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Ultrastructural and Immunocytochemical Assessment of Proliferation and Differentiation Level of Human Keratinocytes *in vitro*

Ultrastrukturalna i immunocytochemiczna ocena stopnia proliferacji i różnicowania ludzkich keratynocytów *in vitro*

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Abstract

Objectives. In this study ultrastructural and immunocytochemical analysis of the changes occuring in cultured keratinocytes during their growth and differentiation *in vitro* was conducted. The influence of extracellular Ca^{2+} concentration on proliferation and differentiation processes of human keratinocytes was investigated.
Material and Methods. The cultures of epidermal cells were carried out in two phases. In phase I populations of high proliferating cells were obtained with the usage of serum-free Keratinocyte SFM containing low Ca^{2+} concentration (0.03 mM). In phase II cells differentiation and stratification was initiated, using DMEM containing high Ca^{2+} concentration (1.2 mM).
Results. Ultrastructural and immunocytochemical comparison of the cells from the first and the second culture phases showed an array of differences, which mainly concerned the placement of specific keratin filaments, formation of characteristic cellular junctions (desmosomes), and the expression of involucrin, specific protein marker of keratinocytes growth and differentiation. These changes were stimulated by various Ca^{2+} concentration in culture medium (**Adv Clin Exp Med 2004, 13, 3, 419–425**).

Key words: keratinocyte culture, ultrastructure, involucrin, Ca^{2+} concentration.

Streszczenie

Cel pracy. W pracy przeprowadzono analizę ultrastrukturalną i immunocytochemiczną zmian zachodzących w hodowanych keratynocytach podczas ich wzrostu i różnicowania *in vitro*. Badano wpływ zewnątrzkomórkowego stężenia Ca^{2+} na procesy proliferacji i różnicowania ludzkich keratynocytów.
Materiał i metody. Hodowle komórek naskórka prowadzono w dwóch fazach. W I fazie otrzymywano populacje wysoko proliferujących komórek stosując wolne od surowicy Keratinocyte SFM zawierające małe stężenie Ca^{2+} (0,03 mM). W II fazie hodowli inicjowano różnicowanie komórek połączone z ich stratyfikacją stosując DMEM zawierające duże stężenie Ca^{2+} (1,2 mM).
Wyniki. Ocena ultrastrukturalna i immunocytochemiczna komórek z pierwszej i drugiej fazy hodowli wykazała wiele różnic, które dotyczyły przede wszystkim położenia swoistych filamentów keratynowych, formowania charakterystycznych połączeń komórkowych (desmosomów) oraz ekspresji inwolukryny, swoistego białka będącego markerem wzrostu i różnicowania keratynocytów. Zmiany te były stymulowane różnym stężeniem Ca^{2+} w medium hodowlanym (**Adv Clin Exp Med 2004, 13, 3, 419–425**).

Słowa kluczowe: hodowla keratynocytów, ultrastruktura, inwolukryna, stężenie Ca^{2+}.

Stratification of epidermis is the result of transition of basal layer cells through subsequent stages of differentiation, which leads to programmed cell death. Keratinocytes stratify forming layers differing in morphology, shape, protein expression, division capability, cytoskeleton con-

struction and evolution of cellular junctions. Keratinocytes differentiation occurs in specific epidermal layers and characteristic structures appearing within the cells are specific markers of this process [1, 2].

There are two main procedures in keratinocyte culture. One of them is organotypic method, established by Breidehl [3], which consists in transferring a skin fragment into an artificial environment and maintaining its viability *in vitro* [4, 5]. The second method based on cells isolation from a skin fragment was developed by Green and Rheinwald in 1975. They worked out the procedure of mice keratinocytes culture, which later has been adapted to human keratinocytes [1, 6]. An essential element, on which this model was based, was growth of keratinocytes on a layer of inactivated mouse fibroblasts 3T3 Balb, which produce components promoting keratinocytes proliferation and compounds inhibiting multiplication of human fibroblasts. Further studies have shown that growth activity of keratinocytes in the presence of mouse fibroblasts result also from a direct contact of cultured cells with fibroblasts 3T3 Balb, not only from the influence of the fibroblasts products [7, 8].

