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Characterization of organotypic keratinocyte cultures on de-epithelialized bovine tongue mucosa

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Summary. Organotypic cultures have been used to study epithelial cell behavior for many years. The aim of this study was to develop an organotypic culture method that better mimics the three-dimensional morphology of interdigitating rete ridges and connective tissue papillae and that also conserves the basement membrane zone (BMZ).

Bovine tongue mucosa connective tissue, separated from epithelium after 1M NaCl incubation, was used as organotypic culture substratum for different human keratinocyte cell lines. Organotypic cultures were characterized by electron and immunofluorescence microscopy for expression of integrin subunits and extracellular matrix components. While spontaneously immortalized mucosal keratinocytes produced highly irregular stratified organotypic cultures, the normal human epidermal keratinocytes (NHEK) demonstrated culture morphology that resembled in vivo epidermis. However, in this model, the histomorphology, expression of differentiation markers involucrin, keratin 10 and 14, and integrins varied significantly between the cell lines. Some cultures appeared to have an extended survival since they were maintained up to 40 days without histological signs of degeneration. The ultrastructure of the BMZ including hemidesmosomes was similar to the normal dermo-epidermal junction. Extracellular matrix molecules, including tenascin, laminin-1 and -5, were expressed in the cultures demonstrating their secretion solely by keratinocytes. Distribution and expression of integrins in NHEK cultures was similar to that seen in vivo skin with the exception of additional expression of 5ß1 and vß6 integrins. Organotypic NHEK cultures show similarities to normal stratified epithelium and are potentially useful for multiple applications for studies on epithelial cell behavior in vitro.

Key words: Organotypic culture, Keratinocytes, Extracellular matrix, Differentiation, Integrins

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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 (Parenteau, 1994). 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 recently shown to produce an organized basement membrane zone (BMZ) including hemidesmosomes (Tomakidi et al., 1998, 1999; Schoop et al., 1999; Stark et al., 1999), but usually lack interdigitating rete ridges (Smola et al., 1998). The epidermal side of de-epidermized human dermis (DED) has been successfully used as culture matrix and these cultures differ from cultures using reticular dermis (Prunieras et al., 1979; Regnier et al., 1981). Cultures on a reticular surface, i.e. dermis without a basement membrane, demonstrated poor stratification and differentiation (Regnier et al., 1981). This indicates a significant influence of the basement membrane in providing a substratum for attachment and growth of keratinocytes. A comprehensive review describing different methods of skin reconstruction has been published recently (Berking and Herlyn, 2001).

Bovine tongue mucosa presents long connective tissue papillae protruding into the epithelium and pronounced connective tissue cores of filiform papillae (Stinson and Calhoun, 1976). The purpose of this study was to utilize this papillary matrix and develop an organotypic culture with a pre-existing BMZ to create an epithelial culture with increased interdigitating rete ridges and connective tissue papillae in vitro. We hypothesized that the interdigitating rete ridges could provide niches for keratinocytes that might facilitate prolonged cell survival as these structures seem to be essential for stem cells in vivo (Watt and Hogan, 2000). For culture characterization, the expression of epithelial integrin subunits, epithelial differentiation markers, and comprehensive extracellular matrix (ECM) and BM molecule distribution was investigated using three keratinocyte cell lines.

Materials and methods

Preparation of bovine tongue

Dorsal tongue mucosa from anterior (tip), middle and posterior area, from freshly sacrificed, 2-3 years old cows, was harvested 2-3 h after sacrifice. Muscle tissue was removed and tissue pieces of approximately 1x0.5x0.25 cm size were incubated in the following conditions: (A) 1 M NaCl aequous solution, 4 °C, 96 h (Scaletta et al., 1978); (B) 0.04 and 0.4% EDTA, 4 °C, 2 h and 24 h; (C) phosphate buffered saline (PBS), 37 °C, 72-96 h (Regnier et al., 1981); (D) 0.25% trypsin, 4 °C, 18 h (Jensen and Mottet 1970).

After incubation the epithelium was separated from the connective tissue with forceps.

Fresh connective tissue specimens were disinfected by washing in 70% ethanol for 1 minute and then washed in Dulbecco's modified Eagle's medium (DMEM, Life technologies, Grand Island, NY) for 1h. Specimens were either stored in DMEM at -80 °C or directly used for cultures.

Cell culture

HaCaT cells, an immortal, spontaneously transformed non-tumorigenic human epidermal keratinocyte line (Boukamp et al., 1988), were routinely cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% gentamycin/streptomycin/amphotericin B. Normal human epidermal keratinocytes from foreskin (NHEK, passage 2) (Clonetics, San Diego, CA) and human gingival keratinocytes (HGK, passage 2) (Mäkelä et al., 1998) were maintained in keratinocyte growth medium (KGM, Clonetics, San Diego, CA). The HGK represent a spontaneously transformed immortal human gingival keratinocyte cell line with partially triploid chromosomes (Mäkelä et al., 1998).

Culture media for organotypic cultures of all cell lines were prepared by a modification of a protocol described by Parenteau (1994). Minimally supplemented basal medium (MSBM), contained a 3:1 mixture of DMEM and Ham's F12, supplemented with 5 µg/ml insulin, 5 µg/ml transferrin, 10^{-4} M ethanolamine, 10^{-4} M phosphorylethanolamine, 20 pM triiodo-thyronine, 0.4 µg/ml hydrocortisone, 5.3×10^{-8} M selenious acid, 0.18 mM adenine, 1% gentamycin/streptomycin /amphotericin B, and 5% FBS. For organotypic raft cultures keratinocytes were trypsinized, counted and seeded onto the BM covered bovine tongue connective tissue (200,000 cells in 10 µl medium per raft). After 2 h, the raft cultures were placed on the permeable membrane of an insert-culture well system (Organogenesis Inc, Canton, MA) and submerged in MSBM. After 6 days, MSBM was replaced by a cornification medium (CM), containing a 1:1 mixture of DMEM and Ham's F12, and the same supplements as in MSBM except 2% FBS. The surface of the connective tissue substrate was raised to the air liquid interface leaving the keratinocytes exposed to air and fed only from below. After another 6 days, CM was replaced by a maintenance medium (MM) with the same composition as CM, but the concentration of FBS was reduced to 1%. The medium was changed every 2nd day for the entire culturing process.

Histology and immunohistochemistry

Immediately after excision, unseparated control specimens from the three dorsal tongue regions were embedded in Histo Prep (Fisher Scientific, New Jersey, NY), snap frozen in liquid nitrogen and stored at -80 °C. The same procedure was applied to split sets of epithelium and connective tissue specimens. Frozen

Table 1. Antibodies, working concentrations, and references.

