Characteristics of Human Neural Stem Cells *In Vitro* and After Transplantation into Rat Brain

M. A. Aleksandrova, O. V. Podgornyi, M. V. Marei*, R. A. Poltavtseva, E. B. Tsitrin, D. V. Gulyaev, L. V. Cherkasova, A. V. Revishchin, L. I. Korochkin***, N. G. Khrushchov, G. N. Sukhikh***

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We studied the effect of culturing conditions on the fate of human neural stem cells after transplantation into rat brain. Human neural stem cells cultured in the presence of mitogens without LIF migrated along the ependyma and cerebral vessels of recipients, but to a great extent degenerated by the 20th day after transplantation. Neural stem cells cultured with LIF migrated, apart from the above mentioned pathways, in the cortex and hippocampus, well survived; proliferating cells were retained 30 days after transplantation.

Key Words: *human neural stem cells; tissue culture, xenotransplantation; immunohistochemistry*

Transplantation of CNS tissues occupies a special place in the problem of organ and tissue transplantation. Since 1970s, when pioneer works on neurotransplantation appeared, thorough knowledge and considerable clinical experience were accumulated in this field. It was demonstrated that cells from embryonic brain (from different mammal species and humans) can develop and integrate into the brain of adult recipients; form interneuronal contacts and express specific neurotransmitters; normalize behavior and restore cognitive functions after damage [1,14]. After the efficiency of neurotransplantation was experimentally proven, more than 300 transplantations is Parkinson's disease, Huntington's chorea, epilepsy, and spinal traumas were performed within the framework of clinical studies [5,8]. Donor material (fragments of the brain) was isolated from human embryos and fetuses obtained during medical abortions. Studies with transplantation of the nervous tissue in Parkinson's disease showed that about $^2/_3$ patients had positive dynamics after transplantation and partial recovery of motor function [10]. However, methodical difficulties (5-6 embryos are required simultaneously for obtaining sufficient amount of the donor tissue) and ethical problems (the use of abortion material) necessitate the search of new alternative sources for cell therapy. A promising approach is transplantation of neuronal and glial stem cells. These cells are present in the brain throughout the life, but their number is maximum during embryogenesis [3,6]. Isolation and *in vitro* culturing of stem cells can solve the ethical problem of using embryonic material, because it provides the possibility of dealing with long-living populations of cells needed for transplantation [7,13]. Stem cells attract considerable interest from both theoretical and practical viewpoints. Many scientists believe that transplantation of human neural stem cells (HNSC) will allow to create new active neurogenic zones in the brain and will be an effective therapeutic approach to the treatment of various neurodegenerative diseases [4,11]. However, success-

Institute of Developmental Biology, Russian Academy of Sciences; *Center of Obstetrics, Gynecology, and Perinatology, Russian Academy of Medical Sciences; **Institute of Ecological and Evolutional Problems, Russian Academy of Sciences; ***Institute of Gene Biology, Russian Academy of Sciences, Moscow. ***Address for correspondence:*** aleksandrova@vigg.ru. M. A. Aleksandrova

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ful transplantation of HNSC depends on migration capacity of these cells and their ability to integrate into the recipient brain. Therefore, the source and conditions of culturing of stem cells are important factors determining the fate of transplanted cells [12].

The aim of the present study is to determine optimal conditions and time intervals for culturing of HNSC isolated from human fetal brain and to characterize their migration and differentiation capacities after transplantation into the brain of intact adult rats and rats exposed to acute hypoxia.

MATERIALS AND METHODS

HNSC were isolated from the whole brain of 9.5 week human embryos obtained during medical abortion. The number of viable cells in the primary suspension was determined by trypan blue exclusion and propidium iodide staining. Viability of suspensions selected for culturing was not less than 60%.

Two methods of culturing were used. The first method (routine) was used for short-term culturing: DMEM medium and F-12 medium (1:1), N2 complement (1:100) with fibroblast and epidermal growth factors (FGF-2 and EGF, 20 ng/ml each), 8 μg/ml heparin, and gentamicin/amphotericin. The second method was used for long-term culturing. The above medium was supplemented with leukemia-inhibiting factor (LIF) and the culturing was performed according to the following scheme: 2 weeks in a medium with bFGF+EGF+LIF (+heparin), 2 weeks in a medium with bFGF (+heparin), 2 weeks in a medium with EGF, 2 weeks in a medium with bFGF (+heparin), and 2 weeks in a medium with bFGF and LIF (+heparin).

