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# Ultrastructural studies of cultured human epithelial sheets used as skin allografts

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**Summary.** In this work the ultrastructural features of cultured epithelial sheets (CES) used as skin allografts in humans are described, before and at various times after grafting. Prior to grafting, CES consisted of 4–5 layers of keratinocytes of a low to moderate degree of differentiation. However, after grafting, the CES developed progressively but rapidly features of a well-differentiated epidermis (including melanocytes and Langerhans' cells) and a dermal-epidermal junction. No evidence of rejection was observed. These results demonstrate the key role of normal dermis in the maturation of the surface epithelium and prove the suitability of CES as skin allografts.

**Key words:** Cultured epithelia-skin allografts-ultrastructure

Recent advances in culture techniques have rendered possible the culture of human epidermal keratinocytes (EK) and the production of thin living epithelia suitable for the coverage of large burns or wounds (Gallico et al. 1979). These were first used as skin autografts; however the absence of Langerhans' cells (LC) and other HLA-class II antigen-bearing cells from these cultured epithelial sheets (CES) makes also possible their use as skin allografts, despite an antigenic donor-recipient histoincompatibility (Thivolet et al. 1986). In this work we report on the ultrastructural differentiation of the CES prior to grafting and at various intervals after allografting.

## Material and methods

The production of CES was carried out as described in detail previously (Thivolet et al. 1986). Briefly, EK from epidermal-cell suspensions from donors were grown onto 3T3 feeder-cell layers, according to the method of Green et al. (1979). The CES obtained in this way were grafted on the graft-taking area (a 120 $cm^2$ area of the forearm) of 10 patients suffering from chronic leg ulcers covered by conventional split-thickness skin autografts. A total of 17 biopsies of the allografts were taken under local anaesthesia at various intervals, starting at day 5 after grafting (see Table 1). These were processed for standard electron-microscopic (EM) examination (fixation in 2% glutaraldehyde, post-fixation with osmiun tetroxide, embedding in epon). Semithin an ultra-thin sections were cut on a Reichert OmU3 microtome, and the latter contrasted with uranyl-acetate and lead citrate and examined under a Philips EM 300 electron-microscope.

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## Results

Cultured epithelial sheets were detached from the culture flasks using dispase. Under EM examination, they were seen to consist of 4–5 rows of EK with features of a moderate to low differentiation degree (Fig. 1a–b): basal EK were grossly cuboidal, had a rather large, centrally located, round or oval-shaped nucleus with marginated chromatin. On their basal pole they presented some microvilli, but neither hemidesmosomes (HD) nor any other basement membrane (BM) components were

**Table 1.** Patients and dates at which biopsies were studied by electron microscopy

| | Days after grafting | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Patients | 5 | 7 | 12 | 14 | 21 | 28 | 60 | 75 | 90 |
| CG | | | | + | | | | | |
| JL | | | | + | | + | | | |
| DM | + | | | + | | + | | | |
| AM | + | | | + | | + | | + | |
| SR | | + | | | | | | | + |
| CM | | | + | | | | | | |
| PL | | + | | | | | | | |
| BP | | | | + | | | | | |
| SC | | | | | + | | | | |
| BM | | | | | | | + | | |

