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Evidence of Increased Keratinocyte Proliferation in Air-liquid Interface Cultures of Non-bullous Congenital Ichthyosiform Erythroderma

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Modern pharmacological and dermatological research requires the use of appropriate in vitro models which permit a faithful reproduction of various aspects of the in situ situation. The air-exposed culture of keratinocytes on dead de-epidermized dermis is one of the best models of in vitro epidermal differentiation known at the moment. In this study, we verified the model's validity for the reproduction of a hyperproliferative genodermatosis: non-bullous congenital ichthyosiform erythroderma. We used subcultured epidermal keratinocytes originating from normal and ichthyotic patients. Light and electron microscopy of pathological cultures disclosed, on day 14, a terminally differentiated epidermis with a marked granular layer and hyperkeratosis which, however, was not dramatically different from the normal controls. On day 25, the normal cultures displayed an even more pronounced hyperkeratosis and hypergranulosis, whereas the reconstructed epidermis of pathological origin presented a considerable reduction of the viable non-keratinized compartment and a focal parakeratosis. Indirect immunofluorescence revealed the expression of several differentiation markers which were not observed in the immersed culture models (e.g. the desmosome- and differentiation-related antigens KM48 and G36–19). Abundant keratohyalin granules were stained with AKH1 antibody and observed even in the deep epidermal layers, but no profilaggrin-filaggrin conversion could be detected biochemically. The cultures displayed some hyperproliferative features, as judged by KL1 and anti-involucrin antibody stainings. The keratinocyte proliferation was estimated by three different methods: 1) the rate of bromodeoxyuridine incorporation, 2) the expression of proliferating cell nuclear antigen and 3) the argyrophilic nucleolar organizer region counts. It proved to be more elevated in additional striking and statistically significant difference was observed all cultures when compared to the normal skin biopsies. An additional striking and statistically significant difference was observed between the normal and ichthyotic cultures on day 14 but was no longer detected after 25 days of culture. Our findings demonstrate that keratinocyte hyperproliferation, a major marker of the ichthyotic epidermis, is maintained in vitro in the emerged culture conditions, even though this model shows a baseline tendency for hyperproliferation. ***Key words: Growth; Ichthyosis; Cultures on dead de-epidermized dermis.***

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In vitro models reproducing in situ conditions are required for pre-clinical pharmacological studies of molecules influencing epidermal differentiation. They permit a simple and rapid screening of the tested drugs and, thus, represent a valuable alternative to the existing animal models. In 1979, Pruniéras et al. (1) developed an in vitro keratinocyte culture system which allowed terminal differentiation of the reconstructed human epidermis, very similar to that observed in vivo. In their model, normal human keratinocytes were seeded on a de-epidermized lethally irradiated dermis and cultured at the air-liquid interface. These culture conditions resulted in the formation of histologically normal granular and horny layers and in the expression of certain differentiation antigens characteristic of a normal epidermis but not observed in the immersed culture models (2). The method was successfully used for in vitro reproduction of some pathologic conditions, such as epithelial cancer or Hailey-Hailey pemphigus, when the neoplasic cell line or lesional skin keratinocytes were employed for culture (3, 4).

All the above-mentioned data indicate that the emerged model of epidermal cell culture on dead, de-epidermized dermis (DED) is a model of choice for studies on terminally-differentiating pure keratinocyte populations.

Non-bullous congenital ichthyosiform erythroderma (NBCIE), a skin disease of purely epidermal origin (5), has been individualized from the group of lamellar ichthyoses on the basis of particular clinical and microscope pictures as well as the characteristic keratinocyte hyperproliferation and epidermal lipid synthesis (6, 7). The keratinocytes from NBCIE maintain their potential for pathological differentiation during cell culture, as demonstrated by the re-expression of the typical microscopic features after grafting of the ichthyotic epidermal sheets onto nude mice (5).

In the present study, we used keratinocytes from skin biopsies of NBCIE for reproduction of the pathological epidermis in vitro, in the air-exposed culture model. The experiment was designed to extend our knowledge of this culture system and to determine its suitability for possible further studies on the biology and pharmacology of reproduced epidermis of normal and pathological origin. Keratinocyte proliferation was investigated using various quantitative methods. In vitro bromodeoxyuridine (BrdU) incorporation assay followed by on-section immunohistochemical detection of BrdU (+) keratinocyte nuclei permitted the evaluation of DNA synthesis rate. Anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody revealed the presence of nuclear proteins associated with cell proliferation (8). Histochemical staining of argyrophilic non-histone proteins linked to the nucleolar organizer regions (AgNOR), hyperexpressed in cells with high turnover rate (8), was also performed. The last two methods, which do not rely on in vitro incorporation of any markers, allowed direct comparisons between normal skin biopsies and cell cul-

