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Use of BD Falcon™ Cell Culture Inserts to Reconstruct a Differentiated Human Epidermis *In Vitro*: Expression of Cell Adhesion Molecules (Integrins)

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Introduction

The epidermis is a highly specialized epithelium that consists of four stratified cell layers. Throughout the epidermis, a delicate balance between proliferation, differentiation, and cell death is maintained.[1] Proliferation only takes place in cells of the innermost basal layer, in contact with the basement membrane that separates the epidermis from the underlying dermis. Following as yet unidentified events, the cells make a commitment to terminal differentiation and subsequently detach themselves from the basement membrane. While undergoing a series of morphological and biochemical changes, they migrate upwards to the surface of the skin. The spatial organization of the epidermis reflects the degree of epidermal differentiation; the further away a cell is located from the basal layer, the closer it gets to the state of terminal differentiation. Changes in integrin-mediated cellular adhesiveness may play a central role in a cell's commitment to terminal differentiation[2] and in cellular migration across the four layers of the epidermis.[3]

Over the past two decades, a number of culture systems have been developed that allow the reconstruction of a human epidermis *in vitro*.[4] Epidermal growth factor and as yet unidentified fibroblast factor(s) have been shown to promote proliferation of epidermal cells *in vitro*,[5,6] but they are not sufficient to induce cell differentiation. Late-stage differentiation requires high concentrations of calcium and low concentrations of retinoids, while early-stage differentiation also requires a system that raises the cells to the air-liquid interface, such as a floating collagen raft or a de-epidermized dermis.[1] Cell culture systems that integrate these factors have been successfully used to obtain an epidermis *in vitro* with many morphological and functional characteristics of an epidermis *in vivo*. However, these systems are complex and difficult to handle. Here, we report the optimization of a simple cell culture system that can be used to reconstruct a human epidermis *in vitro*.

Materials and Methods

Cell and Culture Conditions

Normal epidermal keratinocytes were isolated from neonatal human foreskin (NHEK) as described.[7,8] Cells were grown on 100 mm BD Falcon™ Cell Culture Dishes (BD Biosciences Discovery Labware) in proliferation medium (K-SFM, a serum-free basal medium, supplemented with epidermal growth factor and bovine pituitary extract) (Invitrogen Life Technologies). Second to fifth passage cells were plated on 0.45 µm pore-size BD Falcon Cell Culture Inserts at a density of 60,000 cells per insert (24-well size), and cultured in 1 ml proliferation medium for two days at 37°C in a 5% CO_2 humidified atmosphere. At confluency, cells were cultured at the air-liquid interface, on top of 300 µl proliferation or differentiation medium (DMEM/HAM F12 v/v 3/1, 5% fetal calf serum, 1.5 mM calcium, 1 µM hydrocortisone, 1 µM insulin, 5 ng/ml epidermal growth factor, 1 µM isoproterenol, and antibiotics [penicillin, streptomycin, and kanamycin]); both types of media were replaced every other day.

Histology and Transmission Electron Microscopy (TEM)

Three-week cultures were fixed for two hours in 2% glutaraldehyde - 100 mM cacodylate buffer, pH 7.5, and post-fixed for two hours in 1% osmium tetroxide 100 mM cacodylate buffer. After dehydration, cells were embedded in Spurr (TAAB). Sections were cut perpendicular to the culture substrate and stained with 0.5% toluidine blue in 0.5% borax (1 µm thin sections), or with uranyl acetate and Reynolds' lead citrate formulation (70 nm ultra-thin sections). Sections were viewed using a Philips EM 140 microscope.

Indirect Immunofluorescence

BD Falcon™ Cell Culture Inserts were removed from the plate, gently washed with PBS, fixed and permeabilized in methanol (-20°C for one minute), and washed in PBS for five minutes. To block non-specific binding, the inserts were incubated in 5% normal goat serum for 30 minutes, then incubated in the presence of monoclonal antibodies against specific a integrin subunits (a2 [P_1E_6] Oncogene Science, and a_6 [GoH_3] Monosan), and finally diluted in Evans blue dye (1:10,000) containing diluted goat anti-IgG (1:50) conjugated with fluorescein (Biomedical Technologies, Inc.). Samples were mounted in glycerol: PBS (9:1) and examined using a Leitz epifluorescence microscope; photographs were taken using KODAK™ T-160 film.

Results

Cell Morphology

The formation of a stratified and differentiated epidermis *in vitro* was analyzed over a three-week period using TEM. When cultured on a 0.45 µm microporous membrane at the air-liquid interface in an optimized differentiation medium, the cells began to stratify (up to ten cell layers) and showed foci of differentiation within two to three days after reaching confluency (data not shown). The process of differentiation was completed within three weeks; individual keratinocytes gave rise to stratified colonies that merged with one another at confluency to form a sheet of cells (50-110 µm thick). After three weeks, the reconstructed epidermis was completely covered with a stratum corneum and contained the four histologically differentiated epidermal cell layers: the basal, spinous, granular and horny cell layers, the latter containing 25-40 layers of corneocytes (***Figure 1***). Well organized desmosomes were present throughout the entire reconstructed epidermis, providing intercellular junctions. In the spinous layers, the typical interlacing network of tonofibrils was seen. In the granular layers, keratohyalin granules appeared as amorphous electron-dense material (***Figure 1***). In some cases, this material was in contact with tonofibrils. Cells near the keratinized cell layers acquired increasing numbers of small vesicles that exhibited an internal lamellar pattern.

Terminal epidermal differentiation appeared to be complete, since the stratum corneum consisted of enucleated cells exhibiting features of structural differentiation, including thickened plasma membranes and tightly packed filaments (***Figure 1***).

