Optimum Conditions for Culturing of Human Bone Marrow and Adipose Tissue Mesenchymal Precursor Cells

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We present a technology of culturing of human mesenchymal stem cells under conditions excluding the presence of animal sera or additional growth factors, but preserving high proliferative potential and the capacity of these cells to multilineage differentiation. Human umbilical serum was used as the alternative material. We found that in the presence of human umbilical serum mesenchymal stem cells more effectively proliferate and retain their differentiation capacity. The proposed technology yields 10^9-10^{10} morphologically and functionally identical cells.

Key Words: *mesenchymal stem cells; cell culture; bone marrow; umbilical cord serum; differentiation; phenotype*

During recent decades, mesenchymal (stromal) precursor cells (MSC) are regarded as the potential material for tissue engineering and cell therapy of various diseases and pathological states [2,7,20]. This can be explained by their capacity to effective expansion and differentiation into various cell types both *in vivo* and *in vitro* [3,4,8,10,11,18,20]. MSC can be easily isolated from various postnatal sources, including bone marrow, adipose and placental tissues [1,5,9,13,14,22], and used for obtaining required cell mass or for differentiation triggered by hormones, cytokines, and other factors.

Clinical use of MSC is limited by a number of reasons. One of these reasons is the presence of high concentrations of animal serum (FCS or equine serum) in the culture medium, which determines the risk of transmission of prion agents or zoogenous infection [6], even despite the fact that sera supplied by leading manufacturers is thoroughly tested by many parameters. Immunization of the patient with foreign proteins cannot also be excluded [15,17], which is most dangerous in repeated cell administration.

Thus, there is a need for alternative methods for culturing of human MSC. A possible way is the use of synthetic growth factors [12,16] or autologous or donor serum [15,19].

Here we evaluated the possibility of culturing MSC in the presence of human umbilical cord serum (UCS).

MATERIALS AND METHODS

We used samples of fetal calf serum (FCS) purchased from GIBCO/Invitrogen (2 lots), HyClone (2 lots), and Stem Cell Technologies (2 lots). All sera were stored at -20°C (expire not before 1 year).

Umbilical blood was collected into 50-ml sterile tubes after obtaining the informed consent of the parturient women and in case of negative tests

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for infection diseases (HIV-1/2, hepatitis B, hepatitis C, syphilis). After clot formation, the samples were centrifuged at 600*g* for 15 min. The sera without visible signs of hemolysis were centrifuged at 1000*g*, pooled, filtered through 0.22 μ-membrane filters (Corning), and stored at -20°C.

Isolation and culturing of MSC. Bone marrow and adipose tissue MSC were isolated as described earlier [1]. The cells were cultured in DMEM with low glucose content supplemented with 25 mM HEPES, 2 mM L-glutamine, 1 mM pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin (all reagents were from GIBCO/Invitrogen), and 10% serum. Until passage 2, the cells were cultured in the presence of FSC (a GIBCO/Invitrogen lot). Confluent cultures were washed with Earl solution, removed with trypsin-EDTA, and transferred into new flasks (1:5 or 1:10). The number of adherent cells was counted after 24 h and the medium was replaced with a fresh portion containing UCS or the corresponding lot of FCS. The medium was replaced every 2-3 days. The cultures were examined and photographed using a phase-contrast microscope (Diaphot-TMD, Nikon). After attaining confluence, the cells were reseeded and cultured in the same medium.

For evaluation of possible effects of UCS on adherence and growth of MSC during early passages, a portion of cell material (mononuclear fraction of the bone marrow) was transferred into flasks with USC-supplemented medium immediately after isolation. Further culturing and subculturing were performed as described above.

For construction of growth curves, the number of cells per 1 cm^2 was counted under a microscope in several fields of view.

Analysis of MSC phenotype For flow cytometry analysis, the cells were removed from plastic with trypsin-EDTA and precipitated by centrifugation. Then, MSC were fixed with CellFix solution (Becton Dickinson) and prepared for the analysis as described earlier [1]. Mouse monoclonal antibodies to α-smooth muscle actin (ASMA), CD34, CD45, CD54, CD105, CD106, CD117, HLA-I, HLA-DR, vimentin, von Willebrand factor, myosin of smooth and skeletal muscles, type I collagen, and fibronectin (Becton Dickinson, Chemicon, Sigma-Aldrich) were used. When unlabeled antibodies were used, the preparations after washout were incubated with FITC-labeled antibodies to mouse immunoglobulins (Sigma-Aldrich). Nonimmune immunoglobulins of the corresponding class served as the negative control. The cell suspensions were analyzed on a FACS Calibur flow cytometer using CellQuest software (Becton Dickinson).

