Comparatine Analysis of Cytophenotypes of Cells of Mesenchymal Lineage Isolated from Human Tissues

Yu. G. Suzdal'tseva, V. V. Burunova, N. V. Petrakova, I. V. Vakhrushev*, K. N. Yarygin*, V. N. Yarygin

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The expression of cytoplasmic and surface proteins in cultured human skin fibroblasts, human umbilical cord cells obtained after normal delivery on gestation week 38-40, and mesenchymal bone marrow stem cells was compared by the methods of immunocytochemistry and flow cytofluorometry. Bone marrow mesenchymal stem cells 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.

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

The development of effective biomedical technologies for isolation and therapeutic application of stem, progenitor, and differentiated human cells is now becoming a priority problem of modern medicine. Apart from traditional sources of cell material for transplantations (skin and bone marrow), an actual problem is the search for alternative sources of cells combining the advantages of postnatal sources of stem and progenitor cells for autologous transplantations and preparations obtained from cultured fetal cells allowing allogeneic transplantations. We proposed to use human umbilical cord obtained after normal delivery on gestation week 38-40 as the alternative source of stem and progenitor cells. Cultures of umbilical cord fibroblast-like cells are most technological sources compared to cultures of bone marrow and skin cell cultures and can be easily standardized. The preparations containing umbilical cord cells can be used for allogeneic transplantations and contain a great number of stem and progenitor cells.

Russian State Medical University; *V. N. Orekhovich Research Institute of Biomedical Chemistry, Moscow

The therapeutic potential of cell preparation can be evaluated only in clinical trials. However, valuable information can be obtained in *in vitro* experiments on cell cultures, for example, in studies of the expression on surface marker proteins by cultured cells and cell capacity to differentiation in this of that lineage.

The aim of the present study was to obtain cultures of human skin fibroblasts, human umbilical cord cells (after normal delivery on gestation week 38-40), and mesenchymal bone marrow stem cells and to analyze the expression of proteins in the obtained cultured by the methods of immunocytochemistry and flow cytofluorometry.

MATERIALS AND METHODS

Fibroblast-like cells were isolated from the skin of adult donors, human bone marrow, and from human umbilical cord obtained after normal delivery on gestation week 38-40.

The skin fragment (diameter 2 mm) was minced and incubated in 0.1% type 1 collagenase in serum-free DMEM for 2 h at 37°C. The enzyme was

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neutralized by adding fetal serum. The cell suspension was centrifuged, the pellet was resuspended in DMEM containing 10% fetal serum, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 10 ng/ml basic fibroblast growth factor (b-FGF) to a concentration of 10^4 cells/ml, transferred into culture dishes, and cultured until confluence. The medium was changed 2 times a week.

Human umbilical cord was taken after normal delivery on gestation week 38-40. Umbilical veins were cannulated at both ends and washed first with Hanks solution, then with 0.1% type 1 collagenase in serum-free DMEM for 2 h at 37°C. The vessels were again washed with Hanks solution, the umbilical cord tissue was subjected to gentle mechanical influence, and the detached cells were collected and pelleted by centrifugation. The cell pellet was resuspended in DMEM containing 10% fetal serum, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 10 ng/ml b-FGF to a concentration of 10^4 cells/ml, transferred into culture dishes, and cultured until confluence (the medium was replaced 2 times per week) [4].

Human bone marrow aspirate was collected into vacutainer tubes containing sodium heparin and were transported to the laboratory at 2-8°C. Mononuclear cells were routinely separated in Ficoll density gradient. The cells were suspended in DMEM containing 10% fetal serum, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 10 ng/ml EGF, and 10 ng/ml PDGF-BB, transferred into culture dishes (10^4 cells/ml), and cultured until confluence (the medium was replaced 2 times per week).

Immunocytochemical analysis of the expression of cytoplasmic and surface 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).

For evaluation of the expression of surface markers by the method of flow cytofluorometry, the cell monolayer was suspended with Versene. The cells were stained with FITC-labeled antibodies (Becton Dickinson) and phycoerythrin in PBS containing 1% fetal serum and fixed in 2% formaldehyde. Analysis was performed on a Beckman Coulter Epics XL flow cytofluorometer. The data were processed statistically using WinMDI software.

RESULTS

Adherent cultures of human skin fibroblasts, umbilical cord cells, and bone marrow MSC were obtained. The bulk of cells from the skin (Fig. 1, *a*) and umbilical cord (Fig. 1, *b*) and bone marrow MSC (Fig. 1, *c*) had fibroblast-like morphology. However, colonies of large round cells and colonies of small drop-shaped cells were seen in the culture of bone marrow MSC (Fig. 1, *d*).

