CELL TECHNOLOGIES IN BIOLOGY AND MEDICINE

Capability of Human Mesenchymal Cells Isolated from Different Sources to Differentiation into Tissues of Mesodermal Origin

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We compared the capacity of cultured human skin fibroblasts, human umbilical cord cells obtained after normal delivery on gestation week 38-40, and mesenchymal bone marrow stem cells to differentiation into adipocytes, osteoblasts, and chondrocytes. Our findings suggest that mesenchymal stem cells are multipotent cells and can differentiate into adipose, cartilaginous, and bone tissue. Umbilical cord fibroblast-like cells can differentiate into adipocytes and chondrocytes, and only few cells in this culture can differentiate into osteoblasts. Skin fibroblasts differentiate only into adipocytes.

Key Words: *bone marrow mesenchymal stem cells; umbilical cord fibroblast-like cells; skin fibroblasts*

Technologies of obtaining cell material on the basis of bone marrow multipotent mesenchymal stem cells (MSC) isolated from human postnatal organs and tissues and evaluation of the possibility of their application in clinical practice for the correction of pathological states of various genesis are the main modern trends of fundamental investigations in the field of biomedical cell technologies.

Specific markers or their combinations for *in vitro* or *in vivo* identification of multipotent MSC are unknown. MSC are characterized by a set of morphological, phenotypical, and functional properties, in particular, by the capacity of *in vitro* differentiation into bone, adipose, and cartilaginous tissue. Only few cells within MSC populations obtained from different sources can differentiate into three lineages. Most cells are bipotent or unipotent.

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The aim of the present study was to obtain fibroblast cultures from human skin and umbilical cord after normal delivery on gestation week 38-40 and cultures of human bone marrow MSC, analysis of protein expression in these cells using the methods of immunocytochemistry and flow cytofluorometry, and comparison of the capacity of these cells to differentiation into adipose, cartilaginous, and bone tissues.

MATERIALS AND METHODS

Fibroblast-like cells were isolated from the skin [1], adult human bone marrow (routinely, in Ficoll density gradient), and from human umbilical cord obtained after normal delivery on gestation week 38-40 [5]. The cells were cultured in DMEM containing 10% fetal serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 ng/ml basic fibroblast growth

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factor (b-FGF) until confluence (the medium was replaced 2 times a week).

Immunocytochemical analysis of the expression of cytoplasmic proteins was carried out using primary monoclonal mouse antibodies and second rhodamine- and FITC-labeled antispecies antibodies (Chemicon). Double immunocytochemical staining was performed by simultaneous incubation with primary antibodies obtained from different animals and the corresponding second rhodamine- and FITC-conjugated antispecies antibodies. Cell nuclei were stained with DAPI. The preparation was placed in a special chamber preventing fluorescence quenching and examined under an inverted Axiovert 200 microscope equipped with a Axiocam HRm camera (Carl Zeiss).

Differentiation of the obtained cultures into adipose tissue was carried out in DMEM containing 10% equine serum, 0.5 μM hydrocortisone, 0.5 mM isobutylmethylxanthine, 60 μM indomethacin (Sigma), 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate for 3 weeks (the medium was changed 2 times a week).

Differentiation of the obtained cultures into bone tissue was carried out in a serum-free medium containing 0.2 mM ascorbate, 10 mM β-glycerophosphate, 10^{-7} M dexamethasone, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate for 3 weeks. The medium was changed 2 times a week [4]. On days 7, 14, and 21 in culture, cell capacity to differentiation into osteoblasts was evaluated by the expression of bone-specific proteins: bone sialoprotein and osteonectin (measured immunocytochemically), and endogenous alkaline phosphatase [3], and by accumulation of calcium phosphates in cells (von Kossa staining [4]).

Differentiation of the obtained cultures into chondrocytes was carried out in micromass culture in serum-free DMEM containing 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate 100 ng/ml TGF-β for 3 weeks. After 7, 14, and 21 days in culture, cryotome sections of the obtained micromasses were prepared and stained with 1% toluidine blue in 50% isopropanol [4]. Cell capacity to differentiate into chondroblasts was evaluated by the expression of cartilage tissue-specific proteins (type 1 and 2 collagens and chondroitin).

RESULTS

Adherent cultures of human skin fibroblasts, umbilical cord cells, and bone marrow MSC were obtained. Most cells in these cultures had a fibroblast-like morphology.