Targeted differentiation of MSC. For evaluation of the possibility of differentiation in the adipogenic, osteogenic, and neurogenic directions the MSC cultures during passages 2-3 and 5-7 were incubated in media containing the corresponding inductors [1,22]. The results were assessed by positive staining with Red Oil O (lipid inclusions), detection of alkaline phosphatase activity, or by the presence of neuroinal differentiation markers.

RESULTS

Bone marrow- and adipose tissue-derived MSC cultures during passage 2 (*i.e.* cells used in the experiments as the start cell material) possessed all properties of previously described cells [1]. In the phase of active growth, the mean doubling time was 24-36 h for early passages and 36-72 h during subsequent culturing in the presence of FCS. We revealed no considerable differences in cell morphology and proliferation rate between MSC cultured with standard FCS lots.

The use of sera (chosen and recommended by the manufacturer) increased the rate of culture growth by 20-30% (Fig. 1). A similar effect was observed in cultures grown in the presence of one FCS lot marked as "characterized".

Substitution of FCS with UCS considerably increased proliferative activity of MSC. After 1-2 passages, the rate of culture growth increased 2-3-fold, while doubling time decreased to 18-20 h. During subsequent 1:5 subculturing, the rate of proliferation remained practically unchanged.

In a special experimental series we evaluated the effect of UCS on adherence of MSC to plastic and clonal growth. To this end, mononuclear fraction of the bone marrow obtained after gradient

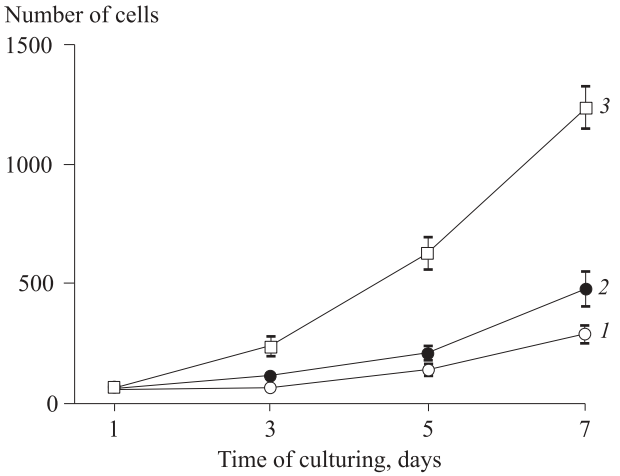

Fig. 1. Number of human bone marrow MCS in cultures grown in the presence of standard FCS (*1*), "chosen" FCS (*2*), and UCS (*3*).

centrifugation on Histopaque 1.077 (Sigma-Aldrich) was divided into two aliquots seeded in a medium with 10% FCS and 10% UCS, respectively. The media were replaced after 48 h and the cells were cultured under the same conditions.

No differences by the number of formed colonies were found. Higher adhesion of cells of the hematogenous origin observed in the medium with UCS (Fig. 2, *a*, *b*) had no effect on the fate of MSC, because leukocytes were completely eliminated during the subsequent washouts and subculturing.

Phase-contrast microscopy showed that cells cultured in the presence of UCS are visually smaller, while the culture looks more homogenous due

