Human Epidermis Reconstructed by Culture: Is It "Normal"?

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Human keratinocytes were grown on a dermal equivalent (or lattice) at the liquid–air interface in an attempt to reconstitute a functional epidermis in vitro. Although the multilayered epithelium thus obtained is well differentiated, as shown by the presence of keratohyaline granules and horny layer, several differences from its in vivo counterpart were also observed: (1) In the reconstructed epidermis, basal keratinocytes do not have the cuboidal shape found in vivo; they synthesize bullous pemphigoid antigen and laminin, but the distribution of these antigens is not linear as in vivo; they contain the plasma-membrane antigens restricted to the basal layer in vivo (VM_1, BC_1), but these antigens are not polarized; lack of polarization is also evidenced by the distribution of actin. (2) Differentiation markers appear but with a topography slightly different from that of epidermis in vivo; the 67-kD keratin does not appear in the first suprabasal layer as in vivo but above; involucrin, which appears in the granular layers in vivo appears as soon as the cells leave the basal layer. (3) ψ_3 antigen and fibronectin found in vivo only in hyperproliferative epidermis (wound healing, psoriasis) are detected. Hyperproliferation would also explain the unexpected staining of basal cells by KL1 monoclonal antibody. Because of the potential clinical or pharmacologic use of artificial epidermis, the question of whether the epidermis obtained in vitro can be considered as "normal" is discussed. *J Invest Dermatol 86:181–186, 1986*

Human epidermis is a stratified squamous epithelium characterized by a high keratin content and by the ability to make cornified envelopes. Schematically, it can be divided into 2 cell compartments: a germinative one consisting of the basal cell layer, and a differentiating one consisting of the suprabasal cell layers, namely the spinous, granular, and horny layers, the latter being in contact with the air. When keratinocytes leave the basal compartment and progress upward in the epidermis, they undergo sequentially several differentiation changes and stop dividing. Morphogenesis and differentiation of epidermis are thus coupled, since most of the cells in the same layer are at the same stage of differentiation. For example, a new set of keratins (acidic 56.5 kD and basic 67 kD), appears in the spinous layers; involucrin, a precursor of the cornified envelopes, usually appears in the granular layers defined by the presence of keratohyaline granules. Cross-linking of involucrin leads to the formation of the cornified envelopes in the horny layers.

Since keratinocytes can be grown in tissue culture, they provide an interesting system to study histogenesis in vitro. So far, most of the information dealing with proliferation and differentiation of keratinocytes has been obtained in conventional cultures where cells are attached to plastic and submerged in the culture medium. More physiologic conditions would therefore require: (1) the attachment of the cells on a matrix; (2) contact of the cultured cells with the atmosphere.

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Abbreviations:
BP antigen: bullous pemphigoid antigen
BSA: bovine serum albumin
MAb: monoclonal antibody(-ies)
PAb: polyclonal antibody(-ies)
PBS: phosphate-buffered saline

To achieve these goals, the first approach consisted in culturing human epidermal cells on air-exposed de-epidermized human dermis to which the basement membrane remains associated [1]. Another approach has also been developed recently: collagen and fibroblasts are combined to form a lattice or dermal equivalent [2] on which epidermal cells are seeded. This culture, after air exposure, results in the reconstruction of a multilayered epidermis on the lattice [3–5]. However, little is presently known about the physiology of keratinocytes in this "artificial" epidermis. For this reason, and because of the interest in artificial skin for surgery [6–8] and for dermatologic and pharmacologic research [5,9], a study of the differentiation and organization of the artificial epidermis was undertaken, in comparison with epidermis in vivo.

MATERIALS AND METHODS

Cultures

Cell Cultures: GM 10 human embryonic skin fibroblasts (from NIGMS human genetic mutant cell repository, Camden, New Jersey) and epidermal cells were both cultured in Eagle's minimal essential medium, supplemented with 10% fetal calf serum [10] (culture media and supplements from GIBCO, U.K.) in the absence of growth factors [11]. Adult interfollicular epidermal cells were isolated [12] from human breast skin and seeded in collagen type I coated dishes (from CERAD, Lyon, France) at a density of $0.2.10^6$ cells/cm^2 and grown in primary cell culture to confluency before use. At this stage, the cells were trypsinized and frozen in liquid nitrogen in the presence of 7.5% dimethyl sulfoxide (Merck, F.R.G.). The procedure was not found to interfere with cell viability or cell attachment.