Keratinocytes growth has been stimulated by various factors such as epidermal growth factor (EGF), cholera toxin, 10–20% fetal serum and an array of hormones and insulin-like growth factors [9]. Besides EGF, also TGF-α and amphiregulin (AR) regulate growth and differentiation of keratinocytes. On the basis of their functional and structural similarity, as well as their binding to the common receptor, these factors are included into the same family [1].

An important factor affecting the process of keratinocytes growth and differentiation is Ca^{2+} concentration. Changing extracellular Ca^{2+} concentration in culture allows for manipulating the process of keratinocytes proliferation and stratification *in vitro.* Such modulation is regarded as reflecting the physiological state, because in normal epidermis *in vivo* there is a well-marked gradient of calcium concentration between the cells of basal and higher layers (extracellular space of keratinocytes in basement layer – low Ca^{2+} concentration, higher layers – high Ca^{2+} concentration). Taking advantage of this phenomenon in culture allows for obtaining strongly proliferating keratinocytes of morphology characteristic for basal cells, or stimulating these cells towards stratification [1, 10].

The aim of this study was the ultrastructural and immunocytochemical analysis of the changes occurring in keratinocytes cultures during their growth and differentiation modified by various concentrations of calcium ions.

Material and Methods

Skin fragments of about 1–2 cm^2 were taken from dead human fetuses from the Department and Clinic of Gynecology in Wroclaw Medical University. The experiments were conducted in agreement with the requirements of Bioethics Commission at Wrocław Medical University. After subcutaneous tissue removal, keratinocytes were isolated mechanically and enzymatically in the process of cold trypsinization. The obtained suspension of epidermal cells was filtrated and condensed by centrifugation. The cell pellet was crushed and placed in plastic bottles in culture fluid Keratinocyte SFM (Gibco, cat. no. 17–005–034) supplemented with epidermal growth factor – EGF (5 ng/ml), extract from bovine pituitary gland – BPE – (50 μg/ml) containing low Ca^{2+} concentration. The cultures were carried out in two phases. In phase I the cells were suspended in the medium described above until full monolayer of keratinocytes was obtained (about 7 days). In phase II the cells were placed in Dulbecco medium (DMEM) supplemented with 10% fetal serum (FCS) containing 1.2 mM Ca^{2+} (about 21 days).

Morphological assessment of keratinocytes was conducted with by inverted phase-contrast microscope and transmission electron microscope (TEM). The cells were fixed and prepared due to the standard procedure.

The degree of keratinocyte differentiation was estimated by involucrin localization (Mouse Monoclonal Anti-Involucrin, Sigma, cat. no. I 9018) using immunoperoxidase method ABC.

Results

Phase-contrast Microscopy

Keratinocytes from the first phase of the culture (low Ca^{2+} concentration) in the initial stage, after about 2 days of growth formed small groups consisted of several to about a dozen cells, and after around 7 days reached full monolayer of not overlapping cells. Contact between the cells was not close. Only some of them were connected through short and thick cytoplasmic bridges. The cells were conforming to one another with shape. A great number of them were circular or oval. The cells cytoplasm was homogeneous, however, in single cells granularities could be observed. The cells nuclei were big and contained variable number of nucleoli (2–3). In some cases divisions were noticed (Fig. 1).

After alteration of culture conditions (high Ca^{2+} concentration), the cells formed layers. The cells of different shape were visible. Deeper

placed keratinocytes were oval, whereas the cells from higher layers got flattened and took spindly shape. These cells were connected through long and thin cytoplasmic bridges. Small nuclei with single nucleoli were observed (Fig. 2).

Electron Microscopy

Cross-section of the first phase of the culture showed one cell layer. Desmosomes were not visible and the only connections were of the nexus type (gap-junction) (Fig. 3). The cells cytoplasm contained numerous ribosomes, single mitochondria and distinct endoplasmic reticulum. Most of the cellular structures were localized in perinuclear part of the cell. The central part of the cell was occupied by a big euchromatic nucleus. Very little dense chromatin was visible underneath nuclear envelope (Fig. 4). Keratin filaments formed thin bundles accumulated under the plasma membrane. Electron dense granulations or membrane limited spherical, oval keratinosomes were not visible in the cytoplasm.

