Mutual Induction of Growth Factor Gene Expression by Epidermal-Dermal Cell Interaction

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Abstract. Epithelial-mesenchymal interactions control epidermal growth and differentiation, but little is known about the mechanisms of this interaction. We have examined the effects of human dermal microvascular endothelial cells (DMEC) and fibroblasts on keratinocytes in conventional (feeder layer) and organotypic cocultures (lifted collagen gels) and demonstrated the induction of paracrine growth factor gene expression. Clonal keratinocyte growth was similarly stimulated in cocultures with irradiated DMEC and fibroblasts as feeder cells. This effect is most probably caused by induction of growth factor expression in cocultured dermal cells. Keratinocytes stimulated mRNA levels for KGF and IL-6 in both mesenchymal cell types and GM-CSF in fibroblasts. The feeder effect could not be replaced by conditioned media or addition of isolated growth factors.

In organotypic cocultures with keratinocytes growing on collagen gels (repopulated with dermal cells), a virtually normal epidermis was formed within 7 to 10 d. Keratinocyte proliferation was drastically stimulated by dermal cells (histone 3 mRNA expression and BrdU labeling) which continued to proliferate as well in the gel. Expression of all typical differentiation markers was provoked in the reconstituted epithelium, though with different localization as compared to normal epidermis. Keratins K1 and K10 appeared coexpressed but delayed, reflecting conditions in epidermal hyperplasia. Keratin localization and proliferation were normalized under in vivo conditions, i.e., in surface transplants on nude mice. From these data it is concluded that epidermal homeostasis is in part controlled by complex reciprocally induced paracrine acting factors in concert with cell-cell interactions and extracellular matrix influences.

THERE is accumulating evidence that epithelial-mesenchymal interactions regulate tissue homeostasis in surface epithelia such as epidermis (6, 7, 9, 13, 19, 20, 30-32, 47). In vivo, these processes are difficult to study due to many variables involved such as the different dermal cell types and superimposed influences of systemic factors of the blood circulation. Thus, in vitro model systems have been developed to mimic epidermal-dermal interactions and to study regulation of epidermal cell proliferation and differentiation (for review see reference 19). It had been shown that coculture on postmitotic fibroblastic mouse 3T3 cells (42, 54) as well as human dermal fibroblasts (30) are required to support human keratinocyte growth at clonal densities in serum containing medium. Under conventional (submerged) culture conditions keratinocyte proliferation is the predominant phenomenon whereas terminal differentiation and tissue organization are reduced or aberrant compared to the in vivo situation (9, 24). Improvement of tissue architecture and induction of terminal differentiation markers have been achieved in organotypic culture systems where keratinocytes are cultured on collagen gels populated with either murine 3T3 cells (27) or normal fibroblasts from various species and different body sites (1, 3, 13, 19, 20, 55). Alternatively, de-epidermized dermis has been used as dermal substitute in vitro with or without interposed collagen gels leading to comparably good results on epidermal organization and differentiation (7, 32, 41).

Under these conditions keratinocytes from different locations, commonly foreskin, exhibited a rather normal morphogenesis by forming a well organized stratified epithelium. Expression and distribution of most differentiation markers were significantly improved largely resembling the situation in normal skin (7, 27). This improvement in keratinocyte growth and differentiation has been attributed (*a*) to the air exposure of the epidermis and thus, a more physiological nutrient flow (from below) (1, 39) as well as the appropriate adjustment of retinoid levels (2, 26, 27), (*b*) to effects of fibroblasts (mediated by diffusible factors) and (*c*) constituents of the extracellular matrix. One possible candidate of a diffusible factor is the recently described keratino-

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cyte growth factor (KGF)[1] (17, 45), but also other members of the FGF family as well as other cytokines. In most other studies, control experiments with keratinocyte cultures on lifted collagen gels without incorporated fibroblasts have not been included. Thus, a clear distinction between the various influences was difficult.

