EXPERIMENTAL BIOLOGY

Differentiation of Bone Marrow Stromal Stem Cells into Cardiomyocyte-Like Cells in Different Mammalian Species

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We describe the possibility of obtaining cardiomyocyte-like cell cultures from rat, guinea pig, and human bone marrow stromal stem cells. The content of troponin I-positive cells attains 35-45% of the total number of cells in the cultures and persists at this level for up to 4 months under differentiation conditions. Spontaneous contractions of cardiomyocyte-like cells were observed after the formation of cell monolayer under differentiation conditions.

Key Words: *bone marrow; stromal stem cells; troponin I*

Multipotent bone marrow stromal stem cells (SSC) were first described by A. Ya. Fridenshtein *et al.* [2,3]. These cells isolated from the bone marrow stroma of adult, newborn, or embryo mammals can adhere, can be cloned, do not phagocytize, and are morphologically similar to fibroblasts. During culturing SSC can differentiate into cells of various mesenchymal tissues (connective tissue, cartilage, bone, fatty tissue, and myelosupporting stroma of lymphoid organs) [4,8]. It was shown [6] that rat SSC can differentiate into myocyte- and cardiomyocyte-like cells (CLC) *in vitro* and *in vivo*. These results gave rise to expectations of using SSC for the treatment of heart diseases. However, the capacity of other mammalian SSC, including human, to differentiate into CLC in culture was not studied. The conditions optimizing the process of proliferation and subsequent differentiation of SSC into CLC are still unknown [5].

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The aim of this study was to confirm the general biological phenomenon of proliferation and differentiation of mammalian SSC into CLC *in vitro* on the rat, guinea pig, and human SSC.

MATERIALS AND METHODS

Bone marrow cells were collected from adult Wistar rats and guinea pigs under ether narcosis; in humans bone marrow cells were collected under intravenous calypsol narcosis by puncture of the ileac bone crest. In animals the cells were washed from the tibial bones with buffered normal saline (BNS) containing 50 U/ml heparin and 250 μg/liter gentamicin (0.5 ml) using a 8G needle attached to a syringe. Suspensions of bone marrow cell were centrifuged at 1500 rpm, the pellet was resuspended in lyzing buffer for 5 min and centrifuged again. Hemolyzed supernatant was removed, and SSC were resuspended in Iskov's growth medium (Gibco) containing 10% fetal calf serum (HyClone) and other additives.

The cells were seeded into Petri dishes (1.5-2.0×10^6 cells/ml) and incubated at 37°C in a CO_2 in-

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cubator with 5% CO_2 at 95% humidity. 5-Azacytidine (Sigma) was added to the cultures at the end of day 3 [7] for preventing spontaneous differentiation of SSC. After 24-h incubation the medium was replaced with growth medium with culturing additives. The medium was replaced every 3 days in all cultures; the duration of culturing of animal and human SSC was more than 2 months. After attaining ~75% monolayer the cultures were subcultured 1:3 using a standard trypsin solution.

For immunohistochemical analysis of cardiomyocyte-like differentiation, SSC were fixed in 100% methanol at 20°C for 20 min, washed with BNS, and incubated with a solution blocking nonspecific immunoglobulin binding and containing 3% equine serum. Then the cells were washed 3 times with BNS and incubated with mouse monoclonal antibodies to cardiospecific troponin I (diluted 1:100) for 12 h at 4°C (antibodies were kindly provided by A. G. Katrukha, Laboratory of Enzyme Chemistry, Biological Faculty of M. V. Lomonosov Moscow University). After thriple washout with BNS, the cells were incubated with FITC-conjugated rabbit antimurine immunoglobulins (1:200) for 45 min at 20°C for detecting primary mouse antibodies bound to cardiospecific tropo-

