INTRODUCTION

Mesenchymal stromal cells (MSC), first found in the stroma of hematopoietic organs (Friedenstein and Luria, 1980), have a status of multipotent stem cells and can give rise to adipogenic, osteogenic, chondrogenic, and other cell lineages. In the human bone marrow, MSC comprise an extremely small cell population (0.01-0.001% of the total cell number) capable of adhering to the surface of cell culture plasticware. Adhesion to the plastic and other substrates, which is essential for MSC proliferation, provides a criterion for their identification (Dominici et al., 2006). Initially, MSC were regarded only as cells of the bone marrow stroma that organize their hematopoietic microenvironment. In addition to the bone marrow, other sources of MSC have been identified. They include fat tissue, umbilical cord blood, and several embryonic tissues.

The International Society for Cellular Therapy established three minimal criteria for defining multipotent MSC (Dominici et al., 2006). These are (1) adhesion to plasticware under standard culture conditions; (2) expression of cell surface antigens CD90, CD105, and CD73; and (3) the ability to differentiate into osteoblasts, adipocytes, and chondroblasts in vitro.

The interest of many researches in MSC is accounted for by the wide differentiation potential of these cells and their ability to support proliferation of hematopoietic stem cells upon their cotransplantation, which offer ample opportunities for using them in cell and gene therapy. However, the use of MSC in therapy makes it necessary to produce them in large amounts. This is possible only by means of long-term in vitro culturing, which, in turn, is an appropriate model for studies on the mechanisms of MSC self-maintenance, directional differentiation, and senescence, which have priority in stem cell biology.

The multipotency of MSC declines in the course of long-term culturing. It was shown, for example, that cultured MSC after 12 passages manifested the signs of senescence and lost the potential for adipogenic differentiation but preserve the potential for osteogenesis. Morphological characters typical of osteogenic differentiation can be observed at the earlier stages of culturing (passages 1–4) but disappear at later stages (passages 9–11), despite mineralization of the extracellular matrix and the expression of osteogenic differentiation markers. A comparative analysis of the proliferation potential of stromal cells has shown that differences in the period of cell population doubling at the early and later stages of culturing are insignificant. An almost complete arrest of cell growth has been observed in the middle of the culture period (passages 5 and 6).

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Comparative Characterization of Mesenchymal Bone Marrow Stromal Cells at Early and Late Stages of Culturing

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Abstract—The mesenchymal stromal cell is a multipotent precursor of osteoblasts, adipocytes, and some other cell types. In this study, a comparative analysis of cultured mesenchymal stromal cells from the rat bone marrow at the early and late stages of subculturing has been performed using molecular genetic and cytological methods. The culture has undergone 11 passages during 140 days. Upon long-term culturing, the mesenchymal stromal cells have proved to lose their potential for adipogenic differentiation but preserve the potential for osteogenesis. Morphological characters typical of osteogenic differentiation can be observed at the earlier stages of culturing (passages 1–4) but disappear at later stages (passages 9–11), despite mineralization of the extracellular matrix and the expression of osteogenic differentiation markers. A comparative analysis of the proliferation potential of stromal cells has shown that differences in the period of cell population doubling at the early and later stages of culturing are insignificant. An almost complete arrest of cell growth has been observed in the middle of the culture period (passages 5 and 6).

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CELL BIOLOGY
Hayflick phenomenon. Morphologically, this is manifested as follows: the cells begin to differ in shape and size; their cytoplasm becomes granular, with many inclusions; and cell debris appears in the culture medium (Bonab et al., 2006).

Replicative senescence of MSC largely depends on the animal species used as their source. According to available data, human MSC are tolerant of 40–50 population doublings (Stenderup et al., 2003), compared to more than 100 doublings in the case of mouse MSC (Meirelles and Nardi, 2003). The proliferation potential of MSC decreases with an increase in the age of cell donors (D’Ippolito et al., 1999, Baxter et al., 2004) and in the number of passages in vitro (DiGirolamo et al., 1999; Bonab et al., 2006).

However, in order to gain a deeper understanding of the role of multiple subculturing in MSC senescence, it is necessary to study in detail the molecular and cytological aspects of changes in the multipotent status of MSC in the course of long-term culturing. Therefore, the purpose of this study was to perform a comparative analysis of rat MSC cultures at earlier and later stages of culturing using cytological and molecular genetic methods.

