Hepatogenic Potential of Human Bone Marrow and Umbilical Cord Blood Mesenchymal Stem Cells

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Conditions of human BM and umbilical cord blood MSC *in vitro* differentiation in the hepatogenic direction were studied. Changes in cell morphology, phenotype, acquisition of the capacity to produce albumin and accumulate glycogen, express cytokeratin, alkaline phosphatase, and albumin mRNA indicated that BM and umbilical cord blood MSC differentiated *in vitro* into immature hepatocyte-like cells.

Key Words: *mesenchymal stem cells; human umbilical cord blood; bone marrow; hepatogenic differentiation; differentiation markers*

The need in liver transplantation steadily increases all over the world. Current method for liver transplantation is sufficiently effective, but extremely difficult and expensive, particularly in patients with terminal stages of liver diseases. In addition, the choice of a donor of the liver is always a problem [10]. Transplantation of hepatocytes derived *in vitro* from patient's own hemopoietic or mesenchymal SC is an alternative to liver transplantation. Stem cells are a potentially unlimited and minimally invasive source for regeneration of hepatocytes and liver in its disease or dysfunction. Mesenchymal SC are a possible source of cells for hepatic tissue regeneration [3]. Several laboratory protocols were used to induce differentiation of MSC of different origin *in vitro* into hepatocyte-like cells. The resultant hepatocyte-like cells have different morphological and functional characteristics [13]. It remains unclear which, specifically, should be the characteristics of the cells for cell therapy used instead of transplantation of allogenic liver or its fragment.

We studied human BM and umbilical cord blood MSC differentiation in the hepatogenic direction.

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MATERIALS AND METHODS

Isolation of MSC from BM and umbilical cord blood. BM was collected from donors at Center for Organ and Tissue Transplantation at State Clinical Hospital No. 9, Minsk. The umbilical cord blood was collected from women who gave informed consent to participation in the study at Regional Maternity Hospital, Minsk. Blood specimens were collected into special containers with heparin. Specimens of the blood and BM were transported at ambient temperature. No more than 3 h passed between the collection of SC-containing specimens and isolation of these cells.

Bone marrow or umbilical cord blood mononuclear cells (MNC) were isolated by 30-min centrifugation (400*g*) on Ficoll density gradient 1.077 (Ficoll-Paque PLUS; GE Healthcare). The collected ring of MNC was washed with PBS (StemCell) and then with Iscov medium (IMDM, Sigma) with 2% FCS (Gibco) for 10 min at 400*g*.

***In vitro* culturing of BM and umbilical MSC.** The suspension of the resultant BM or umbilical cord blood MNC was inoculated for adhesion into T25 culture flasks (Sarstedt) in a concentration of 5×10^5 cell/cm^2 in complete nutrient α-MEM (Sigma) with 10% FCS, 2 mM L-glutamine (Sigma), 100 μg/ml streptomycin, and 100 U/ml penicillin. The flasks with MNC

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were cultured at 37°C in a humid atmosphere with 5% CO_2 for 48 h. Free cells were then discarded, the flasks were washed with PBS, and culturing was carried out in complete nutrient medium until 70-80% confluence. The cells were removed from plastic with 0.25% trypsin-EDTA, washed, and cultured in complete nutrient medium in a concentration of 3×10^3 cell/cm^2. The medium was replaced with a fresh portion every 3-4 days.

Induction of *in vitro* hepatogenic differentiation of BM and umbilical cord blood MSC [7,13]. Bone marrow or umbilical cord blood MSC (passages 2-7) were inoculated in 6-well plates or T25 cm^2 flasks in a concentration of 10,000 cell/cm^2. The cells were cultured in IMDM or DMEM with 10-15% FCS (HyClone), antibiotics, and L-glutamine. After attaining 70-80% confluence, the cells were cultured for 48 h in serum-free IMDM or DMEM-LG with 10 ng/ml main FGF and 20 ng/ml epidermal growth factor before the 2-step protocol. Step 1 (differentiation days 0-7): basal medium, 20 ng/ml hepatocyte growth factor (HGF), 10 ng/ml bFGF (or FGF-4), 4.9 mmol/liter nicotinamide. Step 2 (maturation, days 7-21), basal medium, 20 ng/ml oncostatin M (OSM), 10^{-8} M dexamethasone, 50 mg/ml ITS+premix.

Nutrient medium was replaced with a fresh portion twice a week.

Basal media were IMDM or DMEM-LG with 1 mg/ml BSA, 0.1 mM L-ascorbic acid, 0.25 mM sodium pyruvate, and 2.0 mM glutamine.

