nonviral vectors with the following features and advantages: (1) Safe and easy as a "nonviral" transfection reagent; (2) delivery of various molecules, including plasmid DNA, small interfering RNA, protein, and antisense oligonucleotide; and (3) wide usability from *in vitro* to *in vivo*.[25] In the present study, human HGF was readily detected in both the culture medium of HGF gene-modified MSCs and the CSF from 2 to 16 days after intrathecal injection of HGF gene-modified MSCs, which showed that HVJ-envelope vector had successfully transferred the gene of interest to the cultured MSCs with high efficiency *in vitro*, and the gene-modified MSCs had successfully functioned *in vivo* to express and maintain a high level of the gene of interest.

To detect the neuroprotective efficiency of a combined therapy, human HGF gene was transferred into MSCs *in vitro* and the gene-modified MSCs were transplanted 2 days before spinal cord ischemia in the current study. The results demonstrated that transplantation of HGF gene-modified MSCs significantly attenuated the neurologic injury induced by spinal cord ischemia. In rabbits, irreversible neurologic injury can be induced, even if the period of spinal cord ischemia is as short as 20 min.[11,26] With the extension of ischemic time, more severe neurologic injuries will occur. In the present study, prolonged ischemic periods of 40 and 50 min were enrolled, and significant improvement of neurologic deficits was still detected after transplantation of either MSCs alone or HGF gene-modified MSCs. The most important finding was that transplantation of HGF gene-modified MSCs attenuated the neurologic injury to a much greater extent compared with transplantation of MSCs alone when the ischemic period was extended to 50 min. These data indicate that transplantation of HGF gene-modified MSCs may be a more effective strategy to protect spinal cords than transplantation of MSCs only.

Transplantation of MSCs is presently believed to be an effective way to repair central nervous system injuries and is given more and more considerations. It has been demonstrated that transplantation of MSCs into the brain reduced functional deficits and lesion size associated with cerebral ischemia.[10,11] Improved functional recovery has also been reported after transplantation of MSCs into spinal cords affected by contusion and hemisection injury.[27] In our previous study, intrathecal injection of MSCs 2 days previously significantly attenuated neurologic injuries caused by spinal cord ischemia.[11] In the current study, the neuroprotective effects of MSCs on spinal cords were further confirmed, even when the ischemic time was extended to as long as 50 min. The neuroprotective effects of MSCs may be derived from the production of trophic factors and cytokines,

rather than the replacement or integration of MSCs into the spinal cord.

It has been demonstrated that HGF functions as a powerful angiogenetic factor, as well as a potent neurotrophic factor, and induces versatile neuroprotective effects both *in vivo* and *in vitro*. In cerebral artery occlusion models, HGF has been reported to induce therapeutic angiogenesis, improve blood flow, reduce the infarct volume, and attenuate neurologic injuries,[20,28] which indicates that HGF possesses a potential therapeutic value for central nervous system ischemia. Overexpression of HGF also reduced destruction of the blood-brain barrier without exacerbating cerebral edema in the ischemic brain.[20] Our previous study showed that transfer of the human HGF gene was a powerful neuroprotective method to attenuate ischemia-reperfusion injury of spinal cords. Compared with the control animals, HGF gene transfer significantly improved hind-limb motor function, protected motor neurons, increased the capillary density in the gray matter, and decreased the spinal cord edema.[7] Therefore, it is plausible that the combined therapy of transplantation of HGF gene-modified MSCs induced more powerful neuroprotective effects on spinal cords against ischemia-reperfusion injury than transplantation of MSCs only in the current study. HGF gene-transferred MSCs were intracerebrally transplanted into the rats' ischemic brains, and that combined therapy was consistently shown to be more therapeutically efficient than MSC therapy alone. The combined therapy extended the therapeutic time window from superacute to acute phase.[16]

In the current study, human HGF was detected in CSF and the BrdU-positive cells were identified in spinal cords at least 16 days after transplantation of HGF gene-modified MSCs, indicating that the transplanted gene-modified MSCs functioned and survived throughout the observation period. Although MSCs have been found to survive and express neuronal phenotypic proteins after transplantation into the brain, the engrafted MSCs did not contribute to reestablish normal tissue cytoarchitecture after cerebral ischemia. No clear evidence showed that MSCs differentiated into neurons and developed contacts with other neurons.[18] After ischemia, the nervous tissue reverts to an earlier stage of development and thus becomes highly responsive to stimulation by cytokines, trophins, and growth factors from the invading MSCs.[29] The MSCs may simply serve as the resources of such cytokines, trophins, and growth factors to protect the nervous tissue and stimulate its remodeling. A far more reasonable explanation for the benefit is that MSCs induce cerebral tissue to activate endogenous restorative effects of the brain. In the present study, HGF gene-modified MSCs may also be the resources of not only the extended and abundant exogenous HGF, but also an array of other cytokines and trophic factors over an extended period, which interacted with each other in an anatomically distributed, tissue-sensitive, and temporally ongoing way. All these cytokines and trophic factors may directly protect the neurons against ischemia-reperfusion injury and directly involve promoting plasticity of the ischemic-damaged neurons or stimulating glial cells to secrete neurophins.

The primary limitation of the current study was that the neuroprotective mechanisms of the HGF gene-modified MSCs and the fate of the transplanted cells in the host were not investigated in detail. Although paracrine of implanted MSCs may be the important mechanism for its therapeutic effect, the possible differences of paracrine function between MSCs and HGF-MSCs need to be measured in the further study.

Spinal cord ischemia discussed in the present study is a complication of surgical intervention, and the exact time of its occurrence can be known. Therefore, precautionary measures to protect the spinal cord are necessary and feasible. We have tried a novel prophylactic strategy to prevent spinal cord injury in the current study, and the results demonstrate for the first time that transplantation of HGF gene-modified MSCs induces powerful neuroprotection on spinal cords against ischemia-reperfusion injury and transplantation of HGF gene-modified MSCs is more neuroprotectively efficient than transplantation of MSCs alone. Compared with embryonic stem cells and neural stem cells, MSCs can be readily harvested and expanded *ex vivo*. Moreover, autologous transplantation of MSCs would circumvent potential ethical and immune rejection considerations. Intrathecal injection can be readily performed as a promising way without severe invasion to transplant MSCs into the spinal cord. In addition, transplantation of gene-modified MSCs may be safer than direct gene transfer for *in vivo* treatment. We believe that prophylactic intrathecal injection of HGF gene-modified MSCs possesses a potential clinical value in the prevention of neurologic injury after thoracic aneurysm surgery.

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