Functional Activity of Mitochondria in Cultured Neural Precursor Cells

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We studied mitochondrial transmembrane potential of neural precursor cells forming neurospheres in culture. Uneven energization of mitochondria in neurosphere cells was detected. Heterogeneity of cells by the mitochondrial potential increased with neurosphere enlargement during culturing. Decrease in the mitochondrial potential in the central cells in large spheres, presumably caused by insufficient diffusion of oxygen and nutrients, can provoke their damage and death. Population of cells with high mitochondrial potential responded to addition of the nuclear dye by a decrease in mitochondrial potential, which can indicate functioning of ABCG2 complex in these cells, characteristic of undifferentiated stem cells. These data will help to create optimum conditions for culturing of neural stem cells for the maintenance of their maximum functional and proliferative activity.

Key Words: *mitochondria; transmembrane potential; neurosphere; neural stem cells*

Problems of neural stem cell culturing, multiplication, and differentiation *in vitro* are actively studied in many Russian and foreign laboratories. The interest to biology of neural progenitor cells is primarily due to the prospects of using these cells for medical biotechnology purposes. Transplantation of stem/progenitor cells for the treatment of neurodegenerative diseases, inflammatory injuries to the CNS, spinal and cerebral injuries seems to be the most promising approach [5,10]. Fetal neural stem cells possess a wide spectrum of differentiation potentials and therefore ideally fit for replacement therapy and neurotrophic stimulation [4,6].

Numerous studies in this sphere are devoted to the effects of growth factors, neurotrophins, and other components of culture media on cells in order to obtain pools of constantly proliferating cells or cells differentiating in a certain direction. Phenotyping of neural stem cells during their development and differentiation in culture and after transplantation to experimental animals was described [1,3,7]. Multipotent neural cells divide and form neurospheres during culturing in selective media [13], the number and viability of stem and progenitor cells increasing during these processes.

However, functional activity of stem cells, specifically, their energy status, is virtually neglected in these studies. The status of mitochondria in cultured neurospheres can serve as a criterion of optimum conditions for culturing and isolation of a population of neural stem cells with high proliferative activity and differentiation potential.

We evaluated the status of the mitochondrial system in cultured neural stem/progenitor cells isolated from fetal brain and characterized the energy status of cell mitochondria at different stages of culturing.

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MATERIALS AND METHODS

Neural stem cells were isolated from the brain of 9-10-week human fetuses obtained as a result of medical abortions. Viable cells were counted using Trypan blue and propidium iodide staining; cells with at least 60% viability were used for culturing.

The cells were cultured in DMEM and F-12 medium (1:1) with N-2 factor (1:100), 20 ng/ml fibroblast growth factor (FGF-2), 20 ng/ml epidermal growth factor (EGF), 8 μg/ml heparin, and 0.01% gentamicin. The cell suspension (about 2×10^6/ml) was cultured in 25-cm^2 Corning culture flasks at 37°C in a humid atmosphere with 5% CO_2.

In order to evaluate changes in the size of forming neurospheres, 50% medium in the flasks was replaced every 4 days without dissociating the aggregates by pipetting. In other cases the aggregates were dissociated by repeated pipetting during medium replacement.

The size of neurospheres was analyzed every several days under an inverted phase contrast microscope fitted with a digital camera. The resultant digital images were computer processed, the size of all neurospheres in a visual field was evaluated (at least 10 visual fields).

Transmembrane potential ($\Delta\psi$) of neural cell mitochondria was studied using tetramethyl rhodamine ethyl ester (TMRE). Due to its lipophilic polycation structure this dye penetrates into the mitochondrial matrix and accumulates there in accordance with the $\Delta\psi$ value. This provides imaging of individual cellular mitochondria; the mitochondrial potential can be evaluated by TMRE fluorescence intensity. Neurospheres were incubated with 200 nM TMRE for 40 min at 37°C.

Another mitochondrial dye, JC-1 (Molecular Probes), was used in some experiments. This dye penetrates into mitochondria similarly as TMRE, but its distinctive feature is a shift of fluorescence wavelength in high-energy mitochondria. As a result, cell mitochondria with low potential exhibit green fluorescence and mitochondria with high potential fluoresce red.