While the effects of fibroblastic cells are well documented (6, 13, 20, 30, 31, 47, 50), interactions of endothelial cells with keratinocytes have (to our knowledge) not been investigated so far. In skin, dermal microvascular endothelial cells (DMEC) represent a large cell population providing a multifunctional interface between the blood circulation and surrounding tissue. Capillary endothelium plays an important role in skin physiology and is often involved in pathological processes, e.g., inflammation (12, 25). Whether DMEC, apart from their vascular functions, participate in maintaining epidermal homeostasis by regulating keratinocyte proliferation and differentiation is at present poorly understood.

In this report, we have studied the effects of DMEC and fibroblasts on growth and differentiation of keratinocytes in both conventional and organotypic cocultures. Clonal keratinocyte growth was supported comparably by postmitotic feeder cells of DMEC and dermal fibroblasts. This growth stimulation of keratinocytes was possibly meditated by growth factors that had been induced by keratinocytes in the feeder cells. In organotypic cocultures, human dermal fibroblasts (HDF) and DMEC stimulated keratinocyte growth and differentiation to a comparable extent. In noncontracted collagen gels continued proliferation of both mesenchymal cell types was observed concomitant with hyperproliferation and retarded keratinization of the reconstituted epidermis. This modulation of keratinocyte growth and differentiation by DMEC and fibroblasts, indicative of certain skin disorders, was reversible because it was normalized in the in vivo situation in surface transplants of organotypic cocultures on nude mice. Thus, these experimental systems mimicking normal and diseased epidermis provide models for the detailed study of regulatory mechanisms controlling tissue homeostasis in skin as well as other stratified squamous epithelia.

Materials and Methods

Cell Isolation and Culture

Normal human epidermal keratinocytes were isolated from post mortem (up to 48 h) abdominal skin. Biopsies were incubated for 30 min in betadine solution (10% iodine) and rinsed twice in PBS without Ca^{2+} and Mg^{2+} for 10 min each. Split thickness skin was prepared by a Castroviejo dermatome (Storz Instrument GmbH, Heidelberg, FRG) set at a depth of 0.1 mm and pieces of ~1 × 1 cm were floated on 0.6% trypsin, 0.05% EDTA (1/1) in PBS for 30 min at 37°C. The epidermis was separated from dermis by fine forceps, and gentle aspiration of the epidermal sheets released keratinocytes which were passed through a nylon gauze (0.1 mm mesh width). The cell suspension was counted and seeded at densities of 1-2 × 10^4 cells/cm^2 on feeder layer containing dishes. Instead of murine 3T3 cells (42), irradiated normal human skin fibroblasts were used as feeder cells as published recently (30). Culture medium (called FAD) consisted of 1 part Ham's F12, 3 parts DME, supplemented with 5% FCS (Boehringer, Mannheim, FRG), 5 μg/ml Insulin, 1.8 × 10^{-4} M adenine, 10^{-10} M cholera toxin, 0.4 μg/ml hydrocortisone (all from Sigma Chemical Co., St. Louis, MO) and 10 ng/ml EGF (Collaborative Research, Waltham, MA). For experiments keratinocytes were obtained either from frozen stocks, cryopreserved at first passage, or from fresh isolates after their first and second passage on feeder cells, respectively. Vitality and growth capacity of the respective keratinocytes were routinely tested by cloning assays (see below).

