Transplantation of Cultured Neural Cells from Human Fetuses into the Brain of Rats Exposed to Acute Hypoxia

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Neural stem cells of human brain were cultured for a long time and successfully transplanted into the brain of rats exposed to acute hypoxia. Stem and committed cells, neuroblasts, and astrocytes were revealed in transplants by immunohistochemical assay. The transplants and brain tissue were not separated with a glial barrier. Human neuroblasts widely migrated into regions of neuronal degeneration in the host brain.

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

Oxygen deficiency leads to extensive and severe diseases of the central nervous system (CNS). Hypoxia/ischemia causes encephalopathies during the early postnatal ontogeny (particularly perinatal asphyxia of newborns) and strokes in adults. Etiology of these disorders differs in adults and children. However, disturbances associated with degeneration and dysfunction of neurons have similar pathophysiological manifestations [1,8].

There are no methods for effective therapy of strokes and perinatal encephalopathy. Cells recovery and replacement during degeneration in CNS are an urgent problem. It necessitates the development of new approaches to the therapy of these conditions [5]. According to current notions, there are several approaches to the therapy of these disorders. Stem cells hold much promise in this respect [4,8]. Much attention is given to the search for factors stimulating own stem cells [6,12] and transplantation of cultured neural stem/progenitor cells [7,8,10,11]. The latter approach is of particular importance, since the intensity of reparative processes for own stem cells is extremely low [9]. Transplantation of cultured stem cells provides a new massive neurogenic source in various regions of the damaged brain.

Here we studied the development of human neural stem cells (HNSC) after transplantation into the brain of adult rats exposed to acute hypoxia. This treatment induced diffuse degeneration and death of neurons in the cerebral cortex and hippocampus [1]. We determined the ability of long-cultured HNSC to survive in a pathological microenvironment of the brain in rats after hypoxia.

MATERIALS AND METHODS

Neural stem cells were isolated from the whole brain of human fetuses obtained from healthy women after medical abortion (9.5 weeks' gestation). Primary cultures of dissociated neural cells had 70% viability. The cells (5×10^5 cells/ml) were cultured in DMEM/F12 growth medium with N-2-supplement containing standard growth factors: basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and leukemia-inhibiting factor (LIF). Culturing was performed in media with the following factors: bFGF, EGF, and LIF (+heparin, 2 weeks); bFGF (+heparin, 2 weeks); EGF (2 weeks); bFGF (+heparin, 2 weeks); and bFGF

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and LIF (+heparin, 2 weeks). The cells were phenotyped by the method of flow cytofluorometry. The percent of nestin-positive cells increased from 20 to 40% over the period of culturing. Neural stem cells with these characteristics were used for transplantation.

Experiments were performed on 20 female Wistar rats weighing 250-300 g. The animals were exposed to 3-min hypoxic hypoxia in a special altitude chamber (180 mm Hg) [2]. This procedure caused clonic convulsions in experimental animals; 8 rats died. Survivors were divided into 2 groups (transplantation, group 1; control, group 2). Group 1 rats were anesthetized with 300 mg/kg chloral hydrate on day 1 after hypoxia. The suspension of neural stem/progenitor cells from human brain (3 μl, 1.5×10^5 cells) was stereotactically transplanted into the brain of animals (A 3.5-4 mm; L, 2, 5 mm; V, 5 mm). Transplantation of HNSC was not followed by immunosuppression.