Detection of intracellular albumin. Cells cultured in hepatogenic medium were washed in PBS and fixed in cold 4% paraformaldehyde in PBS for 10 min. The cells were then permeabilized in 0.3% Triton X-100 in PBS (10 min at ambient temperature), washed, and intracellular peroxidase was neutralized according to the instruction for antibody visualization (Dako). Nonspecific binding sites were additionally blocked with 0.2% human immunoglobulin in PBS and 0.1% gelatin (30 min, 37°C). Monoclonal antibodies HSA-11 (Sigma) to human albumin (1:200) in PBS with 0.1% gelatin were added to cells, incubated for 1.5 h at 37°C, and then stained in accordance with the instruction for Dako kit using DAB as the substratum of the enzymatic reaction.

The cells were counted under a Leica inverted-stage microscope. Albumin-positive cells were colored bright brown.

Staining for glycogen. After removal of the medium from the flask the cells were washed in PBS 3 times and fixed in 4% formaldehyde (10 min) or absolute ethanol (10 min). The cells were oxidized with 1% iodic acid (5 min), washed 3 times in deionized water, and treated with Schiff's reagent (10-15 min). The cells were then washed again in deionized water (10 min), stained with hematoxylin (1-2 min), and washed in deionized water [4]. The cells were counted under a Leica inverted-stage microscope. The cells containing glycogen were bright crimson-colored.

Immunophenotypical analysis. The expression of CD45, CD34, CD105, CD90 markers on MSC was evaluated before and after differentiation using monoclonal antibodies (Beckman Coulter). Cell treatment with monoclonal antibodies labeled with FITC and phycoerythrin (PE) was carried out by the standard methods. Monoclonal antibodies (20 μl) were added to the specimen (100,000-200,000 cells) and the specimen was incubated in darkness at ambient temperature for 25-30 min. The cells were then washed in PBS twice, with 5-min centrifugation at 300*g*. Fluorescent-stained cells were analyzed on a FACSCan flow cytofluorometer (Becton Dickinson) using CellQuest software. At least 30,000 cells per sample were analyzed.

TABLE 1. Primer Sequences for Evaluation of Genetic Markers of MSC Hepatogenic Differentiation

Primer	Sequence 3'-5'	Size of cDNA	Marker
αFS	TGCAGCCAAAGTGAAGAGGGAAGA	216	α-Fetoprotein
αFA	CATAGCGAGCAGCCCAAAGAAGAA		
tS	TGAGCAGTCTGTCCACTGCCT	358	Tyrosine aminotransferase
tA	ATGTGAATGAGGAGGATCTGAG		
CK18s	TGGTACTCTCCTCAATCTGCTG	148	Cytokeratin 18
CK18a	CTCTGGATTGACTGTGGAAGT		
P3	TGGAGCTTCAGAAGCTCAACACCA	453	Alkaline phosphatase
P4	ATCTCGTTGTCTGAGTACCAGTCC		
P1	TGAGAAAACGCCAGTAAGTGAC	206	Albumin
P2	TGCGAAATCATCCATAACAGC		

Isolation of RNA and reverse transcription and PCR. RNA was isolated from cultured cells subjected to hepatogenic differentiation and from control specimens using RNeasy Mini Kit (Qiagen). RNA was eluted with nuclease-free water and stored at -20°C. The concentrations of RNA in the samples were measured on a Specord 250 device (Jenna Analytik). The concentration was evaluated by optical density at λ=260 nm.

Reverse transcription (RT) reaction was carried out with 1 μg total RNA of each specimen. The following reagents were used in RT reaction: 4 μl 5× buffer, 2.5 mM $MgCl_2$, 1 mM deoxynucleotide triphosphate (dNTP), 1.25 μM disseminated hexamers, 10 U RNase inhibitor, 20 URT, RNA, and water to a volume of 20 μl. Complementary DNA (cDNA) was synthesized 10 min at 25°C and 40 min at 42°C.

Specimens of cDNA were tested in PCR with specific primer pairs (Table 1). In addition, each specimen was amplified with primers to β-actin for verification of RNA leveling. Reagents for PCR (1 reaction): 5 μl 10× buffer, 1.75 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphate, 0.2 μM reverse and 0.2 μM direct primers, 2.5 U Taq polymerase, 5 μl cDNA, and water to a volume of 50 μl. Amplification conditions were as follows: 5 min at 94°C (30 sec at 94°C, 1 min at 62°C, 1 min at 72°C), 35 cycles; 10 min at 72°C. RT-PCR was carried out on a GeneAmp2700 device (Applied Biosystems).

cDNA specimens after PCR amplification were separated in 1% agarose gel on a Bio-Rad Wide Mini-

