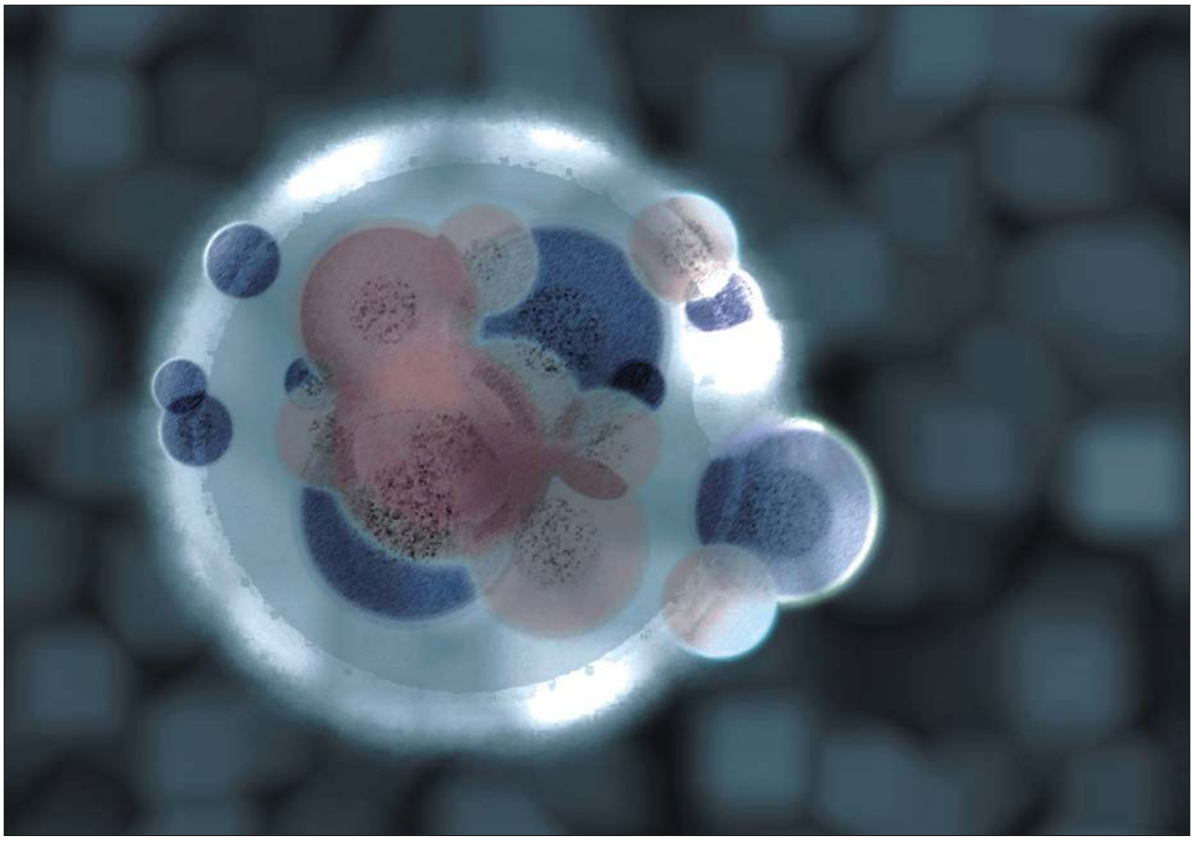

Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Biological Sciences 9 (4): 292-301, 2009
ISSN 1727-3048

Analysis of Primary Human Keratinocytes using Polyclonal Antibodies

O. Spichkina and Yu. Petrov
Institute of Cytology, Russian Academy of Sciences, Sankt-Petersburg, Russia

Abstract: In the present research, the surface properties of keratinocytes have been studied for detection of stem cells subpopulation in epidermis. To reveal all surface properties of the cells the polyclonal antibodies against antigens of their surface have been used. The cell images were analyzed using Image J v.1.41. Area and average staining intensity of the every marked cell were counted. At least 500-1000 cells were analyzed per each sample. Analysis of the results showed that no independent cluster (subpopulation) of the cells among basal keratinocytes *in vitro* was present. However, it was revealed that at transition from basal keratinocytes to differentiating ones all antigenic determinants on a cell surface remain permanent. It is plausible that there is a mechanism of equivalent replacement (exposure-burying) of the antigens of one type by other antigens. Results of this study show that the presence in epidermis of stem cells as autonomous subpopulation is not verified experimentally. Most possibly, the entry of the cells into differentiation is a stochastic process in which all basal keratinocytes participate.

Key words: Cell population, stem cells, differentiation, cell surface, receptors

INTRODUCTION

Special attention of biology and medicine is given now practical use and studying of stem cells (Verfaillie *et al*., 2002; Raikwar *et al*., 2006; Jackson *et al*., 2007; Rowiński, 2007; Deb and Sarda, 2008). It is supposed that renewal of tissues occurs at the expense of a pool of their own stem cells. In some cases, however, convincing evidences of both existence and participation of stem cells in regenerating tissues are absent. In particular, this relate to epidermis cells (Aberdam, 2004; Morasso and Tomic-Canic, 2005; Kaur, 2006). Human keratinocytes could be used for solving of this problem as they have some features facilitating their study. Basal keratinocytes are known to be the precursors for differentiating keratinocytes of the superficial layers of epidermis (Eckert and Rorke, 1989; Lippens *et al*., 2005). Keratinocytes imitate processes of skin morphogenesis when being plated (Watt, 1991; Poumay and Coquette, 2007). Cells laying directly on the substrate can be considered as the basal keratinocytes laying on the basement membrane *in vivo*, where as the cells growing above can be considered as the keratinocytes entering differentiation (the differentiating keratinocytes). The cells of these two types can be easily separated from each other *in vitro* (Jensen and Bolund, 1998).

To reveal skin stem cells the marker proteins such as K19 (Michel *et al*., 1996), ki67 (Gerdes *et al*., 1983), p63 (Pellegrini *et al*., 2001; McKeon, 2004) are used. However, the markers cannot be considered as strictly specific. The aim of the present research was to study the general pattern of a cell surface receptors during a differentiation of keratinocytes and to reveal skin stem cells using the pattern of keratinocyte growth *in vitro*. With this aim we had received polyclonal antibodies against surface antigens of both basal and differentiated keratinocytes. It is obvious that partition of cultured keratinocytes into basal and differentiating cells should increase a percentage of stem cells in fraction of basal keratinocytes. It could be assumed that stem cells having specific antigens on the surface will be marked by polyclonal antibodies.

MATERIALS AND METHODS

This study was performed from 2007 to 2008.

