Preparation and Characterization of Culture of CD146$^+$ Cells from Human Adipose Tissue

A. A. Rzhaninova[1,2], A. V. Kulikov[1], I. A. Spirova[1,2], E. E. Kirienko[1,2], A. V. Volkov[1], and D. V. Goldshtein[1,2]

Translated from *Kletochnye Tehnologii v Biologii i Medicine*, No. 1, pp. 3-9, January, 2010
Original article submitted October 28, 2009

A method for isolation of homogenous culture of cells expressing CD146 marker from adipose tissue lipoaspirate was developed. The resultant clonogenic cultures retained high proliferative activity, immunophenotype, and morphology after numerous passages. The presence of insulin in the medium served as the selective factor for maintenance of the population phenotype. These cultures effectively differentiate into CD31$^+$ endothelial cells and can be used in regenerative medicine.

Key Words: *adipose tissue stromal vascular fraction; insulin; immunophenotype; CD146; CD31*

Stromal vascular fraction (SVF) of human adipose tissue (AT) is now acknowledged to be the most effective source of multipotent stromal cells (MSC) for regenerative medicine, creation of tissue engineering constructions, and cell gene therapy. Experimental and clinical data indicate that *in vivo* MSC are presented by perivascular cells (pericytes) identified by the expression of CD146 marker [4,6].

CD146 (Mel-CAM, MUC18, S-endo-1) is a glycoprotein belonging to the class of cell adhesion molecules. It is expressed on the surface of not only pericytes, but also endothelial progenitor and mature cells, some malignant tumor cells, and some MSC populations [3,7,9,16,17,20]. The origin of the cells carrying CD146 marker on their surface can be identified by their morphology and co-expression of CD31, which labels only endothelial cells [2]. Thus, the CD146$^+$CD31$^+$ phenotype labels the endothelial precursors and mature calls, while the CD146$^+$CD31$^-$ phenotype is characteristic of perivascular cells and some MSC populations [1,10,11].

Increasing interest to *in vitro* isolation of cell cultures expressing CD146 molecule is explained by great potentialities of their use in regenerative medicine. A possible trend of the CD146$^+$CD31$^+$ cell application is vascularization of tissues and tissue engineering constructions, which remains the main problem of regenerative medicine. *In vitro* culturing of endothelial CD31$^+$ cells is difficult, because culturing of endothelial precursors even in specialized media leads to the loss of the expression of specific markers and alteration of cell morphology as soon as after 1-2 passages [8].

The prospects of practical application of CD146$^+$ CD31$^-$ cells are explained by their high osteoinductive potential [14,18]. Isolation of CD146$^+$CD31$^-$ cells from human bone marrow and umbilical cord were described not once [2,19]. It is shown, for example, that bone marrow CD146$^+$ cells are clonogenic osteoprogenitors located in the vascular wall [19]. Nonhemopoietic origin of the umbilical cord CD146$^+$ cells was demonstrated: incubation with antibodies revealed CD146 antigen in the perivascular region and its diffuse distribution in the vascular wall [3]. Umbilical cord cells are characterized by higher osteogenic potential than bone marrow MSC [2]. The isolated cells in both cases were of mesenchymal origin and expressed the CD146$^+$CD31$^-$ phenotype. However, no more than 50% cells expressed CD146; in other words, the studied cultures were heterogeneous.

[1]ReMeTex Company; [2]Medical Genetic Center, Russian Academy of Medical Sciences, Moscow, Russia. ***Address for correspondence:*** rzhaninova@rm7. A. A. Rzhaninova

0007-4888/10/1491-0113

Co-expression of $CD146^+CD31^+$ by proliferating cells was detected in the subpopulation of activated fetal myoblasts, presumably originating from progenitor endothelial cells [5]. These heretofore not described cells were called myoendothelial, because they expressed myogenic markers (desmin and Myo D). A characteristic feature of myoendothelial cells is their fusion in myotubules under conditions of reduced serum concentration in the growth medium. No myotubules formed under similar conditions in the $CD146^+CD31^+$ cell populations isolated from AT in our experiments.