Lattice or Dermal Equivalent Fabrication and Epidermal Cell Seeding: Lattices were prepared according to the principles originally described [2] except that we pooled all solutions but collagen, the last compound to be added (purified type I calf skin collagen from Professor J. Frey, St. Etienne, France, and commercially from CERAD). This procedure allows highly reproducible fabrication of large numbers of dermal equivalents. Details of our routine

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procedure were given previously [5]. Epidermal cells were seeded on the dermal equivalent, using stainless-steel rings as previously described [5].

Tissue Culture: Dermal equivalents seeded with the thawed passaged epidermal cells were cultured for 1 week, submerged in the culture medium to allow epidermal cells to form a confluent monolayer. The cultures were then raised at the liquid–air interface on stainless-steel grids [5]. Another week was found necessary to obtain both stratification and differentiation of the cultures. At this point, samples were collected and processed.

Processing of Tissue Samples and Storage: For histology, cultures were fixed in 10% formalin and embedded in paraffin. For indirect immunofluorescence studies, unfixed samples were rinsed in phosphate-buffered saline (PBS), embedded in Tissue-Tek OCT compound (Miles, Naperville, Illinois), frozen in liquid nitrogen, and stored at −80°C until used. Control samples of human breast skin and psoriatic skin biopsies were treated in the same way.

Histology: Vertical paraffin sections were stained with haemelum-phloxine-saffron.

Immunofluorescence Antisera FN 1213 goat affinity-purified polyclonal antibody (PAb) against human plasma fibronectin was a kind gift of Dr. K. M. Yamada (National Cancer Institute, Bethesda, Maryland). Bullous pemphigoid sera were obtained from Professor J. H. Saurat (Geneva, Switzerland). Rabbit polyclonal antisera, affinity purified against laminin, were obtained from Institut Mérieux (Lyon, France) and Institut Pasteur (Lyon, France). The B11-1 rabbit polyclonal antiserum against desmosomal proteins [13] of bovine muzzle epidermis, the rabbit polyclonal antisera against involucrin, and 67-kD keratin [14] were generous gifts of Dr. M. Steinberg (Princeton University, Princeton, New Jersey), Dr. H. Green (Harvard Medical School, Boston, Massachusetts), and Dr. J. Viac (INSERM, Lyon, France), respectively; ψ_3 monoclonal antibody (MAb), obtained by immunization of mice with Triton-insoluble proteins from psoriatic tissue [15] and VM_1 MAb obtained by injecting mice with keratinocytes from a psoriatic plaque [16] were generous gifts of Dr. J. Mansbridge (Psoriasis Research Institute, Stanford, California) and Dr. V. Morhenn (Stanford University Medical School). BC_1 is a MAb (CIRD, Valbonne, France) prepared by injecting SV40-transformed K14 cells [17] as antigen (Bernard BA, Asselineau D, Schaffar L, Robinson SM, Darmon M, unpublished observation). KL1, a MAb against human keratins [18], was obtained commercially (Immunotech, Marseille, France) and KG8-13, a MAb against bovine keratins [19] was a generous gift of Dr. B. Geiger (Weizmann Institute of Science, Rehovot, Israel). Fluorescein and rhodamine conjugates were purchased from Cappel Laboratories (Downington, Pennsylvania), Nordic (The Netherlands), and Dako (Denmark). Rhodamine phalloidin was purchased at Molecular Probes (Junction City, Oregon).

Immunofluorescence Technique: Vertical frozen sections (5 μm) of tissue samples were washed in PBS containing 3% bovine serum albumin (BSA) to remove the Tissue-Tec OCT compound, and slides were then incubated directly for 30 min at room temperature with the first antibody at the appropriate dilution in PBS containing 3% BSA (ELISA grade, Sigma). After extensive washing, the slides were then incubated for 30 min at room temperature with fluorescein or rhodamine conjugates, diluted in PBS containing 3% BSA. Sections with normal sera were used as controls. After washes in PBS, the slides were mounted in 90% glycerol in PBS containing 5 mM *p*-phenylenediamine [20]. Preparations were examined using a Zeiss standard microscope equipped with UV-epi-illumination, and photographed using Kodak Tri-X film.