In both cases the cell suspension (2 mln/ml) was cultured in the above media in 25 cm^2 flasks (Corning) at 37°C and 5% CO_2. The medium was half-replaced every 4 day, cell aggregates were suspended by repeated pipetting, and the number of viable cells was determined.

Neurospheres grown according to these two methods were fixed after 14 and 65 days for histological and immunocytochemical studies and for electron microscopy. Minor part of these cells was then maintained for 4 days in complete nutrient medium supplemented with 10% fetal calf serum.

Neurospheres and cells were fixed in 4% parform on phosphate buffered saline, washed, and incubated with primary antibodies for 12 h. Antibodies to human nestin (Chemicon, 1:30), glial fibrillary acid protein (GFAP; DAKO, 1:250), class III β-tubulin (Abcam, 1:200), vimentin (NeoMarkers, 1:100), and neurofilaments-70 (Serotec, 1:500) were used. The cells were then treated with secondary antibodies labeled with fluorescent dyes Cy-2 and Texas Red (Jackson).

In the beginning of culturing and before transplantation cytofluorometric analysis was performed using antibodies to nestin, a protein of intermediate filaments of neuroepithelial stem cells, and vimentin, a protein expressed in neuro- and glioblast precursors and radial glial cells. The study was performed on a flow cytofluorometer (Bio-Rad) using the method of double-staining direct immunofluorescence.

The cells cultured according to the above-mentioned protocols were transplanted into the brain of intact rats and rats exposed to acute hypoxia [2]. Experiments were carried out on 25 female Wistar rats weighing 250-300 g. Cell suspension in a volume of 3 μl (1.5×10^6) was stereotactically implanted into the hippocampus (A=3.5-4.0 mm; L=-2.5 mm; V=-4 mm) to intact rats and animals exposed to hypoxia (under chloral hydrate narcosis, 300 mg/kg). After transplantation of HNSC immunosuppression was not performed to recipient rats.

The rats were narcotized on days 10, 20, and 30 after transplantation, the brain was perfused with 4% paraformaldehyde on phosphate buffered saline, and sections were prepared on a freezing microtome. Some sections were stained with hematoxylin and eosin, by the methods of Nissl and Giemsa. Other sections were analyzed by immunohistochemical methods using the above-listed primary antibodies and antibodies against human cell nuclei (anti Human Nuclei, Chemicon, 1:30), neuronal-specific nuclear protein (anti-Neu N, Chemicon, 1:30). The sections were processed according to the above-described technique for cultured cells.

RESULTS

Culturing according to both protocols for 2 days led to the formation of floating cell aggregates consisting of 100 and more cells, which can be easily pipetted to individual cells.

The appearance of small cell aggregates (typical neurospheres) consisting of 6-20 cells was noted on days 5-7 in both cultures. On days 9-10 both cultures contained similar numbers of neurospheres. Then the size of neurospheres increased and they formed ball-shaped structures containing 100 and more cells. Viability of cells in small neurospheres (several tens of cells) was 98-100%. Large neurospheres consisting of 100 and more cells had denser surface cell layer. Trypan blue staining revealed scattered groups of dead cells on their surface.

During culturing according to the second protocol (with FGF, EGF, and LIF) the medium with cell aggregates of different sizes were repeatedly pipetted during medium replacement and the aggregates dissociated into individual cells. Then these cells proliferated and formed new neurospheres. Dead cells were

removed, if necessary. Thus, this technique of long-term culturing yields practically pure culture of newly formed neurospheres.

The cells of 14- and 65-day cultures grown according to the first and second protocol, respectively, were taken from morphological analysis and transplantation into rat brain. Cell composition of whole neurospheres and cultures stimulated for differentiation by adding serum to the culture medium.

Light microscopy of toluidine blue-stained semithin sections of neurospheres and electron microscopy of these sections revealed cells at different stages of mitotic division, cells with growing processes, and apoptotic cells.

