Use of Bone Marrow Mesenchymal (Stromal) Stem Cells in Experimental Ischemic Stroke in Rats

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The effects of human mesenchymal stem cells on neurological functions and behavioral reactions of animals and on damaged brain tissue were studied on the model of focal cerebral ischemia in rats. Homing and differentiation of transplanted mesenchymal stem cells were also studied. Significant regression of neurological disorders after cell transplantation was noted, no appreciable shifts were detected by magnetic resonance tomography. Homing of transplanted cells was detected mainly in the zone of focal ischemia. Some cells died, others exhibited signs of differentiation into neurons and glia.

Key Words: *mesenchymal stem cells; focal ischemia; experimental stroke*

Cell transplantation technologies, aimed at substitution of lost nervous tissue with donor cells or at stimulation of its regeneration, are among the perspective approaches to the treatment of aftereffects of ischemic stroke. However, the potentialities of cell technologies in the treatment of ischemic stroke are poorly studied [5]. Numerous data on the possibility of using stem cells (SC) of different origin for the therapy of ischemia of highly specialized tissues prompted this research.

The history of clinical application of SC for the treatment of stroke started in 1998, when physicians of Petersburg University in the USA transplanted neural cells isolated from teratocarcinoma strain (NT2N) with predominating neural SC into the brain of patients with a history of stroke [6]. This study was carried out in patients with stable motor deficiency resultant from stroke, which occurred from 6 months to 6 years before transplantation; the age of patients was 40-75 years. No malignant transformation of transplanted cells was noted during the observation; a tendency to reduction of motor deficiency was noted.

The therapeutic effects of umbilical blood (UB) transplantation were studied on several models of stroke. One of the early studies showed that intravenous transplantation of hemopoietic $CD34^+$ cells of UB (77-95% in the transplant) to rats with experimental ischemic stroke led to significant regression of neurological deficit. The cells migrated into the brain, mainly into the focus of lesions, and underwent differentiation into neurons and glial cells ($GFAP^+$, $NeuN^+$, MAP^{2+}) [7]. Another study demonstrated the efficiency of $CD34^+$ cells isolated from UB in the treatment of ischemic stroke in immunodeficient mice [12]. Comparative analysis of intravenous and intrastrial transplantations of UB cells to immunosuppressed rats with experimental ischemic stroke was carried out [13]. The results of some functional tests indicated that intravenous injection of cells was therapeutically even more effective than intrastrial transplantation [13].

The study of the effects of transplantation of human UB mononuclear cells on the model of ischemic stroke in rats demonstrated significant dose-dependent improvement of behavioral reactions and

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motor activity of animals 2 and 4 weeks after transplantation. Injection of UB cells in doses of 1 and 10 mln was maximally effective. The injected cells were detected in the brain in the zone adjacent to the stroke focus on the ipsilateral side. Cell transplantation notably reduced the focus of lesions [9].

The therapeutic potential of mesenchymal SC (MSC) was tested by injecting adult bone marrow non-hemopoietic cells labeled with BrdU into the striatum after occlusion of the left middle cerebral artery (LMCA). The mice were sacrificed 28 days after stroke. The BrdU-reactive cells survived and migrated to a distance of about 2.2 mm from the site of transplantation towards the ischemic focus. NeuN and GFAP were expressed in 1 and 8% BrdU-stained cells, respectively. Though the size of infarction zone in experimental animals was similar to that in the controls, functional recovery was better [10].

In one of the studies human MSC were intravenously injected to rats 1 day after LMCA occlusion (LMCAO); the transplantation significantly improved the functional outcome of stroke, evaluated by the somatosensory functions and modified neurological severity score (mNSS), in comparison with rats from the reference group [8]. However, only 1-2% human MSC expressed neurogenic, astrocytic, and endothelial proteins. The absence of complete cell differentiation and integration in tissue was attributed to a short period between the injection and analysis of the results.