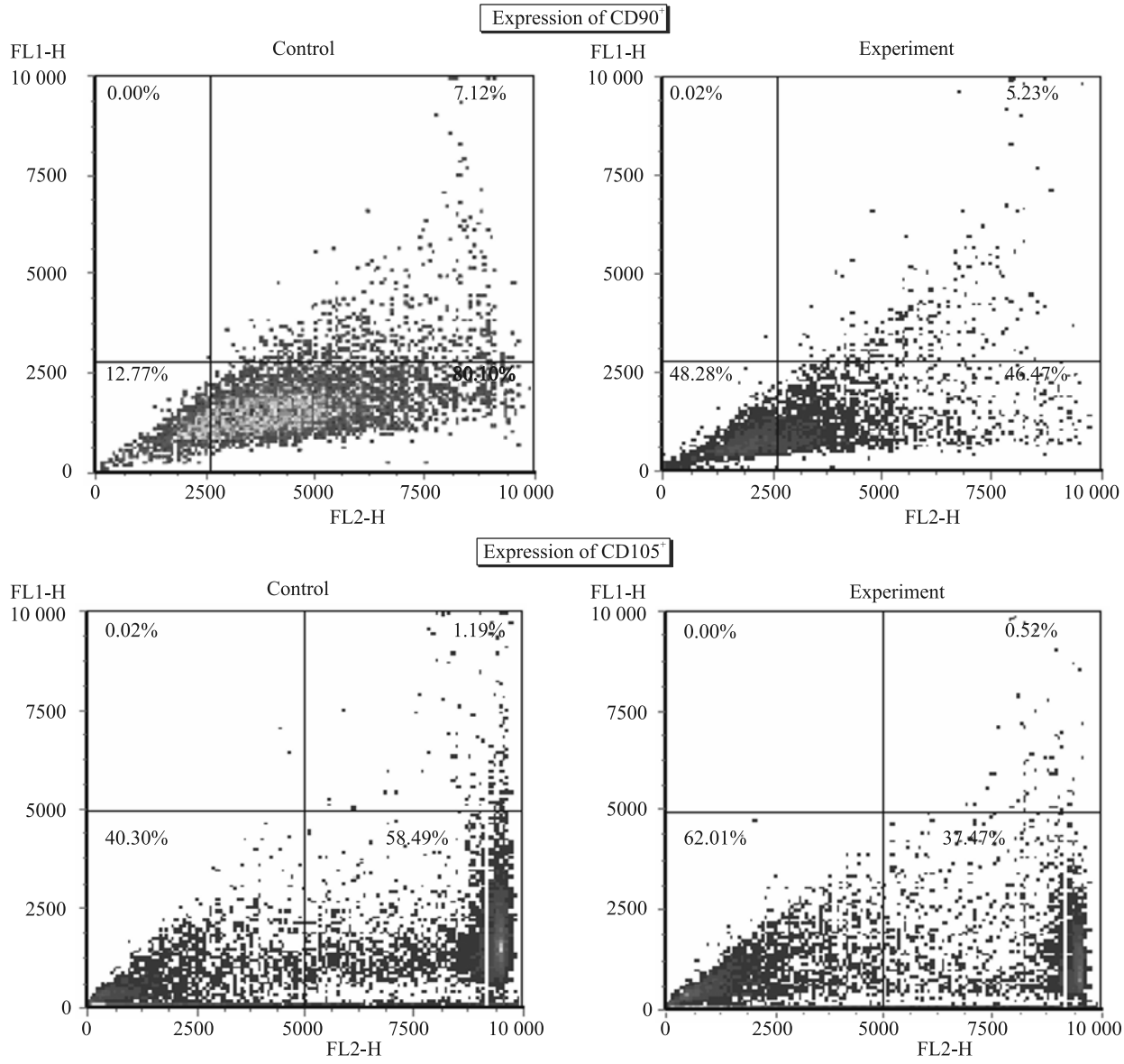

Fig. 1. Expression of $CD90^+$ and $CD105^+$ markers on BM MSC cultured for 3 weeks after the beginning of hepatogenic differentiation (experiment) and without differentiation factors (control).

Sub Cell GT device (Bio-Rad). Agarose gels were analyzed on a Kodak Molecular Imaging Station (Carestream Health).

RESULTS

Ten samples of BM MSC, passages 1-7, and one sample of umbilical cord blood MSC, passages 4-5, were cultured under conditions of hepatogenic differentiation induction.

Differentiation was carried out by 2-step protocol [8,14] until 80-90% confluence. The medium in cell culture was replaced with serum-free medium 48 h before differentiation and EGF and FGF were added to arrest cell proliferation. According to published data, cells differentiated at stage 1 (7 days) and matured into hepatocytes at stage 2 (after 7 days) [8,14]. MSC immunophenotype was evaluated before differentiation and 3 weeks after it.

