Comparative Study of Adult Human Skin Fibroblasts and Umbilical Fibroblast-Like Cells

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Expression of markers, collagens, and HLA-1 by human skin fibroblasts and fibroblast-like cells isolated from the umbilical Wharton's jelly was compared. Skin fibroblasts express collagens (proteins characteristic of differentiated cells of this histogenetic series) and HLA-1, while umbilical cells express, in addition to collagens, juvenile surface markers and almost no HLA-1. This indicates that fibroblast-like cells isolated from different sources are different and can serve as sources for the creation of cell preparations with different characteristics in future.

Key Words: *skin fibroblasts; umbilical fibroblast-like cells; surface markers; collagen synthesis; HLA-1*

The need in biomedical therapeutic technologies based on fundamental and applied data on human stem cells and their derivatives led to adoption in 2002 of a specialized program "New Cell Technologies for Medicine" by the Board of the Russian Academy of Medical Sciences. An important direction of this program is the development of cell technologies for the correction of changes in the skin caused by exposure to unfavorable environmental factors and/or age and developing as a result of disease or surgical intervention.

This paper sums up the results of preclinical studies of two cell types, which can be used in cosmetology: fibroblasts isolated from adult human skin and fibroblast-like cells isolated from newborn umbilical cord. The interest to these cytophenotypes is explained by the special role of fibroblasts in the maintenance of normal structure and function of the skin. Changes in the fibroblastic cells serve are an important component in the processes developing in the skin under the effect of external factors and during aging [5,6]. The decrease in fibroblast number or alteration of their characteristics lead to degradation of collagen and elastic fibers, the "backbone" of the skin, determining its elasticity, width and depth of wrinkles. The decrease in the production of hyaluronic acid decreases the content of humor and determines dryness of the skin, impairs its barrier function, and leads to other negative consequences. Fibroblasts play the leading role in wound healing processes [1,4]. During the proliferative phase they are the main cell population, forming many substances essential for healing (glycosaminoglycanes and collagen). Quantitative insufficiency or functional incompetence of fibroblasts disorders the healing process.

MATERIALS AND METHODS

A skin fragment (2×2 mm or less) collected from the ulnar flexion area was fragmented with scissors and placed for 2 h at 37°C in 0.1% solution of type

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I collagenase in serum-free DMEM. The enzyme was then neutralized by adding 10% serum. The resultant suspension was precipitated by centrifugation. The precipitate was resuspended in DMEM with 10% serum, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, 10 ng/ml basal fibroblast growth factor (β-FGF), transferred into culture dishes, and cultured until confluence, the medium was replaced twice a week. After monolayer formation the cells were reinoculated 1:3.

Human umbilical specimens were collected after normal delivery at weeks 39-40 of gestation. Umbilical veins were cannulated from both ends and washed with Hanks solution and then with 0.1% type I collagenase in serum-free DMEM, after which were incubated for 30 min at 37°C. The vessels were then again washed in Hanks solution, umbilical tissue was subjected to short mild mechanical processing, and separated cells were collected. The resultant cells were precipitated by centrifugation. The precipitate was resuspended in DMEM containing 10% serum, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, 10 ng/ml β-FGF, transferred into culture dishes, and cultured until the formation of a monolayer, the medium was replaced twice a week. When the monolayer formed, the cells were reinoculated 1:3.

For immunocytochemical studies the cells were cultured on slides. After the cells attained the desired growth stage, the slides were washed in phosphate buffer saline (PBS; pH 7.4) and fixed in cold (-20°C) methanol for 2 min. Subsequent operations were carried out at room temperature. Fixed cells were washed in PBS and permeabilized with 1% Triton X-100 in PBS for 10 min. After washing in PBS (3×2 min) the material was exposed for 30 min in buffer A (PBS with 0.5% BSA, 2% normal goat serum, 0.05% Twin 20, 0.01% mertiolate) for blocking nonspecific antibody binding. Specific and fluorescent labeled antispecies antibodies were diluted with buffer A. Incubation with primary antibodies was carried out at ambient temperature for 1.5-2 h, after which the cells were washed 3 times in PBS. Incubation with fluorescent labeled second antibodies was carried out for 45-60 min at ambient temperature and then washed 3 times in PBS. Chemicon primary monoclonal murine antibodies and second antispecies antibodies were used. Cell nuclei were post-stained with DAPI in PBS (1 μg/ml) for 20 min. The preparation was embedded in special medium preventing fluorescence extinguishing and examined in an Axiovert 200 inverted microscope fitted with Axiocam HRm camera (Carl Zeiss).

