In Vitro Growth of Human Umbilical Blood Mesenchymal Stem Cells and Their Differentiation into Chondrocytes and Osteoblasts

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Conditions for culturing and differentiation of human umbilical blood mononuclear cells *in vitro* were studied. The growth of mesenchymal stem cells was attained in 31 of 54 (57.4%) umbilical blood samples and morphological and immunophenotypical authenticity of these cells was confirmed. Stimulatory effects of 20% AB(IV) human serum and transforming growth factor-β (TGF-β) on the growth of mesenchymal stem cells were demonstrated. Osteogenic cells formed in the presence of differentiation factors ascorbic acid, dexamethasone, and β-glycerophosphate, while chondrogenic cells developed in the presence of dexamethasone, ascorbic acid, and TGF-β. Differentiation of mesenchymal stem cells was confirmed by histochemical and molecular genetic tests.

Key Words: *human umbilical blood; mesenchymal stem cells; differentiation; chondrocytes; osteoblasts*

Modern technologies of using mesenchymal stem cells (MSC) for medical and biological purposes involve their *in vitro* culturing. Although umbilical blood is inferior to bone marrow and adipose tissue by the content of MSC [1,4], its availability and high hemopoietic potential prompted creation of an extensive network of umbilical blood banks and development of approaches to their practical use in medicine. However, problems of creating optimal culturing conditions for umbilical blood cells for *in vitro* reproduction of MSC and their differentiation in various directions remain unsolved [5].

We studied optimal conditions for long-term culturing and directed differentiation of umbilical blood MSC into chondrocytes and osteoblasts.

MATERIALS AND METHODS

Umbilical blood was collected in maternity hospitals of Minsk after 39-40-week gestation from healthy puerperae. Written consent to the procedure was obtained. The blood was collected in standard containers with 63 ml anticoagulant (citrate phosphate dextrose adenine; CPDA-1) and transported at 4°C; the period between blood collection and cell isolation was no longer than 4 h.

Umbilical blood mononuclear cells (MNC) were isolated in Ficoll-verografin density gradient (ρ=1.077 g/cm^3). The cells were washed and placed into T25 flasks (Sarstedt) in a concentration of (1-3)×10^6 cell/cm^2 for adhesion. Mononuclears from 3 umbilical blood samples were placed for adhesion into flasks pretreated with AB(IV) human serum for 30 min at ambient temperature.

The umbilical blood MNC were cultured in α-MEM, IMDM (Sigma), and MesenCult (StemCell Technol.) media containing 10% fetal calf serum (FCS; Sigma), 2 mM L-glutamine (Sigma), 100 μg/ml streptomycin, 100 U/ml penicillin. For MSC adhesion, the cultures were incubated for 24 h at 37°C in a humid atmosphere with 5% CO_2. After 24 h the medium was discarded, adherent cells were

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washed twice, and a fresh portion of complete nutrient medium was added. The growth of MSC was stimulated by adding 10 ng/ml FGF-β (Sigma). Culture medium (40%) was replaced every 3-4 days. Mesenchymal stem cells from two umbilical blood samples were cultured in α-MEM with 30% serum (10% FCS and 20% AB(IV) human serum), 10 ng/ml FGF-β, 10 ng/ml transforming growth factor-β_3 (TGF-β_3).

The *in vitro* growth of MSC was monitored under an inverted phase microscope. After attaining 80% confluence, adherent MSC were harvested with 0.25% trypsin and EDTA, washed twice, and repeatedly cultured in a concentration of 200-1000 cell/cm^2.

The expression of the following immunophenotypical markers on cultured cells was studied: CD45, CD14, CD140, CD90, CD105 (Caltag Laboratories). Monoclonal antibodies specific to each marker were added (20 μl) to the sample (100-200 thousand cells) and incubated in darkness at ambient temperature for 25-30 min. The cells were then washed with PSB. The fluorescence was analyzed on a FACSCan cytofluorometer using CellQuest software. At least 10,000 cells were analyzed per sample. Forward and side light scatter was measured.

For induction of osteogenic differentiation, passage 4 MSC in a concentration of 8000 cell/cm^2 were cultured in complete nutrient medium (α-MEM, 10% FCS (Sigma), 2 mM L-glutamine (Sigma), 100 μg/ml streptomycin, 100 U/ml penicillin) at 37°C in a humid atmosphere with 5% CO_2. After attaining 60% confluence, culturing was carried out in a medium for osteogenic differentiation: α-MEM, 10% FCS, 2 mM L-glutamine, 0.1 μM dexamethasone (StemCell), 60 μM ascorbic acid (StemCell), 10 mM β-glycerolphosphate (Fluka), 100 μg/ml streptomycin, and 100 U/ml penicillin. The cells were incubated for 7-10 days, the medium was changed every 3-4 days. Differentiation of umbilical blood MSC into osteoblasts was confirmed histochemically by activity of alkaline phosphatase using the 85L3R-1KT leukocyte alkaline phosphatase staining kit (Sigma-Aldrich) [6,7].

