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Effect of Cooling to Low Temperatures on Viability of Human Skin Keratinocytes at Different Stages of Differentiation

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Abstract—The goal of this study was to conduct a comparative analysis of the degree of resistance to low temperatures of human epidermal cells found at different stages of differentiation. The action of liquid-nitrogen vapors was analyzed in experiments in vitro at various temperature regimes on fragments of the human integral skin and on isolated from them and cultivated keratinocytes. The degree of resistance of keratinocytes to the action of cooling to low temperatures was evaluated by their ability to form a multilayer stratum in culture, which indicates the preservation of the viability of the cells treated with cold. This approach allowed one to reveal the diapason of optimal regimes of the action of low temperatures on cells in the composition of tissue and after their conversion into culture. The quantitative ratios of the epidermal stem, transitory, and differentiated cells in a population of viable cells before and after exposure to low temperatures were determined with antibodies that correspond to their different stages of differentiation. The degree of resistance of keratinocytes to action of cooling to low temperatures was evaluated by their ability to form a multilayer stratum in culture, which indicates preservation of viability of the cells treated with cold. The results of this study show that the resistance of human epidermal cells to low temperature differs depending on their stage of differentiation both in situ and in vitro. The epidermal stem and transitory cells are more stable than the differentiated cells.

Key words: Differentiated, stem, and transitory keratinocytes, human epidermal cells, low temperatures.

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Abbreviations used: K-19, K-14, K-10 are keratins 19, 14, and 10, respectively.

INTRODUCTION

Cryogenic methods of the destruction of pathological neoformations are actively applied in clinical practice; they are used in both esthetic medicine and oncologic situations (Granov et al., 2001). Dermatologists notice that, often, after low-temperature action, no scars remain on the skin, and the histological picture of changes in the zone of cryonecrosis has features of histotypical regeneration (Borkhunova, 2004; Rumyantseva et al., 2005). The cryogenic method has been used successfully to treat keloid scars and hemangiomas (Shafranov, 2009). The full-value recovery of skin after cryodestruction distinguishes this method from high-temperature methods of removing skin neoformations, but the mechanisms that underlie this difference do not yet have explanations. It is also the opinion of cosmetologists that the low-temperature treatment of skin provides a rejuvenating effect (Har-shai et al., 2003). However, in reality, it is not currently known how the cells of the epidermis change or the significance of their degree of differentiation.

The human epidermis is composed of five main cell layers, i.e., basal, spine, granular, pellucid (absent in thin skin), and corneal. The epidermis is constantly renewed due to the proliferation of basal layer cells. During the process of differentiation, the translocation of their to the overlying skin layers occurs with changes in their shape, size, and composition of specific proteins, particularly keratins and involucrin. Cells of the basal layer that contain predominantly stem and transitory keratinocytes are characterized by the presence of keratins 19 (K19) and 14 (K14) (Nelson and Sun, 1983), whereas differentiated cells of the spine layer are characterized by the presence of keratins 1 (K1) and 10 (K10) (Rice and Green, 1979).

One of the approaches to detecting the degree of resistance to cold in keratinocytes at different stages of differentiation is the action of liquid-nitrogen vapors on cultivated cells of the epidermis and subsequent observation of the cell's direct reaction to cooling.

The problem of the selective effect of cold on young and differentiated epidermis cells is important, not only for elucidating the cellular mechanisms of the

rejuvenating effect of liquid-nitrogen vapor, but is also of interest for the development of optimal methods of enriching the heterogeneous population with young cells via the selective action of cold on them. The present work deals with the study of the steady resistance to the cooling to low temperatures of the epidermis cells at different stages of differentiation in the tissue and after their conversion to the culture.

MATERIALS AND METHODS

The objects of the study were keratinocytes isolated from the skin fragments obtained in the course of various plastic correcting operations in the areas of the eyelids, faces, and breasts of healthy women 40–50 years old.

Keratinocytes were isolated by the method of Rheinwald (1980) in our modification (Yudintseva et al., 1999). Skin was cut into small fragments measuring 2×3 mm^2 and incubated for 16–20 h in a mixture of 0.5% dispase II (Roche Diagnostics GmbH Mannheim, Germany) and 0.2% collagenase of hydrobionts (Technology Public Corporation, St. Petersburg) at 4°C. Then, the epidermis was separated from the derma on the basement membrane line with forceps and treated with a mixture of 0.125% trypsin, pH 7.6, and 0.02% versen for 10 min at 37°C. The action of the enzyme was inhibited by the addition of 10% embryonic cattle serum. Then, pieces of the epidermis were pipetted to separate keratinocytes from it. The obtained cell suspension was sedimented by centrifugation at 1000 g for 5 min and the formed pellet was resuspended in a mixture of media DMEM and F12 (3 : 1).

Cells were introduced in the amount of $14–15 \times 10^4$ cells/cm^2 to Petri dishes previously covered with type-I collagen isolated from rat-tail tendons (Chandrakasan et al., 1967) and cultivated in a mixture of DMEM and F12 (3 : 1) media with an addition of 10% embryonic serum and mitogens (EGF, stock ×100, cholera toxin) at 37°C in an atmosphere of 5% CO_2.

