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SHORT-TERM MIGRATION OF TRANSPLANTED *Lin⁻Sca-1⁺c-kit⁺* HEMATOPOIETIC STEM CELLS AFTER HIPPOCAMPAL ISCHEMIC INJURY OF MICE

SUMMARY

The study of migration and differentiation potential of different types of stem cells remains a problem for cell biology and regenerative medicine. The purpose of the study was to evaluate the ability of transplanted hematopoietic stem cells (HSC) of murine fetal liver to migrate into a zone of hippocampal ischemic injury at suboccipital intraventricular injection; and to assess their neural differentiation possibility in the early period after transplantation.

MATERIALS AND METHODS. We modeled an ischemic injury of the hippocampus of *FVB-wt* mice and after 24 hours transplanted suboccipitaly fetal liver *HSC of FVB-Cg-Tg(GFPU)5Nagy/J* fetuses (transgenic by GFP). Sorting of *Lin⁻Sca-1⁺c-kit⁺* HSC fractions was performed by *FACS*. After 7 and 14 days we performed immunohistochemical staining of brain slices for *GFP*, NeuN and *GFAP* markers.

RESULTS. On the 7th day after transplantation injected cells penetrated up to 100 μm from the wall of the 3rd ventricle, and on the 14th day single transplanted cells localized in the ischemic hippocampal *CA1* region. Donor's cells were round shape and did not express NeuN and *GFAP* markers. Features of reactive astrogliosis and neuronal death were kept in the hippocampal CA1 region of experimental animals, similar to the control group.

CONCLUSION. Transplanted *Lin⁻Sca-1⁺c-kit⁺* mice fetal liver *HSC* are able to survive and migrate to the area of hippocampal ischemic injury, but the possibility of their neuronal or astrocyte differentiation in 14-day time was not confirmed.

KEYWORDS: hematopoietic stem cells; fetal liver; hippocampal ischemic injury

Issues of migration potential and plasticity of different types of stem cells continue to arouse considerable interest among researchers in terms of improving the regenerative properties of cell and tissue transplantation [1]. First of all study in this area concerned the scope of bone marrow transplantation, which has been used in clinics for a long time [2, 3]. The ability of hematopoietic cell migration between niches and their transdifferentiation into other germ layer derivatives are the subject of scientific discussions.

Mechanisms of attracting hematopoietic stem cells in the repair of damaged nerve tissue are of particular interest. According to animal experiments and results of the retrospective analysis of some clinical studies of bone marrow recipients there has been demonstrated not only the presence of donor cells in areas of brain damage, but their expression of a number of markers characteristic for neurons and glia [4-8]. These studies suggest that transplanted cells come from blood flow to ischemic areas and differentiate into neural cells. However, there are researches with opposite results that cast doubt on these findings. Although the possibility of fusion of donor cells with recipient cells also remains relevant [9].

Therefore, many factors should be taken into account, including the source, methods of cell's obtaining, preparation and method of transplantation. In particular, the source of stem cells may influence on their maturity and, therefore, the potential for migration and differentiation. The presence of multipotent mesenchymal stromal cells fraction among transplanted bone marrow mononuclear cells does not allow to prove the ability of hematopoietic cells to transdifferentiation.

To characterize hematopoietic stem cells in mice there have been developed a group of immunophenotypic attribute that allow identification of LSK-cells populations expressing stem cell antigen Sca-1 and stem cell growth factor receptor c-kit, with no expression of linearity Lin markers

(*CD45R/220, CD3, CD11b, TER-119, Ly6C/G*) [10]. Transplantation of sorted *Lin⁻Sca-1⁺c-kit⁺* cells in the experiment allows us to estimate the migration and differentiation potential of proper hematopoietic stem cells.

The purpose of the study is to assess the ability of hematopoietic stem cells from murine fetal liver to migrate into the ischemic hippocampal area after suboccipital intraventricular injection.

MATERIALS AND METHODS

Experiments were conducted on male mice *FVB-wt* (wild type) aged 3 months and *FVB-Cg-Tg(GFPU)5Nagy/J* (transgenic by green fluorescent protein gene – *GFP*) from the vivarium of State Institute of Genetic and Regenerative Medicine of Ukrainian NAMS. The mice were housed under standard laboratory conditions and had free access to water and food. All studies with the experimental animals were conducted in compliance with article 26 of the Law of Ukraine «On protection of animals from cruelty» (dated 21.02.2006), «European Convention for the protection of vertebrate animals used for experimental and other scientific purposes» (Strasbourg, 1986) and in compliance with all principles of bioethics and biosafety regulations.

