Mesenchymal Stem Cells from Human Bone Marrow and Adipose Tissue: Isolation, Characterization, and Differentiation Potentialities

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Comparative study of cultured human bone marrow and adipose tissue (lipoaspirate) mesenchymal stem cells was carried out. The main morphological parameters, proliferative activity, expression of surface and intracellular markers of these cells were characterized. Flow cytofluorometry and histological staining showed that both cell types exhibited similar expression of CD105, CD54, CD106, HLA-I markers, were positively stained for vimentin, ASMA, collagen-1, and fibronectin, but not HLA-DR, CD117, and hemopoietic cell markers. The cells underwent differentiation into adipocytes and osteoblasts under appropriate conditions of culturing. Incubation under neuroinductive conditions led to the appearance of a cell population positively stained for type III β-tubulin (neuronal differentiation marker).

Key Words: *mesenchymal stem cells; cell culture; bone marrow; lipoaspirate; differentiation; phenotype*

Mesenchymal (stromal) stem cells (MSC) are rare population of precursor cells capable of maintaining hemopoiesis and differentiating *in vivo* and *in vitro* into three (osteoblasts, chondrocytes, adipocytes) or more (myocytes, cardiomyocytes, neurons, *etc.*) cell types [3,4,7,9,12,15,17]. That is why MSC attract much attention as candidates for replacement or reparative therapy of diseases, gene or cell engineering [1,6,15].

It was considered up to recent time that the main source of MSC in adults humans is the bone marrow, from which these cells can be isolated for experimental and clinical use. Later MSC were isolated from alternative sources, among which are adipose tissue, umbilical and placental tissue, *etc.* [1,2,5,8,13,14,16, 18,19]. In contrast to the bone marrow, these cells are easy available for cell isolation, while the content of MSC in them is not lower, but sometimes even higher than in the bone marrow [2,19]. However, the problem of the identity of MSC derived from different sources remains disputable until now.

The aim of this study was the development of adequate methods for isolation and culturing of human MSC from two sources: bone marrow and adipose tissue, and comparison of their structural and functional parameters *in vitro*.

MATERIALS AND METHODS

Isolation of bone marrow MSC. Aliquots of adult donor bone marrow aspirates (5-10 ml, n=7) were kindly provided by Department of Bone Marrow Transplantation, Hematology Research Center, Russian Academy of Medical Sciences. Two samples were isolated from the femoral head after prosthetic treatment of the hip joint. Cell suspensions were diluted 2-fold with Dulbecco's phosphate buffered saline (DPBS) and

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layered onto a Histopaque 1.077 density gradient (Sigma). After 30-min centrifugation at 400*g* the interface rings containing mononuclears were collected into individual tubes and washed by centrifugation in DPBS. The resultant cell precipitate was resuspended in culture medium and transferred to T25 flasks (Cornig). The medium contained DMEM with low glucose content, 25 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (all reagents from Gibco).

Isolation of adipose tissue MSC. Adipose tissue samples were obtained during cosmetic liposuction and were kindly provided by MeraMed clinic. Lipoaspirate was diluted 3-fold in DPBS and intensely shaken on a vortex for 2-3 min. After centrifugation (10 min at 600*g*) the ring containing adipose cells and supernatant were discarded, while stromal cell precipitate was resuspended in culture medium. The medium composition was similar to the above mentioned. The flasks were incubated in a CO_2 incubator (37°C, 5% CO_2) for 48 h for cell adhesion.

Culturing of bone marrow and adipose tissue MSC. The medium with nonadherent cells was discarded, the cultures were carefully washed in DPBS, and culture medium was replaced with a fresh portion. The medium was then replaced every 2-3 days. The cultures were examined and photographed under a phase-contrast microscope (Diaphot-TMD; Nikon). After attaining a subconfluent state, the cells were removed with trypsin-EDTA (Gibco) and inoculated into new flasks at 1:3-1:10 dilution. Cultures of passages 3-7 were used.