In contrast, cells cultured under proliferating conditions (using a tissue culture flask and proliferation medium) did not stratify, but rapidly detached themselves from the culture substrate after having reached confluency. When culturing the cells on a microporous membrane in the presence of proliferation medium, multiple islands of keratinocytes could be observed. However, these cells did not show a high degree of differentiation. A basal layer of cells with an increased size and five layers of suprabasal cells irregularly occupied the surface, particularly in the center of the membrane. In the basal cell layer, microcytoplasmic projections were the principal cell-to-cell contacts along the basolateral surface (***Figure 1***). While the cells above the basal cell layer were nucleated and contained only a few keratohyalin granules, the extension of intermediate filaments to desmosomes was suggestive of a stratum spinosum (***Figure 1***). No granular or horny layers were formed.

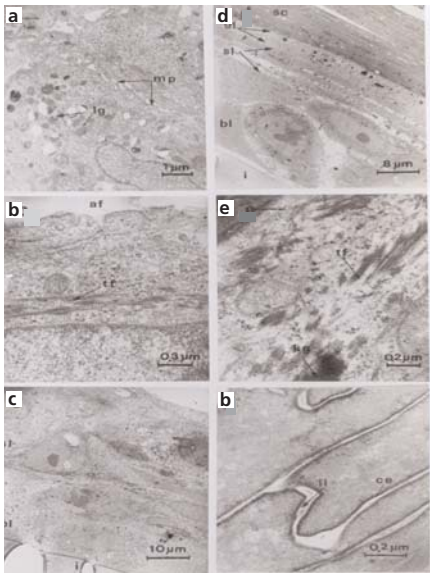

Figure 1*: Transmission electron micrographs of a reconstructed human epidermis. Keratinocytes were cultured for three weeks on a BD Falcon™ Cell Culture Insert in proliferation medium (**a, b, and c**) or differentiation medium (**d, e, and f**) at the air-liquid interface; bl: basal layer; sl: spious layer; gl: granular layer; sc: stratum corneum; kg: keratohyalin granules; tf: tonofilaments; il: intercellular lipids; ce: corneocyte envelopes; mp: microplasmic projections; lg: lipid granules; ap: apical face.*

Expression of Markers of Differentiation

Expression of epidermal differentiation markers was analyzed by indirect immunofluorescence:

1. **Cell Surface Markers (Integrins)**

   Keratinocytes have been shown to express several integrins, mainly a2b1 and a3b1, and to lesser extent a5b1 and a4b1. Following cell commitment to terminal differentiation, the cell adhesion molecules lose their ability to bind extracellular matrix, disappear from the sites of cell-substrate interaction (focal adhesions: FAs), and relocate to sites of cell-cell contact (intercellular adhesions: ICAs).[2,3] Accordingly, immunofluorescence studies showed high-level expression of a2b1 and a3b1, and low-level expression of a6b4 and a5b1 (***Figure 2***).[9]

2. **Intracellular Markers**

   Immunofluorescence analysis using antibodies against various markers of differentiation demonstrated a pattern of expression that is consistent with a stratified and differentiated epidermis (data not shown). Immunohistochemical staining has been found in the basal and spinal cell layers for keratins, the proteins that form the cytoskeletal tonofilaments; furthermore, staining has been detected in the spinal cell layers for involucrin, the protein that forms an insoluble envelope underneath the plasma membrane in the stratum corneum; finally, staining has been seen in the granular cell layers for filaggrin (proteins that bundle tonofilaments to macrofibrils) and transglutaminase (catalyzes cross-linking of involucrin).

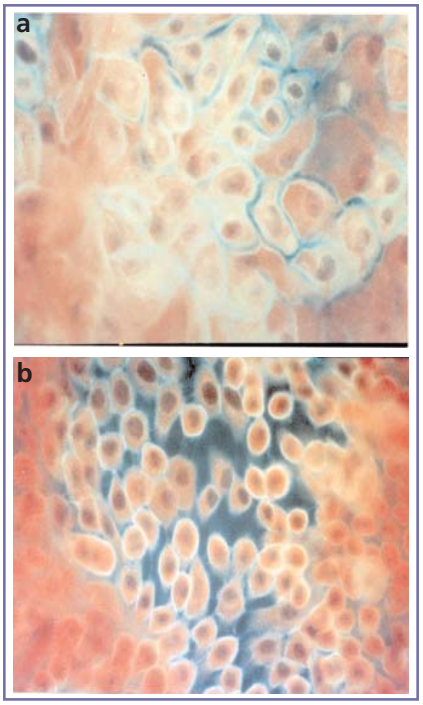

Figure 2: *Immunofluorescent staining of human keratinocytes cultured for two weeks on a microporous membrane of BD Falcon™ Cell Culture Inserts in differentiation medium at the air-liquid interface. Monoclonal antibodies against a2 (a) and a6 (b) specific integrin subunits were used (respective magnifications are 7.6x and 11x).*

Discussion

We have shown that a differentiated epidermis can be reconstructed *in vitro* using an optimized differentiation medium and a simple, membrane-based cell culture system that allows the culture of the cells at an air-liquid interface. This system does not include any dermal components, e.g., fibroblasts or fibroblast-conditioned medium. Using a cell culture insert, the cells can be cultured at the air-liquid interface in the absence of a collagen lattice. The degree of epidermal differentiation has been studied both at the level of cell morphology and function using histological and immunohistochemical analyses. In accordance with earlier studies,[8-11] the potential to differentiate is retained in epidermal keratinocytes when cultured *in vitro* (until at least the fifth passage of the cells) without the need for external signals provided by the microenvironment (e.g., basement membrane mediated receptor activiation).

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