Cell therapy in diseases of the central nervous system can be a promising therapeutic strategy, and somatic SC serve as a potential source of material for replacement therapy or as carriers of neurotrophic substances [1,4].

The use of bone marrow MSC is a promising trend [11].

The aim of this research is comprehensive evaluation of MSC effects on the model of ischemic stroke in rats. We studied the effects of MSC on the size of ischemic focus and severity of neurological deficit in rats with experimental ischemic stroke and investigated the routes of MSC migration into the brain of rats with ischemic stroke after intravenous injection of these cells.

MATERIALS AND METHODS

Experiments were carried out on male Wistar rats initially weighing 260-300 g from Stolbovaya Breeding Center of Russian Academy of Medical Sciences. The animals were kept under standard vivarium conditions: 12:12 light:darkness regimen at 22-23°C with free access to water and food (standard granulated fodder). Veterinarian control of animals was carried out.

MSC were injected intravenously (6 million cells/ml).

Local cerebral ischemia was induced by distal LMCAO [7]. A 2-mm hole was made at the site of the suture between the frontal and zygomatic bones in narcotized (chloralhydrate; 300 mg/kg intraperitoneally) animals, the site of LMCA crossing with the inferior cerebral vein was mobilized. Occlusion of LMCA with a coagulator was carried out proximally from the site of artery bifurcation into the frontal and parietal branches. The wound was sutured by layers. Sham-operated animals were subjected to intervention without LMCAO.

Physiological parameters (blood pressure, heart rate, body temperature) were recorded in animals during the operation.

The neurological status of animals was evaluated by mNSS [7]. The parameters of spontaneous and motor activity of animals hung by the tail, placed on the floor/table, and their capacity to climb a wire grid were recorded. The proprioceptive sensitivity was evaluated by the reflexes from the auricle and cornea and by startle reflex.

The maximum mNSS score is 18; summary score of 13-17 was evaluated as the indicator of mild injury, 7-12 as medium-severe injury, and 1-6 as severe injury.

Integrative activity of the brain was evaluated by animal behavior in the open field and plus-maze tests [2].

The orientation and exploratory behavior of the animals was evaluated in the open field test in A. A. Kamenskii's modification. The animals were placed into the open field (round arena 180 cm in diameter with 40-cm-high wall). The wooden floor of the arena was divided into 48 sectors by 8 lines passing through the center and by two concentrical circles dividing the arena radius into 3 equal segments (26.66 and 53.32 cm from the center). During testing the animal was placed into the center of the arena and its horizontal (number of radial migrations with crossing of the outer and inner circles, respectively) and vertical (rearings) motor activities were recorded over 3 min and the intensity of grooming and number of defecation boluses were evaluated.

Elevated plus-maze is used for studies of anxiety and fear in rats. The experimental box is a plus-maze with 35-cm arms and 20-cm-high walls. Two contralateral arms are dark (with walls at the ends), two others are illuminated and open. The maze is placed at a height of 50 cm above the floor. The rat was placed into the center of the maze and time spent in the dark sections, number of passages

through the maze center, number of rearings and grooming episodes, peeping from closed arms of the maze, and total time spent in the illuminated sections were recorded over 3 min.

Cell culturing. Mesenchymal SC were isolated from human placental amnion by the enzymatic method [14] with some modifications for MSC culturing [3]. The cells were cultured in DMEM-F12 (Gibco) with 10% FBS (HyClone), streptomycin, penicillin, and glutamine (2 mM; all reagents from Gibco) in a CO_2 incubator at 37°C. The initial cell concentration was 2.5×10^5 per flask (area 75 cm^2, Greiner filter). After attaining 80-90% confluence (after 3 days) the cells were removed with trypsin-versene (1:1, 0.25% trypsin:0.02% versene; PanEco) and washed twice in Hanks' buffer (PanEco, 2×5 min at 300*g*).