Keratinocyte isolation and cultivation: Primary keratinocyte cultures were obtained from healthy donors according to the method of Rheinwald (1980) modified by Yudintseva *et al.* (1999). Fragments of facial skin from cosmetic surgery were used as the source of epidermal cells. Pieces were washed with Ca^{2+}, Mg^{2+}-free PBS before subcutaneous tissue was removed. Skin was cut in small fragments of 5-10 mm and incubated in PBS with 0.5% dispase II (Roche Diagnostics GmbH Mannheim, Germany) and 0.2% crab collagenase (Biolot, Russia) at 4°C overnight. The epidermis was then mechanically

Corresponding Author: Yury Petrov, Department of Cell Cultures, Institute of Cytology, Russian Academy of Sciences, 194064, Tikhoretsky av.4, Sankt-Petersburg, Russia Tel: +(812) 247-42-96 Fax: +(812) 247-03-41

separated from derma along the basal membrane line. The epidermis was placed in 0.125% trypsin/0.02% EDTA (Biolot, Russia) for 10 min at 37°C. The enzyme was inhibited by addition of 5% fetal calf serum (HyClone, France). The samples were intensively pipetted and the cell suspension was filtered through nylon mesh. Cell suspension was centrifuged at 1000 rpm for 5 min. Supernatant was removed and the pellet was resuspended in DMEM/F12 mixture (3 : 1) (ICN, France).

For cultivating the cells were resuspended in DMEM/F12 mixture (3 : 1) supplemented with 10% fetal calf serum and plated at the densities of 5×10^6 cells per 5 cm desh. After one day, the medium was replaced by fresh supplemented medium the 10% fetal calf serum 5 μg mL^{-1} insulin (Sigma, France), 5 μg mL^{-1} hydrocortisone (Sigma, France), 10 ng mL^{-1} epidermal growth factor (Sigma, France), 10^{-10} M choleric toxin (ICNFLOW, USA), 5 mg mL^{-1} transferrin (Sigma, France), 1.8×10^{-4} M adenine (Sigma, France) and 2×10^{-11} M lyothyronine (Koch-Light, UK).

Type I collagen was used as substrate for the cultivation. Type I collagen isolated from rat-tail tendon was kindly provided by Dr. L.V. Kuchareva (Institute of Cytology, RAS, St. Petersburg). The bottom of culture dishes were covered with 10 μg mL^{-1} protein and the dishes were incubated at 4°C overnight. Before cell plating, dishes were washed 3 times with PBS. Cells were cultivated at 37°C in 5% CO_2 for 10-14 days until multilayer was formed.

The day before an experiment, the culture medium was exchanged with FAD medium (Biolot, Russia) with low calcium content (0.1 mM) for destratification, i.e., to strip and remove the upper layer of differentiated cells (Jensen and Bolund, 1998). Differentiated cells of supra basal layers were removed by washing with a medium. Basal cells were then washed with 0.02% EDTA and detached from the substrate with 0.125% trypsin and 0.02% EDTA (1 : 1) at 37°C for 2-3 min. After the cells rounded and started to detach from the substrate. The culture medium was added to inhibit trypsin. Suspension was pipetted, transferred into a tube and centrifuged at 1000 rpm for 5 min. Supernatant was removed and the cell pellet was suspended in PBS and centrifuged again. The cell pellets were used to receive plasma membrane specimens of keratinocytes according to method of Haeffner *et al.* (1980). Immunoserums were received against the plasma membrane specimens of basal keratinocytes and supra basal differentiated keratinocytes.

Receiving and absorption of immunosera: Preparation of keratinocyte plasma membrane specimens according to method of Haeffner *et al.* (1980), immunization and reception of specific immunosera were took in laboratory of tumor growth cytology (Institute of Cytology, RAS, St. Petersburg). The antibodies to membrane proteins of basal and differentiating keratinocytes were obtained as described below.

Keratinocyte plasma membrane fractions were obtained by the method of Haeffner *et al.* (1980). The antibodies to membrane proteins of basal and differentiating keratinocytes were obtained as described as follows. The cells were swollen in 12 volumes of 2 mM EDTA, pH 7.0, for 5 min at 5°C and centrifuged at 4000 rpm for 15 min. The packed swollen cells were resuspended up in 6 volumes of 18 mM Tris-HCl buffer, pH 8.0, containing 25 mM NaCl and 0.5 mM $CaCl_2$ and homogenized with 12 up and down strokes in a Daunce homogenizer. The homogenate was centrifuged successively at 2000 rpm for 2 min, at 2500 rpm for 2 min and finally, at 12000 rpm for 15 min. The supernatant fraction was used to immunize rabbits in order to obtain antibodies against membrane proteins of basal and differentiating keratinocytes.

Cross-absorption of the obtained immunosera was performed. To this effect differentiating cell fraction (in volume ratio 2:1) was added to immunoserum contained antibodies to membrane proteins of basal keratinocytes and incubated at 37°C for 30 min and then 4°C overnight. Then cell suspension was centrifuged at 1000 rpm for 5 min and next the differentiating cell fraction was added to immunoserum. Similar immunoserum contained antibodies to membrane proteins vs. differentiating keratinocytes was absorbed by basal keratinocytes.

Interaction of antibodies with keratinocytes Immunofluorescence analysis: To study interactions of antibodies with the cells primary keratinocytes attached to substrate for 10 min were used. The selected cells were incubated 1 h for strong attachment. Fibronectin was utilized as a substrate. It was obtained from human blood plasma (Ruoslahti *et al.*, 1982) and was kindly provided by Dr. I.V. Voronkina (Institute of Cytology, RAS, St. Petersburg). The cover slips were covered with 100 μL fibronectin at concentration of 10 μg mL^{-1} and incubated at 4°C overnight. The cover slips were washed with 3 times PBS, treated with 2% bovine serum albumin for 1 h at 37°C to prevent unspecific binding of the cells with substrate and washed again. Cells in the culture medium were plated on treated cover slips (Biovitrum, Russia).

The cover slips were preliminarily cleaned by incubation in concentrated nitric acid for 30 min and washed by distilled water (acid removal was controlled with test paper). Cleaned glasses washed in 96% ethanol

for 1 min were dried in a thermostat at 37°C. The dried cover slips were placed in Repall-Silan solution (Pharmacia, Sweden) for 20 min to make glasses hydrophobic. Then cover slips were repeatedly washed with distilled water, put in 96% ethanol for 30 sec and dried for 24 h at 20°C (Are *et al*., 2001).