In electronograms of keratinocytes from the second culture phase three cell layers were visible, showing morphological differences. The lowest placed cell layer morphologically corresponded with the keratinocytes was observed in the first phase. Keratinocytes of the higher layers were connected through numerous, regular desmosomes with characteristic dark band in the intermembrane space and electron dense adhesive plates on both sides of the membrane (Fig. 5). Between every two cell junctions wide intercellular spaces were visible. In the cells of the intermediate layers bigger part of the cytoplasm was occupied by thick irregular bundles of keratin filaments directed towards desmosomes. The place of the filaments attachment were electron dense, disc desmosomal plates (Fig. 6). In the peripheral part of the cell a great number of microtubules was accumulated. In the cells of the intermediate and the higher layers numerous, tiny, electron dense granularities and laminar structures called keratinosomes were visible (Fig. 7). In the highest layer cells many myelin-like bodies, vacuoles and electron dense granularities were observed (Fig. 8).

Immunocytochemical Assays

The cells from the first and the second culture phase showed similar involucrin expression. Involucrin was diffused evenly within the cytoplasm (Fig. 9).

Discussion

In the established culture model a significant role in the growth and differentiation processes of epidermis cells plays an extracellular Ca^{2+} concentration and EGF. Both of these factors affect the

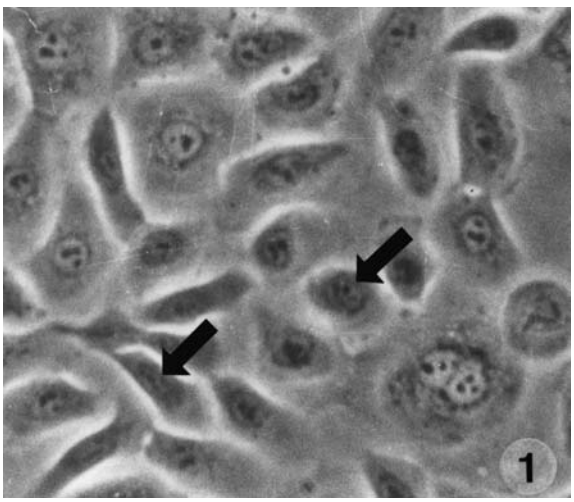

Fig. 1. Keratinocytes from culture phase I – full monolayer. Big part of the cytoplasm is occupied by nucleus with variable number of nucleoli (arrows). Phase-contrast microscope. Magnification 176×

Ryc. 1. Keratynocyty z I fazy hodowli – pełny monolayer. Dużą część cytoplazmy zajmuje jądro komórkowe ze zmienną liczbą jąderek. Mikroskop kontrastowo-fazowy. Pow. 176×

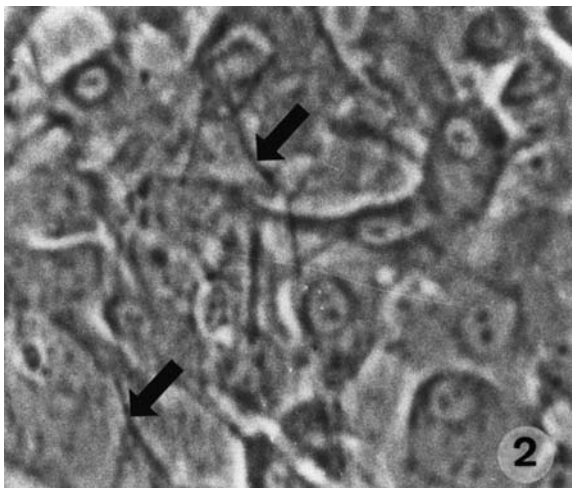

Fig. 2. Keratinocytes from culture phase II. The cells create layers. Deeper placed cells are oval, the higher placed ones are spindle-shaped. The cells are connected through thin cytoplasmic processes (arrows). Phase-contrast microscope. Magnification 176×

Ryc. 2. Keratynocyty z II fazy hodowli. Komórki ułożone warstwowo. Głębiej położone są owalne, wyżej położone – wrzecionowate. Komórki łączą się za pomocą cienkich wypustek cytoplazmatycznych. Mikroskop kontrastowo-fazowy. Pow. 176×