The following objects were submitted to the action of cooling: intact skin fragments, the epidermis after its separation from the derma, and keratinocytes isolated from the epidermis as a suspension or a sediment after centrifugation. The treatment of the studied objects with cold was performed in two ways, i.e., by the direct action of liquid-nitrogen vapors using Petri dishes with perforated lids and by cooling Petri dishes with nonperforated lids. The material was treated with liquid-nitrogen vapors in the chamber of a programmed freezer (Ice cube 1810, CSYLAB, Austria) with electron block control of the cryoconcervation regime. The rate of cooling of keratinocytes to −10 and −20°C was 5°C/min, while that for cooling to −40°C and lower was 12°C/min.

To reveal optimal temperature regimes, the following low temperatures were tested: −10, −20, −30, −40, −50, −60, and −70°C. The optimal temperatures were considered to be those that suppressed the viability of the heterogeneous keratinocyte population; however, at the same time, it did not lead to their complete death. Since the effect of cold could depend on duration of exposition, we checked the action of cooling for 1, 3, 5, and 10 min.

The viability of the keratinocyte population after the action of low temperatures was estimated by their ability to form multilayer strata and the time needed to achieve this process.

The degree of resistance to cold in human skin epidermal cells present at different degrees of differentiation was determined by the ratio of the number of undifferentiated (young) cells of the basal layer and differentiated (old) cells in the isolated population after the action. The degree of keratinocyte differentiation was determined by the method of indirect immunofluorescence using antibodies to the corresponding keratins that are markers of a certain stage of differentiation.

To perform immunofluorescence analysis, coverslips were treated with collagen I at a concentration of 1 μg/ml overnight at 4°C, after which it was washed three times with PBS, pH 7.4, overlaid with the studied cells (3×10^5 per glass), and were kept for 3 or 24 h in a CO_2-incubator at 37°C. Unattached cells were removed, while the spread cells were fixed with 4% formalin (Sigma, United States), treated with 0.1% Triton X–100, washed with PBS, and added for 30 min the 2% bovine serum albumin to prevent unspecific binding of antibodies. As the primary antibodies, the following monoclonal mouse antibodies to human keratins (Novocastra Laboratories Ltd., Great Britain) were used: K14 – marker of transitory cells (dilution 1 : 50, 1 h), K19 – marker of stem keratinocytes (1 : 100, 30 min), and K10 – marker of differentiated cells (1 : 100, 1 h). As the secondary antibodies, we used anti-mouse rabbit antibodies (rabbit anti-mouse (rabbit anti-mouse IgG FITS-Conjugate, Sigma, United States), dilution 1 : 250, 30 min). Then, the cytoskeleton was stained with rhodamine-phalloidin (Invitrogen, United States) for 30 min to visualize all cells.

The ratio of cells at different stages of differentiation was determined by counting stained cells (100 cells in each of 6 different vision fields) using a fluorescent confocal microscope (Leica Carl Zeiss, Germany). The obtained data were treated using software Image J. Differences were considered statistically significant at the $p < 0.05$ level.

RESULTS

To find out whether the cold produces different effects on keratinocytes at different stages of differentiation, it was first necessary to reveal the optimal cooling regimes in which only some of the studied cell

population would lose viability, whereas the remaining cells would preserve their capability for growth and multiplication in culture. For this purpose, the suspension of keratinocytes isolated from skin fragments at a concentration of 4×10^5 cells/ml were submitted to the action of the following temperatures: –10, –20, –30, –40, –50, –60, and –70°C for 1, 3, 5, and 10 min.

Due to the characteristic peculiarity of the basal layer keratinocytes to form dense aggregates in culture, it turned out to be impossible to precisely calculate viable cells after cooling the obtained suspension. The preliminary dissociation of formed aggregates using enzymes leads to cells losing their ability to multiply and form a monolayer, which indicates their viability. Therefore, the preservation of the viability of cells in aggregates was estimated from the ability and time of formation of the multilayer stratum as a result of further cultivation.

The data presented in Table 1 demonstrate that, at –10 and –20°C, the ability of cells to form multilayer strata did not differ from control at 37°C. In both cases, this formation process required 20–25 days. On the contrary, at –50, –60, and –70°C, the majority of cells died, while the number of remaining viable cells was insufficient to form a monolayer, even for a long time.

The most acceptable cooling at which some cells died while the remaining keratinocytes etained their ability to form multilayer strata turned out to be the cooling of the suspension to –30 and –40°C for 5 min. After this treatment, the formation of the stratum occurred after 30–35 and 40 days, respectively.

Experiments also showed that, under the effect of cold for 1 or 3 min the time of formation of stratum by cells most often did not differ from control, while at the same 10-min action too few cells able to form it remained. Hence, the most optimal regime of action of cold on cells turned out to be the exposition for 5 min at –40°C. In subsequent work this regime of treatment with low temperatures served the starting point at comparison of all other variants of experiment. Despite the death of a significant number of cells, those capable of multiplying and subsequently differentiating, i.e., young keratinocytes, remained predominant.

