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HYPERPROLIFERATION OF NORMALLY QUIESCENT KERATINOCYTES IN NON-LESIONAL PSORIATIC SKIN DUE TO HIGH CALCIUM CONCENTRATION

(AN ORGANOTYPIC CULTURE MODEL)

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Calcium plays an important role in the regulation of different functions of keratinocytes. In the present work we studied the effect of different extracellular calcium concentrations (0.01 mM–2.0 mM) on the proliferation and differentiation of human keratinocytes in normal human and non-lesional psoriatic skin. Using explant culture model, the proliferative and differentiated subsets of keratinocytes were detected by specific antibodies related to cell proliferation [beta-1 integrin (CD29), proliferating cell antigen (Ki67), proliferating cell nuclear antigen (PCNA)] and differentiation [differentiated cell cytokeratins (K1/K10) and differentiating cell antigen (lectin Ulex europaius agglutinin, UEA-1)]. After 4 days of culturing at high Ca^{2+} (2.0 mM) we observed marked hyperproliferation among the normally quiescent keratinocytes of non-lesional psoriatic skin. In normal uncultured and cultured skin and in uncultured and two-day-cultured non-lesional psoriatic skin both at normal (1.2 mM) and at high (2.0 mM) Ca^{2+} concentration only one layer of basal $CD29^{+}/Ki67^{+}/K1/K10^{-}/UEA\text{-}1^{-}$ cell was observed. In sections from non-lesional psoriatic skin cultured for 4 days in the presence of high Ca^{2+} (2.0 mM) this cell population has expanded from at least three layers above the basement membrane. This expanded cell population of the 4-day high Ca^{2+} cultured non-lesional skin showed clear PCNA positive staining on frozen sections with the strongest positivity among the most basal localized cells. These data suggest that (i) extracellular Ca^{2+} concentration can influence the proliferation of basal ("stem") keratinocytes, (ii) the proliferative response to high Ca^{2+} concentration of psoriatic non-lesional basal keratinocytes differs from that of normal basal keratinocytes, (iv) changes in the

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extracellular Ca^{2+} milieu might play a role in the induction of the hyperproliferative psoriatic lesion.

Keywords: keratinocytes, psoriasis, hyperproliferation, calcium concentration

Introduction

Ca^{2+} is a universal second messenger responsible for regulating all forms of cellular activity [1, 2]. It has a major role in regulating epidermal functions, including cell proliferation, terminal differentiation and cell-cell adhesion [3, 4]. An interesting aspect of these so-called calcium-mobilizing signals is that many of them are also capable of stimulating the other major intercellular signal pathway based on cyclic AMP. Modification of Ca^{2+} concentration in the culture medium markedly alters the pattern of proliferation and differentiation of cultured keratinocytes. Low extracellular calcium (>0.8 mM) decreases cell-cell adhesion and cell motility, cultured keratinocytes become hyperproliferative and maintain basal epidermal cells morphology [5, 6]. Restoration of the normal Ca^{2+} level [1.2 mM] induces slow proliferation of the cultured epidermal cells and also terminal differentiation [7].

The epidermis traditionally has been divided into three functionally different compartments: germinative, differentiated and cornified. Increasing evidences suggest that the germinative compartment of the epidermis is heterogeneous [8]. In human epidermis, Lavker and Sun described two different proliferating cell types: one consisted of slow cycling, "non-serrated" cells with primitive cytoplasm, termed stem cells and the other consisted of highly proliferative, "serrated" cells with more complex cytoplasmic organisation, termed transiently amplifying cells. Flow cytometric analysis of the proliferative compartment subsets in normal and in psoriatic epidermis revealed a fundamental and robust difference between normal and psoriatic skin. Hyperproliferation in the psoriatic epidermis is due to activation of the normally quiescent cells in the $CD29^{+}$ $K1/K10^{-}$ basal cell compartment [9]. These cells show PCNA positivity in psoriatic skin indicating that they have recently entered cell cycle. These data suggest that stem cells are affected by the hyperproliferative signal(s) in psoriasis.

We are interested in the role of environmental Ca^{2+} changes in the proliferation and differentiation of normal and non-lesional psoriatic epidermis. In order to mimic the *in vivo* environment we used an organotypic culture system and looked at changes in proliferation and differentiation in response to extracellular Ca^{2+} by applying proliferation and differentiation-related markers in immunostaining methods [10, 11].