After staining the neurospheres were placed onto a plastic Petri dish with a poly-L-lysine-coated glass insert and examined under a confocal laser scanning microscope LSM510 (Carl Zeiss) with attached software. Stimulatory laser with λ=543 nm was used for TMRE, collecting emission above 590 nm; for JC-1 stimulatory laser with λ=488 nm was used, collecting emission in the 510-530 nm interval and above 590 nm. All measurements were carried out at 37°C.

In order to evaluate the functioning of ABC complex, the cells loaded with JC-1 were stained with Hoechst 33342 dye in a final concentration of 5 μg/ml. Incubation was carried out at 37°C for 40 min, confocal image of JC-1 fluorescence was recorded every 5 min (one neurosphere was examined). The fluorescence intensity was evaluated by digital image processing using ImageJ software (NIH, Bethesda).

RESULTS

Changes in the linear sizes of neurospheres cultured without mechanical dissociation were studied. Without mechanical fragmentation (pipetting during culture medium replacement) neurospheres in the culture were homogeneous by size during the entire period of culturing. The mean size of neurospheres in culture increased during culturing due to cell proliferation (Fig. 1). Hence, neural stem cells actively proliferated during culturing in selective medium, which led to enlargement of aggregates (culturing without dissociation) or to variations in their size from several cells (30-50 μ) to aggregates of 250 μ and larger (culturing with regular dissociation).

TMRE-stained mitochondria of neurosphere cells had stab or thread-like structure, normally characteristic of mitochondria in the majority of cells. Comparison of confocal images of mitochondria in neurospheres at different terms of culturing showed heterogeneity of TMRE fluorescence in the neurosphere volume. As a neurosphere grew, a zone of weak TMRE fluorescence appeared and increased in its center, with weak TMRE fluorescence in the central part of the sphere (Fig. 2). This was most demonstrative in spheres with a diameter of >150 μ. The decrease of TMRE fluorescence intensity from the periphery to the center of the neurosphere indicated a decrease in the mitochondrial transmembrane potential in neural cells situated in the thickness of the sphere. Presumably, this phenomenon is due to intensive metabolism of surface cells of the sphere, causing a decrease in oxygen and nutrients delivery to the central part of the sphere, as a result of which the cells in the center are unable to maintenance of high energy potential of their mitochondria. In addition, the cells in the center of large aggregates can suffer from growth factor insufficiency, which can lead to their inadequate functioning or death.

However, even in small spheres the cells were heterogeneous by mitochondrial potential, which was detected by JC-1 probe staining. Green fluorescence of mitochondria was observed in some

