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Organotypic Skin Cultures: A Human Model for Basic Studies

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Aim. To produce organotypic skin cultures using human skin samples as a source of keratinocytes and fibroblasts.
Methods. Keratinocytes and fibroblasts from human skin samples were separated by warm trypsine and collagenase, respectively. Keratinocytes were plated in tissue culture dishes in keratinocyte serum free medium supplemented with epidermal growth factor and bovine pituitary extract, and were grown until confluence. Fibroblasts were cultured in Dulbecco's medium (DMEM) supplemented with fetal bovine serum and hydrocortisone. A mixture of fibroblasts, rat dermal collagen type I, E tissue culture medium, and reconstitution buffer were used as a dermal equivalent. Keratinocytes were plated on the top of the dermal equivalent and cultured for 10 days in organotypic culture dishes on stainless steel grids in the supplemented DMEM medium. The cultures were fixed in formaline, embedded in paraffin, stained with hematoxylin and eosin, and immunohistochemically stained with anti-cytokeratin and anti-HLA-DR antibody.
Results. Cultured keratinocytes in organotypic skin cultures expressed the majority of the cytokeratins seen in the normal stratified epithelium. Consistent with previous studies, organotypic skin cultures did not show antigen-presenting Langerhans cells.
Conclusion. Human skin from patients who underwent thoracic surgery can be used to produce organotypic skin cultures. This artificial skin can serve as a basis for future basic science studies and as a skin transplantation model.

Key words: artificial skin; fibroblasts; kerationcytes; organ culture; skin substitutes

Different organotypic culture systems using human foreskin as a source of keratinocytes and fibroblasts have recently been developed as model systems for different basic studies (1-4). These cultures consist of two components. The first is a dermal equivalent made up of a mixture of collagen matrix and fibroblasts, and the second an epidermis that develops from keratinocytes plated on the dermal equivalent. After the exposure of the cultures to the air-liquid interface, keratinocytes differentiate and stratify, and become multilayered, with full reconstruction of the natural skin polarity. The aim of this study was to establish organotypic skin culture, using human skin and a modified system originally developed by Asselineau and Prunieras (3), Kopan et al (5), and McCance et al (6), and based on our previous experience with dog skin cultures (7,8).

Material and Methods

Isolation and Cultivation of Cells

Skin from patients who underwent surgery at the Jordanovac Clinical Hospital was used as a source of keratinocytes and fibroblasts. The biopsy site was aseptically cleansed with multiple alcohol rinses, and full-thickness ventral thoracic skin sections of approximately 1.5 cm2 were removed and put in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% of fetal bovine serum (FBS). After trimming off the connective tissue, the skin section was washed 10 times in phosphate buffer saline (PBS) containing 50 units/mL penicillin, 50 g/mL streptomycin, and 50 g/mL gentamycin, and incubated at 40 °C overnight in 25 mg/mL dyspase (Boehringer, Mannheim, Germany) prepared in PBS containing antibiotics. The following day, the epidermis was removed by forceps and washed 3 times in PBS, incubated in 0.125% trypsin/EDTA for 30 min at 37 °C with periodic gentle agitation. Cells were then pelleted at 200 G for 5 min and harvested by decanting the supernatant. The procedure was repeated several times until no further epidermal cells were obtained. The resulting cell suspension was washed with keratinocyte serum free medium (KSFM) (Gibco, Grand Island, NY, USA), and cultivated in a tissue culture dish at 0.1-0.5x106 cells concentration. Cells were incubated at 37 °C in 5% CO2/95% air atmosphere. Cultivated epidermal cells were routinely washed with PBS and fed every third day with KSFM containing 10 ng/mL of recombinant epidermal growth factor (rEGF) (Gibco) and 50 g/mL of the bovine pituitary extract (BPE) (Gibco). The colonies usually

reached 70-90% confluency 2-3 weeks after seeding. Primary culture plating efficiency was 5.2-27.5%. Growth rate, expressed as the average number of population doublings per day, was from 0.82-0.95. When the cultures reached confluence they were rinsed in PBS, incubated in trypsin for 5 min at 37 °C, and split (1:3 ratio).

In parallel, dermal tissue from the same skin sample was washed in PBS, minced, and incubated overnight at 37°C in 10 mL of 25 mg/mL collagenase (Worthington, Freehold, NJ, USA). Cells were spun at 225 G for 5 min, washed with PBS, and cultured in DMEM supplemented by 10% FBS (Hyclone, Logan, UT, USA), and 0.4 g/mL hydrocortisone (Sigma, St. Louis, MO, USA). Cells were routinely fed every 3 days, and split by warm trypsinization in the 1:3 ratio, until used for organotypic cultures.