Immunocytochemistry and flow cytometry. For immunocytochemical staining, MSC cultures grown in 8-well Lab Tek plates (Nunk) were washed from DPBS and fixed in 1% paraformaldehyde in DPBS for 30 min. The cells were permeabilized with 1% Triton X-100 and repeatedly washed in fresh portions of DPBS with 0.1% bovine serum albumin (DPBS-BSA). Mouse monoclonal antibodies to smooth muscle α-actin (ASMA, Sigma), type 1 collagen (Chemicon), and polyclonal rabbit antibodies to fibronectin (Sigma) serves as primary antibodies. Further staining was carried out by immunoperoxidase method with Biomeda kits.

Cells for flow cytometry were removed with 0.1% EDTA in DPBS and precipitated by centrifugation. Then the cells were fixed in suspension with CellFix solution (Becton Dickinson) and, if intracellular markers were detected, were additionally treated with 0.1% Triton X-100 in DPBS. After washout from DPBS-BSA the cell suspensions were divided into aliquots and incubated for 45 min with appropriate FITC-labeled or native antibodies. Mouse monoclonal antibodies to ASMA, CD34, CD36, CD45, CD62E, CD68, CD71, CD54, CD105, CD106, CD117, HLA-I, HLA-DR, vimentin, von Willebrand factor, smooth and skeletal muscle myosin, type 1 collagen, and fibronectin (Becton Dickinson, Biomeda, Chemicon, Sigma, Immunotech) were used in the study. If unlabeled antibodies were used, the preparations after washout were additionally incubated with FITC-labeled antibodies to mouse Ig (Sigma). Nonimmune IgG of the corresponding class served as the negative control. Cell suspensions were analyzed on a FACS Calibur flow cytometer with CellQuest software (Becton Dickinson).

Directed differentiation of MSC. In order to confirm the possibility of differentiation in the adipogenic and osteogenic direction, MSC cultures of passages 2-3 and 5-7 were incubated in a medium containing the corresponding inductors [18,19]. Neuronal differentiation was carried out as described previously [10]. The results were evaluated by staining of lipid incorporation with oil red O, detection of alkaline phosphatase activity, or presence of neuronal differentiation markers.

RESULTS

Development of methods for MSC isolation from liposuction-obtained adipose tissue samples (a homogeneous mass) showed that no incubation in collagenase solution, used by some authors [2,18,19], was needed. Diluted tissue suspension was easily separated into fatty and stromal elements by centrifugation. Preliminary experiments showed that the yield of MSC under these conditions virtually did not change, while the efficiency of their adhesion to the substrate was even higher than after incubation with enzymes.

The main stages in the formation of primary colonies and culture growth of MSC did not differ from those described previously [1,2,10,18,19]. During passages 2-3 the cultures presented as a morphologically homogeneous population of mononuclear fibroblast-like spindle cells, sometimes with few processes (Fig. 1, *a*, *b*). The mean time of cell population doubling during the logarithmic phase of growth varied from 18 to 36 h during the early passages and decreased to 36-48 h during subsequent culturing.

Immunocytochemical staining showed that MSC derived from the bone marrow and lipoaspirate were almost homogeneous by the content of ASMA and actively produced and released type 1 collagen and fibronectin into extracellular matrix (Fig. 1, *c-e*). Cells positively stained for endothelial cell markers (von Willebrand factor, PECAM-1) were seen only at the early passages in MSC cultures from lipoaspirate; these cells were no longer detected during passages 2-3 (data are not presented).

Phenotype of cultured bone marrow and adipose tissue MSC. Analysis of cell markers by flow

cytometry showed that MSC from both sources contained similar set of surface antigens. The number of cells positively stained for CD105, CD54, CD117, CD106, and ASMA varied from 10 to 75-95% in the cultures (Fig. 2). All cultures expressed high level of HLA-I molecules and vimentin, but were negative by hemopoietic cell markers or HLA-DR. The expression of some MSC markers (CD105, CD54, CD106, CD117) varied within a wide range depending on the stage of culture growth, which can explain different results of MSC phenotyping [2,5,11].

