Integration of Langerhans Cells into a Pigmented Reconstructed Human Epidermis

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The majority of *in vitro* reconstructed human epidermis is composed of keratinocytes only. Recently, the introduction of melanocytes into epidermal reconstructs has enlarged their field of application. The completion of reconstructed epidermis by introducing Langerhans cells remained an important challenge because Langerhans cells, unlike the other epidermal cell types, cannot be subcultured and expanded. To solve this problem, we used cord blood-derived CD34$^+$ hematopoietic progenitors. Seeding these cells, after induction of their differentiation by granulocyte macrophage-colony stimulating factor and tumor necrosis factor-α, onto a reconstructing epidermis, composed of keratinocytes and melanocytes, gives rise to a pigmented epidermis with melanocytes in the basal layer and resident epidermal Langerhans cells located suprabasally. Interestingly, the same result was obtained by co-seeding a mixture of keratinocytes, melanocytes, and nondifferentiated CD34$^+$ hematopoietic progenitors on the dermal equivalent, indicating that keratinocytes provide the environmental conditions for hematopoietic progenitors to differentiate into resident epidermal Langerhans cells, expressing major histocompatibility complex class II molecules, CD1a antigen, and Birbeck granules. ***Key words: hematopoietic progenitors. J Invest Dermatol 109:510–512, 1997***

When normal human keratinocytes, cultured on an appropriate support, are exposed at the air–liquid interface they differentiate and give rise to a multilayered stratified tissue, a reconstructed epidermis. These three-dimensional cultures have provided useful tools, not only to better understand the complex mechanisms of keratinocyte differentiation, but also as valid models in skin pharmacology and toxicology (Schmidt, 1990). Beside the keratinocytes, which account for about 80% of the epidermal cells, the epidermis also contains pigment-producing melanocytes and a specialized dendritic cell type, the Langerhans cell, that plays a major role in the skin immune defense system. Langerhans cells capture exogenous antigens, migrate through the dermis to the regional lymph nodes, and present them to naive T cells (Stingl *et al*, 1980; Moll, 1995).

The absence of melanocytes and Langerhans cells has excluded the use of the epidermal reconstructs in the fields of skin pigmentation and immunology. Recently, melanocytes were successfully integrated into reconstructed epidermis (Bessou *et al*, 1995).[1] The integration of Langerhans cells, however, remained a challenge particularly because these cells, unlike the other two cell types, cannot be subcultured and expanded *in vitro*. Various experiments performed in our laboratory to re-integrate freshly isolated human epidermal Langerhans cells into a reconstructed epidermis failed. The cells never formed dendrites and appeared eventually as pyknotic, round cells in its uppermost layers.

Langerhans cells are bone marrow-derived cells (Katz *et al*, 1979) that circulate in the peripheral blood as progenitors and, at a not yet identified state of their maturation, migrate into the epidermis and become resident dendritic Langerhans cells characterized by the presence of Birbeck granules and expression of the CD1a antigen (Rowden *et al*, 1977; Caux *et al*, 1996).

It is known that exposure of CD34$^+$ hematopoietic progenitors to granulocyte macrophage-colony stimulating factor (GM-CSF) and tumor necrosis factor-α (TNF-α) results in the generation of dendritic cells (DC) containing a subpopulation of Langerhans cells (Caux *et al*, 1992; Strunk *et al*, 1996; Caux *et al*, 1996). We have based our approach on these findings and used either *in vitro* generated DC/Langerhans cells or freshly isolated CD34$^+$ hematopoietic progenitors. The generated DC/Langerhans cells were seeded onto a reconstructing epidermis composed of keratinocytes and melanocytes; whereas the hematopoietic progenitors were co-seeded with the keratinocytes and melanocytes onto the dermal support. Under both conditions, the resulting epidermis contained pigment-producing melanocytes in the basal layer and suprabasally located Langerhans cells.

MATERIALS AND METHODS

Cells and culture conditions Normal human keratinocytes were grown in submerged cultures (Rheinwald and Green, 1975) and used after the third passage. Normal human melanocytes derived from split-thickness human skin were cultured in serum-free medium without phorbol ester and pituitary extract (Olsson *et al*, 1994) until the third passage.