Antigens content on which immunoserums were received was evaluated using indirect immunofluorescence. Actin was revealed by staining with rhodamine-phalloidine. One hundred microliter of cell suspension (100 cells μL^{-1}) were plated on cover slips with immobilized ligands and incubated 1 h at 37°C in 5% CO_2. Non attached cells were washed out with warm PBS. Cells were fixed with 4% formaldehyde (Sigma, USA) for 10 min at 20°C. To perforate the cell membrane, cells were incubated with 0.1% Triton X-100 for 20 min and carefully washed.

Cells were incubated with immunoserums contained rabbit polyclonal antibodies (dilution 1 : 300) for 1 h at 20°C and then washed 3 times with PBS. To visualize the primary antibodies the washed cells were incubated with second antibodies, goat polyclonal antibodies to rabbit IgG, conjugated with FITC (Sigma, USA, dilution 1 : 60) for 30 min and washed with PBS. Then, to reveal actin cytoskeleton rhodamine-phalloidine (Molecular Probes, USA), 10 units mL^{-1} was added for 15 min and washed with PBS. The samples were mounted in mounting medium (Vector Laboratories Inc., USA) and examined under confocal microscope Lieca TCS SL (Germany).

Green fluorescence (FITC) was excited by an argon laser (488 nm), while red fluorescence (rhodamine) was excited by an He-Ne laser (543 nm). FITC fluorescence (500-530 nm) and rhodamine-phalloidine (580-630 nm) were scanned separately by Leica Confocal Software. Objective 63x was utilized. Image size was 1024×1024 pixels.

Image analysis: The cell images were analyzed using ImageJ v.1.41 (Wayne Rasband, National Institute of Health, Maryland, USA, http//rsb.info.nih.govij). Each cell was outlined manually. The program automatically counted area and average staining intensity of the every marked cell. Area was measured in pixels without transforming them into a standard metrical unit, this did not prevent comparison of relative values. The average staining intensity per cell was measured in arbitrary units within 0-255. At least 500-1000 cells were analyzed per each sample.

RESULTS AND DISCUSSION

Overview of the kerttinocytes used in present study is shown in Fig. 1. The same population of primary keratinocytes was divided into two parts. One part was stained with antibodies to basal keratinocytes surface antigens (1b), another one was stained with antibodies to cell surface antigens of differentiating cells (d). Simultaneously, the cells were also stained for actin (Fig. 1a, c) to demonstrate identity of both cellular samples. Noteworthily all cells irrespective of their size have a round shape. Examples of the cells defined as the large, medium and small ones are presented in Fig. 1. They are marked L, M and S, respectively.

As shows the actin staining, all cells have approximately the same low intensity of fluorescence independent of their size (Fig. 2a, c). It is not surprising as all the cells were yet not spread, only a very low level of F-actin staining can be revealed. It is clear that the majority of the cells has a small size. Staining with antibodies against surface antigens revealed, however, qualitative differences between these cells. Large cells were practically not stained with antibodies against basal keratinocytes surface antigens but are intensively stained with antibodies against antigens of the differentiating keratinocytes. Most of the small cells were stained with antibodies to either antigens. Despite a good visualization, Fig. 2 does not give an authentic view about the ratio of different cell types. Histograms in Fig. 3a and b show that the number of large and small cells in both groups is approximately equal as 55-60% of the cells are small cells with the average size of about 2500 pixels and with a standard deviation of about 680 pixels, an other part includes large cells (40-45%) with the average size of about 3500 pixels and with a standard deviation of about 250 pixels.

Both groups of the cells have practically identical and low fluorescence of actin staining (Fig. 3c, d). Also both groups contain about 25% of the cells with 20-25% higher fluorescence intensity than the average.

Staining with antibodies against basal keratinocytes antigens reveals that the cell population under study includes the cells with a very low affinity to these antibodies (Fig. 3e). The average fluorescence of these cells is about 13 units and their total amount comprises 39%. About 42% of the population having greater dispersion were stained more intensively. Their average fluorescence was about 65 unit. In addition, an intermediate group (19%) with moderately low fluorescence (about 25 units) can be revealed in this case.

Two cell subpopulations can be revealed by staining with antibodies to the differentiating keratinocytes (Fig. 3f). Some cells (37%) have moderate intensity of staining (the average is 51 units) and a low dispersion (the standard deviation is 16 units), the others (63%) are stained very intensively (the average is 83 units), but this

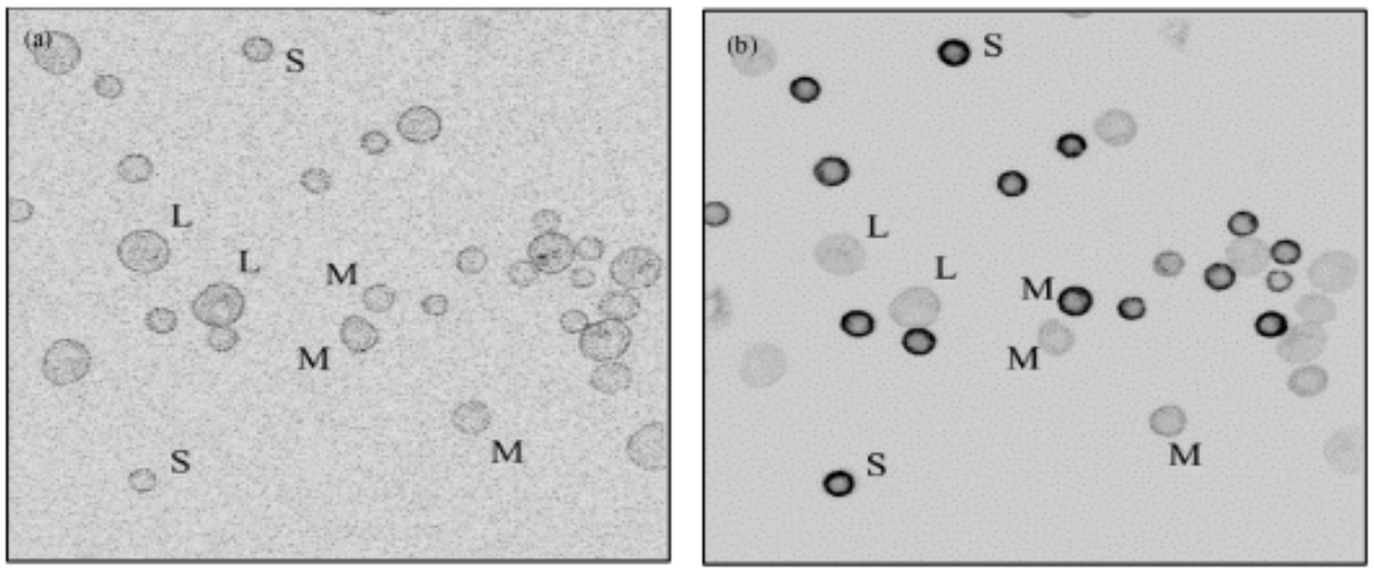

Fig. 1: Confocal microscopy of keratinocytes from a primary culture (the original images are inverted), 10 min after plating. (a and c): Staining with anti-actin antibodies; (b): Staining with antibodies against surface antigens of basal keratinocytes; (d): Staining with antibodies against differentiating keratinocytes surface antigens. L: Large; S: Small; M: Medium cells

subpopulation is more dispersed (the standard deviation is 52 units). In this case no clear intermediate group was revealed.