Table I. *Antibodies used in the IF study*

Antibody	Labelling specificity (on normal human epidermis)	Dilution
AKH1 murine monoclonal Ab (MAb) (Biomedical Technologies Inc. Stoughton, MA, USA)	Filaggrin/profilaggrin: cytoplasmic staining of SG and SC	1:20
KM48 murine MAb (J. Thivolet, Lyon, France)	Keratinocyte membranes (desmosomes) with a positive gradient of expression from basal to granular layer	1:20
G36-19 murine MAb (G. Serre, Toulouse, France)	Corneodesmosome-related antigen (SG-SC interface)	1:50
KL1 murine MAb (Immunotech, Marseille, France)	56.5 kD acidic keratin; suprabasal keratinocytes	1:50
Anti-laminin rabbit polyclonal Ab (Institut Pasteur, France)	Laminin, basement membrane	1:50
Anti-involucrin rabbit polyclonal Ab (Biosys, Compiègne, France)	Human keratinocyte cross-linking envelope; upper spinous and granular layers	
Kp67-2 rabbit polyclonal Ab (J. Thivolet, Lyon, France)	67 kD keratin, suprabasal keratinocytes	1:20
UEA lectin-FITC (EY Laboratories, San Mateo, CA, USA)	α-L-fucose-containing glycoconjugates (1/3 upper spinous layer, granular layer)	1:10

tures. Several stainings of keratinocyte differentiation antigens completed the study, permitting an additional indirect assessment of the differentiation/proliferation state of the examined tissues.

MATERIAL AND METHODS

Skin biopsies

Lesional skin of NBCIE was taken from a hip and an axillar fold of 2 young patients (a 9-year-old boy and a 13-year-old girl). In both cases, the ichthyosis started at approximately the age of 1 month, showing a considerable inflammatory reaction of erythrodermic type and generalized fine white scaling most visible in the axial and inguinal folds, on the flexory faces of elbows and knees, and on the lateral faces of the neck. There was no antecedence of the ichthyotic affliction in their families. Histology showed the acanthotic epidermis with papillomatosis, hypergranulosis and focal parakeratosis in the thickened stratum corneum. The dermal infiltrate, composed of mononuclear cells, was concentrated around the dilated papillary vessels.

Normal human skin from breast and abdomen plastic surgery (female donors aged 21, 32, and 43 years) was used for the control experiments. Additionally, 5 normal skin biopsies (2 men, 3 women; mean age: 32 years) were spared for a routine paraffin embedding and served as controls in the study of the DNA synthesis rate.

Preparation of epidermal cell suspensions

Split-thickness strips of human skin were dissociated in 0.25% trypsin (Gibco BRL, Gaithersburg, MD, USA) at 37°C for 60 min. The epidermis was then separated from the dermis, and epidermal cells were teased apart by gentle pipetting. The cells were counted and placed in a keratinocyte culture medium (3:1 mixture of Dulbecco's modified Eagle's (DMEM)/Ham F-12 medium (Vietech, St Laurent de Mure, France) supplemented with 10% fetal calf serum (Gibco), 0.4 μg/ml hydrocortisone (Sigma, St Louis, MO, USA), 5 μg/ml insulin (Sigma), 10^{-10} M cholera toxin (Sigma), 1% glutamine (Vietech), 100 U/ml penicillin (Vietech), 100 μg/ml streptomycin (Vietech) and 10 ng/ml epidermal growth factor (Sigma)) on the 3T3 mouse fibroblast feeder layer blocked with mitomycin C (Sigma; 4 μg/ml, 30 min, 37°C), according to the method of Rheinwald & Green (9) ($6–8 \times 10^5$ 3T3 cells and 2×10^6 keratinocytes per cm^2). Subconfluent cultures, approximately 8 days old, were dissociated with 0.05% trypsin 0.02% EDTA (1:1) and used for seeding on DED.

Preparation of de-epidermized dermis (DED)

DED was prepared according to the modified method of Pruniéras et al. (1). Sterile split-thickness strips of normal human skin were maintained in magnesium- and calcium-free phosphate buffered saline (PBS) at 37°C for 15 days. The epidermis was then easily removed with forceps. Pieces of the denuded dermis (approx. 2 cm^2) were placed dermal side down in 60 mm Petri dishes and submitted to ten successive freezings and thawings. The obtained DED, devoid of any viable elements, was stored at −20°C.

Emerged keratinocyte cultures on DED

Stainless steel rings, designed to delineate a surface of 1 cm^2, were placed on the thawed DED squares. 5×10^5 keratinocytes suspended in a volume of 250 μl of the culture medium were seeded into each ring. Cells were allowed to attach to the dermis during overnight incubation at 37°C. On the following day the rings were removed, and the cultures were lifted onto fine stainless steel grids and air-exposed. The culture medium was added and changed three times a week until the end of the culture (14 or 25 days). Care was taken so as to maintain the contact between the medium and the dermal substrate through the metal grid only, in order to keep the culture emerged.

Two series of 10 pathologic and 3 series of 20 normal cultures, each from a different donor, could be performed and studied.

In vitro BrdU incorporation

Four hours before the end of emerged cultures, the medium was replaced with a new medium containing a thymidine analogue bromodeoxyuridine (BrdU; Sigma) at a final concentration of 10 μM. Incorporation of BrdU indicated the presence of keratinocytes synthesizing DNA. Samples from each culture were fixed 4 h in Baker's solution and processed for paraffin embedding.