Considerable phenotypic differences between cells in these cultures were revealed. Most umbilical cord cells expressed nestin and type 1 and 2 collagens, about 20% cells expressed endotheliocyte marker (von Willebrand factor). In bone marrow MSC cultures, less than 1% cells expressed nestin,

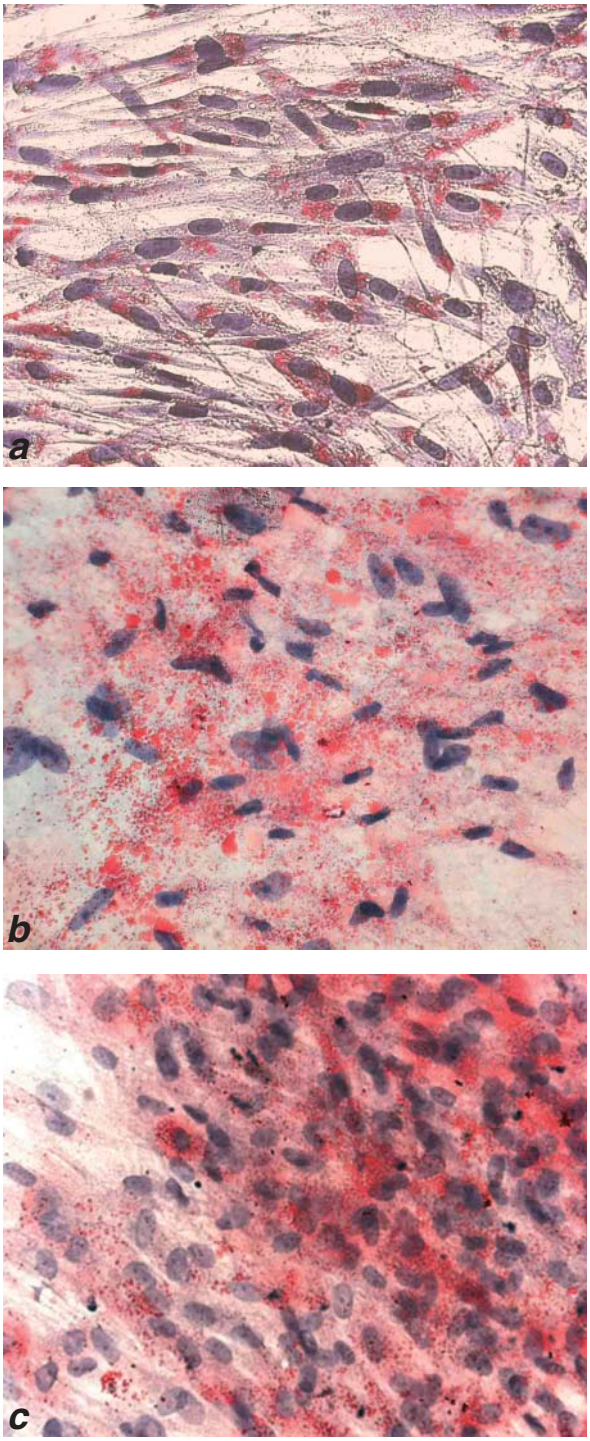

Fig. 1. Differentiation of human skin fibroblasts (*a*), umbilical cord fibroblast-like cells (*b*), and bone marrow MSC (*c*) into adipose tissue. Staining with oil red and hematoxylin.

expression of type 1 collagen was observed during passages 1 and 2 and considerably decreased after the 3rd passage. Bone marrow MSC did not express type 2 collagen and about 10% these cells expressed von Willebrand factor during early passages, but then expression of this factor decreased. Only