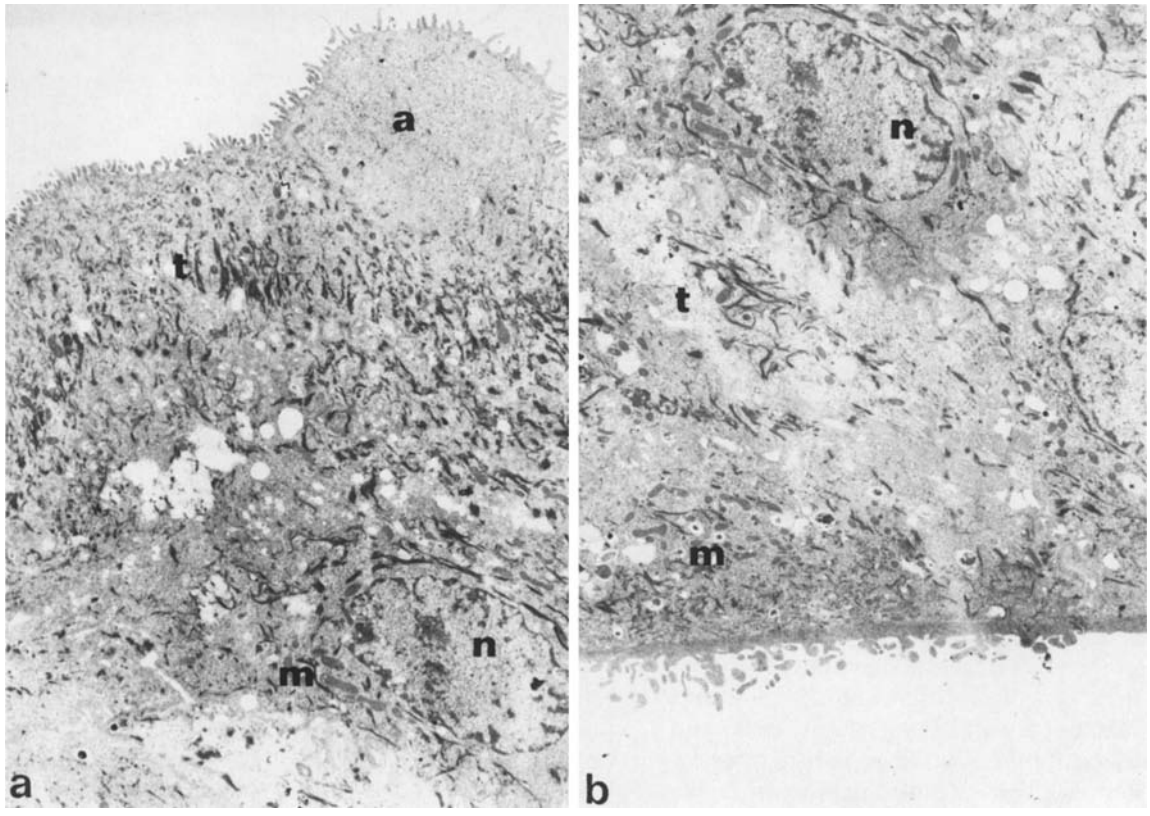


**Fig. 1.** Low-power micrograph of a cultured epithelial sheet (**a** upper half, **b** basal half): it consists of keratinocytes with a large nucleus (*n*), numerous mitochondria with an electron-dense matrix (*m*), sparse bundles of tonofilaments (*t*) and small desmosomes. The uppermost cells are devoid of nucleus (*a*). Note the presence of numerous microvilli, especially on the upper surface ( × 2,050)

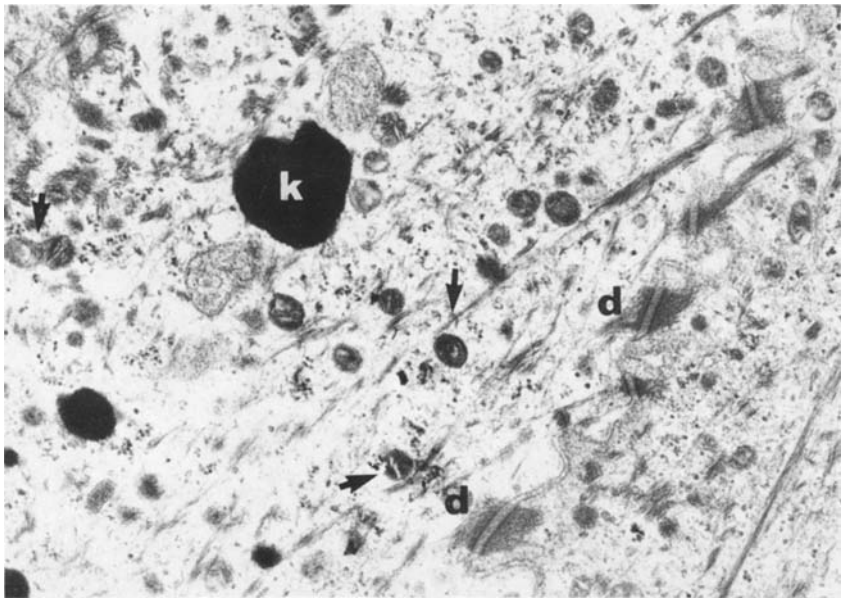


**Fig. 2.** Granular layer of an allograft at D5. Note the keratohyalin granules (*K*), lamellar bodies (*arrowheads*) and small desmosomes (*d*) ( × 16,000)