In all cultures, expression of cytoplasmic proteins was analyzed (vimentin, nestin, type 1 and type 2 collagens). Comparative analysis revealed both similarity and differences between cells in the obtained cultures.

All cells of the obtained cultures expressed vimentin, *i.e.* the protein of cytoplasmic skeleton, which is typical of cells of the mesenchymal lineage [1].

The majority of umbilical cord fibroblast-like cells expressed nestin, a marker of immature and progenitor cells. The number of umbilical blood cells expressing nestin and the level of expression of this protein decreased with increasing the cell monolayer density (Fig. 2, *a*). Expression of nestin by umbilical cord cells can be explained by their origination from fetal trophoblast. Cells of dermal origin and bone marrow MSC did not express nestin.

The most part of umbilical cord cells expressed type 1 collagen. In the culture of skin fibroblast only solitary cells expressed this protein. Bone marrow MSC did not express type 1 collagen [1].

The most part of umbilical cord cells expressed type 2 collagen. The number of umbilical cord cells expressing this protein and the level of expression of this protein increased proportionally to the time of culturing (Fig. 2, *c*). The density of umbilical cord cell monolayer did not affect the level of expression of type 2 collagen. Cells of dermal origin and bone marrow MSC did not express this protein. Intensive expression of type 1 and type 2 collagens by umbilical cord cells can be explained by the function of these cells: formation of special connective tissue, so-called Wharton jelly, surrounding the umbilical vessels.

Double immunocytochemical staining of umbilical cells for type 2 collagen and nestin revealed cells expressing type 2 collagen, nestin, and cells expressing both proteins (Fig. 3).

Many umbilical cord cells expressed endotheliocyte marker, von Willebrand factor (Fig. 4, *b*).

