Heterogeneity of Mitochondrial Potential as a Marker for Isolation of Pure Cardiomyoblast Population

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Typical signs of cardiomyoblasts were determined: high mitochondrial membrane potential and high number of mitochondria in these cells compared to fibroblasts. These signs can be used for identification of these cells. Energy-dependent accumulation of highly specific mitochondrial fluorescent probes applied for visual detection of energized mitochondria allows clear-cut separation of the mixed population: cardiomyocyte population is characterized by higher transmembrane potential than concomitant cells. Using flow cytometry and cell sorting we obtained a population enriched with cardiomyocytes and cardiomyoblasts. Taking into account the fact that the dye has no toxic effect on cells and is rapidly eliminated, the risk of cell damage and death during isolation is considerably reduced compared to traditional methods. These results open possibilities for the development of a new specific method for isolation of cardiomyocyte culture from fetal and embryonic sources for their further use in clinical practice.

Key Words: *fetal stem and progenitor cells; cell therapy; cardiomyocytes, mitochondria*

Cardiovascular diseases such as myocardial infarction and coronary heart disease are accompanied by cardiomyocyte death and impairment of the contractile function of the myocardium. High prevalence of these diseases, on the one hand, and limited capacity of cardiomyocytes for cell division and replacement of damaged cells, on the other, determine urgency of this problem. Cell therapy, a new a promising trend in the treatment of cardiac diseases, is of particular importance in this respect [2]. Numerous experimental and clinical studies [5] demonstrated efficiency of various types of stem and progenitor cells in the restoration of the functions of damaged myocardium. In light of possible therapeutic application of cell technologies for the treatment of various pathologies of the cardiac tissue, isolation of true stem and progenitor cells, including fetal cardiomyoblasts and cardiomyocytes, becomes an urgent problem.

The actual and actively used methods, *e.g.* FACS (fluorescence-activated cell sorting) and MACS (magnetic cell sorting) allow isolation of a certain cell type [6,9]. However, these approaches depend on the type of cells expressing surface marker recognized by the antibody with fluorescent or magnetic label. Maximum efficiency can be achieved in case of markers with absolute cell specificity [3]. However, there is no consensus on phenotypical characteristics of progenitor cells. Moreover, cell isolation by fluorescent or magnetic labeling with antibodies is associated with high risk of cell damage or microbial contamination. In light of this, methods ensuring rapid and selective isolation of cardiomyo-

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blasts and their purification from fibroblasts and other concomitant cells attract special interest [7].

Most modern methods are used for isolation of population of stem cells carrying surface markers for positive or negative selection. There are practically no studies for adaptation of these methods for cardiomyocytes/cardiomyoblasts, probably because their specific markers are located in the cytoplasm and cannot be detected without damage to the cells.

Exotic methods of isolation of cardiomyoblasts, e.g. transduction of stem cells with lentiviral vector carrying fluorescent protein under light myosin chain promoter [4], can be applied only in fundamental studies, but not for clinical cell therapy.

However, functional peculiarities of stem and progenitor cells, e.g. their energetic status, are not taken into account in the development of most methods of cell separation. We previously showed that the state of mitochondria in cultured progenitor cells can serve as a criterion for optimization of culturing conditions and isolation of a population of neural stem cells characterized by high proliferative activity and differentiation potential [11].

Here we studied functional state of mitochondrial apparatus in cultured cardiac stem/progenitor cells isolated from rat embryos. We also verified and confirmed the possibility of using the differences in bioenergetics of embryonic heart cells during specific cardiomyocyte sorting.