Immunophenotypical characteristics of MSC before and after hepatogenic differentiation. Human BM and umbilical cord blood MSC after passage 2 of *in vitro* culturing expressed CD90 and CD105 specific markers, but not markers characteristic of hemopoietic cells (CD34 and CD45). The content of $CD90^+$ cells in the culture was 86-98%, of $CD105^+$ cells 93-100%.

The expression of CD90 and CD105 markers decreased throughout 3 weeks of *in vitro* culturing in experimental samples (hepatogenic differentiation) and in the control (medium without differentiation factors). The loss of these markers was most pronounced in cells of experimental samples (more significant reduction of CD105 expression than of CD90; Fig. 1). In experimental samples, the contents of $CD90^+$ and $CD105^+$ cells by the end of week 3 of differentiation were 33.5 and 29.8% *vs.* 79.7 and 47.5% in control samples, respectively.

The more significant reduction of MSC-specific markers expression in experimental samples could be due to differentiation of some cells into hepatocyte-like cells. In the control, the cells reduced the level of specific proteins expression presumably as a result of long 3-week culturing without reinoculation.

Morphological changes in human MSC during hepatogenic differentiation. Human MSC are characterized by fibroblast-like shape. Changes in cell morphology are determined by several factors: culture medium and serum, presence of growth and differentiation factors, cell concentration, differentiation potential of cells, *etc.* Addition of cytokines inducing hepatogenic differentiation and growth factors does not arrest proliferation, which is an obligatory condition for the beginning of differentiation. Proliferation is arrested only after attaining 100% confluence [12]. Cell density is an important factor for the efficiency of hepatogenic differentiation [9].

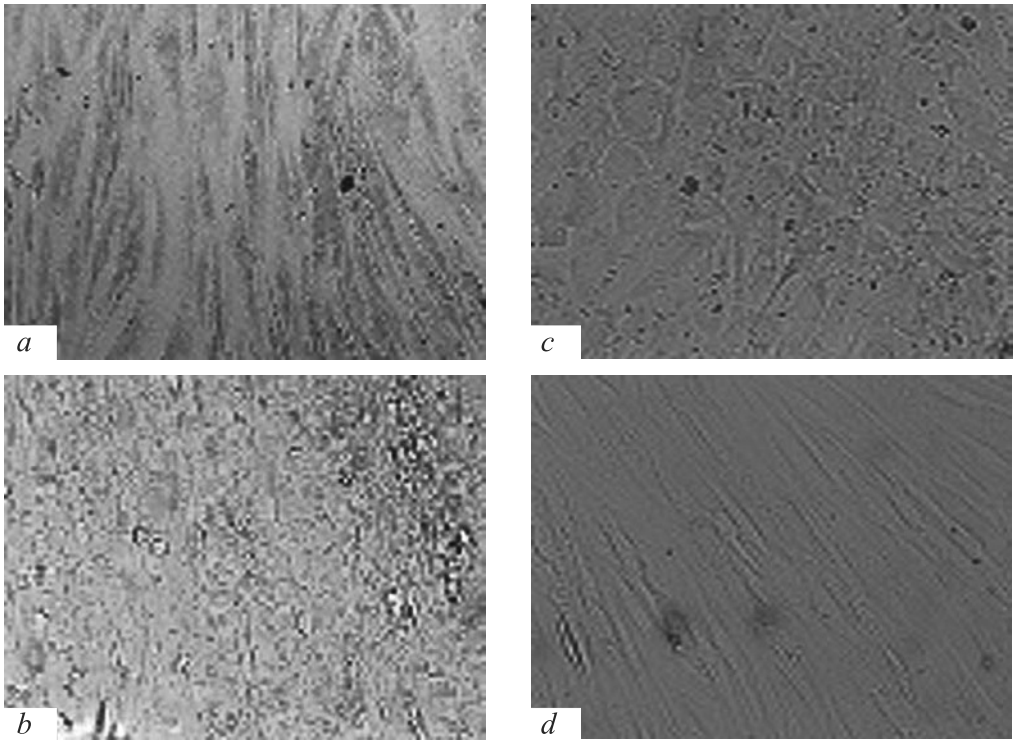

Fig. 2. Changes in BM MSC (passage 2) morphology during *in vitro* hepatogenic differentiation (×100). *a*) day 7; *b*) day 14; *c*) day 21; *d*) control (day 21).