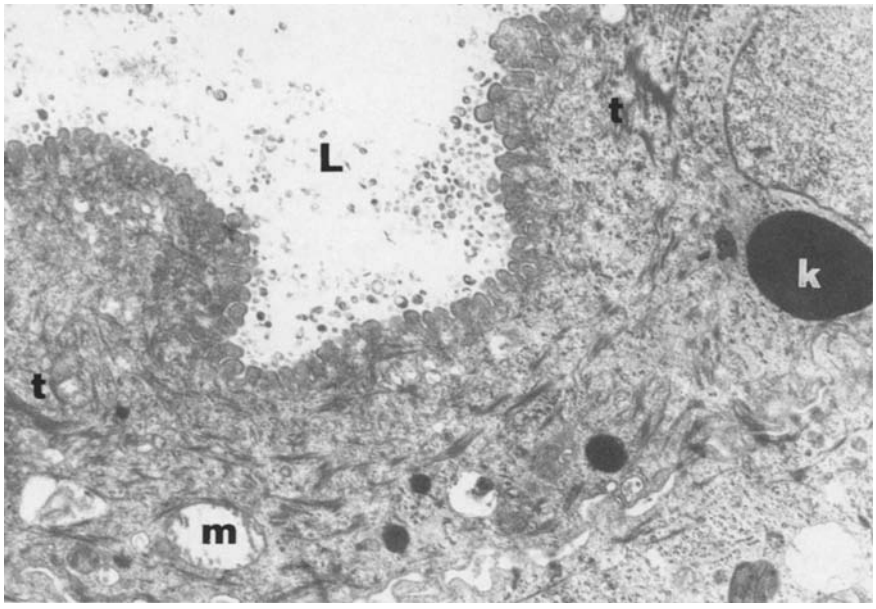


**Fig. 3.** An acrosyringium with typical ultrastructural features is running through an allograft at D14. Note characteristic, rounded keratohyalin granules (*K*), tonofilaments (*t*), mitochondria (*m*) and the lumen (*L*) with a brush-border (×8,200)

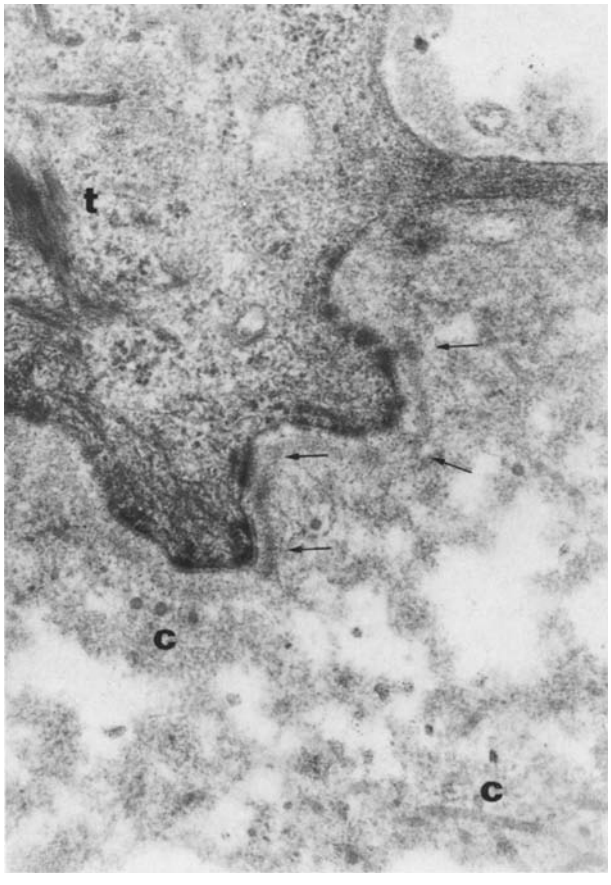


**Fig. 4.** On D12, the dermal-epidermal junction presents hemidesmosomes and a lamina densa (*arrows*) of variable thickness. *t*: tonofilaments; *c*: collagen fibers (×20,000)