MATERIALS AND METHODS

MSC isolation and culturing. Randombred Wistar rats aged 5–6 months and weighing 120–150 g were used in the study. The animals were eutanized to excise the femora and tibiae. Bone epiphyses were cut off, and the contents of the diaphyses were washed out with the α-MEM medium (Sigma, United States) using a 10-ml syringe. The resulting bone marrow samples were suspended in the medium by pumping with the syringe and filtered through nylon gauze. After taking cell count, the suspension (5 × 10⁶ cells/ml) was transferred to plastic culture flasks with a surface area of 75 cm² (Greiner, Germany) and cultured by a standard method in a CO₂ incubator (5% CO₂ at 37°C, using the α-MEM medium without deoxyribonucleotides and ribonucleotides supplemented with 2mM L-glutamine (Sigma, United States) 10% bovine serum (Biolot, Russia), 100 U/ml penicillin, and 100 µg/ml streptomycin. One day after establishing the primary culture, nonadhesive cells were removed, while adhesive cells were washed with two portions of Dulbecco’s modified PBS, pH 7.2–7.4 (Sigma), and the medium was replaced. Thereafter, it was replaced every 3–4 days.

When the cells were grown to 90–100% confluence (after 14–15 days), they were detached with 0.25% trypsin solution (Biolot, Russia) in 1 mM EDTA, transferred to new flasks (3 × 10⁵ cells/ml), and cultured in the α-MEM medium with 8% fetal calf serum but without antibiotics until the cell layer reached 90–100% confluence again. The total culture period was 140 days, including 11 passages.

Induction of osteogenic and adipogenic differentiation of MSC. MSC at a concentration of 2000 cells/cm² were seeded into 25-cm² flasks (Greiner) for isolating total RNA or into 12-well plates with a well area of 3.83 cm² (Greiner) for immunohistochemical and histochemical analyses. To induce osteogenesis, the cells were cultured in the growth medium with 10⁶ M dexamethasone, 50 µg/ml ascorbic acid phosphate, and 10 mM glycerophosphate for 15–18 days, replacing the medium every 3–4 days. To visualize mineral deposits in the extracellular matrix, the cells were washed with PBS, fixed with 70% ethyl alcohol for 1 h, washed with distilled water, and stained with alizarin red (Sigma) at pH 4.1 for 10 min. After washing in two portions of PBS, the cells were poststained with hematoxylin to visualize the nuclei.

To induce adipogenesis, the cells were cultured in the growth medium with 10⁶ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 0.01 mg/ml insulin for 14 days. To detect the appearance of cells with fat droplets, the cultures were regularly examined under a phase-contrast microscope with an Olympus AH-3 camera (Germany). Multilocal adipocytes appeared of day 14 of culturing. To visualize fat droplets, cells were fixed with a formal–calcium mixture for 1 h, washed with tap water for 1 h, rinsed with distilled water and 60% isopropanol, and stained with a fat red O solution in isopropanol (Pearse, 1962). The histochemical reaction was analyzed under the phase-contrast microscope.

Immunocytochemistry. Osteogenic differentiation was studied with monoclonal antibodies to a component of the extracellular matrix, collagen type I (Sigma), diluted 1 : 4000. The multipotent status of MSC was evaluated using monoclonal antibodies to CD90 (Abcam, England) diluted 1 : 50.

MSC cultured in 12-well plates (2000 cells per well) were washed with PBS (37°C) and fixed with 3.7% formaldehyde (for incubating with antibodies to CD90) or chilled acetone (for incubating with antibodies to collagen type I) for 10 min. After fixation, the cells were washed with PBS and consecutively incubated in 0.25% Triton X-100 (Fluka, Germany) solution in Tris buffer (TBS) with 0.1% Twin-20 for 30 min and in the blocking solution (3% bovine serum albumin solution in TBS with 0.1% Twin-20) for 30 min at 20°C. The cells were then washed with PBS and incubated with primary antibodies in the blocking solution at 37°C for 40 min. After washing in four portions of PBS for 5 min, the cells were incubated with secondary antibodies labeled with Alexa Fluor 488 or 568 (Invitrogen, United States) and diluted 1 : 1000 in the blocking solution at 37°C for 40 min. Thereafter, the cells were washed with PBS and embedded under a coverslip in glycerol with 4',6-diamidino-2-phenyl indole (DAPI) (Vector, United States). Specificity of the primary antibodies was tested in control reactions performed with the secondary antibodies alone. Cell preparations were...