ANTIGEN	CLONAL TYPE	DILUTION	REFERENCE/SOURCE
Tenascin (TN)	Monoclonal, BC-24	1:400	Sigma, St. Louis, MO
Laminin-5 (LM-5)	Monoclonal, GB3	1:30	Verrando et al., 1987
Laminin-1 (LM-1)	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
Integrin ß6	Monoclonal, ß6B1	1:10	Huang et al., 1998
Integrin v	Monoclonal, L230	1:10	Houghton et al., 1982
Integrin 2	Monoclonal, MAB 1950Z	1:10	Chemicon, Temecula, CA
Integrin 3	Monoclonal, MAB 1952	1:100	Chemicon, Temecula, CA
Integrin 5	Monoclonal, MAB 1986	1:100	Chemicon, Temecula, CA
Integrin ß1	Polyclonal, 3847	1:500	Roberts et al., 1988
Integrin ß4	Monoclonal, A054	1:400	Gibco BRL, Gaithersburg, MD
Keratin 10 (K10)	Monoclonal, LL002	1:100	Serotec Ltd., Oxford, England
Keratin 14 (K14)	Monoclonal, MAB 3230	1:100	Chemicon, Temecula, CA
Involucrin (INV)	Monoclonal, SY5	1:100	Sigma, St. Louis, MO
Vitronectin (VN)	Monoclonal,	1:100	Nikkari et al., 1995
Fibronectin (FN)	Polyclonal, F3648	1:500	Sigma, St. Louis, MO
Fibronectin EDA	Monoclonal, MAS521	1:100	Harlan Sera-Lab, Loughborough, England

sections (5-7 µm) were mounted on silane (Sigma, St. Louis, MO) coated slides and fixed in acetone for 5 minutes at -20 °C before storing at -80 °C. For routine histology, slides were stained with Harris' hematoxylin and eosin (HE). Samples from HaCaT raft cultures from 5 to 40 days after raising to air liquid interface were stained with a vital dye (PT #6402A, Promega, Madison, WI) and snap frozen in liquid nitrogen. Specimens were stored at -80 °C until sectioning and preparation for immunohistochemistry as described above. Organotypic cultures of HGK or NHEK were terminated 14 days after air exposure (total culture time 20 days) and prepared for immunohistochemistry. Frozen sections of healthy human gingiva (from our laboratory stock) and bovine tongue served as positive controls. A minimum of three sections for each raft was stained with each antibody. Connective tissue substrates without cells were cultured under the exact same conditions to serve as an additional control. Immunohistochemical stainings were performed by indirect immunofluorescence methods using the primary antibodies listed in Table 1 as described previously (Larjava et al., 1993). Briefly, slides were incubated with the primary antibody diluted in PBS/BSA (1 mg/ml) overnight at 4 °C and then washed with PBS/BSA and incubated with an appropriate rhodamine-conjugated secondary antibody (1:50, Boehringer Mannheim Biochemicals, Indianapolis, IN) for 1 h at room temperature. Negative control samples were incubated either with PBS/BSA or an appropriate non-immune serum instead of the primary antibody. After washing and brief drying in air, the samples were mounted with cyanoacrylate glue (Krazy Glue, Elmer's Products, Brampton, Canada). Slides were examined and photographed with a Zeiss Axioskop 20 fluorescence microscope (Zeiss, Jena, Germany).

Electron microscopy

Four glutaraldehyde-fixed specimens of epithelium and connective tissue were prepared for scanning electron microscopy (SEM) using routine protocol (Postek et al., 1980). For transmission electron microscopy (TEM), unseparated mucosa and separated connective tissue specimens were fixed in 2% glutaraldehyde for 2 h, impregnated with 1% osmium tetraoxide for 30 minutes, 2% uranyl acetate for 30 minutes, alcohol-dehydrated and embedded in Epoxy-resin (Epon 812). For TEM, 500 nm thick sections were cut with a glass knife (Microtome MT 6000 Sorvall, Dupont, Wilmington, DE) and stained with 1% tolouidine blue/1% borax for 5 minutes on a hot plate. Then, 80 nm sections were made with a diamond knife and placed on a grid, contrasted with 5% uranyl acetate for 20 minutes and 5 minutes with lead-citrate, and then viewed and photographed with a transmission electron microscope (Philips 300, Philips, Eindhoven, Holland). HaCaT cultures from 20 days after air exposure were prepared for TEM as described above.

Results

Incubation in 1M NaCl resulted in separation of tongue mucosa at the level of lamina lucida

After incubation using protocols A, B and D, the epithelium could be separated from the connective tissue with forceps requiring various degrees of force. Specimens incubated in warm PBS (protocol C) completely disintegrated upon application of any force and were discarded. In the other samples, the localization of tissue separation was evaluated on HE stained specimens. Trypsin and EDTA incubation (protocol B and D) resulted in an intra-epidermal split in all cases (not shown). Tissue prepared in 1M NaCl (protocol A) mostly showed a clean separation between epithelium and connective tissue (not shown). Some 1M NaCl separated specimens (protocol A) revealed clusters of epithelial remnants or interrupted layers attached to the connective tissue (not shown). This was observed more frequently in tissues from the base or tip of the tongue. Therefore, only cold salt separated tissues (protocol A) from the middle of the tongue dorsum were used for further experiments.

Immunohistochemical staining was used to study the localization of BM molecules at the epithelial-connective tissue interface after tissue separation. After separation, type VII collagen, heparan sulfate proteoglycan (HSPG), and type IV collagen were localized at the surface of the connective tissue but there was no immunoreactivity in the epithelium (not shown). Tenascin-C (TN-C) was scarcely localized at the connective tissue surface. Staining for laminin-1 (LM-1) and laminin-5 (LM-5) was reactive for human gingival control tissue but not with the original bovine tissue and remained negative after separation (not shown). Fibronectin (FN) and vitronectin (VN) were present only in the connective tissue compartment and remained unaltered after separation (not shown). In summary, separation of the epithelium from the connective tissue using 1M NaCl incubation appears to occur at the level of the lamina lucida.

Transmission electron microscopy examination (Fig. 1D) confirmed the separation at the lamina lucida. On SEM the surface of the epithelium revealed small irregularities and depressions (Fig. 1A). The connective tissue side showed a papillary surface architecture, free of epithelial cells (Fig 1B). Connective tissue papillae were mostly collapsed and folded, and their surfaces demonstrated multiple folds and grooves.