seen. Tonofilaments (TF) formed slender bundles scattered in the cytoplasm and became more abundant towards the upper cellular layers. Desmosomes (DS) were present throughout the whole thickness of the CES. They presented their characteristic structure, consisting of alternating electron-dense (5) and electron-lucent (4) zones; however they were somehow smaller in size than those of a normal epidermis. Keratohyalin granules (KG) were small and sparse; they had a round, rather than a stellate shape, and were observed in the upper cellular layers. Only few lamellar bodies (LB) were seen in the cytoplasm of the upper cellular layers. Mitochondria were numerous throughout the CES and had an electron-dense matrix. The cells of the uppermost layer were generally devoid of nucleus and presented a relatively electron-lucent cytoplasm and numerous slender surface microvilli. All the cells observed had features of epithelial cells (tonofilaments, desmosomes). Clear cells (melanocytes (MC), Langerhans (LC), Merkel (MK) cells) and melanosomes were absent from all of the specimens studied.

After grafting, the CES progressively developed features of well differentiated epidermis. The following features were observed with time:

*Day 5*: the thickness of EAG increased to reach 12–15 cellular layers. Of these, the horny layer consisted of 6–7 rows of corneocytes with a normal-looking appearance, except that they contained some lipid droplets. KG, although still reduced in

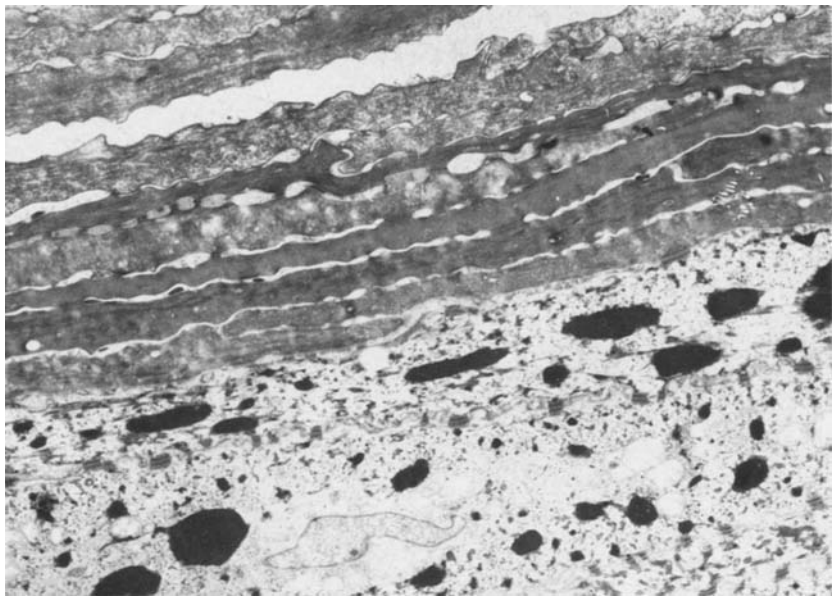

**Fig. 5.** Upper layers of an allograft at D14. Note the abundance of normal-looking keratohyalin granules and the characteristic morphology of corneocytes inbetween which remnants of desmosomal discs are seen (×1,900)