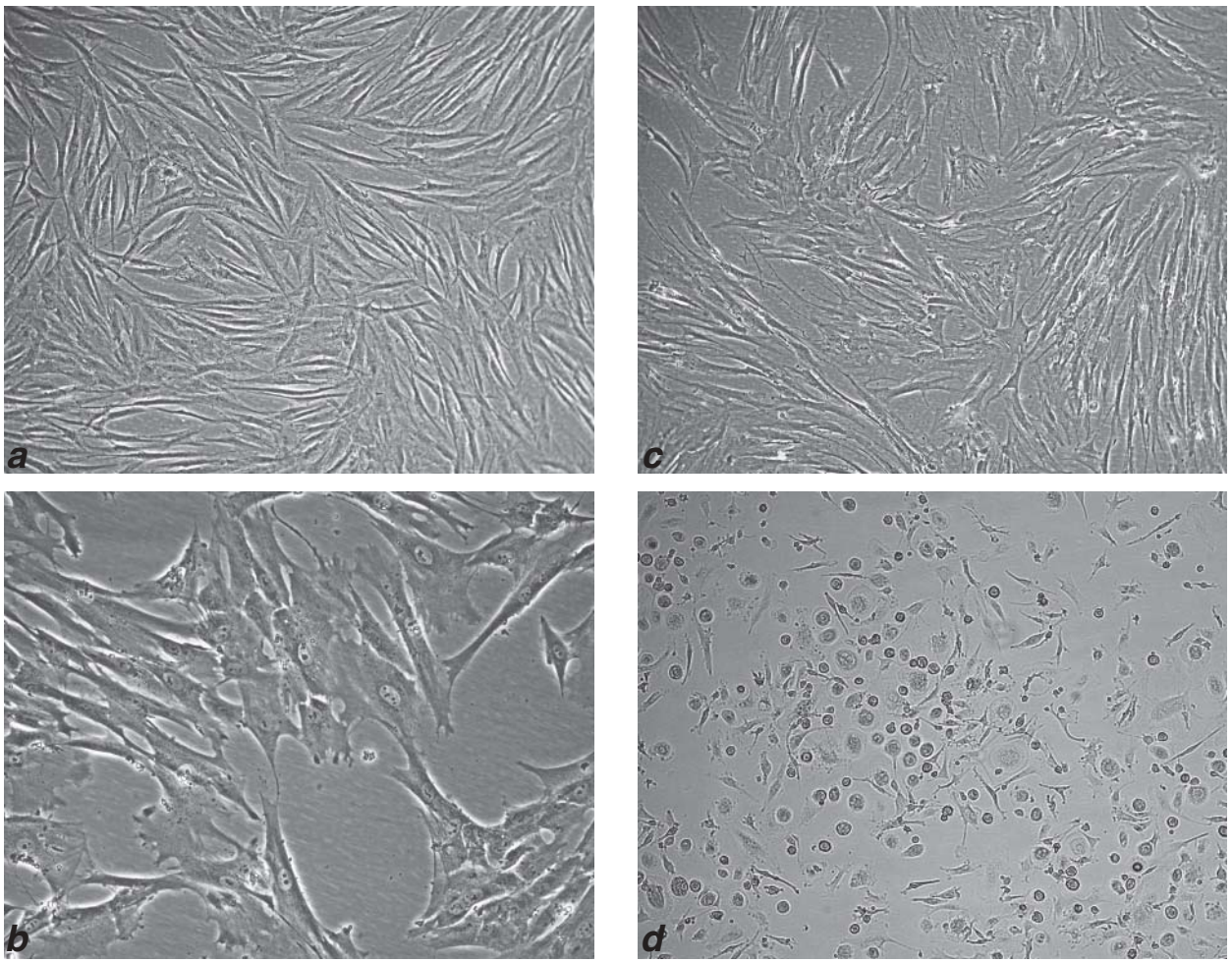

Fig. 1. Cultures of cells isolated from the skin (*a*), human umbilical cord (*b*), and bone marrow (*c, d*), ×200.

The number of cells with low expression of von Willebrand factor decreased 2 fold with increasing the monolayer density: from ~20% in nonconfluent culture to ~10% in confluent culture. Cells with very high expression of this factor appeared (~5%) in dense monolayer, but were absent in low-density monolayer (Fig. 2, *b*). However, the total number of cells expressing von Willebrand factor in the culture of umbilical cells did not change for at least 10 passages. In the culture of bone marrow MSC about 20% cells expressed von Willebrand factor (Fig. 4, *a*). Their number decreased after subculturing, and by the 3rd passage these cells disappeared. Expression of von Willebrand factor in skin-derived fibroblasts was not detected.

These findings attest to differentiation of umbilical cord fibroblast-like cells during culturing: nestin, an immature cell marker, was replaced with mature cell markers type 1 and 2 collagens and then with von Willebrand factor.

The expression of class I major histocompatibility complex proteins in cell cultures is an important parameter, which should be taken into account when determining suitability of cells for transplantation for the treatment of this or that pathology and evaluating the efficiency of therapeutic use of cell preparations. The expression of the major histocompatibility complex proteins (HLA-ABC) was maximum in fibroblasts of dermal origin (Fig. 4, *a*) and medium in MSC culture (Fig. 4, *b*). The culture of umbilical cord cells was heterogeneous: small cells with medium expression of HLA-ABC and large flattened cells expressing these proteins at the background level were seen (Fig. 4, *c*). These findings were confirmed by flow cytofluorometry. The bulk of umbilical cord cells were characterized by medium or high expression of HLA-ABC. It can be hypothesized that umbilical cord cells express a non-classical major histocompatibility complex protein HLA-G, which together with HLA-C, prevents the development of the maternal immune response against the fetus [3]. These results substantiate the possibility of using umbilical cord fibroblast-like cells for allogeneic transplantations because of anti-