Staining of human amniotic MSC with DIL vital dye. Mesenchymal SC cultured under standard conditions were harvested with trypsin-versene and washed twice in Hanks' buffer. DIL vital dye (Sigma) was added to the cell suspension (1 mln/ml) to a final concentration of 1 μg/ml and incubated for 5 min at 37°C and then 15 min at 4°C. After incubation the cells were washed twice in saline.

For further culturing the cells labeled with vital dye were placed in 24-well plates onto slides (10^4 cells/well) and cultured under standard conditions for 2 weeks. The content of DIL vital dye in human amniotic cells was evaluated under a fluorescent microscope (Axioplan 2; Carl Zeiss) using an AxioCam HRc camera with a set of filters by recording the red fluorescence (λ>580 nm) 1, 3, 7, and 14 days after staining.

Cell charging with DIL vital dye did not modify the rate of their proliferation in the culture and capacity to osteogenic, chondrogenic, and adipogenic differentiation.

Immunohistochemical analysis. The left and right brain hemispheres of rats were frozen in liquid nitrogen and stored for 1 month at -70°C. Frontal serial sections (10 μ) of each hemisphere were made on a cryotome (Microm HM560). Human amniotic cells were detected under a fluorescent microscope with a set of filters by recording red fluorescence. Hence, serial sections of the brain were analyzed for rats with a history of ischemic stroke and sham operation 1, 2, 6, and 8 weeks after transplantation of human cells.

In addition, the sections were stained with antibodies to human and rat glial fibrillary acidic protein (GFAP). The sections for GFAP staining were fixed in acetone (10 min at -20°C) and dried at ambient temperature for 30 min. Nonspecific antibody-binding sites were blocked with FBS (30 min at ambient temperature), thoroughly washed in PBS with 1% FBS and 0.1% Twin-20 (solution A), and incubated with the first antibodies for 1 h at 37°C (mouse anti-GFAP, rabbit anti-human GFAP; both from Chemicon). After incubation the sections were washed 3×5 min in solution A and incubated with FITC-labeled second antispecies antibodies for 1 h at ambient temperature. The obligatory washing in solution A was carried out; the nuclei were poststained with DAPI (100 ng/ml DAPI in PBS). The sections were embedded in fluorescence-preserving medium and staining was analyzed under an Axioplan 2 fluorescent microscope (Carl Zeiss).

TABLE 1. Experimental Protocol

Parameter	Period of study												
	Before operation	After operation											
		day 1	day 3	1 week	day 10	2 weeks	day 20	3 weeks	1 month	6 weeks	2 months	10 weeks	3 months
Body weight	+	+	+	+	+	+	+	+	+	+	+	+	+
Neurological status	+	+	+	+	+	+	+	+	+	+	+	+	+
Open field test	+			+		+							
Elevated plus-maze test	+				+		+						
Brain MRT	+			+		+		+	+	+	+	+	+
Brain morphology*			+	+					+		+		+
Brain histology*	+		+	+					+		+		+
Brain immunohistochemistry*				+		+				+	+		

Note. *One animal per period.

Study design. Experiment was carried out on 26 animals divided at random into 4 groups: 1) intact animals (n=3); 2) sham-operated animals (n=3): craniotomy without LMCAO and MSC transplantation; 3) reference group (n=10): animals with LMCAO without MSC transplantation; and 4) main group (n=10): rats subjected to LMCAO and injected with 6 million MSC (LMSAO+MSC).

The duration of the experiment for all groups was 3 months.

The dynamics of brain stroke volume, perifocal edema, and brain edema was evaluated by magnetic resonance tomography of the brain (BioSpec 70/30, 7 T magnetic field). The animals were narcotized intraperitoneally with 300 mg/kg chloralhydrate and fixed in the ventral position on a special platform. The duration of the study did not surpass the duration of narcosis.

The experimental protocol included the terms of tests for evaluation of the neurological and behavioral status (evaluation of neurological deficit, open-field and plus-maze behavior), collection of brain specimens for histological and histochemical studies, and body weight monitoring.

