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Organotypic culture model to study epithelial cell differentiation and function in vitro

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Introduction

Organotypic cultures are three-dimensional tissue cultures used to reconstruct a tissue or organ in vitro, the objective being to allow the cells to exhibit as many properties of the original organ as possible (4). Organotypic cultures have been used for studies of cell behavior, differentiation, drug effects, and cell-matrix interactions. Common techniques use an artificial collagen-fibroblast matrix, which have not shown an organized basement membrane zone (BMZ) including hemidesmosomes. The epidermal side of de-epidermized human dermis has been successfully used as culture matrix and these cultures differ from cultures using reticular dermis (5), which indicates a significant influence of the basement membrane. The purpose of this study was to develop an organotypic culture using a papillary matrix with a pre-existing BMZ to create an epithelial culture with interdigitating rete ridges and connective tissue papillae in vitro.

Material and Methods

a) Donor tissue preparation
3 bovine tongues from freshly sacrificed cows were used. The mucosa from dorsal surface was harvested 2-3 hrs after sacrifice. Tissue pieces of approximately 1 X 0.5 X 0.25 cm size were incubated 96 h in 1 M NaCl aequous solution at 4° C in order to initiate epithelial separation. The epithelium was then separated from the connective tissue (CT) with forceps. Unseparated control specimens and epithelium and CT specimens were embedded in Histo Prep (Fisher Scientific, New Jersey) and snap frozen in liquid nitrogen and stored at -80° C for frozen sections. Fresh CT specimens were disinfected by washing in 70% ethanol for 1 minute and then washed in MEM for 1 h. Specimens were either stored at -80° C or directly used for cultures.
b) Localization of the split
5-7 µm thick frozen sections were made from all samples. The specimens were mounted on silane (Sigma, St. Louis) coated slides and fixed in acetone for 5 minutes at -20° C before being stored at -80° C. Slides were stained with H+E or used for immunohistochemistry (IHC) with the primary antibodies listed in table 1 and methods described previously (3). Briefly, slides were incubated with the primary antibody over night and then washed with PBS/BSA (1 mg/ml) and incubated with secondary rhodamine-labeled antibody (1:50, Boehringer Mannheim Biochemicals, Indianapolis, IN) for 1 h. Non-immune serum and PBS-BSA only for primary incubation served as negative controls. Cover slides were mounted after washing with PBS/BSA and air drying. Slides were examined with a Zeiss Axioskop 20 fluorescence microscope. Two specimens of epithelium and CT were prepared for scanning electron microscopy (SEM) using routine protocol.
c) Culture model
Human HaCat keratinocyte cell line from frozen stock were cultured in DMEM until confluent. Cells were trypsinized and the connective tissue side formerly attached to the epithelium was incubated with 200,000 cells in 10µl medium. After 2 h the specimens were placed on the permeable membrane of an insert - culture well system (Organogenesis Inc, Canton) and cultured by a modification of a protocol described by Parenteau (4). Briefly, minimally supplemented basal medium (MSBM) was used for 6 days and then replaced by a cornification medium (CM). The surface of the CT substrate was raised to the air liquid interface leaving the keratinocytes exposed to air. Supply with medium was maintained through the insert membrane. CM was replaced by a maintenance medium (MM) after 6 days. Samples from culture specimens from 5 to up to 30 days were stained with a vital dye (PT #6402A, Promega) and snap frozen in liquid nitrogen. Specimens were stored at -80° C until sectioned and stained as described above.

Following antibodies/dilutions were used:

Antigen	Clonal type	Dilution	Reference/Source
Integrin Beta 1	Polyclonal, 3847	1:500	Roberts et al., 1988
Integrin Beta 4	Monoclonal, A054	1:400	Gibco BRL, Gaithersburg, MD
Laminin-1 (LM-1)	Monoclonal, GB3	1:30	Verrando et al., 1987
Laminin-5 (LM-5)	Monoclonal, 1924	1:100	Chemicon, Temecula
Type IV Collagen (CIV)	Polyclonal, PS 057	1:100	Monosan, Uden, NL
Type VII Collagen (CVII)	Monoclonal, 1345	1:50	Chemicon, Temecula
Heparan-sulfate-proteoglycan (HSPG)	Monoclonal	1:25	Kemeny et al., 1988
Tenascin (TN)	Monoclonal, BC-24	1:400	Sigma, St. Louis