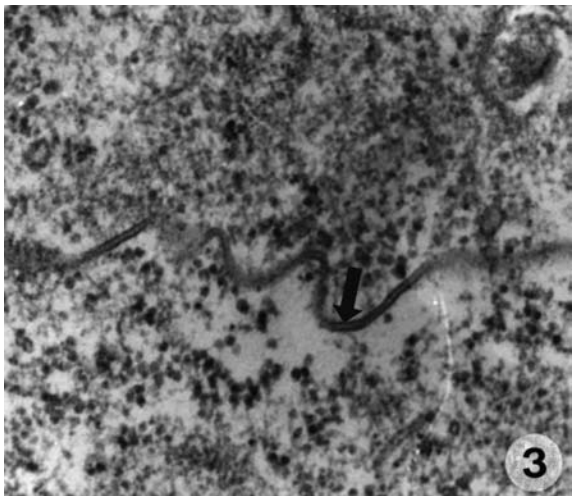

Fig. 3. Keratinocytes ultrastructure from culture phase I. The neighboring cells connected through gap-junction (arrow). The cells cytoplasm contains numerous ribosomes. Magnification 20000×

Ryc. 3. Ultrastruktura keratynocytów z I fazy hodowli. Sąsiadujące komórki połączone za pomocą gap-junction. Cytoplazma komórek zawiera liczne rybosomy. Pow. 20000×

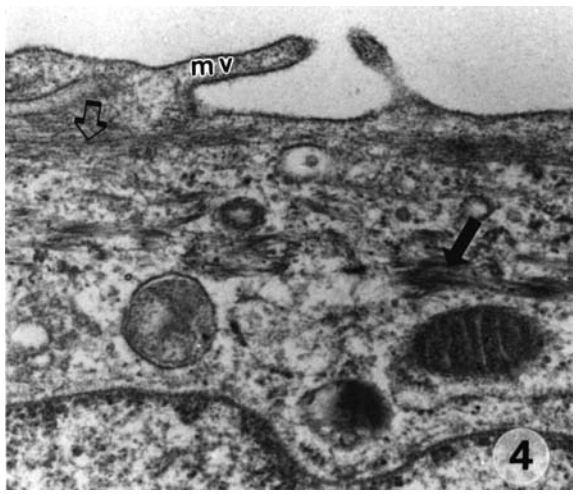

Fig. 4. The ultrastructure of the epidermis cell from culture phase I. In the cytoplasm, in the vicinity of the nucleus mitochondria, lizosomes and multivesicular bodies are visible. The cytoplasm contains keratin filaments bundles (arrow). On the cell surface are numerous microvilli (mv) under which the net of actin filaments (hollow arrow) is present. Magnification 15000×

Ryc. 4. Ultrastruktura komórki naskórka z I fazy hodowli. W cytoplazmie w pobliżu jądra komórkowego są widoczne mitochondria, lizosomy i ciała wielopęcherzykowe. Cytoplazma zawiera pęczki filamentów keratynowych. Na powierzchni komórki są obecne liczne mikrokosmki, a pod nimi sieć filamentów aktynowych. Pow. 15000×.

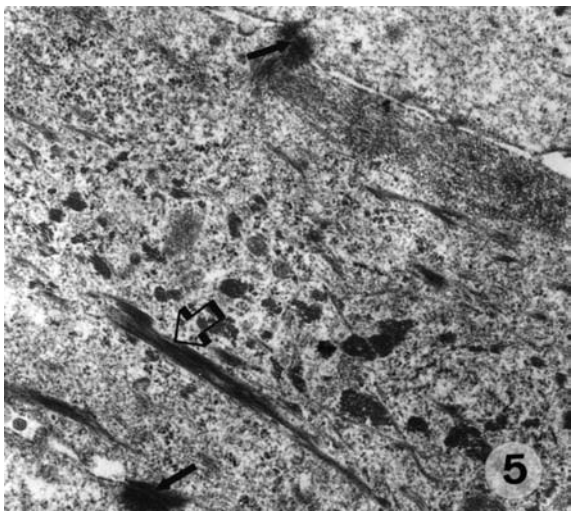

Fig. 5. Keratinocytes ultrastructure from culture phase II. Three layers of the cells connected through spot desmosomes (arrows). The cells cytoplasm is non-homogenous. It contains numerous granularities and great number of keratin filaments (hollow arrow) grouped into bundles. Magnification 15000×