Immunohistochemical study of whole neurospheres from both cultures demonstrated their heterogeneous cell composition (Fig. 1). Staining with antinestin antibodies showed that the number of nestin-positive cells gradually decreased with increasing the size of neurospheres. Nestin-positive cells were arranged in groups or completely covered the neurosphere. They had small round bodies and thin sparsely branching processes. In the majority of neurospheres the cells and processes lay on the surface. Glial precursors, GFAP-immunopositive cells had intensively stained bodies with small processes. The cells containing type III β-tubulin were less intensively stained and had shorter processes. GFAP- and type III β-tubulin-positive cells situated in deep layers of neurospheres and formed compact groups (1-3 groups per neurosphere).

For evaluation of cell differentiation capacity the neurospheres were transferred to flasks with a medium supplemented with 10% fetal calf serum and without substrate. Under these conditions the neurospheres settled on the bottom, adhered, and flattened; some cells migrated from the peripheral zones. On day 4 the cultures were fixed with 4% paraform on phosphate buffered saline and stained with antibodies against nestin, vimentin, and class III β-tubulin (Fi. 2). Immunohistochemical analysis showed that the migration zone contained considerable number of nestin-positive stem cells, many vimentin-positive cells, and cells of glial and neuronal differentiation lineages. The percentage of different cell types in different neurospheres greatly varied. Small neurospheres often contained only neural or only glial cells against the background

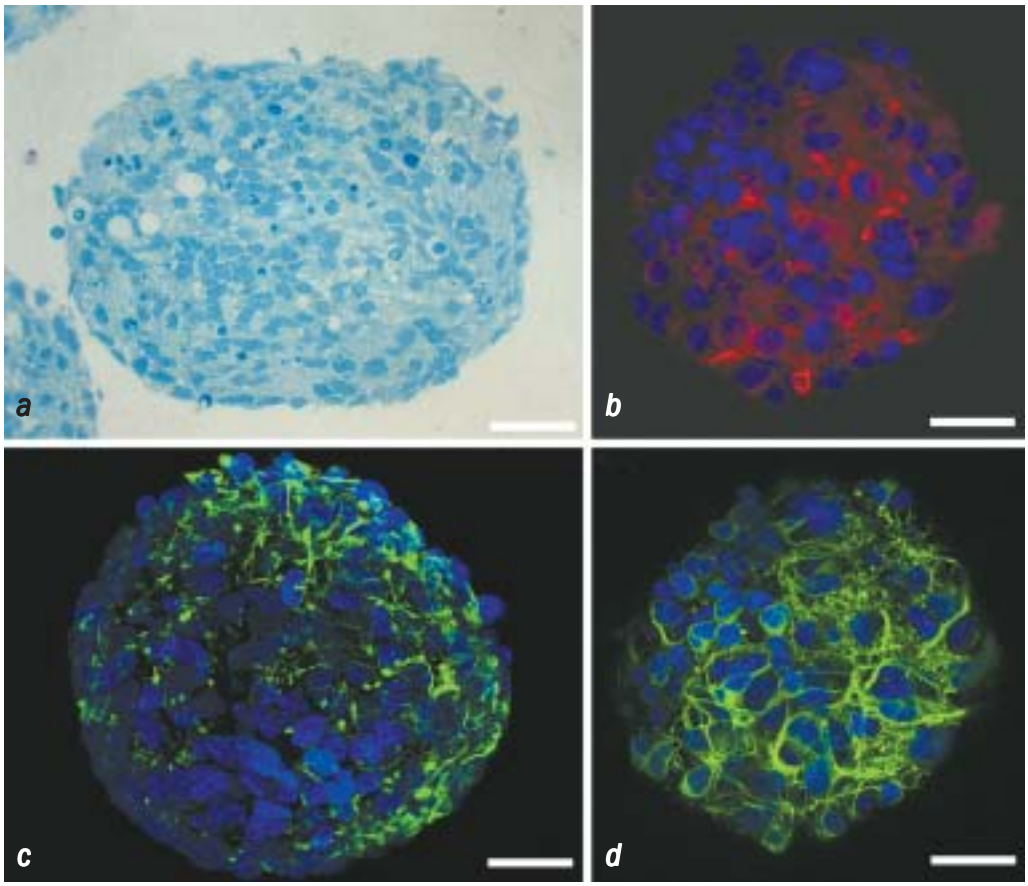

Fig. 1. Cell composition of freely floating neurospheres formed by human neural stem cells(HNSC). Semithin section: multiple viable cells and solitary apoptotic cells; methylene blue staining (*a*); scale — 30 μm. Cell differentiation in neurospheres, confocal microscopy. Staining with antibodies to human nestin (*b*), vimentin (*c*), class III β-tubulin (*d*); cell nuclei are stained with Toto-3; scale — 20 μ.

