Effect of Mesenchymal Stem Cell Transplantation on Cognitive Functions in Rats with Ischemic Stroke

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Translated from *Kletochnye Tekhnologii v Biologii i Medicine*, No. 4, pp. 202-205, December, 2006
Original article submitted October 10, 2006

The effect of intravenous transplantation of mesenchymal stem cells on the recovery of cognitive functions was studied in Wistar—Kyoto rats after brain stroke induced by occlusion of the middle cerebral artery in the left hemisphere. Analysis 2 and 5 weeks after stroke showed that transplantation of mesenchymal stem cells 3 days after middle cerebral artery occlusion reduced the area of cerebral injury, preserved cognitive functions, and decreased mortality in experimental animals.

Key Words: *mesenchymal stem cells; ischemic stroke; cognitive functions*

Cell therapy is a new actively investigated method for the treatment of ischemic stroke. We consider that the most promising material for cell therapy are mesenchymal stem cells (MSC), pluripotent cells capable of differentiating in the osteogenic, chondrogenic, adipocytic, myocytic, cardiomyocytic, neuronal and glial directions [4-6,9-12]. Methods for MSC isolation from human and animal bone marrow, their culturing and reproduction *in vitro* to the needed quantity with retention of their properties were developed and tried, which made possible to use autologous material for cell therapy and to rule out the problems of graft-host immune compatibility. It was experimentally shown that intravenous transplantation of MSC to animals after occlusion of the middle cerebral artery (MCA) inhibited apoptosis of neurons in tissue adjacent to the necrotic focus [2,3] and activated angiogenesis [13]. A significant increase in the surface area of the vascular network in the penumbra was noted [1,3].

We studied the effect of intravenous transplantation of MSC on the cognitive functions of animals after ischemic stroke.

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MATERIALS AND METHODS

Experiments were carried out on 3-4-month-old male Wistar—Kyoto rats (150-170 g; n=37).

Bone marrow suspension was isolated from the femoral bones directly after decapitation. The femoral bone epiphyses were removed under sterile conditions, the diaphyses were washed in culture medium (αMEM, HyClone) with 20% fetal calf serum (Gibco) and 100 μg/ml penicillin or streptomycin (Gibco). The resultant suspension was inoculated in plastic Petri dishes (Sarstedt). Forty-eight hours after bone marrow explantation MSC were washed twice from blood cells in PBS (20 mM PBS, pH 7.4; 0.1 M NaCl). The cells were cultured in a monolayer at 37°C and 5% CO_2 for 6-7 days after explantation, after which the culture was reinoculated every 7 days at an initial density of 1.27×10^3 cell/cm^2. MSC were reinoculated using trypsin and EDTA (HyClone). Nutrient medium was replaced every 3 days.

Rat MSC phenotyping was carried out by flow cytofluorometry on a FACSscan cytofluorometer (Becton Dickinson). MSC were stained with antibodies to negative marker CD45 (Becton Dickinson) and positive marker CD90 (Becton Dickinson). In order to stain MSC with antibodies to sur-

face markers, the cells were removed from dishes with trypsin and EDTA (HyClone), washed twice in PBS (20 mM phosphate buffer, pH 7.4; 0.1 M NaCl), transferred for 1 h into solution of fluorochrome-conjugated monoclonal antibodies (1:20), washed twice in PBS, and fluorescence intensity was measured. Phenotyping was carried out after the first, second, and third reinoculation of the culture.

Experimental ischemic stroke was induced by MCA occlusion. The rats were intraperitoneally narcotized with ketamine (125 mg/kg). During surgery until the end of narcosis body temperature was maintained at 37°C. Animal head was fixed in a stereotaxic device with ear cores and dental clamps. The skin on the left side from the lateral end of the orbit to the auricle was cut and the edges of the wound were pulled apart; this manipulation exposes the oval foramen of the trigeminal nerve, which was extended (4×4 mm) with a dental drill under the control of an operation microscope. The dura mater was opened, MCA was separated with a fine steel hook fixed in a micromanipulator, and electrocoagulation of 2-3 mm of the artery was carried out, after which the operation wound was sutured layer-by-layer.

The animals were divided into 3 groups. Group 1 were sham-operated rats (n=10); in these animals the trigeminal nerve foramen was extended, but no electrocoagulation was carried out. In group 2 (control; n=13) stroke was induced, but no therapy was carried out. In group 3 animals (cell therapy; n=15), MSC transplantation was carried out 3 days after MCA occlusion (5 million cells in 100 μl αMEM were injected into the caudal vein). All animals were decapitated on day 42 after MCA occlusion.

The animals were perfused with 4% paraformaldehyde in PBS through the left heart ventricle. Directly after decapitation the brain was removed and a segment including the visible zone of injury and intact marginal zones was resected. The resected block was routinely fixed in paraformaldehyde.