Materials and methods

After obtaining informed consent approved by the Institutional Review Board untreated non-lesional psoriatic skin was taken by 4 mm ∅ punch biopsies from the arm of the patients using local anaesthesia. Normal skin was obtained from adult individuals undergoing plastic surgery of the breast or abdomen. Tissue materials were collected and transported in Hank's balanced salt solution (Gibco, Scotland) with 100 units/ml penicillin and 100μg/ml streptomycin (Gibco).

Explant culture

Skin explants were cultured as described previously [12–14]. The top layer of skin of preferable thickness was prepared from breast and abdomen material by using an electrical or a hand dermatome. Explants were cut by a 4 mm ∅ biopsy punch causing minimal trauma. The punch biopsies from non-lesional psoriatic skin or normal skin were placed on cellulose acetate/cellulose nitrate filters, with 2.2 μm porosity (Millipore), on a stainless steel grid platform in a 25 mm^2 glass Petri dish. Each Petri dish contained six skin explants and had enough medium to wet the filters without covering the explants. The culture medium consisted of DMEM (Gibco) with 10% inactivated normal human serum (CLB, The Netherlands), 2 mM Glutamine (Gibco) 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) and $CaCl_2$ in the range of 0.01–2.0 mM. The above procedure enabled the tissues to be maintained at the air/liquid interface during the culture period. The explants were cultured at 37 °C at 5% CO_2 atmosphere. Explants were snap frozen in liquid nitrogen without culturing, after 2 and 4 days of culturing and stored at –80°C. Six μm cryostat sections were cut and the expression of different proliferation and differentiation markers were detected by indirect three-steps immunoperoxidase staining.

Three steps immunoperoxidase staining

Indirect immunostaining was performed at room temperature under humid conditions. Non-specific staining was prevented by pre-incubation with 10% normal swine serum (Dacopatts, Denmark). In the first step, acetone fixed 6 μm thick frozen sections were incubated with a primary antibody at established working dilutions for 60 min. After this, endogen peroxidase activity was blocked using 0.1% methanol-H_2O_2 solution. In the second step, peroxidase-labeled rabbit anti-mouse antiserum (Dacopatts, Denmark) was used in a dilution of 1:50 for 30 min. Finally, peroxidase-labeled goat-anti-rabbit antiserum (Dacopatts, Denmark) was used in a dilution of 1:100 for 30 min. Peroxidase activity was detected using 3,3-amino-9-ethylcarbazole (Sigma, The Netherlands) as a substrate.

Results

Normal epidermis

In normal human skin CD29 a mAb to the β1 subunit of the CD49-CD29 integrin complex and Ki67 (proliferating cell antigen) stained the basal layer, occasionally the second cell layer in the uncultured epidermis (Figs 1, 2) and the normal Ca^{2+} (1.2 mM) cultured epidermis (data not shown). Anti-K1/K10 (Fig. 3) and UEA-1 positive staining, on the other hand, was limited to upper layers of the epidermis, generally, but not exclusively, sparing the basal layer in both uncultured and normal Ca^{2+} (1.2 mM) cultured epidermis.

High Ca^{2+} concentration in the culture medium did not change the staining of anti-CD29 and anti-Ki67. Only one or occasionally two $CD29^{+}/Ki67^{+}$ cell layers were observed which was comparable to the results obtained from uncultured and low Ca^{2+} cultured skin (data not shown). At all conditions used in our experiments there were no signs of hyperproliferation of the $CD29^{+}/Ki67^{+}/K1/K10^{-}/UEA\text{-}1^{-}$ keratinocytes in normal skin.

Non-lesional psoriatic epidermis

Under normal Ca^{2+} condition (1.2 mM) the same overall pattern of K1/K10 and UEA-1 expressions were observed in non-lesional psoriatic skin sections as in normals: in both K1/K10 and UEA-1 were localized mainly above the $CD29^{+}/Ki67^{+}$ layers (data not shown). The CD29 and Ki67 staining were the same in normal and in non-lesional psoriatic skin under normal Ca^{2+} condition after 2 and 4-day cultured skin.