Tab.1: Antipodes and working concentrations

Results

a) Separation
On HE stained specimens and IHC the complete epithelial detachment was observed with basal cell remaining attached to the epithelial half (Fig. 1). SEM examination (Fig. 3) confirmed those findings showing a complex CT surface. Multiple connective tissue papillae of various length and diameter were seen without evidence of epithelial cell remnants. The epithelial part showed the basal cell surface with rete ridges. IHC (Fig. 1&2) showed positive staining for integrin beta 4 and beta 1 at the basal epithelial side before and after separation and positive staining of the separated CT surface for HSPG, type IV and VII collagen. This indicates that the split occurred at the level of the lamina lucida of the basement membrane zone (BMZ). Reaction with LM-1 and LM-5 were negative for unseparated and split bovine tissue (not shown). TN was localized within the BMZ of the CT at selected connective tissue papillae only (not shown).
b) Cell culture
All culture rafts remained vital as indicated by vital dye until end of the experiment after 30 days. Stratification of keratinocytes was observed 5 days after raising the culture to the air-liquid interface. Some morphologic differentiation could also be seen with flattening of cells in the superficial layers (Fig. 4). Maximum stratification was observed from day 18 on with up to 15 cell layers. The epithelium appeared well organized and differentiated with a distinct basal layer. There was a tendency towards vertical orientation of basal cell nuclei and basal cell palisading. No major changes occurred in the period from 18-30 days. Epithelial migration was seen at the lateral borders of the raft. The migrating layer usually consisted of 2-3 cell layers (not shown).
IHC (Fig. 5) showed positive staining at the BMZ for type IV and VII collagen and HSPG. Antibodies to LM-1 (not shown) and LM-5 that were not reactive to bovine tissue decorated the BMZ in the cultures, which suggests that human epithelial cells were able to deposit BMZ components onto the existing bovine BMZ. Basal aspects of basal keratinocytes expressed integrin beta 4. TN was present in a wide band at the BMZ (not shown).

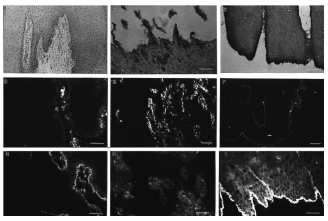

Fig. 1. H+E staining and IHC of bovine tissue before and after separation: (A, D, G) unseparated BMZ; (B, E, H) connective tissue; (C, F, I) epithelium. (A-C) H+E; (D-F) integrin beta 1; (G-I) integrin beta 4. Bar 200 µm.

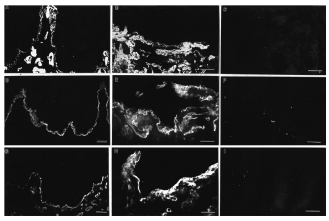

Fig. 2. IHC of bovine tissue before and after separation: (A, D, G) unseparated BMZ; (B, E, H) connective tissue; (C, F, I) epithelium. (A-C) type IV collagen; (D-F) type VII collagen; (G-I) HSPG. Bar 200 µm.

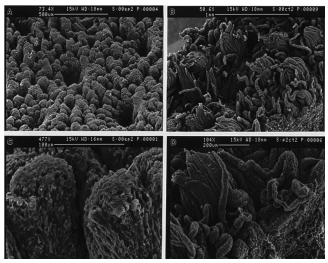

Fig. 3. Scanning electron microscopy after separation showing the formerly attached surfaces facing up: (A,C) epithelium; (B,D) connective tissue.

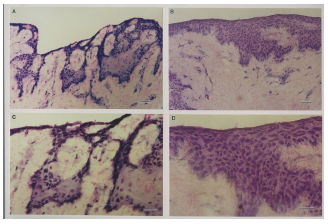

Fig. 4. H+E staining of HaCat keratinocyte cultures: (A,C) 5 day culture; (B, D) 18 day culture. Bar 200 µm.

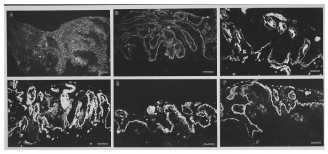

Fig. 5. IHC of 18 day HaCat keratinocyte cultures: (A) integrin beta 1; (B) integrin beta 4; (C) type IV collagen; (D) HSPG; (E) type VII collagen; (F) LM-5. Bar 200 µm.

Discussion and Conclusions

The applied separation technique produced a basement membrane covered CT substrate. The BMZ components appear to remain unaltered during this procedure. The preexisting basement membrane of the CT substrate supports the formation of stratified squamous epithelium of HaCat keratinocyte cultures. BMZ components appear to remain relatively intact during the study period. However changes in their distribution and intensity occurred. This might be due to cellular deposition or alterations of the substrate caused by the culturing process. The rafts resemble normal epithelial histology. There is evidence that HaCat cells cultured in such manner produce the basement membrane components LM-1 and LM-5. Secretion of other basement membrane components appears likely and is indicated by positive staining of areas where cells migrate, which were not part of the old BMZ (not shown). Formation of basement membrane by HaCat cells was observed in surface transplants (1) and by human keratinocytes cultured on collagen-fibroblast matrix (7). The positive reaction for integrin beta 4 indicates a possible presence of hemidesmosomes, which remains to be confirmed by transmission electron microscopy. Hemidesmosomes were observed by SEM in keratinocytes cultured on human de-epidermized dermis by Regnier et al. (5). Furthermore clear cornification and keratohyalin granules were described, which could not be observed in this model. This may be due to using HaCat cells, although keratinization was seen by HaCat cells transplanted onto mice (1). The differentiation patterns of this model remains to by clarified. In summary, our raft culture model using human keratinocytes on top of bovine tongue basement membrane appears to closely resemble normal tissue structure and may be used for multiple applications in studying epithelial cell biology.

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