Ryc. 5. Ultrastruktura keratynocytów z II fazy hodowli. Trzy warstwy komórek są połączone ze sobą za pomocą desmosomów punktowych. Cytoplazma komórek jest niejednorodna. Zawiera liczne ziarnistości i dużą ilość filamentów keratynowych zgrupowanych w pęczki. Pow. 15000×

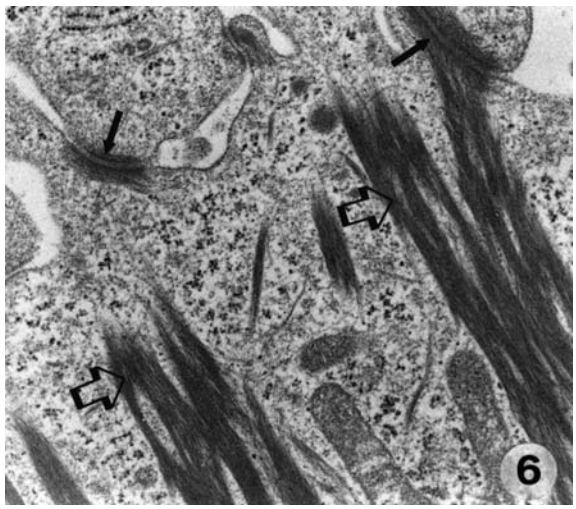

Fig. 6. Keratinocytes ultrastructure from culture phase II. The layers cells connected through numerous spot desmosomes (arrows). In the cytoplasm are visible thick bundles of cytokeratin filaments (hollow arrows), which anchor in desmosomes. The cytoplasm contains numerous mitochondria. Magnification 20000×

Ryc. 6. Ultrastruktura keratynocytów z II fazy hodowli. Komórki warstw są połączone za pomocą licznych desmosomów punktowych. W cytoplazmie są widoczne grube pęczki filamentów cytokeratynowych, które zakotwiczają się w desmosomach. Cytoplazma zawiera liczne mitochondria. Pow. 20000×

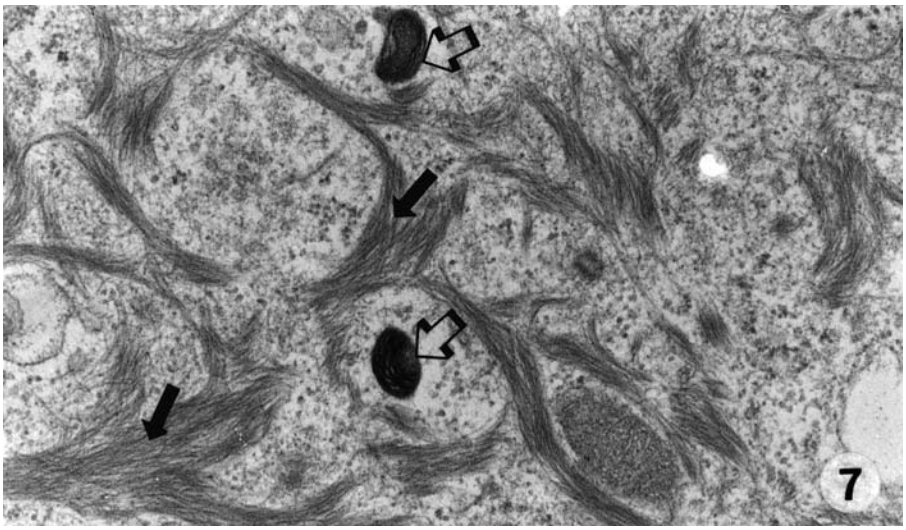

Fig. 7. The ultrastructure of the cell from culture phase II. In the cell cytoplasm are visible numerous bundles of cytokeratine filaments (arrows) and two laminal bodies – keratinosomes (hollow arrow). Magnification 20000×

Ryc. 7. Ultrastruktura komórki z II fazy hodowli. W cytoplazmie komórki są widoczne liczne pęczki filamentów cytokeratynowych oraz dwa ciała blaszkowate – keratynosomy. Pow. 20000×