The rats were narcotized 27 days after transplantation. The brain was perfused with 4% paraformaldehyde in phosphate buffer. Brain sections were prepared on a freezing microtome. Brain sections were treated with Nissl stain, hematoxylin and eosin, and Giemsa stain. Other sections were assayed immunohistochemically using primary antibodies against human nestin (anti Human Nestin, 1:20, Chemicon), neurofilaments 70 (anti Neurofilament-70, 1:500, Serotec), human cell nuclei (anti Human Nuclei, 1:30, Chemicon), gliofibrillar acid protein (GFAP, anti GFAP, 1:250, DAKO), anti β-tubulin III-β-III tubulin (Abcam, 1:200), vimentin (anti Vimentin Santa Cruz, 1:100), and differentiated neuron nuclear protein (anti-Neu N, 1:30, Chemicon). Samples were treated with biotinylated secondary antibodies (Jackson ImmunoResearch Laboratories, dilution 1:200) and stained by streptavidin labeled with fluorescence dye Texas Red for 1 h (Jackson). Secondary antibodies labeled with fluorescence dyes Texas Red and Cy-2 (Jackson) were used for double immunocytochemical staining. After washing the samples were clarified with 50% glycerol in phosphate buffer and examined in luminescent or combined light.

RESULTS

Acute hypoxia induced degeneration of neurons in layers II-IV of the neocortex and pyramidal layers of the hippocampus. These zones contained cells in the state of chromatolysis, hyperchromatic neurons, and shrunken and lysed cells. Despite pronounced generative changes in the surrounding tissue, HNSC transplants were characterized by good survival in the brain of rats exposed to hypoxia. HNSC arranged in a column were found in the cortex, hippocampus, and partially in the thalamus (Fig. 1). Transplanted cells were small (7-9 μ). Transplants were vascularized with vessels from the host brain. Non-scarred zones and regions with moderate glial scars were revealed at the boundary between the transplants and host brain tissue.

Human antinuclear antibodies clearly identified HNSC and served as the marker for human cells in rat

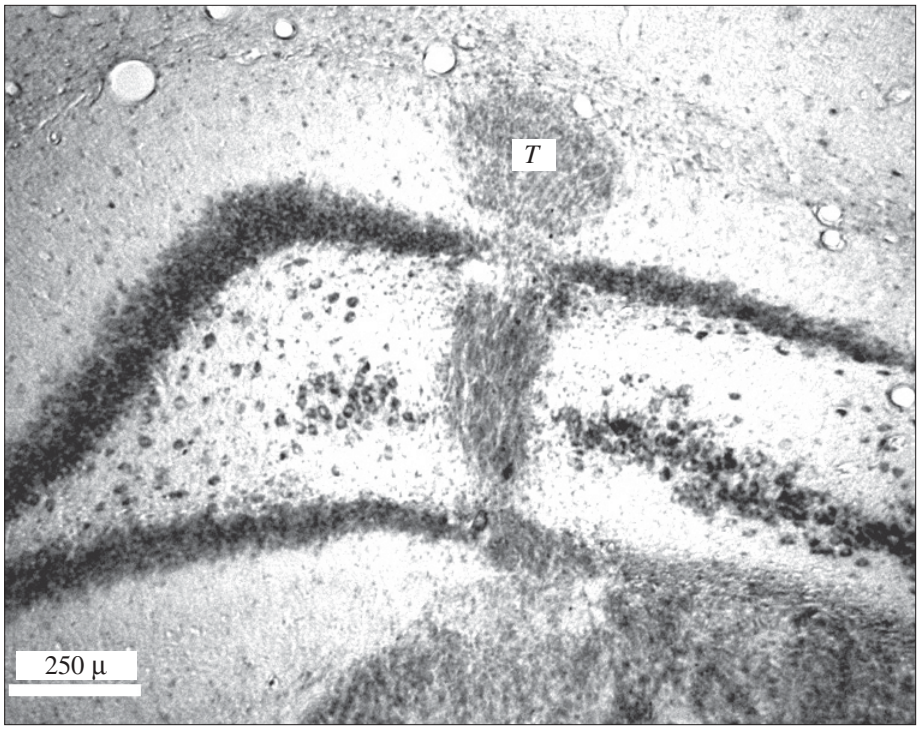

Fig. 1. Transplant of human brain neural stem cells (*T*) in the hippocampus and thalamus of rats exposed to acute hypoxia: cresyl violet staining.

brain. Treatment with these antibodies revealed pronounced migration of cells (up to 2 mm) from the transplants into the neocortex (Fig. 2) and hippocampus. Cells migrated to layers III-IV of the cerebral cortex. Neurons localized in this region are most susceptible to hypoxic injury. Transplanted cells also migrated over the suprapyramidal layer in the hippocampus. It should be emphasized that transplanted cells spread in the brain parenchyma and blood vessels, but not in fibers.