Raft cultures of HaCaT keratinocytes on bovine tongue matrix remain vital up to 40 days

HaCaT raft cultures remained vital as indicated by vital dye staining until end of the experiment after 40 days (not shown). HaCaT cells migrated into irregularities of the connective tissue surface filling out all empty spaces at 5 days (Fig. 2A). A highly

corrugated epithelium-connective tissue interface was formed, resembling rete pegs and connective tissue papillae. Collapsed and folded connective tissue papillae appeared as round or ovoid structures surrounded by epithelium. Stratification of keratinocytes was observed five days after raising the culture to the air-liquid interface (Fig. 2A). Maximum stratification of epithelium was observed from day 18 on and epithelium consisted of up to approximately 15 cell layers (Fig. 2B). The epithelium appeared well organized and differentiated with a distinct basal layer, but there was no formation of a stratum granulosum or stratum corneum. There was a tendency towards vertical orientation of basal cell nuclei and basal cell palisading. No major changes occurred in the period from 18 to 40 days (not shown). HaCaT epithelial migration was seen at the

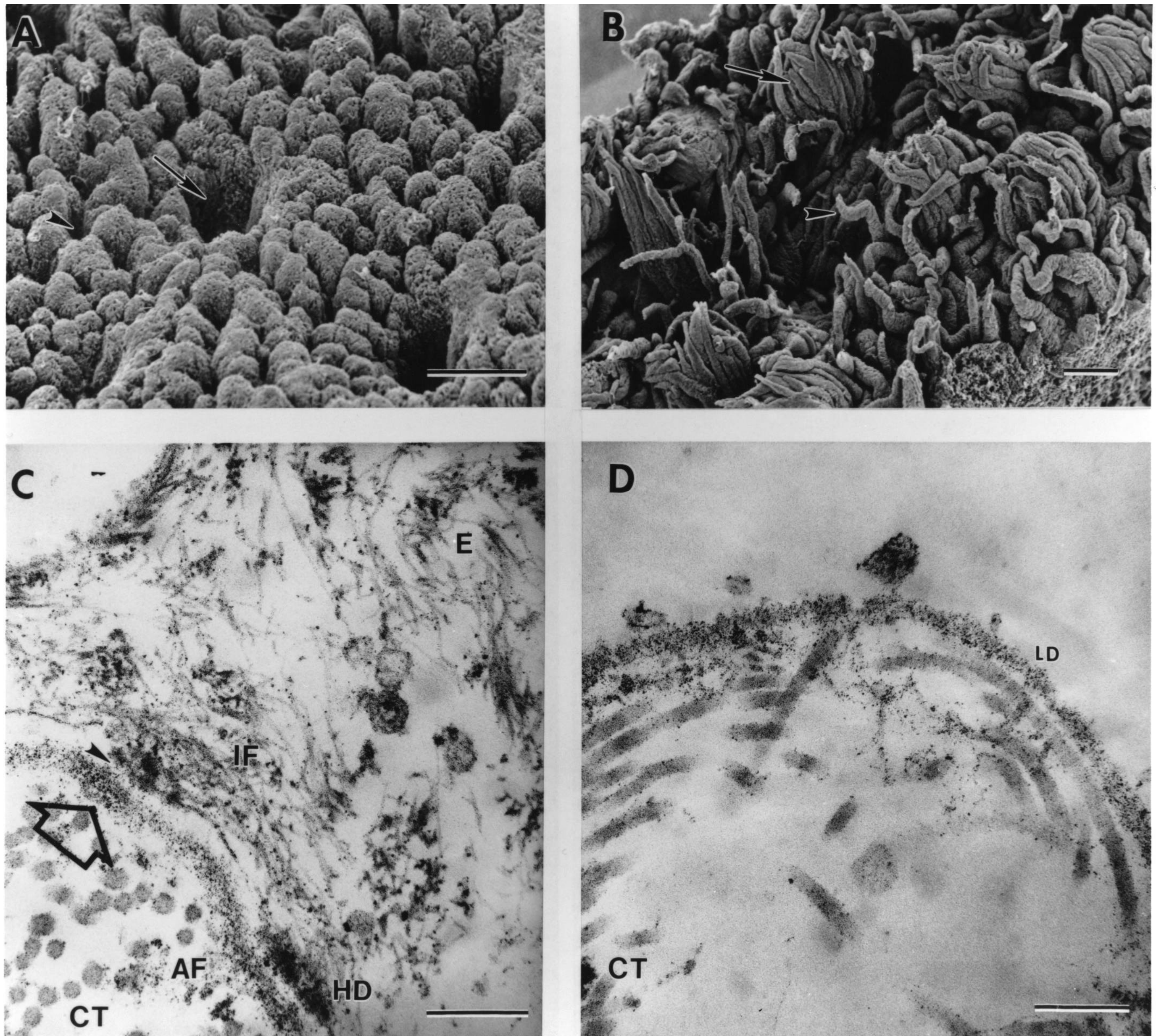

Fig. 1. Scanning **(A, B)** and transmission **(C, D)** electron microscopy of bovine tongue mucosa BMZ. **A.** Separated epithelium shows former location of connective tissue core of filiform papillae (arrow) and former location of connective tissue papillae (arrowhead) between rete pegs. **B.** Separated connective tissue shows connective tissue core of filiform papilla (arrow) and connective tissue papilla (arrowhead). Unseparated specimen **C** shows hemidesmosomes (HD), anchoring filaments (arrowhead), increased thickness and density of the lamina densa subjacent to hemidesmosomes (open arrow), and anchoring fibrils in the sublamina densa (AF). Intermediate filaments (IF) appeared to be associated with the cytoplasmic hemidesmosomal plaque. **D.** Separated connective tissue is covered by lamina densa (LD). e: epithelium; ct: connective tissue. Bar: A, 250 µm, B, 200 µm, C-D, 500 nm.

lateral borders of some rafts. The migrating layer usually consisted of 2-3 cell layers (not shown). There were no histological changes evident in the connective tissue at any time point (not shown).

Raft cultures of immortalized human gingival keratinocytes

HGK cultured on bovine tongue matrix resembled corresponding HaCaT cultures in the rete ridge-like pattern of the newly formed epithelium, but HGK cultures showed less stratification and some flattening of the most superficial cells only (Fig. 2C,D). HGK cultures also lacked a stratum granulosum and corneum. In the HGK cultures the number of cell layers was much smaller and resembled early HaCaT cultures. The cell layers in the HGK cultures were also less organized compared with HaCaT cultures (Fig. 2A,B). There was a wide variety in nuclear shape, size and density (Fig. 2D). Like HaCaT cultures, HGK cultures also showed signs of epithelial migration at raft margins (not shown).