Tissue examinations

Routine histology, standard electron microscopy and indirect immunofluorescence were performed on all the DED cultures.

Electron microscopy. Tissue fragments destined for standard electron microscopy were fixed with 2% glutaraldehyde in sodium cacodylate buffer, pH 7.4, washed, post-fixed in 1% osmium tetroxide, dehydrated in ethanol and embedded in epon. Ultrathin sections were contrasted with uranyl acetate and lead citrate.

Immunofluorescence (IF). Immunoreagents used in the IF studies are detailed in Table I. Briefly, 5 μm frozen sections were incubated 30 min with a primary antibody, washed with PBS (twice for 10 min), and reacted with an appropriate conjugate for 30 min. After the final wash in PBS, the sections were mounted in Fluoprep (BioMérieux, Marcy l'Etoile, France) and examined on a Zeiss fluorescence microscope.

Evaluation of keratinocyte proliferative activity

Anti-BrdU labelling. For detection of the BrdU-positive cells, the deparaffinized tissue sections were preincubated in 4N HCl (15 min, 37°C) for DNA denaturation and washed in 0.1 M borax buffer (5 min) before use of the anti-BrdU primary antibody (Becton-Dickin-

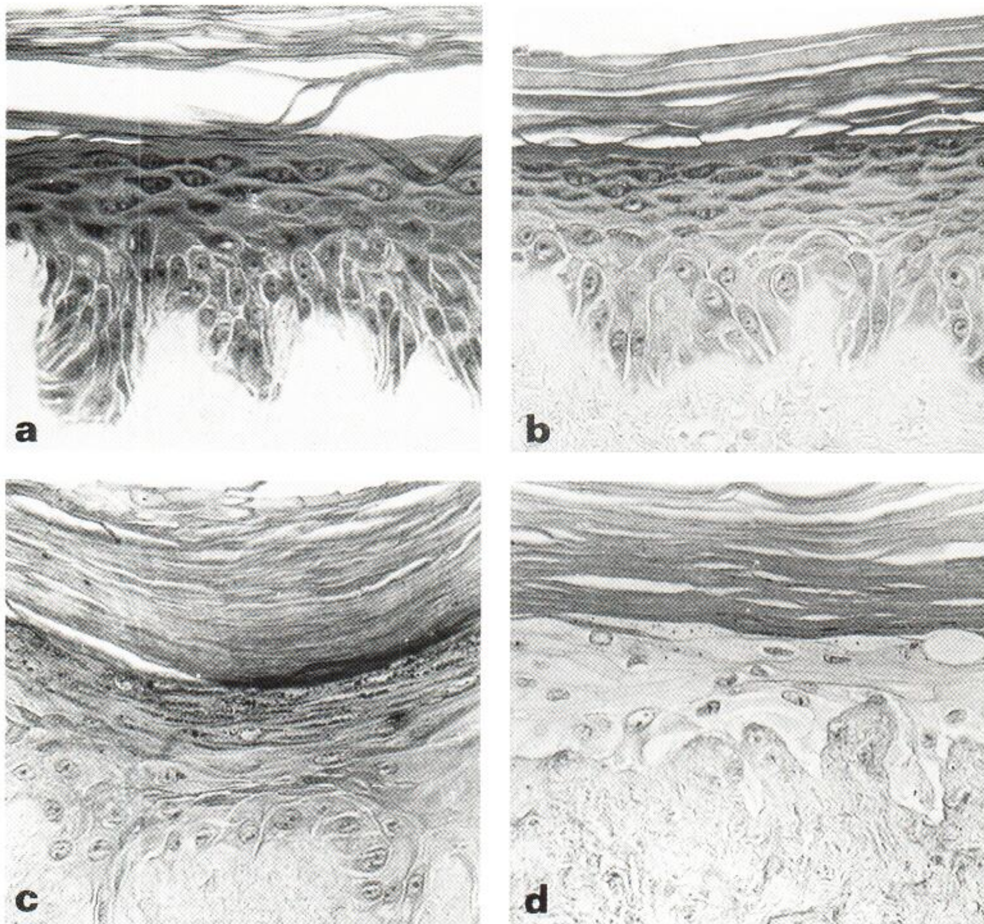

Fig. 1. Histology of normal (*a, c*) and pathological (*b, d*) keratinocytes cultured on DED. (*a, b*) 14 days of emerged culture: the reconstructed epidermis well imitates a normal appearance in situ. Note the complete absence of cellular elements in the dermis. The granular layer is thick and KHG are numerous. (*c, d*) 25 days of emerged culture: (*c*) SC and SG are thickened; (*d*) SC is thicker but SG contains only rare KHG (×25).

son, San Francisco, CA, USA), diluted 1:10. After an overnight incubation at +4°C and washing in tris-buffered saline (TBS), the immunoreactivity was visualized with an avidin-biotin-alkaline phosphatase technique (ABC kit, Vector Lab, Burlingame, CA, USA) (10).

The counting of positive cells was performed according to the methods described previously (11), using a semiautomatic image analyser (Mini-Mop, Kontron, Germany). The rate of keratinocyte DNA synthesis was expressed as the mean number of the BrdU-positive cells counted per 1 mm of section length, measured along the limit between the granular and horny layers.