High (2.0 mM) Ca^{2+} concentration

After 4 days of culturing at high Ca^{2+} concentration we observed a marked hyperproliferation of the $CD29^{+}/Ki67^{+}$ keratinocytes of non-lesional psoriatic skin. Three or more rows of cells were very strong $CD29^{+}/Ki67^{+}$ (Figs 4, 5) and $K1/K10^{-}/UEA\text{-}1^{-}$ (Figs 6, 7). Among these layers the undermost keratinocytes clearly expressed PCNA, showing that they lost their quiescent state (Fig. 8).

Low Ca^{2+} (>0.01 mM) concentration

Using low Ca^{2+} concentration in the culture medium, after 2 days of culturing both normal epidermis and non-lesional psoriatic epidermis were degenerated (data not shown).

Fig. 1. Anti-CD29 moAb stained the basal layer in normal human skin and occasional the second layer cells in the uncultured epidermis

Fig. 2. Anti-Ki67 moAb stained the basal layer in normal human skin and occasional second layer cells in the uncultured epidermis

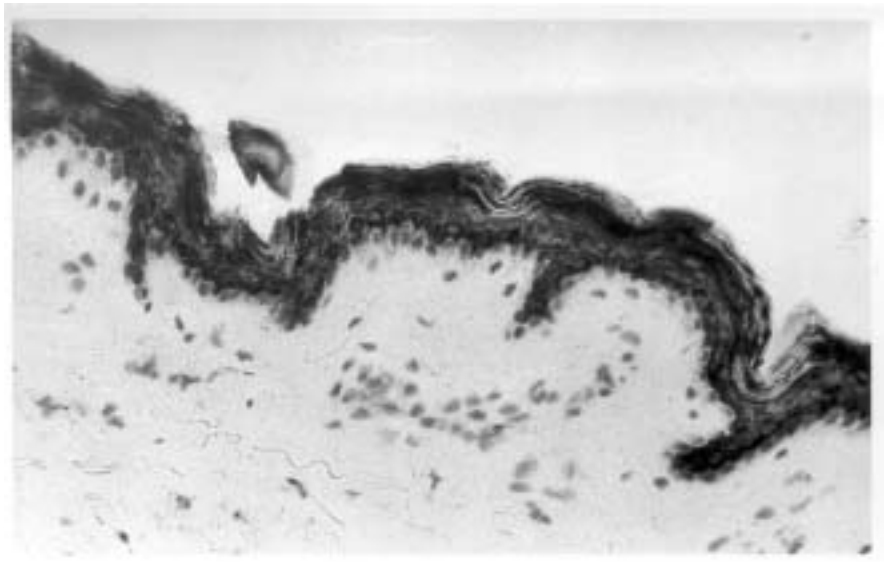

Fig. 3. Anti-K1/K10 positive staining was limited to upper layers of the epidermis in both uncultured and normal Ca^{2+} cultured epidermis

Fig. 4. In the non-lesional psoriatic epidermis three or more rows of the basal layers showed very strong CD 29 positivity after 4 days culturing at high Ca^{2+} concentration

Fig. 5. After 4 days culturing at high Ca^{2+} concentration at least three rows of basal layers showed very strong Ki67 positivity in the non-lesional psoriatic epidermis

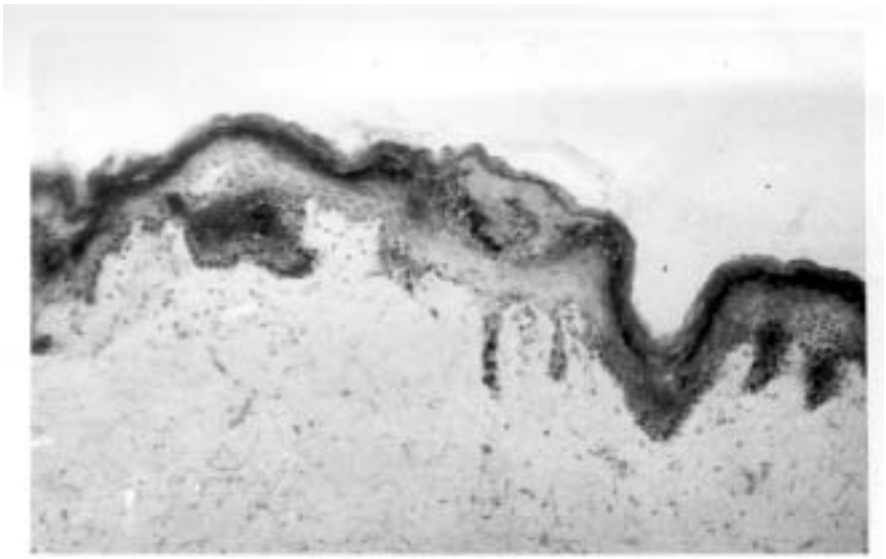

Fig. 6. In the non-lesional psoriatic epidermis at least three rows of cells showed very strong K1/K10 positivity in the differentiated layers of the epidermis after 4 days culturing at high Ca^{2+} concentration