Histological analysis of the material collected from 7 animals of each group was carried out. Necrotic area in the left hemisphere and damaged structures were evaluated. Brain structures were identified as described earlier [8].

Two and five weeks after MCA occlusion the animals were tested in Morris water maze for 6 days by a modified method [7]. This test characterizes the animal capacity to spatial orientation. The rat capacity to find the platform hidden under the water by the exterior check-points was evaluated. A basin with plastic walls 145 cm in diameter and 50 cm deep was placed on a tray at the height of 50 cm above the floor in the center of the room. Check-points for animals (blue and green rectangles) remained on the walls during the entire period of training. The room was illuminated diffusely with two 250-W lamps. The basin was filled with water (24±1°C) to a height of 25 cm.

The animals were trained before testing. During 6 days each rat was placed in the basin and the time of searching the platform was recorded. If the animal failed to find the platform over 120 sec, the experimenter placed the rat on it. The animal stayed on the platform for 30 sec. Each rat performed 4 attempts from different starting points daily. The swimming trajectory was videotaped.

RESULTS

Flow cytofluorometry showed that cell culture prepared for transplantation consisted of 5-8% $CD45^+$ cells (hemopoietic cells) and 90-95% $CD90^+$ cells (MSC proper).

Transplantation of MSC after MCA occlusion 1.7-fold increased animal survival (Table 1) and considerably modulated animal behavior in Morris water maze (Figs. 1, 2). Group 3 animals retained learning capacity after MCA occlusion: the time of searching the platform during the first training session (2 weeks after MCA occlusion) decreased from day 1 to day 6. Controls (group 2) spent several-fold more time for finding the platform than animals receiving cell therapy (group 3). By day 5 of training (session 1), group 3 rats developed an optimal strategy of searching the platform and effectively used it 2 weeks later during session 2. Group 2 animals attained learning criterion only by day 5 of test session 2. These data suggest that MSC trans-

TABLE 1. Number of Animals Dead during the Study in Different Groups

Group	Number of animals	Total mortality, %	Number of animals included in analysis
Sham-operation (1)	10	0	10
Control (2)	20	50	10
Cell therapy (3)	15	30	10

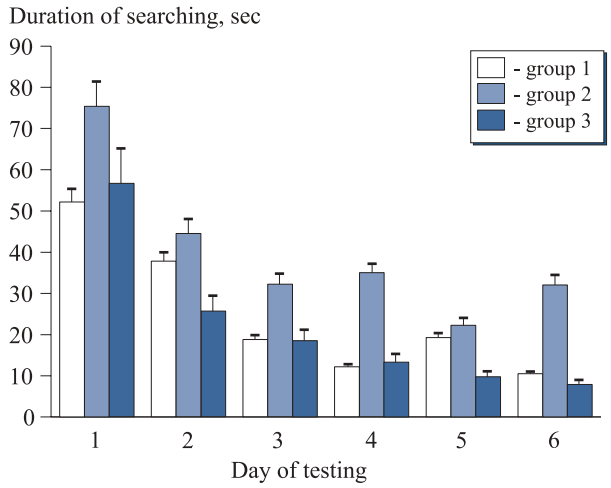

Fig. 1. Testing in Morris water maze 2 weeks after MCA occlusion.

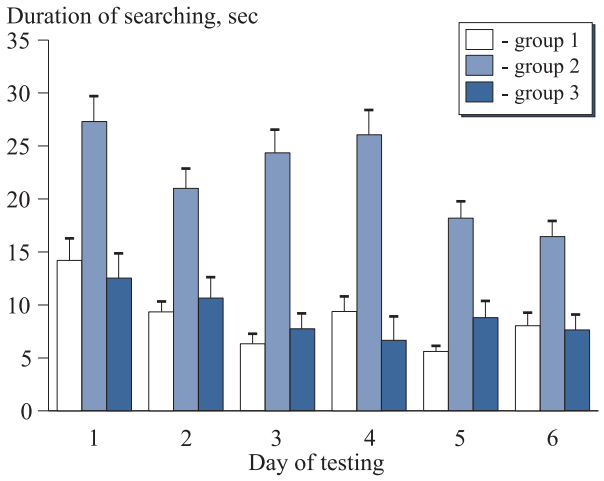

Fig. 2. Testing in Morris water maze 5 weeks after MCA occlusion.

plantation produced a positive impact on cognitive functions and preserved learning capacity in experimental animals at the level of sham-operated rats.