During week 1 in culture (passages 2-3), the cells little changed their morphology during hepatogenic differentiation of BM and umbilical MSC. The cells retained fibroblast-like shape during this period, but were more flattened in comparison with the control. Cell morphology changed significantly. They acquired polygonal or cuboid shape after addition of oncostatin M and dexamethasone after 7 days of differentiation (step 2 of the protocol; Fig. 2).

BM and umbilical cord blood MSC of passages 5-7 virtually did not change during differentiation. This could be due to reduction of cell differentiation potential.

It was found that MSC lost fibroblast-like morphology in the presence of hepatocyte growth factor and FGF [9]. Umbilical cord blood MSC acquired cuboid morphology of hepatocyte-like cells during week 2 of differentiation. This morphology was observed until days 21-28 of culturing in hepatogenic medium. Mature differentiated cells were characterized by the presence of numerous granules in the cytoplasm [9]. Previous studies showed the appearance of small round cells among umbilical cord blood MSC after 1 week of differentiation [6]. By day 28, about 64% cells were small, round, epithelioid, while in the control all cells had a fibroblast-like shape. According to previous data, morphological changes in MSC isolated from the placenta were minimum during culturing on surfaces not treated with poly-L-lysine [5]. MSC isolated from the placenta acquired polygonal shape only during culturing in differentiation medium in dishes treated with poly-L-lysine [5]. Human MSC exhibited no morphological and phenotypical characteristics of hepatocytes during exposure in a cocktail of hepatocytic factors [12]. Successive treatment of human MSC with fibroblast and hepatocyte growth factors, ITS, and dexamethasone, similar to secretion during the liver ontogeny *in vivo*, significantly stimulated accumulation of glycogen from day 8. From days 8-9 the cells expressed cytokeratin 18 (cytoskeleton filament in hepatocytes). However, the morphology of differentiated cells did not correspond to that of hepatocytes [12].

Synthesis of intracellular albumin by hepatocyte-like cells. Intracellular albumin was detected using HSA-1 mouse monoclonal antibodies to human albumin (Sigma). Visualization was carried out using Universal LSAB tm2 to mouse/rabbit antibodies (Dako) with horseradish peroxidase, DAB as substrate, and Huh-7 highly differentiated human hepatocarcinoma cells as positive control. Albumin expression was found in differentiated cells at all terms (days 7, 14, 21 of differentiation). Intracellular albumin was expressed in negligible levels in undifferentiated cells. The difference between intracellular albumin levels in differentiating and control cells was shown by the immunohistochemical method as early as on day 8 and became obvious after 3 weeks of *in vitro* hepatogenic differentiation (Fig. 3).

The percentage of albumin-positive cells after 3 weeks of differentiation of MSC was evaluated by flow cytofluorometry. The level of albumin-positive