Clonogenic $CD146^+$ cells of perivascular origin were detected in cultures isolated from the endometrium and were characterized by the MSC-like phenotype [15].

Cells expressing CD146 and CD31 are detected in primary culture of AT SVF. However, it is not clear, which subpopulations they label. Three and more passages under standard conditions lead to elimination of cells with these markers or to reduction of their count to the basal level [5].

We developed a method for isolation of AT SVF perivascular cells carrying the CD146 marker and characterized the resultant population.

MATERIALS AND METHODS

The study was carried out on biopsy specimens of subcutaneous AT obtained by liposuction from 15 donors aged 39±13.97 years.

AT SVF was isolated from donor lipoaspirate. AT was disaggregated with a mixture of collagenase-1 and dispase for 1 h at 37°C. The resultant suspension was centrifuged (10 min, 1000*g*) and the supernatant was discarded. The cell precipitate was resuspended in a small volume of DMEM/F12 and the number and viability of the isolated cells were evaluated by trypan blue staining. The cell suspensions with viability ≥80% were cultured. The cells were cultured in DMEM/F12 (1:1) with 10% FCS, 2 mM L-glutamine, 10 ng/ml recombinant fibroblast growth main factor (FGF-2), and 8 U/ml heparin. Recombinant human insulin was dissolved in 1 N hydrochloric acid and used in a final concentration of 5 ng/ml. The cells were inoculated in 90-mm plastic Petri dishes (Cornig) at a density of 1500-2000 thousand cell/cm^2 flask surface. The cells were cultured in a CO_2 incubator (37°C, 5% CO_2). After 3-day incubation, floating cells were transferred into fresh dishes and the growth medium was replaced. The cultures were incubated until 70% confluence, after which they were removed with trypsin and inoculated into fresh dishes at the same density.

In order to count the colonies, the cells were reinoculated into plastic Petri dishes (90 mm) at a density of 50 cell/cm^2. The cultures were incubated at 37°C and 5% CO_2 in growth medium until the formation of colonies of at least 2 mm in diameter. After incubation, the medium was discarded, the dishes were washed with Hanks' solution, and the cells were stained with 5% crystal violet in 70% ethanol (5 min at ambient temperature). The cells were then washed twice with distilled water and stained colonies (≥2 mm in diameter) were counted. The means were calculated by the results of analysis of at least 3 dishes per sample.

The culture doubling time was calculated by the formula: [duration of culturing (days)]×[$\ln_2$]/ln [number of cells inoculated/number of cells collected]. The mean was calculated from the results of 3 estimations.

The culture doubling number was calculated by the formula: [$\ln_2$/ln [number of cells inoculated/number of cells collected].

The expression of specific surface antigens in the studied cultures (immunophenotype) was analyzed on a FACS Calibur flow cytofluorometer (BD Biosciences). Mouse monoclonal antibodies (PharMingen, Chemicon) were used. Nonspecific mouse (rabbit) IgG (the same firms) served as the negative control. The results were processed using WINMDI 2.8 software.

RESULTS

According to the standard technology, population of cells highly adherent to plastic (cells adhering from the primary cell suspension within no more than 2-3 days) is used in culturing. However, after incubation of cultures during this period, only nonlymphoid nuclears are present in the suspension; the adhesion properties of these cells are less pronounced and they adhere to the plastic later. These cells remain usually not analyzed. Predominant growth of fibroblast-like cells is observed during culturing by the standard method.

Insulin maintains the growth and replication of many cell types, including fibroblasts. In sensitive cells, insulin potentiates the capacity of FGF to stimulate multiplication of cells arrested in phase G1 as a result of serum removal from the medium. However, this model describes the processes in 3T3 fibroblasts, and it is not proven that it is universal.

In our experiments, addition of insulin to the growth medium during AT SVF culturing led to proliferation inhibition, enlargement of the cells, and appearance of adipogenic differentiation foci (lipid droplets). Long-term growth of AT SVF in media with insulin resulted in preterm aging of cultures and discontinuation of proliferation after 7-8 passages. However, the expression profiles of surface antigens in cultures grown with and without insulin did not differ. More than 90% cells in all the studied cultures expressed

CD13, CD29, CD73, CD44, CDC90, and CD166. Endothelial cell markers were detected in no more than 10% cells or were not detected at all.

