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Immunocytochemical evaluation of reorganisation of keratinocyte cytoskeleton induced by change in Ca^{2+} concentration in culture medium

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Ca^{2+} level-induced changes in the arrangement of cytoskeleton of cultured keratinocytes were estimated immunocytochemically, by evaluating expression of specific cytokeratins, desmoplakin and tubulin. Keratinocytes were isolated from fragments of skin of dead human foetuses. Culture of epidermal cells was performed in two phases: phase I yielded cells of high proliferation abilities in serum-free Keratinocyte SFM of low Ca^{2+} level (0.03 mM); in phase II differentiated cells were obtained in Dulbecco medium of a high Ca^{2+} concentration (1.2 mM). Immunocytochemical evaluation of phase I and II cells revealed an array of differences which involved mainly expression and distribution of specific cytokeratins, distribution of tubulin, testifying to a different microtubule arrangement and distribution of desmoplakin and indicating a tendency to form desmosomes. The changes were induced by the changes in Ca^{2+} level in the culture medium.

key words: keratinocytes, culture, cytoskeleton, desmosomes

INTRODUCTION

The epidermis represents a tissue, 90% of which is formed of one kind of cells — keratinocytes. The cells are directly responsible for the properties and stratified structure of epidermis. The remaining cells, represented singly in particular layers of the epidermis (Langerhans cells, Merkel cells and melanocytes), do not affect the stratification and the resulting functions [2]. The multi-layered structure of the epidermis is a result of basal layer cells undergoing consecutive stages of differentiation, the ultimate stage of which involves being apoptosis [3,13].

Basal layer keratinocytes undergo specialisation and pass into keratinised cells, the structure of which is totally different from the original one. The cells form layers of different morphology, shape, ability to divide, cytoskeleton structure and intercellular junctions. The differentiation of the basal layer cells results in great differences between particular zones. Besides the synthesis of specific proteins (involucrin, filagrin, loricrin), the most important changes accompanying this process are changes in the cytoskeleton structure in the cells [8].

The epidermis is a dynamic tissue undergoing constant changes. This is possible due to the balance between the frequency of divisions and differentiation, and constitutes the basis of the normal functioning of epidermis *in vivo*. Maintaining this homeostasis *in vivo* and *in vitro* depends on many internal and external factors that induce changes in the epidermis cells during their growth and differentiation. An important factor affecting these processes is Ca^{2+} concentration. A change of extracellular Ca^{2+} level *in vitro* makes it possible to manipulate

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proliferation and stratification of keratinocytes. Such a modulation is regarded as corresponding to the normal physiological process, since in a normal epidermis a distinct calcium level gradient can be noted between the basal and higher layers. Intercellular spaces of the basal layer are characterised by a low Ca^{2+} concentration (0.03–0.1 mM), in contrast to such spaces in the upper layers where the concentration is clearly higher (1.0–1.2 mM) [4,6,15]. These differences can be applied to design an *in vitro* culture model which makes it possible to obtain strongly proliferating cells with a morphology characteristic of the basal layer, or to stimulate stratification of these cells. It permits a full morphological analysis of these cells and an evaluation of changes which take place in the keratinocytes during their growth and differentiation.

The objective of this paper was an immunocytochemical analysis of the reorganisation of cytoskeleton of cultured keratinocytes induced by changes in Ca^{2+} concentration in the culture medium. Expression of specific cytokeratines, desmoplakin and tubulin was estimated in epidermis cells cultured at a low or high Ca^{2+} concentration.

MATERIAL AND METHODS

Original cultures were based on fragments of human skin of 1–2 cm^2 surface area, taken from dead foetuses. The cells were isolated mechanically and then enzymatically, through cold trypsinisation during 24 hrs. The keratinocyte culture was performed in two phases. In phase I the cells were suspended in Keratinocyte SFM of low Ca^{2+} concentration (0.03 mM), with addition of 5 ng epidermal growth factor (EGF) and pituitary gland extract (BPE) to initiate cell proliferation. In phase II the cells were placed in Dulbecco medium (DMEM) with addition of 10% foetal serum (FCS), containing 1.2 mM Ca^{2+} to initiate their stratification. The analysis of cytoskeleton of *in vitro* cultured epidermis cells was performed for both culture phases, employing the immunoperoxidase ABC method or the indirect immunofluorescence method. Cytokeratins, desmoplakin and tubulin were localised immunocytochemically, with the following anti-human murine monoclonal antibodies: CK5 diluted 1:50, CK10 diluted 1:50, CK14 diluted 1:100, desmoplakin I+II at a concentration of 5 μg/ml, anti β-tubulin diluted 1:100.

