Umbilical Cord Blood Mesenchymal Stem Cells

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We studied umbilical cord blood mesenchymal stem cells and compared mesenchymal stem cells derived from umbilical cord blood, adipose tissue, and skin. Umbilical cord blood mesenchymal stem cells were characterized morphologically, cytofluorometrically, and by their differentiation potential. Umbilical cord blood mesenchymal stem cells did not differ from cells isolated from adipose tissue and skin by the main parameters (by morphology, expression of surface markers, and differentiation potential). A specific feature of umbilical cord blood mesenchymal stem cells is their low count per volume of the initial material and very low proliferative activity.

Key Words: *mesenchymal stem cells; umbilical cord blood; adipose tissue; skin*

Umbilical cord blood is one of the most perspective sources of stem cells used in clinical practice. By the present time sufficient experience is gained in the collection, storage of umbilical cord blood, and isolation of stem cells from it.

Mesenchymal stem cells (MSC), attracting the greatest interest in modern regeneration medicine, are present in virtually all human tissues, including the umbilical cord blood, together with hemopoietic stem cells. However, umbilical cord blood MSC are little studied. Only few reports present comparative characteristics of umbilical cord blood MSC and MSC from other sources [7,9,10].

Umbilical cord blood has some obvious advantages in comparison with other sources of stem cells: it is a source of two types of stem cells (hemopoietic and mesenchymal) and can be collected by a noninvasive method.

Sufficient experience is gained in transplantation of umbilical cord blood and isolation of hemopoietic cells from it. MSC from the umbilical cord blood are now intensively studied. Umbilical cord blood attracts the attention of scientists and clinicians also as a perspective source of MSC for autologous and allogeneic transplantation.

We studied the umbilical cord blood MSC and compared these cells with MSC isolated from adipose tissue and skin.

MATERIALS AND METHODS

Umbilical cord blood for the study was collected after normal delivery. Adipose tissue collected by liposuction and small skin fragments collected during plastic surgery in donors (mean age 30 years) giving consent for the use of their material (adipose tissue and skin) for scientific purposes, were used for comparative characterization.

The umbilical cord blood was collected directly after delivery. For each experiment, 30 ml blood from one donor was used. Stem cells were isolated no later than 15 h after collection of the material.

The fraction of mononuclear cells from the umbilical cord blood was isolated under standard conditions in Ficoll density gradient, diluted with an equal volume of PBS, applied onto Ficoll paque (Pharmacia), and centrifuged at 400*g* for 30 min at 10°C. The middle fraction of cells was collected, washed in PBS, centrifuged at 200*g* for 10 min at ambient temperature, erythrocytes were lysed with

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160 mM NH_4Cl, washed again, and transferred (5×10^8/cm^2) to uncoated plastic (Costar). The fragments of analyzed adipose tissue and skin were washed in PBS, minced, and treated with 0.075% collagenase (30 min at 37°C). The enzyme was inactivated with DMEM containing 10% fetal calf serum (FCS; Gibco), centrifuged at 200*g* for 10 min at ambient temperature, and washed several times in PBS; erythrocytes were lysed with 160 mM NH_4Cl, if necessary. The fragments of lysed tissue were removed by filtration through 100-μ Nylon filters (Millipore). The cells were plated (1×10^6/cm^2) on uncoated plastic. The medium was replaced on the next day. In all cases MSC were cultured under identical conditions in DMEM (Gibco) with low glucose content (1 g/liter), 10% FCS (Gibco), 2 mM glutamine, 1% penicillin and streptomycin. The cells were cultured under standard conditions at 37°C and 5% CO_2. The medium was replaced every 3-4 days. After 80% confluence was attained, the cells were harvested with 0.25% Trypsin solution with EDTA and subcultured (6-10 cells/cm^2). After the first passage, all cultures were frozen in liquid nitrogen using 10% dimethylsulfoxide and 30% FCS. The first cytofluorometric analysis was carried out after 2 passages, the second after 5 passages. The samples were analyzed on a FACSCalibur flow cytofluorometer (Becton Dickinson) using logarithmic scale of fluorescence and CellQuest software. Fluorescence was excited with Innova-90-6 argon laser (Coherent). A kit of FITC-labeled direct monoclonal CD44 antibodies, APC-labeled CD105 (indirect), CD90, CD34, and Cy2-labeled second anti-mouse antibodies (Beckman Coulter and Coltak) were used. Before the analysis on a flow cytofluorometer, the cells were harvested with 0.25% Trypsin solution with EDTA and washed in PBS with 0.1% FCS. Cell aliquots (10^6) were incubated in a buffer containing monoclonal antibodies; before staining with indirect antibodies the cells were washed in PBS with 0.1% FCS and incubated for 30 min with second antibodies. Dead cells were excluded from the analysis using propidium iodide (PI) staining and by scatter parameters. The data were processed using WinMDI 2.8 software.