The data presented in Fig. 1 and 2 allow us to assume that the extent of intensity of the staining with antibodies is associated to the cell size. To verify this suggestion we have arbitrarily divided the cell population into three groups containing small, large and medium cells. Examples of such cells are presented in Fig. 1 and their area averages are shown in Fig. 4 both for the non-selected groups and for the selected groups. It is clear that the average size of the cells in both groups is the same (negligible differences are due to technical errors). Differences in the area of the cells between groups is statistically reliable, no differences within the groups were practically observed. This means that selection of the cells for area was performed correctly, the groups are unimodal, therefore they can be compared by other parameters.

The average staining densities for the antibodies of both types have been calculated for each cells group. Analyzing staining of different cell subgroups for actin, one can see that differences is not revealed in any case (Fig. 5a). However, the clear correlation between the staining intensity and the cell size is shown in Fig. 5b. While, the small cells are stained with both antibodies with identical intensity, the large cells prefer to be stained with antibodies to differentiating cells. The medium cells fill the place of an intermediate position.

Analyzing the data in Fig. 5, we can assume that the summarized staining with both antibodies does not depend on cell size and it is a constant. It can be seen that such procedure gives approximately the same value in each case (slightly exceeding 100 units).

The aim of present study was to reveal regular changes in the pattern of cell surface receptors during keratinocytes differentiation. With this aim we have directed polyclonal antibodies against surface receptors of undifferentiated and differentiating human skin keratinocytes isolated from the basal and supra basal layers of primary keratinocytes culture. These antibodies were exploited to visualize and quantitate the ratio of various surface receptors in primary human skin keratinocytes subcultures separated according to their adhesion properties.

Present results showed that the cluster of small cells is well detected upon staining with antibodies to basal keratinocytes (Fig. 2b). The result is in agreement with the published data that the undifferentiating skin cells are

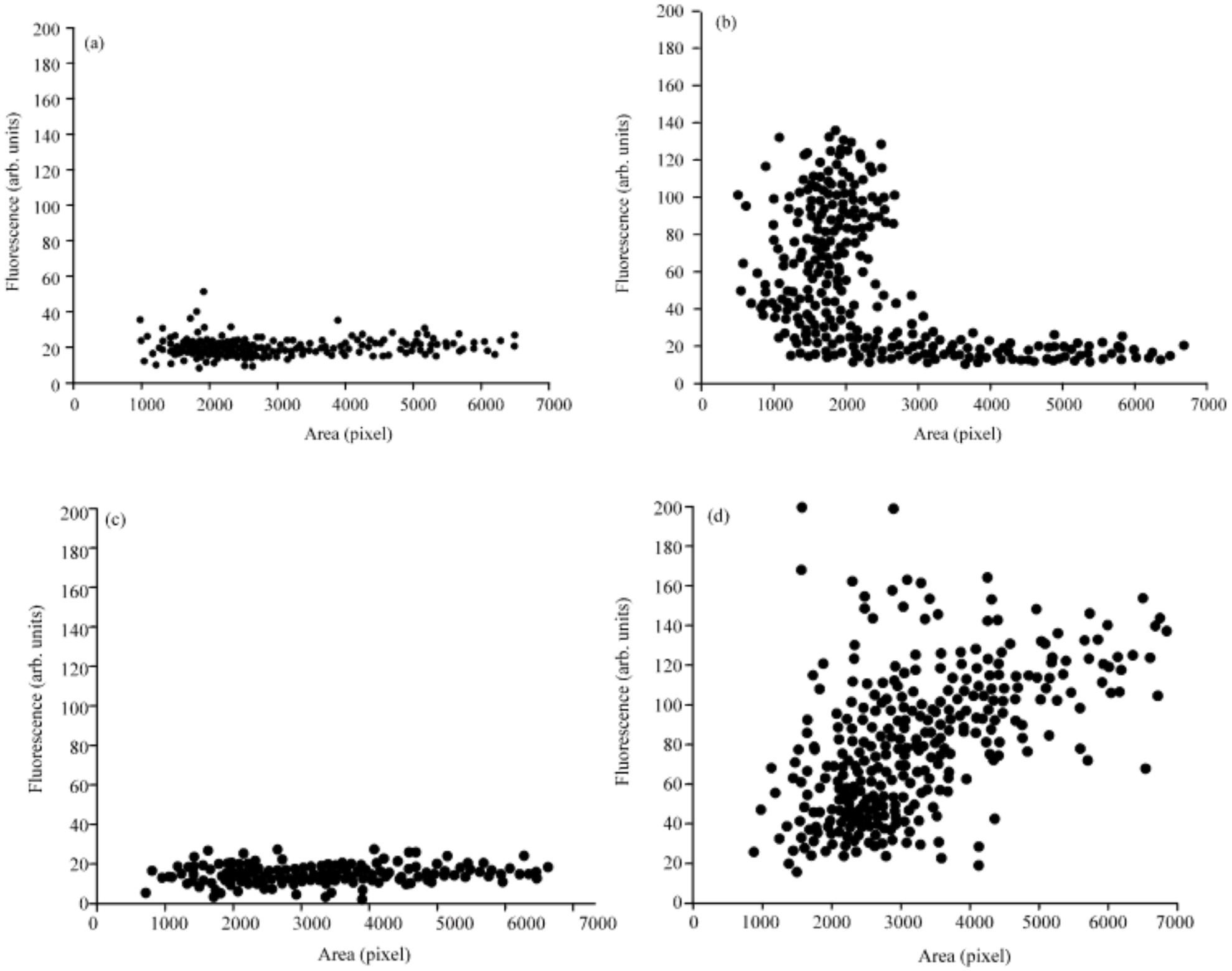

Fig. 2: Interrelation between the staining intensity of primary keratinocytes by antibodies to cell surface antigens and the cell size; (a and c) staining of keratinocytes with antibodies to actin; (b): staining with antibodies to the basal keratinocytes cell surface antigens; (d): staining with antibodies to the antigens of the differentiating keratinocytes cell surface. Abscissa is the projection area of the cells on the substrate, Area (pixels). Ordinate is the average staining intensity per cell (fluorescence, arbitrary units within 0-255)

smaller than those of the differentiating ones (Barrandon and Green, 1985). We could not evaluate homogeneity of large cells on this staining because it was rather faint. In addition, we observed a pseudo-cluster of small cells which were stained more intensively than the large cells but clearly less intensively than the majority of the small cells. These cells may probably represent an intermediate group of cells.