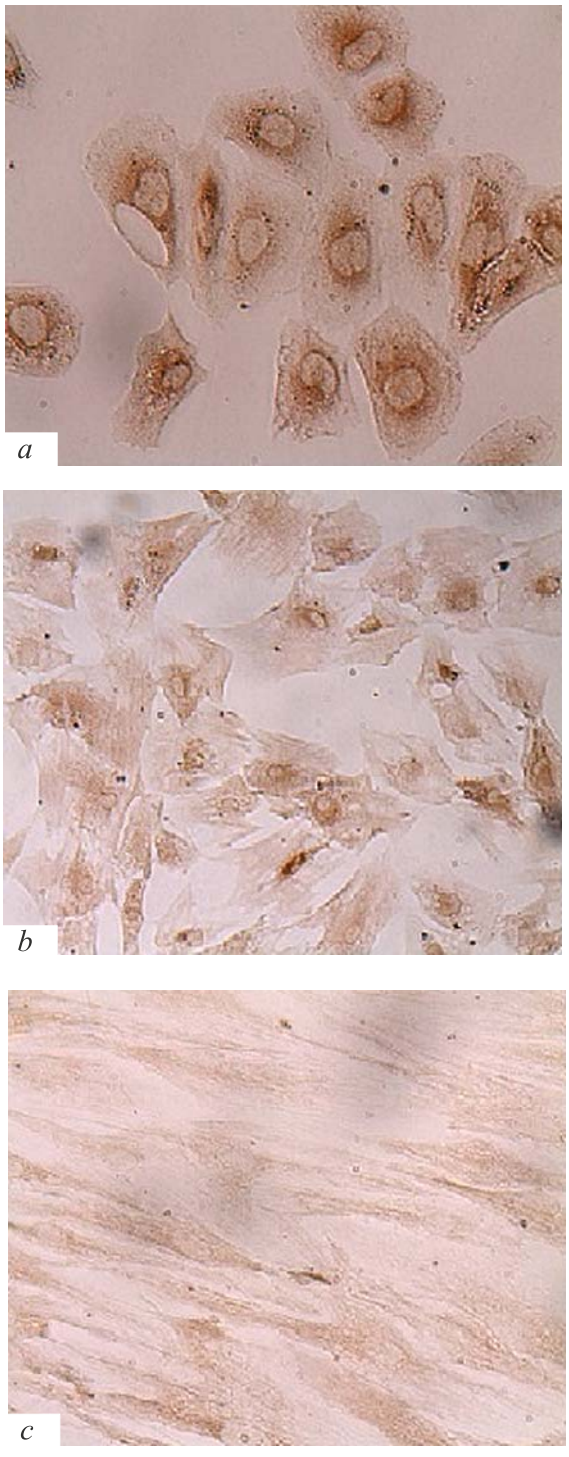

Fig. 3. Staining of umbilical cord blood MSC for intracellular albumin after 3 weeks of *in vitro* hepatogenic differentiation (×100). *a*) control Huh-7 cells; *b*) differentiated MSC; *c*) undifferentiated MSC.

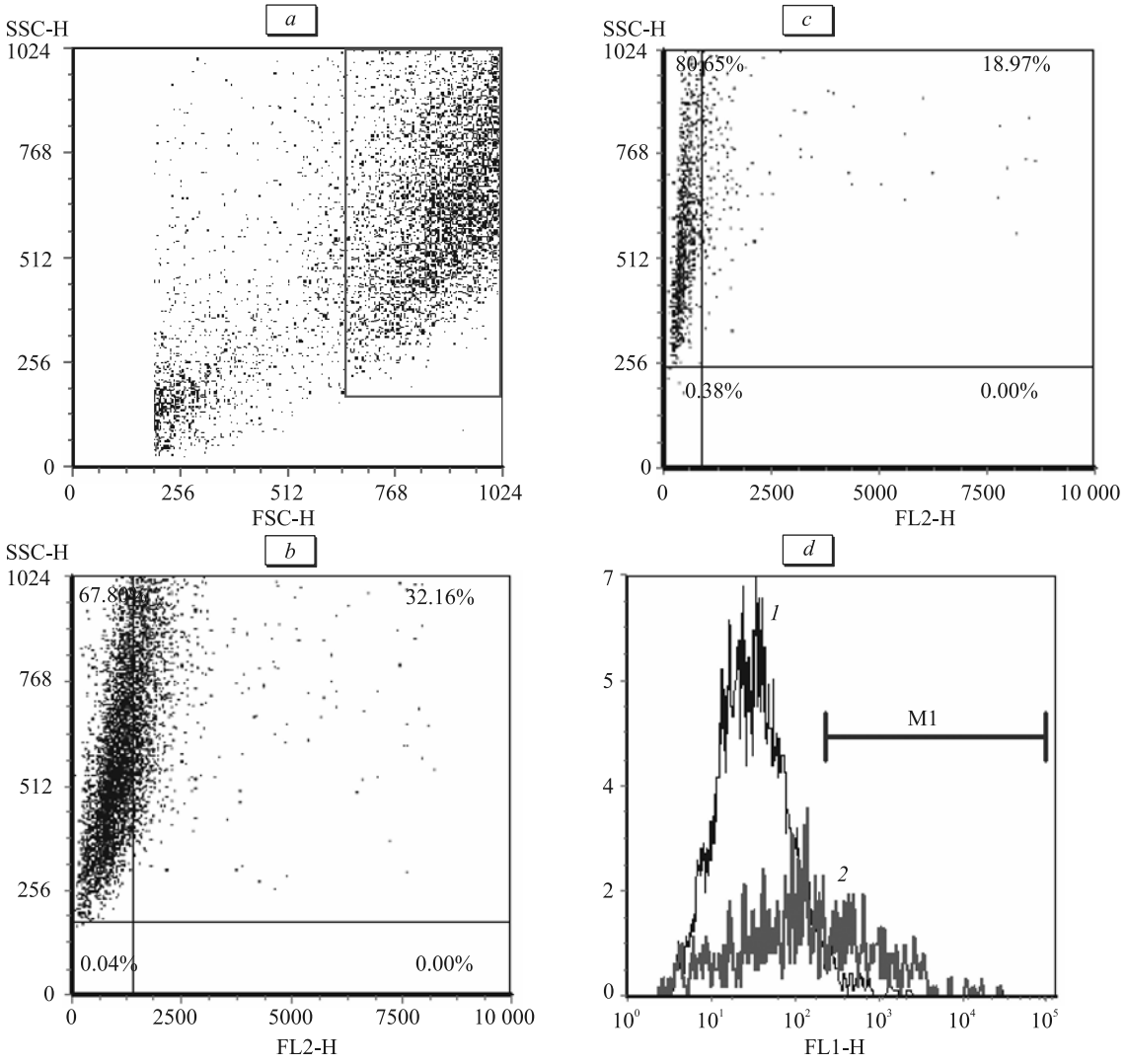

Fig. 4. Levels of albumin-positive cells in BM MSC culture after 3 weeks of *in vitro* hepatogenic differentiation. *a*) MSC distribution by direct and lateral diffusion after hepatogenic differentiation; *b*) differentiated cells; *c*) undifferentiated cells; *d*) histogram: *1*) control; *2*) experiment.