RESULTS

The artificial epidermis was compared with the epidermis in vivo, namely, epidermis of human breast skin from which the cells were isolated for culture (see *Materials and Methods*). Therefore, human breast epidermis represents the in vivo counterpart to the epidermis reconstructed in vitro and is taken here as the reference for "normality."

Morphology of the Artificial Epidermis Stained paraffin sections of the artificial epidermis obtained in vitro can be compared with that of epidermis in vivo (Fig 1). Although in the reconstructed epidermis multilayering as well as terminal differentiation can be seen (keratohyaline granules and horny-like layers), some differences with epidermis in vivo can be detected: (1) the tissue obtained during the time allotted for stratification and differentiation (7 days) is thicker than epidermis in vivo; this is in good agreement with our previous studies showing that the reconstructed epidermis continues to stratify for as long as 20 days [5]; (2) the cell density is probably smaller, since the size of the cells seems to be greater; and (3) the nuclei at the level of the basal layer are flattened, suggesting that the basal cells lack a cuboidal shape.

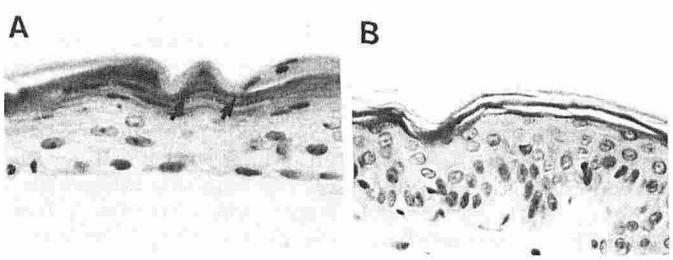

Figure 1. Morphology of (*A*) human epidermis reconstructed in culture and (*B*) epidermis of human breast skin. In the former, *arrows* point at keratohyaline granules. Vertical paraffin sections were stained with haemalum-phloxine-saffron. × 200.

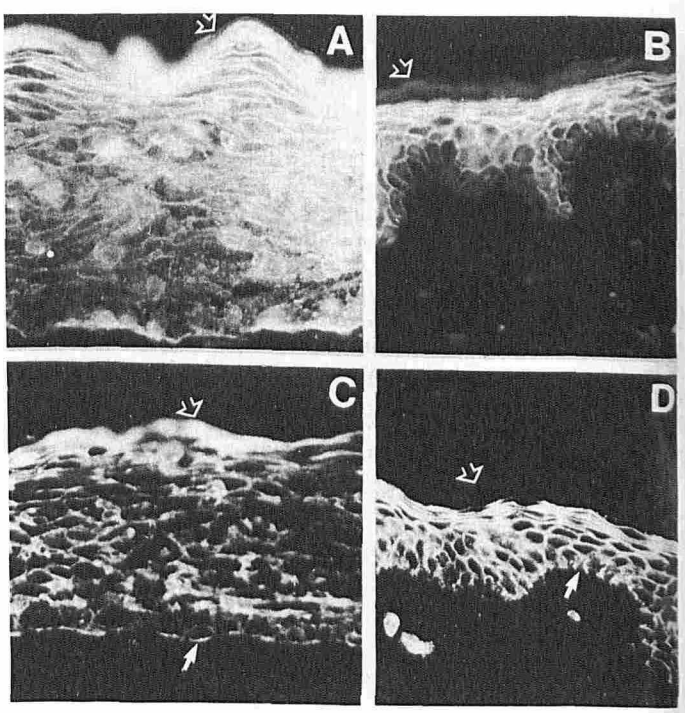

Figure 2. Distribution of desmosomes (*A,B*) and actin (*C,D*) in epidermis reconstructed in vitro (*A,C*) and epidermis in vivo (*B,D*). Frozen sections were reacted with rabbit antiserum against desmosomes and rhodamine phalloidin. *Empty arrows* point at the edge of cornified layers (*A–D*) and *solid arrows* point at the basal pole of basal cells (*C,D*). × 200.