Human DMEC were isolated from post mortem abdominal skin biopsies essentially as described by Davison and Karasek (14). In short, 5x5 mm large split thickness skin pieces (0.2 mm dermatome setting) were incubated in 0.3% trypsin, 1% EDTA (1/1) in PBS for 30 min at 37°C. The epidermis was removed by fine forceps, the remaining dermis rinsed with PBS and transferred to fresh culture medium (M 199, Sigma Chemical Co.). Microvascular endothelial cells were released from the vessels into the medium by lateral application of moderate pressure on the dermal surface with a blunt scalpel blade. The DMEC were pelleted by low speed centrifugation (200 *g* for 15 min), resuspended in 1 ml culture medium and seeded on fibronectin (10 μg/ml fibronectin; Boehringer Mannheim, FRG) coated petri dishes. Contaminating fibroblast-like cells were mechanically removed with a gauge fitted to a syringe under the phase contrast microscope after 24 h. The remaining small endothelial cell colonies were further cultivated in M 199 supplemented with 10^{-9} M cholera toxin, 20% FCS, 20 ng/ml endothelial cell growth factor (ECGF), 90 ng/ml heparin (ECGF and heparin: Boehringer Mannheim, FRG) and medium was changed every 2 or 3 d. DMEC were subcultured at confluence by incubation in trypsin/EDTA (0.1% and 0.05%, respectively) and replated on fibronectin coated dishes. Frozen stocks were prepared from which cells were thawed and subsequently used at passages 5 to 7.

Cultured DMEC were identified by positive staining in indirect immunofluorescence (IIF) for factor VIII related antigen (VIIIr:Ag), collagen type IV and laminin, while being negative for smooth muscle actin. As an additional marker, accumulation of acetylated low density lipoprotein labeled with fluorescent 1,1'-dioctadecyl-1-3,3,3',3'-tetramethyl-indo-carbocyanine perchlorate (DiI-Ac-LDL) (Paesel and Lorei, Frankfurt, FRG) was tested by incubation for 4 h in medium containing fresh DiI-Ac-LDL (10 μg/ml). After fixation with buffered formalin, cells were viewed on an epifluorescence microscope with a rhodamin filter combination. Cells from frozen stocks were routinely tested once more for factor VIIIr:Ag.

Normal human skin fibroblasts were isolated from post mortem abdominal skin explant cultures in DME containing 10% FCS. At passage 4, frozen stocks were prepared and in all experiments fibroblasts from these stocks were used at passages 5 to 7. These cells could be maintained as proliferating cultures up to passage 12 when they were still able to grow at clonal densities.

Conditioned media of dermal feeder cells and epidermal-dermal cell cocultures were obtained at 2, 4, 6, 8, and 10 d after seeding (and 48 h incubation time), sedimented at 1,000 *g* and used either immediately or stored frozen. Keratinocytes were seeded at clonal densities (1 × 10^4/60-mm dishes) and incubated with normal and conditioned medium, respectively, for 10 d and colonies fixed and stained.

Keratinocyte Cloning Assays

Cell cloning assays were performed on growth inhibited DMEC, HDF, or a 1:1 mixture of both. Dermal cells were irradiated (70 Gy, ^{141}Cs source) and seeded at 1 × 10^5 cells/60-mm dish. After 1 to 3 d keratinocytes were added at 5 × 10^2, 10^3 and 10^4 cells/60-mm dish and cultured for 10 d in FAD without medium change. After fixation in buffered formaldehyde colonies were stained with Mayer's Hematoxylin and Rhodamin B, the latter for visualizing heavily keratinized colonies, and counted.

Organotypic Cultures

Organotypic cultures were prepared with collagen gels. Collagen type I was isolated from rat tail tendons by solubilization in 0.1% acetic acid after removal of blood vessels and peritendineum. After centrifugation, the cleared solution was lyophilized. On SDS polyacrylamide gels each preparation showed the typical banding pattern of collagen type I. To prepare collagen gels 8 vol of ice cold collagen solution (4 mg/ml in 0.1% acetic acid) were mixed with one volume 10× Hank's balanced salt solution at 4°C and neutralized with 1 N NaOH. To produce cell-free gels, 1 vol of FCS was added, whereas in the case of gels repopulated with mesenchymal cells (3), DMEC or HDF were suspended in 1 vol FCS. One ml of this solution (containing a total number of 1 × 10^5 dermal cells) was poured into each well