cells in different experiments was 3-9-fold higher than in the control: 11-36% (median 22.6%; Fig. 4).

Control cultures contained 1.2-5.3% albumin-positive cells (median 3.4%).

According to previous data, 70% umbilical cord blood MSC were positively stained for albumin by the immunofluorescent method as early as 1 week after differentiation induction [9].

Accumulation of glycogen by hepatocyte-like cells. The synthesis and deposition of glycogen are a specific characteristics of liver cells. Accumulation of glycogen was evaluated in BM and umbilical cord blood MSC cultures throughout 3 weeks of hepatogenic differentiation by cell staining with Schiff reagent. Figure 5 presents differences between the control and experimental cell cultures.

Glycogen is secreted in differentiated cells starting from day 14 [6] or from days 24-28 [8]. In our experiments, solitary cells positively stained with Schiff reagent were detected after 7 days of differentiation. After 21 days the greater part of cells were positively stained for glycogen.

Detection of molecular genetic markers of hepatogenic differentiation. The expression of molecular markers of hepatocytes (α-fetoprotein, alkaline phosphatase, albumin, cytokeratin 18, tyrosine aminotransferase) was studied in seven specimens of differentiated human BM MSC and two specimens of umbilical cord blood MSC after 1, 2, and 3 weeks of hepatogenic differentiation. Human highly differentiated hepatocarcinoma Huh-7 RNA served as the positive control. Human undifferentiated MSC RNA served as the negative control. Specimens of control cultures were taken for the analysis during the same periods of culturing as the experimental specimens. β-Actin was used to verify total RNA leveling of specimens.

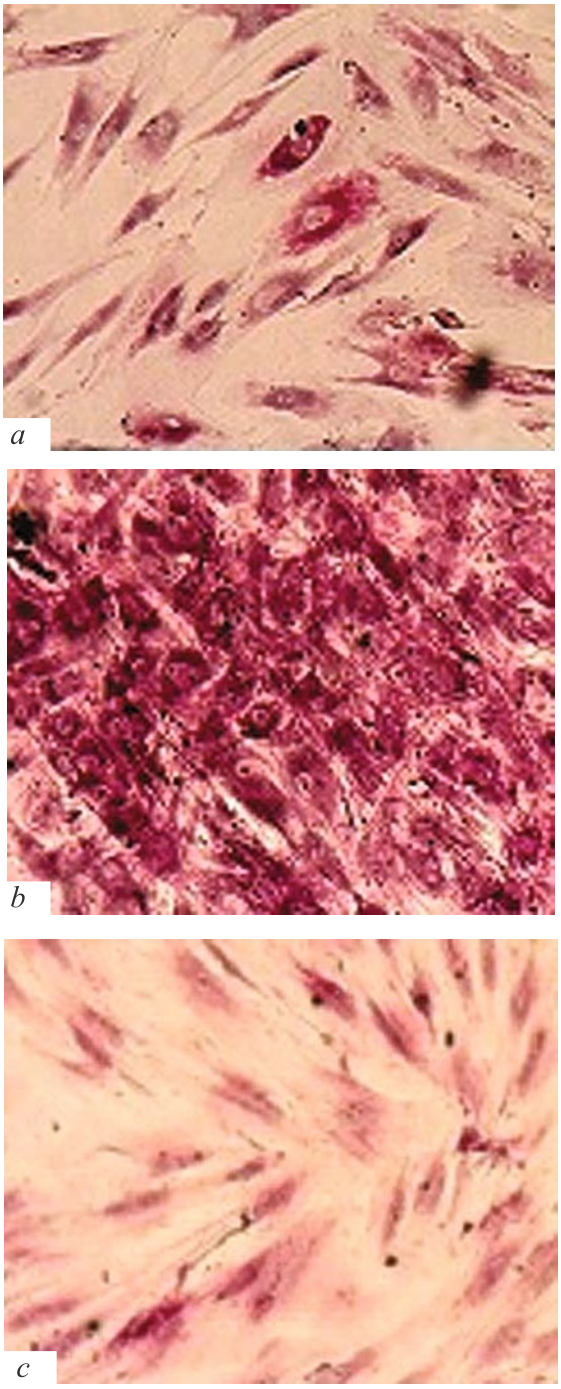

Fig. 5. Glycogen accumulation by hepatocyte-like cells during hepatogenic differentiation of BM MSC (staining with Schiff's reagent; ×100). *a*) day 7; *b*) day 21 (experiment); *c*) day 21 (control).