Statistical data processing. The data are presented as means±standard error in the means. Kruskal—Wallis nonparametric test was used for comparison of the groups. ANOVA and Friedman's statistics were used for evaluation of the time course of parameters in behavioral tests (open field and elevated plus-maze). Newman—Keuls test was used for subsequent comparison of the groups. The differences were considered significant at p<0.05. The results were processed using Statistica 6.0 software [2].

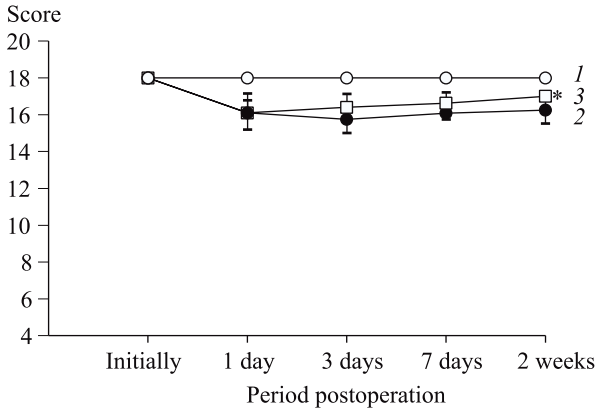

Fig. 1. Dynamics of the neurological status of rats with experimental ischemic stroke. *1*) sham-operated; *2*) LMCAO (reference group); *3*) main group. *Improvement of the neurological status of animals in comparison with the reference group (p=0.034).

RESULTS

After the operation, all animals of the reference and main groups developed a typical picture of focal cerebral ischemia manifesting by significant changes in the neurological status in comparison with intact animals (p<0.034) and by clearly discernible (according to MRT) infarction zone in the frontoparietal brain area.

Monitoring of the neurological status (by mNSS) during the postoperative period showed that neurological deficit (evaluated by spontaneous motor activity and proprioceptive sensitivity) decreased more rapidly in rats of the main group in comparison with the controls, the difference became significant on day 14 (p=0.034; Fig. 1).

Based on the previous data [5,6], we delayed testing of higher nervous activity to days 7-10 in order to rule out postoperative effects of focal cere-

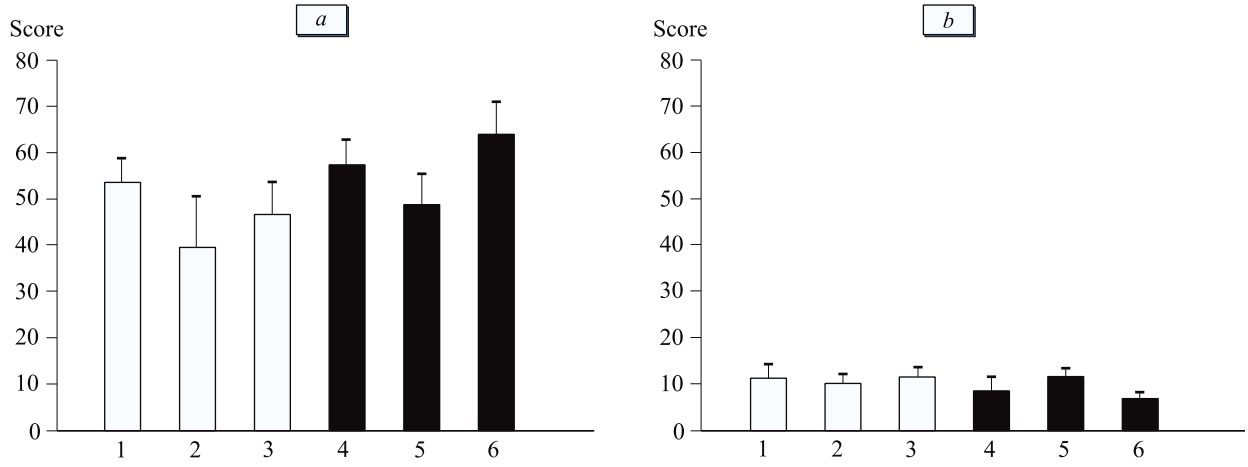

Fig. 2. Comparative analysis of the open field test results. *a*) runs; *b*) rearing. *1-3*) reference group animals (LMCAO without MSC): *1*) before operation; *2*) day 7 postoperation; *3*) day 14 postoperation. *4-6*) animals of the main group: *4*) before operation; *5*) day 7 postoperation; *6*) day 14 postoperation.