Cell populations with poor adhesion to plastic were cultured similarly (with and without insulin). A population presented by fibroblast-like cells predominated after 2-3 passages in both cases. The growth of the predominant population was also inhibited by adding insulin to the medium. However, 2 weeks after inoculation of cultures in medium with insulin, we observed the appearance of foci of compact growth of small epitheliocyte-like cells differing by the morphology from the main population (Fig. 1). These cells were characterized by high mitotic activity in the presence of insulin and FGF-2. Cytofluorometric analysis detected changes in the expression of CD146 antigen: the percent of positive cells reached 53% in this culture compared to <1% (basal level) in the main population cultured without insulin (Fig. 2, *a*, *b*). These data suggested that the colonies of actively dividing cells growing with insulin expressed CD146 marker. The expression of CD31 remained low (6%), not higher than in cultures grown without insulin (1-8%). The colonies of small epitheliocyte-like cells ("plaques") were mechanically isolated and were cultured separately with insulin. Similarly as during the early stages of the study, the antigenic profiles of the separated cells were identified. The expression of CD146 and CD31 markers reached 91 and 49%, respectively (Fig. 2, *c*). The culture was characterized by very rapid growth: cell count doubled after <18 h, while the populations cultured in parallel by the standard protocol ($CD146^{-}$) doubled after 32-45 h (Fig. 3; data for 3-6 passages are presented). In contrast to $CD146^{-}$ cells, the growth of isolated positive populations was not inhibited by insulin: the time of culture doubling in the presence of insulin was just slightly shorter in comparison with cultures growth in basic medium without additives. Hence, insulin virtually did not modify the rate of $CD146^{+}$ cell growth, but served as the factor of selection for them (at the expense of inhibition of $CD146^{-}$ populations proliferation).

Mitotic globular cells were selected from the test cultures during the logarithmic growth phase and in-