Staining with antibodies to differentiating keratinocytes showed that also in this case small cells form a distinct cluster, i.e., they were segregated as a true subpopulation (Fig. 2d). These small cells were stained with antibodies to differentiating keratinocytes less intensively than the large cells but still intensively enough. The large cells did not form any distinct cluster again. They are heterogeneous both on their size and staining. It is noteworthy, however, that intensity of the staining increased gradually as the size of the cells increased. No indication for clustering of these cells on this parameter was revealed. Moreover, there is an impression that the cells form some tail area outgoing from the basic cluster of the small cells as their size is increased. We assume that such distribution of the cells probably reflects a gradual differentiation (maturation) of keratinocytes. If so this process has no intermediate steady states. In other words, the phenotype changes

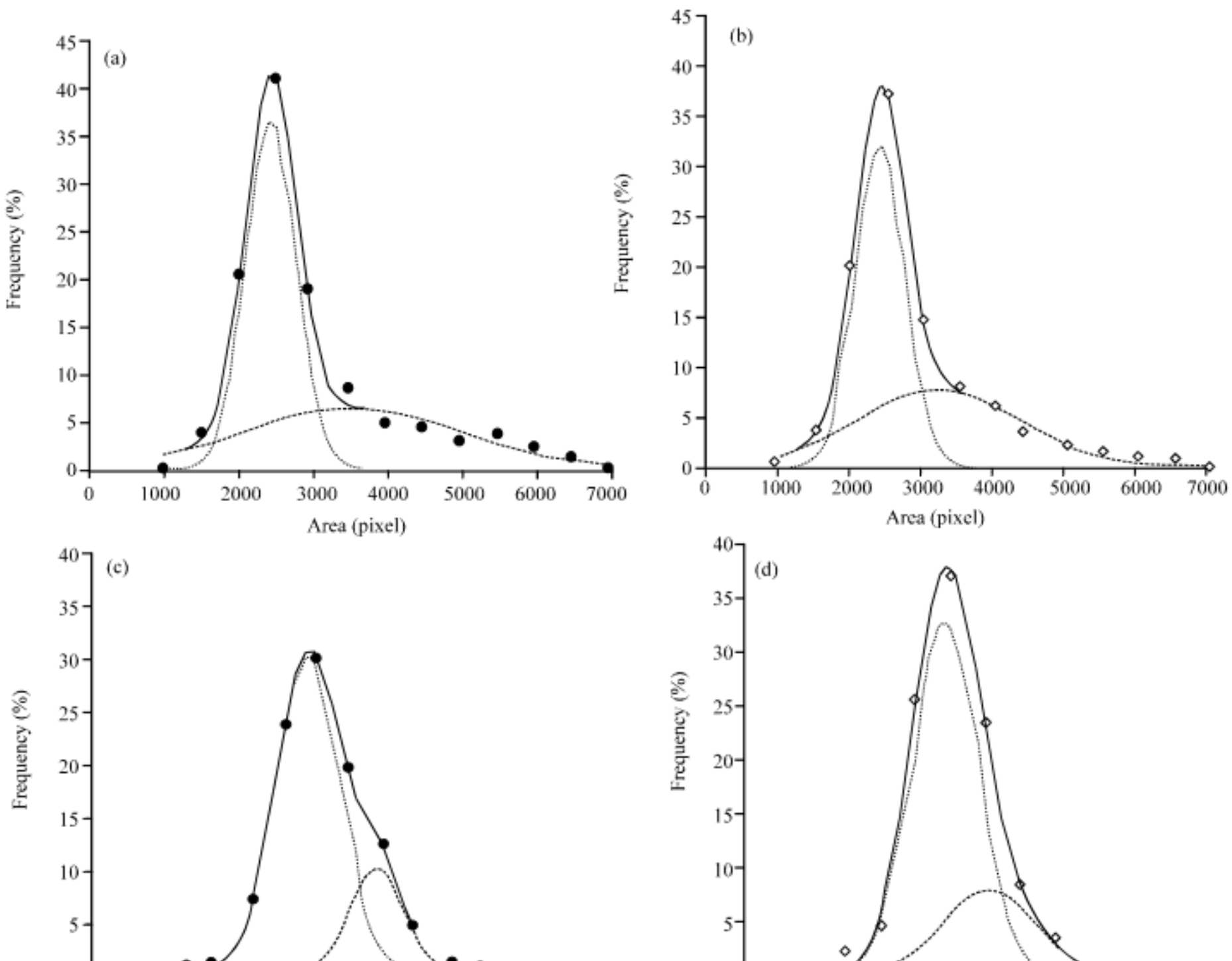

Fig. 3: Distribution of primary keratinocytes for area (a, b), actin staining (c, d), staining with antibodies to basal and differentiating keratinocytes (e and f, respectively). Ordinate is frequency, %. Abscissa is the projection area of the cells on the substrate, area, pixels (a, b), the average staining intensity per cell for actin (c, d); the average staining intensity per cell with antibodies against surface antigens of basal and differentiating keratinocytes, fluorescence arbitrary units (e and f, respectively)

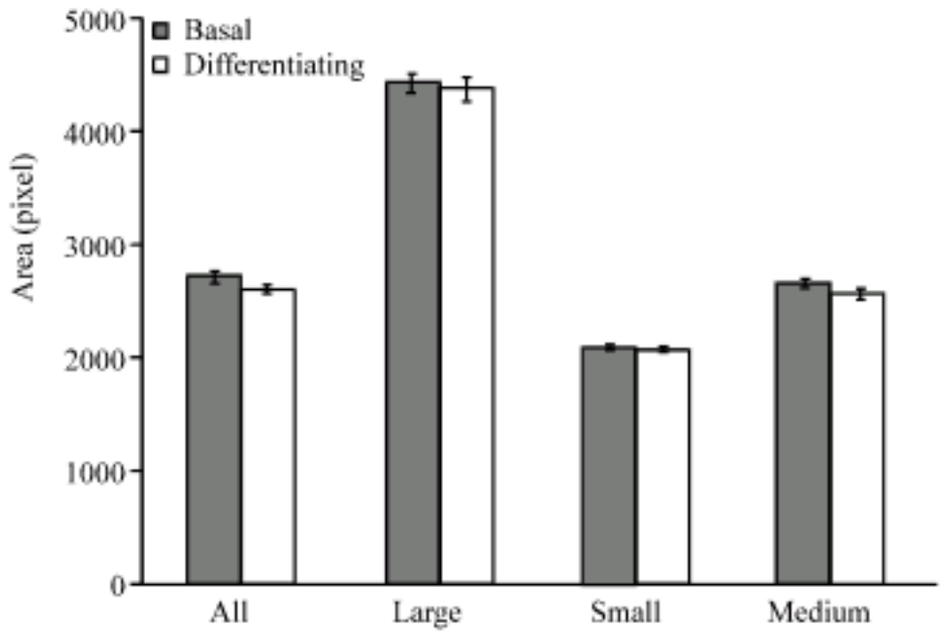

Fig. 4: Average value of projections of keratinocytes on the substrate (area) for the whole cell sample and for the large, small and medium cells. The cells stained with antibodies to the basal keratinocytes antigens and the cells stained with antibodies to the antigens of differentiating keratinocytes