Immunohistochemical staining for human nestin showed that transplants included a considerable number of cells. They expressed nestin, which serves as the marker for stem cells. These cells had small round bodies and long oppositely directed processes. Some processes were directed along the long axis of the transplant. Sometimes they formed bundles or irregular network. At the site of contact with the host brain, processes of human stem cells were positioned perpendicular to the boundary of the transplant and grew into the adjacent tissue (Fig. 3). In the host brain, stem cell fibers were found near vessels and in axon bundles. Fibers of nestin-positive cells grew into the brain tissue. However, these cells did not migrate from the transplants. Study of preparations revealed only single stem cells migrating from the transplants into the neocortex.

Antibodies to β-III-tubulin (marker of early fibroblasts) revealed a considerable number of cells in the transplants. They were regularly distributed in the transplants and usually had a bipolar shape with small processes. Many cells migrated from the transplants and were found in the host brain parenchyma. Double immunocytochemical staining for nestin and β-III-tubulin showed that these proteins are synthesized by different cells. Numerous fibroblasts were seen between nestin-positive cells. These cells had short processes rarely leaving the transplants.

Staining with antibodies against glial fibrillary acid protein showed reaction of host glial cells and differentiation of astrocytes in the transplants. Rat brain astrocytes were slightly activated in response to xenotransplanted human cells. No continuous glial scars were revealed at the host-transplant boundary. Only in some regions, glial fibers lay along the boundary. Double immunocytochemical staining with antibodies to human nuclei and glial protein did not reveal glial reaction around migrating human cells. Processes of host glial cells grew into transplants and were localized between transplanted cells or in the perivascular space.

Only a small number of transplanted cells underwent differentiation into astrocytes. Double immunocytochemical staining with antibodies to human nuclei and glial protein identified several groups of 5-10 cells expressing both markers. Astroblasts were morphologically similar to nestin-positive cells in the transplants.

Double immunocytochemical staining with antibodies to vimentin (marker of astrocyte precursors and neurons) and differentiated neuron nuclei showed that