Preparation of Organotypic Skin Cultures

Dermal equivalent was first made for organotypic skin cultures (raft cultures). Confluent dermal fibroblast cultures were trypsinized, washed in PBS, and resuspended in PBS to a final concentration of 1-2x106 cells/mL. Seven parts of rat dermal collagen type I (Collaborative Research, Bedford, MA, USA) in a concentration of 3 mg/mL, one part of E tissue culture medium (3DMEM:1F12), one part of reconstitution buffer (2.2% NaHCO3, 0.05% NaOH, and 200 mmol/L HEPES), and one part of fibroblasts in PBS, were mixed on ice. Two milliliters of the collagen mixture were pipetted into 35 mm plastic dishes, and allowed to solidify at 37 °C in humid 5% CO2 atmosphere for one hour. The cultures were then overlayed with 0.5x106 keratinocytes in 2 mL DMEM containing 10% PBS, 0.4 g/mL hydrocortisone, 0.1 nM cholera toxin (Sigma), 50 units/mL penicillin, 50g/mL streptomycin, 250 g/mL Fungisone (Gibco), 5g/mL transferrin (Sigma), 2 nM 3,3,-5, tri-iodo-thyronine (Sigma), 10 ng/mL rEGF, and 5 g/mL insulin (Sigma).

The cultures were submerged for 5-6 days and fed daily with supplemented DMEM. In such cultures, the organotypic skin cultures become elastic, shrink to about a half of their original diameter, and become 2-3 times thicker. Six days later, composite collagen gels were removed from culture dishes, washed gently in PBS, and floated onto stainless steel grids. The grids were placed onto the center well of a 60 mm organ culture dish (Falcon 3037, Becton Dickinson, San Jose, CA, USA) containing 4 mL supplemented DMEM. The cultures were fed daily for 6-8 additional days, then harvested, fixed in 10% formaldehyde, paraffin embedded, sectioned, and stained with hematoxilin and eosin. Sections were also stained with anti-human HLA-DR a–chain antibody (Dakopatts, Kopenhagen, Denmark), anti-cytokeratin antibody (MNF-116; Dakopatts), that reacts with cytokeratins 5,6,8,17, and probably 19 in human epitelial tissues. Standard avidin-biotin detection method was used to detect positive structures.

Results

A product closely resembling normal skin morphology composed of or containing the dermis and epidermis, with well-defined basal, spinous, granular, and cornified layers was reproduced several times using the described technique (Fig. 1). Morphologically, the major difference between these cultures and normal skin was that organotypic cultures did not contain the minority cell types of epidermis, such as melanocytes and Langerhans cells. They also did not contain vasculature and peripheral nerves in the dermis. Additionally, they did not form rete ridges or hair follicles. Immunohistochemical staining with anti-cytokeratin antibody recognizing most of the basal and suprabasal keratins (Fig. 2) showed strong positivity of cultured cells. There were no MHC class II positive cells (Langerhans cells) stained with anti-HLA-DR antibody (Fig. 3a), in contrast to the normal skin (Fig. 3b).

Figure 1: Cross-section of formalin-fixed, paraffin-embedded organotypic skin culture stained with H–E. The cultures have well–developed, stratified epidermis (E), and dermis (D). Original magnification approximately x200. [view this figure]

Figure 2: Cross-section of formalin-fixed, paraffin-embedded organotypic skin culture stained with anti-cytokeratin antibody, counterstained with hematoxyilin. Positive reaction is visible in the whole thickness of the epidermis (arrow). Original magnification approximately x200. [view this figure]

Figure 3: Cross-section of formalin-fixed, paraffin-embedded organotypic skin culture. Expression of MHC class II antigen detected by immunohistochemistry. Langerhans cells were not found in cultivated epidermis (E). Original magnification approximately x200. [view this figure]

Figure 4: Cross-section of formalin-fixed, paraffin-embedded normal human skin stained with an MHC class II antibody. Langerhans cells in the basal skin layer are present (arrow). Original magnification approximately x200. [view this figure]

Discussion

Epidermal and dermal tissue from the human skin as a source of keratinocytes and fibroblasts was used to produce a structure closely resembling morphologically normal skin with the dermis and epidermis. Keratinocytes expressed cytokeratin antigens but not MHC class II antigens, suggesting that Langerhans cells as antigen-presenting cells are not present in this product.

These structures may be a suitable transplantation model of reconstituted living human skin. As reviewed by Phillips in 1991 (9), some studies demonstrated that grafted allogeneic human cultured epidermis could survive permanently. In some studies, allografts of human cultured epidermis survived permanently as a consequence of the absence of Langerhans cells from epidermis (7). However, other animal studies showed that Langerhans cell-free allogeneic cultures which were initially accepted were invariably rejected at a certain point after grafting (9-11). Additionally there is some evidence that cultured skin allografts do not survive permanently after the transplantation, and that the beneficial effect of the transplanted allogenic cultures results rather from effect on wound healing, than formation of permanent graft. It has also been shown in clinical practice that these cultures can be used as an autologous skin replacement following extensive skin loss (12), but producing the cultures is a time-consuming process, and an allogenic graft would have a much wider application.

Therefore, the organotypic skin model may be suitable for testing engraftment, rejection, and replacement of the graft by host epidermis in the animal model, where the fate of the donor skin can be followed by markers that distinguish the donor from the host. Additionally, these cultures also represent a promising model for many basic studies in the fields of immunopathology, dermatology, and pharmacology.

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