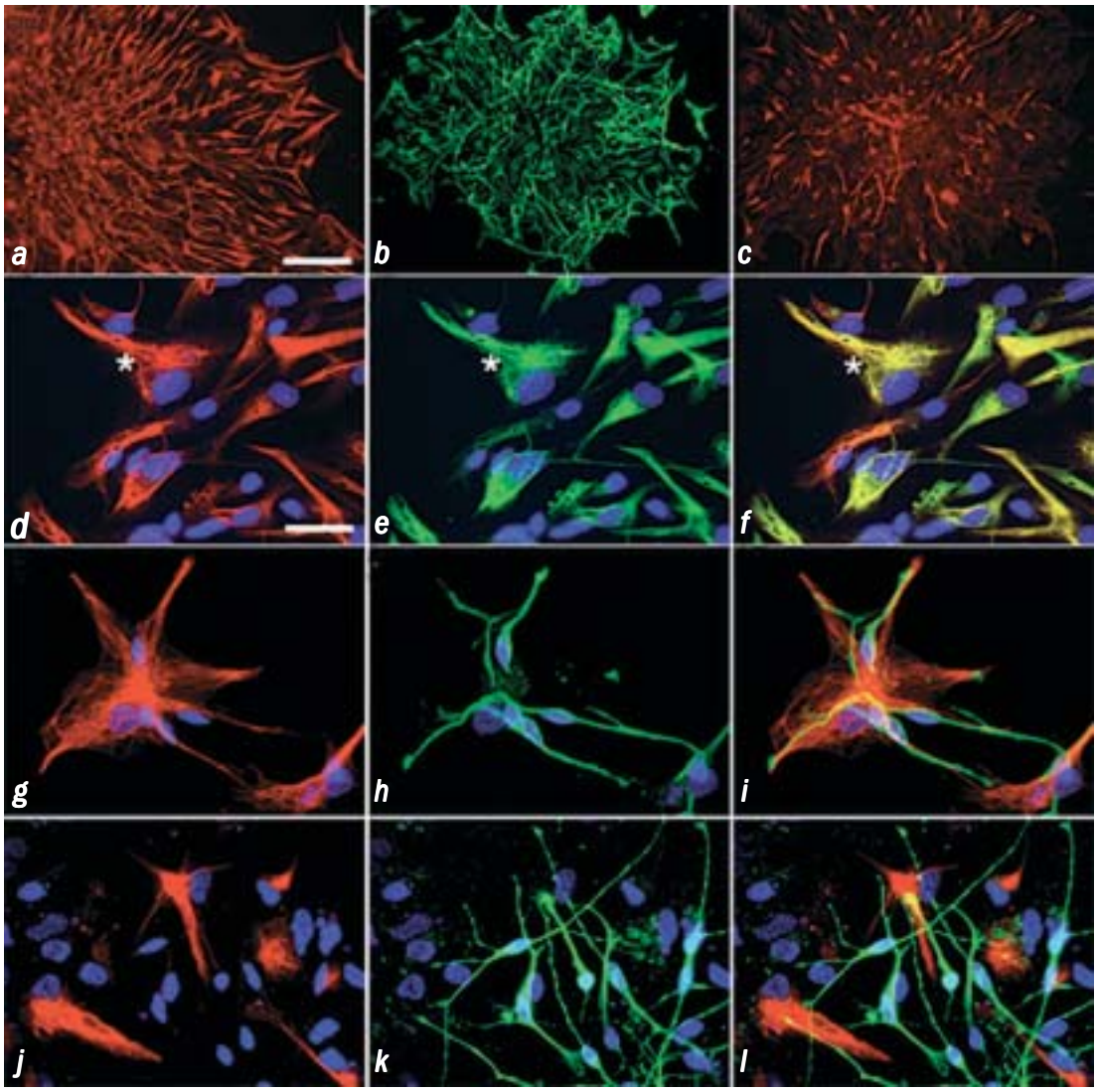

Fig. 2. Heterogeneity of cells in settled neurospheres. Cells of flattened neurospheres stained with antibodies to human nestin (*a*), class III β-tubulin (*b*), GFAP (*c*); scale — 100 μ. Double staining with antibodies to human nestin (*d*) and vimentin (*e*), overlay image (*f*); cells expressing both protein are clearly seen (asterisk). Double staining with antibodies to human nestin (*g*) and class III β-tubulin (*h*), overlay image (*i*). Double staining with antibodies to glial protein (j) and class III β-tubulin (*k*), overlay image (*l*). Cell nuclei are stained with Heochst 33342, scale 40 μ.

of nestin- and vimentin-positive cells. Large neurospheres usually contained all cell types, but their quantitative ratios greatly varied.