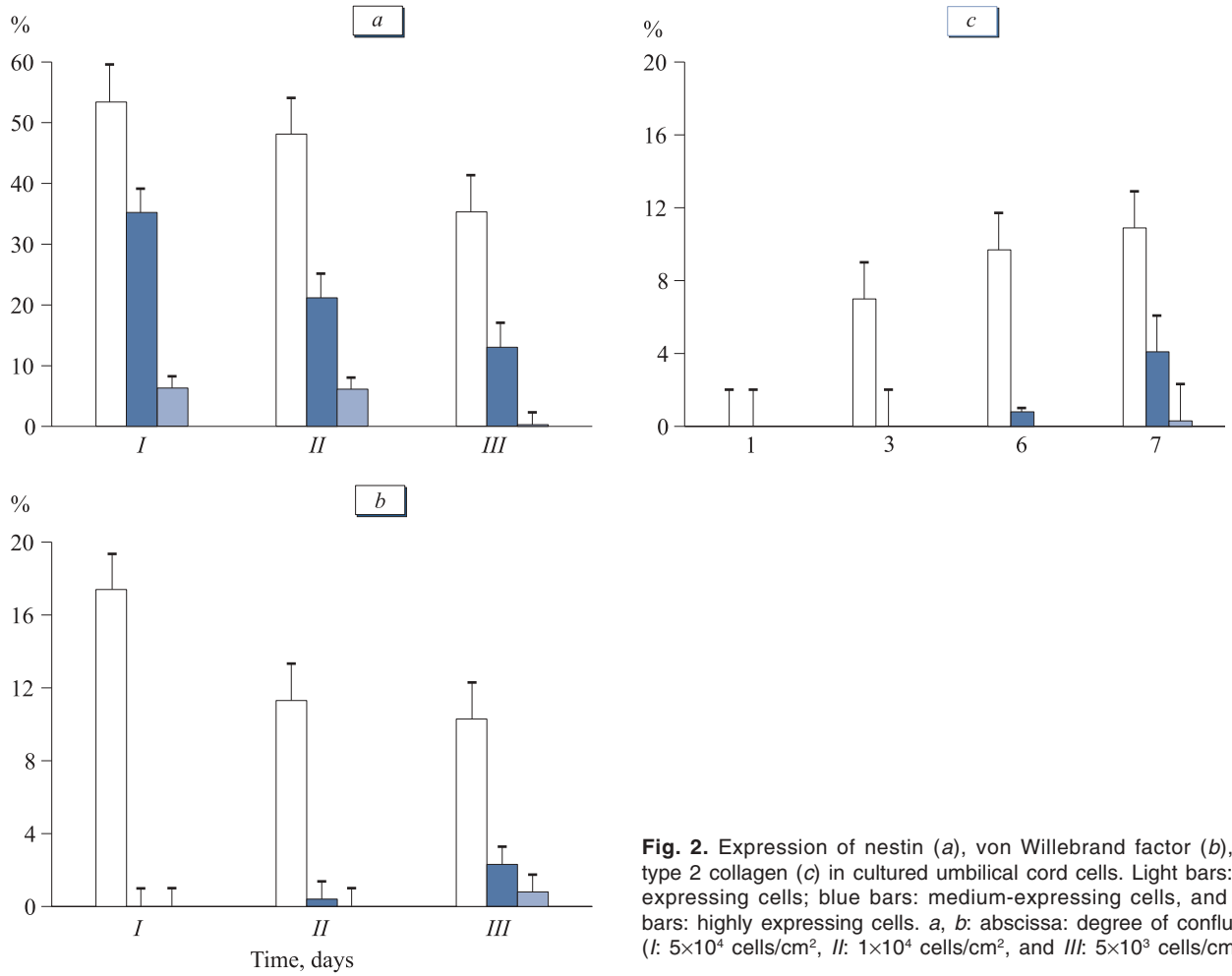

Fig. 2. Expression of nestin (*a*), von Willebrand factor (*b*), and type 2 collagen (*c*) in cultured umbilical cord cells. Light bars: low-expressing cells; blue bars: medium-expressing cells, and gray bars: highly expressing cells. *a*, *b*: abscissa: degree of confluence (*I*: 5×10^4 cells/cm^2, *II*: 1×10^4 cells/cm^2, and *III*: 5×10^3 cells/cm^2).

cipated low level of the immune response to these cells. High expression of class I major histocompatibility complex proteins by fibroblasts of dermal origin and bone marrow MSC restricts their application for allogeneic transplantations, but leave the opportunity for autologous transplantations.

The obtained cultures were characterized by the expression of surface stem cell marker proteins CD34, CD44, CD45, CD49b, CD54, CD90, CD105, CD106, CD117, HLA-DR by the method of flow cytofluorometry (Table 1).

All cultures were negative by the expression of hemopoietic stem cell markers CD34, CD45, and HLA-DR.

High expression of cell adhesion molecules CD44, CD49b, CD54, CD90, CD106 playing a critical role in processes of cell migration and homing is typical of cells of the mesenchymal lineage.

Protein complex CD44, apart from adhesion molecules, contains pgp1 protein, a representative of transporter proteins responsible for cell multidrug resistance. A great number of these molecules maintaining intracellular homeostasis and high proliferation level even under unfavorable conditions was observed in stem and progenitor cells. Cultures of human skin fibroblasts, umbilical cord cells and MSC little differed by the expression of CD44. The expression of CD44 was high in cultured skin fibro-