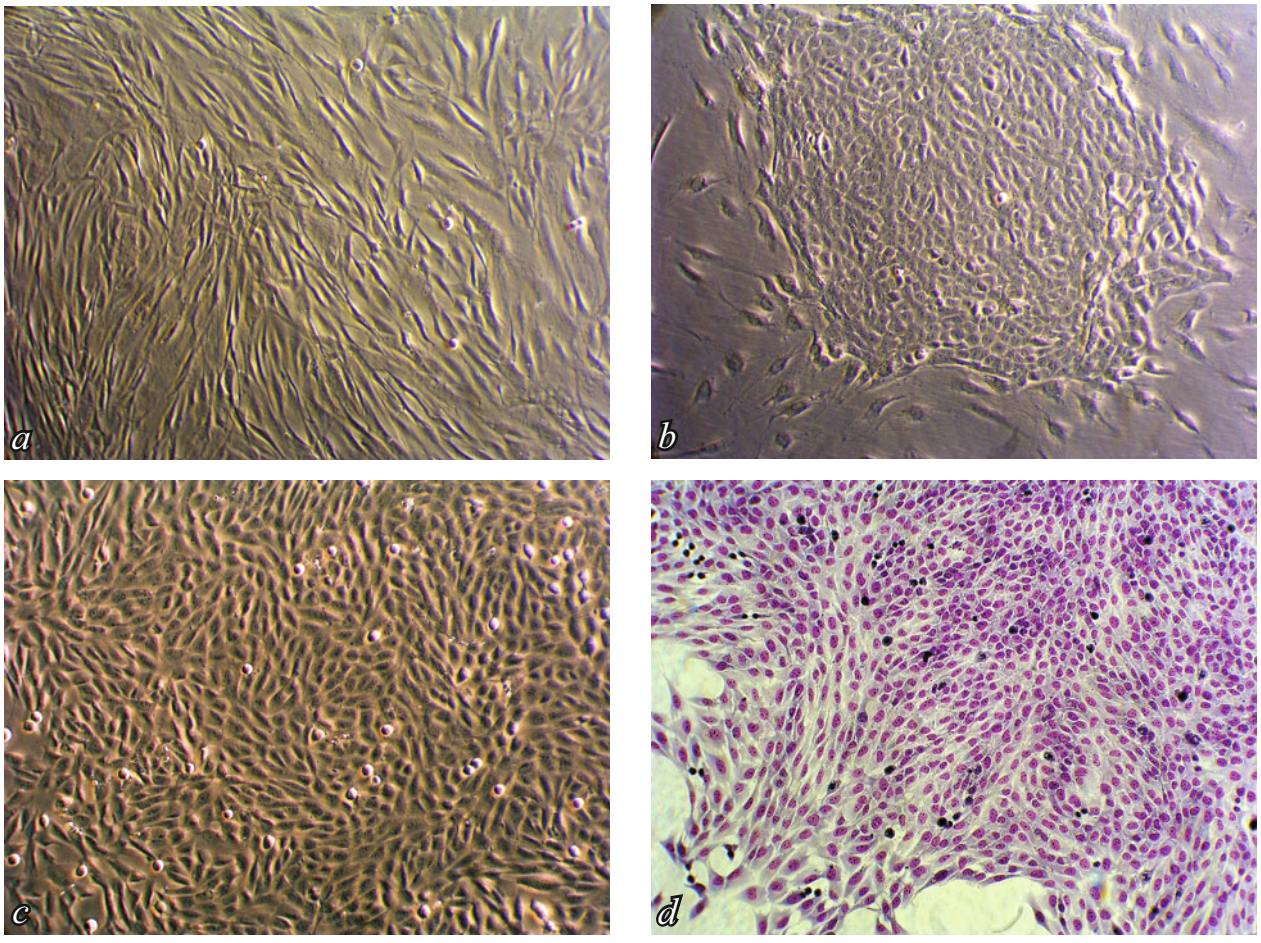

Fig. 1. Changes in the morphology of AT SVF culture during selection of $CD146^{+}$ population. *a*) AT SVF culture obtained by the standard method, passage 2; *b*) AT SVF culture with poor adhesion to plastic; growth of endotheliocyte-like cells in the presence of insulin; *c*, *d*) isolated $CD146^{+}$ cell population. Relief phase contrast (*a*-*c*) and Pappenheim's staining (*d*; ×100).