Anti-PCNA labelling. Streptavidin-biotin immunoperoxidase reactions (LSAB kit, Dako, Glostrup, Denmark) were performed on deparaffinized tissue sections after a preliminary incubation with a monoclonal mouse anti-PCNA antibody (Dako), diluted 1:10. PCNA-positive cells were quantified as described for the BrdU incorporation study.

AgNOR staining. Staining of the argyrophilic nucleolar organizer regions (AgNOR) was performed according to the technique of Ploton et al. (12). Briefly, the silver staining solution was freshly prepared by mixing 1 volume of 2% gelatin solution in 1% formic acid with 2 volumes of 50% aqueous solution of silver nitrate. The solution was filtered before use, put onto the deparaffinized tissue sections, and left for 20–25 min in the dark. The sections were then washed, dehydrated, and mounted in dibutyl phtalate xylene (DPX) medium.

All black nuclear dots that could be separated by appropriate focusing were counted as individual AgNOR. The cells examined were randomly chosen within the basal and spinous layers. A total of at least 95 cells was taken into account in each case.

Statistics

The statistical analysis of the obtained results was performed with the Mann-Whitney U-test.

RESULTS

Examination by light microscopy after 14 days of emerged normal cultures revealed that the newly-formed epidermis was closely attached to the dead de-epidermized dermis (Fig. 1a). The stratum Malpighi was composed of several keratinocytes with well-preserved nuclei and clear cytoplasm. The relatively thick stratum granulosum (SG) was constituted of 3–5 cellular layers. The orthokeratotic stratum corneum (SC) consisted of 10–15 horny cells. No major differences were observed between the cultures of normal and pathological origin on day 14 (Fig. 1b).

After 25 days, the granular layer was even more developed and the SC was clearly hyperkeratotic (Fig. 1c), whereas the reconstructed ichthyotic epidermis presented a considerable reduction of the viable, non-keratinized compartment and a focal parakeratosis (Fig. 1d).

Ultrastructurally, whatever the origin of the 14-day cultures, the dermal-epidermal junction (DEJ) was intact: lamina lucida, lamina densa, anchoring fibers and hemidesmosomes were evident. Keratin filaments were evenly disposed around the cell nuclei, and the keratinocytes were linked together by well-structured desmosomes. Keratohyalin granules (KHG),