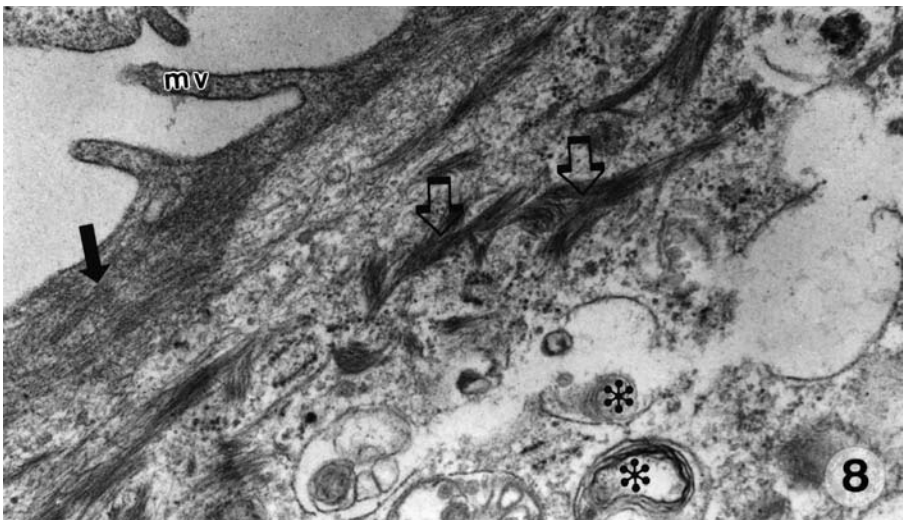

Fig. 8. The ultrastructure of the keratinocyte from the highest layer. The plasma membrane forms single microvilli (mv). Under the membrane well-formed actin filaments (arrow) net is visible. The cytoplasm contains also numerous tonofilaments (hollow arrows) and myelin-like bodies (asteriks). Magnification 20000×

Ryc. 8. Ultrastruktura keratynocytu z warstwy najwyższej. Błona komórkowa tworzy pojedyncze mikrokosmki. Pod błoną widoczna dobrze wykształcona sieć filamentów aktynowych. Cytoplazma zawiera także liczne tonofilamenty i ciała mielinopodobne. Pow. 20000×

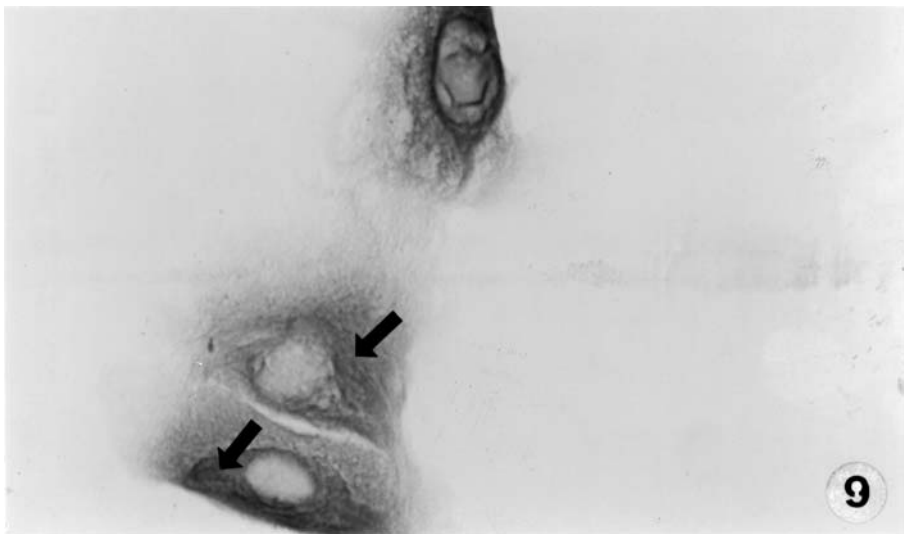

Fig. 9. Immunocytochemical localization of involucrin in keratinocytes from the first culture phase. Strong immunoreaction observed in the whole cytoplasm (arrows. Mouse monoclonal antibody against involucrin. ABC method). Magnification 160×