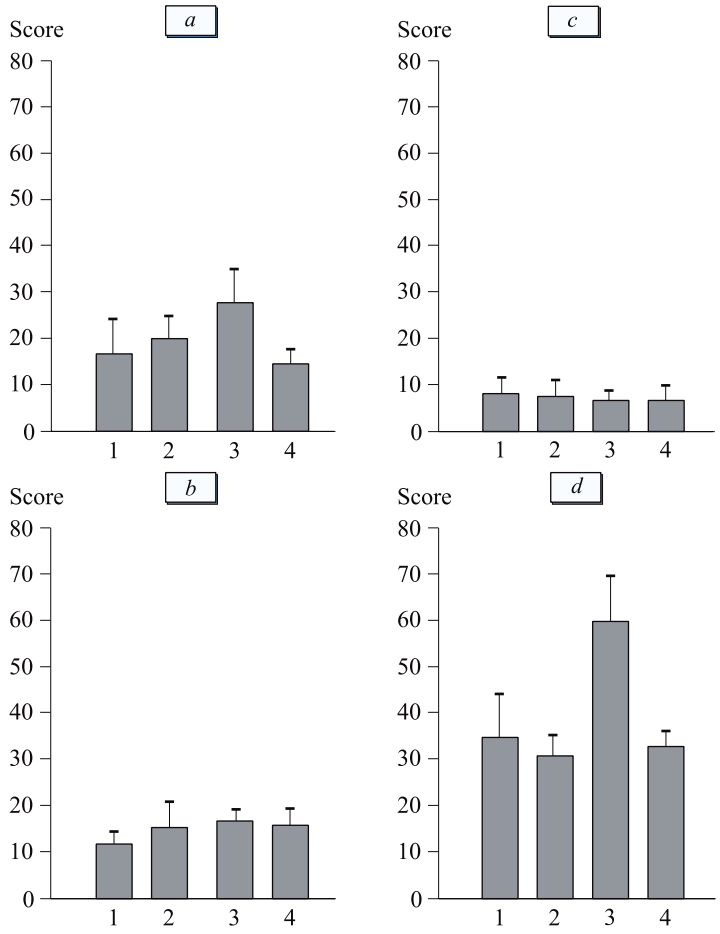

Fig. 3. Comparative analysis of the elevated plus-maze test results. *a*) latency; *b*) rearings; *c*) total time: *d*) peeping out episodes. *1*) reference group animals before operation; *2*) main group animals before operation; *3*) reference group animals on day 10 postoperation; *4*) main group animals on day 10 postoperation.

bral ischemia, while the neurological status of animals was monitored starting from day 1 of LMCAO.

Horizontal activity of rats in the main group evaluated by the number of the central circle crossings increased significantly in comparison with the reference group (LMCAO without MSC; p=0.01) on days 7 and 14 after experimental stroke; vertical activity also significantly increased on day 7 (p=0.04).

No significant differences between the groups were detected in the elevated plus-maze test before the operation. On day 10 postoperation, the latency (p=0.04) and total time spent in light arms (p=0.004) increased significantly in the main group in comparison with the reference group.

Hence, motor activity of animals with LMCAO injected with MSC increased significantly according to the results of behavioral tests (Figs. 2, 3).

The groups virtually did not differ by the volume of brain lesion foci (hyperintensive sites on T2 suspended MRT images); by day 14 virtually complete regression of MRT changes was observed in both groups.

Hence, neurological disorders regressed and parameters of behavioral tests normalized more rapidly during the first 2 weeks postoperation in animals injected with MSC. The data of MRT showed no differences in the rate of regression of focal changes in the brain matter, depending on MSC injection.