Alkaline phosphatase transcripts were evaluated in the specimens after weeks 2 and 3 of differentiation (Fig. 6). Cytokeratin 18 expression was found in BM MSC after 1 week of differentiation; after 2 and 3 weeks the level of expression decreased to a level of negative control. In umbilical cord blood MSC, cytokeratin 18 was detected after 3 weeks of differentiation. Albumin expression was observed throughout 3 weeks of differentiation in BM and umbilical cord blood MSC specimens. No expression of α-fetoprotein and tyrosine aminotransferase was detected in any of analyzed MSC specimens subjected to hepatogenic differentiation and in the control samples.

According to published data, specific liver proteins are divided into 3 groups by the time of their expression. Early proteins are HNF3β and α-fetoprotein (day 7), medium proteins are albumin (day 8) and cytokeratin 18 (day 9), and late proteins are HNF1α (day 13), MRP2 (day 17), C/EBPα (day 19), and tyrosine aminotransferase (day 28) [12].

One of the early markers of endodermal differentiation is α-fetoprotein, a large serum protein produced at first by the yolk sac visceral endoderm and by hepatoblasts and better differentiated fetal liver cells. α-Fetoprotein is not expressed by all SC. Adult SC, including human umbilical cord blood and BM MSC, do not express α-fetoprotein. However, other progenitor cells, for example, rat BM MSC and pluripotent SC precursors of peripheral blood monocytes, do express α-fetoprotein [8]. α-Fetoprotein-producing cells isolated from embryonic SC differentiate into mature hepatocytes [8]. The absence of α-fetoprotein expression in our experiments presumably indicated higher degree of maturing of BM and umbilical cord blood MSC in comparison with fetal liver cells.

Negative results of evaluation of tyrosine aminotransferase expression can be attributed to the fact that it was late marker of hepatogenic differentiation not detected before day 28. The maximum period of differentiation in our experiments was 21 days.

The expression of hepatogenic markers α-fetoprotein, cytokeratin 18, and albumin by differentiated MSC was previously found only in their co-culturing with fetal liver cells [7].

Hence, BM and umbilical cord blood MSC, induced to hepatogenic differentiation *in vitro*, changed

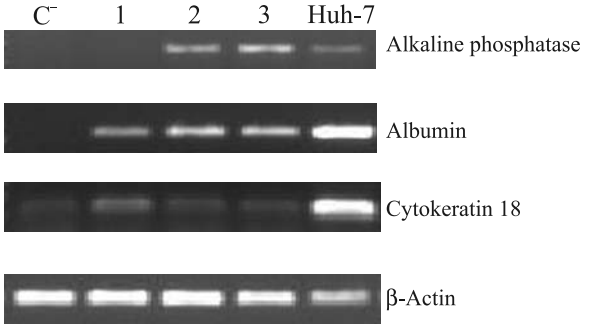

Fig. 6. Expression of genetic markers by human BM MSC during *in vitro* hepatogenic differentiation. K^-: negative control; 1, 2, 3: specimens of weeks 1, 2, and 3 of differentiation, respectively; Huh-7: positive control.

their morphology from fibroblast to hepatocyte-like, increased the production of albumin and accumulation of glycogen, and expressed such hepatocyte markers as albumin, alkaline phosphatase, and cytokeratin 18. However, some authors mentioned [5] that the expression of hepatocyte-specific markers (mRNA) and the protein level did not yet mean that the differentiated hepatocyte-like cells acquired the functional activity intrinsic of hepatocytes. Because of low incidence of MSC isolation from the umbilical cord blood, their use in regenerative medicine seemed doubtful [1]. Undifferentiated MSC, transplanted into the liver, transformed into myoblasts with the development of fibrous tissue [2]. B. Parekkadan, *et al.* (2007) expressed an opposite opinion [11]. According to these authors, MSC had an immunomodulating effect on stimulated hepatic stellate cells through paracrine mechanisms, providing the protective function in liver inflammation and fibrosis. Therefore, the therapeutic potential of pre-differentiated cells for regenerative medicine deserves further studies, *i.e.* on experimental models, among other things, because of the probability of a favorable therapeutic effect in patients with liver diseases and functional incompetence and because of possible side effects of cell therapy with human MSC.

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