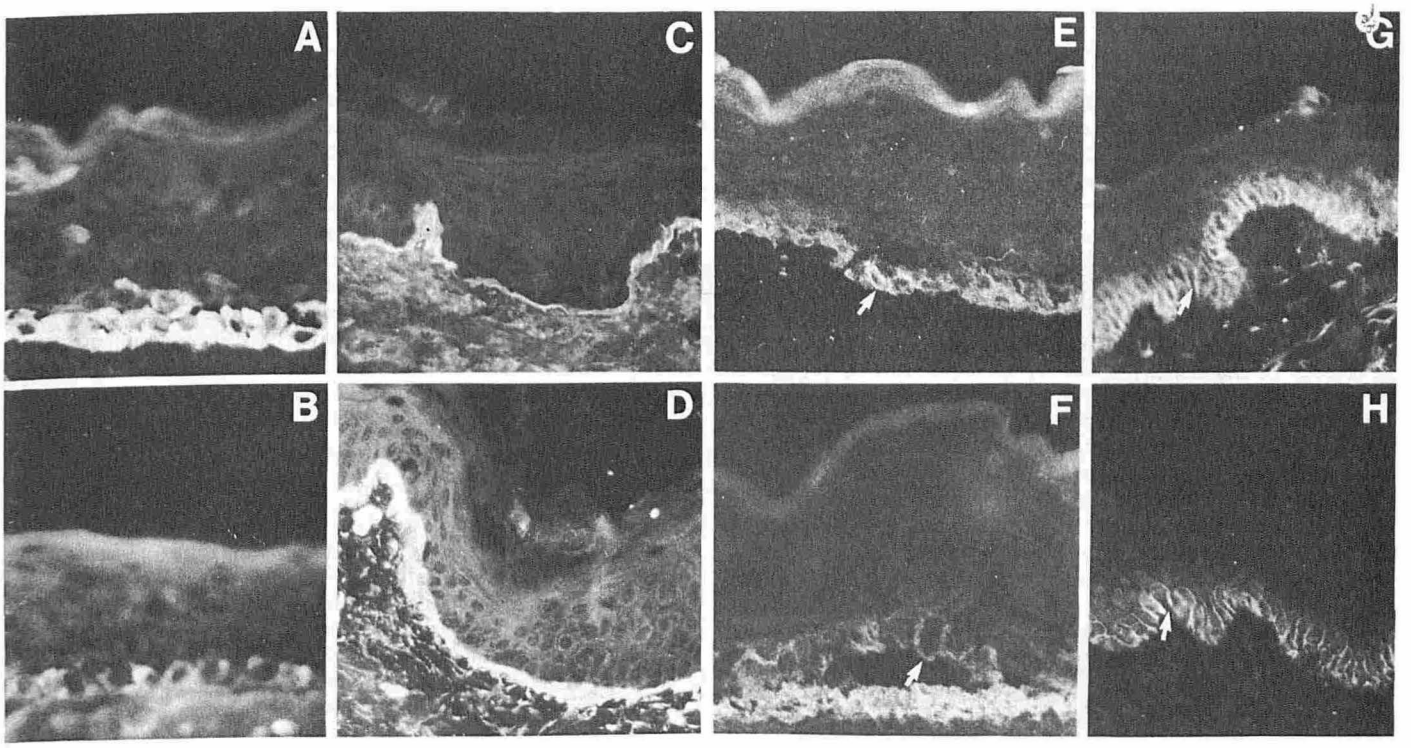

Figure 3. Localization of BP antigen (*A,C*), laminin (*B,D*), and VM_1 (*E,G*) and BC_1 (*F,H*) antigens in epidermis reconstructed in vitro (*A,B,E,F*) and in epidermis in vivo (*C,D,G,H*). Frozen sections were reacted with BP serum, rabbit antiserum against laminin, and VM_1 and BC_1 MAb. *Arrows* point at the basal pole of basal keratinocytes (*E–H*). × 200. Note that the basal pole is stained in the artificial epidermis, but not in the in vivo epidermis.