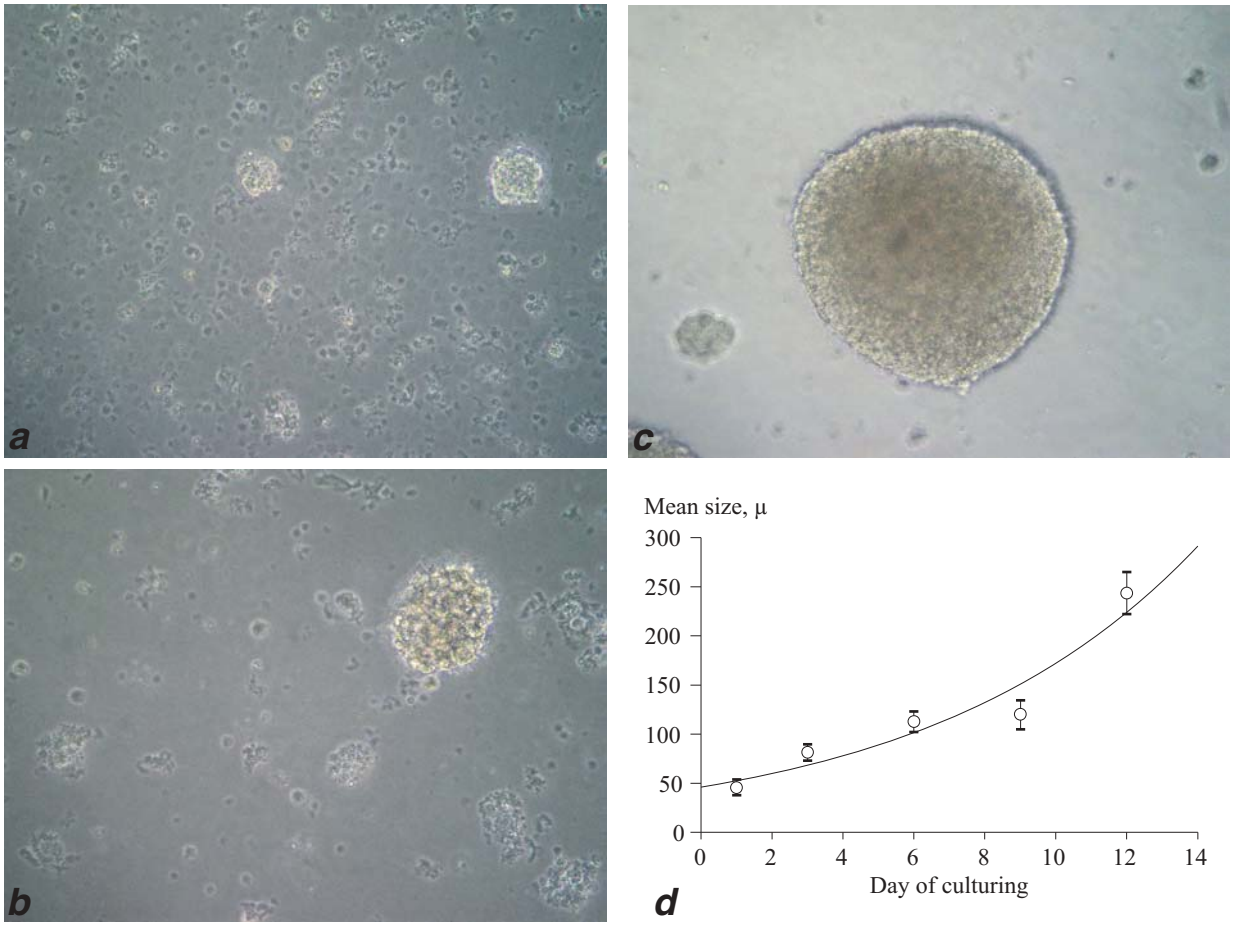

Fig. 1. Size of neurospheres cultured in selective medium. Typical images of neurospheres after 1 (*a*), 6 (*b*), and 12 (*c*) days of culturing. *d*) approximating curve of the mean size of aggregates. At least 10 visual fields per term. Phase contrast; scale 100 μ.