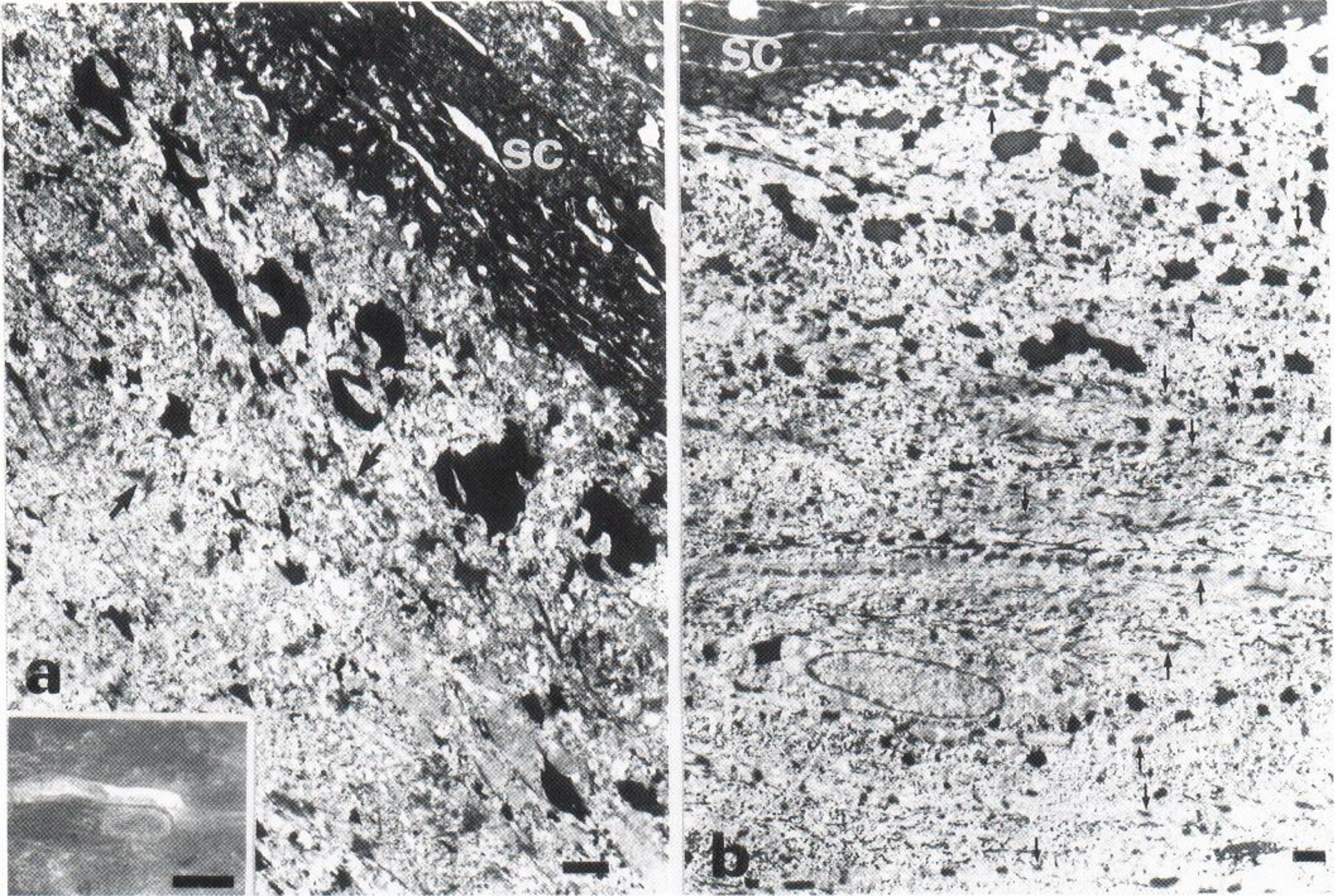

Fig. 2. Ultrastructure of the epidermal cultures on DED. (*a*) A 14-day normal culture presenting the same morphological features as its counterpart of ichthyotic origin. Note the presence of large KHG and numerous lipid vacuoles. *Arrows* indicate some desmosome junctions between consecutive SG keratinocytes (bar, 1 μm). *Inset* (bar, 0.2 μm): a normally-structured lamellar body from SG. (*b*) After 25 days of the control culture, the keratohyalin granules are visible in numerous cell layers, deep in the epidermis. Approximately 10 flattened keratinocytes are visualized (desmosomes at the consecutive cell borders are indicated by *arrows* at the right side of the picture) (bar, 1 μm).

of medium and large size, were present in the cytoplasm of the 3–5 most superficial nucleated cell layers of the living epidermis (Fig. 2a). Normal lamellar bodies were abundant in the cytoplasm of SG keratinocytes. Some of these organelles persisted also in the SC. Lipid vacuoles were clearly visible in the SC. In the 25-day control cultures, the SC and SG appeared much thicker: very large KHG were observed and small KHG could be seen even in the deep epidermal layers (Fig. 2b).

The pathological cultures of the same age displayed a thin granular layer with very rare and small KHG. The cytoplasm of keratinocytes contained non-aggregated peripherally disposed keratin filaments. Numerous lipidic vacuoles were visible in the SC and also present in the SG. Some nuclei persisted in the keratinocytes of the horny layer.

Expression and distribution of differentiation markers

Laminin was present at the denuded surface and around the blood vessel cavities of DED even before the culture. The anti-laminin antibody labelled the DEJ over its entire length (Fig. 3a).

The high molecular weight 67 kD keratin (K1, according to Moll et al.) (13) was present in all the suprabasal layers including SC, as revealed by cytoplasmic staining with Kp67 polyclonal antibody (Fig. 3b).

The KL1 monoclonal antibody, which reacts with the suprabasal 56.5 kD keratin (K10) in normal epidermis, labelled all living layers in the cultured epidermis (Fig. 3c). However, in some cultures, the basal layer appeared less intensely stained.

The anti-involucrin antibody (Fig. 3d) labelled the spinous and granular layers in a pericellular pattern. Often, the labelling started directly in the first suprabasal layer.

The AKH1 antibody (anti-profilaggrin/filaggrin) stained the SG with a decreasing gradient of intensity towards the deeper cell layers (Fig. 3e). On day 14, the cytoplasmic fine-granular staining concerned one third of the living epidermis. On day 25, it affected already one half of the Malpighian layer and, in places, even the directly suprabasal cells.

Staining obtained with KM48 was focal on day 14 and became more regular after 25 days of culture. The typical punctate labelling of intercellular spaces was observed in the basal and spinous layers, whereas higher SG keratinocytes were always negative (Fig. 3f).