1. *Abbreviations used in this paper*: BrdU, 5-bromodeoxyuridine; CRD, combi ring dish; DiI-Ac-LDL, 1,1'-dioctadecyl-1-3,3,3',3'-tetramethyl-indo-carbocyanine perchlorate-acetylated low density lipoprotein; DMEC, dermal microvascular endothelial cells; GM-CSF, granulocyte macrophage colony stimulating factor; HDF, human dermal fibroblasts; IIF, indirect immunofluorescence; IL-1, 3, 6, interleukin 1, 3, 6; KGF, keratinocyte growth factor.

of 24 well plates and allowed to polymerize for at least 30 min at 37°C giving rise to gels of 2–3 mm thickness. To these gels, 1 ml DME (containing 10% FCS) was added and left for 1 d for equilibration. For organotypic cocultures of keratinocytes and dermal cells the 1 ml DME was aspirated and 7×10^5 keratinocytes were added and, after 24 h of additional submerged culture, gels were placed on metal grids at the air medium interface. Alternatively, organotypic cultures were prepared in culture chambers consisting of two concentric teflon rings (Combi Ring Dish, CRD [35]; Renner KG, 6701 Dannstadt, FRG). For CRD cultures, the collagen lattice (with or without dermal cells) was mounted between the rings and 24 h later, keratinocytes (2.5×10^5/CRD, culture surface: 0.5 cm^2) were plated. The following day, the confluent keratinocyte cultures were air-exposed by aspiration of the medium inside the CRD and transferred to Stanzen Petri dishes (with 1-mm circular rims molded at the bottom, Greiner and Söhne, Nürtingen, FRG) thus allowing free access of the medium to the lower surface of the culture. Generally, medium was changed daily and consisted of DME/Hank's F12 (3:1) supplemented with 10% FCS, 0.4 μg/ml hydrocortisone, 10^{-10} M cholera toxin and 50 μg/ml ascorbic acid (Sigma Chemical Co.). In both assays, when DMEC and HDF were incorporated separately or grown together within the gel, the total number of dermal cells was kept constant (5×10^4 cells/CRD, 1×10^5 cells/gel on grids).

Transplantation of Organotypic Cultures onto Nude Mice

Before transplantation, the above described organotypic cultures mounted in the CRD device (b/c' ring pair) were covered with the outer hat-like part of a silicone transplantation chamber (Renner KG, 6701 Dannstadt, FRG) and transplanted *in toto* onto dorsal muscle fascia of nude mice as described (7). After 1–3 wk, transplants were dissected en bloc and further processed for histology and kryostat sectioning.

Indirect Immunofluorescence

Antibodies, their origin and dilutions used in this study are listed in Table I. Specimens were frozen in embedding medium (Reichert und Jung, Nußloch, FRG) and kryostat sections made at a 6-μm setting and allowed to air dry on glass slides. Sections were incubated with primary antibody for 1–12 h in a moist chamber, washed three times in PBS before FITC conjugated secondary antibodies (all affinity purified, obtained from Dianova, Hamburg, FRG) were applied for 30 min. For double IIF, sections were incubated with both primary keratin antibodies (anti K1 [raised in guinea pig] and anti K10 [in rabbit]) (both kindly provided by Dr. Denis Roop, Houston, TX) concomitantly followed by biotinylated anti-rabbit antibody and subsequent incubation with FITC-labeled anti-guinea pig antibody together with Texas red conjugated streptavidin. Finally, samples were embedded in Aqua Mount (BDH Chemicals, Poole, England) and photographed on a Zeiss inverted microscope (IM35) equipped with epifluorescence.

Histology

Specimens were prefixed in buffered formalin (3.7%), embedded in agar (3%) to prevent dissociation of the epithelium from the underlying collagen gel, and postfixed in formalin. After paraffin embedding, 5-μm sections were made and stained with hematoxilin and eosin.