Raft cultures of normal human epidermal keratinocytes resemble normal epidermis

Organotypic NHEK cultures in general showed the complete histomorphology of a keratinized stratified squamous epithelium (Fig. 2E). There was a basal layer with columnar or cuboidal basal cells, a few layers of spinous cells, one or two layers of granular cells and a distinct keratin layer mostly free of nuclei (Fig. 2E). Keratohyalin granules were present in the granular cell layer (not shown). However, some cultures or culture sections lacked a stratum granulosum and corneum (Fig. 2F). Instead a fine eosinophilic line covered the surface. The rete ridge pattern of NHEK cultures seemed to be

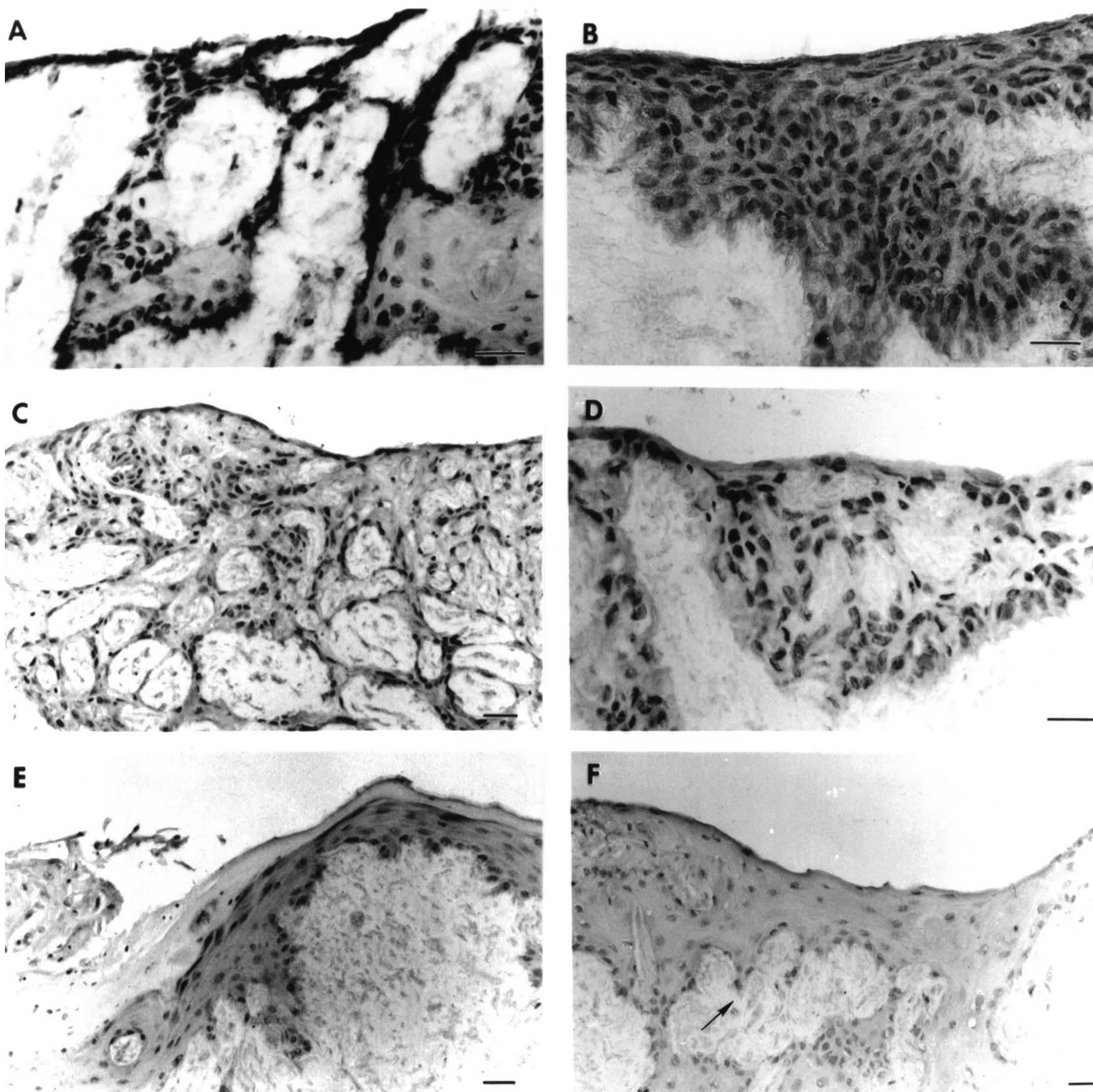

Fig. 2. Hematoxylin and eosin staining of raft cultures: HaCaT keratinocyte culture, five days **(A)** and 18 days **(B)** after air exposure. **C.** Human gingival keratinocyte (HGK) cell culture 14 days after air exposure. **D.** A higher magnification of HGK culture 14 days after air exposure. **E.** Normal human epidermal keratinocyte (NHEK) cell culture 14 days after air exposure, presence of a stratum corneum. **F.** NHEK culture section without stratum cornum 14 days after air exposure with saw-tooth like rete ridges (arrow). Bars: 200 µm.