Figure 1 presents photographs of keratinocytes attached to the substrate during their multiplication and the formation of a monolayer in the control and after exposure to cold. This shows that, in both in the control and after exposure to cold (–20°C), 7 days after seeding on a background of spread cells, numerous colonies of round cells are seen (Fig. 1b). In the case of cooling to –40°C there are observed the greater interaction between cells and arrangement of cells in chains (Fig. 1c). For 7 days the monolayer is not formed, large colonies are absent, and a large number strands of cell chains are formed. After 14 days, during the further cultivation of these cells, the formed network is filled with new cells and large cell clusters are formed, which seems to be the basis for the further formation of a multilayer stratum after 35–40 days (Fig. 1d).

Table 1. Formation of multilayer plate by keratinocytes after 5-min-long cooling in suspension and sediment

Temperature, °C	Time after cooling, days	
	in suspension	in sediment
37 (control)	20–25	20–25
–10	20–25	20–25
–20	20–25	20–25
–30	30–35	20–25
–40	40	30–35
–50	n.f.	30–35
–60	n.f.	40
–70	n.f.	40

Note: For Tables 1 and 2, n.f. means not formed.

Many years of experience worldwide in work with cell cultures shows that single cells are more sensitive to external negative actions than populations of cells that interact with each other. Previously, based on the example of the transformed cell strain HeLa, we showed (Shubin et al, 1999) that the degree of damage of cultivated cells under conditions of low temperatures depends on their degree of interaction with one another. After treatment under similar conditions with liquid-nitrogen vapors, cells present in the suspension died, while a significant part of cells present in pellet after preliminary centrifugation prior to cooling remained viable and formed rapidly the monolayer (Shubin et al., 1999).

Since keratinocytes in the epidermis interact closely with one another, it was necessary to determine the degree to which this interaction affects the result of cooling. In connection with this, the keratinocytes isolated from tissue were sedimented by centrifugation, while the obtained sediment was cooled by liquid-nitrogen vapors in the same regime as cells in suspension. Then, we suspended the cells introduced into Petri dishes and observed their ability to form a multilayer stratum.

The obtained results showed that the previously revealed tendency of cells to have higher resistances in sediment under the action of low temperatures is also characteristic of keratinocytes. These cells formed the stratum for 40 days of cultivation even after cooling at –60 and –70°C for 5 min. After cooling cell sediment to –40 or –50°C, the stratum was formed for 30–35 days. At cooling temperatures of –10, –20 or –30°C, the cell reaction did not differ from that in the control and the stratum was formed in 20–25 days (Table 1). Thus, keratinocytes in sediment turned out

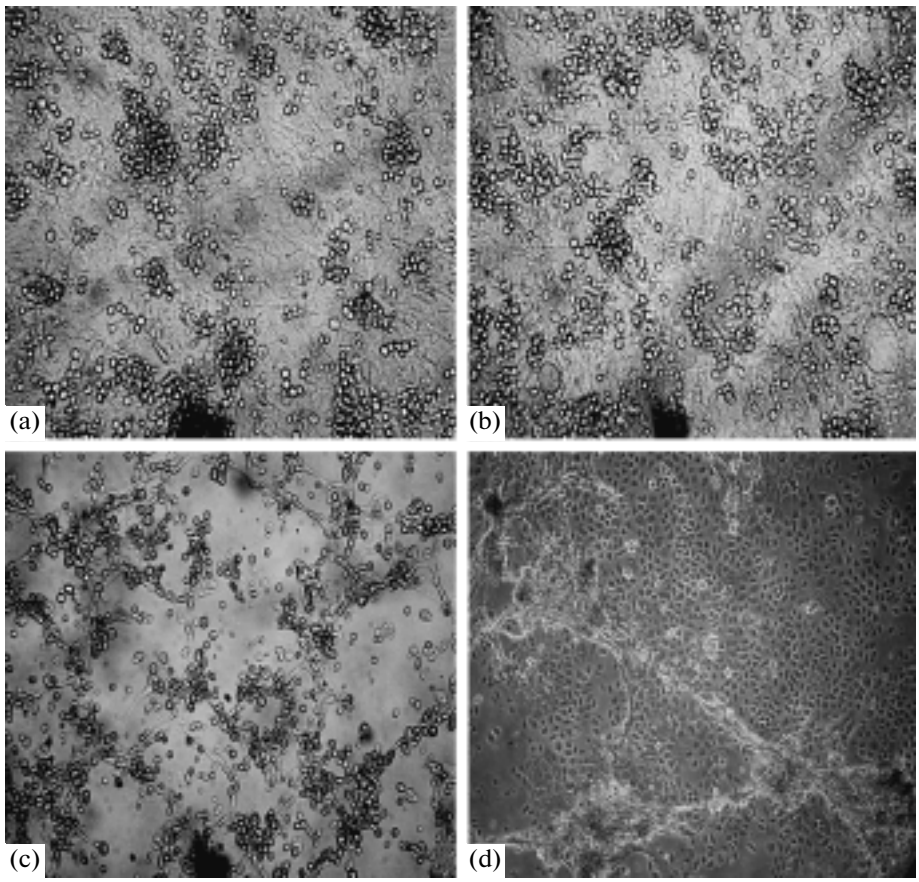

Fig. 1. Process of forming monolayer by keratinocytes for 7 days of cultivation (a–c) after their cooling in the suspension state with liquid-nitrogen vapors. (a) 37°C (control), (b) (–20°C), (c) (–40°C), (d) (–40°C) after 14 days.

to be more resistant to the action of even the lowest temperatures.