Cell differentiation was carried out by standard protocols. After 2 passages MSC isolated from all sources were plated (15-20 cell/cm^2) and cultured in DMEM with 4 g/liter glucose, 2 mM glutamine, 1% antibiotic and antimycotic, 10% FCS for 3 weeks with addition of 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, 10 μM insulin, and 200 μM indomethacin (adipogenic differentiation) or 0.1 μM dexamethasone and 50 μM ascorbate-2-phosphate (osteogenic differentiation).

After 3 weeks of culturing, differentiated cells were washed twice in PBS and fixed. Adipocytes were fixed in 4% formaldehyde solution (60 min at ambient temperature), after which they were incubated in 0.5% Oil Red (20 min). Osteoblasts were fixed in 70% ice-cold ethanol for 1 h and stained with Alizarin Red (40 mM, pH 4.10) for 10 min. Microphotographs of stained cells were made using a phase contrast microscope (Olympus CK 40 M) and Image-Scope Lite software.

RESULTS

Cells morphology was typical of MSC. Generally, the population was highly heterogeneous by cell size (from 10 to 300 μ) and shape. Apart from spindle-shaped and fibroblast-like cells, there were round and triangular cells (Fig. 1, *a*). Large cells (often multinuclear) predominated during culturing.

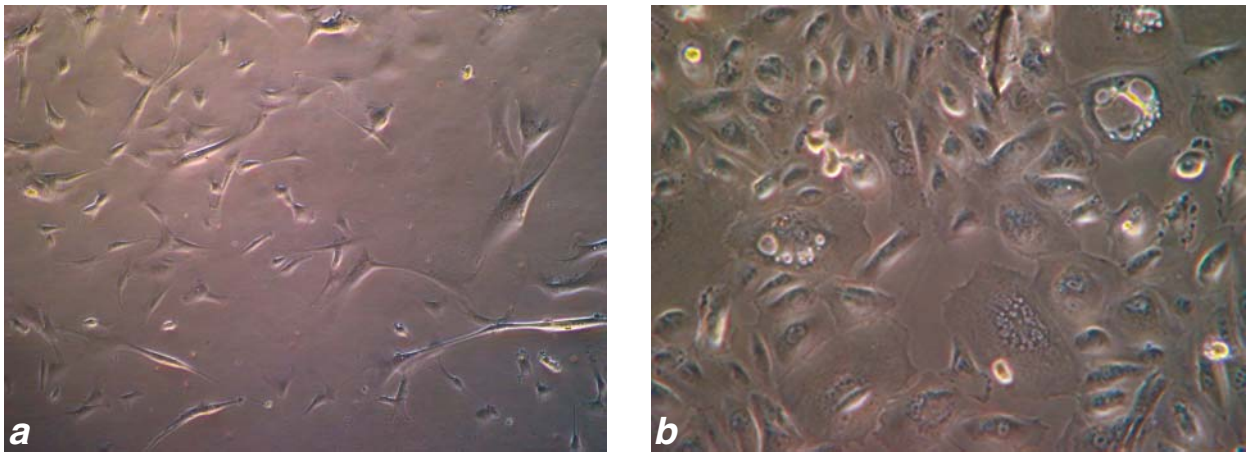

Fig. 1. Umbilical cord blood MSC. *a*) typical umbilical cord blood MSC; *b*) specific colonies of umbilical cord blood cells adhering to plastic.

A specific feature of the culture was the presence of several cell populations differing from each other, at early stages of culturing. Apart from typical MSC there were numerous colonies of smaller and round cells. Very large multinuclear cells, often with high content of secretion, were also seen in these populations (Fig. 1, *b*). During the first 3 weeks of culturing, the number of these colonies and typical MSC colonies was about the same. The cells were under standard conditions of selective MSC culturing. Later, the cells in these colonies died, while MSC survived. Detailed description of all cells isolated from the umbilical cord blood and adhering to plastic was not planned in this study.

A specific feature of umbilical cord blood MSC is their low percentage in the initial material. Our data indicate that no more than 1-2 MSC clones per 10^8 mononuclear cells of umbilical cord blood can be seen.