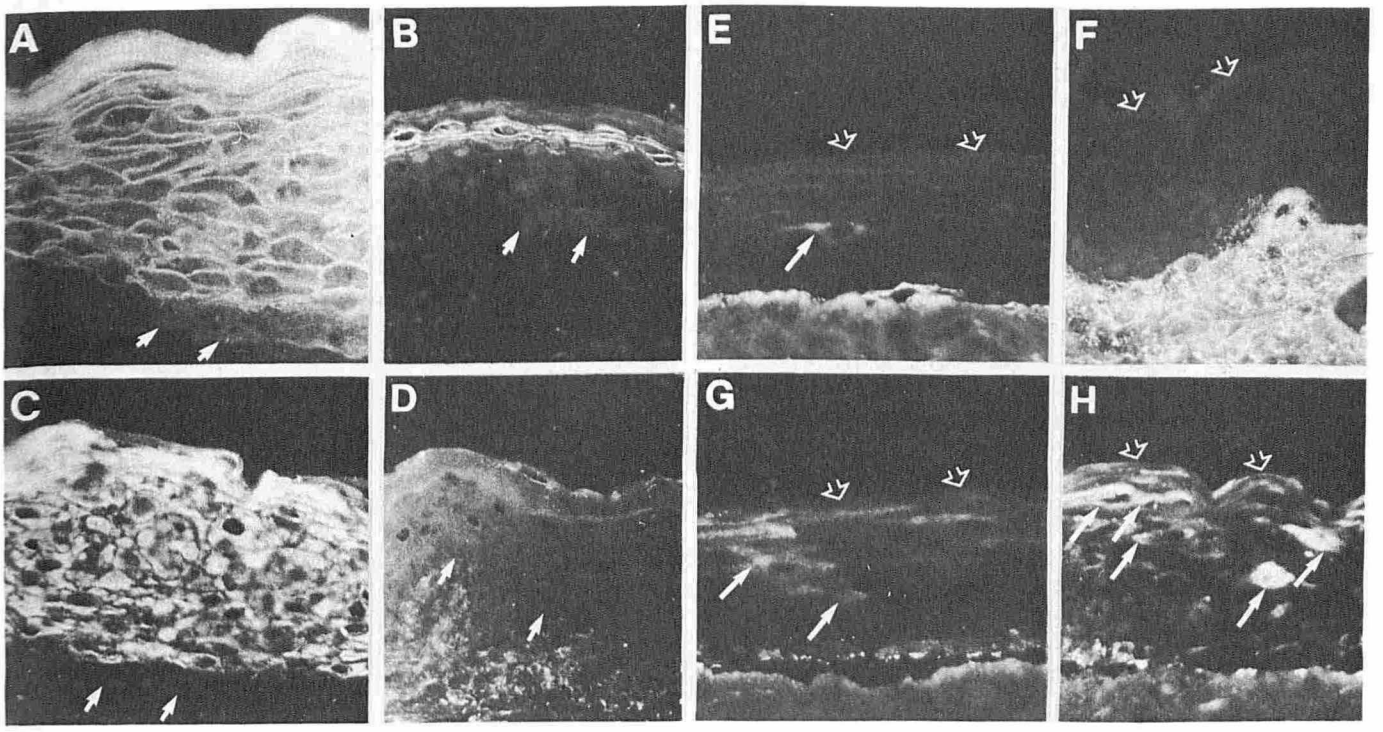

Figure 4. Distribution of involucrin (*A,B*), ψ_3 antigen (*C,D*), and fibronectin (*E–H*) in epidermis reconstructed in vitro (*A,C,E,G,H*) and epidermis in vivo (*B,D,F*). Frozen sections were reacted with rabbit antiserum against involucrin, ψ_3 MAb, and goat antiserum against fibronectin. *Short solid arrows* point at the dermal-epidermal junction (*A–D*), *long solid arrows* indicate fibronectin labeled epidermal cells (*E,G,H*) and *empty arrows* point at the edge of the upper layers (*E–H*). × 200.