Nestin- and vimentin-positive cells are morphologically similar; two cell types can be distinguished among them: large flattened astrocyte-like cells with oval nucleus and short processes (type 1) and cell with round or elongated bodies, large nucleus, and long processes (type 2). Coexpression of nestin and vimentin in the same cell was often noted.

Many migrating cells differentiated by the neuronal type. This was confirmed by positive reaction with antibodies against type III β-tubulin. These cells had uni-, bi-, and multipolar shape with long processes and were arranged in groups.

The cells differentiating by the glial type contained GFAP and also had long processes. Both GFAP-positive and type III β-tubulin-positive cells were arranged in more of less dense groups.

Cytofluorometry showed that cultures grown by the first protocol initially contained 39% nestin-positive and 15% vimentin-positive cells and after 14-day culturing 34% nestin- and 35% vimentin-positive cells; for cultures grown according to the second protocol the percentage of nestin- and vimentin-positive cells was 20 and 6%, respectively, at the initial stage, and 31.5 and 13% after 65-day culturing. These data suggest that by the moment of transplantation the percentage of stem cells was similar in these cultures. However, in cultures grown by the first protocol the

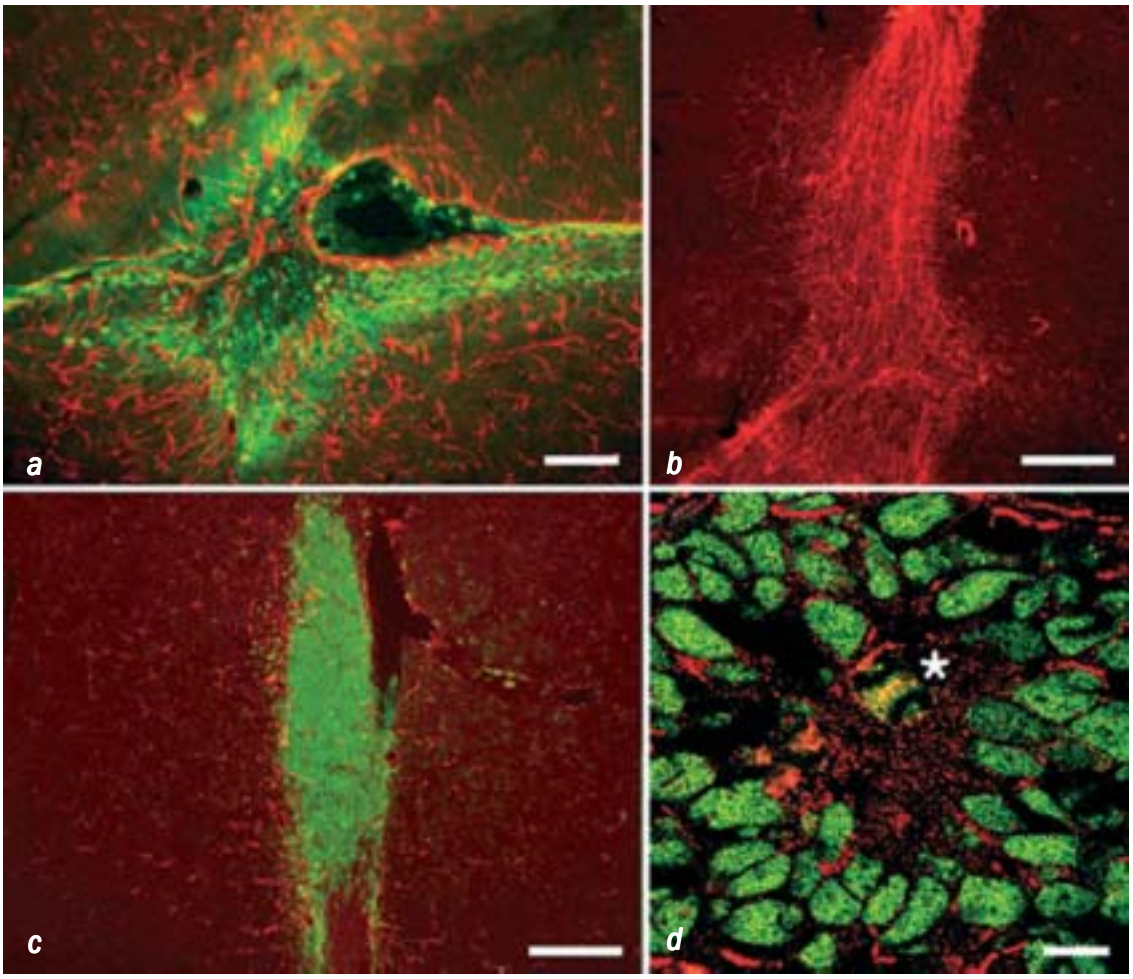

Fig. 3. Cultured HNSC after transplantation into rat brain. Cells grown in cultures with mitogens without LIF degenerate after 20 days and are replaced with glial cells of the recipient (*a*). Staining with antibodies against human cell nuclei (green) and glial protein (red); scale — 40 μ. HNSC cultured with LIF well survived after transplantation; stem cells are present in the transplants (*b*). Staining with antibodies to nestin; scale — 100 μ. The transplants do not induce glial reaction over 30 days (*c*); scale 100 μ. Cells in the transplants proliferate (*d*; asterisk). Staining for nestin and human nuclear protein, confocal microscopy; scale — 10 μ.

content of stem cells tended to decrease, while the number of progenitors increase. Culturing according to the second protocol led to considerable accumulation of stem cells and minor accumulation of progenitor cells, which agrees with published data [15].

The cell suspensions (both protocols) were injected into the hippocampus of intact rats and rats exposed to acute hypoxia. Microscopic examination on days 10, 20, and 30 confirmed the presence of HNSC in the brain of all recipients. Transplanted cells detected by staining with antibodies to human nuclei were seen in the cortex, hippocampus, and, partially, in thalamic structures. The majority of transplanted cells were located in the needle