MSC differentiation. We failed to reveal crucial differences in the capacity of bone marrow and adipose tissue cells to differentiate into adipocytes. In response to stimulation up to 95-98% cells within 2 weeks formed lipid incorporations clearly seen after staining with oil-soluble stain (Fig. 3, *a*).

High alkaline phosphatase activity was detected in bone marrow and adipose tissue cells in response to osteogenic induction after 1-2 weeks (Fig. 3, *b*). Solitary positively stained MSC were seen in control cultures.

The potentiality of MSC to neuronal differentiation was studied in experiments with monoclonal antibodies to the following markers: β-tubulin-III, CNPase, S-100, neurofilament 68 (NF-68), GFAP (Sigma), NewN, Tau (Chemicon). Positive results were obtained only for β-tubulin-III, CNPase, and NF-68. The number of cells positively stained for β-tubulin-III (Fig. 4) varied from several to tens of percent, reaching 75-90% after 2-3 repeated cycles of induction (Fig. 5). The number of cells carrying other neural markers (NF-68, CNPase) varied and usually not surpassed 5-10% of the total number of cells. Virtually all (95-99%) cultured bone marrow and adipose tissue MSC cells were positive S-100 irrespective of the neuronal induction (Fig. 1, *f*).

Hence, MSC derived from the "classical" (bone marrow) and "alternative" (adipose tissue) sources virtually do not differ by behavior under conditions of culturing, set of surface and intracellular markers, and capacity to directed differentiation in at least three directions. Other authors investigating these cell types in independent experiments reported similar data [8-10,18,19].

Lipoaspirate is superior to the bone marrow by the yield of stromal cell [2]. This can be due to peculiarities of blood supply to the adipose tissue and high density of capillaries. However, though the precipitated fraction after centrifugation contains endothelial cells, pericytes, tissue fibroblasts, and some other cell types [19] adherent to plastic, of all these cells only MSC seem to find necessary conditions for active growth. After several passages the cultures are free from "ballast" cell types and become almost homogeneous.

In addition to the biological characteristics, one of specific features of adipose tissue MSC is the avail-