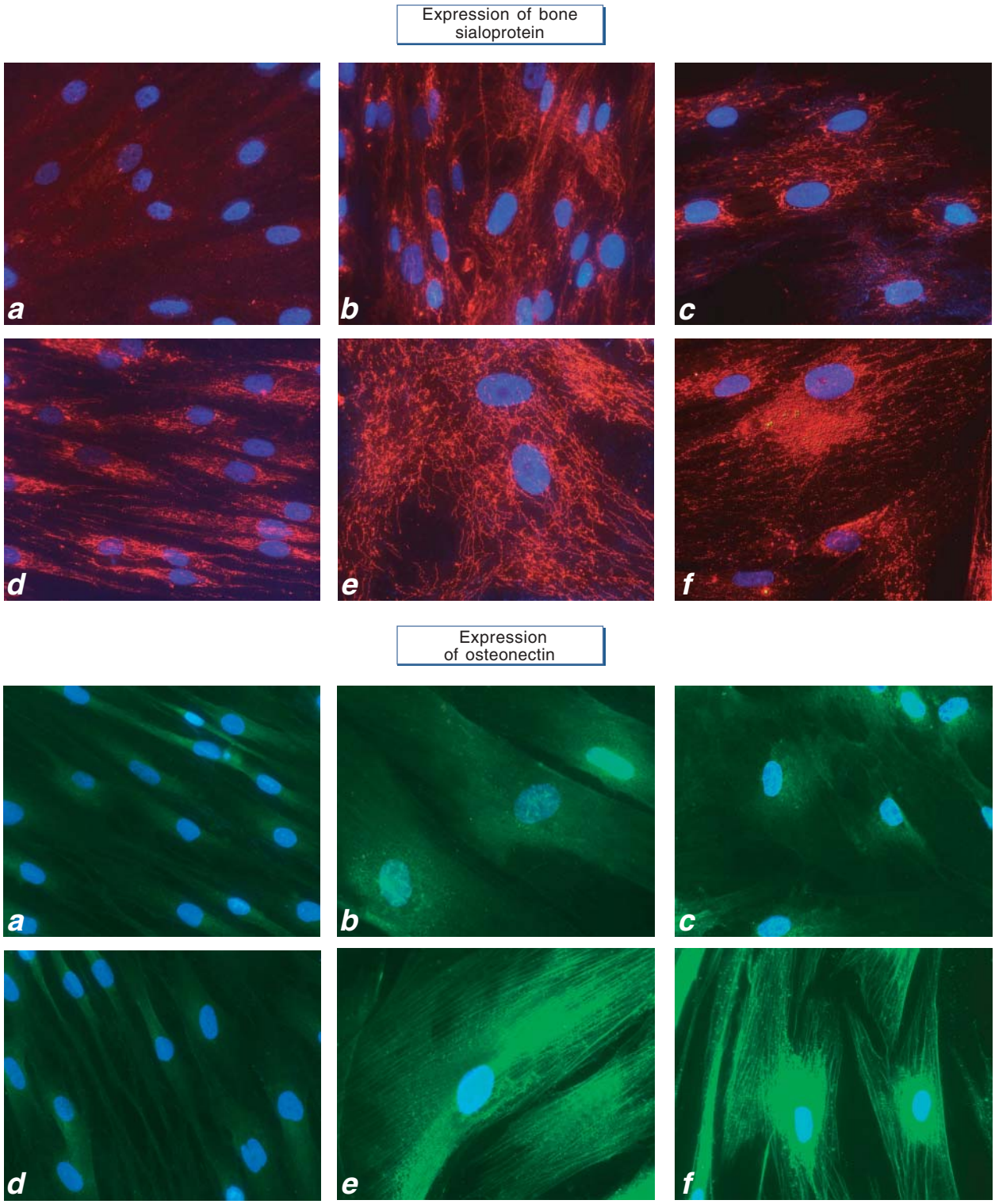

Fig. 2. Expression of bone sialoprotein (Rhod) and osteonectin (FITC) in skin fibroblasts (*a, d*), umbilical cord fibroblast-like cells (*b, e*), and bone marrow MSC (*c, f*) on days 1 (*a-c*) and 21 (*d-f*) of culturing in a medium stimulating osteoblast differentiation. Nuclei were stained with DAPI, ×630.

solitary cells of dermal origin expressed type 1 collagen; type 2 collagen and von Willebrand factor were not expressed by these cells.

Expression of progenitor and stem cell markers in cells of the obtained cultures was studied by the method of flow cytofluorimetry. It was found that none cultures expressed markers of hemopoietic cells CD34, CD45, and HLA-DR. Skin-derived cells had the following cytophenotype: $CD13^+$, $CD44^+$, $CD49b^-$, $CD54^-$, $CD90^+$, CD105low, $CD106^-$, $CD117^-$. Fibroblast-like cells from the umbilical cord expressed $CD13^+$, $CD44^+$, CD49blow, $CD54^+$,

