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Electron Probe Microanalysis of Potassium and Sodium in Clonogenic Culture of Human Neural Stem Cells

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The mean potassium and sodium concentrations and distribution of potassium in clonogenic culture of human neural stem cells (neurosphere) were estimated by means of electron probe microanalysis. High sodium concentration was typical of undifferentiated cells. Potassium was irregularly distributed in the test structure. Our results confirm published data on heterogeneous morphological structure of neurospheres.

Key Words: *neurosphere; potassium; sodium; electron probe microanalysis*

The study of the origin and development of neural stem cells (NSC) possessing multipotent activity and characterized by clonal growth and self-renewal is a priority direction in cell biology. Much attention is paid to various fundamental problems, including localization of NSC in the central nervous system (CNS), expression of cell markers, differentiation, and use of NSC grafts in the therapy of CNS pathologies [2,5,8, 9,14]. High therapeutic effectiveness of neurotransplantation was demonstrated in Parkinson's disease, spinal cord traumas, and disseminated sclerosis. Cell grafts can be used for the therapy of patients with stroke. This disease is a leading cause of disability and mortality. Apart from neuronal death in the central infarction area an important role in the pathogenesis and pathokinesis of strokeis is played by penumbra, where cascade pathobiological events induce degeneration of viable cells. The use of high-activity cultures from NSC holds much promise for timely correction of ischemic stroke [2].

NSC can be isolated from embryonic or adult brain. Cultured NSC form spherical clones (neurospheres) [6,8]. Therapeutic activity of clonogenic cultures of NSC depends on the ratio of low differentiated and proliferating cells and progression of intraclonal apoptosis and necrosis. Neurospheres are heterogeneous by their ultrastructural characteristics [7,12]. Differences were revealed in the size of cells, cytoplasmic contents, and presence and distribution of active mitochondria [12]. Clonal cells are characterized by asynchronous development and exhibit various phases of the cell cycle, mitosis, and apoptosis. It depends on spatial distribution of cells in the neurosphere.

A relationship was found between intracellular ion balance and mitotic activity [1,10]. Initiation of mitosis is associated with changes in the transmembrane potential. It was hypothesized that these changes are associated with variations in intracellular Na^+/K^+ balance [10].

Flame photometry studies showed that potassium and sodium concentrations undergo significant changes in proliferating Ehrlich ascitic tumor cells [9]. In contrast to proliferating cells, differentiated cells are characterized by high potassium concentration and low sodium content in the cytoplasm [10]. This balance is maintained by Na^+/K^+-ATPase, which compensates oppositely directed passive transport of K^+ and Na^+ cations along the electrochemical gradient. Variations in the Na^+/K^+ ratio serve as a criterion for functional changes in the cell.

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Here we studied the concentration and distribution of potassium and sodium in clonogenic neurosphere. Taking into account that the size of this structure is similar to that of the early mammalian embryo, the study was performed by the method of electron probe microanalysis (EPMA) [1,3].

MATERIALS AND METHODS

Human neurospheres were cultured at the Institute of Stem Cell and Cell Technologies. Human embryonic brain was obtained during abortion (6-10 weeks' gestation). Embryonic forebrain was prepared, minced, and incubated with 2% dispase (Invitrogen) for 30 min in a CO_2 incubator. The mixture was pipetted at 10-min intervals. After complete dissociation of tissue fragments the cells were pelleted at 200*g* for 7 min. The ratio of viable cells was estimated by trypan blue elimination. Culturing was performed with cell suspensions containing 60-100% living cells.

Culturing was performed in 6-mm Petri dishes (Corning) at 37°C and 5% CO_2 [5-9]. The initial density of inoculated cells was 2×10^6 cells/ml. The growth medium was prepared from DMEM/F12 1:1 medium (Invitrogen) and contained 10 ng/ml FGF-2, 20 ng/ml EGF (Sigma), complex of mitogens N2 (1:100, Invitrogen), and 8 U/ml heparin. The growth medium (50%) was replaced at 3-day intervals. Neurospheres were not dissociated.

Small neurospheres appeared on days 5-7 of culturing and then their size increased. After 2-week culturing, the medium included neurospheres that could be classified by size (small, medium, and large neurospheres) and homogeneity in phase contrast microscopy (light homogenous neurospheres of regular shape; and dark granular neurospheres with irregular boundaries). Neurospheres were collected with a micropipette and classified under an inverted microscope.

The elemental composition of each neurosphere was estimated on semithin sections by the method of EPMA. The samples were obtained by preparative low-temperature dehydration [14]. Neurospheres were frozen in liquid propane and lyophilized at low temperature (200°K) under high vacuum conditions (10^{-4} Pa). After low-temperature dehydration, dried cells were heated in a vacuum chamber of the lyophilizer to 300°K. The samples were transferred to capsules with a mounting medium from epoxy resin Epon 812. This procedure was performed in inert gas medium. Further polymerization of resin was conducted at 330°K.

The sections (2-μ width) were prepared from resin-embedded neurospheres using a Reichert microtome with a dry glass knife. For electron microscopy, these sections were mounted on a copper grid. Carbon was layered (40 nm) on the surface of each section by means of thermal spraying under vacuum conditions. Spraying was performed in a Micro BA 3 vacuum device (Balzers).