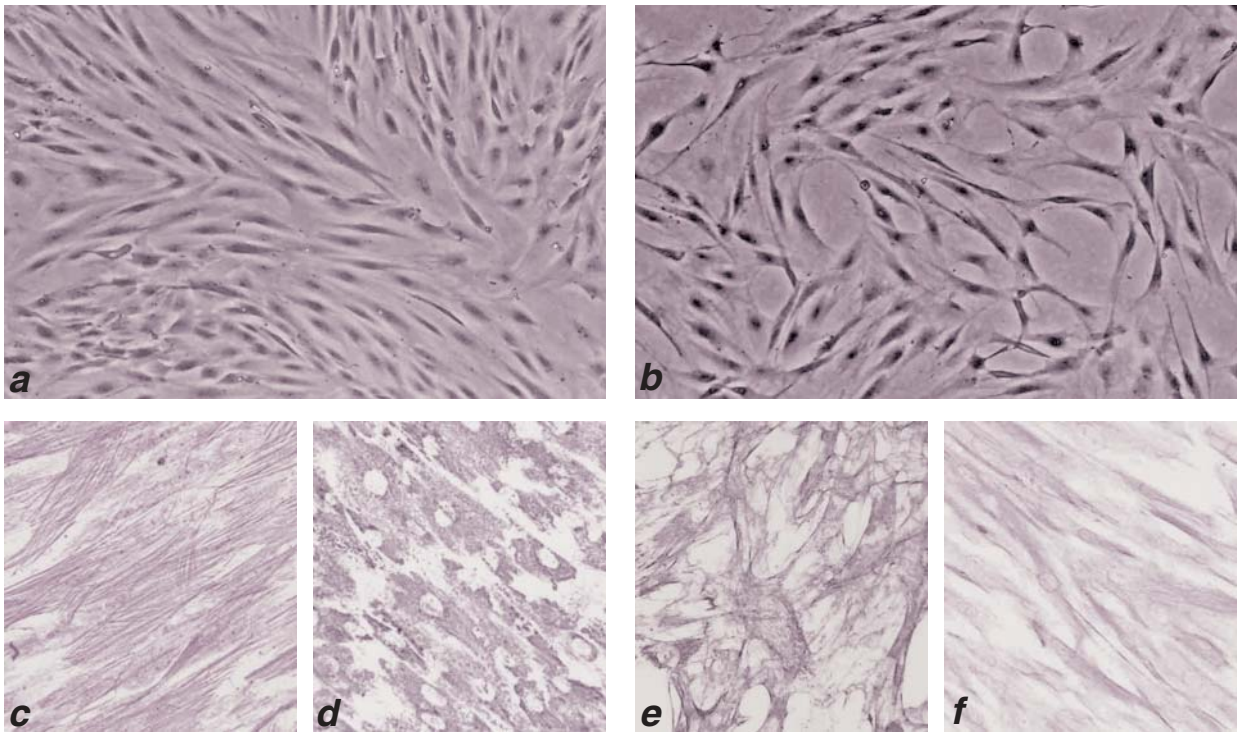

Fig. 1. Human bone marrow and adipose tissue mesenchymal stem cell (MSC) cultures. *a*) bone marrow; *b*) lipoaspirate, phase contrast, passage 3, ×200; expression of ASMA (*c*), collagen 1 (*d*), fibronectin (*e*), and S-100 (*f*) in human adipose tissue MSC culture, immunoperoxidase technique, ×200.

ability of the material for subsequent cell isolation. Cosmetic liposuction is now becoming more and more popular among even young patients; tens of thousands of such operations are carried out annually only in Moscow. Under these conditions, adipose tissue can become not only an extra source of postnatal (adult)