To check whether the keratinocytes that interact with each other in tissue have increased resistance to the effects of low temperatures, we cooled the epidermis separated from the derma, as well as the cell suspension isolated from the intact skin fragment. After cooling the epidermis at different temperatures, keratinocytes were isolated from it and their ability to form a multilayer stratum was determined. Contrary to expectations, we did not reveal differences in viability between cells submitted to low temperatures in the epidermis and suspension. As can be seen in Table 2, these and other cells formed a multilayer stratum 20–25 days after cooling at –10 and –20°C for 5 min. The cells exposed to –30°C for 5 min formed a stratum in 30–35 days, whereas, after cooling to temperatures below –40°C (5 min), they were unable to form a stratum, even in 40 days.

Table 2. Effect of 5-min-long cooling on time of formation of multilayer plate by keratinocytes cooled in suspension and in composition of the epidermal layer

Temperature, °C	Time after cooling, days	
	of suspension	of epidermal layer
37 (control)	20–25	20–25
–10	20–25	20–25
–20	20–25	20–25
–30	30–35	30–35
–40	40	40
–50	n.f.	n.f.
–60	n.f.	n.f.
–70	n.f.	n.f.

Hence, the basal layer cells cooled both in the suspension state and in the state of isolated epidermis did not differ by resistance to low temperatures. In connection with this, it was important to determine the effect of cold on the integral skin fragments. In this series of experiments, the isolated bioptates were cooled in two ways, i.e., in normal Petri dishes and in dishes with perforated lids. In the latter case, the bioptates were submitted to the direct action of nitrogen vapors, similar to the action on human skin for medical purposes.

In even the first experiments, we revealed that cells of skin bioptates taken from different skin areas differ in their resistance to the effects of low temperatures. Therefore, we performed a systematic comparative study of the action of low temperatures on the viability of keratinocytes of three types of bioptates, i.e., eyelid, face, and breast. To check the ability of the studied cells to form the multilayer stratum, as in previous cases, we used different temperatures and different exposures; however, in Table 3, we only presented the final results that characterize the optimal cooling regimes established for different studied bioptates.

It turned out that the different resistance of keratinocytes to low temperatures depended both on the localization of the area in the skin tissue and the conditions of the cold treatment of the bioptates. The data in Table 3 show that cells from the eyelid area are less resistant to cold than cells of the face and breasts. Resistance to cold was significantly affected by the method of cooling with the liquid-nitrogen vapors directly on the bioptate or through the nonperforated lid. The eyelid cells treated with cold at temperatures below −30°C (5 min) in the closed dish did not form a multilayer stratum, even after 40 days; it was only formed after exposure to cold at −30°C for 5 min. At the same time, keratinocytes from the bioptate that cooled in the perforated dish could only withstand −20°C for the same time, with the stratum being formed in 30–35 days. After treatment with liquid-nitrogen vapors at −10°C, the stratum was formed in 20–25 days, which differed insignificantly from the control. The face cells in the nonperforated dish withstood −40°C for 5 min, whereas, in the perforated dish, it only withstood 3 min at the same temperature.

Breast skin cells turned out to be the most resistant to these treatment conditions, as they wtithstood the that endured the 10-min cooling to −50°C in the nonperforated dish and the same cold, but only for 5 min in the perforated dish. In both cases, the cells formed a stratum in 40 min. At −60°C for 5 min under the same conditions, a stratum was not formed for 40 days. At −40 and −30°C, 30–35 days were sufficient for the formation of a stratum. As in the control, at −10 and −20°C, the stratum was formed in 20–25 days (Table 3). The lower resistance of keratinocytes from skin bioptates treated in the perforated dish can be explained by the destructive effect of the direct action of liquid nitrogen on the tissue.

The obtained results indicate that, under the effects of cold on cells or tissues in the optimal regime (5 min at −40°C), the cells that remain viable multiply and form a multilayer stratum in almost all experimental variants. This allowed us to believe that differentiated cells predominantly submit to death.

To make sure that this suggestion is true, it was necessary to confirm that the stem and transitory keratinocytes were more resistant to the action of low temperatures. For this purpose, we performed an immunofluorescent analysis of cells labeled with antibodies to

Table 3. Conditions of cooling for formation of plate by keratinocytes from skin bioptate of different localization for 40 days

Parameter	Petri dishes			Perforated Petri dishes		
	Eyelids	Face	Breast	Eyelids	Face	Breast
Temperature, °C	−30	−40	−50	−20	−40	−50
Time, min	5	5	10	5	3	5

Note: Formation of plate with control cells (37°C) did not depend on localization and amounted to 20–25 days. In the case of cooling of eyelids in perforated Petri dishes, the time of plate formation amounted to 30–35 days.