The concentrations of potassium and sodium in lyophilized and resin-embedded neurospheres were measured by EPMA. The study was performed on a JSM-U3 scanning electron microscope/microanalyzer (JEOL) equipped with crystal diffraction X-ray spectrometers. We recorded the most intensive Ka line of a characteristic X-ray spectrum for potassium and sodium.

Microanalysis of potassium and sodium was performed under the following conditions: accelerating voltage 25 kV; probe electron current 5 nA; and pulse storage time 20 sec [13]. To estimate the average concentration of each element, the count of K_α line pulses was recorded from a square raster inscribed in a contour of the neurosphere. The spatial distribution of potassium was determined by the scanning line drawn along the neurosphere. It was conducted during recording of the characteristic X-ray curve. Morphology was evaluated in a passed electron mode.

RESULTS

Experiments were performed with homogenous light neurospheres with a diameter of 80 μ (Figs. 1 and 2).

The concentrations of potassium and sodium in cell cytoplasm significantly varied in neurospheres (130±30 and 100±10 mM, respectively). These data illustrate differences in physiological activity of examined clones. Sodium concentration was high. Even with consideration for extracellular space, sodium concentration was much higher compared to that typical of differentiated cell. For example, cytoplasmic sodium concentrations in the primary culture of cardiomyocytes and fibroblasts are 35 [13] and 25 mM, respectively [4].

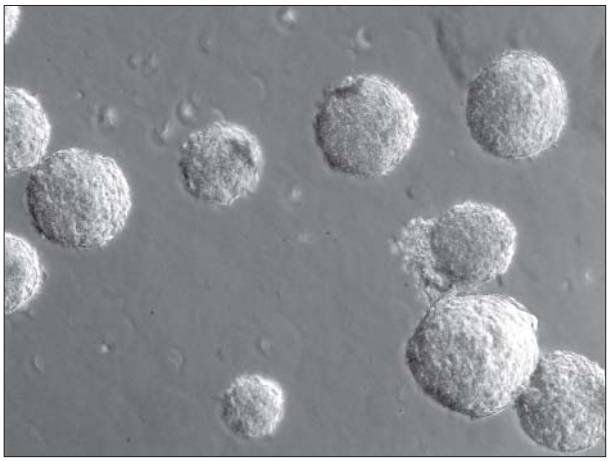

Fig. 1. Human neural stem cells (neurospheres, 14-day culture) used to preparing samples for electron probe microanalysis. Relief phase contrast, ×200.

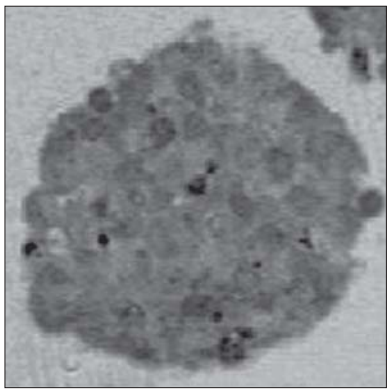

Fig. 2. Section of human clonogenic neurosphere stained with methylene blue (2 μ).

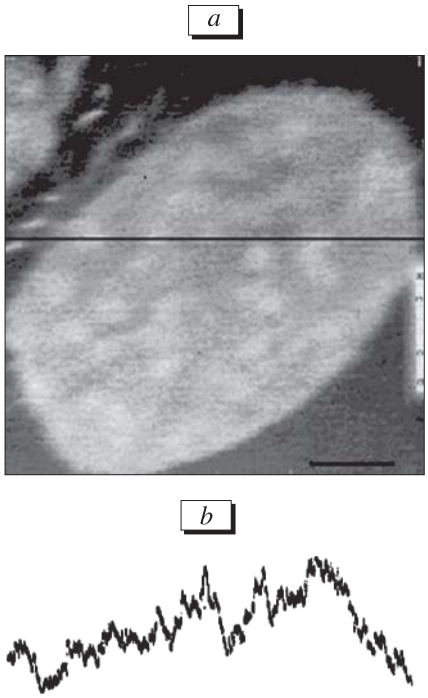

Fig. 3. Qualitative electron probe microanalysis of human clonogenic neurosphere. Section (2 μ) under a scanning electron microscope (passing electron mode), scanning line (*a*); potassium distribution along the scanning line (*b*).

Potassium was irregularly distributed in the test structure (Fig. 3). These results agree with published data on ultrastructural and morphological heterogeneity of neurospheres [7]. Let us consider the regulation of cell division by changes in cytoplasmic potassium balance. Under these conditions, variations in potassium concentration in human neurosphere probably correspond to the unsynchronized state of the cell. Morphological signs for various phases of the cell cycle, mitosis, and apoptosis were revealed in the clone [7]. The value of intracellular potassium concentration under physiological conditions illustrates normal function of Na^+/K^+-ATPase.

Our results indicate that EPMA is an effective method for evaluation of physiological activity of clonogenic neurotransplants by the concentration of main cytoplasmic electrolytes.

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