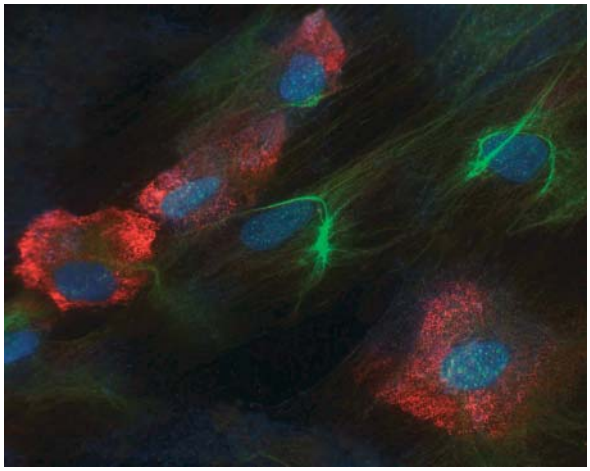

Fig. 3. Expression of type 2 collagen (Rhod) and nestin (FITC) in cultured umbilical cord cells. Here and on Fig. 4-6: nuclei were stained with DAPI, ×630.

TABLE 1. Results of Immunocytochemical Analysis of Bone Marrow MSC and Skin and Umbilical Cord Fibroblast Cultures

Marker	Bone marrow MSC	Umbilical cord fibroblast-like cells	Skin fibroblasts
CD34	—	—	—
CD45	—	—	—
HLA-DR	—	—	—
HLA-ABC	±	±	+
CD44	±	±	+
CD49b	—	±	±
CD54	+	+	—
CD90	+	+	+
CD105	±	±	±
CD106	+	—	—
CD117	±	±	—

Note. + high expression, ± low expression, — no expression.

blasts and medium in umbilical cord cells and MSC.

The obtained cultures differed considerably by the expression of CD54 containing ICAM (intercellular adhesion molecule), a ligand for β_2-integrins. Expression of CD54 normally increases in cells differentiating into supporting tissues (bone cartilage) [2,5]. High expression of CD54 was observed in the culture of umbilical cord cells, while large flattened cells in these cultures expressed these proteins at the background level. Medium expression of CD54 was detected in bone marrow MSC culture, while skin fibroblasts practically did not express this marker. Cytofluorometry showed (Fig. 5) that the population of umbilical cord cells consisted of ~50% cells with high expression of CD54, ~30% cells with medium expression of CD54, and ~10% cells with low expression of this factor. In MSC culture, high, medium, and low expression of CD54 was noted in 20, 40, and 20% cells, respectively. In the culture of skin fibroblasts, we detected only ~10% cells with weak expression of this

