In Vitro Development of Neural Progenitor Cells from Human Embryos

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Behavior of stem/progenitor cells from the brain of human embryos during *in vitro* culturing was studied. Cultured cells from human embryonic brain developed and formed neurospheres heterogeneous by their cell composition. In a serum-containing medium some cells underwent differentiation by the neuronal pathway, while others remained in the stem state.

Key Words: *neural stem/progenitor cells in human brain; tissue culture; development; differentiation*

In vitro and *in vivo* identification of self-renewing neural progenitor cells capable of differentiating into neurons, astrocytes, and oligodendrocytes allows us to study cellular mechanisms underlying reparation and development of the central nervous system (CNS) [2, 3,6,8]. Neural progenitors are an invaluable source of cells for clinical transplantation, which opens new vistas in the therapy of neuropathological disorders [1,4,6,9,10,12,14]. Isolation of undifferentiated neural progenitors from human embryonic brain holds much promise in this respect [4,5,7,11-13]. These immature cells possess higher plasticity and proliferative activity than their analogues from adult brain.

Of primary importance is the choice of optimum culturing conditions of neural cells providing sufficient biological material for transplantation. There are no standard methods for isolation, growth, and culturing of neural cells from human fetuses, which would maintain them in the undifferentiated state [5,14].

Here we studied the behavior of neural stem/progenitor cells from human embryonic brain during *in vitro* culturing and developed optimum conditions for their self-renewal and differentiation.

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MATERIALS AND METHODS

The materials after abortions were obtained from licensed medical institutions (Russian Ministry of Health). Abortions were performed for reasons that fell into approved general categories (legislation on health protection of citizens of the Russian Federation). We used nonviable human fetuses weighing less than 500 g and obtained from healthy women, which terminated pregnancy at 17-21 weeks gestation by social prescriptions (recommendations of the World Health Organization approved by the Russian Ministry of Health).

The materials were placed on ice in a sterile container and transported to the autopsy department within 3 h after induced abortion. The fetuses without visible developmental defects and skin injuries were treated with a disinfectant. Brain regions evolutionarily enriched with neural stem cells were isolated: paraventricular and subventricular zones of the forebrain, dentate gyrus of the hippocampus, and olfactory bulbs. Brain membranes were thoroughly removed. Tissue samples were placed in 60-mm Petri dishes (Costar) with 2 ml medium F-12 and mechanically disintegrated by pipetting to obtain a suspension of individual cells. Cell aggregates were removed. The primary cell suspension was centrifuged at 800 rpm for 5 min and washed 2 times with medium F-12. The

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cells were counted, and their viability was estimated by staining with trypan blue and propidium iodide. Cell suspensions containing not less than 60% viable cells were used for culturing.

Two media were used for cell culturing: NPBM medium (Clonetics) supplemented with standard growth factors (fibroblast growth factor, neuronal survival factor, and epidermal growth factor, EGF), gentamicin, and amphotericin B (NPMM, Clonetics) and a medium developed at the laboratory of Snyder [7] containing DMEM and F-12 (ratio 1:1), N2 (1:100), 20 ng/ml fibroblast growth factor, 8 μg/ml heparin, 250 μg/ml penicillin or streptomycin, and (in some series) 20 ng/ml EGF.

The cells (2×10^6/ml) were grown in suspensions; the medium was replaced (50%) every 4 days. The culture was repeatedly pipetted to prevent cell aggregation. The cells were cultured at 37°C and 5% CO_2 for 12-16 days and then transferred into a medium with 10% fetal bovine serum (FBS) to induce differentiation. After 4-day culturing these cells were studied by immunocytochemical methods. The cells were fixed with 4% paraformaldehyde in phosphate buffer for 20-30 min at room temperature, washed, and treated with 0.3% Triton X-100. Primary antibodies to nestin (Biogenesis, 1:20) and β-tubulin III (ICN, 1:100) were added. After overnight incubation the cells were treated with biotinylated secondary antibodies for 1 h (dilution 1:200, Vector Laboratories) and stained with DiI-labeled streptavidin (Molecular Probes).

RESULTS

Floating aggregates consisting of more than 100 cells were found in both media after 2-day culturing. The cells were counted, and their viability was estimated during pipetting and medium replacement; 30% cells died over the first week of culturing.

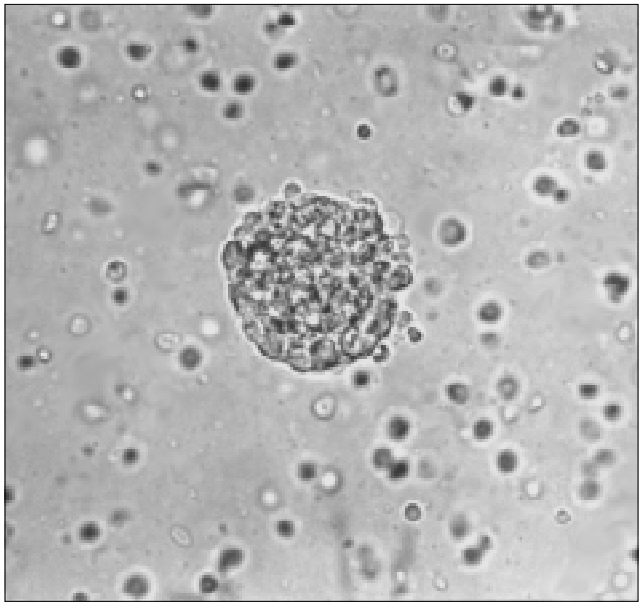

Fig. 1. Small neurosphere on day 7 of culturing on NPBM medium. Unstained preparation (×200).