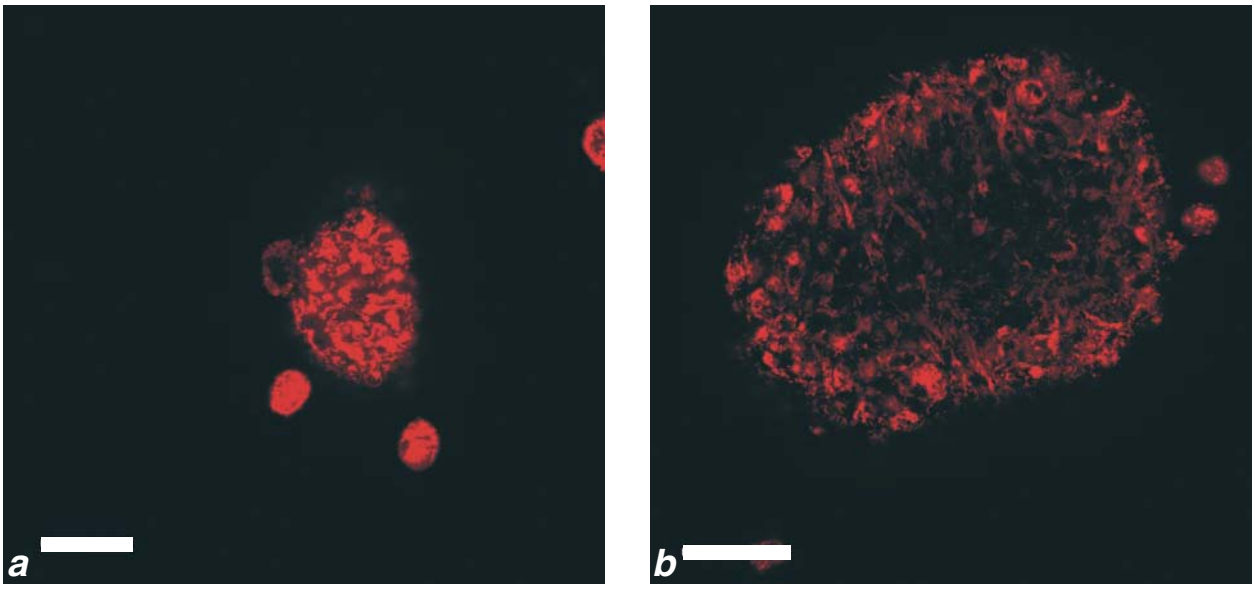

Fig. 2. Mitochondrial potential of TMRE-stained cells in a neurosphere (red fluorescence). Small aggregates are evenly stained (*a*). TMRE fluorescence is lower in the central part of large neurospheres (*b*); scale: 50 μ.