Ryc. 9. Immunocytochemiczna lokalizacja inwolukryny w keratynocytach z I fazy hodowli. Silna immunoreakcja jest obserwowana w całej cytoplazmie. Mysie przeciwciało monoklonalne przeciwko inwolukrynie. Metoda ABC. Pow. 160×

maintenance of balance between proliferation and differentiation of keratinocytes *in vitro*, although the molecular mechanism of this regulation is not fully known. Medema [11] showed in his studies mutual dependence between EGF and Ca^{2+} concentration. In epidermal cells EGF induces expression of protooncogene $p21^{ras}$ and augments entry into the cell cycle. Increase in Ca^{2+} concentration in medium causes decrease in $p21^{ras}$ activity and hence proliferation inhibition. The control of protooncogene $p21^{ras}$ may play a key role in taking a decision about proliferation or differentiation of keratinocytes [9, 12, 13].

Our studies confirmed that change of Ca^{2+} concentration in culture medium causes changes in epidermal cells, stimulating proliferation or differentiation. The change in Ca^{2+} concentration in medium induces several morphologically separate phenomena in epidermal cells [11, 13]. In our previous paper it has been shown that human keratinocytes proliferated in low Ca^{2+} concentration (0.03 mM), whereas 1.2 mM concentration had an inhibitory effect on cellular divisions [14]. Similar results have been obtained in the studies concerning growth and differentiation of mice keratinocytes *in vitro* [8]. Frequency of cellular divisions of keratinocytes is negatively correlated with Ca^{2+} concentration in culture medium, inversely than in fibroblasts. Low Ca^{2+} concentration in culture medium affects DNA synthesis increase and proliferation of human keratinocytes probably through increase in cAMP concentration in the cell [15].

Ultrastructural picture of the cells incubated in low Ca^{2+} concentration resembles morphologically poorly differentiated basal cells *in vivo*. Keratin filaments in the cells from culture phase I are placed around nucleus, which has been shown mainly in cytochemical studies [14]. This way of arrangement differs from tonofilament arrangement in basement cells *in vivo* and seems to be a consequence of the lack of desmosomes between the cells cultured in low Ca^{2+} concentration. The lack of these junctions between cells corresponding with basal cells has also been found in other studies [16].

The only cellular junction type from the first culture phase were nexus junctions (gap-junction). Most organelles of these cells are concentrated around nucleus, which is probably the result of perinuclear localization of cytoskeletal structures, mainly microtubules.

Watt [6] has proved that increase in Ca^{2+} concentration in culture medium affects the migration into higher layers, primarily of the cells synthesizing involucrin. The studies conducted on mice keratinocytes suggest that epidermal cells in low Ca^{2+} concentration do not synthesize involucrin but proliferate gaining monolayer. Terminal differentiation is induced through increase of calcium ions concentration in culture medium [17].

Increase of calcium ions concentration in culture medium induces changes in epidermal cells connected with their differentiation [17]. The first stage is cells stratification and desmosomes formation. Ultrastructural analysis has shown the presence of properly formed desmosomes in these cells. Ultrastructural studies prove that just after several hours following Ca^{2+} concentration increase in the medium, full desmosomes are created. As a consequence of the junctions formation changes in an arrangement of intermediate filaments occur; the filaments anchor in desmosomal plates [17, 18]. Increase in Ca^{2+} concentration in the medium changes keratin filaments arrangement; they disperse radially from the nucleus to the plasma membrane [6]. The observed changes as well as the presence of electron dense granules keratohialin and keratinosomes suggest a great variety of cultured keratinocytes. The cells from the second culture phase show clear features of differentiation, although it is impossible to distinguish layers analogical to those occurring *in vivo.* Observed in the highest layer numerous myelin-like bodies and secondary lizosomes suggest degeneration processes in keratinocytes.

Our studies suggest that *in vitro* (under medium conditions) involucrin is already synthesized in undifferentiated keratinocytes. This is different *in vivo* conditions where the protein is expressed only in differentiated cells. The explanation to this phenomenon can be that calcium ions may induce involucrin expression.

Ultrastructural and cytochemical analysis suggests that cultured epidermal cells can reach different levels of differentiation. An important role in stimulation of proliferation and differentiation of these cells *in vitro* plays Ca^{2+} concentration.

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