RESULTS

The changes in the cytoskeleton structure involved different expression of cytokeratins and desmoplakin, as well as the different arrangement of microtubules. The immunoreaction was observed to differ between the cells cultured at low Ca^{2+} concentration (culture phase I) and those kept at the high Ca^{2+} level (phase II). Using three different monoclonal antibodies: CK5, CK10 and CK14, directed against cytokeratins of a different mass (40–70 kD), a strong expression of cytokeratins CK5 and CK14 was found in the cells of phase I (Fig. 1, 2). No immunoreaction for CK10 cytokeratin was observed. In the cells in which stratification had been initiated, the immunoreaction for cytokeratin CK10 was clearly strong. No presence of cytokeratins CK5 and CK14 was found in such cells. The cells of culture phases I and II differed also in their desmoplakin expression. In proliferating cells, desmoplakin was concentrated only around the nucleus (Fig. 3). No presence of this protein was detected in the peripheral part of cytoplasm. At a higher Ca^{2+} concentration in the culture medium, a strong immunoreaction involved almost the whole cytoplasm (Fig. 4). Immunocytochemical evaluation of β-tubulin in the keratinocytes of culture phases I and II revealed distinct differences in their microtubule organisation. Figure 5 shows keratinocytes growing at a low Ca^{2+} concentration, in which the presence of β-tubulin was demonstrated by immunofluorescence. The microtubules formed a distinct ring around the nucleus. A small fraction was directed towards the plasmalemma. Increased Ca^{2+} concentration in the medium induced a change in the microtubule concentration. A dense microtubule network, forming a characteristic ring around the nucleus, underwent dispersion, spreading radially to the peripheral parts of cytoplasm (Fig. 6).

DISCUSSION

Extracellular Ca^{2+} concentration plays a significant role in the growth and differentiation of keratinocytes *in vitro* [15,18]. Its change in the medium induces several morphologically distinct changes in the epidermal cells. The observations are confirmed by our studies on the cytoskeleton reorganisation in keratinocytes, depending upon a change in Ca^{2+} concentration. Similar results were obtained by Zamansky [19,20]. However, the author studied changes in relations between microfilaments, keratin filaments and microtubules. Considerable differences in the cytoskeleton arrangement were observed shortly after the change from low (0.15 mM) to high (1.05 mM) Ca^{2+} concentration in the culture medium [12]. In this study, we estimated mainly the changes in expression of cytokeratins and desmoplakin, as well

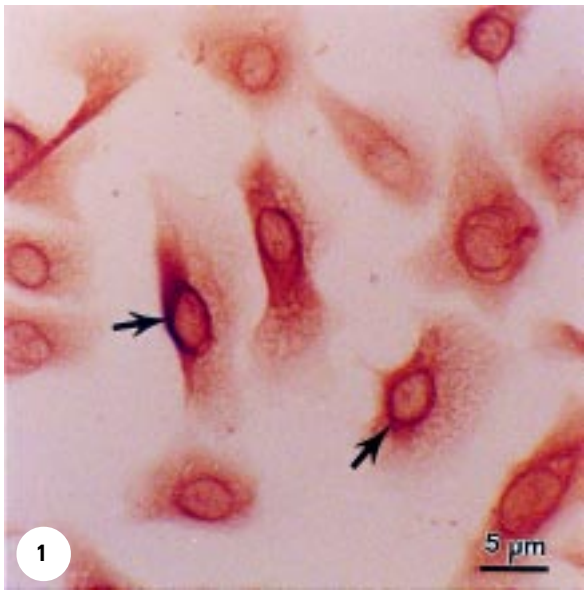

Figure 1. Immunocytochemical localisation of cytokeratins in epidermal cells of culture phase I. Cytokeratin CK14 forms a distinct ring around the nucleus (arrows). ABC method.

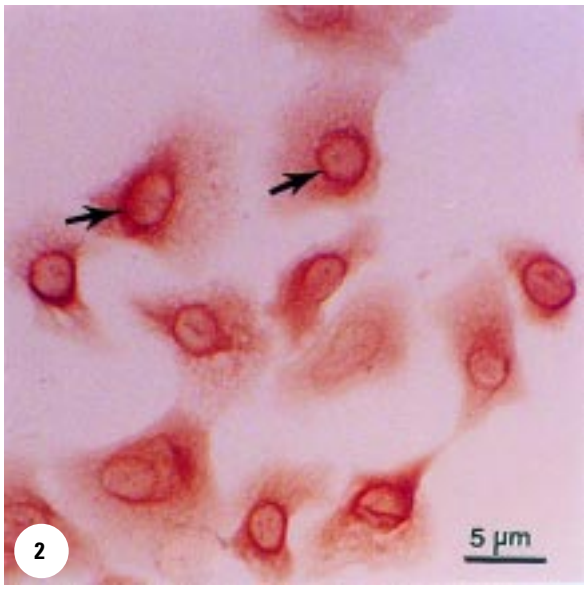

Figure 2. Immunocytochemical localisation of cytokeratins in epidermal cells of culture phase I. Cytokeratin CK5 forms a distinct ring around the nucleus (arrows). ABC method.