MATERIALS AND METHODS

Isolation of primary culture of cardiac cells from rat embryos. Primary culture of cardiac cells was isolated from rat embryos on gestation days 15-17. The uterus and embryos were isolated under aseptic conditions. The hearts isolated under sterile conditions were minced, transferred into Hanks balanced buffered solution containing 140 mM NaCl, 5.4 mM KCl, 1.25 mM $CaCl_2$, 0.44 mM KH_2PO_4, 0.58 mM Na_2HPO_4, 0.81 mM $MgSO_4$, pH 7.4, and washed from blood. The tissue was then treated with 0.1% type 2 collagenase (Gibco) for 50 min at 37°C for tissue dissociation into cells. The resultant suspension was twice washed from collagenase and centrifuged at 1000*g* for 3 min. The cell pellet was resuspended in DMEM/F12 (4:1; Gibco) with 15% equine serum and 5% FCS (Gibco). The cells were cultured at 37°C and 5% CO_2 in a humid atmosphere for 2-4 days until the appearance of adherent spontaneously contracting associates, which were used in further experiments.

Evaluation of the state of cell mitochondria. Mitochondrial transmembrane potential ($\Delta\psi$) was evaluated in living cells using tetramethylrhodamine ethyl ester (TMRE, Molecular probes), a potential-sensitive probe with lipophylic monocationic structure with delocalized charge; due to these properties the probe crosses the mitochondrial membranes and penetrates into the matrix according to $\Delta\psi$ on the inner mitochondrial membrane [8]. The difference between the extra- and intramitochondrial concentrations of TMRE allows clear-cut visualization of individual normally energized mitochondria.

The cell culture was stained with 200 nM TMRE in DMEM/F12 with 20 mM HEPES for 40 min and then was washed from dye excess with the same medium.

Microscopic examination of cells. Microscopic examination of the mixed cell culture was performed under a LSM510 confocal laser scanning microscope (Carl Zeiss) with proprietary software. Fluorescence was excited with an argon laser (488 nm) and helium-neon laser (543 nm), emitted fluorescence was recorded after passing through a system of standard cutoff filters. The image was obtained on a computer after averaging four scannings. The rate of scanning, amplification of the signal and resolution of the obtained images were similar for all experiments in each series. The thickness of the confocal section was ~1.5 μ (pinhole 150 μ). For visualization of TMRE and TRITC-phalloidin we used an exciting laser (λ=543 nm) and measured fluorescence in a range of λ=560-590 nm. For visualization of FITC-conjugated antibodies, we used an exciting laser (λ=488 nm) and measured fluorescence in a range of λ=505-530 nm. The images were processed using ImageJ.

Immunocytochemical staining of cells. Actin cytoskeleton was studied using TRITC-phalloidin fluorescent dye (Sigma), a natural toxin conjugated with fluorescent label and selectively binding to actin molecules.

The cells were fixed in 4% formalin for 15 min at 4°C, then treated, and washed with PBS with BSA. The prepared cells were incubated in 10 μM TRITC-phalloidin for 1 h at 25°C, then washed with PBS, and embedded into 40% glycerin on PBS under a coverslip.

Antibodies to cytochrome oxidase were used for evaluation of the number of mitochondria in cells. The cells were fixed, permeabilized (0.2% triton-X-100 for 40 min at 4°C), and specifically stained for actin. The preparations were then incubated for 2 h at 4°C with mouse monoclonal antibodies against cytochrome oxidase subunit IV (BD Bioscience) diluted in a blocking buffer (PBS with 0.1% BSA) at 1:200 ratio. After incubation with

primary antibodies, the sections were washed with PBS (3×15 min) and incubated with secondary FITC-labeled rabbit antimouse antibodies (Sigma) in blocking buffer at 1:100 ratio. After 3-fold washout, PBS sections were transferred on a slide and embedded into 40% glycerin on PBS. The sections were covered with coverslips, fixed with a lacquer, and examined under confocal microscope.

Flow cytofluorometry. Comparative analysis of mitochondrial membrane potential in various populations of cultured cardiac cells was performed using a flow cytofluorometer (Beckman Coulter) after cell staining with TMRE. Fluorescence was excited with a laser (λ=488 nm) and measured in a range of λ=560-590 nm.

