Growth and Adipogenic Differentiation of Mesenchymal Stromal Bone Marrow Cells during Culturing in 3D Macroporous Agarose Cryogel Sponges

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We studied the possibility of population of macroporous agarose cryogel sponges by mesenchymal stromal bone marrow cells with their subsequent adipogenic differentiation. After 7-day culturing of mesenchymal stromal cells in agarose cryogel, the level of cell proliferation was 35%. After 3-week culturing in a medium inducing adipogenesis we observed accumulation of intracellular neutral lipids positively stained with Oil Red O. These findings can be used for the development of bioengineering constructions of the adipose tissue on the basis of spongy carriers.

Key Words: *mesenchymal stromal cells; macroporous agarose cryogel sponges; adipogenic differentiation; Alamar Blue*

Implantation of autologous adipose tissue [9] or synthetic materials (primarily, silicon implants) are used for reconstruction of soft tissues. However, these approaches have some drawbacks: insufficient neovascularization of the implant, painful sensations related to isolation and healing of the donor tissue, and loss of the volume and shape (by on average 40-60%) in delayed period after transplantation [11]. Transplantation of synthetic materials is fraught with the risk of breaks, leakage of the content, or displacement of the implant. Moreover, the applied materials are often characterized by insufficient biocompatibility. These popular approaches are primarily aimed at replacement of the damaged tissue, but do not provide functional recovery.

Methods and approaches of tissue engineering are promising for reconstruction of the shape and function of the adipose tissue. Polymer ester matrices can be used as the carriers for 3D culturing of cells [12]. Moreover, there are reports on the use of carriers of the basis of hyaluronic acid [7], collagen [15], and chemically modified alginate [10]. These matrices rapidly degrade *in vivo*, which can be associated with the loss of the volume and shape of the implanted tissue engineering constructions.

The materials on the basis of agarose do not degrade in the organism of mammals due to the absence of agarolytic enzymes, which can provide long-term maintenance of the shape and volume of the agarose-based fat transplant. Macroporosity is a typical feature of agarose cryogels (ACG) [2]. The pores of the polymeric matrix are interrelated [3]. Moreover, this polymer after transplantation can induce neovascularization in subcutaneous areas [16].

Multipotent mesenchymal stromal cells (MSC) of the bone marrow were used as biological components. Bone marrow MSC are used for engineering of the bone [5] and cartilage [8] tissue, but creation of bioengineering constructions of the adipose tissue is little studied.

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The aim of the present study is evaluation of the capacity of bone marrow SC to proliferation and adipogenic differentiation under conditions of culturing in macroporous spongy ACG matrixes.

MATERIALS AND METHODS

Human bone marrow was isolated from the iliac crest under local anesthesia. The studies were approved by Ethical Committee of Institute of Cryobiology and Cryomedicine Problems, National Academy of Sciences of Ukraine. The cell suspension was isolated from bone marrow aspirate by multiple washout with medium M-199. The cell suspension was centrifuged at 150*g* for 10 min and suspended in DMEM containing 15% FCS, 50 U/ml penicillin, 50 mg/ml streptomycin, and 2 mmol/ml L-glutamine. The cells were cultured at 37°C, 5% CO_2 and 95% humidity. The medium was changed after 72 h and then every 3-4 days. After attaining 70% confluence, the adherent fraction was harvested with trypsin—Versene (1:4) and subcultured at 1:3 ratio.

Immunophenotyping was performed by flow cytofluorometry. To this end, passage 4 cells were stained with monoclonal antibodies to CD29, CD34, CD44, CD45, CD73 and CD105 and analyzed on a FACS Calibur flow cytometer (BD Biosciences).

ACG disks (2 mm thickness, 10 mm diameter) were prepared as described previously [2] with subsequent activation of the polysaccharide matrix with divinylsulfone and covalent binding of type A gelatin (Sigma) to the walls of macropores as described elsewhere [4]. The amount of bound gelatin was 0.5-0.8 mg protein per 1 g wet ACG.

The spongy disks were saturated with culture medium 1 h before population with the cells. Bone marrow MSC of passage 4 in a concentration of 5×10^5 cell/ml were transferred into tubes with ACG. Population of ACG with the cells was stimulated by creating partial vacuum in the tube [14]. The cells were incubated at 37°C under these conditions for 3 h. Then, the ACG disks with cells were transferred into wells of a 24-well plate for further culturing. The cells were cultured in ACG disks for 4 weeks.