Blood specimens from mothers of newborn donors of umbilical cord and from skin donors was tested for antibodies to HIV-1, HIV-2, hepatitis B surface antigen, summary antibodies to hepatitis C virus (by enzyme immunoassay — EIA) and for hepatitis C virus RNA, hepatitis B virus DNA, types 1 and 2 herpes simplex viruses DNA, cytomegalovirus DNA, Epstein—Barr virus DNA, ureaplasma DNA, chlamidial DNA, mycoplasma DNA, and toxoplasma DNA (by PCR). Cultures grown *in vitro* were analyzed by PCR for infectious agents. The presence of human immunodeficiency virus or any of hepatitis viruses or syphilis agent in the blood and/or culture and detection of antibodies to HIV, hepatites B or C viruses, or *Treponema pallidum* ruled out operations with the material. Infection of donor blood by any of the above mentioned agents necessitated thorough testing of the resultant cell cultures by PCR. The cells were used in experiments only if the absence of infectious agents was validated. Infection of the initial material and cultures by common microflora was controlled visually by the formation of bacterial colonies during culturing.

Cell viability was evaluated by the colorimetric method based on the ability of live (but not dead) cells to transform soluble 3-(4,5-dimethylthiasol-2-yl)-2,5-tetrasolium bromide (MTT, yellow) into insoluble purple-blue crystals of MTT Formasan (MTT-F) [7]. The intensity of MTT transformation into MTT-F reflects the total level of dehydrogenase activity in the cells and is modulated by the activities of the priority conjugated enzymatic systems, such as the respiratory chain, electron transfer chain, *etc.*

Saturated MTT solution was prepared in Hanks' solution and stored in a dark glass flask at 4°C no longer than for 1 month. The cells were cultured in a 96-well plate in 200 μl growth medium, after which 20 μl saturated MTT solution was added into the wells and the culture was incubated for 4 h at 37°C; supernatant was discarded and 150 μl DMSO was added into each well for dissolving intracellular MTT-F crystals. Optical signal was measured at λ=450 nm (the well containing only 150 μl DMSO served as the zero level). Enzyme activity (cell viability) was evaluated by comparing optical densities in the experiment and control.

After 3-5 *in vitro* passages fibroblasts were removed from the substrate at the stage of active growth (50-60% confluence) by trypsin treatment, washed twice in sterile saline, and suspended in sterile saline in a concentration of 5×10^5 or 5×10^6 cells/ml. Female Wistar rats (n=40; 150-175 g) received 10 subcutaneous injections (0.1 ml) of the

preparation into the lateral surface of the abdomen. Ten rats were injected with human skin fibroblast preparation in a concentration of 5×10^5 cells/ml, 10 received the same preparation in a concentration of 5×10^6 cells/ml. Ten rats were injected with human umbilical fibroblast preparation in a concentration of 5×10^5 cells/ml, and 10 other animals received the same preparation in a concentration of 5×10^6 cells/ml. Animal status and skin status at the site of injections were evaluated visually on days 1, 7, and 14 after injections.

RESULTS

Enzyme treatment of adult human skin samples yielded several morphologically different cell types from the skin, with round cells (presumably keratinocytes) and fibroblast-like cells predominating. After 5-7-day culturing only adherent fibroblast-like cells were left in dishes. During the first stages multiplication of these spindle-like axonal cells was slow and uneven on the dish surface. Culture growth rate sharply increased after the formation of one or several aggregations of adherent cells ("growth islets"). The start of active growth and time of attaining confluence varied for cells originating from different donors; we failed to detect a clear-cut correlation between these parameters and donor age (by the present time we have data on at least 100 donors of both sexes aged from 25 to 79 years). Cell growth could be stimulated by incubation medium conditioning by adding the medium from dishes with actively growing fibroblasts.

Cells of different morphology were isolated from the umbilical cord: along with fibroblast-like cells, there were droplet-like cells, presumably endotheliocytes, and small round hemopoietic precursor cells. Fibroblast-like cells from the umbilical cord morphologically differed from skin fibroblasts. Instead of spindle cells predominating in the skin, more spread cells with almost the same size in the longitudinal and transverse directions predominated here. After passages mainly fibroblast-like cells were left, which, similarly as skin fibroblasts, started actively dividing only after the formation of growth islets. Conditioning of the medium also led to growth stimulation and contraction of the period from the beginning of active growth till attaining the confluent state.