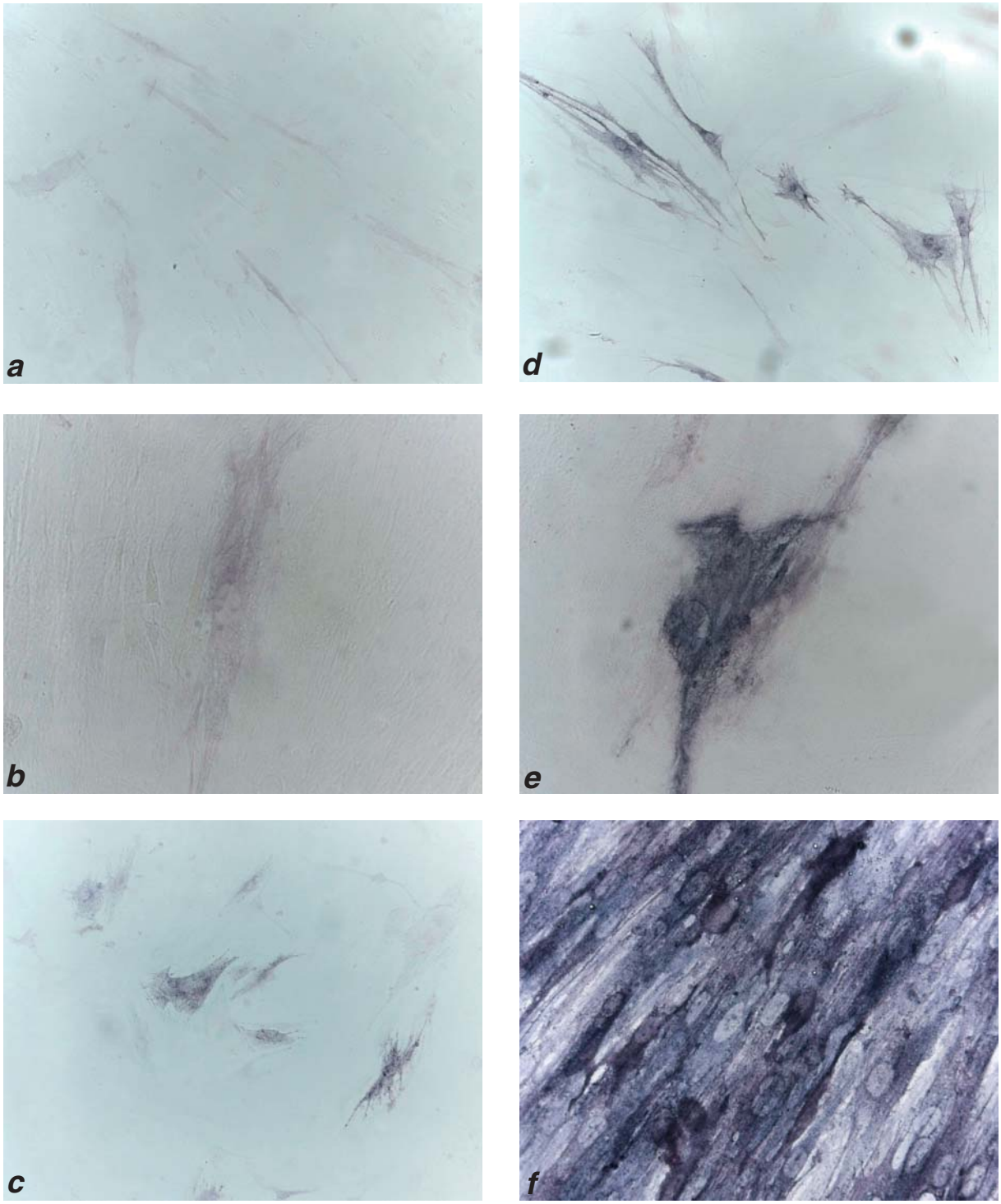

Fig. 3. Activity of alkaline phosphatase in skin fibroblasts (*a, d*), umbilical cord fibroblast-like cells (*b, e*), and bone marrow MSC (*c, f*) on days 1 (*a-c*) and 21 (*d-f*).

CD90$^+$, CD105low, CD106$^-$, CD117$^+$, while bone marrow MSC expressed CD13$^+$, CD44$^+$, CD49-blow, CD54$^+$, CD90$^+$, CD105low, CD106$^+$, CD117$^-$. Cells of the obtained cultures differed by the expression of class I major histocompatibility complex proteins (HLA-ABC). The expression of HLA-ABC was maximum in fibroblasts of dermal origin and medium in bone marrow MSC. In the culture of umbilical cord cells we saw small cells with medium expression of HLA-ABC and large flattened cells expressing these proteins at the background level.