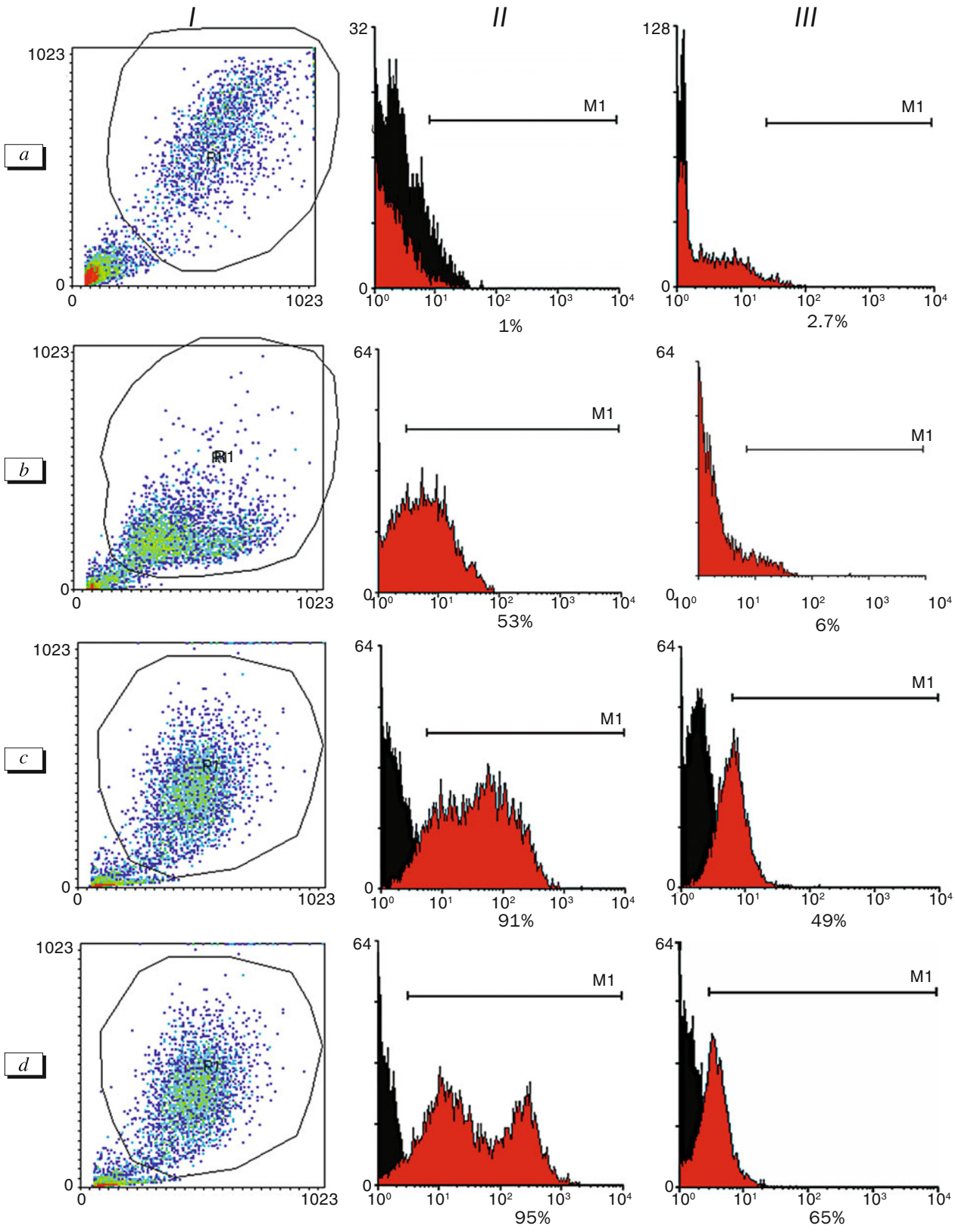

Fig. 2. Cytofluorometric analysis of the expression of CD146 and CD31 surface antigens in AT SVF cultures. *I*) distribution of cell populations in forward and side light scatter; *II*) histograms reflecting expression of CD146; *III*) histograms reflecting expression of CD31. *a*) standard culturing; *b*) mixed population grown with insulin; *c*) isolated colony of endotheliocyte-like cells; *d*) culture derived from cells selectively multiplying in the presence of insulin. Histograms: black peak: autofluorescence of cells cultured with isotypical control; red peak: fluorescence after staining with antibodies (PE: phycoerythrin). Ordinate: cell number; abscissa: fluorescence intensity.