Immunocytochemical study of fibroblast cultures isolated from the skin and umbilical cord was carried out in order to detect specific markers: collagen 1, collagen 2, nestin, vimentin, CD34, CD45, CD133, and von Willebrand factor (Figs. 1-4). Fibroblasts of dermal origin expressed only type 1 collagen (Fig. 1), type 2 collagen (Fig. 2), and vimentin (Fig. 3), all cells were stained with antibodies to vimentin (in contrast to antibodies to collagen). In the umbilical cord collagens were expressed by the majority of cells (Figs. 1, 2), while vimentin was expressed by all cells (Fig. 3). An appreciable part of umbilical cells expressed nestin (Fig. 4, *a*), solitary cells of the first passages expressing von Willebrand factor (endotheliocyte marker; Fig. 4, *b*) and stem and progenitor hemopoietic cell markers CD34 (Fig. 4, *c*), CD 45 (not shown), and CD133 (Fig. 4, *d*).

Immunocytochemical study of fibroblast cultures isolated from the skin and umbilical cord was carried out in order to evaluate the level of HLA-1 expression (Fig. 5, *a*, *b*). The level of HLA-1 expression was high in dermal fibroblasts but negligible in umbilical cells. HLA-1 is a receptor mediating the interactions between cytotoxic lymphocytes and target cell. These data suggest that cells of umbilical origin can induce a lesser number of

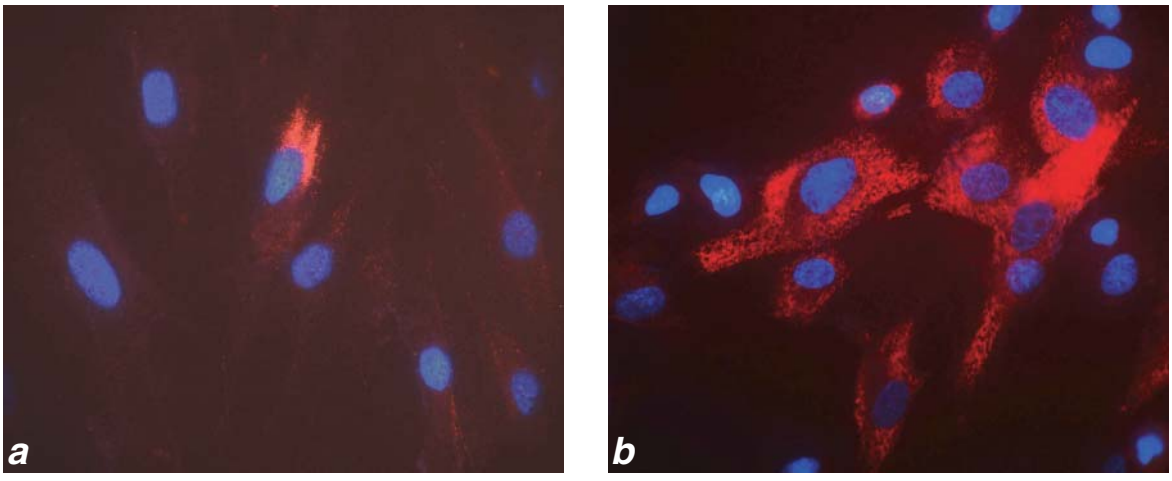

Fig. 1. Expression of collagen-1 in culture of fibroblasts isolated from adult human skin (*a*) or newborn umbilical cord (*b*).