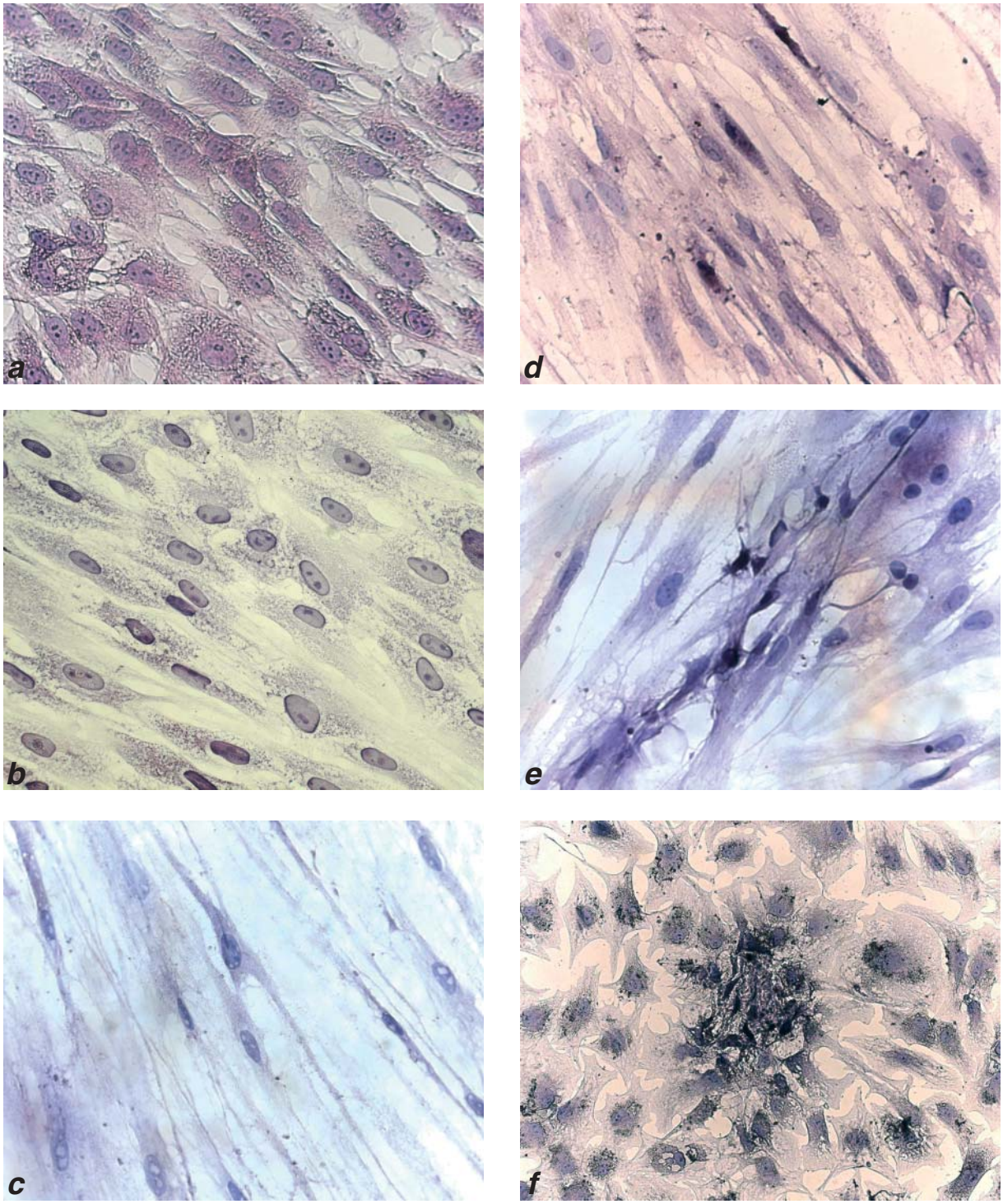

Fig. 4. Activity of calcium phposphate in skin fibroblasts (*a, d*), umbilical cord fibroblast-like cells (*b, e*), and bone marrow MSC (*c, f*) on days 1 (*a-c*) and 21 (*d-f*). Staining by the method of von Kossa.