After 7-day culturing in NPBM medium we found small cell aggregates, which looked like neurospheres (Fig. 1). Probably, these aggregates were true neurospheres stemming from one cell (these structures are below designated as neurospheres). In Snyder medium these neurospheres were formed on days 8-9 of culturing and were less abundant than in NPBM medium. The formation of neurospheres was more intensive on days 9-10 of culturing. Neurospheres progressively in-

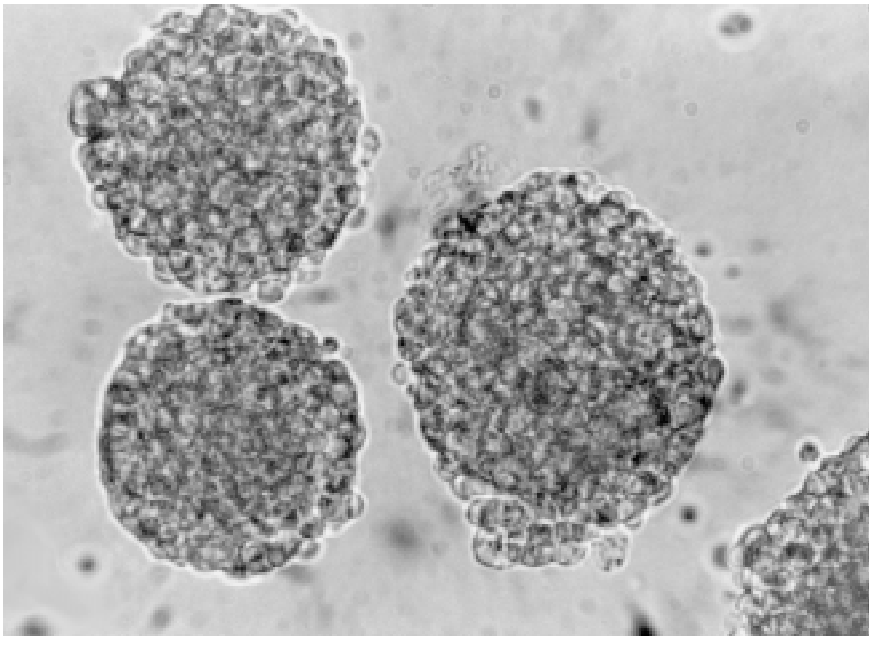

Fig. 2. Large neurospheres on day 10 of culturing on NPBM medium. Unstained preparation (×200).

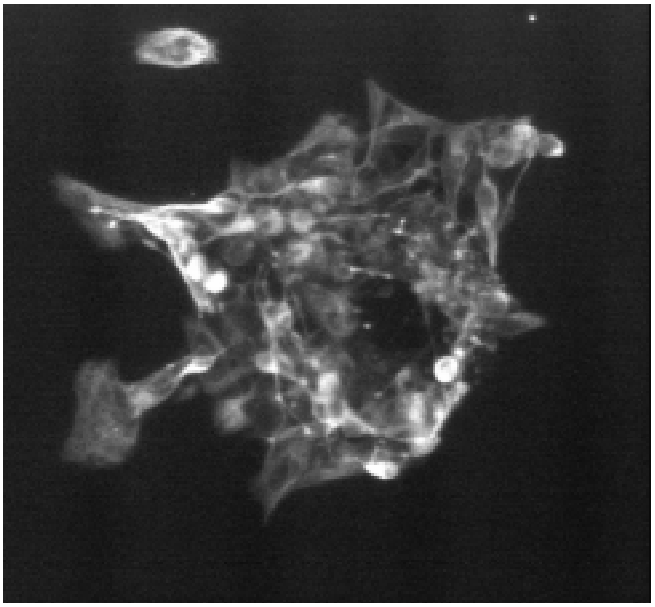

Fig. 3. Cells migrating on flask bottom in a differentiating culture after transition into complete nutrient medium. Bright β-tubulin-positive cells. Immunohistochemical staining for β-tubulin (×400).

creased in size and looked like round structures consisting of more than 30-100 cells in diameter (Fig. 2). Small neurospheres containing tens cells were most viable. Large neurospheres consisting of hundreds cells were surrounded by a dense membrane. Trypan blue staining revealed patchy distribution of dead cells in some large neurospheres.

On days 7-9 of culturing the formation of neurospheres was accompanied by the appearance of large cell aggregates, especially in Snyder medium. Cultured cells were repeatedly pipetted, and aggregates were disintegrated into individual cells that formed small neurospheres. Dead cells were removed by centrifugation or precipitation, which allowed obtaining a pure neurosphere culture. After addition of EGF the cell growth in Snyder medium was similar to that in NPBM medium.

For evaluation of cell differentiation capacity in neurospheres, the cells were cultured in a medium supplemented with 10% FBS. In this medium neurospheres adhered to the plastic and the cells entered differentiation. Staining with antibodies to nestin and β-tubulin III revealed the presence of individual migrating nestin-positive stem cells. Staining with β-tubulin antibodies showed that most cells underwent differentiation by the neuronal type (Fig. 3). These bipolar cells had elongated processes. Various growth cones were found.

Our results indicate that *in vitro* cultured brain cells isolated from human fetuses at 17-21 weeks gestation developed and formed neurospheres. Cell growth and formation of neurospheres were more intensive on NPBM and EGF-containing Snyder media, while on EGF-free Snyder medium these processes were delayed. In a FBS-supplemented medium some cells underwent differentiation by the neuronal type, while others remained in the stem state.

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