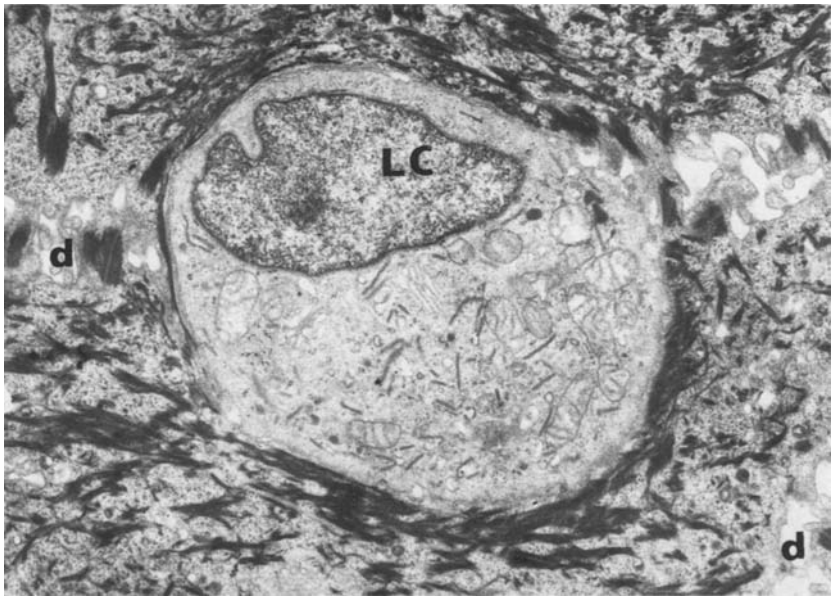


**Fig. 6.** A Langerhans cell (*LC*) containing numerous Birbeck granules, mitochondria and lysosomes is seen in an epithelial allograft on D28. The surrounding keratinocytes contain dense bundles of tonofilaments and well-developped desmosomes (*d*) (×5,600)

size and number as compared to a normal epidermis, were larger and more numerous than in CES, and so were LB that presented a characteristic internal lamellar structure (Fig. 2). At the level of the lower epidermis the intercellular spaces were slighty widened and a few necrotic EK were seen. No clear cells were seen in the epidermis. Basal EK presented at their basal pole regular densifications of their plasma membrane, highly reminiscent of the attachement plaque of HD. At places a lamina densa-like material of medium electron-density was seen, parallel to the undersurface of the epidermis. The superficial dermis contained a finely fibrillar material and in the mid-dermis an inflammatory (mainly lympho-histiocytic) cellular infiltrate of moderate intensity, along with occasional extravasated erythrocytes was seen.

*Day 7*: KG were much more abundant at the level of the granular layer, and so were LB that were observed both inside the cytoplasm and opening to the extra-cellular space. A striking feature was the presence, in one case, of a well developed acrosyringium running through the epidermis; it presented a characteristic structure with a narrow,