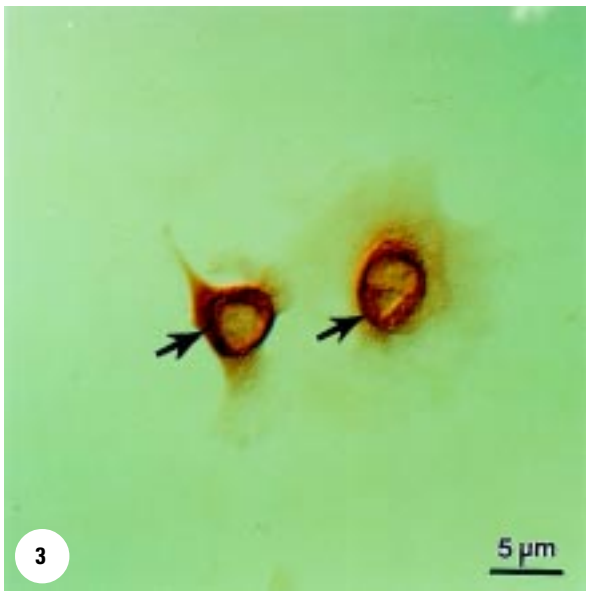

Figure 3. Immunocytochemical localisation of desmoplakin in epidermal cells of culture phase I. Desmoplakin concentrated around the nucleus (arrows). ABC method.

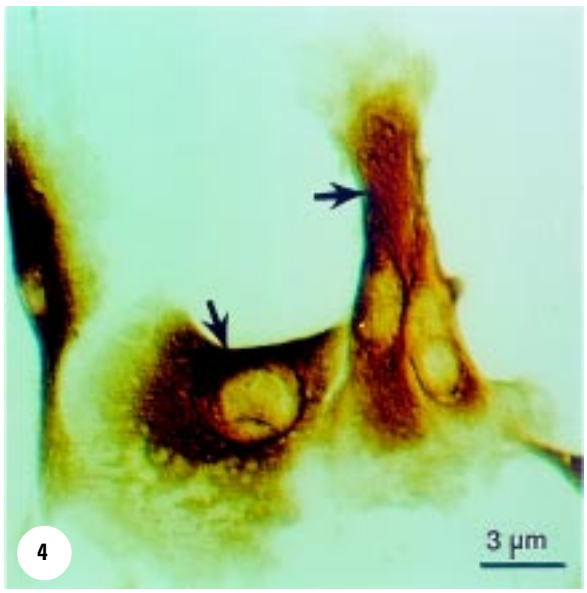

Figure 4. Immunocytochemical localisation of desmoplakin in epidermal cells of culture phase II. Strong immunoreaction involves almost entire cytoplasm (arrows). ABC method.

as the microtubule arrangement. Basal cells of stratifying epithelia are poorly differentiated and synthesise cytokeratins of 40–50 kD range (CK5, CK14) [12,14]. The cells of culture phase I show no positive reaction for cytokeratin CK10 since it is synthesised in higher epidermal layers and is an important marker of keratinocyte differentiation *in vitro*. In the cells kept at low Ca^{2+} concentration (0.03 mM), proteins typical of basal epidermal layer *in vivo* have been localised. Some authors also demonstrated, albeit in smaller quantities, the presence of cytokeratin CK15 in proliferating cells [9,14]. The perinuclear arrangement of cytokeratin filaments in the cells kept at low Ca^{2+} concentration was observed also at the ultrastructural level [5,17]. The arrangement departs from the arrangement of tonofilaments in the basal

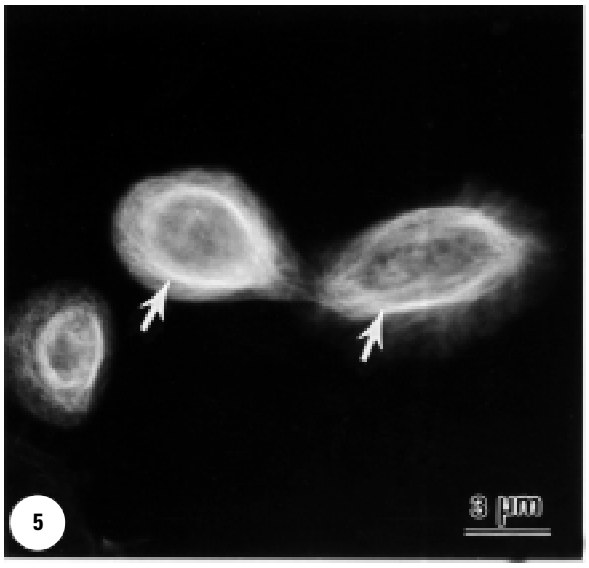

Figure 5. Immunocytochemical localisation of β-tubulin in epidermal cells of culture phase I. β-tubulin forms a distinct ring around the nucleus (arrows). Indirect immunofluorescence.