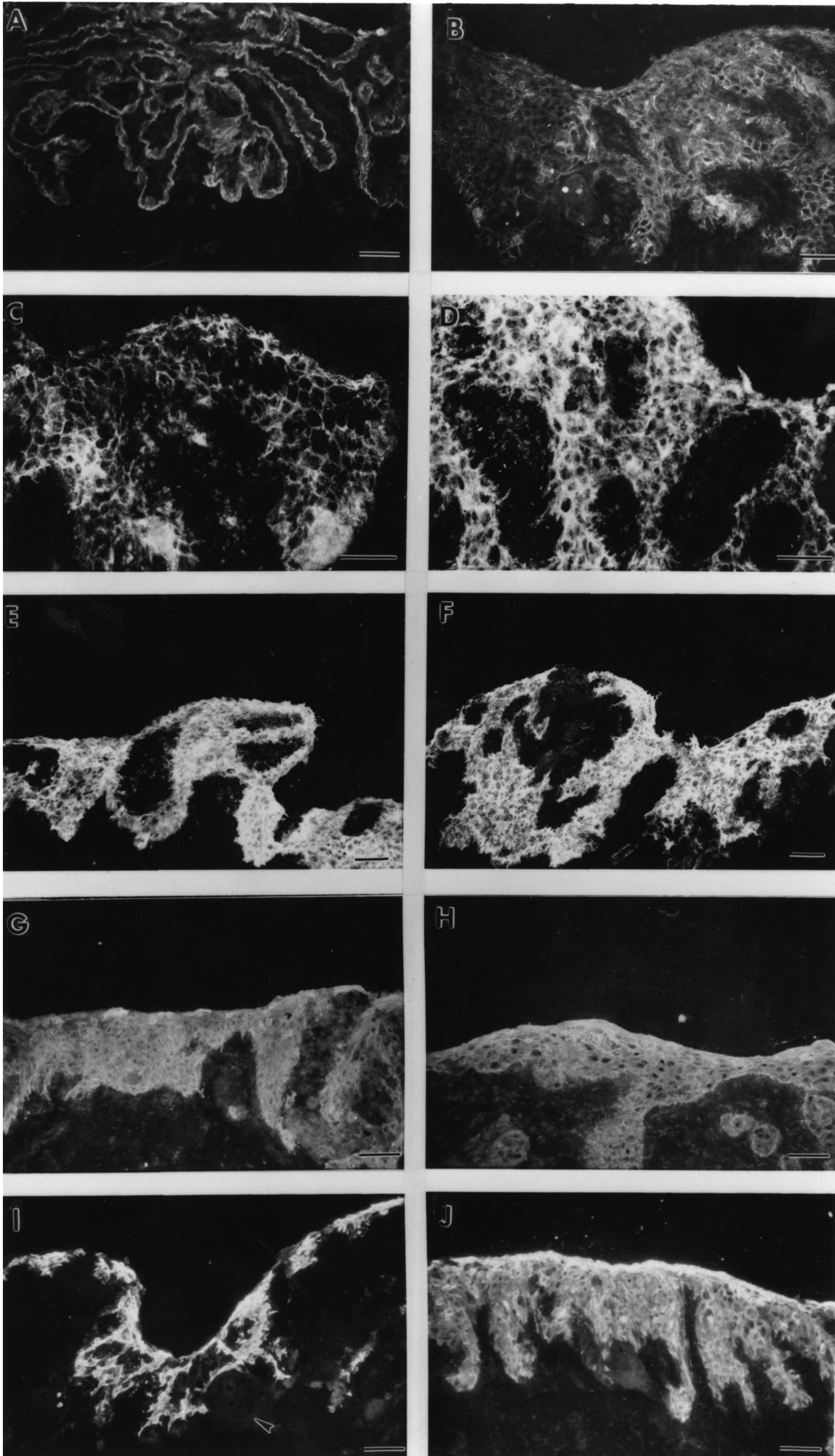

Fig. 3. Immunolocalization of integrin subunits and differentiation markers in HaCaT raft cultures, 18 days after air exposure. **A.** Integrin ß4. **B.** Integrin ß1. **C.** Integrin ß6. **D.** Integrin av. **E.** Integrin 2. **F.** integrin 3. **G.** K14. **H.** Involucrin. **I.** K10, 5 day old culture with non-reactive basal cells (arrowhead). **J.** K10, 18 day old culture. e: epithelium; ct: connective tissue. Bars: 200 µm.

distinct from the original tongue epithelium, and from corresponding HaCaT (Fig. 2B) or HGK (Fig. 2D) cultures. The tips of rete ridges had a saw-tooth like appearance rather than being rounded (Fig. 2F).

Localization of basement membrane zone components, integrins and differentiation markers in the raft cultures

HaCaT keratinocytes

Control connective tissue substratum, cultured parallel without cells and analyzed simultaneously with cultures, remained generally unaltered during the culture process and showed immunoreactivity for type IV and VII collagen at the BMZ (not shown). TN-C was expressed in a few areas of the BMZ as a line at the former connective tissue surface (not shown). HSPG showed the same pattern of localization but with somewhat reduced intensity at the connective tissue surface compared to split mucosa before culturing, whereas blood vessels within the connective tissue showed similar intensity as before the culturing process (not shown). Negative reactivity for LM-1 and LM-5 persisted (not shown). FN and VN expression was similar to connective tissue specimens immediately after tissue separation (not shown).

In general, there was no difference between young (5 days) and more mature (>18 days) cultures in respect to ECM molecule expression. Immunofluorescence staining of raft cultures demonstrated reactivity at the BMZ for HSPG, type VII and type IV collagen (not shown). Antibodies to LM-5 and LM-1, that were not reactive with bovine tissue, decorated the BMZ in the cultures (not shown). LM-1 was also expressed scattered throughout the epithelium. Staining of vascular BM was seen for HSPG, and type IV collagen only. TN-C was present in a wide band at the BMZ (not shown), which was distinct from control tissue without keratinocytes (not shown).

Integrin subunit ß4 was expressed linear at the BMZ (Fig. 3A) where it localized at the basal aspect of HaCaT keratinocytes (not shown). Integrin subunits ß1 (Fig. 3B), ß6 (Fig. 3C) and v(Fig. 3D) were present throughout all layers of the epithelium. Integrins 2