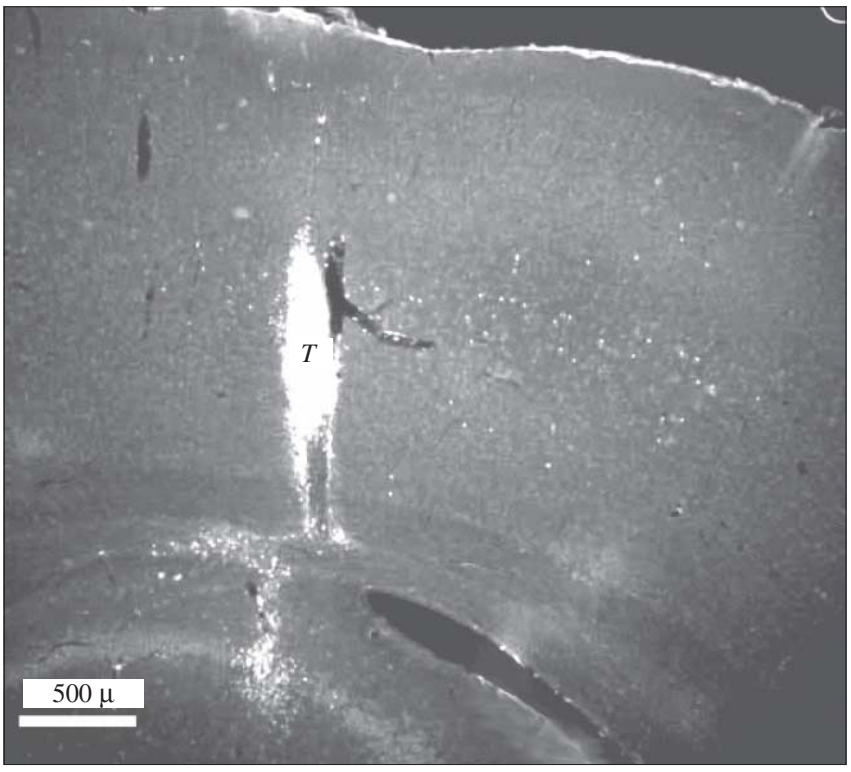

Fig. 2. Migration of human neural cells from the transplant (*T*) to layers III-V of the neocortex and CA-1 field of the hippocampus in rats exposed to hypoxia: staining with antibodies to human cell nuclei (Human Nuclei).

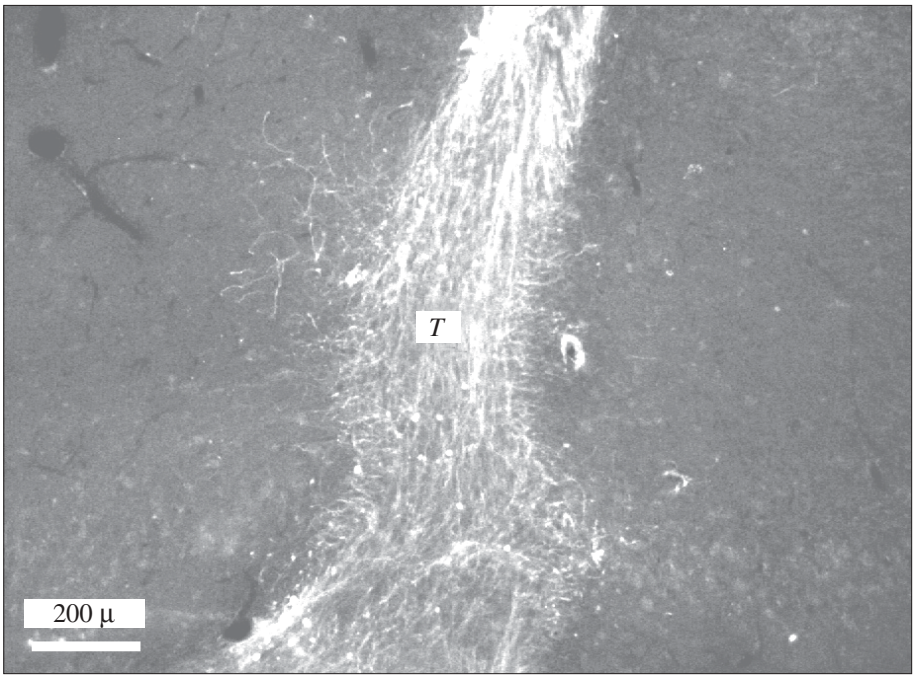

Fig. 3. Transplant of human neural stem cells (*T*) in rat hippocampus: staining with antibodies to human nestin.

transplants contain a considerable number of committed neural cells, but not differentiated neurons.

We revealed for the first time that long-cultured human brain neural stem cells survive in the brain of rats after acute hypoxia. Acute hypoxia induced damage to the blood-brain barrier [3]. However, transplanted cells did not cause the immune response and were not rejected for at least 27 days. The transplants contained different cell populations, including stem cells, committed cells, early fibroblasts, and astrocytes. A considerable number of stem and committed cells survived for a long time, which attested to high differentiation potential of transplanted cells. The glial barrier between the transplants and host brain tissue was absent, which promoted the growth and migration of transplanted cell fibers. Neuroblasts had the highest migration activity and spread in regions of neuronal degeneration in the host brain.

Our study showed that cultured HNSC survive, retain multipotent activity, and migrate in the brain with extensive neuronal degeneration. Transplanted stem cells hold much promise for replacement therapy of neurodegenerative brain diseases associated with hypoxia/ischemia.

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