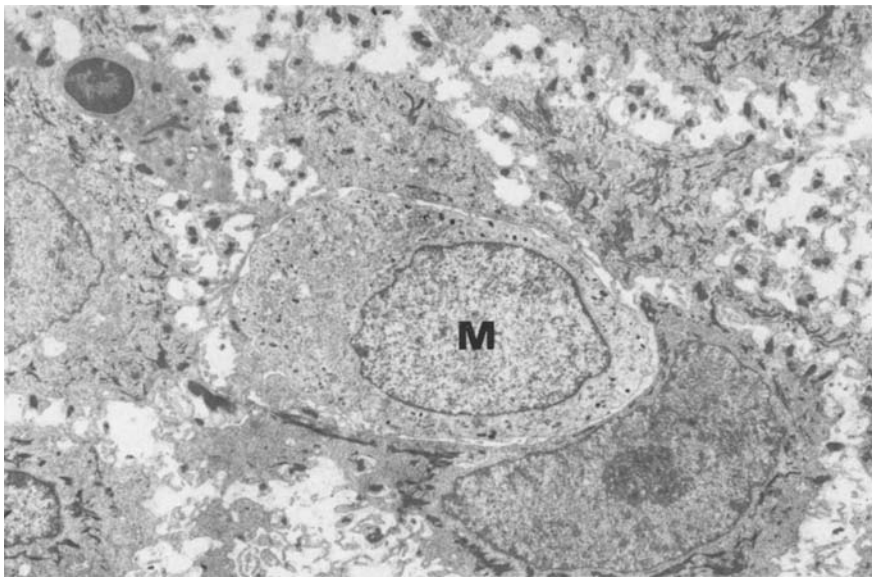


**Fig. 7.** A suprabasal melanocyte (*M*), containing numerous melanosomes, can be seen in an allograft on D14 (×3,300)

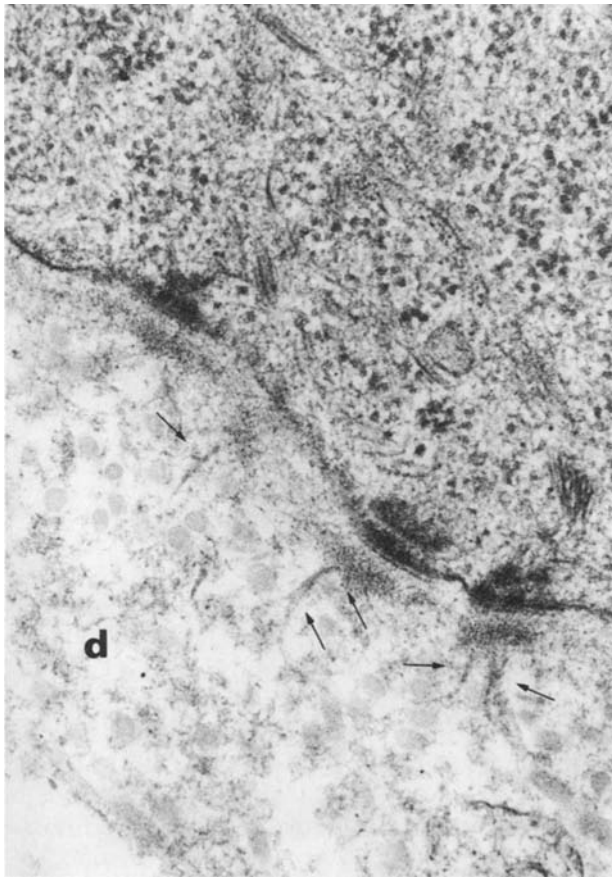


**Fig. 8.** On D14, at the level of the epidermal-dermal junction hemidesmosomes, the lamina lucida, lamina densa and anchoring fibrils (*arrows*) can be seen; *d*: dermis (×33,000)

slightly branching lumen with a brush border. The acrosyringial keratinocytes contained round KG, sparse bundles of TF and were interconnected to each other through small DS (Fig. 3).

*Day 12*: KG were increased in size and stellate; they were present in the uppermost 4–6 cellular layers of the viable epidermis. In the lower epidermal layers, some cells with features of indeterminate cells were seen (cells with a clear cytoplasm lacking TF, DS, MS or Birbeck granules (BG), and possessing a convoluted nucleus). However, no LC or MC were observed. At the level of the epidermal-dermal junction (EDJ), the lamina densa (LD) was distinctly visible (Fig. 4), although of variable thickness.

*Day 14*: by this time the EAG had assumed features of a fully-differentiated, relatively thick epidermis: the horny layer comprised 6–8 rows of corneocytes in between which desmosomal discs were seen. The granular layer consisted of 3–5 cellular layers containing typical KG and LB (Fig. 5). TF and DS had a normal appearance, although the intercellular spaces were slightly widened. Rare inflammatory cells (lymphocytes and polymorphonuclear leucocytes) were seen in some biopsies. Indeterminate cells were seen in most of the specimens studied. Furthermore, some rare LC with typical ultrastructural features (BG, sometimes bound to the cell membrane) were seen in a suprabasal location (Fig. 6). MC were also seen in a suprabasal location; although they were not nu-