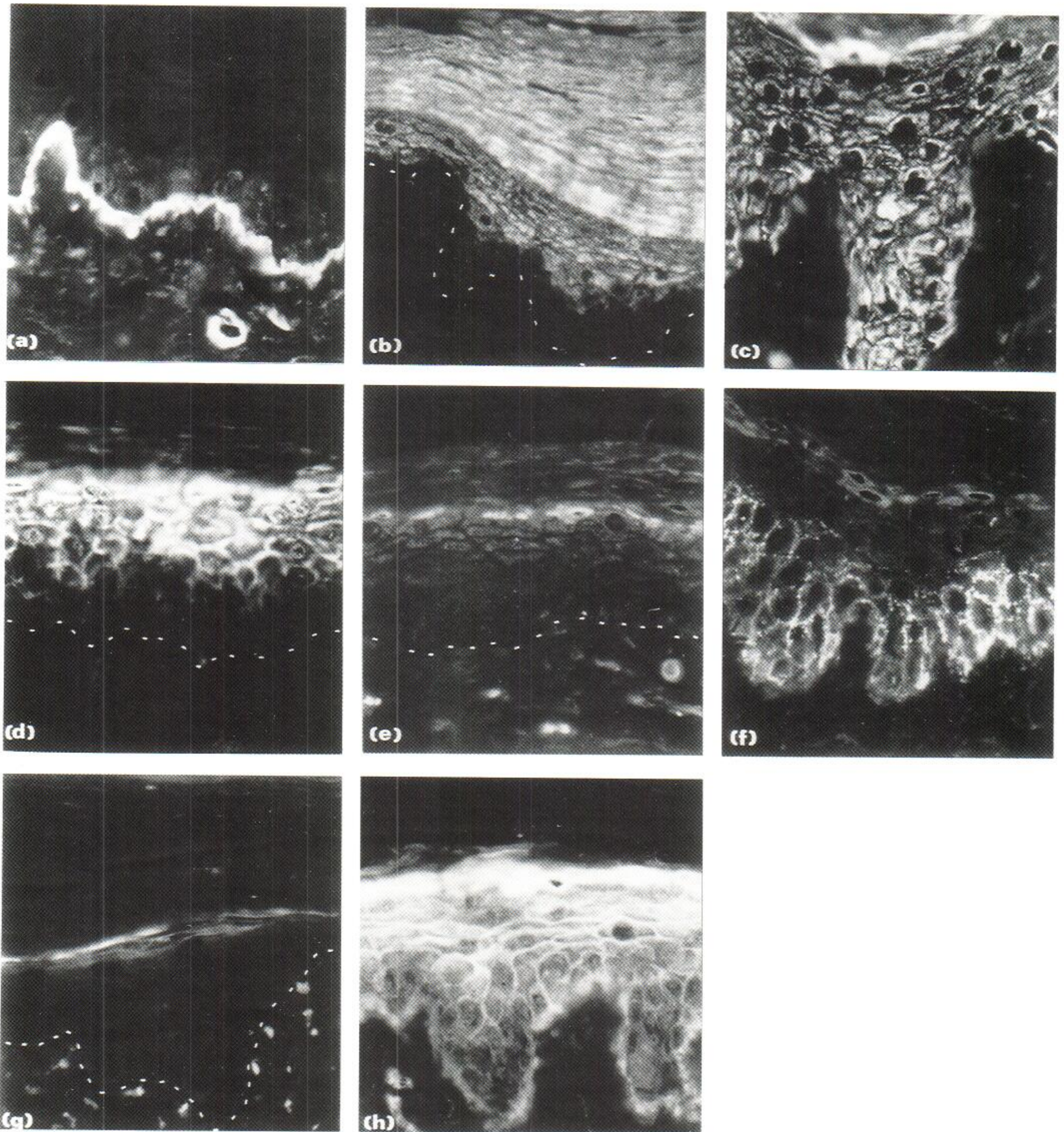

Fig. 3. Expression and distribution of differentiation markers in a 14-day culture of normal epidermis reconstituted on DED. Indirect immunofluorescence with antibodies anti-laminin (*a*), Kp67 (*b*), KL1 (*c*), anti-involucrin (*d*), AKH1 (*e*), KM48 (*f*), G36-19 (*g*). Direct immunofluorescence with the lectin UEA-FITC (*h*). A comparable pattern of immunofluorescence was also observed in air-exposed cultures of ichthyotic origin (not shown). In (*a*), a weak intracellular staining suggests laminin synthesis in basal keratinocytes. Note the positivity of basement membranes of the blood vessels with laminin (*a*) and UEA lectin (*h*). *Broken lines* indicate the dermal-epidermal junction.

The G36–19 monoclonal antibody gave a pericellular staining, limited to the uppermost part of the SG (Fig. 3g).

UEA lectin, specific for fucose-rich cell membrane glycoconjugates, stained the whole living epidermis in a pericellular pattern (Fig. 3h).

Distribution of the differentiation antigens revealed with the same antibodies was not qualitatively modified in the ichthyotic epidermis, except for a decreased staining observed with AKH1 on day 25.

