Journal of Bioscience and Bioengineering
VOL. 118 No. 3, 323–326, 2014

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NOTE

Keratinization induced by air exposure in the reconstructed human epidermal model: An *in vitro* model of a cultured epithelial autograft

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Received 22 November 2013; accepted 21 February 2014
Available online 14 April 2014

A reconstructed human epidermis, an *in vitro* model of a cultured epithelial autograft, was used to examine the formation of a stratum corneum induced by exposure to air. A prolonged wet condition and excess application of petrolatum on the dressing reduced efficient production of the stratum corneum.

[**Key words:** Cultured epithelial autograft; Reconstructed human epidermis; Keratinocytes; Stratum corneum; Keratinization]

The cultured epithelial autograft (CEA), a graft of cultured epidermis prepared from autologous keratinocytes, was used for closing large burn wounds for the first time in the early 1980s (1). In recent years, CEAs are more clinically available from suppliers such as Epicel (Genzyme) and JACE (Japan Tissue Engineering, Gamagori, Aichi, Japan). CEAs have been utilized for patients suffering large skin burns (usually greater than 30%), who do not have enough unburned skin available to cover the wounds. To prepare a CEA, a skin biopsy from the patient is minced, trypsinised, and cultured in a flask containing irradiated mouse 3T3 cells for 2–3 weeks, with subculturing if needed (2). After placing it on the wound bed, the CEA is covered with a fine mesh gauze and a dry dressing. Because the CEA does not possess a stratum corneum, it is transparent and invisible on the wound bed until the stratum corneum develops (1), which interferes with recognizing the graft.

Although it has been empirically known that exposing CEA to air is important for developing the stratum corneum (3), a concrete examination has been absent thus far. In basic biological research, by simplifying experimental procedure the reconstructed human epidermis (RHE) has been developed to study biological phenomena. The RHE is constructed by culturing keratinocytes at the air/liquid interface on a de-epidermized dermis, a gel of collagen, acetate cellulose or polycarbonate filter, on which cells proliferate, differentiate, and eventually forms a fully differentiated epidermis, including the stratum corneum [reviewed by Poumay and Coquette (4)].

In this study, we utilized an RHE as a model which mimics the CEA in order to study differentiation of keratinocytes, especially the formation of the stratum corneum by air exposure. It was found that prolonged sustaining of the RHE in a submerged condition delayed successive formation of the stratum corneum after air exposure. In addition, we found that dressings did not affect the differentiation of keratinocytes, but excess petrolatum in the dressing depressed production of the stratum corneum. Our findings provide valuable information on clinical as well as biochemical aspects concerning keratinization of the cultured epidermis.

A three-dimensional cultured human epidermal model was constructed using the LabCyte EPI-KIT, which is comprised of materials needed for preparing the RHE: normal human epidermal keratinocytes (cat. no. C-001-5C, Cascade Biologics, Portland, OR, USA), cell culture inserts having a microporous PET membrane with 0.4 μm diameter pore (cat. no. 353095, BD Biosciences, San Jose, CA, USA), 12-well multiwell culture plates (cat. no. 353043, BD Biosciences) and a culture medium containing fetal bovine serum (cat. no. 402250, Japan Tissue Engineering). Cryopreserved keratinocytes grown in the presence of irradiated 3T3 cells (5) were thawed and suspended in a culture medium. Cell aliquots were seeded into cell culture inserts placed in multiwell culture plates where each well was filled with 1.5 ml of the culture medium and incubated at 37°C in a 5% CO_2 incubator with saturated (~95%) relative humidity. The culture medium in the well was changed every 2 or 3 days. Keratinocytes seeded in the cell culture insert are situated in a submerged condition (named L/L) when the medium exists within the insert. Upon taking off the medium in the insert, keratinocytes are exposed to the air on the upper side, resulting in a culture at the air/liquid interface (named A/L).

Portions of the epidermal model were fixed in 10% neutral buffered formalin or 4% paraformaldehyde and embedded in paraffin. Tissue sections (thickness; 5 μm) were treated by staining with Mayer's hematoxylin and 0.1% eosin or immunofluorescent staining with antibodies after deparaffinization. A stratum corneum is stained in red color with eosin. Proteolytic digestion was carried out with proteinase K (cat. no. S3020, Dako, Glostrup, Denmark) for immunostaining. The primary antibodies used were cytokeratin-1/10 (dilution 1:50; cat. no. CBL226 Millipore, Billerica, MA, USA) and involucrin (dilution 1:50; cat. no. K5390-9950, AbD Serotec, Kidlington, UK). The secondary antibody used was Alexa Fluor 488 F(ab′)$_2$ Fragment of goat anti-

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2014.02.019

mouse IgG (dilution 1:300; cat. no. A11017, Invitrogen, Carlsbad, CA, USA). The specimens were observed under an optical microscope (Nikon ECLIPSE 50i) with FITC filter (Ex. 465–495 nm, Em. 515–555 nm) and UV-2A filter (Ex. 330–380 nm, Em. 420 nm).

CEA consists of only keratinocytes in the form of a stratified squamous structure (1). After 4 days of culturing keratinocytes overlaid with the medium in the cell culture insert, keratinocytes formed a stratified squamous structure similar to a CEA (Fig. 1A, day 4). The medium within the cell culture insert was taken off at day 4 to begin a culture at the air/liquid interface. The stratum corneum was first observed at day 7 (Fig. 1A, A/L, day 7) and its production proceeded according to a prolonged culture. The thickness of the stratum corneum reached about 35 μm at day 14 (Fig. 1A, A/L, day 14 and Supplementary Fig. S1). In contrast to the culture at the air/liquid interface, the submerged culture repressed formation of the stratum corneum even at day 14 (Fig. 1A, L/L, day 14), although cells in the suprabasal layer became squamous. The formation of the stratum corneum in the cultured epidermis was induced by air exposure but repressed by the blocking of air exposure.