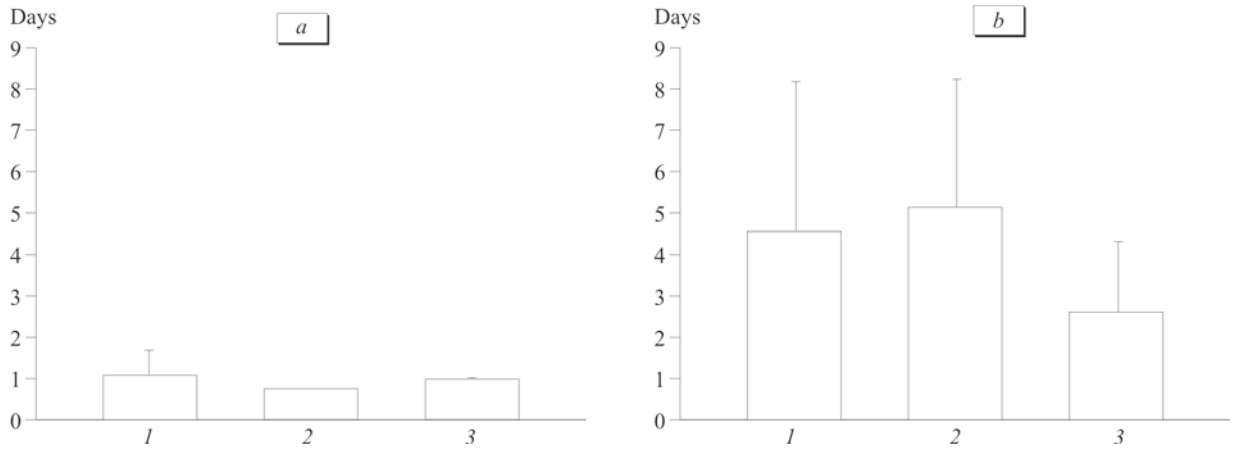

Fig. 3. Effects of insulin and FGF-2 on doubling time of CD146$^+$ (*a*) and CD146$^-$ (*b*) cell populations. The means of 3 measurements are presented. For *a*: *1*) serum-free medium; *2*) medium with FGF-2; *3*) with insulin and FGF-2. For *b*): *1*) serum-free medium; *2*) medium with insulin; *3*) with insulin and FGF-2.

cubated with insulin (inoculation density 50 cell/cm^2). As a result, cell cultures selectively multiplying in the presence of insulin were obtained.

According to published data, endothelial cells expressing CD31 are characterized by short lifespan *in vitro* and are dedifferentiated or eliminated after several divisions. The results of cytofluorometric analysis of the isolated population indicate that the CD146 marker is expressed by 95% cells, while the percent of CD31-positive cells reached 65% (Fig. 2, *d*).

Simultaneous growth of two colony types was observed in the studied cultures. One type was presented by the endothelial morphotype cells, while the other type were smaller cells with short processes (Fig. 4).