Proliferative activity of normal and pathological keratinocytes

BrdU incorporation by cultured keratinocytes was detected

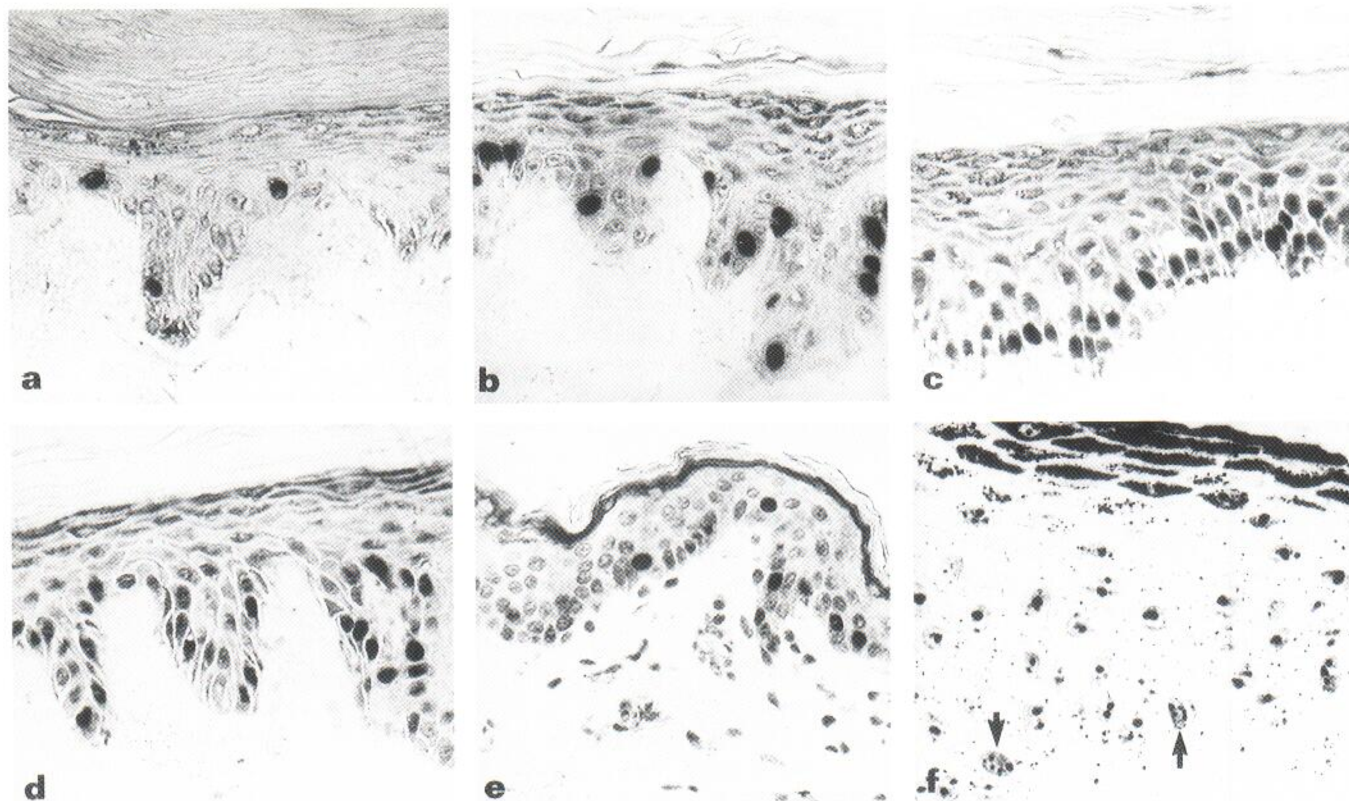

Fig. 4. Proliferative state of normal and pathological keratinocytes. Distribution of (*a, b*) BrdU- and (*c, d, e*) PCNA-positive keratinocytes (dark nuclei) (×25) and (*f*) AgNOR staining within cell nuclei (5 AgNOR are contained in the nuclei indicated by *arrows*) (×40). *a, c*: 14-day normal keratinocyte culture on DED. *e*: normal skin biopsy. *b, d, f*: 14-day NBCIE culture on DED.

Table II. *Number of BrdU and PCNA-positive keratinocytes and AgNOR in DED-cultured epidermis: cell proliferation study*

	Number of positive nuclei per 1 mm of epidermal section length (mean ± SD)		AgNOR counts
	BrdU-(+)	PCNA-(+)	
Normal cultures			
14 days ($n = 21$)	5.8±1.3	30.1±3.1	1.8±0.2
25 days ($n = 9$)	5.4±1.3	34.7±1.6	1.2±0.1
NBCIE cultures			
14 days ($n = 9$)	12.4±2.7*	53.2±4.5**	2.0±0.1
25 days ($n = 8$)	4.7±1.0	23.4±1.4	ND
Normal skin biopsies			
($n = 5$)	ND	18.6±3.2**	1.05±0.1**

* $p < 0.01$; ** $p < 0.05$, when the value is compared to the 14-day normal culture; (Mann-Whitney U-test); ND: not done

with anti-BrdU/alkaline phosphatase labelling, which gave a clear-cut nuclear staining of DNA-synthesizing cells (Fig. 4a,b). The PCNA (Fig. 4c–e) and AgNOR (Fig. 4f) expression provided further information on the keratinocyte proliferation potential. The quantitative results are summarized in Table II. The number of BrdU and PCNA-positive cells was significantly increased in 14-day NBCIE cultures when compared to normal controls. After 25 days of culture, the values obtained with NBCIE keratinocytes regained the range characteristic of normal cultures of 14 or 25 days.

The two evaluation methods, independent of the marker incorporation, permitted the comparison between control keratinocyte cultures and normal skin biopsies. The values obtained with both PCNA labelling and AgNOR staining were significantly higher in the cultured tissue.

DISCUSSION

Emerged cultures on dead de-epidermized dermis permitted us to reconstitute in vitro a stratified human epidermis of normal and ichthyotic origin. Histological and ultrastructural studies show that the overall morphology of the cultured tissue is very similar to the in situ appearance of epidermis in man. This is in agreement with previously published observations (3, 14).