Chondrogenic differentiation was induced as follows. Mesenchymal stem cells (2-2.5×10^5 cells) were centrifuged in a 15-ml polypropylene tube for 5 min at 1500 rpm and 1 ml medium for chondrogenic differentiation containing α-MEM, 10% FCS, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 0.1 μM dexamethasone, 60 μM ascorbic acid, and 10 ng/ml TGF-β_3 (Sigma) was added to the tube without resuspending the precipitate. The cells were cultured for 2-3 weeks; the medium was replaced with a fresh portion every 4th day. Differentiation of MSC into chondroblasts was confirmed by hematoxylin staining of tissue sections [2,3].

For detection of genetic markers of osteogenic and chondrogenic differentiation, total RNA was isolated from cells using RNAqueous-Micro Kit (Ambion Inc.) according to manufacturer's instruction. Subsequent reverse transcription and PCR were carried out using GeneAmp Gold RNA PCR Reagent Kit (Applied Biosystems) according to the description (Catalogue No. 4308206). Alkaline phosphatase (osteogenic differentiation) and aggrecan genes (chondrogenic differentiation) served as the markers. The results of reverse transcription PCR were analyzed by electrophoresis in 2% agarose gel using molecular weight markers (GeneRuler 50 b.p. DNA Ladder; Fermentas).

The data were statistically processed using Student's t test and χ^2 test. The differences were significant at $p<0.05$.

RESULTS

Umbilical blood MSC were isolated from 31 (57.4%) of 54 studied specimens. The mean volume

TABLE 1. Relationship between MSC Growth and Culturing Conditions

Culturing conditions		Total number of specimens	Number of specimens with	
			MSC growth	monolayer formation
Media	α-MEM (10% FCS): control	54	31	5
	MesenCult (10% FCS)	16	8	2
	IMDM (10% FCS)	4	1	—
Pretreatment of plastic by human AB(IV) serum		11	10	3
α-MEM with high serum content (10% FCS+20% AB(IV) serum)		11	10	1
α-MEM with	FGF-β	31	15	—
	FGF-β+TGF-β_3	10	9	4