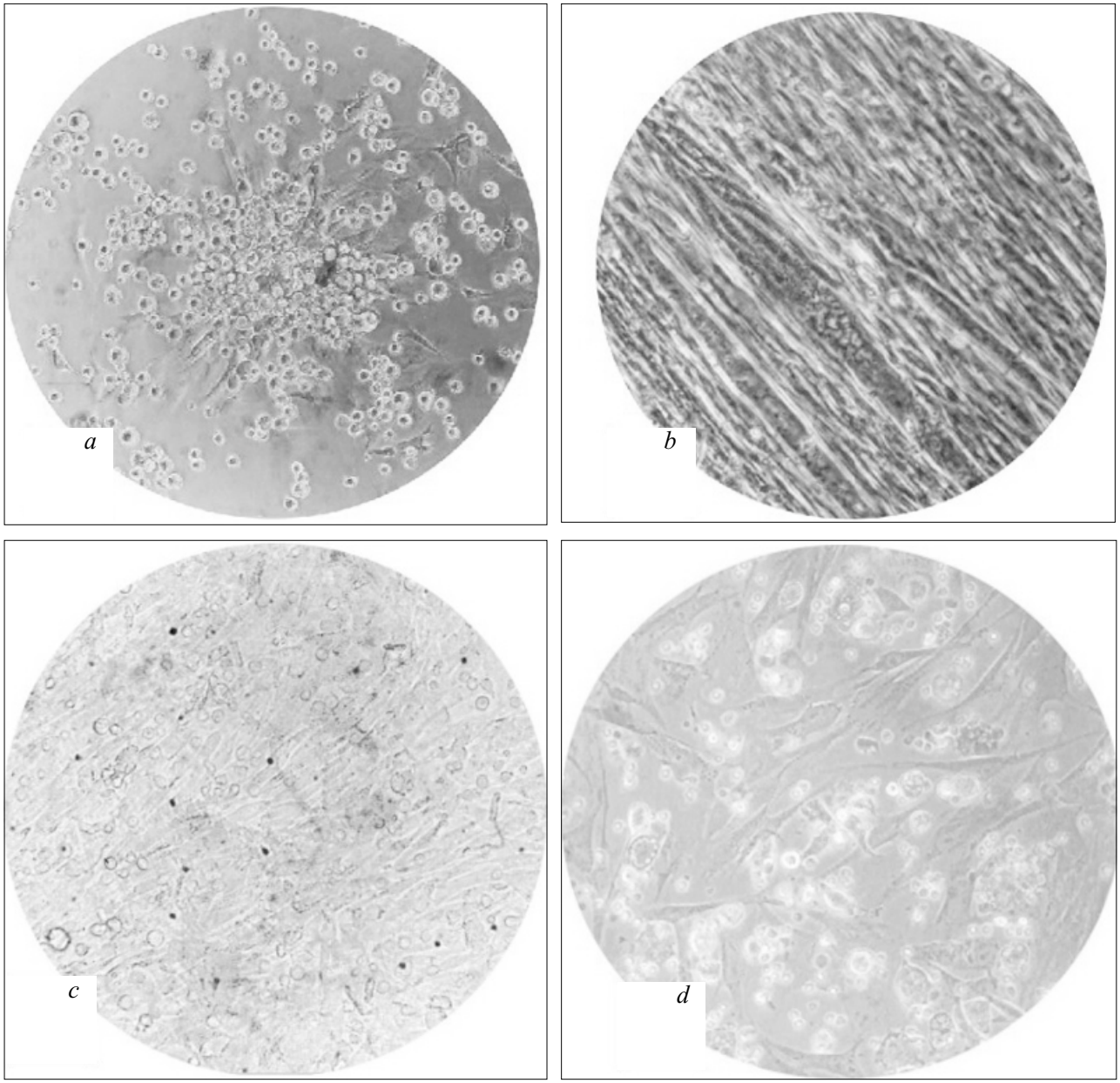

Fig. 1. Bone marrow stromal cell cultures. Phase contrast microscopy. *a*) day 4 of culturing, fibroblast-like morphology of a growing rat cell clone, ×100; *b*) rat cells, week 4 of culturing, myotubule-like morphological elements appear against the background of a fibroblastoid cell monolayer, ×400; *c*) guinea pig cells, week 2 of culturing, ×200; *d*) human cells, week 4 of culturing, ×400.

nin I. After washout with BNS, 60% glycerol was applied onto the preparations and they were covered with slides. Phase contrast and fluorescent microscopy were carried out using a Laborlux microscope (Leika). Microphotographs were made using Fujicolor color negative film.

RESULTS

Culturing of SSC from the rat, guinea pig, and human bone marrow in a medium supplemented with 10% fetal serum, insulin, and dexamethasone led after 2-3 weeks to the appearance of spontaneous differentiation towards the formation of CLC. The number of troponin-I-positive cells is usually 15-25% of the total number of cells in the culture. However, the presence of factors inducing myocytic differentiation increased in the number of troponin-I-positive cells to 35-45% by the same term. Figure 1 presents 1-month cultures of rat, guinea pig, and human SSC grown in the presence of differentiation factors.