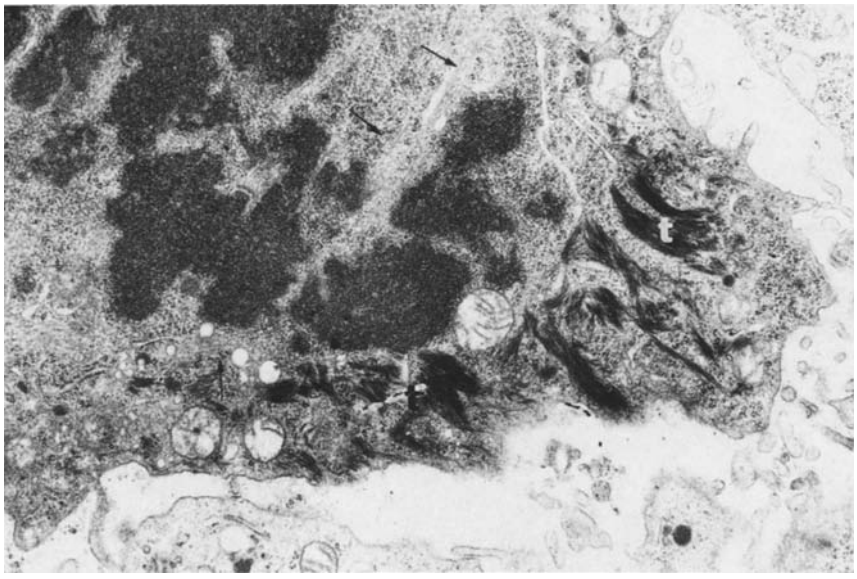

**Fig. 9.** A basal keratinocyte undergoing mitosis in an epithelial allograft is seen an D28. Note the presence of microtubules associated to the mitotic spindle (*arrows*); *t*: to-nofilaments (×6,800)

merous, they were unmistakable thanks to small but otherwise typical MS contained in their cytoplasm (Fig. 7). Basal EK contained typical hemidesmosomes and pinocytotic vesicles. The lamina lucida (LL) and the LD were well observable. Anchoring fibrils were relatively rare, but still discernable (Fig. 8). The underlying dermis contained some inflammatory cells, occasional myofibroblasts and fibroblasts with a well-developped endoplasmic reticulum.

*Days 21, 28*: LC were seen much more frequently than on previous biopsies; they were present not only in a suprabasal location but also higher in the epidermis, and contained numerous BG. In the basal-cell layer EK undergoing mitosis (Fig. 9) as well as occasional mast-cells were seen. The EDJ had an almost normal appearance, although at places small subepidermal vesicles were seen.

*Days 60, 75 and 90*: in the epidermis a normal number of dendritic LC rich in BG, mitochondria and lysosomes was seen. MC containing MS of various levels of maturation were seen, and the latter were also observed in adjacent EK. The EDJ presented a characteristic structure with HD, LL, LD and anchoring fibrils. Furthermore, the basal surface of basal ED presented small indentations protruding into the dermis, giving thus the EDJ an undulating appearance. In the papillary dermis small collagen fibers with a characteristic periodic striation were observed.

## Discussion

CES have been successfully used as autografts for the permanent coverage of burn wounds (Gallico et al. 1984). The suitability of the CES as allografts relies on the fact that these in vitro-grown epithelia are devoid of LC and other HLA-class II antigen-bearing cells. The absence of rejection of the allografted CES that was obvious upon clinical follow-up of the patients was underlined by the results of mixed epidermal-lymphocyte cultures, since epithelial cells from CES did not stimulate peripheral blood lymphocytes from the recipients (Thivolet et al. 1986). The absence of rejection was further substantiated upon light and electronmicroscopic examination by the morphological evolution of the grafted CES: despite a few necrotic EK and some inflammatory cells in exocytosis observed occasionally on early biopsies, the CES were not only maintained and present in all subsequent biopsies, but they also rapidly developed features of a well-differentiated epidermis. Mitotic figures infrequently observed among basal EK proved that these CES were capable of regeneration. The occasional presence of mast cells in the basal-cell layer as noted sometimes in the normal epidermis (Zelickson 1982) also points to normal differentiation of the allografts. The maturation of the CES was achieved progressively but rapidly after grafting. Indeed, the most striking morphogical changes were observed between the day of grafting and day 5 after grafting: by that date the number of cellular layers had increased threefold and the distinct epidermal compartments (str. basale, spinosum, granulosum and corneum) were already conspicuous, although all the features of a normal epidermis were not quantitatively present until later dates. The horny layer initially contained some lipid droplets (as observed in some keratinization disorders), but these disappeared from later