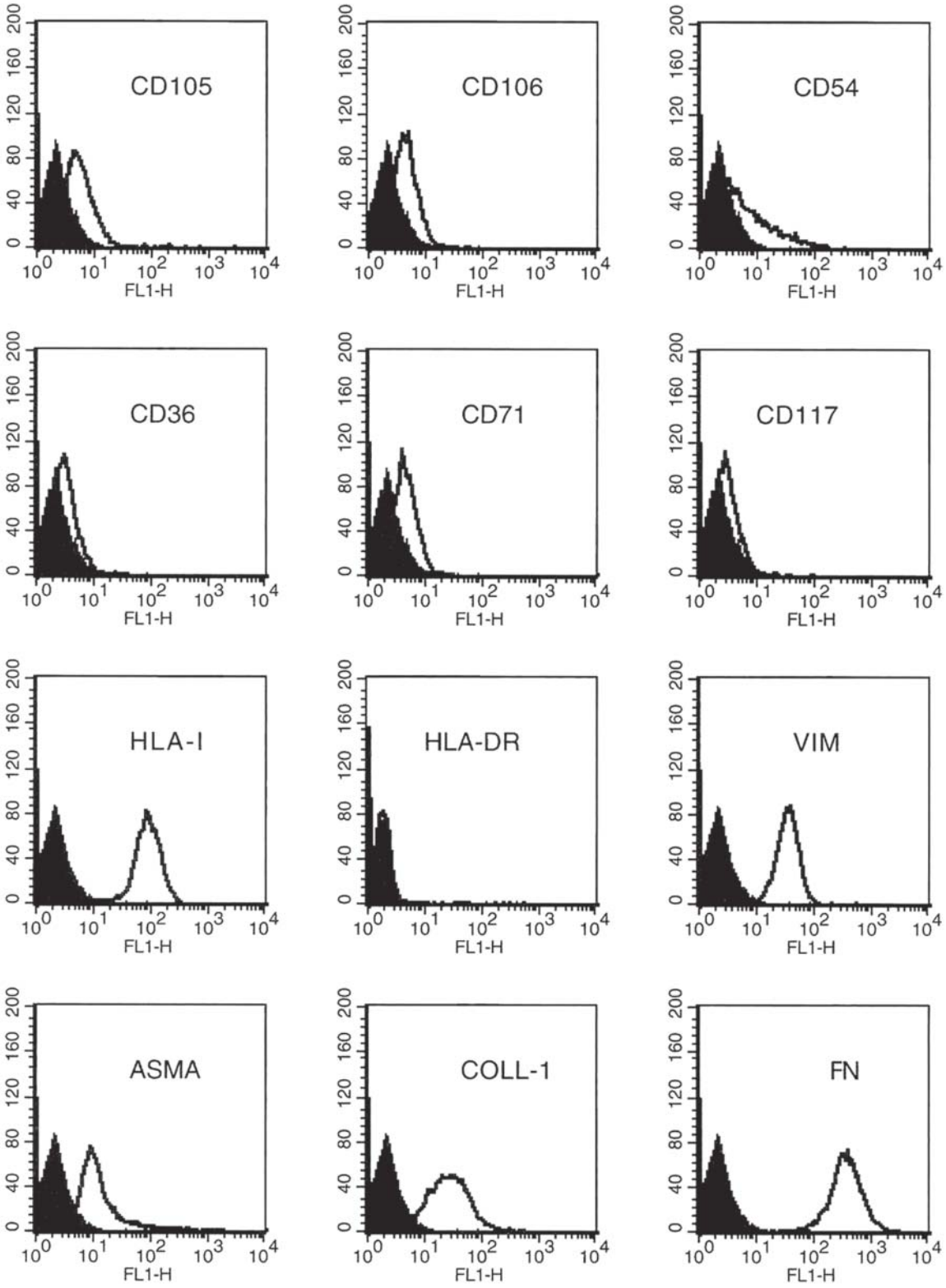

Fig. 2. Cultured human bone marrow and adipose tissue MSC phenotype. VIM: vimentin; ASMA: smooth muscle α-actin; COLL-1: collagen 1; FN: fibronectin.

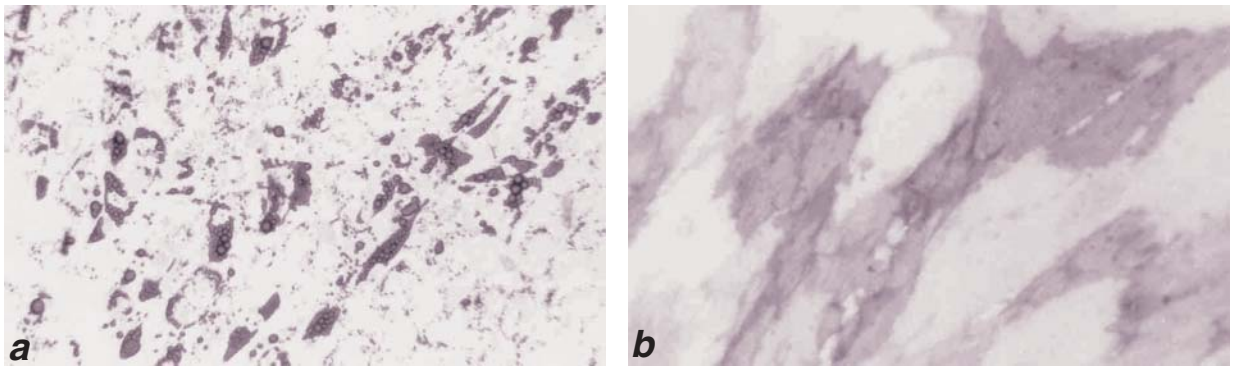

Fig. 3. Results of adipogenic (*a*) and osteogenic (*b*) differentiation of cultured human adipose tissue MSC. Oil red O staining, ×200 (*a*); detection of alkaline phosphatase, ×450 (*b*).