The source of fetal liver *HSC* was 12.5 dpc fetuses of *FVB-Cg-Tg(GFPU)5Nagy/J* mice. Pregnant females were euthanized by cervical dislocation under ether anesthesia. In sterile conditions fetuses were isolated from the uterus and fetal liver was minced in *RPMI-1640* medium (*Sigma*, USA) by passing through needles of decreasing diameter. The fraction of mononuclear cells containing HSC was isolated by centrifugation for 15 min at 380 g and 4 °C on *Ficoll-Paque* (*Sigma*, USA) 1.077 g/cm^3 density gradient and washed in *RPMI-1640* medium. Subpopulation of *Lin⁻Sca-1⁺c-kit⁺* cells was identify by flow cytometry using Lineage Panel for 5 monoclonal antibodies (*CD3e, CD11b, CD45R/B220, Gr1, Ly-76*) conjugated with *PerCP-Cy5.5; anti-Sca-1* (*PE-Cy7*) and *anti-c-kit* (*APC-Cy7*) *antibodies* (all - *Becton Dickinson*, USA). For sorting the concentration of mononuclear cell was adjusted to $2 \cdot 10^7$ cells/ml, added to the antibodies in a working concentration of 0.5 µg per 10^6 cells, and incubated for 30 minutes at 4 °C. After twice washing in a *RPMI-1640* medium suspension was passed through 70 µm cell strainer and adjusted to a concentration of $5 \cdot 10^6$ cells/ml. Sorting of LSK cells was performed under aseptic conditions with cell sorter *BD FACSAria* (*Becton Dickinson*, USA) using *FACS Diva 6.1.2* software. The relative content of viable cells, which was determined using of 7-aminoactinomycin D (*7-AAD*), after sorting was 88.5 ± 3.8 %.

Ischemic stroke in *FVB-wt* mice (n = 4) was modeled by a 20-minute occlusion of both common carotid arteries. Before operation and cells transplantation the mice were anesthetized via intraperitoneal administration of *Calypsol* (75 mg/kg) and *Xylazine* (2 mg/kg). In 24 hours after hippocampal ischemia $2 \cdot 10^5$ selected HSC in 20 µl *DMEM+F12* (*Sigma*, USA) were transplanted into 3rd ventricle of the brain through suboccipitial region. Control group include animals with simulated ischemic brain damage, which were administered with only 20 µl *DMEM+F12* medium (n = 2), and pseudooperated animals (n = 2).

Immunohistochemical study of the brain was performed at the Department of Cytology of Bogomoletz Institute of Physiology NAS Ukraine. In 7 or 14 days after transplantation, mice were anesthetized intraperitoneally (75 mg/kg Calypsol) and by inhalation of ether. Fixation of brain tissue was performed by transcardial perfusion (4 % paraformaldehyde solution on 0.1 M phosphate buffer, pH 7.4) for 20 minutes. The mice were decapitated; the brain was treated and fixed in 4 % paraformaldehyde solution for 12 hours at 4 °C. Frontal 50 µm sections were prepared with 752/M Vibroslice tissue cutter (*Campden Instruments Ltd*, UK). After that they were incubated in a mixture of primary antibodies for 48 hours at 4 °C: rabbit *anti-GFAP* antibody (1:1500) (*DAKO*, Denmark) + mouse *anti-NeuN* (1:1000) (*Chemicon*, USA) or *anti-NeuN* (1:1000) (*Chemicon*, USA) + goat *anti-GFP* (1:5000) (*Novus biologicals*, USA). After the washing off the primary antibodies, the sections were incubated with secondary anti-rabbit antibody for *GFAP* conjugated with *Alexa Fluor 594* or *Alexa Fluor 350* (1:1000) (*Molecular Probes Inc*., USA) or *anti-goat* (for *GFP*) antibodies conjugated with *Alexa Fluor 488* (1:1000) (*Molecular Probes Inc*., USA) on 0.1 M phosphate buffer. Sections were placed onto a glass slides and covered with balsam *Vectashield Mount Medium* (*Vector*, USA). Histological analysis was performed using a confocal laser scanning microscope *FV 1000-BX61 Wi* (*Olympus*, Japan).

RESULTS AND DISCUSSION

An ischemic stroke developed after an external 20-minute occlusion of carotid arteries in the control group animals without cell transplantation, the maximum changes were observed in the hippocampal *CA1* region. On the 8th day after the modeling a characteristic feature of ischemic hippocampus was reducing of pyramidal neurons number up to 70 % with their degeneration and reactive astrogliosis.