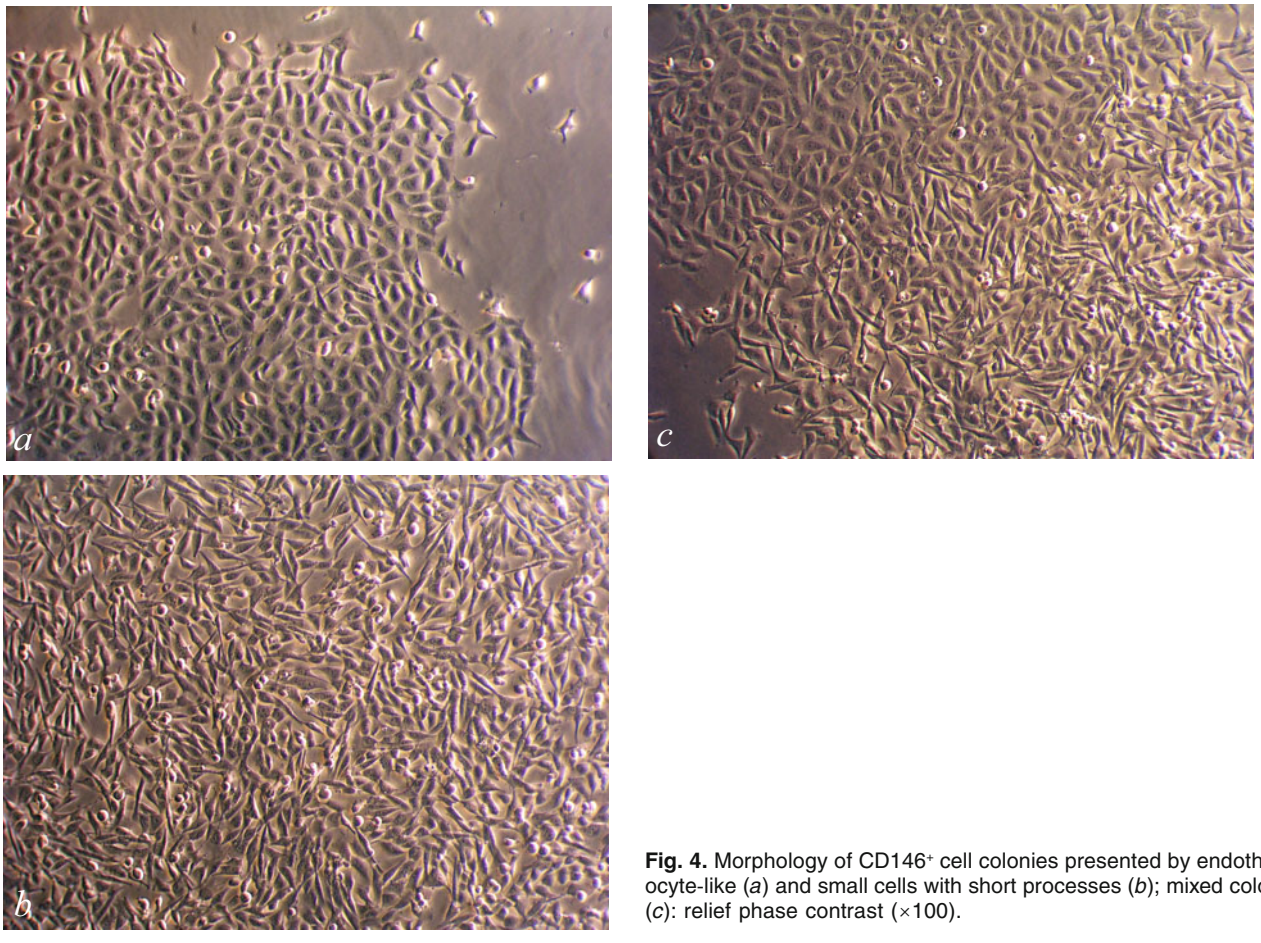

Fig. 4. Morphology of CD146$^+$ cell colonies presented by endotheliocyte-like (*a*) and small cells with short processes (*b*); mixed colony (*c*): relief phase contrast (×100).

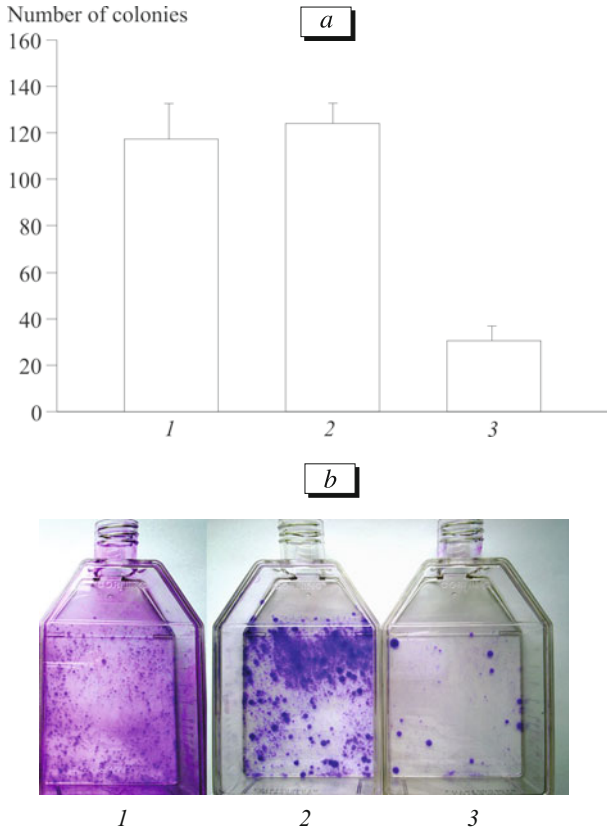

Fig. 5. Effects of insulin and FGF-2 on the number (*a*) and size (*b*) of CD146$^+$ cell colonies. *1*) insulin; *2*) insulin+FGF-2; *3*) FGF-2. *b*) crystal violet staining. The means of 3 measurements are presented.

Inoculation of mitotic globular cells resulted in growth of cultures of the same phenotype during subsequent passages. A total of 18 passages were carried out during the study; the cultures were cryopreserved. The presence of two CD146$^+$ cell populations in the primary suspension of FR SVF was demonstrated previously, but their origin and behavior in culture are unknown [1].