In order to verify a response of RHE model to air exposure, we further examined whether the formation of the stratum corneum depends on a period of air exposure. After 4 days in a submerged condition, the medium within the cell culture insert was taken off for 0, 8, 16, or 24 h per day. The stratum corneum was first observed at day 8 in the RHE with air exposure for 8 h per day, whereas the stratum corneum was observed at day 6 in the RHE with air exposure for either 16 or 24 h per day (Fig. 1B). The formation of the stratum corneum depended on the period of air exposure. Taken together, we concluded that our RHE model is suitable for studying the response to air exposure.

To test whether a delayed start of the culture at the air/liquid interface affects the formation of stratum corneum, we examined three different culture conditions. Culture medium within the cell culture insert was preserved until day 4, 7, or 11 and after that cells were exposed to the air/liquid interface. In the case of the culture at the air/liquid interface from day 4, the stratum corneum was observed after 3 days (Fig. 2A, day 7) as with the above experiment (Fig. 1A) and accumulated during prolonged culturing. In the case of the culture at the air/liquid interface from day 7 or 11, tissue structure at the start of the air/liquid interface was similar to that of the culture at the air/liquid interface from day 4. Stratum corneum was observed after 4 days or 7 days in RHE starting air exposure from day 7 or day 11, respectively (Fig. 2A, day 11 and day 18). The development of the stratum corneum was delayed by the late start of culturing at the air/liquid interface. In addition to the delay in the appearance of the stratum corneum, production of it was apparently repressed. In the case of air exposure from day 11, even after 10 days culturing at the air/liquid interface, the stratum corneum was slightly thicker (Fig. 2A, day 21).

In order to examine protein expression associated with differentiation in the epidermal model, immunofluorescent staining of differentiation markers was performed. Two proteins associated with terminal differentiation in the epidermis were selected. One is keratin 1 and 10, which are expressed during cornification (6), and the other is involucrin, which increases as the cell stratifies (7). In the case of immunofluorescent staining using monoclonal antibody

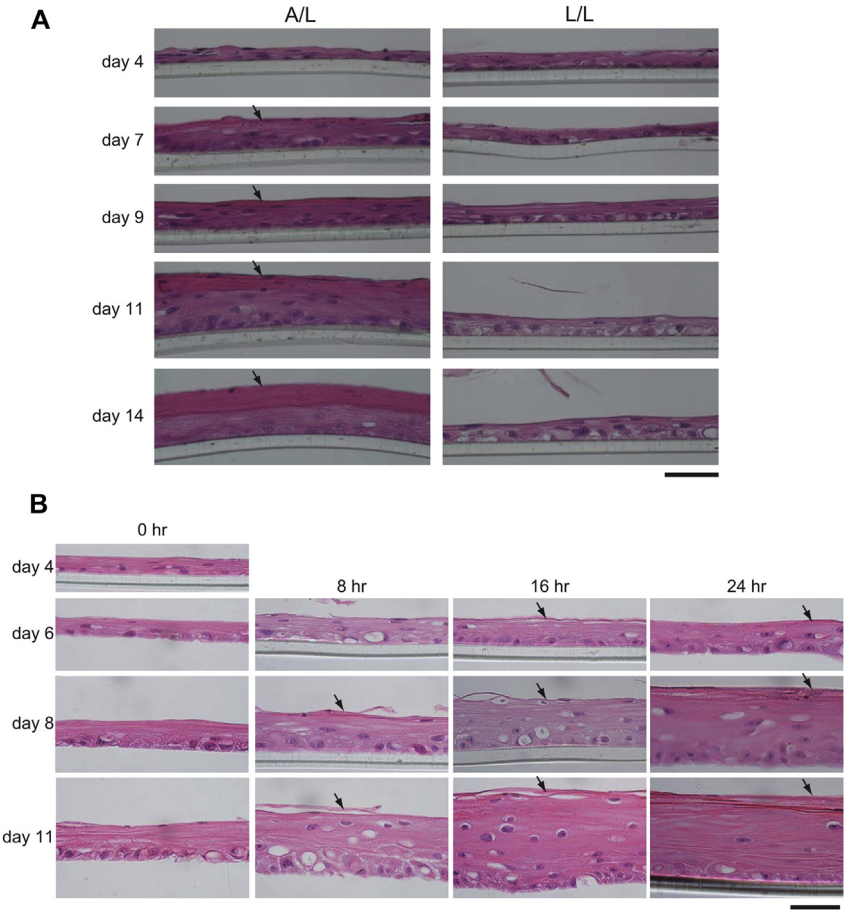

FIG. 1. (A) Culturing at the air/liquid interface (A/L) or in the submerged condition (L/L). Sections were stained with hematoxylin and eosin after fixation of RHE with formaldehyde and embedding in paraffin. Arrows indicate the stratum corneum in the RHE. (B) Culturing at the air/liquid interface in different periods. The culture medium within the cell culture insert was removed for 0, 8, 16, or 24 h per day between day 4 and day 8. Sections were stained with hematoxylin and eosin after fixation of RHE with formaldehyde and embedding in paraffin. Arrows indicate the stratum corneum in the RHE. Membrane on the bottom of some RHEs was missed in the preparation of section. Scale bar: 50 μm.