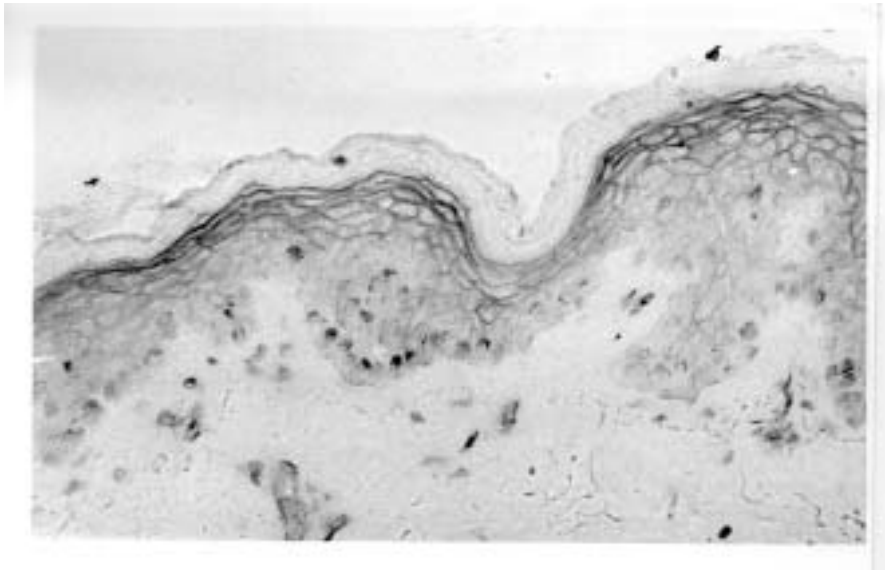

Fig. 7. After 4 days culturing at high Ca^{2+} concentration in the non-lesional psoriatic epidermis at least three rows of cells showed UEA-1 positive in the differentiated layers of the epidermis

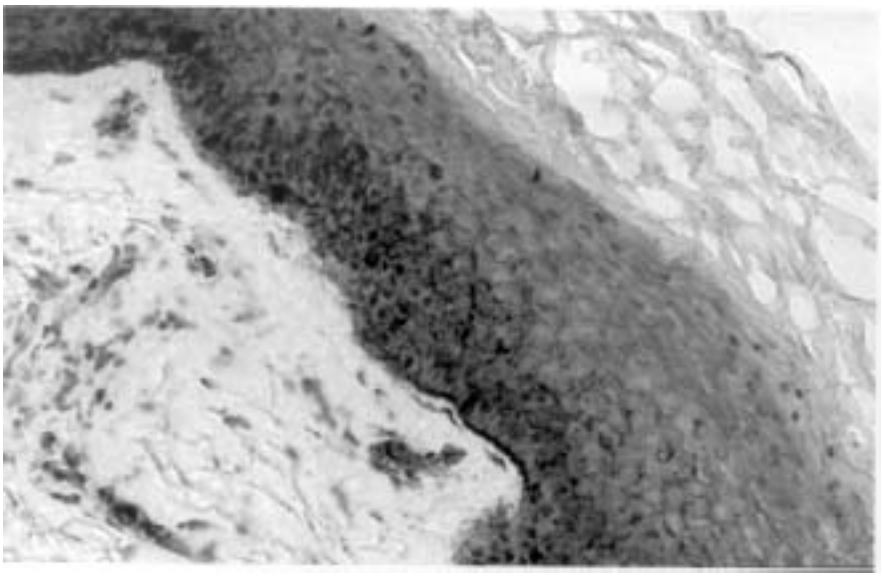

Fig. 8. After 4 days culturing at high Ca^{2+} concentration in the non-lesional psoriatic epidermis among the basal layers at least three rows of cells showed PCNA positive cells