Proliferative activity of umbilical cord blood MSC was evaluated visually. The lag period for all cultures was 24-48 h, and at this stage there were virtually no differences between MSC cultures. The periods of exponential growth (logarithmic phase) differed significantly. Umbilical cord blood cells cultured for 6 months passed only 3 passages. The first monolayer (80% confluence) of umbilical cord blood MSC was obtained after 2 months of culturing, while adipose tissue cells formed monolayer after 7-9 days of culturing and skin cells formed it on day 9-10. The population doubling rate during the exponential growth period was calculated by the formula: $\log N_1/\log N_2$, where N_1 is the number of cells in the confluent layer and N_2 is the initial number of cells. Cell growth intensity virtually did not change with time under these culturing conditions. Proliferative activity of any MSC type depends primarily on the quality of initial material. However, all umbilical cord blood samples analyzed in our study were characterized by extremely low proliferation in comparison with adipose tissue and skin MSC and bone marrow MSC, cultured in parallel.

All cells were analyzed twice on a flow cytofluorometer by the expression of CD44, CD90, CD45, and CD34 during 6 months of culturing (after passages 2 and 5). Background fluorescence did not exceed 3%. The expression of CD44 and CD90 was virtually the same in all analyzed cells and was very high (90-94%; Fig. 2), virtually not changing during culturing. Deviations from basal CD34 expression during culturing were 9.9% (Fig. 2), which was presumably due to the quality of antibodies. The expression of CD45 marker did not surpass the background level.

Differentiation into adipocytes and osteoblasts was associated with morphological changes in cells from all tissues. At the initial stages it was simpler to trace and visually evaluate changes in the cultures during differentiation into adipocytes, because the cells started accumulating lipid inclusions. Dif-

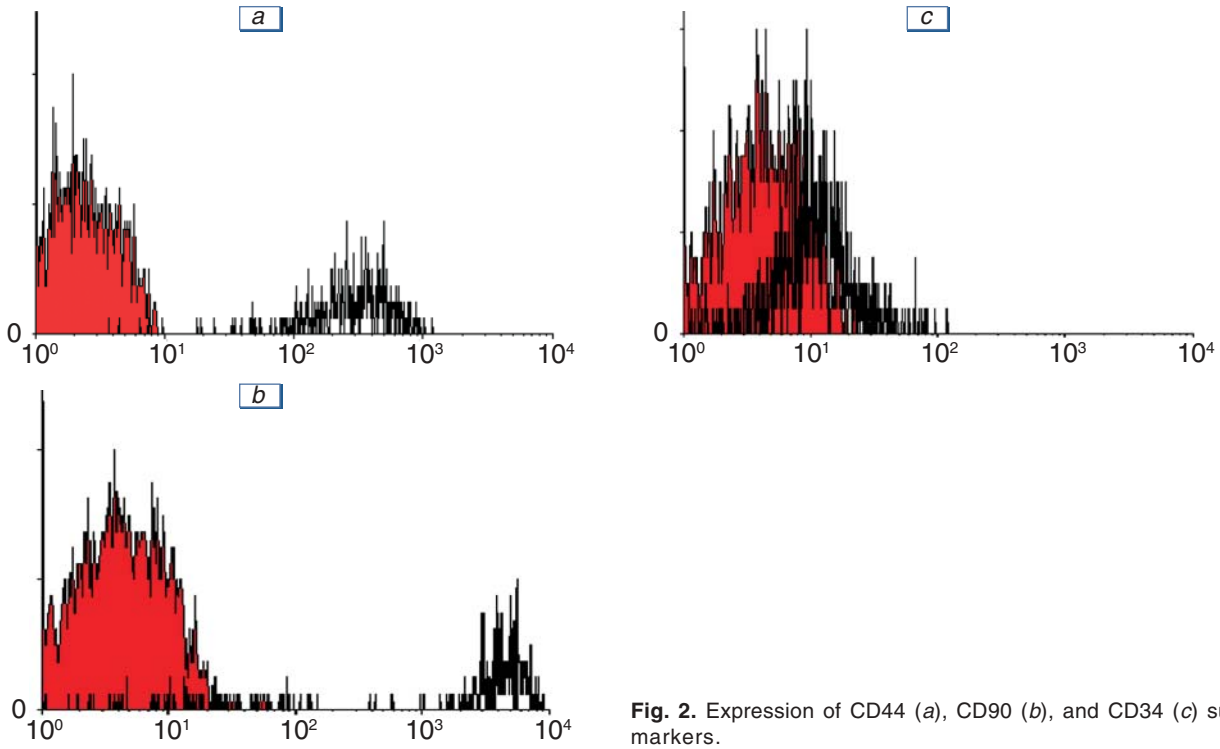

Fig. 2. Expression of CD44 (*a*), CD90 (*b*), and CD34 (*c*) surface markers.