The perfectly normal appearance of the DEJ and basal layer keratinocytes contrasts, however, with the slight modifications

which can be discerned on light and electron microscopy in the more superficial layers after 14 or 25 days of emerged culture. Numerous KHG, some of them excessively large, were noted already in 2-week cultures. After 25 days, the hyperexpression of KHG became even more evident, with numerous small keratohyalin deposits visible ultrastructurally even in the deep layers of epidermis. These findings concorded with the IF pattern of labelling with AKH1 MAb.

Despite the increased thickness of SG, some other keratinocyte antigens, normally expressed at this level of epidermal differentiation, could not be co-localized in all the cells containing KH granules. Labelling with G36–19 Mab was not increased and concomitant with the profilaggrin expression as has been described in normal epidermis (15, 16) but limited to the most superficial SG keratinocytes. The KM48 desmosome-related antigen was absent from the upper SG, where it is most strongly expressed in normal epidermis (17). Such a dissociation in the expression of various differentiation markers underlines the necessity of detailed studies using multiple criteria for comparison between the in vitro models.

The exclusively suprabasal expression of keratins recognized by Kp67 polyclonal antibody in the epidermal cultures on DED was originally put forward as a major criterion of proof, speaking for the close resemblance of this model to the in vivo situation (18). Labelling of the cultured epidermis with the KL1 Mab revealed, however, an atypical staining pattern which comprised the basal layer positivity. In normal human epidermis, KL1 antibody recognizes a suprabasal acidic 56.5 kD keratin (K10) but has been shown to react also with a basic 56 kD keratin (K6) expressed in the hyperproliferative epithelia (18, 19). Indeed, in our cultures, KL1 revealed K6 (2D-immunoblot electrophoresis, results not shown). Some other findings also indicate a hyperproliferative state of the epidermis cultured on DED. An early suprabasal expression of involucrin (3, 20) and the presence of the fucose-rich cell-membrane glycoproteins in the basal layer (21, 22) are all characteristic of epidermal hyperproliferation and are observed in the lesional skin in psoriasis. As described by Mansbridge & Knapp (23), the lesions of psoriasis, epidermal wound healing, and submerged keratinocyte culture show a number of features in common that distinguish them from normal epidermis. It may be hypothesized that the pattern of epidermal differentiation observed in our cultures on DED reflects the situation encountered in vivo during the healing of epidermal wounds.

We could confirm that the model of culture on DED is associated with an increase of keratinocyte proliferation using quantitative evaluation of PCNA-positive cells and AgNOR enumeration. The PCNA is an acidic nuclear protein inducible in proliferating cells and defined by reaction with an antibody found in an autoimmune disease, systemic lupus erythematosus (24, 25). The expression of PCNA correlates with the proliferating state in human keratinocytes (26), so that the anti-PCNA mouse monoclonal antibody provided us with a simple method of recognizing proliferating cells in fixed tissue sections (culture or biopsy). A simple silver staining technique allows the visualization of non-histone argyrophilic proteins, named AgNOR, which are associated with nucleolar organizer regions (NOR) (27). NOR represent loops of DNA coding for ribosomal RNA. The function of these nucleic acid-protein complexes is not fully understood, but recent studies suggest that AgNOR counts provide a means of evaluating cell kinetic activity and may be of diagnostic value in differentiating between tumours (28, 29).

Both methods revealed more labelled keratinocytes in the cultured epidermis than in the normal human skin biopsies, so we can conclude that imperfect epidermal differentiation and a tendency for hyperproliferation appear to be two major drawbacks of the culture model studied.

Despite its baseline hyperproliferative state, the model allowed for reproduction in culture of one of the major hallmarks of NBCIE, namely increased keratinocyte proliferation (6). The re-expression of this disease was previously obtained after grafts of the ichthyotic cell cultures on athymic nude mice (5). Also, recombinant grafts composed of lamellar ichthyosis epidermis (a non-hyperproliferative variety) and normal dermis indicated the purely epidermal origin of this group of ichthyoses (30). Our 14-day cultures on DED displayed some characteristic histological signs of NBCIE variety of autosomal recessive ichthyosis, i.e. hypergranulosis, hyperkeratosis, and focal parakeratosis, even if at this culture time, no major macroscopic differences nor any marked variations in epidermal immunoreactivity were observed between the cultures of normal and pathological origin. By contrast, a significant increase of the BrdU incorporation rate and of the PCNA-positive nuclei count characterized the 14-day NBCIE keratinocytes. The third method of evaluation, the AgNOR enumeration, confirmed this tendency but the difference was not statistically significant.

The 25-day NBCIE cultures were very hyperkeratotic, with a strikingly thinned viable epidermis. No hypergranulosis or hyperproliferation could be found any more. A similar phenomenom named "dying-back" of the cultured epidermis has already been observed by Chapman et al. in the emerged porcine skin organ cultures on nylon mesh (31). This may represent a precocious ageing of the epithelial tissue of NBCIE, compared to its normal counterpart.

In conclusion, our results demonstrate that the emerged epidermal cell cultures on DED represent a useful model for potential reproduction of skin diseases related to epidermal differentiation and hyperproliferation. However, the imperfect differentiation program and increased keratinocyte proliferation, inherent in this model, may still create some difficulties in the interpretation of results.

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