MICROBIOLOGY AND IMMUNOLOGY

Human Fetal Neural Stem Cells in Rat Brain: Effects of Preculturing and Transplantation

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 139, No. 2, pp. 181-184, February, 2005
Original article submitted July 14, 2004

The fate of human fetal stem/progenitor cells transplanted into rat brain depends on conditions of preculturing (long or short) and state and site of transplantation. Human nestin-positive stem cells cultured according to the short protocol did not migrate into hypoxic and normal brain after transplantation, but actively migrated in damaged spinal cord. After transplantation of long-cultured cells into the brain mainly committed neuroblasts and solitary nestin-positive cells migrated from the site of transplantation into the brain.

Key Words: *human brain neural stem cells; xenotransplantation; hypoxia; immunohistochemistry; spinal cord*

Modern cell technologies stimulate the development of novel methods for the treatment of CNS diseases associated with massive neuronal death. Studies of stem cells are considered as the most promising [4,5]. The search for new methods for stimulation of host cerebral stem cells [7,10] and for transplantation of cultured neural stem/progenitor cells is now in progress [4,9,12,13]. This latter approach is the most perspective, because reparative potential of human brain is very limited [11], while transplantation of cultured stem cells can create new massive reparative resources in damaged areas.

In order to carry out effective cell therapy, we should know how cultured stem cells behave after transplantation into different regions of the brain. We studied the effect of the status of recipient brain and the regimen of culturing of the transplanted material on the behavior of human fetal cells transplanted into rat brain [1-5,7,12].

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MATERIALS AND METHODS

Precultured stem/progenitor cells from human fetal brain were transplanted into intact or damaged brain (hypoxic hypoxia), or into mechanically damaged spinal cord of adult rats [1-5,7].

The cells from the brain of 9-12-week human fetuses (obtained during medical abortion) were dissociated and inoculated into growth medium in a concentration of 2×10^6 cell/ml [12]. Two culturing protocols were used: short (14 days) and long (more than 65 days). Short-term culturing was carried out in DMEM and medium F-12 (1:1 ratio) supplemented with N2 complement (1:100), 20 ng/ml fibroblast growth factor (FGF-2), 20 ng/ml epidermal growth factor (EGF), 8 μg/ml heparin, and gentamicin/amphotericin. The same medium with leukemia inhibition factor (LIF)

0007-4888/05/1392-0213

was used for long-term culturing. Long-term culturing was carried out by 2-week periods in medium with bFGF+EGF+LIF (+heparin), medium with bFGF (+heparin), medium with EGF, medium with bFGF (+heparin), and medium with bFGF and LIF (+heparin).

The cell suspension was cultured in these media in culture flasks at 37°C and 5% CO_2. Half of the medium was replaced every 4th day. Cell aggregations were dissociated by repeated pipetting.

Morphological studies of 14-day and 65-day cultures was carried out and cell composition of whole neurospheres and cultures stimulated to differentiation by adding serum was evaluated by cytofluorometry and immunocytochemical analysis. The following antibodies were used: human antinestin (Chemicon 1:30), anti-glial fibrillary acid protein (GFAP; DAKO; 1:250), anti-β-III-tubulin (Abcam, 1:200), antivimentin (NeoMarkers, 1:100), antineurofilaments-70 (Serotec, 1:500). By the moment of transplantation the cultures contained similar percentage of nestin-positive stem cells. However, culturing by the short protocol was associated with a trend to a decrease in the percentage of stem cells and increase in the count of progenitors, while culturing by the long protocol (with LIF) led to an appreciable increase in the percentage of stem cells and a slight decrease in progenitor cell count.

The cells cultured by both protocols were transplanted into the brain of intact rats and rats exposed to hypoxia [1,3]. Experiments were carried out on adult female Wistar rats. The suspension of human neural stem/progenitor brain cells (HNSC) was stereotactically transplanted (3 μl, ~1.5×10^5 cells) into the hippocampus (coordinates A=-3.5-4.0 mm, L=2.5 mm, V=4 mm). Immunosuppression was not performed.

Ten, 20, and 30 days after transplantation the rats were narcotized, the brain was perfused with 4% paraformaldehyde in phosphate buffer, and sections were made on a freezing microtome. Some sections were stained by the methods of Niessl, Giemsa, with hematoxylin and eosin, and other sections were studied by immunocytochemical methods using anti Human nuclei (Chemicon; 1:30) and anti-Neu N antibodies to the nuclear protein of differentiated neurons (Chemicon; 1:30) as first antibodies. The sections were processed as described previously [1-5,7].

Only cells cultured by the short protocol were transplanted into damaged rat spinal cord. In Wistar rats (250-300 g) 30 operations with dosed spinal injury and subsequent transplantation of human fetal stem/progenitor brain cells and 20 operations with dosed spinal injury without cell transplantation (control series) were performed.

In experiments with cell transplantation into damaged spinal cord the animals were subjected to laminectomy under calypsol narcosis, after which contusion of the spinal tissue was inflicted by dropping a 10-g load from the height of 6.5 cm. Directly after the injury, fetal stem/progenitor human brain cells were transplanted into the spinal cord (injected with a microsyringe proximally and distally at a distance of 5 mm from the focus of injury to a depth of 1.5-2 mm, 6×10^5 cells, 3 μl from each side). No immunosuppression was carried out.

RESULTS

The cells cultured by the short and long protocols were injected (as a cell suspension) into the hippocampus of intact rats and animals exposed to hypoxia [1,3,7]. The results of microscopic examination 10, 20, and 30 days after transplantation showed HNSC in the brain of all recipients. Transplanted cells detected by staining with antibodies to human nuclei were distributed in the cortex, hippocampus, and partially in the thalamic compartments of the brain. The majority of transplanted cells were located compactly in the injection track; other cells migrated to different distances.