RESULTS

Spontaneously contracting cell associates were formed 1 day after cell isolation. Depending on cell density in the culture we observed contraction of individual cells and multicellular islets, in the later case the contraction was coordinated and periodic. The isolated culture was heterogeneous: apart from contracting cells it contained a considerable number of non-differentiated cardiofibroblasts and other non-contracting cells. The culture was stored under these conditions for up to 2 weeks.

For visualization of transmembrane potential of cell mitochondria we used TMRE (Fig. 1). A total of 10 cultures obtained from rat embryos were analyzed. The intensity of TMRE fluorescence in spontaneously contracting cells, cells adjacent to contracting agglomerates, and in other cells of the culture (primarily, cardiofibroblasts) was recorded. It was found that a considerable fraction of heart cells exhibited extremely intensive TMRE fluorescence, whereas in other cells the intensity of staining in mitochondria was more than 4-fold lower (155±10 and 40±5 rel. units, respectively). A strict correlation was revealed between spontaneous contraction and intensive TMRE fluorescence: the majority of cells had either both or none signs. We hypothesized that the cells characterized by intensive TMRE fluorescence belong to cardiomyocyte and/or cardiomyoblasts population. Counting of cells with different mitochondrial potential on confocal images showed that cardiomyocytes with highly intensive fluorescence of the mitochondrial probe constitute about 50% cells in the primary culture, while other 50% cells constitute cardiomyoblasts and other concomitant cells with low TMRE fluorescence.

The differences in TMRE staining can be explained by higher potential or greater number of cardiomyocyte mitochondria. Moreover, the two