Metabolic and proliferative activity of cells in ACG disks was determined on days 1, 4, and 7 using REDOX indicator Alamar Blue (AB). To this end, 10% AB was added at different terms of culturing. After 2 h, the medium containing AB was taken and the degree of AB reduction was determined using a Tecan GENios plate reader at excitation and emission wavelengths of 550 and 590 nm, respectively. The data were presented as the difference between the experimental and blank sample (without cells) and expressed in arbitrary units of fluorescence.

For induction of adipogenic differentiation, bone marrow MSC of passage 4 in confluent culture or embedded into ACG were cultured for 3 weeks in DMEM containing 10% adipogenic stimulatory factors (Stem Cell Technologies Inc.), 50 U/ml penicillin and 50 mg/ml streptomycin. Cell cultured without inductors were used as the control.

The direction and degree of cell differentiation was evaluated by accumulation of intracellular neutral lipids positively stained with Oil Red O. To this end, the cells in monolayer culture or in ACG sponge were fixed with 4% buffered neutral formalin (20 min at 4°C), washed with 60% isopropyl alcohol, and stained with Oil Red O for 30 min at room temperature.

RESULTS

Bone marrow cells were cultured under selective conditions, which according to previous data are

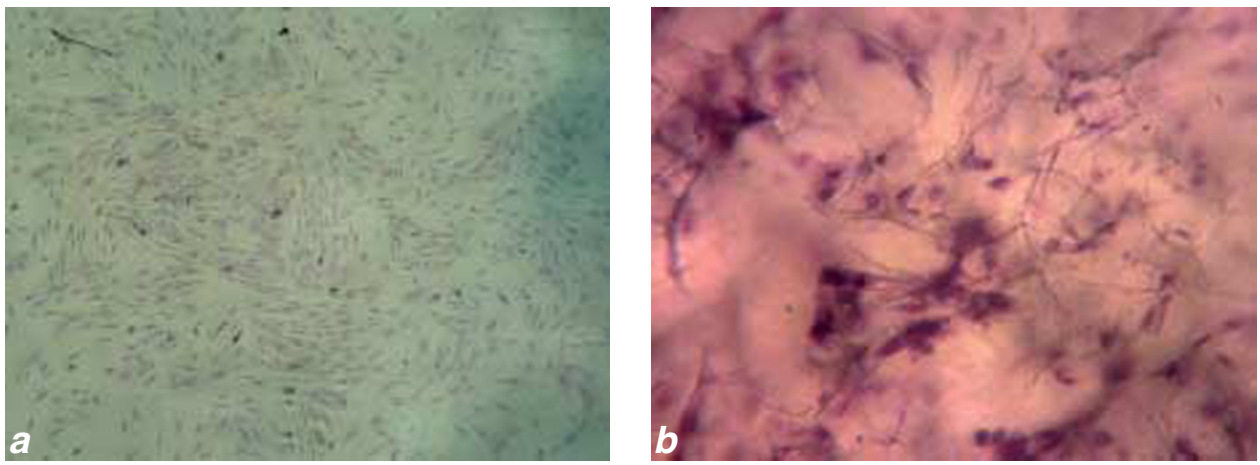

Fig. 1. Bone marrow MSC on day 7 of culturing in monolayer culture (*a*; ×40) and in macropores of ACG disk (*b*; ×200). Azure and eosin staining.

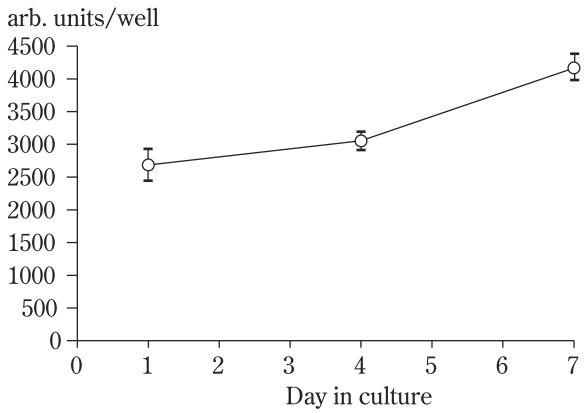

Fig. 2. Metabolic activity of MSC under conditions of 3D culturing in ACG sponge evaluated by AB reduction.

optimal for MSC expansion [1] over 4 passages. Passage 4 cells had MSC phenotype $CD29^+$, $CD44^+$, $CD73^+$, $CD105^+$, $CD34^-$, $CD45^-$.

Bone marrow MSC of passage 4 were embedded into macropores of ACG sponge and after 7 days the samples cultured in monolayer and embedded in ACG were subjected to histological analysis. During monolayer culturing, cell morphology unified, in particular, they acquired spindle shape typical of fibroblasts (Fig. 1, *a*). MSC adsorbed on the walls of ACG macropores had normal fibroblast-like morphology and were evenly distributed in the sponge from the periphery to the center (Fig. 1, *b*).