Desmosomes and Actin Staining of desmosomes with antidesmosome PAb as well as staining of actin with phalloidin [21], seem at first glance similar in the artificial and the in vivo epidermis (Fig 2). However, a closer examination reveals that whereas the horny layers are not stained in vivo, they are stained in vitro both with antidesmosome antibodies and phalloidin. Thus, some impairment of terminal differentiation probably exists in the artificial epidermis. In addition, particularly interesting is the fact that a staining of actin can also be observed at the basal pole of basal keratinocytes in the artificial epidermis (Fig 2*C*) contrary to what is found in vivo (Fig 2*D*). This result suggests that the polarity of basal keratinocytes might be altered in vitro.

Attachment and Polarity of the Basal Keratinocytes in the Artificial Epidermis

Localization of Bullous Pemphigoid (BP) Antigen and Laminin: Frozen sections of artificial and normal skin were reacted with human BP antisera and affinity-purified antilaminin PAb. We observe an intracellular staining of the basal cells of the artificial epidermis (Fig 3*A,B*), instead of the linear staining found in vivo (Fig 3*C,D*).

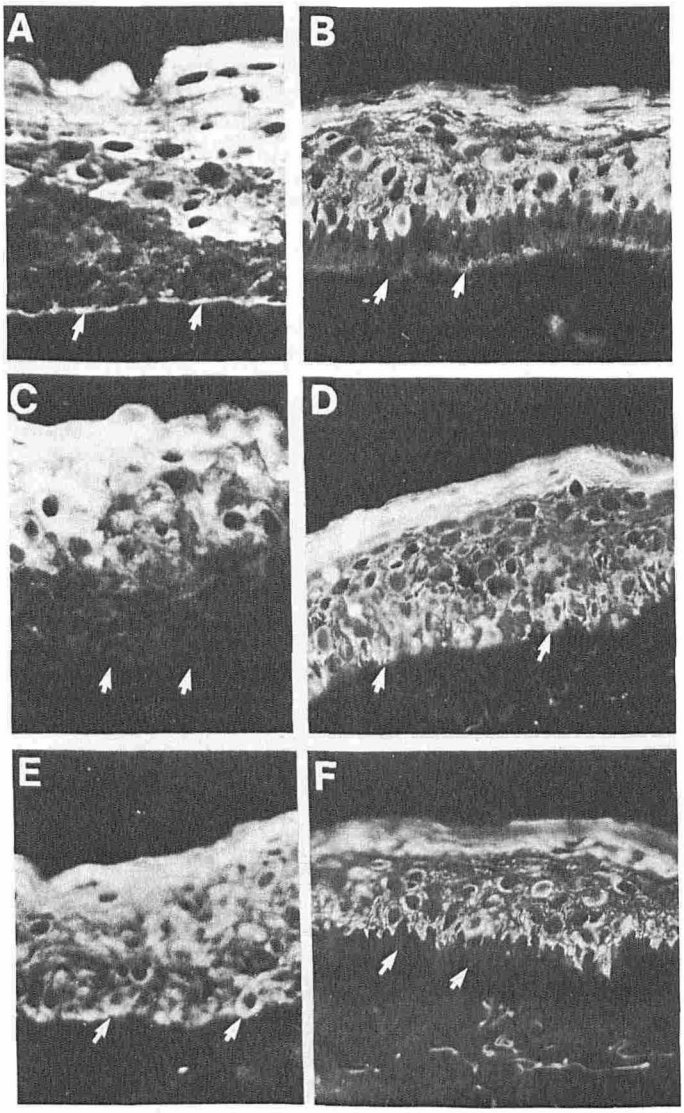

Figure 5. Staining of keratins in epidermis reconstructed in vitro (*A,C,E*) and epidermis in vivo (*B,D,F*). Frozen sections were reacted with rabbit antiserum against the 67-kD keratin (*A,B*), and KG8.13 (*C,D*) and KL1 (*E,F*) MAb. *Arrows* point at the dermal-epidermal junction. × 200.

Staining of basal cells with VM_1 and BC_1 MAb: VM_1 [16] and BC_1 (Bernard, Asselineau, Schaffar, Robinson, Darmon, unpublished observation) are 2 MAb recognizing specifically plasma membrane antigens of basal cells in vivo (Fig 3*G,H*). VM_1 and BC_1 antigens are also restricted to the basal layer of the artificial epidermis (Fig 3*E,F*). The fainter labeling of the cells grown on the dermal equivalent might be due to the fact that these cells are generally larger in size as compared with basal cells in vivo. Thus, an equal amount of plasma antigen would be diluted in the enlarged cells. In addition, in vivo the staining is restricted to the apicolateral faces of the cells, whereas in the artificial epidermis, the basal face of the membrane is also stained. These results seem to confirm that the polarity of basal cells is altered in vitro.