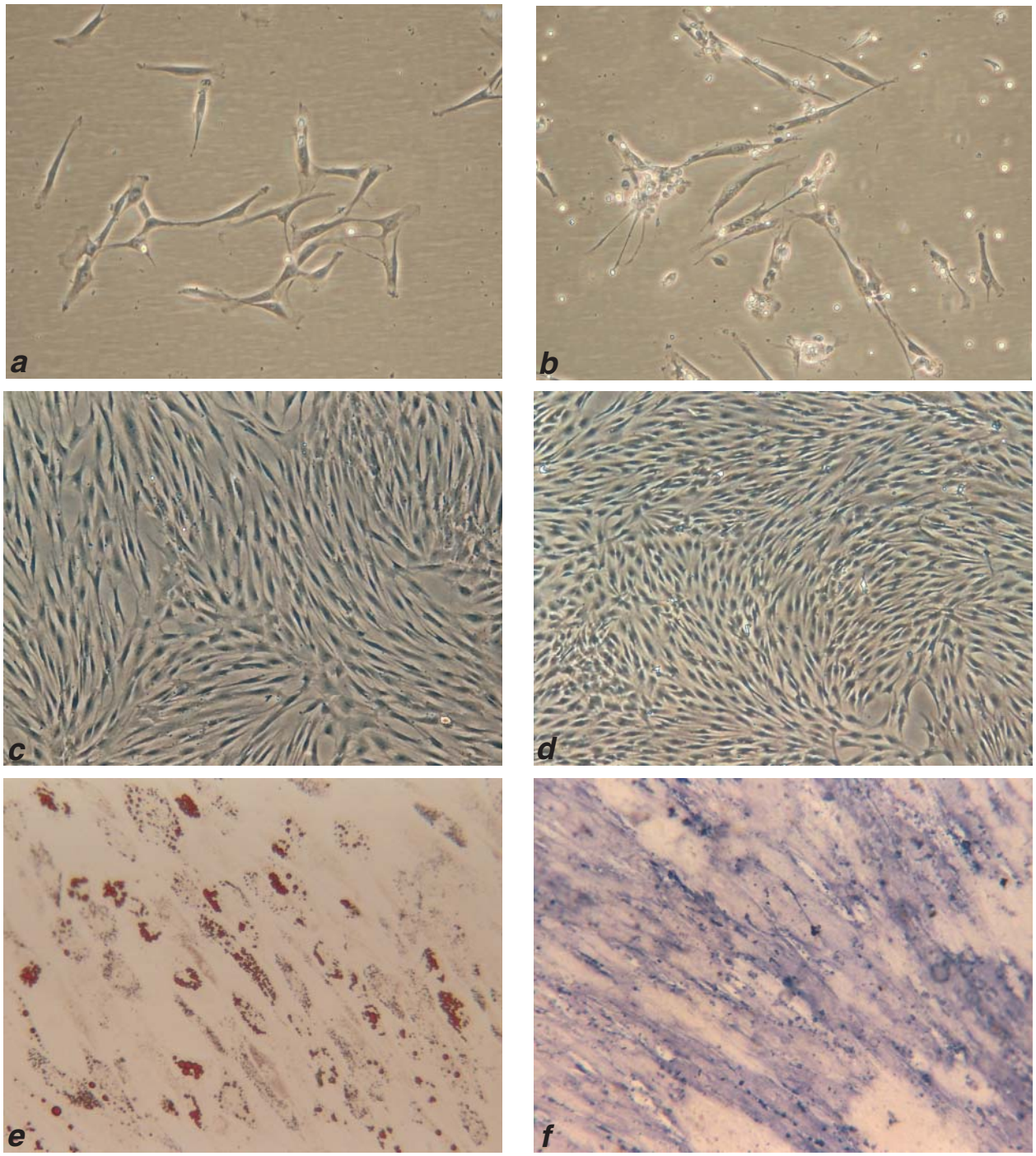

Fig. 2. Morphology and differentiation capacity of human bone marrow and adipose tissue MSC cultured in the presence of FCS and UCS. *a, b*) primary colonies of bone marrow MSC on day 3 after seeding in FCS and UCS, respectively; phase contrast (×250). *c, d*) morphology of bone marrow and adipose tissue MSC cultured in the presence of UCS, passage 4; phase contrast (×100). *e, f*) adipogenic and osteogenic differentiation of bone marrow MSC cultured in the presence of UCS, passage 4, ×250.

to the prevalence of regular spindle-shaped cells with low number of processes (Fig. 2, *c, d*). Parallel cultures grown in the presence of FCS contained a considerable number of large flattened cells or cells with irregular shape. Visual differences between the cultures were confirmed by flow cytometry (analysis of direct and lateral light scattering).

Analysis of expression of surface markers confirmed mesenchymal properties of the obtained cultures. However, expression of markers of differentiated cells (ASMA, CD54, CD105, CD106) was lower in cultures grown in the presence of UCS, compared to parallel cultures grown in the presence of FCS (Fig. 3). We found no reliable phenotypic differences between cultures of passages 3-4 and 7-8. Spontaneous (without preliminary neuronal induction) expression of β-tubulin-III was observed in some cultures grown in the presence of UCS. In none cultures markers of hemopoietic, endothelial, and smooth muscle cells were detected.