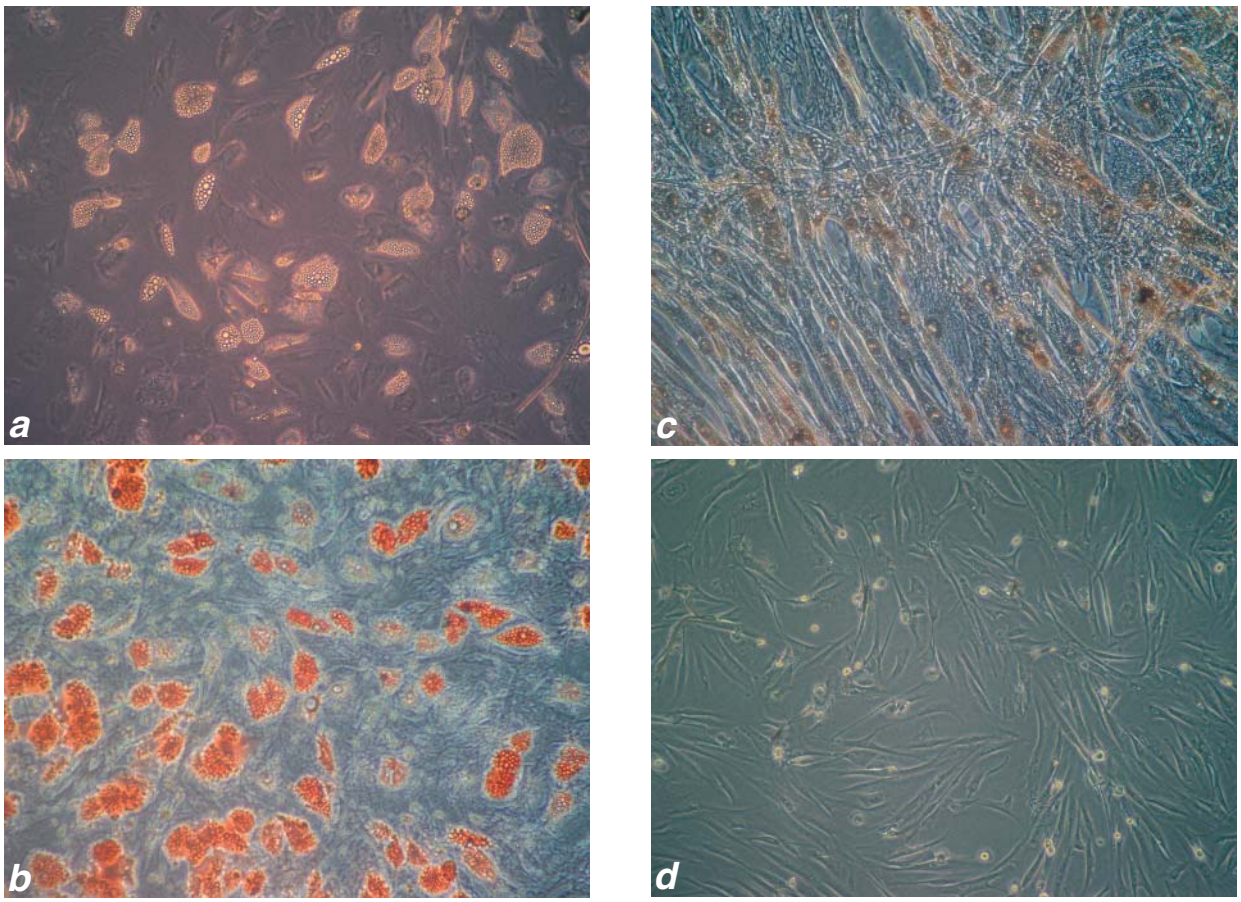

Fig. 3. Differentiation of umbilical cord blood MSC. *a*) adipogenic differentiation of umbilical cord blood MSC (no stain); *b*) adipogenic differentiation of umbilical cord blood MSC; Oily Red staining; *c*) osteogenic differentiation of umbilical cord blood MSC; Alizarin Red staining; *d*) control (umbilical cord blood MSC).

ferentiation into adipocytes was first noted in cell cultures isolated from adipose tissue, then in umbilical cord blood cells. The first lipid inclusions appeared in umbilical cord blood cultures after 3-4 days (Fig. 3). After 1 week 30-40% adipose tissue MSC and 20-30% umbilical cord blood MSC were differentiated (Fig. 3). By this time the first differentiated cells appeared in cultures originating from the skin.