Human nestin-immunopositive stem cells were small, round, with long processes. Stem cell fibers were detected in the recipient brain near vessels and in axon bundles.

Cells immunopositive to early neuroblast marker β-III-tubulin were evenly distributed in the transplants; they were as a rule bipolar with small processes.

Rat brain astrocytes detected with anti-GFAP were poorly activated in response to human cell transplantation. There were no solid glial cicatrix at the host—graft interface. The recipient glial cell processes grew into the transplants and were situated in the bulk of transplanted cells or in perivascular zones. Just a negligible portion of transplanted cells was differentiated into astrocytes. The reaction of rat brain astrocytes to transplantation of cells cultured by the long protocol was much weaker.

Survival of transplanted cells and their migration in the recipient brain depended on culturing protocol, but not on the recipient brain damage. Twenty days after transplantation of cells cultured by the short protocol the number of nestin-positive cells in recipients notably decreased, while human stem/progenitor cells cultured by the long protocol survived 30 days.

The use of antibodies to human cell nuclei showed appreciable (up to 2 mm) migration of cells from the transplants to the neocortex and hippocampus of hypoxic rats. Short-cultured cells after transplantation into intact and damaged brain migrated along the ventricular ependyma and cerebral vessels of the recipients. Long-cultured cells migrated also in the cortical and hippocampal parenchyma and well survived

there; proliferating cells were found 30 days after transplantation.

β-III-Tubulin-immunopositive neuroblasts exhibited the highest migration capacity. Despite the fact that nestin-positive cell fibers grew into recipient brain tissue, the cells virtually did not migrate from the transplants. The behavior of nestin-positive cells in intact and damaged recipient brain was similar.

Our results indicate that transplanted human fetal neural stem/progenitor cells not only survive, but exert a neuroprotective effect on degenerative neurons in the brain of adult rats pre-exposed to hypoxic hypoxia. This effect manifested in normalization of learning and facilitation of acquisition of active avoidance behavior in the shuttle-box test in rats exposed to hypoxic hypoxia and receiving xenotransplants of cultured human fetal stem/progenitor cells [3]. The transplants were injected into recipient brain 24 h after the exposure which seemed to prevent the development of traumatic posthypoxic effect. Hence, we can speak about stimulation of the compensatory reparative processes in the CNS of rats exposed to hypoxia under the neurotrophic effect of transplanted cells.

Only cells cultured by the short protocol were transplanted into damaged spinal cord [2,5]. Preparations of the spinal cord 1 week after surgery contained notably damaged tissue areas and extensive hemorrhagic zones. Glial reaction and sites of neovascularization were seen at the edges of mechanical injuries.

Two weeks after the injury and transplantation the zone of spinal injury increased. The traumatic area with a vast necrotic zone and foci of hemorrhages involved the entire thickness of the spinal cord and occupied about 1 cm along its axis. A pronounced glial reaction was seen at the edges of the traumatic zone.

One and two months after the injury and transplantation of cultured stem cells the damaged zone was 3-4 cm, without edema or cyst formation; glial reaction was pronounced, but there were no glial cicatrices. Numerous new capillaries were seen at the site of injury. Neurons with normal morphological structure were seen distally and proximally to the damaged area of the spinal cord.

Active revascularization without cyst formation at the site of injury (characteristic of control animals receiving no transplantation) was observed in all animals after transplantation of cultured stem cells. This suggests that human stem/progenitor cells had a positive impact on the posttraumatic processes in the spinal cord of adult rats.

Transplanted human brain stem/progenitor cells survived at least 3 months and retained capacity to migration and differentiation in damaged spinal cord. Human cells migrated along fibers, vessels, and were detected in necrotic zones. Nestin-positive stem cells were detected among implanted cells up to 2 months post-transplantation, but were not detected in animals 3 months after transplantation. Nestin-positive cells in injured rat spinal cord extensively migrated from the site of injection, in contrast to cells transplanted into hypoxic and intact brain, where migration of the nestin-positive cells was minimum. Disappearance of nestin-positive cells in animals survived 3 months after transplantation can be due to elimination of non-differentiated cells, which found no partners for cell-cell interactions, and to differentiation of stem cells.

After mild spinal injury stem cells were located compactly at the site of their injection into the spinal cord, which can be due to insufficiency of "trauma" factors stimulating migration and differentiation.

Transplantation of cultured human fetal brain stem cells into the spinal cord of rats after mechanical injury led to revascularization, but not to the formation of cysts and glial cicatrix. This confirmed the results of other investigations [15] and indicated a protective effect of implanted stem and progenitor cells, presumably, at the expense of a wide spectrum of neurotropic factors released by them [8].

Duration of survival in the recipient brain, capacity to migration, and reaction of the recipient tissue depend on the characteristics of human fetal transplanted cells and on the status and site of transplantation. Long-cultured cells survived longer in rat brain and more extensively migrated from the site of injection causing less pronounced glial reaction of the recipient brain tissue compared to short-cultured cells. On the other hand, migration of human nestin-positive stem cells was minor in the brain, but more pronounced in damaged spinal cord. Hence, for possible clinical application of human fetal neural stem/progenitor cells the material should correspond to the therapeutic purpose of treatment and location of transplantation. Transplantation of short-cultured human fetal neural cells can be used mainly for the realization of their neurotrophic potential. If these cells are transplanted into the spinal cord, they can be also used for replacement cell therapy. Long-cultured cells are preferable for replacement of dead cells in the brain.

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