Discussion

The importance of extracellular Ca^{2+} milieu is well documented in the regulation of multiple functions of keratinocytes [1]. Changes of Ca^{2+} concentration alter proliferation and differentiation of cultured keratinocytes [2]. Low Ca^{2+} in the culture media favors keratinocyte proliferation while the cells maintain basal cell morphology, higher Ca^{2+} concentration on the other hand induces keratinocyte differentiation together with suppressed proliferation. In order to mimic the *in vivo* keratinocyte milieu we used organotypic cultures and monitored the Ca^{2+}-related changes in the epidermis with proliferation and differentiation markers. Ca^{2+} concentration under physiological levels (< 0.01 mM) was not sufficient to maintain cell survival in the explants, both normal and psoriatic uninvolved epidermis degenerated by the second day of culturing. Ca^{2+} concentrations at normal levels led to intact epidermal structure after 2 and even 4 days of culturing without apparent differences between normal and psoriatic uninvolved cultured skin relative to the uncultured samples.

In agreement with previous reports [15–17] in normal skin CD29 (β1 integrin) and Ki67 marked the basal cells with occasional second layer cells. K1/K10 staining was localized to suprabasal cells generally, but not exclusively. In case of non-lesional psoriatic skin we documented the sparing of K1/K10 staining to the basal layers (Fig. 7). UEA-1 positivity was detected above the first layer of $K1/K10^+$ cells, indicating that K1/K10 expression precedes UEA-1 in the differentiation pathway of keratinocytes. High Ca^{2+} concentration in the culture had no apparent effect on normal skin after 2 or 4 days of culturing. However, psoriatic uninvolved skin by 4 days of culturing in the high Ca^{2+} environment exhibited marked changes in the staining pattern of the above markers. Three or more layers of $CD29^+$ $Ki67^+$ cells appeared in the psoriatic uninvolved skin explant indicating the expansion of this cell population due to a hyperproliferative response. The staining pattern clearly resembled the psoriatic lesional-skin in that the $CD29^+$ $Ki67^+$ cells did not express K1/K10. Based on flow cytometric analysis, it has previously been shown that the major difference in proliferation between normal and psoriatic lesional skin lies in the $CD29^+K1/K10^-$ cell population of the epidermis. The majority of the $CD29^+K1/K10^-$ cells in the normal epidermis are $PCNA^-$ indicating quiescent (G_0) state in the cell cycle, while all $K1/K10^-$ keratinocytes show PCNA positivity in lesional psoriatic skin, suggesting that they are or have been recently in a cycling state [18–20]. It is of interest to note the differential staining of Ki67 and PCNA. On frozen sections of non-hyperproliferative tissue we could not localize PCNA staining to individual cells, however in the hyperproliferative epidermis of non-lesional high-Ca^{2+} cultured skin using similar technique, $CD29^+$ $K1/K10^-$ cells showed clear PCNA positivity. Based on numerous published data,

PCNA expression seems to be closely related to cell cycle stages. PCNA expression peaks in G_1/S phase cells and is localized in the nucleus. In mitotic cells PCNA staining appears to be cytoplasmic and after cell division starts to vanish, while nuclear staining reappears in cells cycling again [21–23]. Our data indicate that on frozen sections PCNA can only be detected in cells, which have nuclear PCNA indicating that the cells are in G_1/S phase or have recently completed mitosis and still retaining cytoplasmic PCNA.

In psoriasis it is well documented that the major pathological changes of the epidermis relates to excessive keratinocyte proliferation and disturbed differentiation [24, 25]. The level of calmodulin (a protein which binds to and modulates Ca^{2+} actions) is increased in lesional psoriatic skin 2–3 fold compared to normal skin [26], T cell lymphokines (specifically IFN-γ) have been indicated to be a crucial regulatory factor in the initiation and maintenance of psoriatic lesions [27–28]. Among other effects IFN-γ has been shown to stimulate Ca^{2+} influx by receptor mediated Ca^{2+} channel and activate the calmodulin branch of the Ca^{2+} messenger system [29–30]. These data suggest that changes in extracellular Ca^{2+} in the keratinocyte environment might be an important factor among others in the pathomechanism of psoriasis.

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