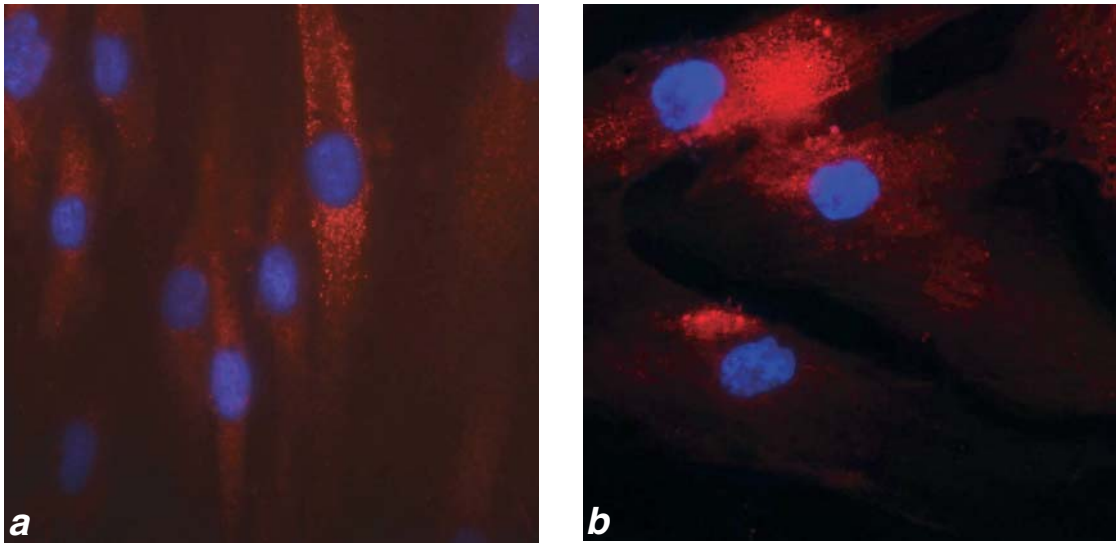

Fig. 2. Expression of collagen-2 in culture of fibroblasts isolated from adult human skin (*a*) or newborn umbilical cord (*b*).

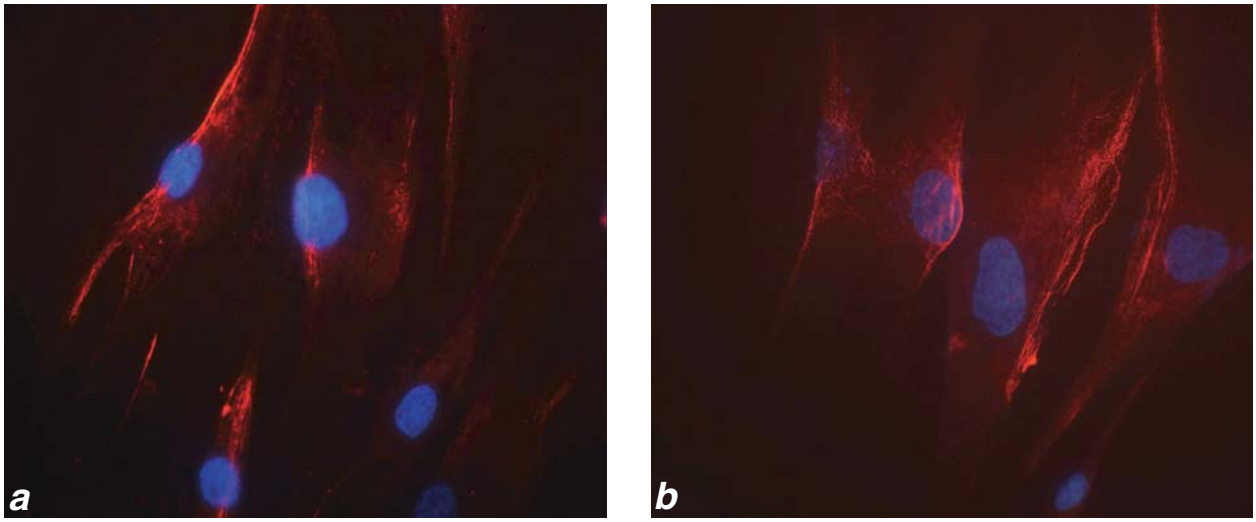

Fig. 3. Expression of vimentin in culture of fibroblasts isolated from adult human skin (*a*) or newborn umbilical cord (*b*).

immune reactions in xeno- or allotransplantation than skin fibroblasts.

After expansion in culture fibroblasts should be washed from fetal cattle serum before using in preclinical or clinical tests, transferred into medium fit for injection to experimental animals (transportation medium — TM), and transport to the place of use. The longest possible survival of cells in TM should be attained.

Cell viability in three TM was studied: saline for intravenous injections, saline for intravenous injections with 2 g/liter glucose, and saline for intravenous injections with 10% human serum; two thermal variants of transportation (at room temperature and at 4°C) were used. Cell viability in TM was evaluated in MTT test in comparison with cells not transferred into TM (Table 1).

The presence of human serum and low temperature appreciably improved fibroblast viability in normal saline. Glucose also had a stabilizing effect on the cells. However, fibroblasts retained sufficiently high viability during the first hours even in common saline for injections.