(on the given parameter) not only directionally but also gradually. Therefore, subpopulation of the cells with the given staining is observed only for small cells rather than for the large ones.

The histograms display the same result but they also allow us to estimate the ratio of the cells with different staining as a function of their size (Fig. 3). It is necessary to notice that the right peak in Fig. 3e but the left peak in Fig. 3f correspond to the small cells. As the small cells are mostly stained with antibodies against basal cell surface receptors they should corresponds to basal keratinocytes of epidermis. Therefore, the question arises whether these cells are stem cells. Because today it is assumed that a part of basal keratinocytes are skin stem cells.

Skin stem cells are suggested to have specific set of markers including cell surface markers (Li *et al*., 1998; Watt, 1998). The number of these cells, according to different data, varies from 0.5% up to 10-12% of all basal keratinocytes (Kaur and Li, 2000; Alonso and Fuchs, 2003). It means that the stem cells should be autonomous being a part of basal keratinocytes that displays properties of subpopulation. We assume that polyclonal antibodies against surface receptors of basal keratinocytes cover all spectrum of surface antigens of these cells and they should recognize stem cells too. As stem cells should differ from basal keratinocytes by antigenic determinants. We should find a separate cluster (subpopulation) of these cells as a part of the cluster of small cells . However, no such thing was revealed in present experiments. We could assume that the number of

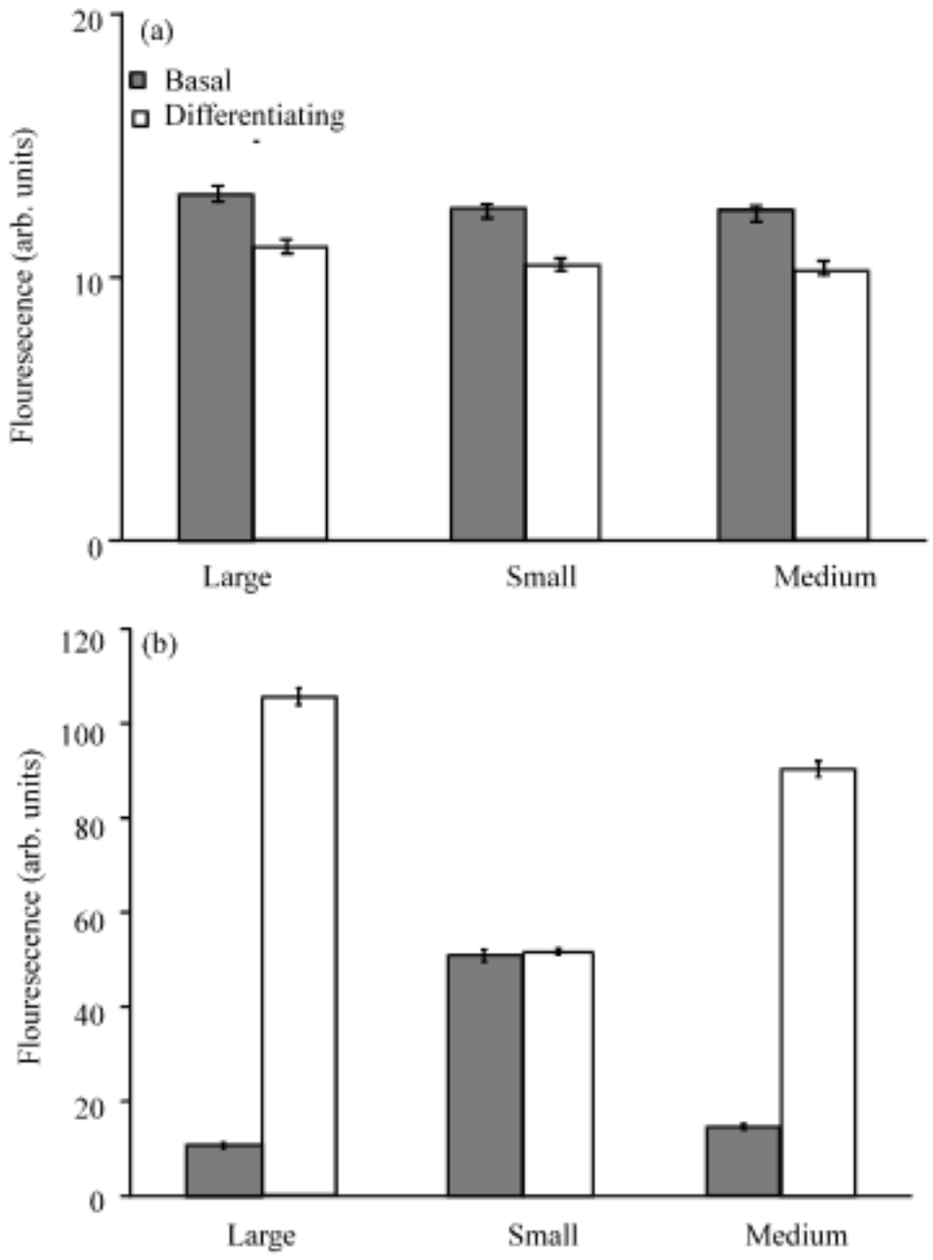

Fig. 5: Comparison of primary keratinocytes from the same cell population stained with antibodies to cell surface antigens of basal and differentiating, keratinocytes. (a): The average density of actin staining (fluorescence, arbitrary units) for the cells with different area. (b): The average density of staining with antibodies to the cells with different area

stem cells is insignificantly and they cannot be found by the antibodies used in present study. However, this is unlikely. Now, there are no univocal evidence for of the presence of stem cells in skin (Poumay and Coquette, 2007; Spichkina *et al*. 2008a, b). Therefore, it is more possible to assume that all the basal cells have the differentiation potential and there is no independent pool of specialized cells segregated as subpopulation. Entry of basal keratinocytes into differentiation is a stochastic process. This process depends on numerous factors which determine at which time and what basal keratinocyte should start to differentiate. In other words, any cell from the basal stratum can start to differentiate with the certain probability and, therefore, can imitate the stem cells.