K19 (marker of stem cells), K14 (marker of basal or transitory cells), and K10 (marker of differentiated keratinocytes) after their treatment with cold and a subsequent transition to culture. Based on the results of previous experiments, the whole bioptate was not submitted to cooling for 5 min at −20 and −40°C, only the epidermis after separation from the derma. To reveal all cells that adhered to and spread on substrate, the cytoskeleton was stained with rhodamine-phalloidin.

An immunofluorescent analysis of the heterogeneous keratinocyte population in culture was performed 3 and 24 h after the action of cold. These time intervals were chosen in connection with the data obtained by Spichkina et al. (2006) that cells of different degrees of differentiation in the heterogeneous population of basal keratinocytes differ by the rate of adhesion to the substrate. After 3 h, only some of the cells with higher affinities to the basement membrane proteins (in this case, to collagen) adhere, while, after 24 h, all cells of the population are attached to the substrate.

An analysis of the adhered cells has shown that, in the control (37°C), along with stem cells brightly stained for K19, a significant amount of differentiated keratinocytes is also present. Three hours after exposure to cold (at −20°C), the total amount of the attached cells in preparations decreased and the relative amount of undifferentiated cells rose. A further decrease in temperature to −40°C was accompanied by the marked predominance of undifferentiated cells in preparations. The calculation of the ratio of stem and differentiated cells shows that the number of stem cells accounts for 32% of the total number of cells attached to the substrate for 3 h. After treatment with cold at −20°C, their number increases nearly twofold (up to 63%). After cooling at −40°C, this amount becomes somewhat higher (up to 67%), but these differences are statistically insignificant (Fig. 3a). Thus, despite a decrease in the total amount of resistant cells that rapidly adhered to the substrate, at a decrease in temperature to −40°C, the ratio between the stem and