The intensity of AB fluorescence increased during culturing (Fig. 2). On day 1 in culture, this parameter was 2684±235 arb. units/well, on day 4 it increased by 12% (3053±116 arb. units/well), while on day 7 it attained 4167±198 arb. units/well (*i.e.* surpassed the result obtained on day 1 by 35%). Bearing in mind that AB is an integral parameter reflecting activity of redox enzymes in cells, increased level of its reduction during culturing attests to an increase in the number of cells, *i.e.* their proliferation [6,13]. Thus, our findings suggest that bone marrow MSC cultured in the ACG carrier have normal morphology, retain metabolic activity, and can proliferate.

For induction of adipogenic differentiation of bone marrow MSC in ACG, the cells after 7-day culturing were transferred into a medium containing adipogenesis-stimulating factors. The samples were cultured under these conditions for 3 weeks, then the cells were fixed and stained with Oil Red O for detection of neutral lipids (Fig. 3).

Culturing of MSC without inductors was not accompanied by accumulation of neutral intracellular lipids (Fig. 3, *a*). Culturing in the presence of adipogenic factors for 4 weeks with adipogenic factors stimulated cell differentiation in both monolayer culture (Fig. 3, *b*) and under conditions of 3D culturing (Fig. 3, *c*).

Thus, human bone marrow MSC cultured in 3D macroporous ACG sponges proliferate and are cap-

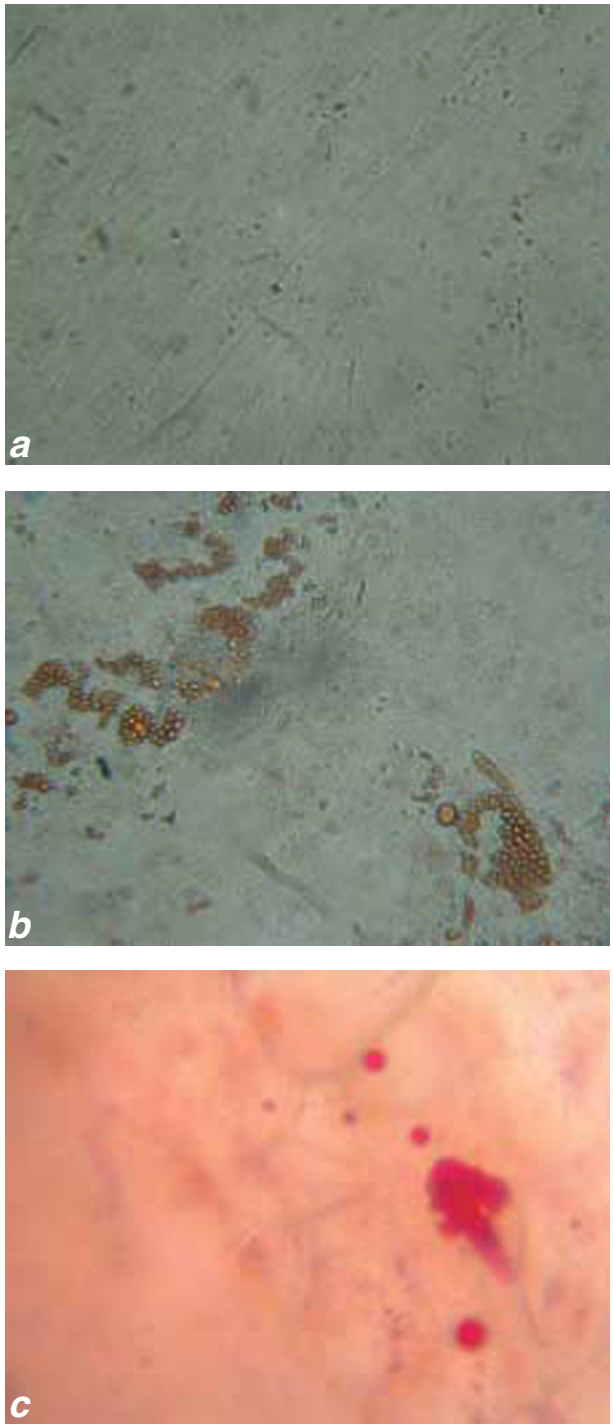

Fig. 3. Adipogenic differentiation of bone marrow MSC. *a*) cells cultured without differentiation factors; *b*) adipogenic differentiation of bone marrow MSC in monolayer culture; *c*) adipogenic differentiation of bone marrow MSC in 3D culture; Oil Red O staining, ×100.

able of targeted adipogenic differentiation. These findings can be used for the development of bioengineering constructions of the adipose tissue on the basis of spongy carriers.

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