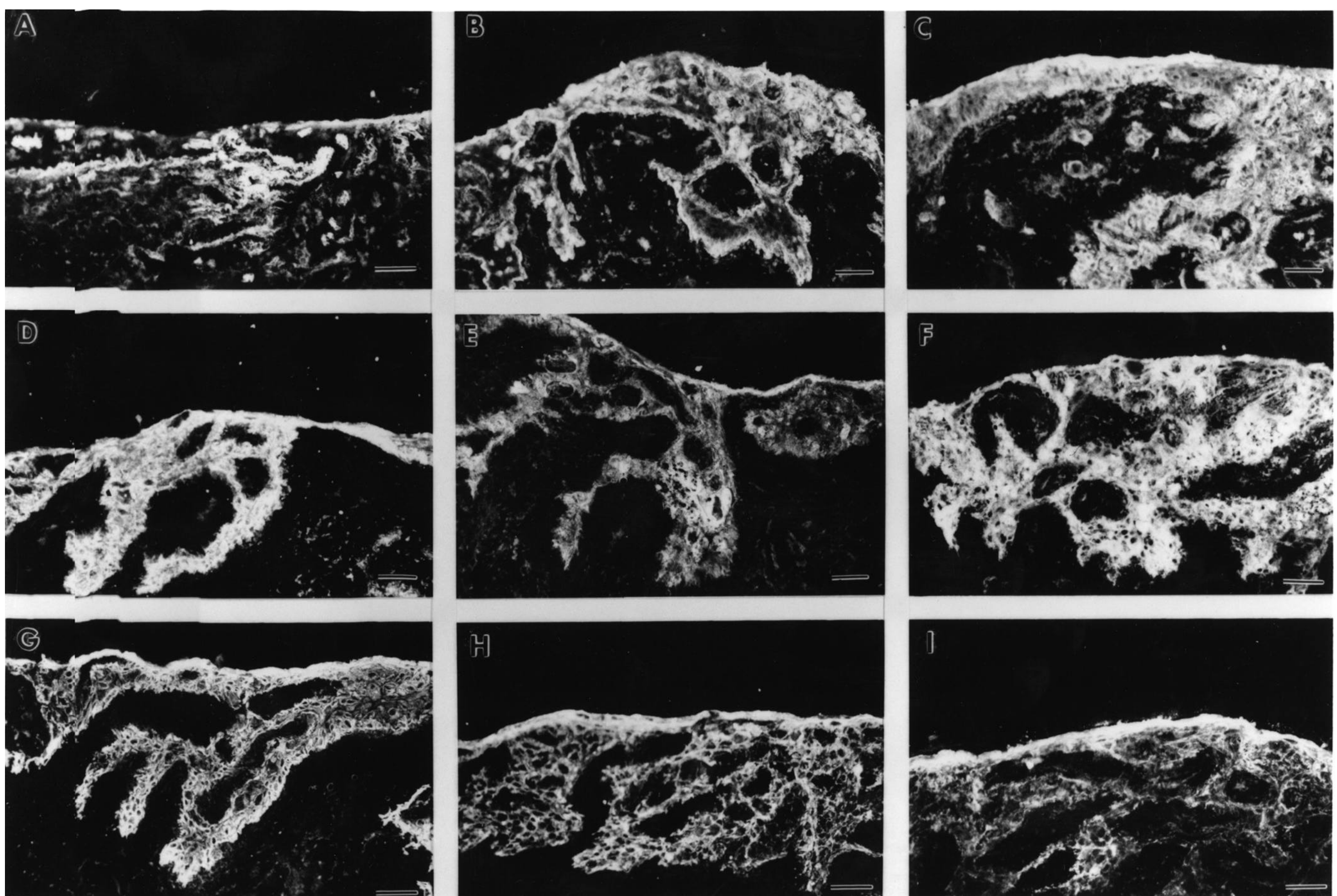

Fig. 4. Immunolocalization of integrin subunits, TN-C, and differentiation markers in HGK raft cultures, 14 days after air exposure: **A.** TN-C. **B.** Integrin ß4. **C.** Integrin ß1. **D.** Integrin 3. **E.** Integrin v. **F.** Integrin ß6. **G.** K14. **H.** K10. **I.** Involucrin. e: epithelium; ct: connective tissue. Bars: 200 µm.

(Fig. 3E) and 3 (Fig. 3F) were also located in all epithelial layers, but no immunoreactivity for 5 integrin was observed (not shown).

Keratin 14 (K14) was expressed in all epithelial layers (Fig. 3G). Keratin 10 (K10) appeared predominantly in suprabasal layers at day 5 after air exposure (Fig. 3I) but was present in all epithelial layers after 18 days (Fig. 3J). Expression was, however, more intense in suprabasal cell layers than in basal layers. Staining for involucrin was seen throughout all layers and most intense at the epithelial surface layer (Fig. 3H).

Immunohistochemistry for negative controls showed no specific reaction (not shown).

Toluidine Blue stainings of thin sections (not shown) used for transmission electron microscopy showed no signs of bovine epithelium. Ultrastructural observations of the BMZ in HaCaT cultures (not shown) demonstrated basal cell cytoplasma projections interdigitating with basal lamina protrusions. Furthermore, a normal appearance of a lamina lucida, lamina densa and numerous hemidesmosomes were seen.

Immortalized human gingival keratinocytes

Like HaCaT raft cultures, HGK cultures demonstrated immunoreactivity for type IV and VII collagen, HSPG, LM-1, LM-5 and TN at the BMZ. Expression of LM-1, LM-5 and TN-C again was new and distinct from control connective tissue without keratinocytes (not shown). TN-C expression was slightly different from HaCaT cultures demonstrating a small band under the basal cells in some areas (Fig. 4A) but not consistently in all culture sections. Integrin ß4 was localized linear at the BMZ or diffusely widespread (Fig. 4B). Integrin subunits ß1 (Fig. 4C), 3 (Fig. 4D), v(Fig. 4E), and ß6 (Fig. 4F) demonstrated a diffuse expression in all epithelial cell layers, whereas immunoreactivity for 5 integrin was negative (not shown). K14 (Fig. 4G), K10 (Fig. 4H) and involucrin