In the literature we have found only one similar study claiming presence of the stem cells subpopulation among the basal keratinocytes (Li *et al*., 1998). The

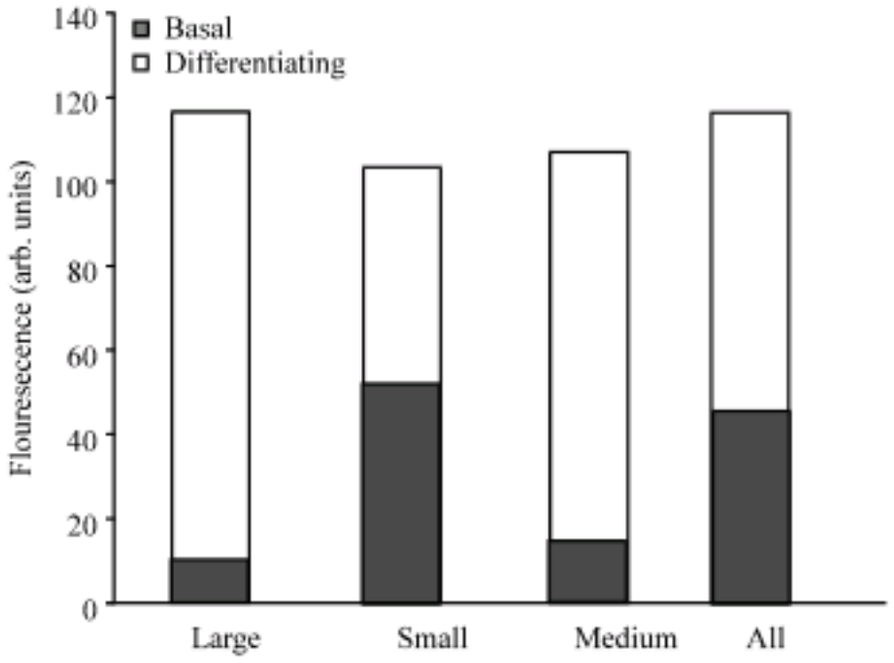

Fig. 6: The total contribution of staining by different antibodies for primary keratinocytes in culture. Basal: The cells stained with antibodies to surface antigens of basal keratinocytes. Differentiating: The cells stained by antibodies to surface antigens of the differentiating keratinocytes. Ordinate is the summarized staining with both antibodies (fluorescence, arbitrary units)

researchers have shown that the part of the basal keratinocytes (10%) specifically interacted with the antibodies directed against cell surface antigens of malignant keratinocytes. Such interpretation seems to be inadequate.

Furthermore, the data presented in this study allow us to draw some conclusions on the type and distribution of surface receptors on keratinocytes starting to differentiate. To this end, we have quantitatively analyzed staining of the same population of cells with antibodies against basal and differentiating keratinocytes keeping in mind that small and large cells seem to correspond to basal and differentiating keratinocytes, respectively. Approximately the same amount of small, medium and large cells stained with antibodies of each type was processed. Analysis of the data (Fig. 5, 6) was based on the assumption as follows. (1) positioning of keratinocytes in culture simulates a structure of a native epidermis, therefore, we believe that the general pattern of surface receptors of the keratinocytes *in vivo* and *in vitro* is the same too, (2) we believe that during 1 h in culture there is no synthesis of new surface receptors (antigens) on the keratinocytes, (3) for this time, no evident redistribution of available receptors can take place too. In other words, the number and composition of the receptors of each cell transferred into the culture are not changed, (4) basal and differentiating keratinocytes of epidermis expose both similar and different (specific) receptors and (5) total number of receptors (antigens) in basal and differentiating keratinocytes cannot be the same.

The result presented in Fig. 5 shows that small and large cells were stained differently by both antibodies. The medium cells occupy an intermediate position. This implies that these cells sharply differ by the pool of exposed antigens (receptors). However, summation of both staining intensities shows that the total amount of all exposed antigens for both small and large cells is the same (Fig. 6). Extrapolating these data to a single cell we come to conclusion that small and large cells have the same amount of the antigens exposed. As mentioned above the probability of such event is very low. This discrepancy requires an appropriate interpretation.

In the a literature, we have not found any data about redistribution of cell surface receptors at early stages of keratinocytes differentiation.

It is obvious that both small and large cells share a part of the receptors (antigens) which will be recognized by both types of antibodies. Other receptors (antigens) will be specific for the cells of different types. The higher is the difference between the two cells in intensity of staining with different antibodies the more they differ by the specific receptors. If we would assume that these specific receptors are distinguished both structurally and functionally, the probability that the number of specific receptors for basal and differentiating keratinocytes is the same, tends to zero with increasing the number of such receptors on each type of the cells. However and on the contrary, if the number of specific receptors for each type of the cells is large but the total amount of receptors is the same, this unequivocally indicates that these are the same structures. These structures can be identical either morphologically or functionally. If the status is functional, it requires very strong mechanisms to synchronize switching of spatially separated receptors during differentiation. We believe that such synchronic functioning of morphologically different structures is unlikely.

It is easier to explain the revealed phenomenon from other positions. Any receptor (antigen) can have several sites to which the appropriate antibodies can be directed. If we assume that during differentiation both exposure and burying of different sites of the same receptors (antigens) happens then it becomes clear why the total staining intensity by different antibodies is approximately the same for both basal and differentiating cells.

Such phenomenon of redistribution of various sites of the same morphological structures (receptors, antigens) can probably be both the cause and the effect of keratinocytes differentiation. This is a subject for further studies. This does not apparently imply, however, that surface properties of keratinocytes during their metamorphosis variate only in such manner (that is, at the expense of activation of different antigens of the same

receptors rather than at the expense of synthesis of the new ones). We shall remind that the fraction of highly adhesive keratinocytes (only the cells capable to attach to a substratum for 10 min) has been used in this study. As concerns adhesion these cells are practically identical. Therefore, it can be assumed that under the given conditions only those keratinocytes, which are at the first stages of differentiating, fell into the experiment. It is possible that a real change in the number of surface receptors (antigens) will occur at the next stages.

In this connection it is necessary to pay attention to the following. The number of the keratinocytes selected into a subgroup of large cells is 10-15%. Though, this is relatively small amount, nevertheless, Fig. 6 shows that the total number of receptors on the cells of this group is approximately 10% higher, than in the whole population. Hence, about 1% of the cells in the fraction (10 min) of keratinocytes studied has more receptors than a basic bulk of the cells. This may be connected with an additional pool of the receptors specific for the very large cells, which are more advanced ones during differentiation.