Acute toxicity of fibroblast preparation injected to laboratory rats was evaluated using saline as TM (in order to rule out evaluation of human serum effects and thus simplify evaluation of the results). The cells remained in TM at 4°C no longer than 6 h.

Study of acute toxicity of fibroblast preparation from adult human skin and umbilical cord injected subcutaneously to rats showed that the animals remained active during all terms of the study. No abnormalities in their status and behavior were observed. Weak inflammatory reaction (edema) was

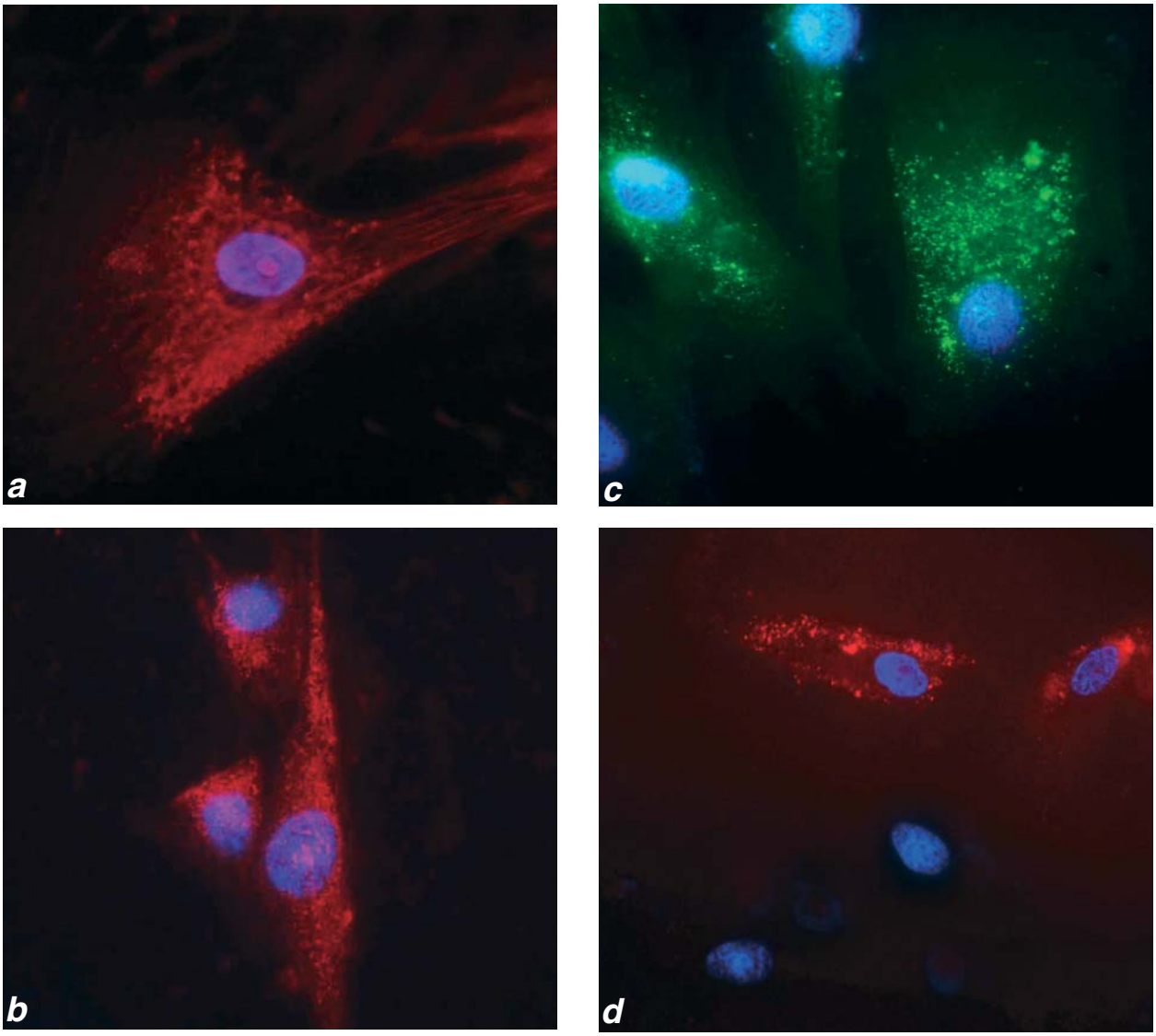

Fig. 4. Expression of nestin (*a*), von Willebrand factor (*b*), CD34 (*c*), and CD133 (*d*) in culture of fibroblasts isolated from newborn umbilical cord.