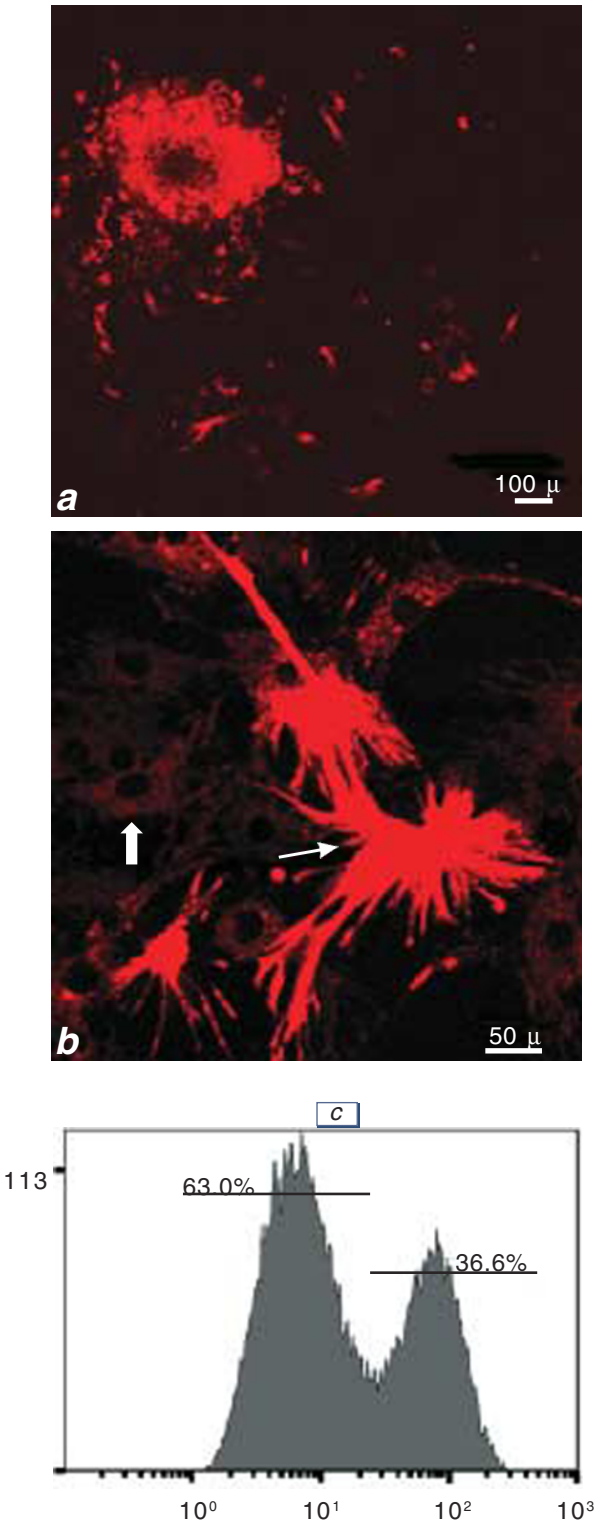

Fig. 1. Heterogeneity of mitochondrial potential of cells in culture of embryonic rat heart tissue. Staining with 200 nM TMRE. Confocal microscopy (*a, b*), and cytometry (*c*). High intensity of fluorescence corresponds to cardiomyocytes (thin arrows). *a*) cell agglomerate with coordinated contractions; *b*) individual contracting cells surrounded by fibroblasts (thick arrows); *c*) separation of populations with different TMRE fluorescence intensity on a flow cytofluorometer.

component of transmembrane mitochondrial electrochemical potential, proton gradient (ΔpH) and electrochemical potential (Δψ), can differ in different cells: while TMRE accumulation reflects Δψ only.

For verification of this hypothesis, in special experimental series the cells were treated with nigericin modulating ionic currents and transforming the concentration component ΔpH into Δψ. However, the difference between fluorescence of the accumulated probe in cardiomyocytes and fibroblast-like cells did not disappear under these conditions. This attested to more intensive accumulation of fluorescent cationic probes in cardiomyocytes.

The identified cell populations with different mitochondrial potential were analyzed quantitatively and separated by FACS on a flow cytofluorometer (Fig. 1, *c*). The TMRE fluorescence intensity peaks for cell populations differed by 8-9 times, which allowed clear-cut separation of these populations on a cell sorter. The ratio of cells with high and low TMRE fluorescence estimated on the fluorometer was close to that obtained during confocal microscopy. Cultures obtained in different periods contained from 50% to 30% cells with high mitochondrial potential (Fig. 1, *c*).

Examination of the actin skeleton in different cell types of the isolated culture confirmed cell heterogeneity and the presence of at least two populations. Cross-striation of actin fibrils was clearly seen in some cardiomyocytes (Fig. 2, *a*). This organization of actin is a typical sign of contractile apparatus: regularly structured arrangement of actin