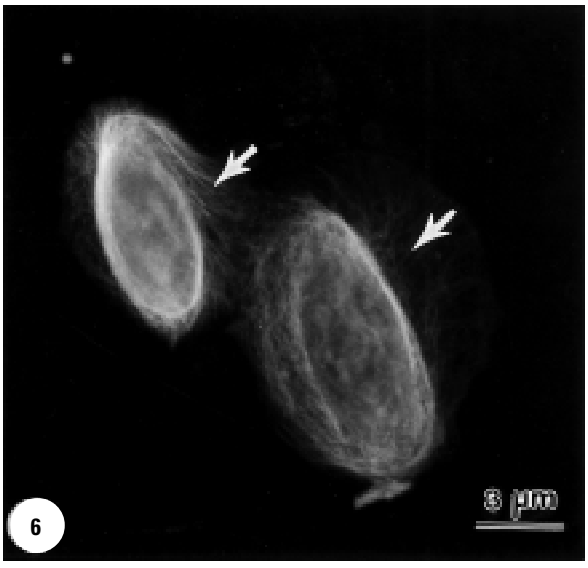

Figure 6. Immunocytochemical localisation of β-tubulin in epidermal cells of culture phase II. β-tubulin disperses (arrows). Indirect immunofluorescence.

cells *in vivo* and seems to be a consequence of the lack of desmosomes between the neighbouring cells of culture phase I. Earlier TEM studies [5,7,17] demonstrated that the cells cultured at a low Ca^{2+} concentration did not join each other forming desmosomes. These data were confirmed immunocytochemically, by estimating desmoplakin expression in the epidermal cells of phase I. A strong immunoreaction for desmoplakin in the perinuclear part of cytoplasm indicates that in the keratinocytes cultured at a low Ca^{2+} level no desmosomes are formed. Similar results were obtained by Watt et al. [17], also in their studies on the cell line of normal human keratinocytes HaCaT [16]. Epidermal cells *in vitro*, morphologically corresponding to the cells of basal layer, have a definite arrangement of cytoskeleton-forming proteins, and the results of immunocytochemical analysis provide evidence that their microtubules, like cytokeratin filaments, form a regular ring around the nucleus. Such an arrangement testifies to a poor differentiation of the cells. Most organelles are located around the nucleus, which is a consequence of the perinuclear location of the cytoskeleton components, mainly microtubules. The studies on cultured keratinocytes indicate that endosomes, lysosomes, Golgi apparatus and endoplasmic reticulum form a network closely linked with the cytoskeleton components [4]. These observations find confirmation not only in studies on human keratinocytes, but also on mouse macrophages [10]. The increase in Ca^{2+} concentration in the culture medium induces several morphologically distinct changes in epidermal cells, associated with their differentiation. The first stage involves cell stratification and desmosome formation. Immunocytochemical localisation of desmoplakin indicates that the increased Ca^{2+} concentration induces desmosome formation. In the cells of culture phase I, desmoplakin has been localised only in the perinuclear part of cytoplasm. Watt et al. [17] and Jones et al. [7] located desmosome-forming proteins in the peripheral parts of cytoplasm as early as 15 min after the increase in Ca^{2+} concentration to 1.2 mM. For this reason desmoplakin in culture phase II of our studies has been detected in the entire cytoplasm. Changes in the arrangement of cytokeratin filaments which anchor in adhesion plates of desmosomes are a consequence of desmosome formation. Cytokeratins in the cells of culture phase I have shown a characteristic perinuclear arrangement. The increase in Ca^{2+} level in the medium changes the cytokeratin arrangement, and the filaments spread radially from the nucleus to the plasmalemma. Immunocytochemical analysis of cytokeratin synthesis in the cells of culture phase II has revealed an expression of CK10 which is characteristic of differentiated cells. Analogous cytokeratins are synthesised in mouse keratinocytes cultured under similar conditions [11]. Chatuverdi et al. [1] demonstrated that cytokeratin K1 was an early marker of keratinocyte differentiation.

Immunocytochemical localisation of tubulin in the cells of culture phase II indicates that the increased Ca^{2+} level induces changes in its position. The radial arrangement of tubulin indicates that the microtubules have been formed, which are arranged radially toward the plasmalemma. Peripheral arrangement of microtubules is characteristic of differentiated cells. Similar results were obtained by Girolomani et al. [4]. Our studies show that cultured epidermal cells can reach various stages of differentiation, and an essential influence on the cytoskeleton organisation during these changes is exerted by the extracellular calcium level.

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