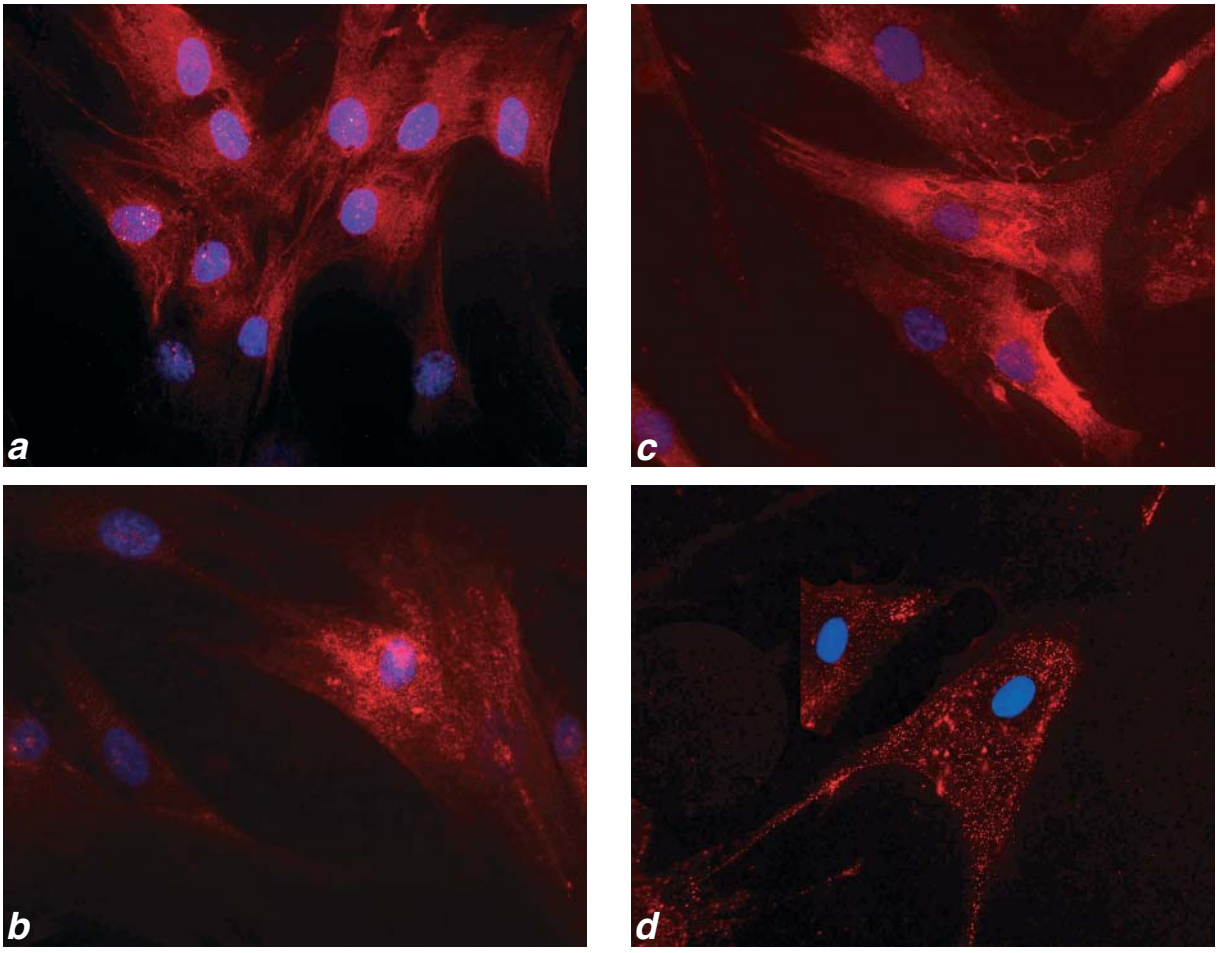

Fig. 4. Expression of von Willebrand factor (Rhod) in bone marrow MSC (*a*) and umbilical cord cells (*b*), expression of CD106 (Rhod) in bone marrow MSC (*c*), and expression of CD117 (Rhod) in umbilical cord cells (*d*).