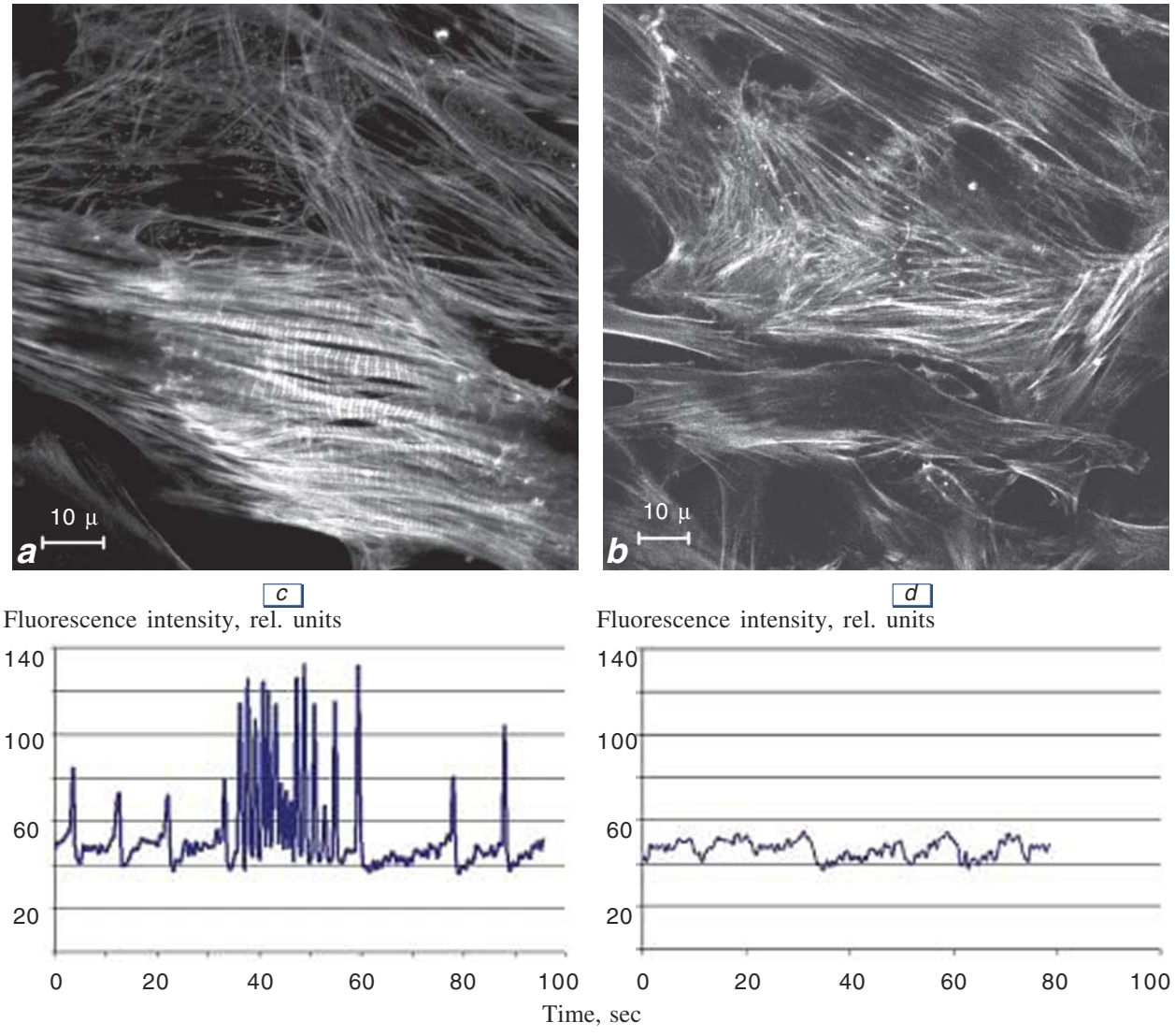

Fig. 2. Differences in contractile apparatus of cardiomyocytes (*a, c*) and fibroblast-like cells (*b, d*) in primary heart culture. *a, b*) fixed cells stained for actin; *c, d*) a scan of calcium probe Fluo-3 fluorescence in cells.

and myosin in myofilaments. Analysis of confocal images suggests that organization of actin in contracting cells corresponds to sarcomeres. Another cell population had actin cytoskeleton typical of fibroblasts (Fig. 2, *b*): sross-striation (and hence contractile apparatus) was absent; actin threads were irregular and represented usual stress-fibrils.