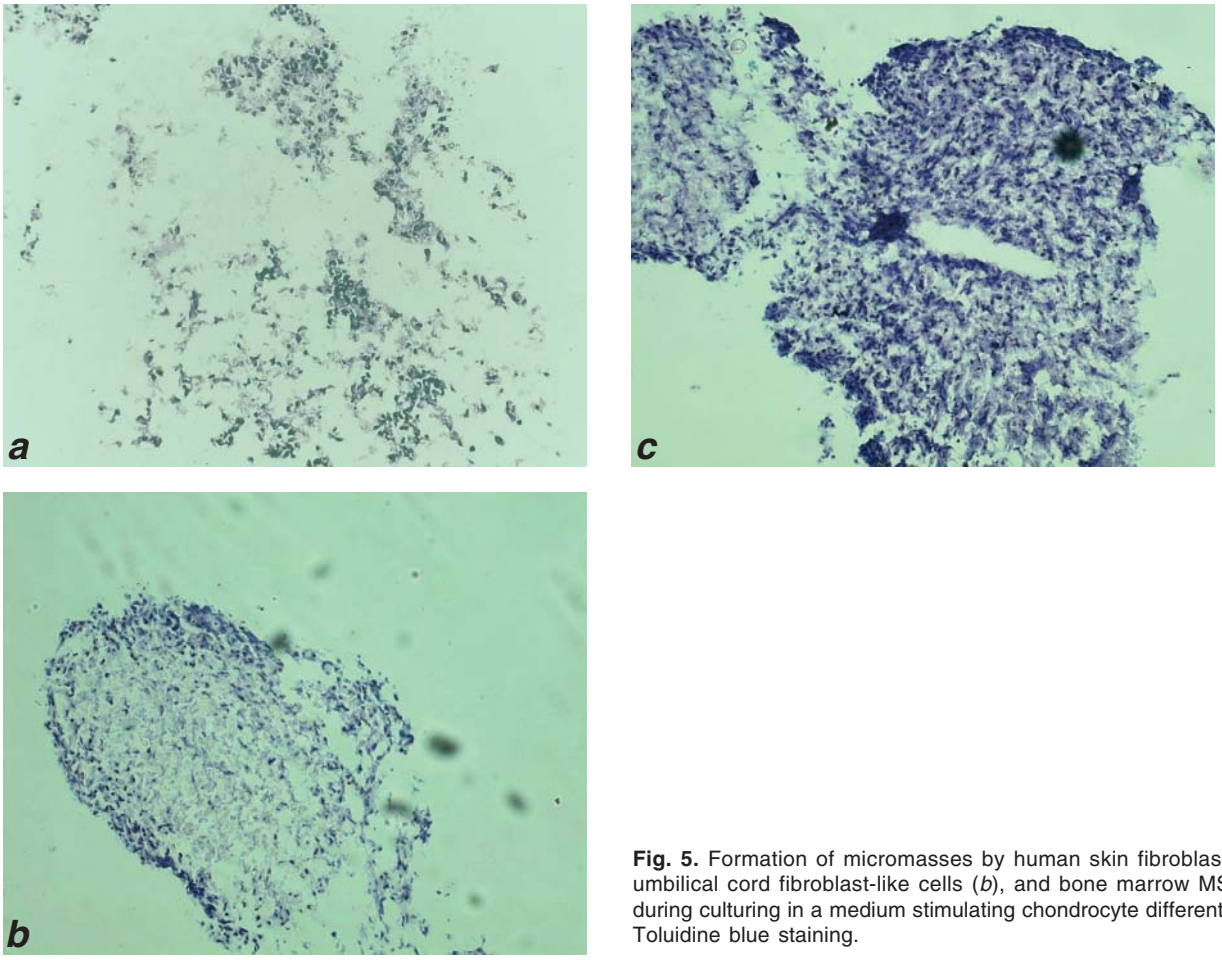

Fig. 5. Formation of micromasses by human skin fibroblasts (*a*), umbilical cord fibroblast-like cells (*b*), and bone marrow MSC (*c*) during culturing in a medium stimulating chondrocyte differentiation. Toluidine blue staining.

Bone marrow MSC expressed a great variety of marker proteins typical of stem and progenitor cells and did not express proteins typical of differentiated cells; fibroblast-like umbilical cord cells expressed markers of both stem cells and differentiated cells. Fibroblasts of dermal origin were characterized by intensive expression of proteins typical of differentiated cells.

Thus, by its phenotypical profile the culture of fibroblast-like cells from the umbilical cord was intermediate between bone marrow MSC and skin fibroblasts.

In order to verify the assumption on the presence of progenitor and stem cells in the obtained umbilical cord cell and MSC cultures, we evaluated the amount of multipotent cells (*i.e.* cells capable to differentiate into adipose, bone and cartilage tissues) in these cultures.

Differentiation of the obtained cultures into adipose tissue was carried out in a medium containing hydrocortisoze, isobutylmethylxanthine, and indomethacin. By day 14 of culturing in the medium stimulating adipocyte differentiation, 100% cells of the dermal origin, umbilical cord fibroblast-like cells, and bone marrow MSC accumulated lipid vacuoles (visualized by oil red staining, Fig. 1).