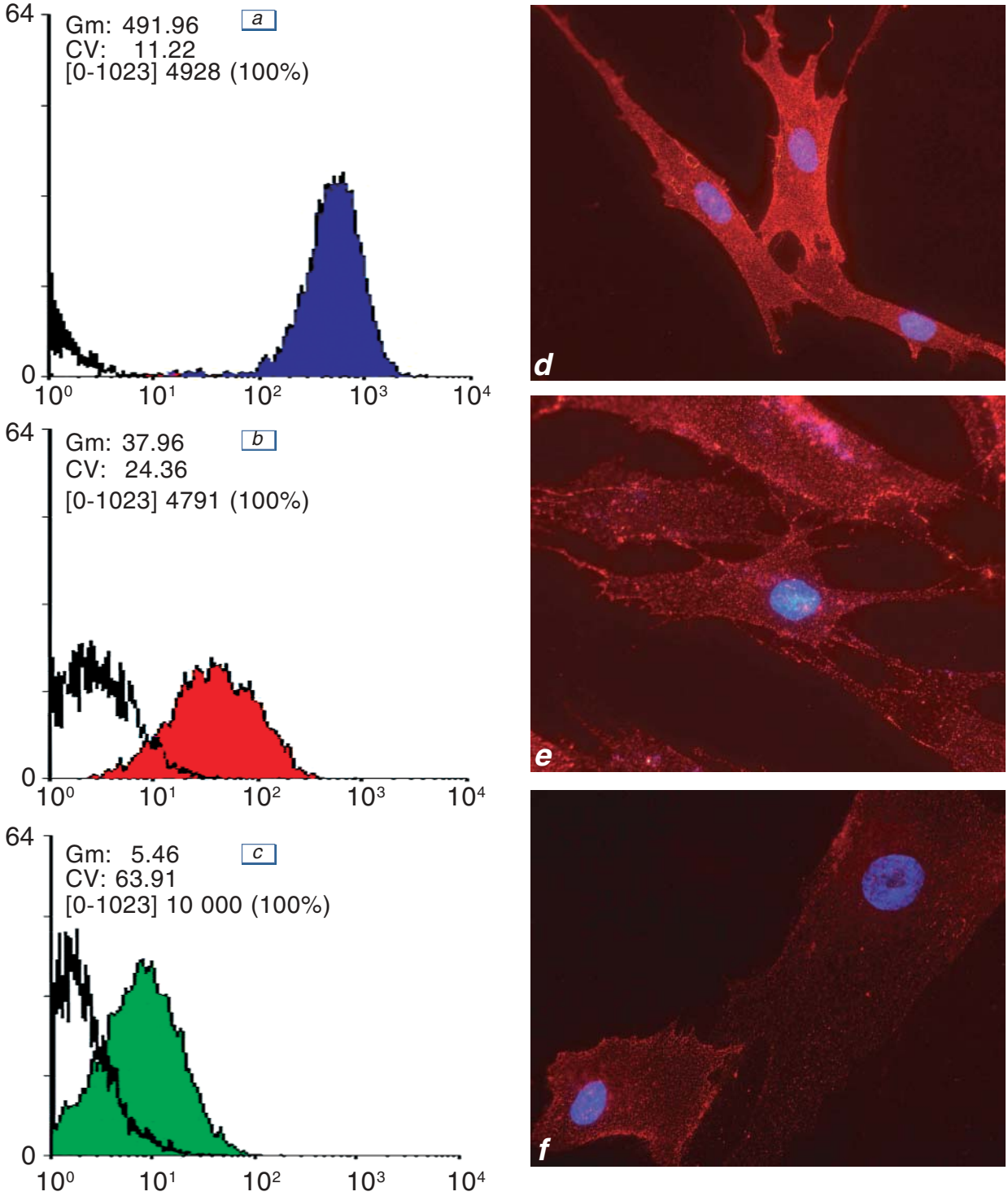

Fig. 5. Expression of major histocompatibility complex proteins (HLA-ABC) by skin fibroblasts (*a, d*), bone marrow MSC (*d, e*), and umbilical cord cells (*c*, *f*). *a*, *b, c*: abscissa: fluorescence intensity, ordinate: number of events (cells). Isotypic control is not stained; *d*, *e*, *f*: HLA-ABC (Rhod).

factor. It can be hypothesized that high expression of intercellular adhesion molecules by cells and the great number of highly expressing cells in the culture determine their capacity to form dense structures, which agrees with the data obtained in studies of the capacity of umbilical cord fibroblasts and MSC to differentiation into bone and cartilage tissue.

Apart from von Willebrand factor, bone marrow MSC expressed endotheliocyte adhesion mole-