As seen from Fig. 1, rat and human CLC are characterized by fibroblast-like spindle shape, while guinea pig CLC look like normal cardiomyocytes. We found that at acid pH (6.8-7.0) some cells in the monolayer differentiated into adipocyte-like cells containing typical lipid inclusions; under hypoxic conditions of culturing some cells differentiated into chondrocyte-like cells. These results persuaded us that high level of troponin I-positive CLC in the culture (Fig. 2) can be maintained by strict maintenance of adequate differentiation conditions. We maintaining these conditions during long-term (up to 4 months) culturing and showed that the percentage of CLC in the culture remained at the level of 35-45% of the total cell number, but did not increase.

Our results are in line with the data of other authorities [1], who demonstrated impossibility of 100% cell differentiation in the same preset direction. We believe that this is explained by the autoregulatory effect of the total cell population in the culture (some cells provide adequate microenvironment for differentiating cells). Obviously, the appearance of spontaneous contractility of CLC, which we observed during weeks 2-4 of culturing after attaining of the monolayer and which was retained after replacement of the nutrient medium, was the result of adequate microenvironmental conditions (Fig. 3). These contractions disappeared after subculturing and reappeared after attaining of a new monolayer.

Hence, our study revealed a common regularity of the formation of CLC in a culture of SSC derived from different mammalian species. It was shown that stem cell cultures differentiating into cardiomyocytes always