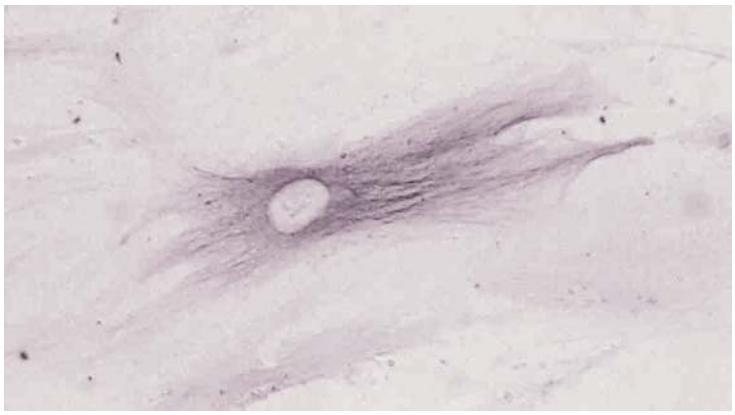

Fig. 4. Detection of β-tubulin-III in cultured human bone marrow and adipose tissue MSC after neuronal differentiation. Immunoperoxidase technique, ×450.

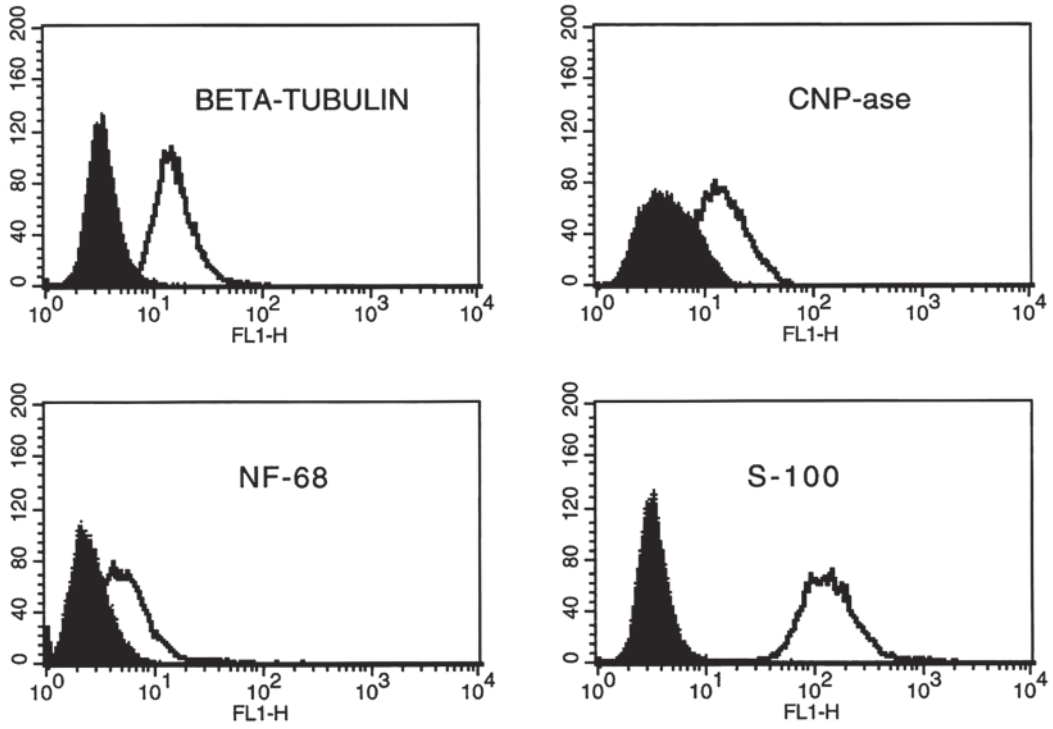

Fig. 5. Neuronal differentiation markers in bone marrow and adipose tissue MSC after two cycles of induction.

MSC for biomedical studies, but become a real alternative to the bone marrow, isolation of which is fraught with certain technological and medical difficulties. It does not, however, mean that the main efforts should be concentrated on the study of only this source.

There is another available tissue of postnatal origin, which, according to our findings and published data, can be used for effective isolation of pluripotent stromal cells: umbilical and placental tissue [5,8,13, 14,16]. Umbilical/placental stromal cells are characterized by biological "youth", possibility of pluripotent differentiation characteristic of MSC from other sources, and lower probability of life-time contamination with viral agents, can also become potential candidates for the development of cell preparations.

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