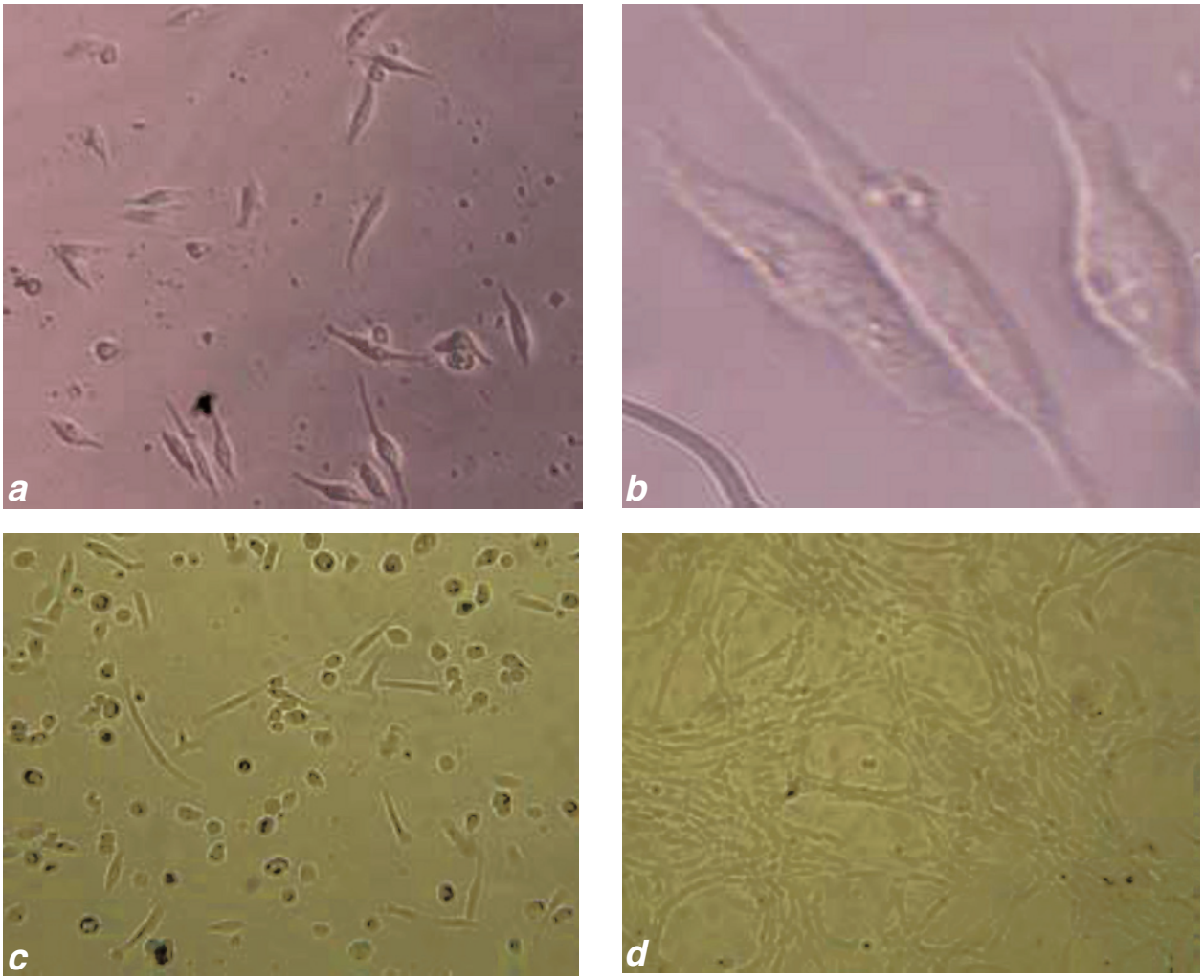

Fig. 1. Morphology of umbilical blood MSC. *a*, *b*) fibroblast cells on day 4 of umbilical blood culturing (*a*: ×100, *b*: ×400); *c*) MSC 2 days after passage 1: heterogeneous cultures (×100); *d*) monolayer formation within 7 days after passage 2 (×100).

of umbilical blood sample was 72.6±22.7 ml (25-140 ml), the count of nuclear cell $12.2\pm8.6\times10^8$ (3.1-38). The count of MNC isolated in density gradient was $8.87\pm4.78\times10^5$ (25-212) cells.

Fibroblastoid cells typical of MSC appeared on days 2-5 of MNC culturing. Cell adhesion to the bottom of culture flasks pretreated with AB(IV) human serum promoted MSC formation in comparison with control samples (Table 1).

Culturing of umbilical blood MNC in α-MEM, MesenCult, and IMDM with 10% FCS showed similar growth efficiency of culturing in α-MEM and MesenCult and lower efficiency in IMDM.

Increasing serum content in the medium to 30% (10% FBS+20% AB(IV) serum) increased the number of umbilical blood samples with MSC growth in comparison with the control (p=0.036; Table 1).

Addition of FGF-β stimulates proliferation of MSC [4]. We observed MSC growth in 15 of 31 samples. On the other hand, addition of FGF-β together with TGF-β_3 increased the number of umbilical blood samples with MSC growth compared to addition of FGF-β alone (p=0.02). This was paralleled by a 10-fold increase in proliferative activity of MSC evaluated by the number of cells in a visual field.

The morphology of primary cultures of umbilical blood cells and passage 1 cultures was different; along with fibroblastoid cells, round cells of different size were seen in the culture. Expansion of umbilical blood MSC did not result in colony formation; the cells diffusely spread on the plastic surface (Fig. 1, *a-c*).

In vitro growth of MSC was maximum on days 7-10 of culturing. The mean count of MSC increased 6-10-fold, in 5 of 54 samples cell number increasing more than 10-fold. In one umbilical blood sample, the count of MSC increased 300 times (6 passages). Proliferative activity decreased by days