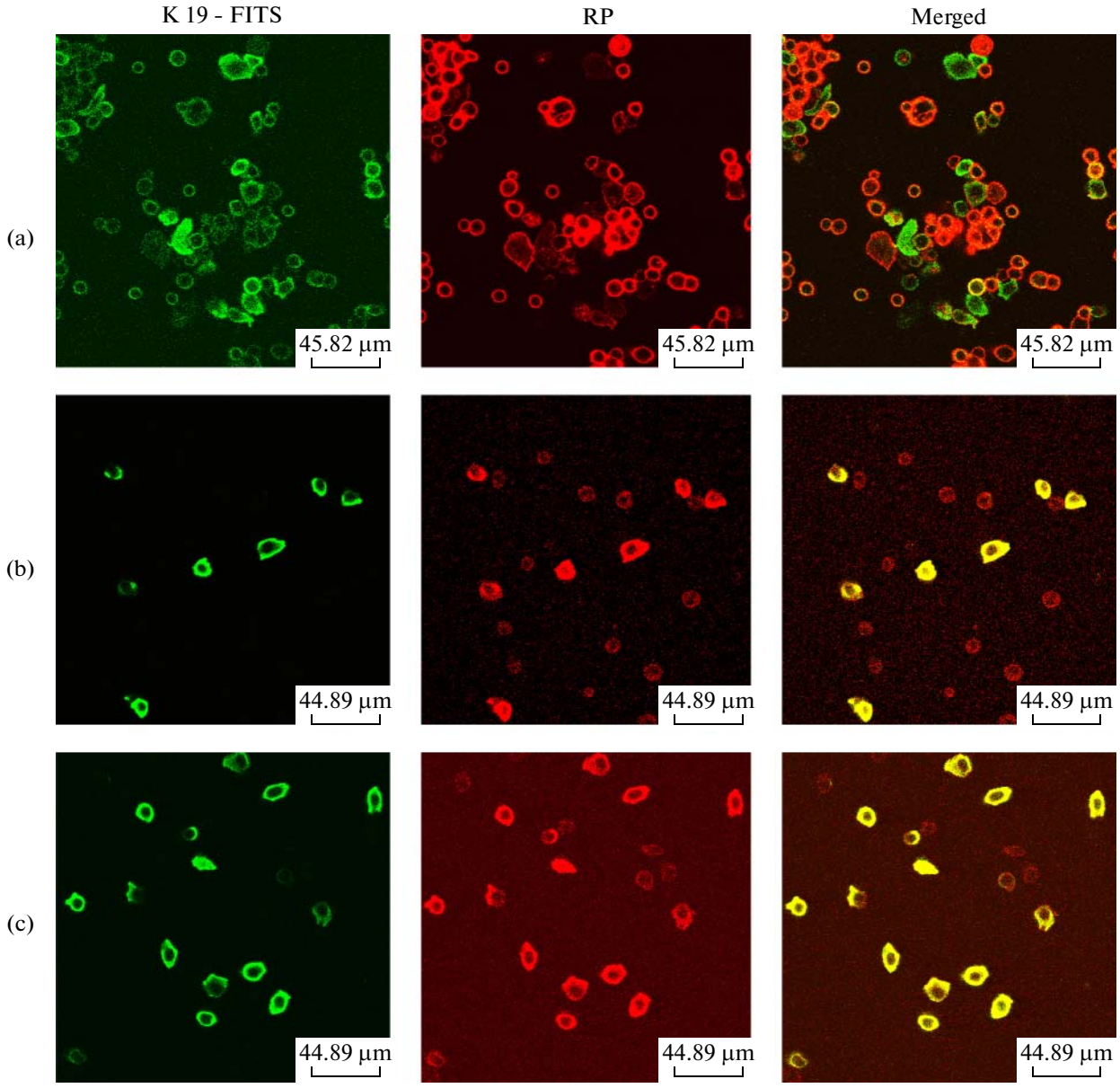

Fig. 2. Change of ratio of stem and differentiated cells in population of keratinocytes attached to substrate 3 h after cooling to different temperatures. (a) 37°C (control), (b) (–20°C), (c) (–40°C). Here and in Fig. 4, cells were labeled with antibodies to keratin 19 (K19-FITC), cytoskeleton was stained with rhodamine-phalloidin (RF) for visualization of all cells.

differentiated keratinocytes remains the same, which is shown in Figs. 2 and 3a.

In analyzing the composition of cells that attached to the substrate 24 h after seedling, as expected, an increase was observed in the total amount of cells in the preparations exposed to cold at the same temperatures compared to their number after 3 h of cultivation. This increase in the number of adhered cells both in control and after action of cold occurred at the expense of stem keratinocytes (Fig. 4).

In control the proportion of stem cells amounted to 42%; after cooling at –20°C it rose to 65% and reached 70% after –40°C. As in the case of the attachment of cells for 3 h after cooling, the greatest change in the cell composition towards a rise in the portion of stem cells in the population by almost twofold occurred at a temperature of –20°C (Fig. 3b).

Our previous studies have revealed that K19 is not the exclusive marker for stem keratinocytes; it is also present in smaller amounts in transitory cells and gradually disappears when keratinocytes transition to the differentiated state. Therefore, we additionally labeled the cells with antibodies for K14 that interact predominantly with transitory cells and, for K10, a characteristic marker of differentiated cells. This labeling was intended to specify the degree to which

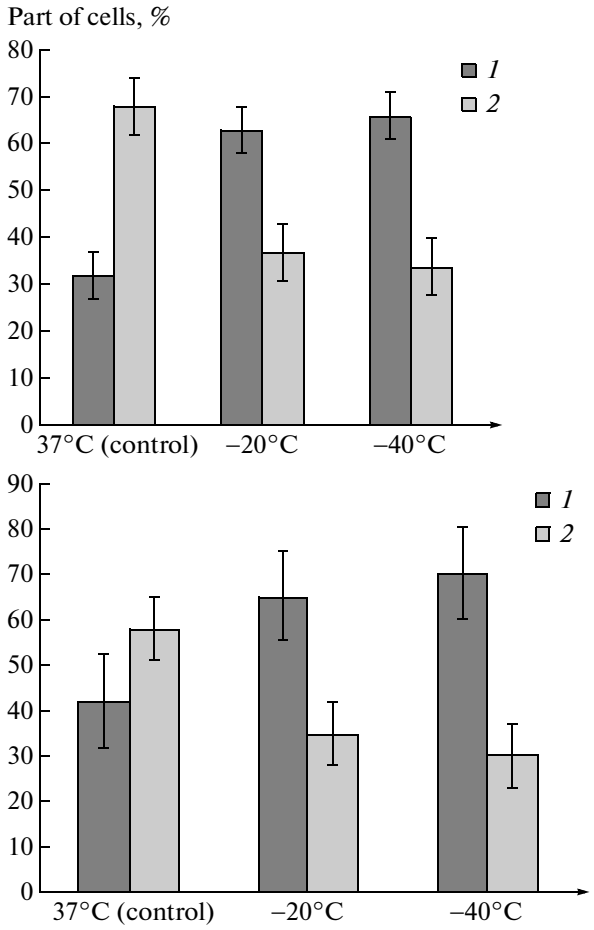

Fig. 3. Histogram of ratio of stem (1) and differentiated (2) cells in population of keratinocytes 3 (a) and 24 (b) h after cooling.

the previous labeling of cooled cells for K19 corresponds to the proportion of stem keratinocytes.

Figure 5a shows that the proportion of transitory cells in the control 3 h after seeding is 47%. After treatment with cold at –20 and –40°C, it rises up to 65 and 75%, respectively. An increase in the time of cell adhesion to 24 h leads to insignificant changes in the number of attached cells. For this time, in the control, the portion of transitory cells barely changes (49%); after cooling at –20°C, it still amounts to 65%, while at –40°C, it decreases to 68% (Fig. 5b), which can be the result of the beginning of the process of differentiation. Thus, the results show that, in the population of remaining viable cells, after cooling at a low temperature, young (stem and transitory) keratinocytes are predominant.

DISCUSSION

The performed study has shown the selective damaging action of cold on various cells differing by the degree of differentiation. In all variants of experiments at optimal regimes of treatment with liquid-nitrogen vapors of cells in suspension, in sediment, in composition of epidermis or the entire skin bioptate, in population of the remaining viable keratinocytes invariably predominant are stem and transient cells. It has been established that the optimal regime that preserves a sufficient amount of viable cells capable of forming a multilayer stratum in the culture for a suspension of keratinocytes from the whole bioptate and keratinocytes of the epidermis basal layer (after its separation from derma) is the 5-min cooling at –40°C.

