TRANSPLANTATION OF NEURAL PROGENITOR CELLS STIMULATES ENDOGENOUS NEUROGENESIS IN MICE AFTER ISCHEMIC STROKE

ABSTRACT

The researchers have currently been actively investigating the possibilities for transplantation of the stem cells of various sources for treatment of the ischemic and degenerative diseases of the nervous system. Influence of transplantation of the hippocampal neural progenitor cells (NPCs) on endogenous neurogenesis in the mice after brain ischemia-reperfusion induced by 20 min occlusion of both carotid arteries has been studied. Following 24 hours after occlusion the NPCs isolated from the hippocampus of the FVB-Cg-Tg(GFPU)5Nagy/J mice transgenic by the GFP gene were transplanted stereotactically into hippocampal CA1 area of the experimental animals. For evaluating neurogenesis in the hippocampus of the ischemic animals we used immunohistochemical staining of the brain slices for BrdU and doublecortin (DCX). It has been found that transplantation of neural progenitor cells increased the number of BrdU- and DCX-positive cells in the dentate gyrus of the hippocampus after short-term global ischemia. These data allow admit that NPC transplantation to the ischemic animals influences on endogenous adaptation processes in the brain and on the neurogenesis, in particular.

KEYWORDS: neural stem cells, stereotaxic transplantation, brain ischemia, neurogenesis, hippocampus.
The experiments were carried out on the adult FVB wild-type (12-week-old) and FVB-Cg-Tg (GFP)Nagy/J mice, transgenic by green fluorescent protein (GFP) gene. The mice were given by the European molecular-biological laboratory (Monterotondo, Italy). All parameters of the space for keeping animals were observed: air temperature 22 °C, air humidity 40-60%, lightening 50 lux, and 12-hour light/dark cycle. The animals had free access to water and food.

The FVB wild-type mice were randomly allocated to one of the three groups. The 1st group (control) included three sham-operated animals which were operated except carotid arteries occlusion and without transplantation of NPCs. The 2nd and 3rd groups comprised of the brain ischemic animals which 24 hours following ischemia were stereotaxically injected culture media (2nd group, n = 3) and freshly isolated GFP-positive NPC (3rd group, n = 5).

All the experiments were conducted in keeping with “The European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes” and following the norms of bioethics and biological safety.

**Global short-term brain ischemia**

Ischemia was modeling in the narcotized (2,2,2-tribromethanol 125-240 mg/kg, intraperitoneally) FVB wild-type animals by occlusion of both common carotid arteries during 20 min and following unclicping and renewal of perfusion. The sham-operated animals of the control group underwent only artery preparation and they stayed under narcosis during 20 min without clipping.

Evaluation of regional cerebral blood flow (rCBF) and ischemia confirmation were done by means of the Moor-VMS-LDF-1 laser Doppler blood flow monitor (Moor Instruments, Great Britain) prior to and after occlusion and immediately after reperfusion. The obtained data were analyzed by the moorLAB software (Moor Instruments, Great Britain). In further experiments, we used only the animals whose rCBF was not lower than 15% of the normal base level before occlusion.

**Obtaining of neural progenitor**

In the FVB-Cg-Tg(GFP)Nagy/J GFP-transgenic mice we prepared under sterile conditions the hippocampus from the brain of 17-18 dpc fetuses. The fetal neural tissue was mechanically dissociated by means of Paster pipett of varying diameter in the Neurobasal culture medium (Gibco, USA). The obtained suspension of cells was passed through 40 μm cell filters (Falcon, USA). The purified fraction of NPCs was obtained by centrifugation of cells suspension in the density gradient (22% Percoll). The NPCs washed in the medium were transplanted to the ischemic animals. The percentage of the viable cells in suspension were identified by the flow cytometry on the cell sorter BD FACSAria (Becton Dickinson, USA) after incubation of the cell suspension with 7-aminoactinomycin D (7-AAD).

**Transplantation of NPCs**

The suspension of GFP-positive NPCs (2-2.5·10⁴ cells in 2 μl of Neurobasal medium) was stereotaxically transplanted into the hippocampus of experimental animals (coordinates from bregma: lateral ± 1.5 mm, posterior 2.0 mm, dorsoventrally 1.7 mm) under combined 2,2,2-tribromethanol narcosis (125 mg/kg intraperitoneally) 24 hours after ischemia/reperfusion. The sham-operated animals were injected 2 μl of Neurobasal medium into the same coordinates.

**Injection of BrdU**

For identification of the proliferating cells, the animals of all experimental groups were injected 5-bromodesoxyuridin (BrdU) prior to tissue obtaining for morphological study. BrdU (50 mg/kg) were done intraperitoneally twice a day during 2 days prior to brain extraction.

**Immunohistochemical staining**

Tissue obtaining for immunohistochemical analysis was done on day 14 after NPCs transplantation. Prior to brain extraction the mice were narcotized by intramuscular injection of Calispsol (75 mg/kg) and ether inhalation. Tissue fixation was done using transcardial perfusion-fixation in 4% paraformaldehyde solution on 0.1 M phosphate buffer (PB) at pH 7.4.

The frontal brain 40 μm slices were made using the VT1000A Vibratom (Leica, Germany). After washing in 0.1 M PB, the slices were blocked in 0.1 M phosphate buffer (pH 7.4) with addition of 0.5% bovine serum albumin (BSA) and 0.3% Triton X-100. For identification of the astrocytes we used the antibodies to GFAP (1:1500, DAKO, Denmark); for donor cells – the antibodies to GFP (1:750, Molecular Probes Inc., USA); for neuronal precursors – the antibodies to doublecortin DCX (1:100; Santa Cruz Biotechnology, USA); for proliferating cells – the antibodies to BrdU (1:100, Oxford Biotech, Great Britain). Prior to the immunohistochemical BrdU staining the slices were incubated during 30 min at 37 °C in 2N HCl solution for DNA denaturation and further according to standard protocol.