track. Other cells migrated at different distances from the track. It was found that HNSC survival and their migration in the brain of the recipient correlated primarily with methods of culturing, but did not depend on brain damage in the recipient. In intact rats and rats exposed to acute hypoxia HNSC cultured according the first protocol migrated only along the cerebroventricular ependyma and cerebral vessels. Twenty days after transplantation we observed considerable degeneration of these cells in the injection site. In contrast, HNSC cultured according the second protocol well survived (up to 30 days without signs of degeneration) and migrated not only along the ependyma and vessels, but also in the gray matter of the neocortex and hippocampus. In the cortex of rats subjected to acute hypoxia human cells were located in layers III-V at a distance of up to 2 mm from the injection site. In the hippocampus they migrated in the suprapyramydal layer. The transplants in the dense part were vascularized with vessels grown from the recipient brain.

In none experiments tumor formation was noted.

The fate of transplanted HNSC was studied using staining with antibodies to human cell nuclei and specific differentiation markers: human cell nestin, vi-

mentin, class III β-tubulin, neurofilaments-70, GFAP, and neuron-specific protein (Fig. 3). Double staining with antibodies to human nuclei and nestin revealed a considerable number of survived stem cells 10 days after transplantation in all series. However, on day 20 the content of nestin-positive cells considerable decreased in the brain of recipients receiving cells cultured according to the first protocol. At the same time, HNSC grown according to the second protocol well survived even for 30 days. These transplants contained many nestin-positive cells at different stages of mitotic division. In all cases nestin-positive cells had round bodies and long thin processes extended along the transplant or growing into the recipient brain. Migrating stem cells are usually associated with vessels, ependyma, and sometimes with fiber tracts. In some cases nestin-positive cells adjacent to vessels were seen far from the transplantation site. The behavior of nestin-positive cells in the intact and damaged brain was similar.

Antibodies to class III β-tubulin (marker of early neuroblasts) revealed numerous cells with bipolar shape with small processes evenly distributed along the transplants. The content of class III β-tubulin-positive cells in group 1 transplants decreased, because many transplanted cells degenerated by the 20th day after transplantation. In group 2 neuroblasts survived much better. Many cells migrated outside the transplant and were detected in the brain parenchyma.