In order to better study the differentiation pattern of the artificial epidermis, an immunofluorescence study of specific differentiation markers was undertaken.

Differentiation of Suprabasal Keratinocytes in the Artificial Epidermis

Distribution of Involucrin and ψ_3 Antigen: Involucrin, one of the soluble precursors of the cross-linked envelopes of horny cells [22–24] can be stained with specific rabbit PAb. Fig 4*A,B* shows that involucrin is revealed only in the granular layers of human epidermis, although all the suprabasal layers of artificial epidermis can be stained with the antibody. A similar distribution of involucrin has recently been described in psoriatic epidermis [25,26] and in epidermis during the wound healing process [27]. To further investigate these similarities, we used ψ_3 MAb, which recognizes a protein found in the suprabasal layers of epidermis in psoriasis and other hyperproliferative diseases, and in wound healing [15], which, however, is not detected in normal epidermis (Fig 4*D*). Fig 4*C* shows that, as in psoriasis, ψ_3 MAb decorates the suprabasal layers of artificial epidermis.

Presence of Fibronectin in the Suprabasal Cells of the Artificial Epidermis: In normal skin, fibronectin is essentially located at the level of the papillary dermis [28]. Its presence in the basement membrane zone is still discussed [29], but it is not normally found in epidermis. In the present study, the absence of fibronectin in keratinocytes of normal skin was confirmed (Fig 4*F*), but intracellular fibronectin was found in single cells or small clusters of cells in the artificial epidermis (Fig 4*E,G,H*). It is noteworthy that similar observations have been reported for psoriatic skin [26,30].

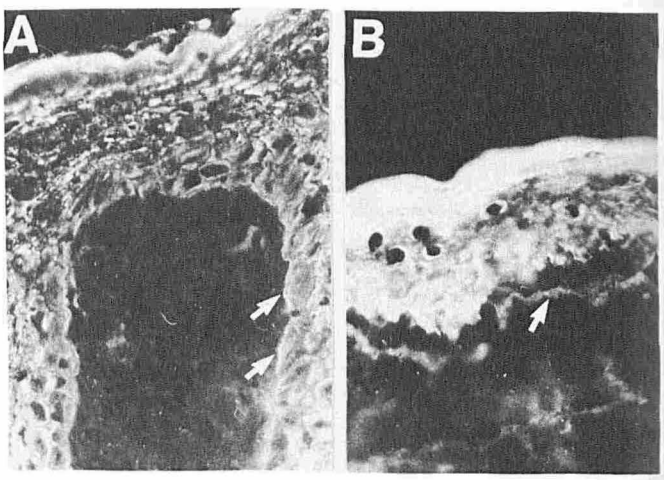

Figure 6. Double-immunofluorescence staining of the basement membrane *(arrows)* and keratins in a stable psoriatic lesion (*A*) and in normal epidermis (*B*). Frozen sections were reacted both with BP serum and KL1 MAb. Note that in the stable psoriatic lesion (*A*) all basal cells are KL1-positive while in normal skin (*B*) basal cells are KL1-negative. × 200.

Staining of Keratins with Anti-67-kD PAb, KG8.13 and KL1 MAb: The anti-67-kD PAb stains all suprabasal layers of the epidermis in vivo (Fig 5*B*), but only the upper suprabasal layers of the artificial epidermis (Fig 5*A*). This result confirms that keratinocytes undergo a terminal differentiation program in the artificial epidermis. Biochemical demonstration of the synthesis of the 67-kD keratin polypeptide in the artificial epidermis is given elsewhere [31].

KG8.13 MAb stains homogeneously all layers of human epidermis in vivo (Fig 5*D*), but in artificial epidermis, the staining is essentially observed in the superficial layers (Fig 5*C*). As shown in Fig 5*F*, KL1 MAb stains specifically the suprabasal layers of interfollicular epidermis in vivo [18], but in addition to suprabasal layers, this antibody also stains the basal layer of the artificial epidermis (Fig 5*E*). A similar pattern was observed with KL_1 MAb in the stable lesion of psoriatic skin (Fig 6) and in wound healing [27].