Estimation of the number of colonies of CD146$^+$ cells grown with and without insulin showed that the count of colony-forming cells increased more than 3-fold in the presence of insulin. Hence, the presence of FGF-2 is obligatory for colony growth (Fig. 5). FGF-2 accelerates doubling of CD146$^+$ and CD146$^-$ populations, but CD146$^+$ cells need FGF-2+insulin combination for retaining clonogenic activity and effective growth. The biochemical mechanism of the effect of insulin on cell replication is unclear. Presumably, it is based on the anabolic effect of the hormone. The effects on absorption of glucose, phosphate, type A neutral amino acids, and cations may be essential. The hormone can stimulate or inhibit replication by stimulating or inactivating enzymes through regulation of the rate and degree of protein phosphorylation or regulation of enzyme synthesis. However, the use of insulin as a selective factor for isolation of individual cell populations has never been described.

Hence, we developed a method for isolation and long-term culturing of CD146$^+$ cell populations from AT. The population of CD146$^+$ cells in AT is less than in the bone marrow, umbilical cord, and some other more abundantly vascularized tissues and organs [2, 13]. However, high proliferative activity of these cells, clonogenic activity and stability of their phenotype during culturing according to our protocol make it possible to produce cultures for transplantations in the needed volumes.

The study was supported by the Federal Agency for Science and Innovations of the Russian Federation (State Contract No. 02.512.11.2221 of July 4, 2008) and Russian Foundation for Basic Research (grant No. 09-04-00724-a).

REFERENCES

1. G. Astori, F. Vignati, S. Bardelli, *et al.*, *J. Trans. Med.*, **5**, 55 (2007).
2. D. Baksh, R. Yao, and R. S. Tuan, *Stem Cell*, **25**, No. 6, 1384-1392 (2007).
3. N. Bardin, V. Moal, F. Anfosso, *et al.*, *Thromb. Haemos.*, **90**, No. 5, 915-920 (2003).
4. A. I. Caplan, *J. Pathol.*, **217**, No. 2, 318-324 (2009).
5. M. Cerletti, M. J. Molloy, K. Tomczak, *et al.*, *J. Cell Sci.*, **119**, Pt. 15, 3117-3127 (2006).
6. M. Crisan, S. Yap, L. Casteilla, *et al.*, *Cell Stem Cell*, **3**, No. 3, 301-313 (2008).
7. B. Delorme, J. Ringe, N. Gallay, *et al.*, *Blood*, **111**, No. 5, 2631-2635 (2008).
8. L. S. Ferreira, S. Gerecht, H. F. Shieh, *et al.*, *Circ. Res.*, **101**, No. 3, 286-294 (2007).
9. B. Guezguez, P. Vigneron, N. Lamerant, *et al.*, *J. Immunol.*, **179**, No. 10, 6673-6685 (2007).
10. K. Lin, Y. Matsubara, Y. Masuda, *et al.*, *Cytotherapy*, **10**, No. 4, 417-426 (2008).
11. P. Mancuso, P. Antoniotti, J. Quarna, *et al.*, *J. Clin. Cancer Res.*, **15**, No. 1, 267-273 (2009).
12. J. B. Mitchell, K. McIntosh, S. Zvonic, *et al.*, *Stem Cells*, **24**, No. 2, 376-385 (2006).
13. D. A. Rider, C. Dombrowski, A. A. Sawyer, *et al.*, *Ibid.*, **26**, No. 6, 1598-1608 (2008).
14. B. Sacchetti, A. Funari, S. Michienzi, *et al.*, *Cell*, **131**, No. 2, 324-336 (2007).
15. K. E. Schwab, P. Hutchinson, and C. E. Gargett, *Human Reprod.*, **23**, No. 4, 934-943 (2008).
16. B. M. Seo, M. Miur, S. Gronthos, *et al.*, *Lancet*, **364**, 149-155 (2004).
17. I. M. Shih, T. L. Wang, and W. H. Westra, *Clin. Cancer Res.*, **2**, No. 3, 569-575 (1996).
18. A. Sorrentino, M. Ferracin, G. Castelli, *et al.*, *Exp. Hematol.*, **36**, No. 8, 1035-1046 (2008).
19. C. Tripodo, A. DiBernardo, M. P. Ternullo, *et al.*, *Haematologica*, **94**, No. 1, 127-130 (2009).
20. H. Zhang, V. Vakil, M. Braunstein, *et al.*, *Blood*, **105**, No. 8, 3286-3294 (2005).