The capacity of the obtained cells to differentiate into osteoblasts was also evaluated. Unlike skin-derived cells not synthesizing bone sialoprotein under normal conditions, umbilical cord fibroblast-like cells and bone marrow MSC are characterized by initially high expression of this protein (Fig. 2, *a-f*). Expression of bone sialoprotein increased in cells of all cultures over 3 weeks of culturing under conditions stimulating bone differentiation (this increase was minimum in cells of dermal origin and maximum in bone marrow MSC). At the initial stages of differentiation, bone sialoprotein in umbilical cord fibroblast-like cells and bone marrow MSC was seen in the cytoplasm among cytoskeleton proteins, but at later terms this protein accumulated in the endoplasmic reticulum (in exocytozed vacuoles). Bone sialoprotein was also found in the extracellular matrix. If the cells moved over the period of observation, a protein trace appeared.

Skin fibroblasts, umbilical cord cells, and bone marrow MSC did not express osteonectin at the initial

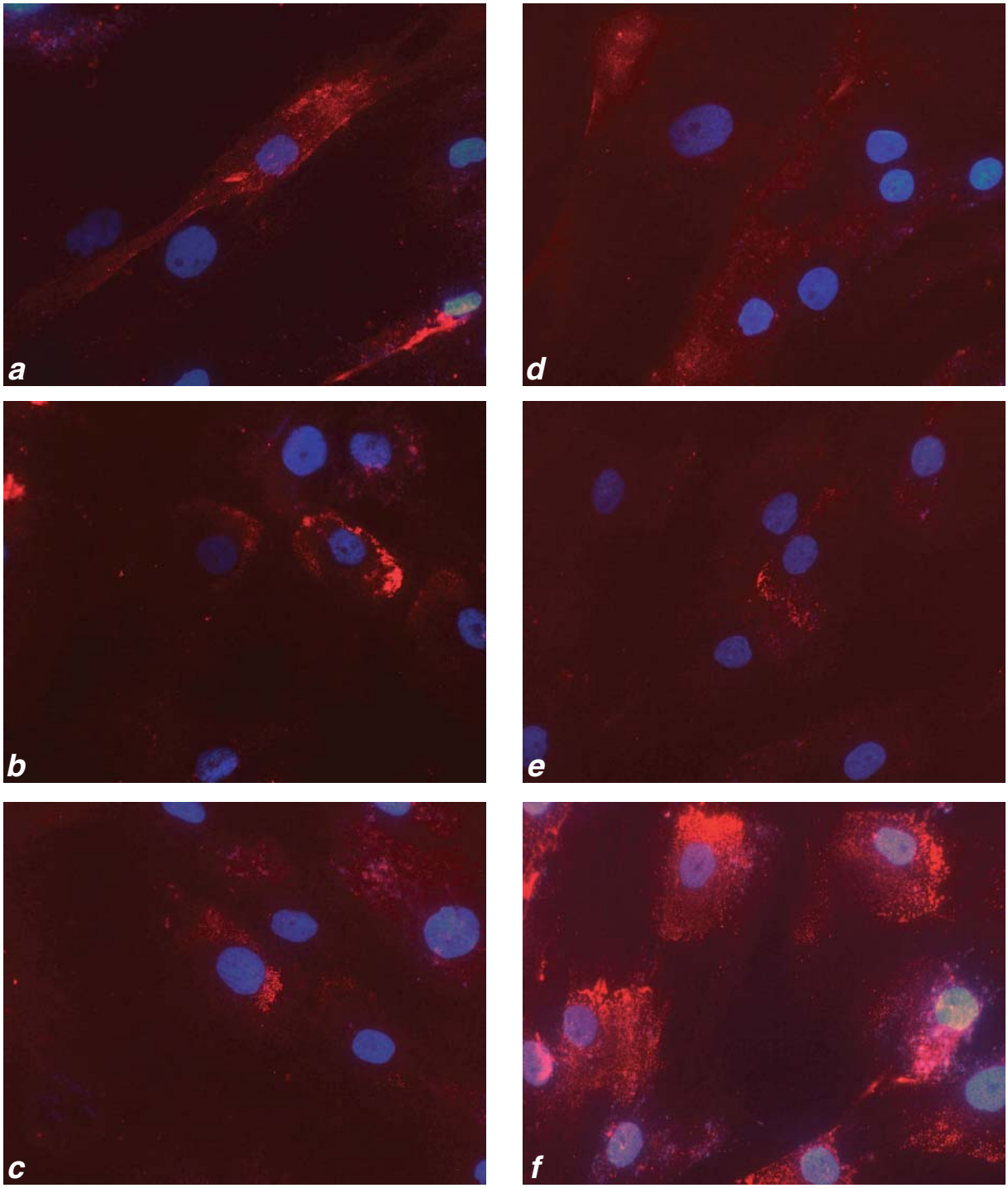

Fig. 6. Accumulation of chondroitin (Rhod; *a, d*), type 1 collagen (Rhod; *b, e*), and type 2 collagen (Rhod; *c, f*) in bone marrow MSC cultured in chondrocyte differentiation medium in the presence (*a-c*) and absence (*d-f*) of TGF-β. Nuclei were stained with DAPI, ×630.

stages of differentiation into osteoblasts (Fig. 2). Expression of osteonectin in umbilical cord cells and bone marrow MSC increased over 3 weeks under conditions stimulating bone differentiation. In cells of dermal origin osteonectin was not detected in the extracellular matrix, but was present in cell cytoplasm among cytoskeleton proteins.

At the initial stages, low activity of endogenous alkaline phosphatase in cultures of skin fibroblasts, umbilical cord cells, and bone marrow MSC was detected in only solitary cells (Fig. 3). Activity of alkaline phosphatase increased during further culturing under conditions stimulating cell differentiation into osteoblasts. However, in culture of skin

fibroblasts this enzyme was detected in only solitary cells. In the umbilical cord culture, the cells with high alkaline phosphatase activity formed rare separate clusters. Only in bone marrow MSC culture activity of endogenous alkaline phosphatase critically increased in practically all cells.

Calcium phosphate was not detected in cells at the initial stages of culturing (Fig. 4). In skin fibroblasts cultured under conditions stimulating cell differentiation into osteoblasts, calcium accumulated in vacuoles of solitary cells (black staining appeared). In the culture of umbilical cord cells, solitary clusters of cells morphologically similar to osteoblasts were seen. In MSC culture, intensive accumulation of calcium phosphate in vacuoles and formation of a considerable number of ossification foci were noted, which initiated differentiation of adjacent cells into osteoblasts.