DISCUSSION

Results reported here strongly suggest that, as in normal skin, keratinocytes grown on a dermal equivalent at the liquid–air interface stratify and enter the terminal differentiation pathway since: (1) basal cells express bullous pemphigoid (BP) antigen and laminin, and display VM_1 and BC_1 antigens on their plasma membrane; (2) suprabasal cells synthesize involucrin and the 67-kD keratin; (3) keratohyaline granules are present in a recognizable "granular layer"; and (4) a horny layer is formed.

The differences between the epidermis reconstructed in vitro and its in vivo counterpart do not affect the presence or absence of the differentiation markers, but only slightly alter their topology. These topologic differences are the following. The presence of laminin and BP antigen in the cytoplasm instead of their linear localization apparently suggests that attachment of the basal layer of the artificial epidermis to the lattice is altered. However, recent studies have demonstrated that striking regional variation occur in vivo in human epidermis in the expression of the BP antigen [32]. The loss of polarity that we observed (modified pattern of actin and of VM_1 and BC_1 antigens) might be related to this altered attachment. Altered attachment may also be responsible for the precocious expression of some suprabasal characters in the artificial epidermis: for instance, involucrin normally detected in the granular layers of epidermis in vivo appears in the artificial epidermis as soon as the cells leave the basal layer, showing that under these conditions, commitment to synthesize involucrin occurs when they lose contact with the substrate. Although precocious involucrin synthesis has been reported in pathologic conditions such as psoriasis [25,26] or squamous cell carcinomas [25,33], it is also well-established that the epidermal layer in which involucrin synthesis begins varies considerably in nonpathologic squamous epithelia [34]. On the contrary, the appearance of the 67-kD keratin which is induced as soon as the cells leave the basal layer in normal epidermis probably is delayed in the artificial epidermis. Although masking phenomena might be taken into account [35], these observations suggest a shift in the timing of the differentiation program as evidenced by the 67-kD keratin and involucrin induction in the artificial epidermis.

The antikeratin MAb KL1 decorates only suprabasal layers in normal epidermis, but all layers in artificial epidermis. This suggests either that a component normally appearing in vivo in the suprabasal layers is already present in the basal layer of artificial epidermis, or that a particular keratin polypeptide absent in normal epidermis is synthesized in the basal keratinocytes of the artificial epidermis. Since one-dimensional immunoblots have previously shown that this MAb recognizes a 56-kD keratin [18], one possibility is that the unexpected labeling of keratins observed here in the basal cells of the artificial epidermis is due to the abnormal presence of the 56.5-kD acidic keratin which in vivo appears in suprabasal cells associated to the basic 67-kD keratin. However, this is unlikely since we show that the 67-kD keratin cannot be detected with anti-67-kD PAb in the basal layer of either normal or artificial epidermis. Another possibility is that KL1 labeling of the basal cells is due to the presence of the 56-kD basic keratin, a keratin associated with the 48-kD acidic keratin in normal hyperproliferative epidermis such as sole epidermis [36]. This possibility is more likely since we have demonstrated biochemically that the acidic 48-kD keratin is synthesized in the artificial epidermis [31]. The fact that similar KL1 immunofluorescence patterns were obtained with psoriatic skin as shown here or during wound healing of human skin [27] is in good agreement with this interpretation, especially since it is now demonstrated that psoriatic epidermis, contrary to normal nonhyperproliferative epidermis, contains the 56-kD basic keratin [36]. Thus, it appears that the artificial epidermis shares some features with healing or psoriatic epidermis. The presence of fibronectin, involucrin, and ψ_3 antigen in the suprabasal layers is in agreement with this interpretation. However, one important difference with psoriasis is that we observe the presence of keratohyaline granules in the artificial epidermis.

In conclusion, the in-vitro system described here represents a promising tool both for basic and clinical or pharmacologic research. Future studies will tell whether modifications of the culture conditions allow the reconstruction of a more "normal" epidermis.

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