Keratinocytes in sediment after the centrifugation of the suspension withstand cooling up to –70°C for 5 min. It should also be noted that the high resistance of young keratinocytes to the action of low temperatures is determined by their properties rather than by their spatial disposition in tissue, since their predominance in a population of viable cells is retained after both the suspension and the entire epidermis are cooled at approximately the same level. The significantly higher resistance to the action of cold in the cells in the composition of the whole skin bioptates is remarkable, as are the variations in this resistance depending on the body area from which this bioptate was obtained. However, keratinocytes of the epidermis basal layer (after separation of bioptate into epidermis and derma) isolated from skin of different localizations respond identically to the effects of cold of the same temperature.

The cause of this phenomenon is still unclear in the course of subsequent studies; however, it is quite probable that the revealed differences are due to changes in the thickness of skin in different areas of the body. To reach the same temperature in tissue fragments of different volumes, different times are required in the same regime of cooling. The substantiation of this suggestion is confirmed by the data of the destructive cold action at direct contact between bioptates and liquid-nitrogen vapors.

In the process of cultivation of keratinocytes submitted to cooling, in the formed multilayer stratum the amount of cell aggregates increases, while the number of cells spread on substrate decreases. Since the process of aggregation is characteristic of the functioning of stem cells, this might serve as an additional confirmation of the selective preservation of stem and transitory cells after cooling.

In the evaluation of the time of the formation of a multilayer stratum of keratinocytes submitted to cooling at different temperatures, an increase in time upon a consecutive decrease in the acting temperatures correlated directly with the number of dead cells in the population. Meanwhile, the results of the immunofluorescent analysis of the ratio of young and differentiated cells in the population indicate that the greatest changes in favor of undifferentiated cells occur already after cooling to –20°C, whereas a further decrease in temperature to –40°C does not significantly change the composition of the population. A comparison of