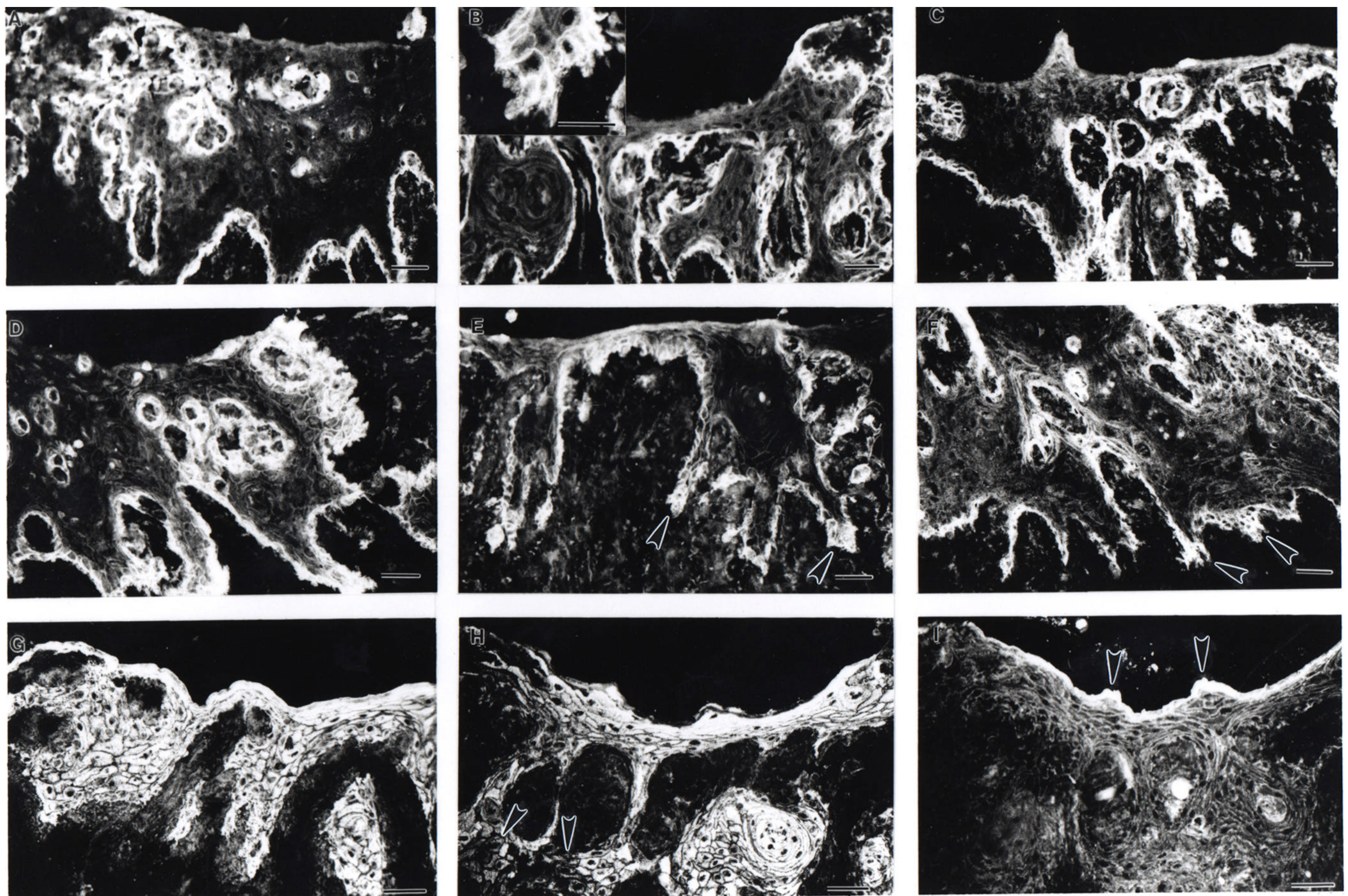

Fig. 5. Immunolocalization of integrin subunits and differentiation markers in NHEK raft cultures, 14 days after air exposure. **A.** Integrin ß4. **B.** Integrin ß1 (higher magnification at the rete peg area in the insert). **C.** Integrin 2. **D.** Integrin 3. **E.** Integrin v, increased intensity and suprabasal cell staining at rete pegs (arrowheads). **F.** Integrin ß6, increased intensity and suprabasal cell staining at rete pegs (arrowhead). **G.** K14. **H.** K10, localized basal cells with reduced reactivity (arrowheads). **I.** Involucrin, increased intensity at the epithelial surface (arrowheads). Bars: 200 µm.

(Fig. 4I) were expressed in all epithelial layers.

Normal human epidermal keratinocytes

In NHEK cultures, type IV and VII collagen, HSPG, LM-5 and LM-1 were located at the BMZ (not shown). TN-C was distributed at the BMZ as a band (not shown), comparable to that seen in HaCaT cultures.

Integrin subunit ß4 was expressed linear at the BMZ (Fig. 5A) and ß1 was located on lateral and basal cell membranes of basal cells (Fig. 5B). Integrin 2 (Fig. 5C) and 3 subunits (Fig. 5D) were localized on cell membranes around basal cells. Expression of integrin 5 subunit was limited to the cell membrane of basal cells (not shown). Integrins v (Fig. 5E) and ß6 (Fig. 5F) also demonstrated expression around basal and some suprabasal cells especially at the tips of the rete ridges.

K14 was also expressed in all epithelial cell layers (Fig. 5G). K10 was weak in some basal cells and more intense in the whole cytoplasm of all suprabasal cell layers (Fig. 5H). Involucrin staining was most intense at the superficial cell layer (Fig. 5I).

Discussion

The tissue separation techniques used for this organotypic culture model have been described previously with different tissues. The intraepithelial separation seen with cold trypsinization is similar to observations made by Skerrow (1980) but different from results described by Jensen and Mottet (1970) and Woodley et al. (1983), who observed a dermo-epidermal separation. Separation attempts with EDTA at 4 °C failed in this study despite prolonged incubation periods. A possible explanation for different outcomes with EDTA and trypsin pretreatment might be the epithelial thickness. The tongue mucosa used here has longer rete ridges/connective tissue papillae and is considerably thicker than previously used epithelia like mouse ear skin (Harris et al., 1980), mouse tongue (Mackenzie and Hill, 1981) or human oral mucosa (Scaletta and MacCallum, 1972). For tongue mucosa, the separation by 1M NaCl occurred within the lamina lucida, which is in agreement with previous observations (Scaletta et al., 1978; Willsteed at al., 1990; Karpati et al., 1991). ECM components investigated remained unaffected as judged by immunofluorescence. This confirms and supplements the findings by Woodley et al. (1983). We conclude that the NaCl induced split is the most reliable and results in minimal morphologic or biochemical alterations, which is in agreement with conclusions by Willsteed et al. (1991).

De-epithelialized tongue mucosa appeared to have similar culture histomorphology as de-epidermized dermis (DED) (Regnier et al., 1981), which is distinct from fibroblast/collagen lattice co-cultures (Garlick et al., 1996) as they lack a rete ridge pattern. One major difference between DED and BM covered lamina propria of bovine tongue is the presence of longer connective tissue papillae in the bovine tongue mucosa. Additionally, the pattern of keratinization in the original tissue was different. It has been demonstrated that in vivo the underlying connective tissue determines whether epithelium becomes keratinized or not (Karring et al., 1975, Mackenzie and Hill, 1984) and that the connective tissue determines histodifferentiation of the epithelial cells (Squier and Kammeyer, 1983). However, in some cases transplanted epithelium may maintain its original morphology (Billingham and Silvers, 1967; Gipson et al., 1986). There may be separate connective tissue influences on epithelial architecture and differentiation and there seems to be a regionally-related variation in the competence of epithelia to respond to these influences (Mackenzie and Hill, 1984).

A viable dermal substrate might be necessary for the formation of a basal lamina under explant outgrowth (Woodley et al., 1980), although, in recombination culture, a basal lamina was formed on devitalized dermis (Briggaman et al., 1971). More recent studies have shown that a living dermal substrate is not required for normal histomorphology, keratinization (Mackenzie and Fusenig, 1983, Noel-Hudson et al., 1995) or formation of a basement membrane (Tinois et al., 1991). In the absence of living dermal components, features of the cell lines seem to determine histomorphology. Only NHEK were capable of producing a stratum corneum, although not consistently. This variation may be explained by the fact that foreskin is a source of a mixed keratinocyte cell population. Fluctuations of the culture conditions may favor either the expression of a skin type or mucosa type culture. In this model, the potential for keratinization of HaCaT is indicated by the expression of K10, which is typically expressed in keratinized epithelium (Bloor et al., 1998). Keratinization of HaCaT cells has been demonstrated in vitro (Schoop et al., 1999) and after transplantation of cultures onto nude mice (Breitkreutz et al., 1998). Regarding keratinization of HaCaT cultures, the lack of viable mesenchymal cells in our model might be the key difference between our cultures and keratinocyte-fibroblast co-cultures. Therefore, extrinsic mesenchymal factors (Schweizer et al., 1984) or intrinsic differentiation programs (Mackenzie and Hill, 1981; Boukamp et al., 1990; Lindberg and Rheinwald, 1990) may determine differentiation in these cultures.

HGK did not produce cultures with normal histomorphology, having only a few cell layers without distinct epithelial layers. Abnormal stratification and aberrant integrin expression was seen in other transformed keratinocytes grown on collagen/fibroblast lattice (Kaur and Carter, 1992). The poor histodifferentiation, nuclear polymorphism and aberrant diffuse integrin expression may be attributed to their spontaneous transformation and partially triploid chromosomes in HGK (Mäkelä et al., 1998).