It seems that MSC transplantation after MCA occlusion reduced in the area of brain tissue defect and preserv protected brain structures. This was shown by histological studies of the brain preparations from animals tested in Morris water maze (Fig. 3). Adhesions of the pia mater and slight impairment of the neocortical surface caused by tissue

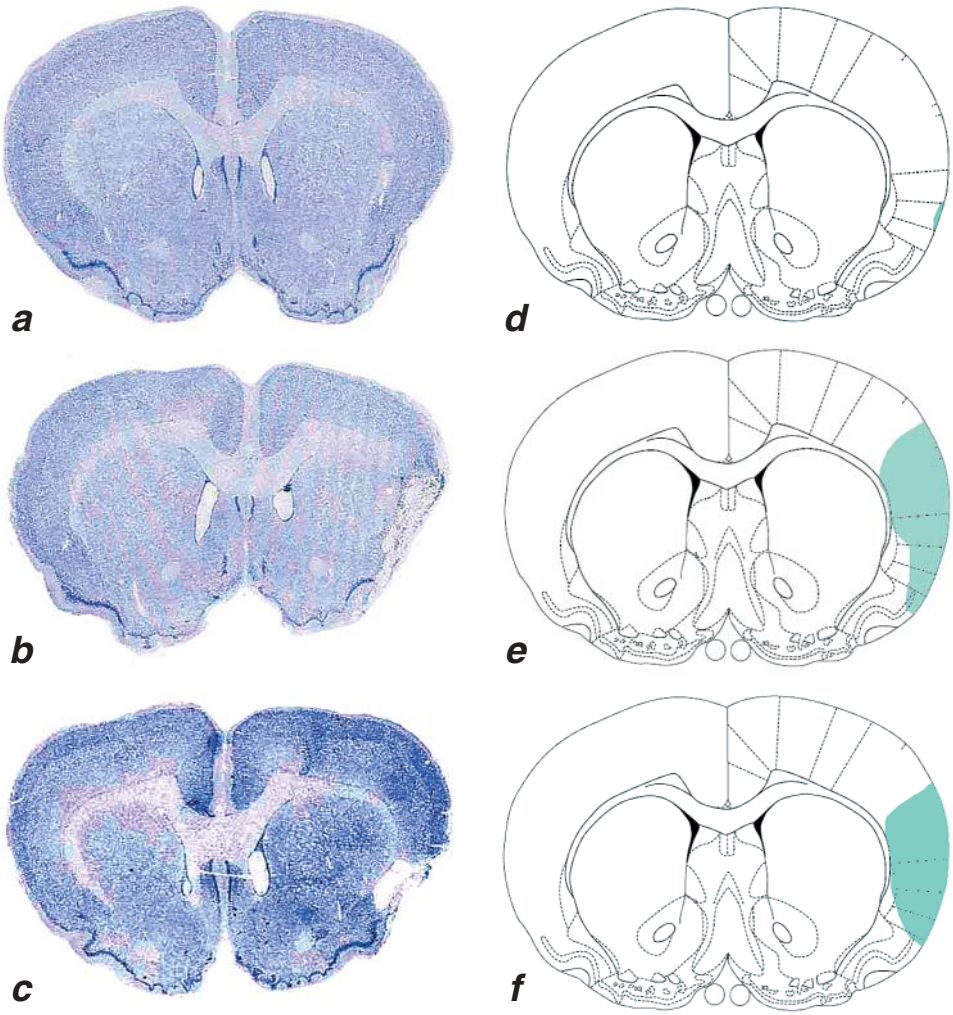

Fig. 3. Cerebral tissue defect 6 weeks after MCA occlusion. *a*, *b*, *c*) histological preparations, Nissl staining; *d*, *e*, *f*) schemes of the same brain sections. *a*, *d*) group 1 (sham-operated animals); *b*, *e*) group 2 (control); *c*, *f*) group 3 (MSC transplantation).

compression during the intervention were detected in sham-operated animals (group 1; Fig. 3, *a*, *d*).

Significant defects of the neocortex and external capsule of the brain were detected in group 2 animals (control). About one-third of the primary and secondary somatosensory and more than half of the insular and periform cortex were lacking. Dying cerebral neurons were detected in an area reaching the lateral ventricular wall. Gliosis developed in these cerebral areas. The caudate nucleus was partially damaged. Morphologically intact neurons were detected only at an appreciable distance from the interface of damaged area (Fig. 3, *b*, *e*).

The neocortical defect in group 3 animals (cell therapy) was significantly less pronounced and confined to a small part of the somatosensory and insular cortex. Zones with intact or little changed neurons were detected in the neocortex at the interface with tissue defect, which was not seen in group 2 animals. Nerve cells, though morphologically changed (hyperchromatic), were seen at the very interface with the cerebral tissue defect. A well-formed capsule was detected at the interface of the marginal defect: it consisted of 3-6 cell layers (astrocytes, meningocytes, fibroblasts). Caudate nuclei neurons were preserved. Numerous vessels were seen near the the defect boundary in all structures (Fig. 3, *c*, *f*).

Hence, we showed that MSC can serve as neuroprotectors maintaining neuronal viability in the penumbra and promoting recovery of cognitive functions after ischemic stroke. It was experimentally proven that transplantation of MSC activated angiogenesis in ischemic zones of the brain. Hence, in parallel with the neuroprotective effect, MSC restore blood circulation in tissue damaged after stroke.

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