CD34$^+$ hematopoietic progenitor cells were isolated from cord blood cells through positive selection by indirect immune staining and immunomagnetic mature cell depletion (Caux *et al*, 1992). The CD34$^+$ were used either directly or after 6 days of culture in the presence of GM-CSF, 200 ng per ml, 2×10^6 U per mg (Sandoz, Rueil-Malmaison, France), and TNF-α, 2.5 ng per ml, 1×10^7 U per mg (Genzyme, Cambridge, MA) (Caux *et al*, 1992).

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Abbreviations: DC, dendritic cells; GM-CSF, granulocyte macrophage-colony stimulating factor; MHC-II, major histocompatibility complex class II; TNF-α, tumor necrosis factor-α.

[1] Régnier M, Schmidt R: Reconstitution of an epidermal melanin unit by cocultures of normal human keratinocytes (NHK) and normal human melanocytes (NHM). *J Invest Dermatol* 102:596, 1994 (abstr.).

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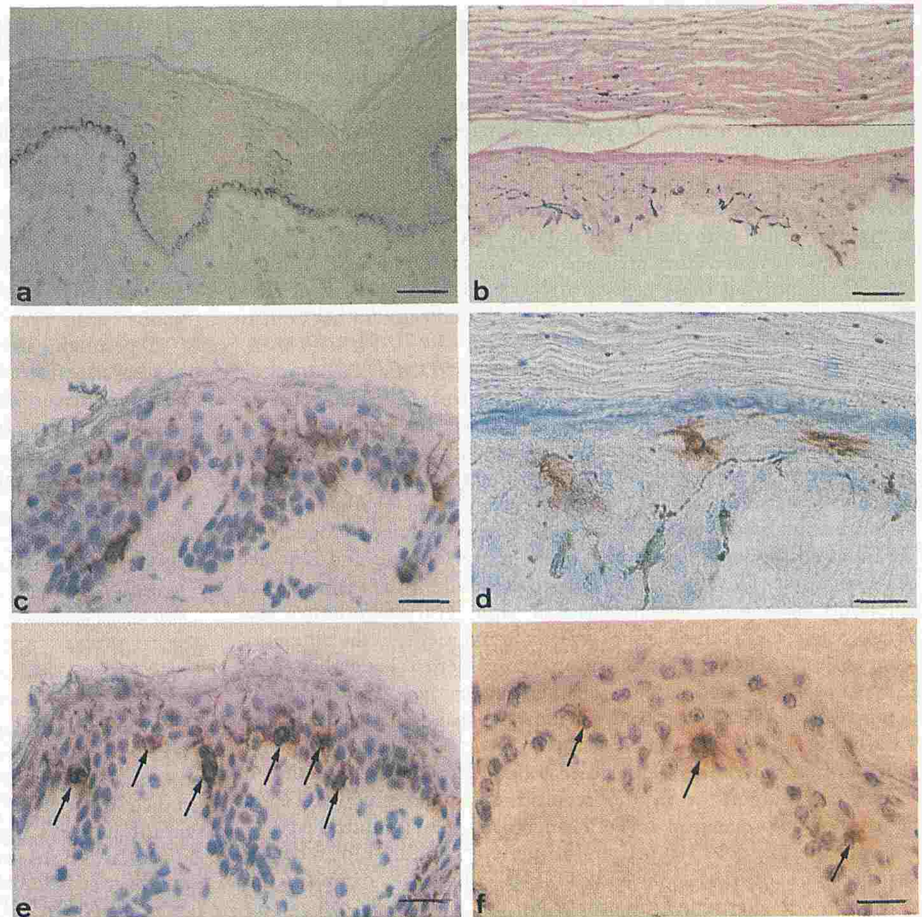

Figure 1. Melanocytes and Langerhans cells in normal human epidermis and reconstructed epidermis. Histochemical and immunohistochemical analysis of normal human skin (*a,c,e*) and reconstructed epidermis (*b,d,f*) containing melanocytes and Langerhans cells. Fontana Masson staining (*a,b*) to reveal the presence of melanin. Anti-CD1a (*c,d*) and anti-Lag (*e,f*) staining with hematoxilin counterstaining on cryostat sections was performed to identify Langerhans cells (→ indicate Lag$^+$ cells). *Scale bars*, (*a,b*) 100 μm, (*c–f*) 50 μm.