In the present study, the expression of differentiation markers was different than that seen in vivo skin, in which K14 is mostly restricted to basal cells and K10 is

expressed in suprabasal cells (Dale et al., 1990). Suprabasal expression of K14 and expression of K10 in basal layers occurred in all cultures in the present study. Expression of K14 in all epithelial layers has been described in keratinocyte cultures grown in porcine skin in vitro previously (Matouskova et al., 1998). In HaCaT and NHEK raft cultures, expression of K14 was mostly restricted to basal cells and K10 to suprabasal cells (Breitkreutz et al., 1998). An extended distribution of keratin mRNA compared to the corresponding keratin has been described in vivo (Bloor et al., 1998). Involucrin appears to be present in immediate suprabasal layers of keratinocyte raft cultures from various origins (Banks-Schlegel and Green, 1981). Matouskova et al. (1998) reported involucrin and transglutaminase expression in the granular and horny layer. Normal expression of involucrin and late differentiation markers was also reported for cultures of epidermal keratinocytes and outer rooth sheath cells (Limat et al., 1991). In our study, all cultures showed varying degrees of immunostaining for K10, K14 and involucrin and was considered abnormal with expression in all cell layers.

Integrin expression in HaCaT and HGK keratinocyte raft cultures was not restricted to the basal cell layers except for the ß4 integrin subunit that was localized at the basal membrane of basal cells as in gingiva and original bovine tongue mucosa. The basal location of integrin ß4 in HaCaT and NHEK cultures indicate the stationary epithelial status of basal cells (Larjava et al., 1993). Integrin subunit v was found in normal oral epithelium by some investigators (Jones et al., 1993) but not by others (Larjava et al., 1993; Zambruno et al., 1995). In this study, integrins v and ß6, 3 and ß1 were present in all layers of HaCaT and HGK organotypic cultures. It seems likely that vß6 heterodimers were formed, although it cannot be ruled out that vß5 or vß1 integrins were also expressed since vß1 (Koivisto et al., 1999) and vß5 integrins (Zambruno et al., 1995) have been reported to be present in cultured keratinocytes. Continuous normalization of integrin expression and basal cell expression for integrin v, which became interrupted at 3 weeks, has been described in organotypic gingival cultures on collagen/fibroblast lattice (Tomakidi et al, 1999). The ß6 subunit has not been investigated in organotypic culture models previously. It is expressed in epithelial cells during wound healing, and in premalignant and malignant epithelia (Haapasalmi et al., 1996; Häkkinen et al., 2000; Hamidi et al., 2000). The integrin vß6 augments the proliferation of colon carcinoma cells in collagen gels (Dixit et al., 1996). Since both vß6 and 5ß1 integrins were present in NHEK raft cultures, it is possible that they mimick epithelium typical for late wound healing where basement membrane is formed. It remains to be shown whether these cultures remain positive for these integrins when cultured for extended time.

Suprabasal expression of 3 and 2 subunits is seen during oral mucosal (Larjava et al., 1993) wound healing and psoriasis (Hertle et al., 1992) and can be induced in vivo by retinoids (Häkkinen et al., 1998). This pattern could be observed in our HaCaT organotypic cultures. NHEK organotypic cultures showed integrin expression that resembled normal epidermis in many respects. All 2, 3, 5, and ß1 integrin subunits were located pericellularly around basal cells. Basal cell expression of 2, 3, 6 and ß1 integrins has been observed previously in cultures of normal epidermal keratinocytes and outer root sheath cells on fibroblast/collagen lattice (Limat et al., 1995). Expression of 6ß4, 2ß1, 3ß1, and avß5 was observed in cultured human skin keratinocytes (Marchisio et al., 1991).

It has been well established that epithelial cells are capable of producing major BMZ components, which has been demonstrated in various keratinocyte-fibroblast co-cultures (Fleischmajer et al., 1993; Breitkreutz et al, 1998; Tomakidi et al., 1998, 1999). In our culture model, only keratinocytes were present to deposit ECM as there were no vital mesenchymal cells. Deposition of BMZ components by HaCaT cells has been demonstrated in organotypic culture transplants onto nude mice (Breitkreutz et al., 1998). When HaCaT cells are injected into athymic mice they form cysts with peripheral deposition of BMZ components (LM-5, type IV and VII collagen) (Kainulainen et al., 1998). LM-5 was clearly expressed in all cultures of our model, which is reflected by the formation of anchoring filaments in the lamina lucida as judged by TEM. In our study LM-1, LM-5 and tenascin were solely produced by keratinocytes.

In organotypic cultures, BMZ components are secreted and form an ultrastructurally normal BMZ both in vitro (Woodley et al., 1980; Regnier et al., 1981, 1990, Smola et al., 1998; Schoop et al., 1999; Stark et al., 1999; Tomakidi et al., 1999) and when transplanted onto mouse skin (Breitkreutz et al., 1998). When the lamina densa is present it seems to be utilized and integrated into the new BMZ (Regnier et al., 1981). Our EM observations imply that hemidesmosomes form in the areas of existing anchoring fibrils which is in agreement with observations from transplants on denuded corneal basal lamina (Gipson et al., 1983). Formation of anchoring fibrils has also been observed at one week in transplanted HaCaT-fibroblast co-cultures (Breitkreutz et al., 1998) and around skin explant cultures (Woodley et al., 1980). In both cases, a viable dermal substrate is present which was thought to be essential for anchoring fibril formation (Briggaman and Wheeler, 1975). As nidogen is exclusively produced by fibroblasts (Fleischmajer et al., 1998) the formation of a functional BMZ may depend on fibroblast presence unless the pre-existing matrix provides these elements.

In summary, organotypic cultures using bovine tongue connective tissue matrix and NHEK show some features of in vivo skin, but HaCaT cultures may benefit from their low maintenance, rapid growth and so far extended life span in our model. HaCaT cultures remained vital and histologically unaltered for up to 40 days after raising the culture to the air liquid interface.

The majority of studies on organotypic cultures are limited to a few weeks. No attempts to grow organotypic cultures for prolonged time periods are currently known. The significant lifespan of our HaCaT cultures may be extended even further. This may be attributed to the immortality of HaCaT cells. The presence of deep rete ridges, which may favor the formation of niches for increased numbers of stem cells (Watt and Hogan, 2000), might prolong the life span of NHEK cultures. This remains to be confirmed by prolonged culture experiments and proliferation assays. An extended longevity of an organotypic culture expands its application to more time requiring experiments and long-term observations. Transformed HGK produce rather poor histomorphology and show many abnormalities in keratin and integrin expression. However, these cultures may still be valuable for studying and comparing various pathologic conditions.

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