Culturing of bone marrow and adipose tissue MSC in the presence of UCS had no effect on their potency to differentiate in the adipogenic and osteogenic directions. Most cells (~95-98%) formed

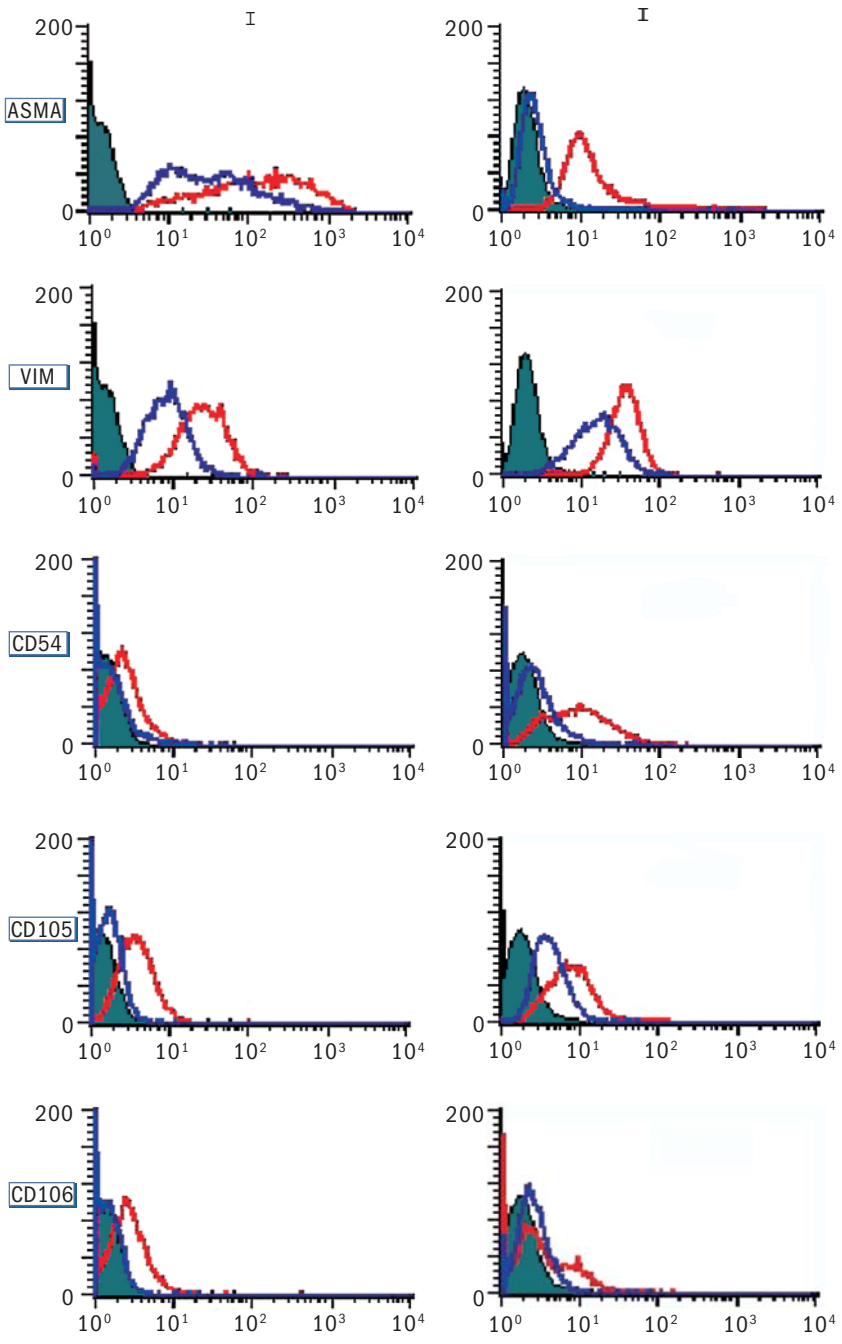

Fig. 3. Comparative analysis of phenotype of human bone marrow MSC (*I*) and adipose tissue MSC (*II*) cultured in the presence of FCS (red line) and UCS (blue line). ASMA, α-smooth muscle actin, VIM, vimentin.