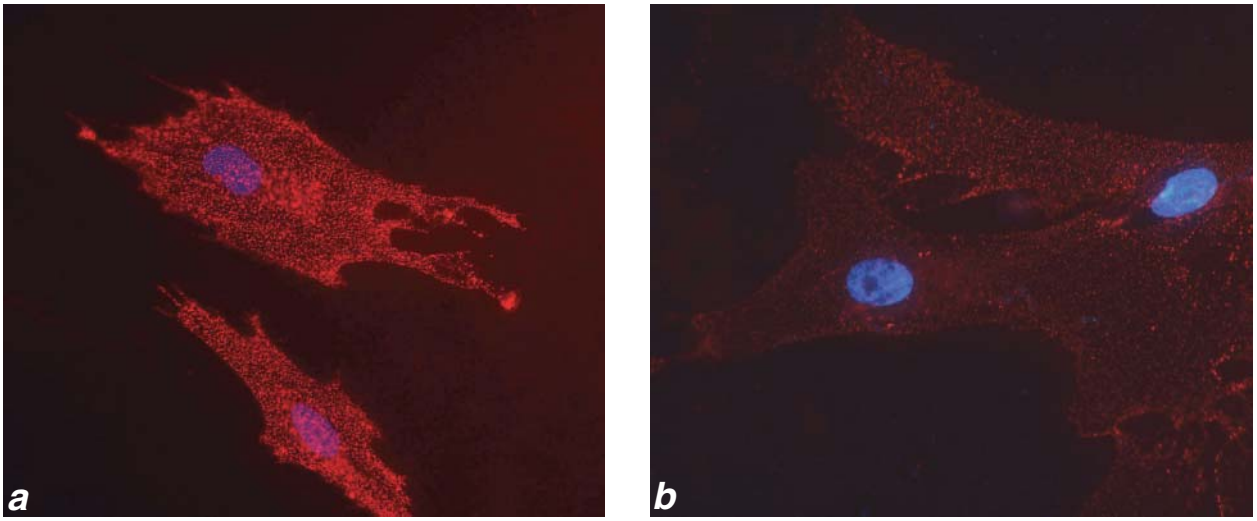

Fig. 5. Expression of HLA-1 by cultured skin (*a*) and umbilical fibroblasts (*b*).

TABLE 1. Viability of Human Umbilical Fibroblasts in Transportation Media of Different Composition on the Cold and at Room Temperature (%)

Transportation conditions		Transportation medium composition		
		saline	saline+2 g/liter glucose	saline+10% serum solution
0 h		100%	100%	100%
6 h	4°C	82±13	92±5	95±3
	20°C	25±8	42±11	73±18
24 h	4°C	14±6	23±9	88±5
	20°C	0	0	34±7
48 h	4°C	0	0	81±9
	20°C	0	0	21±12
72 h	4°C	0	0	73±6
	20°C	0	0	7±5

observed 1 and 7 days postinjection at the site of injection in one rat injected with 5×10^6 skin cells/ml. After 14 days no disorders in the skin status were seen in this rat (at all terms in the rest rats).

A method for isolation and culturing of fibroblasts from human skin and umbilical cord was developed and conditions were selected at which undamaged cells can be transported to the place of use or be stored as ready-for-use preparation for 3 days.

Using this method, it is possible to isolate differentiated fibroblasts from adult human skin and reproduce these cells in culture; a mixture of fibroblasts, including endotheliocyte precursors and, presumably, hemopoietic stem and progenitor cells can be isolated from the umbilical cord. Umbilical fibroblasts (or fibroblast-like cells) express simultaneously types 1 and 2 collagens (proteins charac-teristic of differentiated cells) and nestin (immature cell marker). Fibroblasts from both sources express vimentin. The morphology of cultured skin and umbilical fibroblasts is different. Evaluation of HLA-1 complex expression suggested that umbilical cells are weakly immunogenic. Dermal fibroblasts are more immunogenic, and transplantation is fraught with problems of immune incompatibility.

Testing of the preparation on animals showed that it is nontoxic even in doses *a priori* higher than the doses which can be used in clinical trials, and hence, it can be used in further studies. Some data indicate that the use of cultured fibroblasts in accordance with certain protocols can be effectively used for repair of human skin structure and function [2,3].

These data indicate a variety of properties of fibroblast-like cells isolated from different sources, which should be taken into consideration when planning preclinical and clinical trials of technologies based on the use of these cells.

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