biopsies. The KG that were small, sparse and rounded on CES became progressively larger, more abundant and stellate, and looked normal by D12. The number of LB also increased rapidly, and the fact that by D7 they were seen not only in the cytoplasm of the cells of the upper malpighian layers but also in the extracellular space, suggested that a normal barrier function for the epidermis was possible. The DS increased rapidly in size and number and the TF became more abundant throughout the epidermis, whereas a numerical decrease of the mitochondria was observed. The differentiation of the surface epithelium also concerned the intraepidermal sweatduct unit (acrosyringium). This was observed as early as D7, and by that date it presented characteristic ultrastructural features. Its development most likely resulted from the multiplication of cells of the underlying dermal sweat-ducts of the recipient, as has already been suggested (Christophers and Plewig 1973). This was demonstrated by immunohistochemical studies that showed the acrosyringial keratinocytes to bear the recipient's blood-group antigens (Mauduit et al. 1987). A further differentiation feature of these acrosyringia was the expression of carcinoembryonic antigen and of HLA-DR antigens (Kanitakis et al. 1987). The presence of structurally normal acrosyringia suggests that a normal sweating function of the grafted area was possible by D7.

With respect to non-epithelial dendritic epidermal cells (LC, MC, MK) these were absent from the CES examined. For better evaluation of LC, immunohistochemical studies using antibodies to OKT6 and HLA-class II antigens further demonstrated their absence from the CES (Thivolet et al. 1986). After grafting, LC with a typical morphology-including Birbeck's granules-were first seen by EM on D14, although by IF studies these could be detected in small numbers on D7 (Faure et al. 1987). As a matter of fact, cells with features of indeterminate cells were seen already on D12; although the precise nature of these cells in debated, these cells could represent immature precursors of LC (Czernielewski et al. 1983). The number of intraepidermal LC increased with time and on later dates they were also seen at the level of the midmalpighian layers. The appearance of LC in the allograft is most likely due to the colonization of the surface epithelium by a circulating pool of these cells originating in the bone marrow (Katz et al. 1979). With respect to MC, these were first seen by EM in the grafted CES on D14, and contained already mature MS. On later biopsies, more and more dendritic processes in these cells were observed in the intercellular spaces insinuating between EK. By D75, MS were also observed in the cytoplasm of EK, a finding indicating that melanization of the epidermis was taking place. The origin of these MC is uncertain. It has recently been found that a small number of unpigmented premelanocytes may be present in cultures (Bennet et al. 1985), and therefore the possibility that these MC developed from premelanocytes present in cultures cannot be ruled out. However, another likely explanation is that the MC colonized the surface epithelium from underlying adnexal (follicular) structures (Staricco 1963). If that were the case, it would indicate that a MC-EK cooperation (in terms of MS transfer) can take place despite genetic heterogeneity of these cells. Merkel cells were not observed in any of the specimens studied, but this is not surprising if one considers the low density of these cells in the normal epidermis, especially of the arm (Dubois et al. 1986).

A final point of interest is the reconstitution of the EDJ. On CES, no components of the normal EDJ were observed by EM examination. Through IF studies, just prior to grafting, the CES showed a linear fluorescence at their basal surface when tested for the bullous pemphigoid (BP) antigen, but neither laminin (L) nor type IV collagen were found expressed on CES detached with dispase from the culture flasks. These antigens were detected as early as at D5 after grafting (Faure et al. 1987). By EM examination, the earliest discernable morphological component at the level of the EDJ was the plasma membrane of basal EK comprising densifications reminiscent of the attachement plaque of HD. These structures were present already at D5, even at places where the other components of the EDJ were completely lacking. The LL, LD and anchoring fibrils were first distinctly observed on day 12 and day 14, and by the latter date pinocytotic vesicles were seen on the basal pole of basal EK, pointing out to the functional integrity of these cells. It seems therefore that the antigenic differentiation of the EDJ preceeded the morphological one, as often happens when one deals with the BMZ. The traumatism due to the biopsy may well account for the dermo-epidermal cleavage observed in some early biopsies. However, despite relative early fragility, the structure of the EDJ was completely normal on later biopsies (D60 onwards), where its characteristic undulations were present.

In conclusion, this study demonstrates the key role of the normal dermis in the maturation and differentiation of the surface epithelium. This becomes obvious when the morphogical features of the CES prior and after grafting are considered. The epidermis reconstituted in this way comprises

all the morphological features of terminal differentiation, including LC, MC and acrosyringia, and can thus be considered mature also from a functional point of view. Taken together with the absence of rejection, this fact demonstrates the suitability of the CES as skin allografts.

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