Osteogenic differentiation was more difficult to observe visually. The cells gradually acquired a round shape, specific depositions appeared in cells. After 3 weeks of culturing the cells in all cultures were stained with Alizarin Red. Bone marrow MSC differentiated into osteoblasts most actively; adipose tissue MSC differentiated less intensely. Umbilical cord blood MSC differentiated into osteoblasts not so actively. After 3 weeks of culturing in osteogenic medium, about $^1/_3$ of umbilical cord blood cells differentiated into osteoblasts (Fig. 3).

According to published data, umbilical cord blood MSC were to be obtained from at least 30 ml material collected no later than 15 h after labor [1].

The main characteristics of MSC are fibroblast-like morphology, adhesion to plastic, clonogenic activity, morphological heterogeneity of the population, capacity to differentiate into various cell types under certain conditions, expression of marker genes, such as CD44, CD90, CD105, CD13, Stro-1, and the absence of expression of marker genes characteristic of hemopoietic cells (for example, CD45, CD34). Umbilical cord blood MSC fully meet all these requirements. Umbilical cord blood MSC are positive for SH2 (CD105), SH3 (CD73), CD13, CD29 (β_1-integrin), CD49e (α_5-integrin) and negative for CD3, CD14, CD19, CD34, CD45 [3,5], express class I HLA and do not express class II HLA [1]. Some differences in the expression of surface marker by umbilical cord blood and bone marrow MSC were reported. For CD105 the umbilical cord blood expression was reported as 53.6±4.3%, bone marrow expression as 84.4±2.8%. Analysis of transcriptome showed that umbilical cord blood MSC virtually did not differ from bone marrow MSC [4]. Comparative analysis of umbilical cord blood mononuclear fraction and its MSC subpopu-

lation showed a significant difference in the gene expression. For example, expression of 47 genes was 50-fold more intensive in MSC. These were mainly genes encoding for intercellular matrix, cytokine, growth factor proteins and some genes coding for membrane and nuclear proteins. According to flow cytofluorometry, 22 surface antigen markers showed no differences between bone marrow, umbilical cord blood, and adipose tissue cells [10].

The umbilical cord blood MSC contain two different populations of clonogenic cells [2]. They differ morphologically by shape: some cells (greater part) are flat fibroblasts, the others (lesser part) are spindle-shaped cells. The fraction of spindle-shaped cells did not express CD90 and during differentiation exhibited a lesser trend to adipogenesis.

One more characteristic feature of umbilical cord blood MSC is the presence of fraction of the so-called multipotent progenitor (or dormant) cells with a high differentiation potential; these are small (up to 10 μ) cells, capable of differentiating in all three germinal layers [9].

Umbilical cord blood MSC differentiated not only into adipocytes and osteoblasts, but also into neural cells [4,6,11], hepatocytes [8], and skeletal myoblasts expressing myogenic markers [4].

Comparison of differentiation of the bone marrow, umbilical cord blood, and adipose tissue MSC in a previous study showed that umbilical cord blood MSC did not differentiate into adipocytes [7]. Our data prove the opposite fact and experimentally confirm the capacity of umbilical cord blood MSC to differentiate into adipocytes.

The majority of scientists noted low or, according to some data, extremely low count of MSC in the mononuclear cell population in the umbilical cord blood [1] and difficulties in isolation and expansion of MSC from this source. The count of MSC-like cells varied from 0 to 2.3 clones per 10^8 mononuclear cells of umbilical cord blood [1]. During 8 passages the cell population doubled at least 20 times. According to our data, the count of MSC in the umbilical cord blood mononuclear fraction is extremely low in comparison with their population in the bone marrow, adipose tissue, and skin and *in vitro* expansion of MSC is really associated with some difficulties. Umbilical cord blood cells are characterized by low proliferative activity, high sensitivity to the quality of the serum and other components of the medium, low survival after freezing in comparison with other sources. All this limits the possibility of using umbilical cord blood as an adequate source of MSC in allogeneic transplantation, not ruling out, however, its use for autologous transplantation due to extremely easy method for collection of umbilical cord blood.

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