Results of this study show that the presence in epidermis of stem cells as the autonomous subpopulation is not verified experimentally. Most possibly, the entry of the cells into differentiation is a stochastic process in which all basal keratinocytes participate. However, this result does not exclude the possibility that in culture any basal keratinocyte can manifest itself as a true stem cell. In other words, an adequate induction *in vitro* can stimulate basal keratinocytes to differentiate not only into specialized skin cells but also into the cells of other tissues. Further experiments should solve this problem.

ACKNOWLEDGMENTS

We wish to thank Dr. V.A. Ivanov and Dr. I.I. Tyuryaeva for immunization and reception of specific immunosera, Dr. S.Yu. Khaitlina for discussions and help in preparation of the manuscript.

REFERENCES

Aberdam, D., 2004. Derivation of keratinocyte progenitor cells and skin formation from embryonic stem cells. Int. J. Dev. Biol., 48: 203-206.

Alonso, L. and E. Fuchs, 2003. Stem cells of the skin epithelium. Proc. Natl. Acad. Sci. USA., 100: 11830-11835.

Are, A., G. Pinaev, E. Burova and U. Lindberg, 2001. Attachment of A-431 cells on immobilized antibodies to the EGF receptor promotes cell spreading and reorganization of the microfilament system. Cell Motil. Cytoskeleton, 48: 24-36.

Barrandon, Y. and H. Green, 1985. Cell size as a determinant of the clone-forming ability of human keratinocytes. Proc. Natl. Acad. Sci. USA., 82: 5390-5394.

Deb, K.D. and K. Sarda, 2008. Human embryonic stem cells: Preclinical perspectives. J. Translat. Med., 29: 1-8.

Eckert, R.L. and E.A. Rorke, 1989. Molecular biology of keratinocyte differentiation. Environ. Health Perspect., 80: 109-116.

Gerdes, J., U. Schwab, H. Lemke and H. Stein, 1983. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int. J. Cancer, 31: 13-20.

Haeffner, E.W., K. Kolbe, D. Schroeter and N. Paweletz, 1980. Plasma membrane heterogeneity in ascites tumor cells. Isolation of a light and a heavy membrane fraction of the glycogen-free Ehrlich-Lettré substrain. Biochim. Biophys. Acta, 603: 36-51.

Jackson, L., D.R. Jones, P. Scotting and V. Sottile, 2007. Adult mesenchymal stem cells: Differentiation potential and therapeutic applications. J. Postgrad. Med., 53: 121-127.

Jensen, P. and L. Bolund, 1998. Low Ca^{2+} stripping of differentiating cell layers in human epidermal cultures: An *in vitro* model of epidermal regeneration. Exp. Cell Res., 175: 63-73.

Kaur, P. and A. Li, 2000. Adhesive properties of human basal epidermal cells: An analysis of keratinocyte stem cells, transit amplifying cells and postmitotic differentiating cells. J. Invest. Dermatol., 114: 413-420.

Kaur, P., 2006. Interfollicular epidermal stem cells: Identification challenges, potential. J. Invest. Dermatol., 126: 1450-1458.

Li, A., P.J. Simmons and P. Kaur, 1998. Identification and isolation of candidate human keratinocyte stem cells based on cell surface phenotype. Proc. Natl. Acad. Sci. USA., 95: 3902-3907.

Lippens, S., G. Denecker, P. Ovaere, P. Vandenabeele and W. Declercq, 2005. Death penalty for keratinocytes: Apoptosis versus cornification. Cell Death Differ., 12: 1497-1508.

McKeon, F., 2004. p63 and the epithelial stem cell: More than status quo? Genes Dev., 18: 465-469.

Michel, M., N. Török, M.J. Godbout, M. Lussier, P. Gaudreau, A. Royal and L. Germain, 1996. Keratin 19 as a biochemical marker of skin stem cells *in vivo* and *in vitro*: Keratin 19 expressing cells are differentially localized in function sites and their number varies with donor age and culture stage. J. Cell Sci., 109: 1017-1028.

Morasso, M.I. and M. Tomic-Canic, 2005. Epidermal stem cells: The cradle of epidermal determination, differentiation and wound healing. Biol. Cell, 97: 173-183.

Pellegrini, G., E. Dellambra, O. Golisano, E. Martinelli, I. Fantozzi, S. Bondanza, D. Ponzin, F. McKeon and M. De Luca, 2001. p63 identifies keratinocyte stem cells. Proc. Natl. Acad. Sci. USA., 98: 3156-3161.

Poumay, Y. and A. Coquette, 2007. Modeling the human epidermis *in vitro*: Tools for basic and applied research. Arch. Dermatol. Res., 298: 361-369.

Raikwar, S.P., T. Mueller and N. Zavazava, 2006. Strategies for developing therapeutic application of human embryonic stem cells. Physiology, 21: 19-28.

Rheinwald, J.G., 1980. Serial cultivation of normal epidermal keratinocytes. Meth. Cell Biol., 21A: 229-254.

Rowiñski, W., 2007. Future of transplantation medicine. Ann. Transplant., 12: 5-10.

Ruoslahti, E., E.G. Hayman, M. Pierschbacher and E. Engvall, 1982. Fibronectin: Purification, immunochemical properties and biological activities. Methods Enzymol., 82: 803-831.

Spichkina, O.G., G.P. Pinaev and Y.P. Petrov, 2008a. Analysis of heterogeneity of human keratinocytes interacting with immobilized fibronectin and collagenes I and IV types. Tsitologiya, 50: 210-217.

Spichkina, O.G., G.P. Pinaev and Y.P. Petrov, 2008b. Analysis of heterogeneity of human keratinocytes population by their adhesion to substrate and the keratin 19 to actin ratio. Tsitologiya, 50: 218-227.

Verfaillie, C.M., M.F. Pera and P.M. Lansdorp, 2002. Stem cells: Hype and reality. Am. Soc. Hematol. Edu. Program, 1: 369-391.

Watt, F.M., 1991. Cell culture models of differentiation. FASEB J., 5: 287-294.

Watt, F.M., 1998. Epidermal stem cells: Markers, patterning and the control of stem cell fate. Phil. Trans. R. Soc. Lond., 353: 831-837.

Yudintseva, N.M., Y.V. Gorelik, I.A. Diakonov, N.V. Kalmykova, M.I. Blinova and G.P. Pinaev, 1999. Transplantation of allogenic epithelium layers on burning wounds. Tsitologiya, 41: 324-328.