Since function and contraction of myocytes, including cardiac myocytes, are associated with permanent fluctuations of cytoplasmic calcium, we analyzed the primary culture of heart cells for this sign. Heterogeneity of cell cultures by the distribution of intracellular calcium ions was studied by analyzing confocal images of cells loaded with Fluo-3 calcium probe. Contraction of cardiomyocytes was accompanied by periodic changes in the intensity of Fluo-3 green fluorescence corresponding to changes in intracellular calcium concentration, which attested to periodic generation of action potential on the cell membrane. No fluorescence fluctuation was revealed in cardiofibroblasts and other blast cells. The use of a special

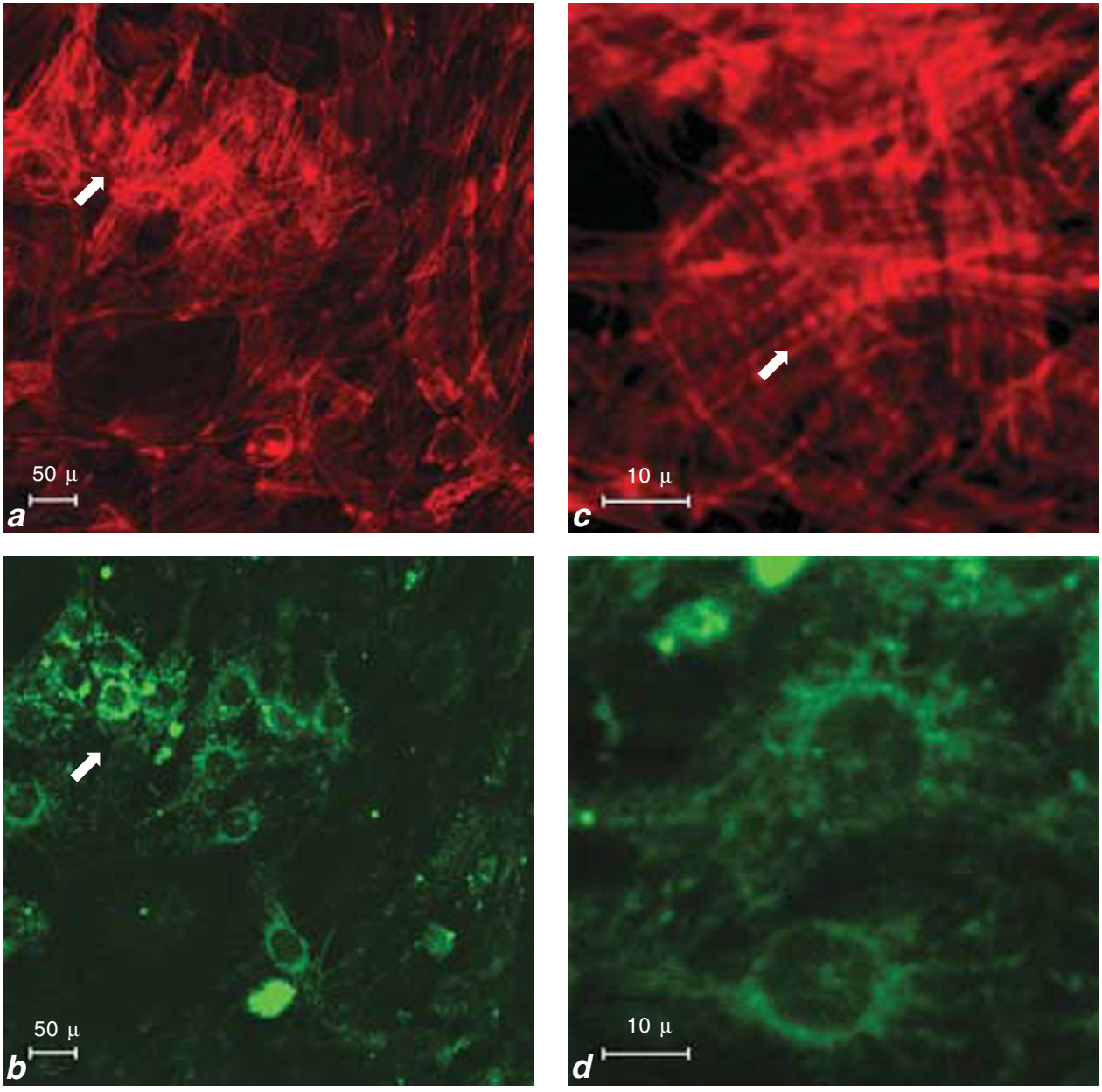

Fig. 3. Correlation between the intensity of staining for specific mitochondrial protein and structure of actin cytoskeleton in cultured heart cells. Cells are stained for actin cytoskeleton (*a, c*) and cytochrome oxidase (*b, d*) as mitochondrial marker. *a, b*) low magnification; *c, d*) a site with maximum intensity of cytochrome oxidase-positive staining. Arrows show: elements of cytoskeleton (*a, c*) and more intensive fluorescence (*b*) typical of cardiomyocytes and cardiomyoblasts.

regimen of confocal microscopy, scanning along a predetermined line with a certain frequency, made it possible to record real-time fluctuations of Fluo-3 fluorescence. When scanning cardiomyocyte, we recorded Fluo-3 fluorescence on a 100-μ segment passing along the whole cell every 2 sec over 100 sec. Changes in Fluo-3 fluorescence along the entire segment were revealed. The intensity of fluorescence of Fluo-3 AM varies with time and changes uniformly in the whole cell, *i.e*. fluctuation of cytoplasmic calcium concentration occur (Fig. 2, *c*). No changes in the concentration of calcium ions were observed in cells lacking the contractile apparatus (Fig. 2, *d*).

Since differences in the intensity of TMRE fluorescence can be explained by high number of mitochondria in cardiomyocytes, we studied the content of the main mitochondrial marker cytochrome oxidase in different cells in the culture. Maximum intensity of cytochrome oxidase-positive staining corresponded to cells with cross-striation of fibrils (Fig. 3). In the primary culture of heart cells we observed considerable heterogeneity by the content of cytochrome oxidase (Fig. 3), which confirmed our assumption on different number of functioning mitochondria in different cells in the culture. To identify cells with maximum content of cytochrome oxidase and, hence, mitochondria, we carried out double staining for actin cytoskeleton and cytochrome oxidase. It was found that the intensity cytochrome oxidase-positive staining was maximum in cells with cross-striated actin filaments (Fig. 3).