On the 7th day after transplantation in the animals injected with fetal liver HSC, donor cells were found at the site of injection in the walls of the 3rd brain ventricle. The cells localized in the tissue at a depth of 10 µm and kept a rounded shape. (**Fig. 1**)

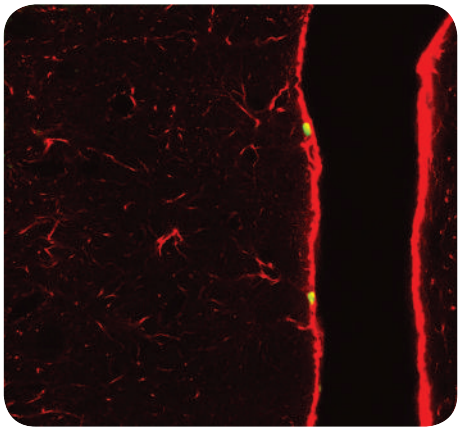
In some brain slices we identified a group of cells that were in the tissue at different depths up to 100 µm from the ventricle wall, which may indicate an active migration of transplanted cells to the direction of ischemized zone, reached of injured hippocampus (**Fig. 2**).

On the 14th day single transplanted cells, maintaining a rounded shape without morphological signs of differentiation, were detected in the hippocampal CA1 region. But donor cells were not found in other analyzed brain structures (data are not shown).

Thus, on the 7th and the 14th day after cell transplantation (8th and 15th day after the modeling of ischemic brain injury, respectively), experimental animals kept signs of reactive astrogliosis and loss of neurons in hippocampal *CA1* region, similar to parameters in the control group.

At immunohistochemical study on the 7th and the 14th day after injection transplanted cells did not express neuronal marker *NeuN* and astrocytes marker *GFAP*, confirming and supplementing the other authors' data. In particular, Massengale M. et al. (2005) demonstrated that transplanted hematopoietic cells expressed only microglial *Iba1* and *Mac1* markers in damaged brain [11]. *Roybon L. et al.* (2006) after stereotactic transplantation of labeled *LSK* cells into the striatum and cerebellum did

Fig. 1. Frontal section of the 3rd ventricle wall of mouse brain, 7 days after fetal liver HSC transplantation, confocal laser scanning microscopy. Transplanted HSC are labeled with anti-GFP antibodies (green), astrocytes – anti-GFAP-Alexa594 (red), x200.



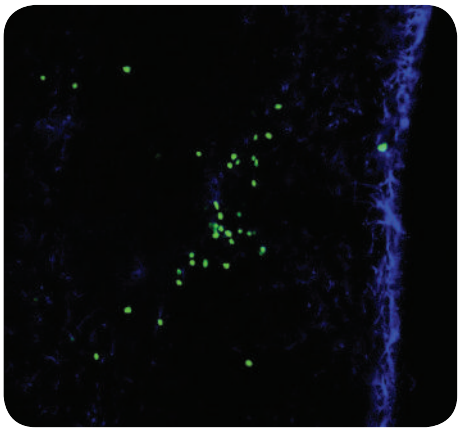
not detect their expression of *NeuN* and calbindin (markers specific to Purkinje cells), and even Iba1 [12]. *Wehner T.* et al. also found that bone marrow cells, transplanted into mice with focal cerebral ischemia, do not differentiate into astrocytes [13].

Although our study did not reveal signs of morphological improvement in hippocampal ischemic areas as well as changes of morphology and phenotype of donor cells, the fact of transplanted hematopoietic cells migration from the ventricular cavity into the area of ischemic brain damage confirms the assumption of many researchers that donor stem cells of different types, including hematopoietic, are able to respond to signals of ischemic tissue and actively migrate to the area of injury [14, 15].

CONCLUSION

THUS, THERE WAS DEMONSTRATED AN ABILITY OF TRANSPLANTED HEMATOPOIETIC STEM CELLS WITH *Lin⁻Sca-1⁺c-kit⁺* PHENOTYPE, DERIVED FROM MICE FETAL LIVER TO SURVIVE AND MIGRATE FROM THE CAVITY OF THE BRAIN VENTRICLES INTO AREA OF HIPPOCAMPAL ISCHEMIC DAMAGE IN VIVO, WHICH MAY INDICATE AN ACTIVE HOMING OF HEMATOPOIETIC STEM CELLS IN RESPONSE TO SIGNALS OF TISSUE DAMAGE, WHICH DIFFER IN ORIGIN IN ONTOGENESIS. IN A 14-DAY PERIOD AFTER TRANSPLANTATION THERE WERE NOT MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL FEATURES OF AN INTRODUCED CELLS DIFFERENTIATION INTO NEURONS OR ASTROCYTES.

Fig. 2. Frontal section of the 3rd ventricle wall of mouse brain, 7 days after fetal liver HSC transplantation, confocal laser scanning microscopy. Transplanted HSC are labeled with anti-GFP antibodies (green), astrocytes – anti-GFAP-Alexa 350 (blue), x200.



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