Visualization of primary antibodies was done by using secondary antibodies conjugated with Alexa Fluor 488 and Alexa Fluor 555 (1:1000, Molecular Probes Inc., USA). The stained slices were covered by ImmumOUNT medium (Thermo Scientific, USA). The immunohistochemically stained slices were examined under FV1000-BX61WI microscope (Olympus, Japan).

**Quantitative and statistical analyses**

The numbers of BrdU- or DCX-positive cells were counted in the dentate gyrus of hippocampus in each fifth frontal slice of the brain (coordinates: from 1.7 mm to 2.3 mm posterior from bregma). Altogether 5 slices per animal were examined and the total number of the BrdU- or DCX-positive cells were given as means ± standard error.

Data were statistically analyzed by means of the Statistica software (version 5, StatSoft). For non-parametric analysis was done by means of the Kolmogorov-Smirnov criterion. The differences between the values at p < 0.05 were assumed as statistically significant.

**RESULTS AND DISCUSSION**

For creating experimental global ischemia we used two-vessel occlusion of the common carotid arteries in the FVB wild-type mice. It was showed early that such model led to injury of the pyramidal neurons of the hippocampus along with activation of the glial cells [17]. As known, the hippocampus is the brain structure where neurogenesis is dynamically regulated throughout an entire life course [18, 19]. Therefore we have chosen the hippocampus for exploring effects of ischemic injury on the neurogenesis.

Following 24 hours after two-sided occlusion of the carotid arteries in 3rd group experimental animals we stereotaxically transplanted freshly isolated GFP-positive neural progenitor cells into the hippocampus. The transplanted cells were visualized by immunohistochemical staining of the brain slices with the use of antibodies to GFP. The GFP-positive cells were identified in the hippocampal CA1 area and did not migrate far from the injection site (Fig. 1).

Two days prior to tissue collection for morphological study the mice of all experimental groups were injected BrdU – synthetic nucleozide capable replace thymidine in the process of DNA replication being integrated into new DNA that allowed identify the proliferating cells pool (Fig. 2).

The immunohistochemical study of the brain slices with the use of the antibodies to BrdU showed that the hippocampus of sham-operated animals demonstrated the basic level of BrdU inclusion into the cells of the subgranular zone of the dentate gyrus and the number of BrdU-positive cells was 24.3 ± 2.1 (Figs. 3A; 4).
After experimental brain ischemia-reperfusion we observed the increase of the number of BrdU-labeled nuclei making 37.7 ± 2.3 (Fig. 3B, 4).

Stereotaxic transplantation of neural progenitor cells led to a two-fold increase in the number of BrdU-positive cells in the subgranular zone of the dentate gyrus compared with the 2nd group animals making 76.4 ± 3.3 (Figs. 3C: 4).

The BrdU-positive cells in mice of all experimental groups formed proliferative clusters in the subgranular zone (Fig. 5) that is characteristic of the dentate gyrus precursors [20].

To analyze the phenotype of cells which formed proliferative clusters in the dentate gyrus of the hippocampus we used immunohistochemical staining on doublecortin. This phosphoprotein associated with microtubules is expressed in immature cells and used as the marker of neurogenesis [8, 21].

The immunohistochemical analysis has showed that DCX-positive cells were seen in the subgranular zone the dentate gyrus. These cells formed numerous clusters and had well-developed processes which were directed into molecular layer of the dentate gyrus (Fig. 6).

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mice and gerbils [6, 25, 26]. However, it is well known that such potential of newly formed cells for differentiation by neural phenotype after ischemic brain injury is considerably decreasing with age [4]. This can be linked with a decrease in production of the neurogenic factors such as fibroblasts growth factor, insulin-like growth factor 1, neurogenesin-1 and vascular endothelium growth factor in the neurogenic zones of the brain [27-29].

It is for this reason that so active research work has been done with the aim of finding possibilities for transplantation of neural cells for compensation of the brain ischemic injury outcome at the expense of activation of the organism’s own reparative mechanisms [16]. It was showed that transplantation of the CNS fetal tissue containing cells of the hippocampal CA1 area reduced cognitive impairments caused by the injury of pyramidal CA1 zone neurons in the adult rats following global ischemia [14]. After transplantation into adult brain, the NPCs differentiated into mature neurons with morphological and biochemical peculiarities typical of the surrounding neurons of recipient’s brain. This indicates that CNS stem cells are capable to respond to microenvironmental signals and influence recipient’s tissue [30, 31].

The obtained data have shown that transplantation of NPCs following ischemic brain injury significantly increased the number of both BrdU- and DCX-positive cells in the subgranular zone of the dentate gyrus. We can assume that transplantation of neural progenitor cells in the ischemic hippocampus may stimulate endogenous neurogenesis in the hippocampal subgranular zone by secretion of various growth factors, contained in high concentrations in fetal nervous tissue.

**CONCLUSION**

THE NEURAL PROGENITOR CELLS TRANSPLANTED INTO THE HIPPOCAMPUS OF THE ISCHEMIC MICE HAVE POTENTIAL TO STIMULATE ENDOGENOUS NEUROGENESIS IN THE SUBGRANULAR ZONE AND THEREBY PROMOTE RECOVERY OF LOST FUNCTIONS.
REFERENCES