Thus, culture of embryonic rat heart tissue is characterized by a number of signs. Cardiomyocytes and cardiomyoblasts, in contrast to ballast cells are capable of spontaneous contraction, possess developed contractile apparatus, and exhibit typical fluctuation of cytoplasmic calcium concentration. The intensity of probe fluorescence reflecting mitochondrial membrane potential in these cells several fold surpassed the corresponding parameters in cardiofibroblasts. A clear-cut correlation between the presence of specific signs of cardiomyocytes and high content of mitochondria and mitochondrial membrane potential was revealed. The culture obtained by standard method contained 40-50% these cells (cardiomyocytes and cardiomyoblasts). This proportion remained unchanged despite the use of standard methods [12] of elimination of fibroblasts and other non-myocyte cells by adhesion to plastic during short-term incubation in the nutrient medium during isolation. Increasing the time of this incubation, apart from removal of fibroblasts, reduced the relative yield of cardiomyocytes.

We showed that staining of the primary culture of heart cells with highly specific fluorescent mitochondrial dye TMRE provides clear-cut separation of the mixed population into cardiomyocytes with intensive TMRE fluorescence and concomitant cells with weak fluorescence. Using flow cytometry and FACS we obtained a population enriched with cardiomyocytes and cardiomyoblasts. Taking into account the fact that the dye has no toxic effect on cells and is rapidly eliminated, the risk of cell damage and death during isolation is considerably reduced compared to traditional methods.

Recent studies showed that mitochondria play an important role in differentiation of stem cells into cardiomyocytes [10]. The present study supplements our notions [1] on participation of mitochondrial apparatus in vital activity of cardiomyocytes and opens possibilities for the development of a new specific method of isolation of pure cardiomyocyte culture from fetal and embryonic sources for further use in clinical and experimental studies.

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