Umbilical cord cells and bone marrow MSC cultured under conditions stimulating chondrocyte differentiation formed structures characterized by the presence of a considerable amount of extracellular matrix. In skin fibroblasts cultured in micromasses we observed the appearance of loose structures easily degrading to small fragments (Fig. 5).

The extracellular matrix of micromasses obtained during culturing of umbilical cord fibroblast-like cells consisted of equal amount of type 1 and type 2 collagens, while in micromasses of bone marrow MSC the extracellular matrix consisted primarily of type 2 collagen.

During long-term culturing of bone marrow MSC in a medium stimulating chondrocyte differentiation without TGF-β these cells synthesized and accumulated primarily type 1 collagen, while expression of type 2 collagen and chondroitin remained at the background level. This proportion between type 1 and type 2 collagens is typical of the fibrous cartilage tissue (Fig. 6, *d-f*).

Thus, long-term culturing of MSC in a chondrocytes differentiation medium containing TGF-β, the intensity of type 1 collagen synthesis decreased, while the intensity of type 2 collagen and chondroitin synthesis increased, which is characteristic of hyaline cartilage (Fig. 6, *a-c*).

Thus, the presence or absence of TGF-β in bone marrow MSC culture medium is a factor determining the strategy of differentiation of these cells into cartilage tissue of different types. Culturing of umbilical cord fibroblast-like cells in the presence of TGF-β in the chondrocyte differentiation medium had no effect on the proportion between type 1 and type 2 collagens.

Our findings suggest that only bone marrow MSC can differentiate into adipose, cartilaginous, and bone tissue. Thus, taking into account morphological, phenotypical, and functional properties of bone marrow MSC these cells can be regarded as multipotent stem cells. The bulk of umbilical cord fibroblast-like cells can differentiate only into adipocytes and chondrocytes, and only few cells in this culture can differentiate into osteoblasts. Thus, only some cells in the obtained culture are multipotent stem cells. The majority of umbilical cord fibroblast-like cells are bipotent stem cells. Skin fibroblasts differentiate only into adipocytes, and, hence, are unipotent cells. Information on cell phenotype and differentiation capacities of cell cultures can help to determine the sphere of their application in medical practice.

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