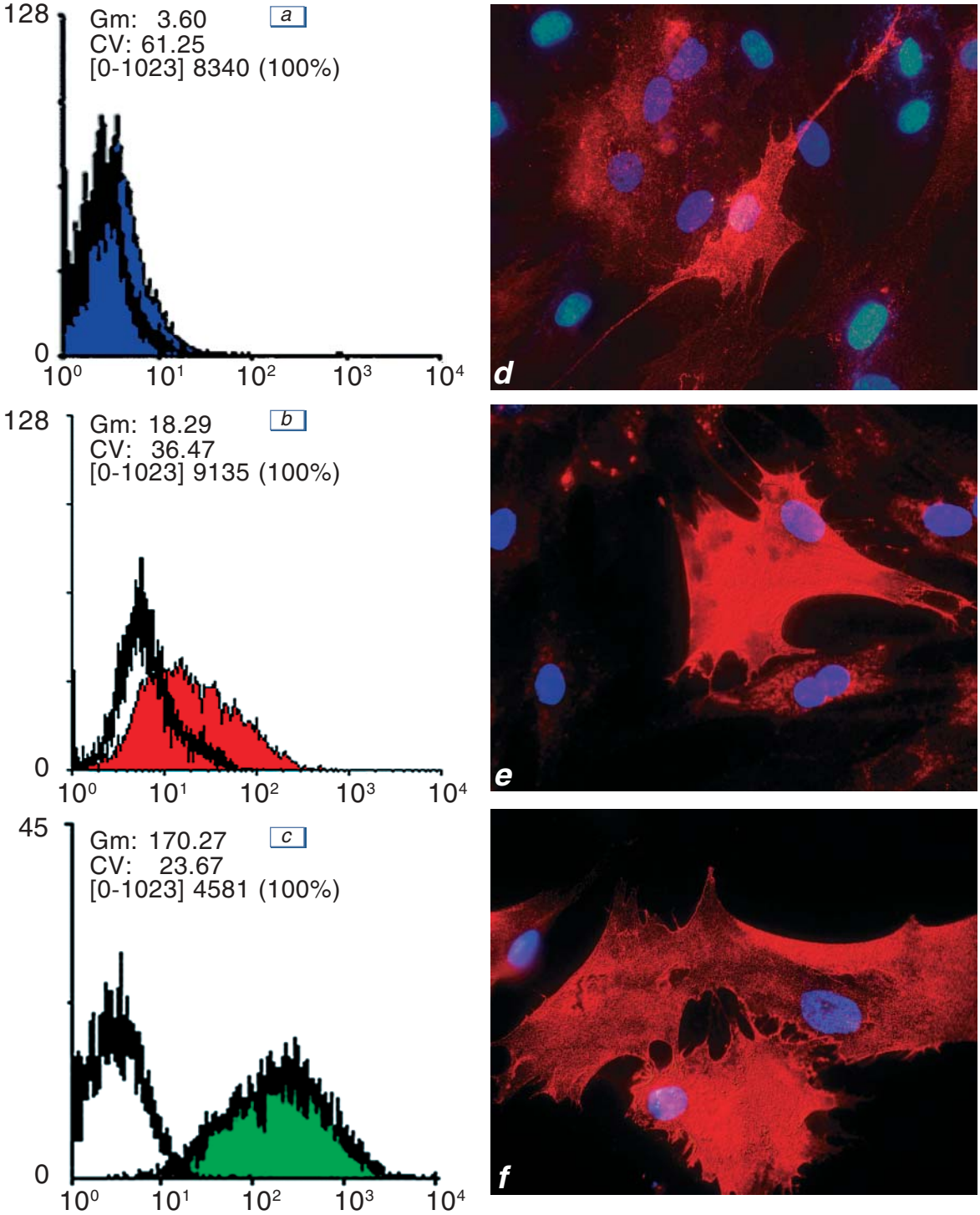

Fig. 6. Expression of CD54 by skin fibroblasts (*a*, *d*), bone marrow MSC (*d*, *e*), and umbilical cord cells (*c*, *f*). *a*, *b, c*: abscissa: fluorescence intensity, ordinate: number of events (cells). Isotypic control is not stained; *d*, *e*, *f*: CD54 (Rhod).

cule, VCAM (CD106). Cells of dermal origin and umbilical cord cells did not express this protein.

The expression of CD49b ($\alpha_2\beta_1$-integrin) was similarly low in skin fibroblasts and umbilical cord cells. Bone marrow MSC did not express this marker.

The cells of all obtained cultures were characterized by high expression of CD90 belonging to the family of immunoglobulin-like receptors.

Expression of receptors for growth and transforming factors is an important sign attesting to the

presence of stem and progenitor cells in the obtained cultures.

The product of *c-kit* gene (CD117) is a receptor for stem cell factor (SCF) regulating mitotic division and triggering antiapoptotic processes in progenitor cells. Umbilical cord cells and MSC were characterized by medium expression of CD117. Skin fibroblasts were not stained with antibodies against this protein. These findings confirm the presence of stem and progenitor cells in cultures obtained from the bone marrow and umbilical cord.

The obtained cultures did not differ by the expression of CD105 (endoglin), a receptor for TGF-β triggering a reaction cascade changing cell morphology and adherence characteristics. CD105 was expressed at the medium level in all cultures.

Bone marrow MSC expressed a great variety of surface proteins typical of stem and progenitor cells and did not express cytoplasmic proteins typical of differentiated cells. The population of MSC was homogenous by the expression of the studied markers. The culture of umbilical cord fibroblast-like cells was heterogeneous and contained at least 3 subpopulations: cells expressing only surface and cytoplasmic marker proteins of MSC and progenitor cells, cells expressing only differentiated cell markers, and cells expressing both MSC and progenitor cell markers and differentiated cell markers. Skin-derived fibroblasts expressed only some surface markers typical of MSC and progenitor cells, and to a great extent, marker proteins of differentiated cells.

Thus, by its phenotypical profile the culture of fibroblast-like cells from the umbilical cord is intermediate between practically homogenous bone marrow MSC culture and culture of skin fibroblasts exhibiting properties of highly differentiated cells. However, only comparative analysis of cell capacities to differentiate into adipose, cartilage and bone tissue can prove the presence of stem and progenitor cells in the culture.

REFERENCES

1. *K. N. Yarygin, Yu. G. Suzdal'tseva, V. V. Burunova, et al., Kletochn. Tekhnol. Biol. Med*., No. 1, 53-59 (2006).
2. J. Diaz-Romero, J. P. Gaillard, and S. P. Grogan, *J. Cell Physiol*., **202**, No. 3, 731-742 (2005).
3. P. K. Lalla, Before We Are Born: Essentials of Embriology and Birth Defects. (2003), pp. 103-104.
4. Y. A. Romanov, V. A. Svintsitskaya, and V. N. Smirnov, *Stem Cells*, **21**, 105-110 (2003).
5. Y. Tanaka, I. Morimoto, Y. Nakano, *et al., J. Bone Miner Res*., No. 10, 1462-1469 (1995).