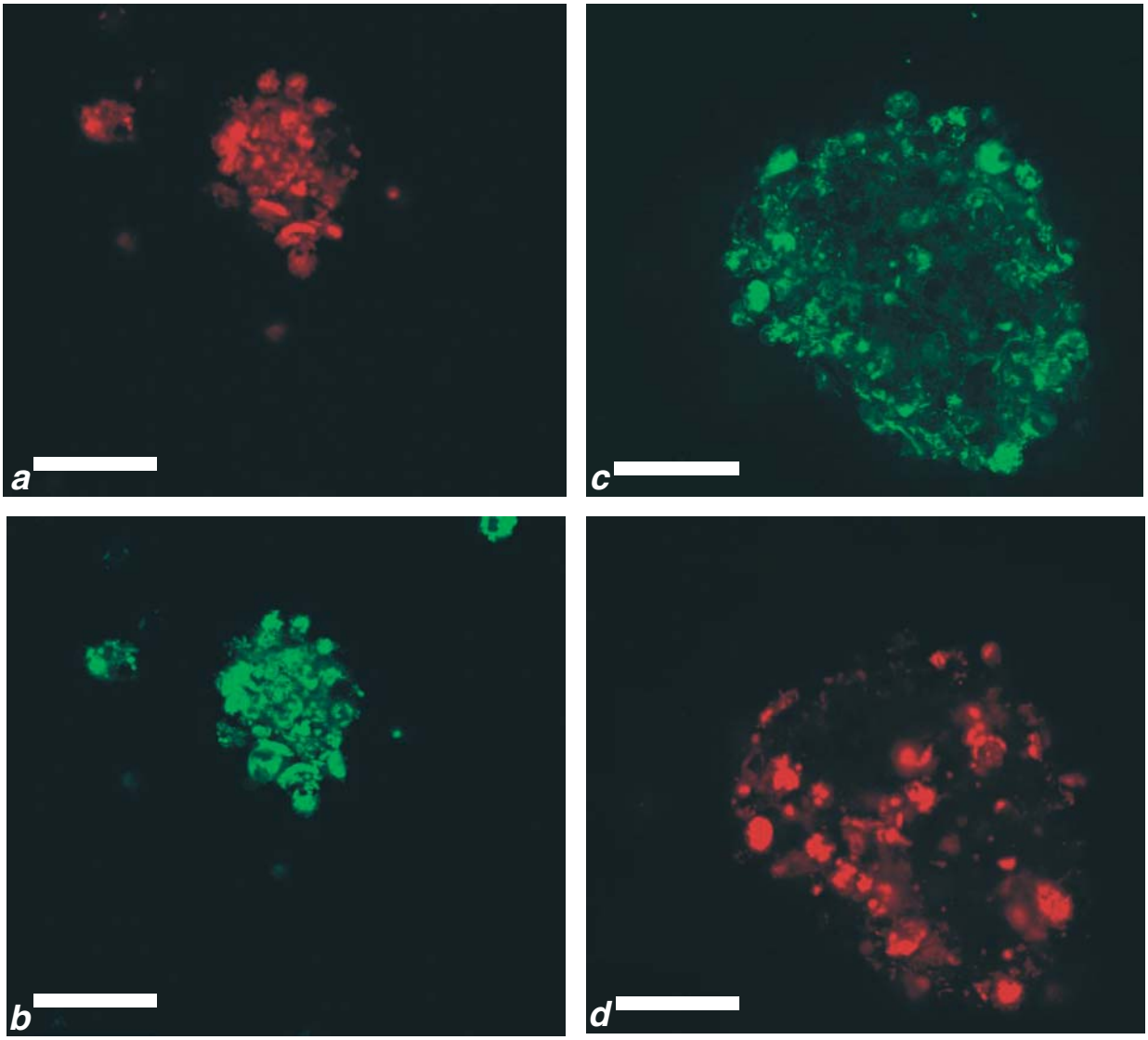

Fig. 3. Cell heterogeneity by the mitochondrial potential. JC-1 staining. Total staining of mitochondria (*a*, *c*): green fluorescence. High-energy mitochondria (*b*, *d*): red fluorescence. Mitochondria with a high potential in small neurospheres (*a*, *b*); in larger neurospheres only some cells with high potential (*c*, *d*); scale: 50 μ.

cells in neurospheres (low membrane potential), while other cells exhibited red fluorescence of mitochondria (significantly higher potential; Fig. 3). The content of cells with high mitochondrial potential decreased with enlargement of neurospheres, while in small aggregates virtually all cells possessed high-energy mitochondria (Fig. 3).

These data clearly correlate with previously detected distribution of nestin-positive cells in aggregates of different size [2]. Since nestin is a marker of stem neural cells, we suggested a relationship between mitochondrial function and the status of neural precursor cells.

In addition to nestin expression, a characteristic sign of neural stem cell population is expression of ABCG2 membrane transporter complex [9]. This protein complex is responsible for the release of foreign substances from cells, specifically antitumor drugs, anthracyclines, campothecins, DNA binding agents [8]. In stem cells this system protects DNA from injuries and mutations and maintains the cells in an undifferentiated state [14]. Presumably, high energy potential of some cells in neurospheres ensures functioning of this complex and hence, can serve as a marker of neural stem cells.

We carried out preliminary studies of the relationship between functional activity of mitochondria and ABC complex functioning. The capacity of ABCG2 transporter to release Hoechst 33342 nuclear dye [12] was used for detecting this relationship. It is obvious that ABCG2 functioning spends ATP energy, resynthesized by using the

mitochondrial membrane energy potential [11]. Presumably, activation of ABCG2 in these cells involves reduction of $\Delta\psi$. Incubation of neurospheres with Hoechst 33342 (5 μg/ml) really led to reduction of mitochondrial potential in some cells. Digital analysis of confocal images showed that the intensity of red fluorescence (JC-1) of the cell population with high-energy mitochondria decreased by 30% after 40-min incubation with Hoechst 33342. The intensity of green fluorescence of JC-1 did not decrease in this population (data not shown), as this effect was not due to dye release or its bleaching under the effect of stimulatory laser.

The possibility of ABCG2 effect on mitochondrial dyes, which can also be transported from the cell, should be taken into consideration. Therefore, for detailed characterization of the relationship between ABCG2 complex functioning and mitochondrial status a more thorough inhibitor analysis is needed. The results obtained at this stage of investigation indicate almost zero effect of ABCG2 complex on JC-1 accumulation. One of the methods of ABCG2 inhibition is cell exposure to excess substrates actively transported by this transporter (for example, Hoechst 33342). At the expense of competition the transporter releases predominantly this compound without touching other ones (in our case JC-1). However, if ABCG2 transporter removes the mitochondrial dye from the cell, during its inhibition (addition of Hoechst 33342) the intensity of JC-1 red fluorescence can be expected to increase, but not decrease, as was shown in our study. We therefore suggest that the effect of ABCG2 transporter on intracellular concentration of JC-1 can be neglected.

Hence, in this study we showed that mitochondrial membrane potential in cells of neurospheres helps to evaluate cell function and can serve as the criterion for selecting culturing conditions for optimum supply of oxygen, nutrients, and signal substances to cells in the culture. It was found that enlargement of neurospheres during culturing can have a negative impact on the status of central cells in large aggregates. Neural cells with high mitochondrial potential possessing characteristics of true stem cells were detected in the culture. The number of these cells decreases with enlargement of neurospheres, presumably because of differentiation of some cells into more mature neural cells. Further experiments should be aimed at more complete characterization of ABCG2 transporter and its energy provision in neural cells.

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