As soon as 1 week after intravenous injection these human MSC accumulated in the ischemic focus in the left hemisphere, while just solitary fluorescent cells were detected in other compartments of the left and right hemispheres. Many cells in the ischemic focus were at different stages of degradation. Two weeks after transplantation numerous labeled cells were still present in the ischemic zone (Fig. 4, *a*). Solitary fluorescent cells were still detected beyond the ischemic focus (Fig. 4, *b*).

Six weeks after transplantation, the transplanted cells lost the fluorescent label. However, immunohistochemical staining of the ischemic focus cells with antibodies to human GFAP detected solitary positively stained cells (Fig. 5, *a*) and their accumulations (Fig. 5, *b*). This indicates that some

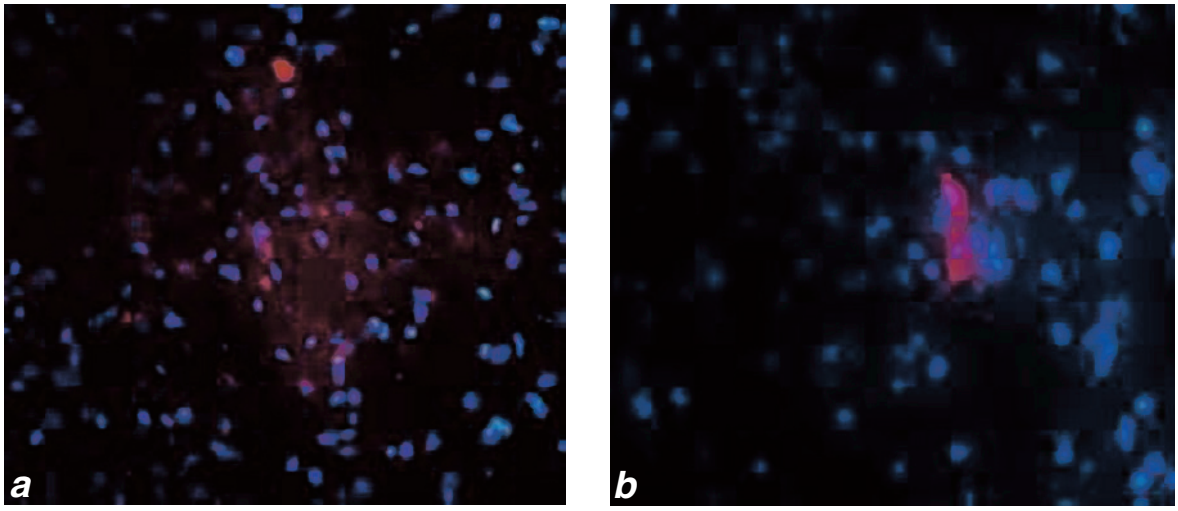

Fig. 4. Cryosection of the left (*a*) and right (*b*) hemispheres of the brain of a rat after ischemic stroke: 2 weeks after MSC injection. Red fluorescence: human cells labeled by DIL (nuclei poststained by DAPI).