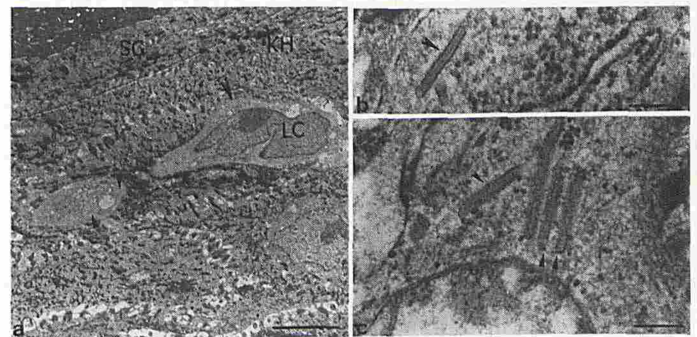

Figure 2. Electron microscopy of a Langerhans cell in reconstructed human epidermis. The electron micrograph (*a*) shows a Langerhans cell (LC) in the reconstructed epidermis. The Langerhans cell is located beneath the stratum granulosum (SG), characterized by the presence of numerous keratohyalin granules (KH). Magnifications of the Langerhans cell (*b,c*) reveals the presence of Birbeck granules characteristic for epidermal Langerhans cells. *Arrowheads* depict Birbeck granules. *Scale bars*, (*a*) 5 μm, (*b,c*) 0.1 μm.

Reconstruction of a pigmented epidermis and integration of Langerhans cells To obtain a pigmented reconstructed epidermis, a mixture of heterologous, cultured (third passage) human keratinocytes and melanocytes (10:1) was seeded onto dead de-epidermized acellular human dermis (Régnier *et al*, 1981, 1986) and cultured for 6 d in Dulbecco's modified Eagle medium/Ham F12 (3:1), containing 10% fetal calf serum, 10 ng epidermal growth factor per ml, 400 ng hydrocortisone per ml, 10^{-6} M isoproterenol, 5 μg transferrin per ml, 2×10^{-9} M triiodothyronine, 1.8×10^{-4} M adenine, and 5 μg insulin per ml. Thereafter, the cultures were lifted at the air–liquid interface and isoproterenol, transferrin, triiodothyronine, and adenine were removed from the culture medium. At day 2 of the air-exposure, a stage where the reconstructing epidermis is composed of 4–5 cell layers and keratinocyte differentiation has not yet resulted in formation of a stratum corneum, cord blood derived CD34$^+$ hematopoietic progenitor cells were seeded either directly or after 6 d of culture in the presence of GM-CSF and TNF-α onto the reconstructing epidermis (seeding density 5×10^5 cells per cm^2). Histologic examinations of the reconstructed epidermis were performed after 2 wk of culture.

In some experiments heterologous keratinocytes, melanocytes, and freshly isolated CD34$^+$ precursors were co-seeded (10:1:10) onto the de-epidermized dermis and cultured as described above.

Histochemistry and immunohistochemistry Fontana Masson staining was performed to reveal the presence of melanin. Indirect immunohistochemical staining on cryostat sections with hematoxilin counterstaining was performed using the labelled streptavidin-biotin (LSAB) assay (LSAB^{R}2 Kit, Dako, Carpinteria, CA). Monoclonal antibody CD1a (clone BL-THY1) was obtained from TEBU (Le Pezzay-en-Yralines, France) and used at a 1:50 dilution. The monoclonal antibodies CD14 (clone TÜK 4) and CD68 (clone Pb-M1) were purchased from Dako, and used at 1:10 and 1:50 dilutions, respectively. The monoclonal Lag antibody was kindly provided by S. Imamura and used as described (Kashihara *et al*, 1986). The number of CD1a$^+$ cells was determined by means of a fluorescence-activated cell sorter.

Electron microscopy Samples, 1–2 mm^2, of reconstructed epidermis were fixed in Karnovsky's formaldehyde glutaraldehyde solution, postfixed in 1% (wt/vol) osmium tetroxide, dehydrated through alcohol and propylene oxide, and embedded in epon. Sections were cut on a TM60 (Reichert Jung, Leica, Vienna, Austria) with diamond knives, stained with uranyl acetate and lead citrate to be examined with a TEM 902 electron microscope (Zeiss, Oberkochen, Germany).