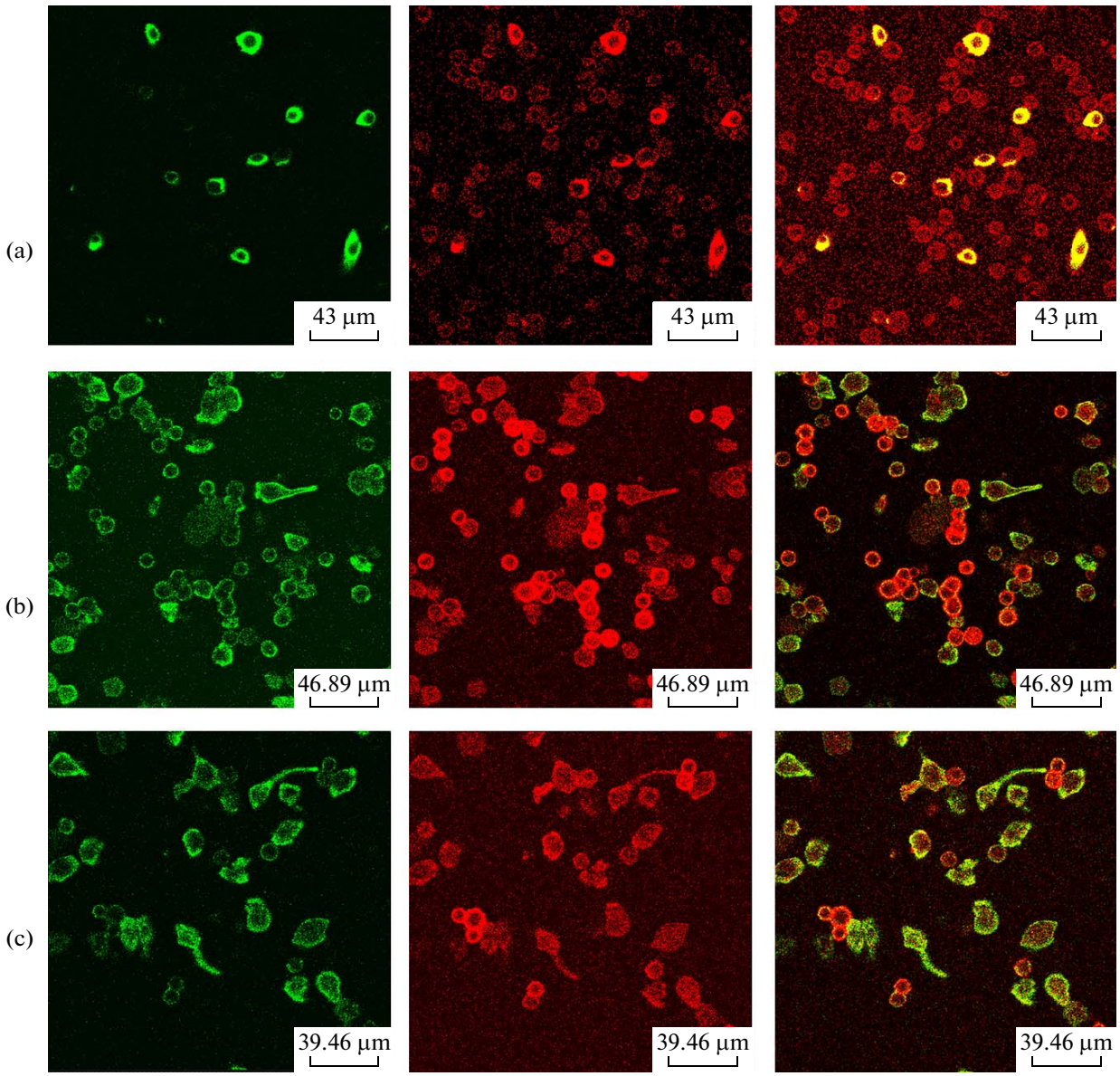

Fig. 4. Change in ratio of stem and differentiated keratinocytes stained 24 after staining. (a) 37°C (control), (b) (–20°C), (c) (–40°C).

these data leads to the conclusion that a twofold increase in the time of formation of a multilayer stratum as a result of cooling to –40°C seems to be connected with the change of the physiological state of the stem and transitory keratinocytes themselves, rather than with the additional death of differentiated cells. The particular molecular mechanisms that provide the resistance of undifferentiated cells to the action of low temperature remains unclear.

Hence, for the selective choice of young (undifferentiated) cells, it is sufficient to cool the heterogeneous cell population to the temperature of –20°C. One day after cooling, these cells accumulate in the population approximately twofold more than differentiated cells.

It should be noted that, apart from terms of the formation of a multilayer stratum after skin bioptates or keratinocyte suspension are cooled, changes were also observed in the morphology of the newly formed stratum in which, compared with the control, keratinocytes were observed to have a greater inclination to aggregate, which is characteristic of stem cells. These aggregates were not in a disrupted state; rather, they were connected to one another by stretched cells and, as a result, formed a branched network on the surface of dish.

The performed study has again confirmed our previous observations, which indicate that finding K19 and K14 in cells using antibodies, in spite of the commonly accepted opinion, is not the basis for ascribing

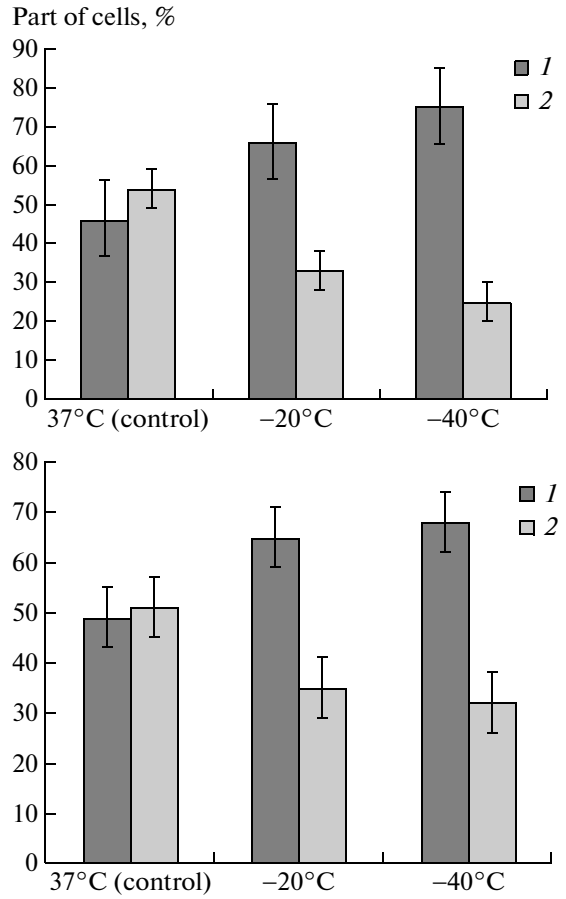

Fig. 5. Histogram of ratio of transitory (*1*) and differentiated (*2*) keratinocytes 3 (a) and 24 (b) h after cooling.

them exclusively to stem or transitory keratinocytes. In the analysis of the preparations presented in Figs. 2 and 4, it is clear that the intensity of staining in cells labeled with antibodies for K19 ranges from bright to barely visible. Cooling leads to the almost complete disappearance of weakly stained population cells, while the brightly stained cells remain. Most likely, this means that weakly stained cells are keratinocytes that already enter the pathway of differentiation. On the other hand, a comparison of the quantitative ratio of the stem, transitory, and differentiated cells labeled with corresponding antibodies shows that data of calculations for stem and transitory cells overlap. This means that this type of cell corresponds to the maximal staining, which does not disappear completely in the cells' transition to the next maturation stages, but decreases gradually until the complete disappearance. For this work, this circumstance was not of decisive significance, since young cells clearly differed from differentiated keratinocytes.

In summary, it should be emphasized that the obtained results can serve as grounds for the further analysis of the mechanisms of the effects of cooling to lower temperatures on stem and differentiated cells. Furthermore, the obtained results can also be used to select cells under the effects of cold on other heterogeneous populations in the composition of tissue or in culture.

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