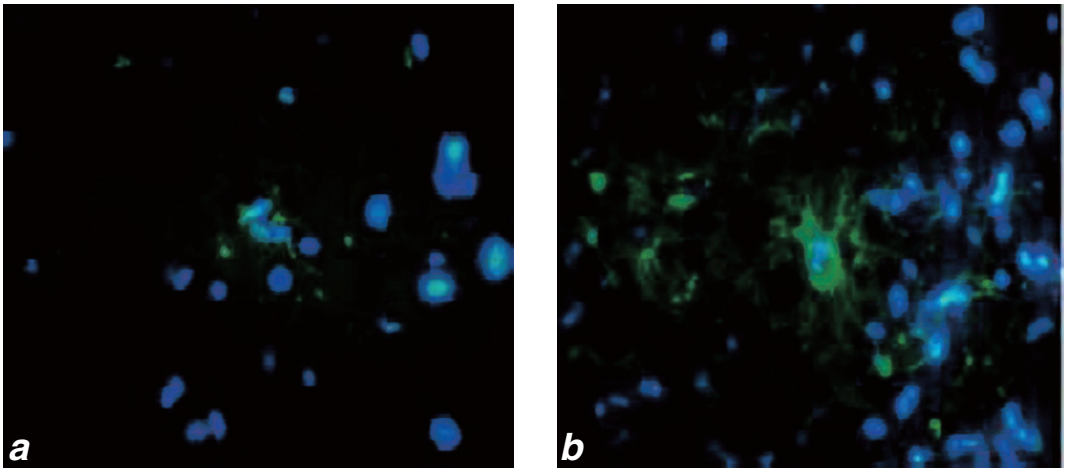

Fig. 5. Cryosections of the left hemisphere of rats with LMCAO: 45 days after MSC injection; staining for human GFAP.

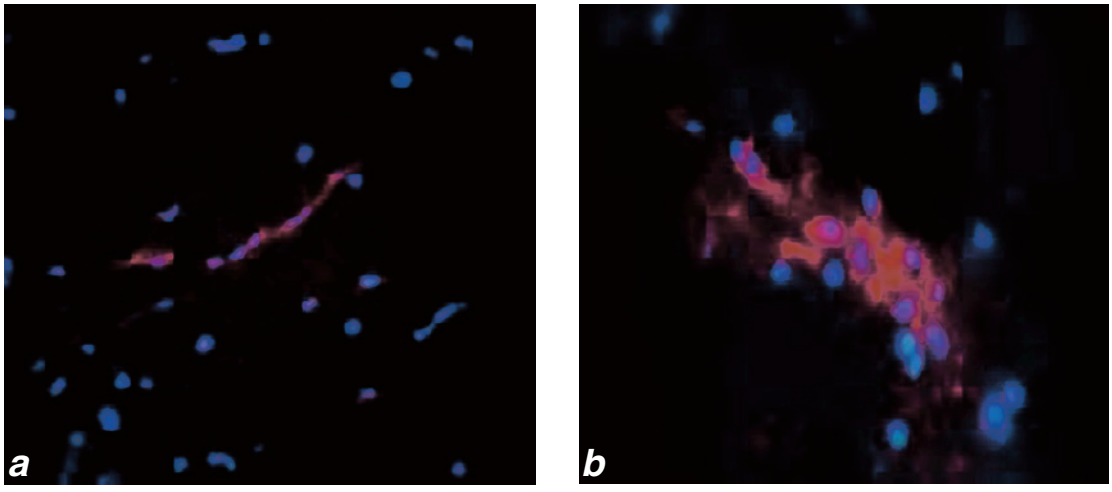

Fig. 6. Cryosections of the left hemisphere of rats with LMCAO: 60 days after MSC injection; staining for human GFAP (*a*) and βIII tubulin (*b*).

cells in the ischemic zone exhibit signs of differentiation towards the glia. Unfortunately, at this stage of the study we have no data indicating whether these cells were the transplanted ones or their descendants after mitoses. However, it is clear that these are human cells, because we used specific antibodies to human GFAP.

During the later stage of recovery after injury we observed signs of neuronal differentiation of transplanted cells or their descendants. Signs of differentiation into glial cells were retained (Fig. 6).

Our data indicate that human MSC injected intravenously to rats with experimental ischemia of the left hemisphere penetrated through the blood-brain barrier and were homed in the ischemic zone. Some of these cells exhibited signs of neural and glial differentiation. The immunohistochemical data do not indicate clearly whether these cells become functionally full-value neurons and gliocytes.

Hence, our experiments represent stage 1 of a comprehensive study of the neuroprotective effect of MSC on the model of acute focal cerebral ischemia. A series of experiments on animals with "intraluminal" focal cerebral ischemia is planned (when cortical injuries are paralleled by involvement of the subcortical structures). Development and mastering of methods for life-time monitoring of transplanted cells are planned.

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