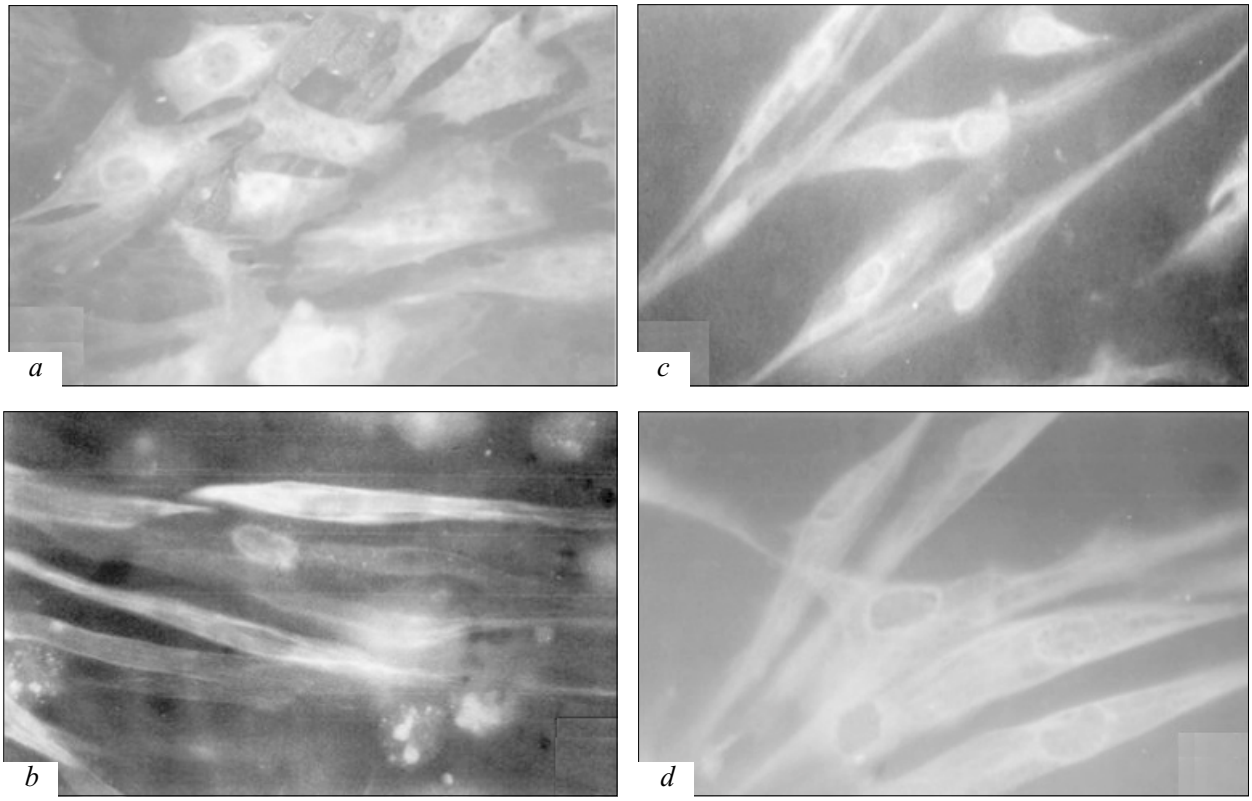

Fig. 2. Stromal stem cell culture at the end of week 3 of culturing. Immunofluorescent staining for troponin I by mouse monoclonal antibodies and development by FITC-conjugated rabbit antiserum. *a*, *b*) guinea pig culture, ×400 (*a*) and ×200 (*b*). *c*, *d*) human cell culture, ×200.

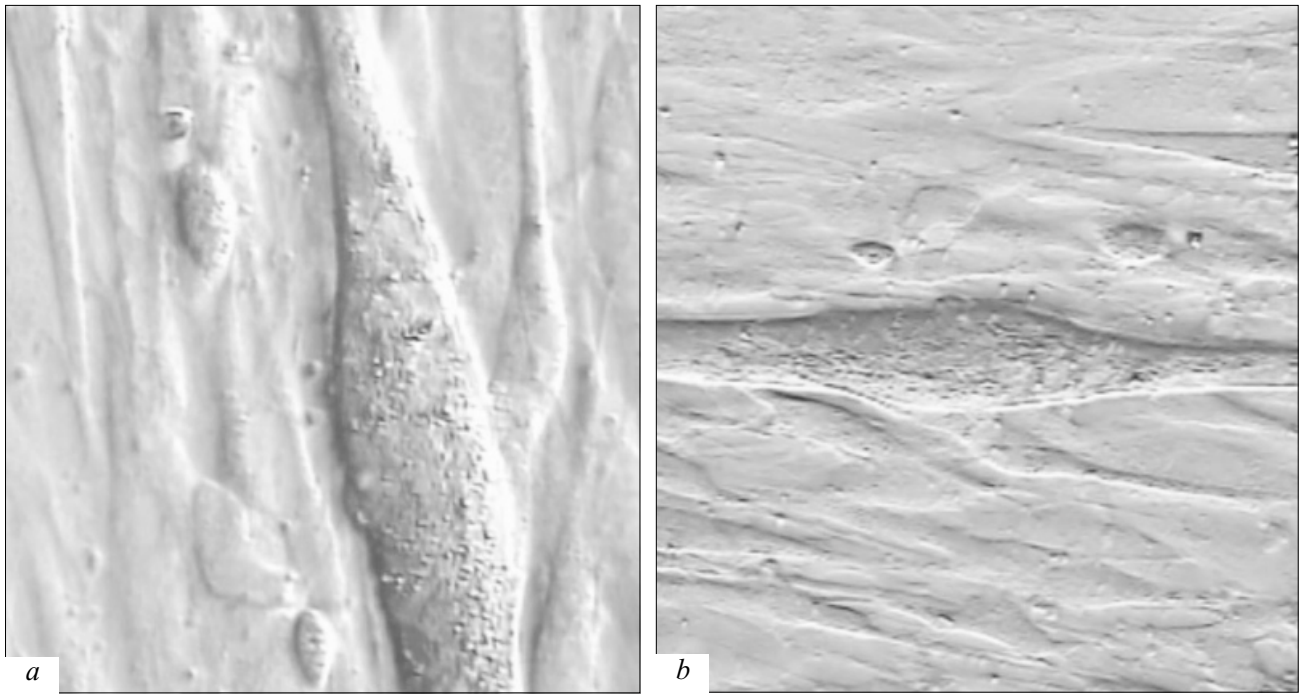

Fig. 3. Rat (*a*) and human (*b*) contracting cardiomyocyte-like cells in bone marrow stromal stem cell culture. Week 6 of culturing after 24 h preincubation in medium containing 3 μM 5-azacytidine. Phase contrast microscopy, ×400.

contain a portion of cells differentiating into stromal cell types and responsible for the maintenance of conditions needed for the existence of a pool of differentiated cells and their specialized functioning.

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