Immunohistochemical staining with antibodies to vimentin, a marker of astrocyte and neuron precursors, and to nuclear proteins of differentiated neurons showed that group 2 transplants contained a considerable number of committed vimentin-positive cells, but had no differentiated neurons.

Differentiation by the glial type was detected with antibodies to GFAP. It was founds that in all groups of transplants only small number of cells differentiate into astrocytes. Double staining with antibodies to human nuclei and GFAP revealed groups consisting of several tens of cells expressing both markers.

Moreover, staining with antibodies to GFAP showed reaction of recipient glial cells to xenotransplants (human cells). Group 1 transplants provoked gliosis in the surrounding brain tissue. As the transplanted cells underwent degeneration, processes of host astrocytes grew into dense areas of the transplant. In none cases the formation of continuous glial scar at the host-graft boundary was observed. Solitary processes of recipient glial cells grew into the transplants and lay among the transplanted cells or in spaces around vessels also grown from the recipient brain. Double immunochemical staining with antibodies to human nuclei and GFAP revealed no glial reaction around migrated human cells.

Thus, both methods used by us for culturing of HNSC led to the formation of typical neurospheres. Immunochemical analysis confirmed heterogeneous composition of neurospheres. They were formed by cells at different stages of differentiation: stem cells, progenitors, and neuro- and glioblasts. Though the presence of different cell types in neurospheres greatly varied, no differences between cultures grown according to different protocols were revealed at the morphological level. Considerable differences were detected in the behavior of these cultures after transplantation into rat brain. HNSC cultured according to the first protocol (short-term culturing) though migrated along the ependyma of cerebral ventricles and blood vessels in the recipient brain, considerably degenerated by the 20th day after transplantation into both intact and damaged brain. At the same time, HNSC cultured for a long time in the presence of LIF migrated (apart from the above mentioned pathways) in the cortex and hippocampus, which agrees with published data [8]. These cells well survived; proliferating cells persisted in the transplants even on day 30 after transplantation.

These data suggest that transplantation of short-cultured HNSC can be used mainly for the realization of their neurotrophic potential, while transplantation of long-cultured HNSC (in a medium with LIF) can be used for both neurotrophic stimulation and replacement cell therapy.

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REFERENCES

1. M. A. Aleksandrova, *Ontogenez*, **32**, No. 2, 106-113 (2001).
2. L. V. Polezhaev, M. A. Aleksandrova, V. N. Vitvitskii, *et al.*, Transplantation of Brain Tissue in Biology and Medicine [in Russian], Moscow (1993), p. 238.
3. R. J. Armstrong and C. N. Svendsen, *Cell Transplant*., **9**, No. 2, 139-152 (2000).
4. R. A. Barker, M. Jain, R. J. Armstrong, *et al.*, *J. Neurol. Neurosurg. Psychiatry*, No. 74, 553-557 (2003).
5. A. Bjorklund and O. Lindvall, *Nat. Neurosci*., **3**, No. 6, 537-544 (2000).
6. F. Gage, *Science*, **287**, 1433-1438 (2000).
7. F. Gage, J. Ray, and L. J. Fisher, *Annu. Rev. Neurosci.*, No. 18, 159-192 (1995).
8. T. Freeman, F. Cicchetti, R. Hauser, *et al.*, *Proc. Natl. Acad. Sci.* USA, **97**, No. 25, 13,877-13,882 (2000).
9. M. Jain, R. Armstrong, S. Elnei, *et al.*, *Neuroreport*, **14**, No. 9, 1257-1262 (2003).
10. O. Lindvall and P. Hagell, *Clin. Chem. Lab. Med.*, **39**, No. 4, 356-361 (2001).

11. T. Qu, C. Brannen, H. M. Kim, *et al.*, *Neuroreport*, **12**, No. 6, 1127-1132 (2001).
12. K. Sugaya, *Cell Mol. Life. Sci.*, **60**, 1891-1902 (2003).
13. C. N. Svendsen and M. A. Caldwell, *Prog. Brain. Res.*, No. 127, 13-34 (2000).
14. *Transplantation into the mammalian CNS*, Eds. D. M. Gash, Jr. Sladek, Amsterdam, New York. (1988) p. 663.
15. L. Wright, J. Li, M. Caldwell, *et al.*, *J. Neurochem.*, No. 86, 179-195 (2003).