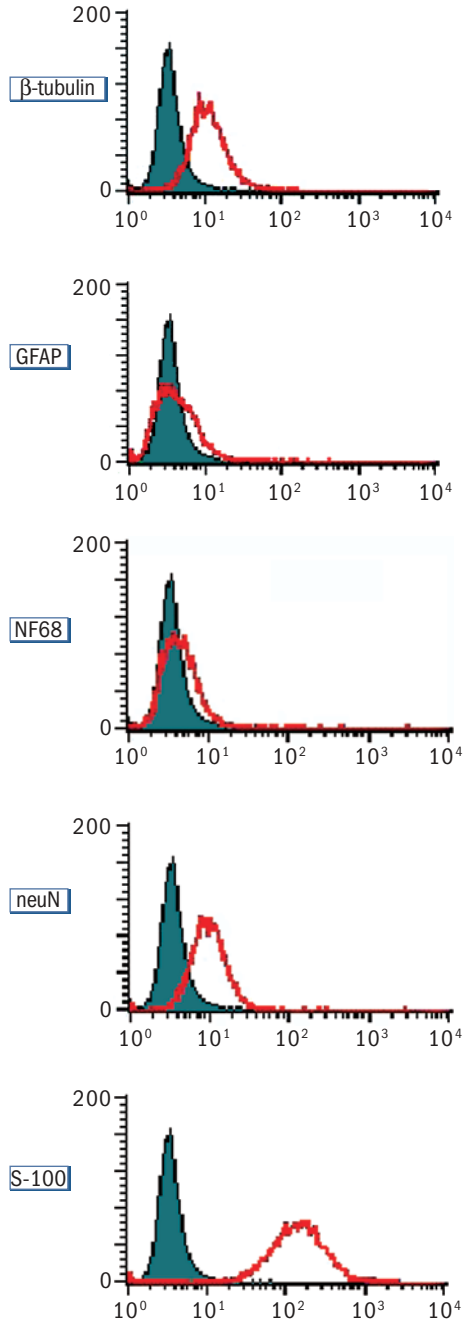

Fig. 4. Markers of neuronal differentiation of human adipose tissue MSC (passage 3) after two induction cycles in the presence of UCS.

lipid inclusions or expressed alkaline phosphatase within 2 weeks after the corresponding stimulation (Fig. 2, *e, f*).

For evaluation of the neuronal differentiation potency of MSC we used monoclonal antibodies to β-tubulin III, CNPase, S-100, NF-68, GFAP (Sigma-Aldrich), and NeuN (Chemicon). Expression of β-tubulin III, CNPase and NF-68 was noted after two consecutive induction cycles (Fig. 4). Solitary cells demonstrated GFAP-positive staining. Practically all bone marrow and adipose tissue MSC (95-99%) expressed S-100 irrespective of the serum used.

Development of protocols of clinical use of human MSC necessitates improvement of methods for cell isolation and culturing. However, the majority of existing methods are based on the use of components of animal origin for the culture media. FCS is more often used for these purposes; its concentrations usually varies from 10 to 20%. This is sufficient for effective expansion of cells and their multilineage differentiation under experimental conditions, but this approach cannot be used in clinical practice, because of high risk of transfer of foreign proteins not completely eliminated by cells. The number of administered cells is tens and hundreds of millions, and therefore milligrams of foreign protein can be transferred [17]. FSC proteins can be removed by cell washout before injection or by cell culturing in a serum-free medium, but the risk of immunization cannot be excluded. The risk of infecting the cells and patient with viral or prion agents should be also taken into account when developing protocols of obtaining biologically safe cell preparations. An alternative method is cell culturing is special serum-free media containing synthetic growth factors and hormones [15,19], but this approach did not find wide application in clinical practice.

UCS can be used as effective analog of FCS for preparing culture media for human MSC. It was found that UCS has no effect on the adherence of freshly isolated MCS and the rate of formation of primary cell colonies, but considerably stimulated further cell growth. The advantages of UCS over FCS become apparent few days after changing medium composition and peak after 1-2 passages. High rate of culture growth is retained during subsequent culturing. This is probably related to the predominance of MCS population with high proliferative potential in the culture, which leads to its rejuvenation: the cells become smaller and more homogenous and acquire the capacity to active and uniform growth after inoculation with lower density (to 1:10). The phenotype of MSC is also changed; expression of markers of differentiated cells decreases. Culturing in the presence of UCS had no negative impact on MSC differentiation into adipocytes, osteoblasts, and neural cells. In this case the signs of differentiation appear sooner and are more pronounced compared to culturing under standard conditions.

The use of UCS considerably accelerates the procedure of obtaining the therapeutic dose of cells. According to our data, when the cells are subcultured in 1:5-1:10 ratios, the intervals between passages are 7-9 days and 100 mln. MSC can be ob-

tained after about 6 weeks. During the subsequent 2-3 weeks the cell mass increased by 100 times (to 10^{10} cells).

Thus, technologies of effective culturing of human cells suitable for clinical use can be developed under conditions of strict adherence to requirements to blood products and principles of aseptics.

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