RESULTS AND DISCUSSION

Two weeks after seeding CD34$^+$ hematopoietic progenitors, cultured for 6 days in the presence of GM-CSF and TNF-α, onto the reconstructing epidermis, histochemical analysis revealed the presence of a stratified epidermis, covered with a compact stratum corneum, and dendritic, melanin containing melanocytes in the basal cell layer (**Fig 1*b***). To detect the presence of Langerhans cells, we used three antibodies directed against antigens that are specifically expressed in

functional epidermal Langerhans cells: (i) major histocompatibility complex class II (MHC-II); (ii) CD1a (Rowden *et al*, 1977); and (iii) Lag (Kashihara *et al*, 1986), the latter representing a 40-kDa glycoprotein associated with the Birbeck granules, the most characteristic morphologic marker of epidermal Langerhans cells. The suprabasally located DC within the reconstructed epidermis express the CD1a (**Fig 1*d***), the Birbeck granule associated Lag antigen (**Fig 1*f***), and exhibit anti-MHC-II reactivity, the latter with a pattern comparable with that revealed with the CD1a antibody (result not shown). Electron microscopy revealed the presence of numerous Birbeck granules (**Fig 2**), identifying these cells clearly as epidermal Langerhans cells. The dermal substrate, in general free of nucleated cell, did not contain CD1a or MHC-II positive DC.

The same result was obtained when purified $CD34^+$ hematopoietic progenitor cells, not exposed to GM-CSF and TNF-α, were co-seeded directly with keratinocytes and melanocytes onto the dermal equivalent. The resulting reconstructed epidermis showed the same characteristics as presented in **Fig 1**, with melanocytes in the basal layer and suprabasally located Langerhans cells. To see whether melanocytes are implicated in the maturation of the hematopoietic progenitors, we repeated the same experiment without melanocytes and obtained the same result. The fact that the culture medium for the reconstructed epidermis does not contain GM-CSF and TNF-α indicates that keratinocytes are involved, either directly or indirectly, in the differentiation of the hematopoietic progenitors into epidermal Langerhans cells. It remains to be determined whether under these conditions the maturation of $CD34^+$ progenitors into DC/Langerhans cells is also induced by GM-CSF and TNF-α.

A control experiment revealed that no DC are present, either in the dermal support or in the reconstructed epidermis, unless no hematopoietic progenitors or DC/Langerhans cells are introduced.

Before seeding 5×10^5 progenitors per cm^2, cultured for 6 days in the presence of GM-CSF and TNF-α, onto the reconstructing epidermis, we determined the proportion of $CD1a^+$ cells to be about 15%. A semiquantitative, comparative analysis, after immunohistologic staining of equal size sections obtained from normal human skin and reconstructed epidermis, revealed an average of 350 $CD1a^+$, 340 Lag^+, and 50 $CD14^+/CD1a^-$ cells per section in normal human skin, and 70 $CD1a^+$, 60 Lag^+, and 10 $CD14^+/CD1a^-$ cells per section in the reconstructed epidermis. No $CD68^+$ cells were detectable in both types of sections. This shows that our reconstructed epidermis exhibits essentially the same proportions, but five times less DC than normal human epidermis. We are actually trying to increase the number of Langerhans cells in the reconstructed epidermis by preselecting $CD1a^+$ cells from cultured $CD34^+$ progenitors after cytokine-induced maturation.

The use of skin organ culture models to study Langerhans cells behavior is hampered by the fact that the resident Langerhans cells emigrate spontaneously from the epidermis (Lukas *et al*, 1996), a process that normally occurs *in vivo* after antigen internalization, e.g., during contact sensitization (Kripke *et al*, 1990). As deduced from a semiquantitative analysis of histologic sections taken over a culture period of 4 wk, Langerhans cells in the reconstructed epidermis, unlike in organ cultures, seem not to emigrate spontaneously. Future experiments to evaluate the function of Langerhans cells in reconstructed epidermis will show whether the fact that our Langerhans cells do not emigrate from the epidermis can be interpreted positively, or whether it indicates that the $CD1a^+$, Lag^+ cells do not reflect normal Langerhans cells.

This advanced model of a reconstructed epidermis composed of keratinocytes, melanocytes, and Langerhans cells provides many new research opportunities, e.g., to study some open questions concerning the complex mechanisms of epidermal cell–cell interactions, and in particular, the role of each cell type in the skin immune response.

Very recent experiments show that not only cord blood derived $CD34^+$ hematopoietic progenitors, but also progenitors obtained from the peripheral adult blood give rise to resident Langerhans cells in the reconstructed epidermis, enlarging considerably the availability of $CD34^+$ cells.

Experiments are in progress comparing the Langerhans cells behavior of the *in vitro* model with the *in vivo* situation, especially their responsiveness to known contact sensitizers, to validate the system for its use as a model for immuno-pharmaco-toxicology. In the future, this model could help to replace tests that today have still to be performed on animals or humans.

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