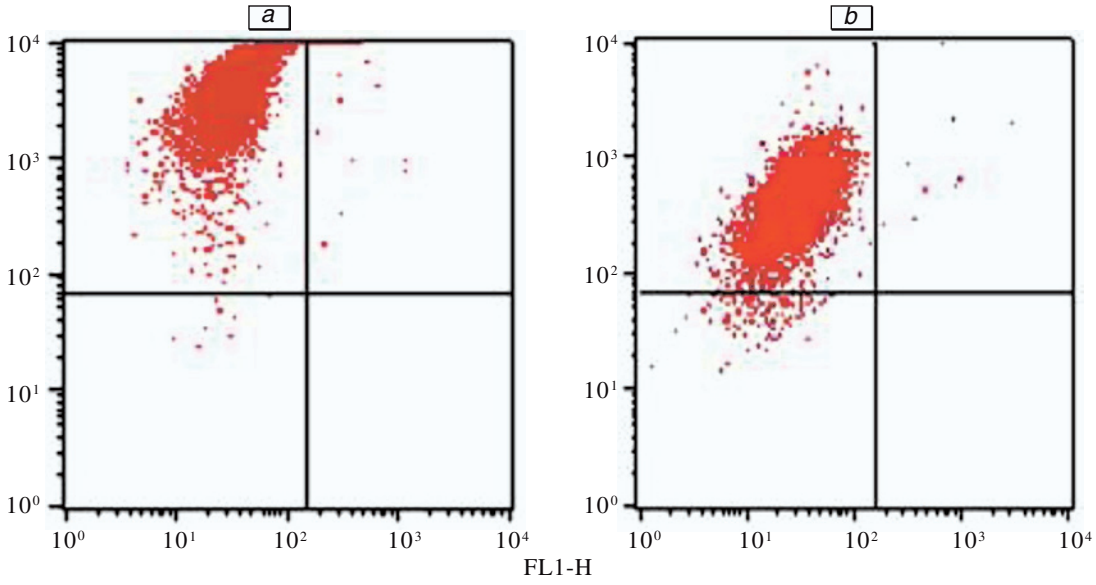

Fig. 2. Expression of CD90 (*a*) and CD105 (*b*) on umbilical blood MSC.

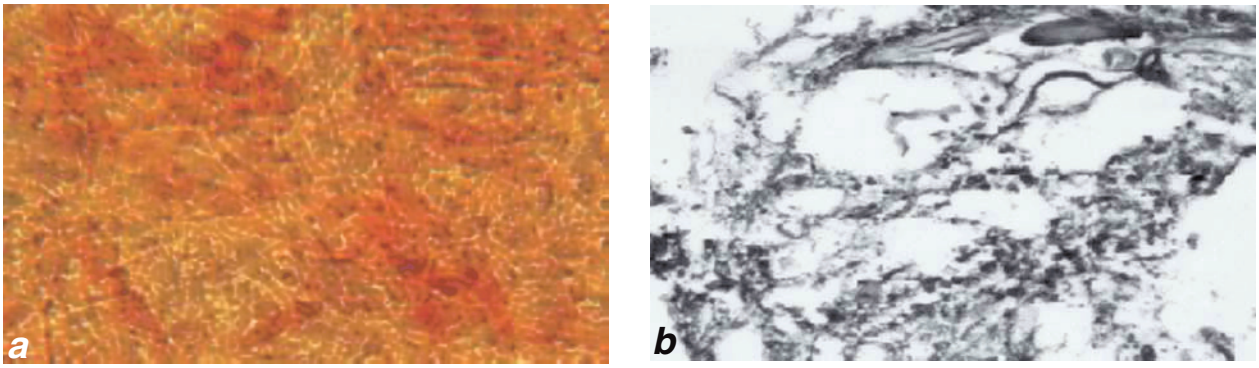

Fig. 3. Differentiation of umbilical blood MSC. *a*) umbilical blood MSC monolayer after osteogenic differentiation (alkaline phosphatase staining, ×100); *b*) section of loose fibrocellular tissue, formed *in vitro* as a result of chondrogenic differentiation of umbilical blood MSC (hematoxylin staining, ×400).

14-16 of culturing and signs of apoptotic cell death appeared: fragmentation of cell nuclei, cytoplasm granularity, large round cells adhered to the plastic, and small nonadherent cells were seen.

Immunophenotyping of cells after passage 3 detected expression of markers characteristic of MSC: 98% $CD90^+$, 98% $CD105^+$, 14% $CD140^+$, less than 1% $CD45^+$, and less than 1% $CD14^+$ cells (Fig. 2).

Osteogenic and chondrogenic differentiation of passage 4 MSC *in vitro* was evaluated using induction protocols [2,3,6,7]. Alkaline phosphatase (osteoblast marker) was detected 10-14 days after addition of differentiation factors. MSC retained spindle shape during osteogenic differentiation (Fig. 3, *a*). Differentiated cells expressing alkaline phosphatase were stained red. Osteoblast nuclei were stained blue. The majority of the monolayer cells differentiated into osteoblasts by day 14 of culturing with ascorbic acid and β-glycerolphosphate.

Chondrogenic differentiation of MSC was attained by adding TGF-β, dexamethasone, and ascorbic acid (Fig. 3, *b*). Osteogenic and chondrogenic differentiation of three specimens of umbilical blood MSC was confirmed by PCR, by the presence of alkaline phosphatase and aggrecan mRNA, respectively.

Hence, MSC were isolated from more than 50% umbilical blood specimens and their growth was maintained without growth factors until passage 1. However, further reinoculation inhibited proliferative activity and led to cell apoptosis. Pretreatment of culture flasks with serum and addition of FGF-β and $TGF\text{-}\beta_3$ to the culture medium as well as the presence of 20% human AB(IV) serum improved the efficiency of isolation, expansive growth, and *in vitro* culturing of umbilical blood MSC. These results indicate that the use of optimal techniques of culturing made it possible to produce sufficient

amounts of MSC from the umbilical blood for experimental and practical purposes.

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