Proliferation Assays

In addition to the analysis of histone 3 mRNA on Northern blots (as a marker for DNA replication), DNA synthesizing cells were identified in organotypic cocultures after simultanous incorporation for 24 h of equimolar concentrations (65 μM) of 5-Bromodeoxyuridine (BrdU) and Deoxycytidine (both Sima) and subsequent labeling of frozen sections with a BrdU specific monoclonal antibody (Partec AG, CH-4144 Arlesheim; 1:100 in PBS + 0.5% Tween 20) linked to a fluoresceinisothiocyanat labeled (Sigma Chemical Co.) secondary antibody. Total cell number was visualized by staining cell nuclei with 5 μg/ml Bisbencimide (Hoechst).

Proliferation of DMEC and HDF in collagen gels was quantitated by cell counting over a period of 14 d after complete digestion of the collagen gels by incubation in 2 μg/ml collagenase (Sigma Chemical Co.) in a 30°C water bath for 35 min, followed by centrifugation (10 min at 2,000 rpm) and resuspension of the dermal cell pellet in PBS, as described (47).

RNA Isolation and Hybridization

Total RNA was isolated as described by Chomczynski and Sacchi (10). In the case of feeder layer cocultures, before lysis dermal cells were separated from half-confluent keratinocyte cultures by gentle pipetting of a 0.1% EDTA solution which detached dermal cells while keratinocytes remained tightly adherent. Separation of the epidermal from the dermal component in organotypic cocultures was achieved by mechanical removal of the epidermis from the underlying dermal equivalent with fine forceps to allow assignment of positive signals to either compartment. Histological control of the specimens confirmed complete separation. In addition, Northern blots using probes for collagen type I (for fibroblasts) and keratin 14 (for keratinocytes) confirmed the clean separation of both compartments (not shown here). Samples containing 5 μg total RNA were fractionated in a 1.2% agarose gel and transferred to a nylon membrane (Hybond N, Amersham). UV cross-linking and hybridization (18–24 h) were performed as described by Church and Gilbert (11). Inserts of histone 3 DNA (3' coding region [38]), IL 3 (15), GM-CSF (33), IL 6 (56), interstitial collagenase (personal gift from B. Stein, Karlsruhe, FRG) and GAPDH (kindly provided by Thomas Krieg, Köln, FRG) were random-prime labeled with ^{32}P-alpha-dCTP (3000 Ci/mmol, Amersham) according to Feinberg and Vogelstein (16). After hybridization, filters were washed in 2 × SSC (SSC: 0.15 M NaCl, 0.015 M sodiumtricitrate), 0.1% SDS, followed by 0.1 × SSC, 0.1% SDS at 65°C and exposed at −70°C with intensifying screens.

Construction of Keratinocyte Growth Factor Probe

A KGF specific probe was constructed based upon the published sequence of human KGF. After RNA extraction from fibroblasts and reverse transcription the cDNA was amplified by PCR employing as 5' primer ATGCACAAATGGATACTGACA and as 3' primer AGTTATTGCCATAGGAAGAAA (personal gift from W. Weinig) according to the published sequence (17). The resulting 582-bp fragment was identified by: (*a*) the EcoRI restriction site generating a 345-bp and 237-bp fragments and (*b*) after subcloning into pCR 1000 vector (Invitrogen, San Diego, CA) by partial sequencing.

Results

Characterization of Microvascular Endothelial Cells

DMEC were isolated from post mortem trunk skin. After trypsinization of the epidermis, the remaining dermal slices consisted of the stratum papillare and upper parts of the stra-

Table I. List of Antibodies

Antigen	Antibody specification	Dilution	Reference
K14	LH 8	undiluted	(40)
K 1 (sequence specific)	polyclonal (guinea pig)	1:500	(44)
K 10 (sequence specific)	polyclonal (rabbit)	1:500	(44)
Involucrin	polyclonal (rabbit)	1:250	(51)
Collagen type IV	polyclonal (rabbit)	1:100	(18)
Laminin	polyclonal (goat)	1:100	(18)
Factor VIIIr:Ag	polyclonal (chicken)	1:200	Sera Lab
alpha-smooth muscle cell actin	1A4	1:200	Sigma Chemical Co.
BrdU	monoclonal	1:100	Partec AG

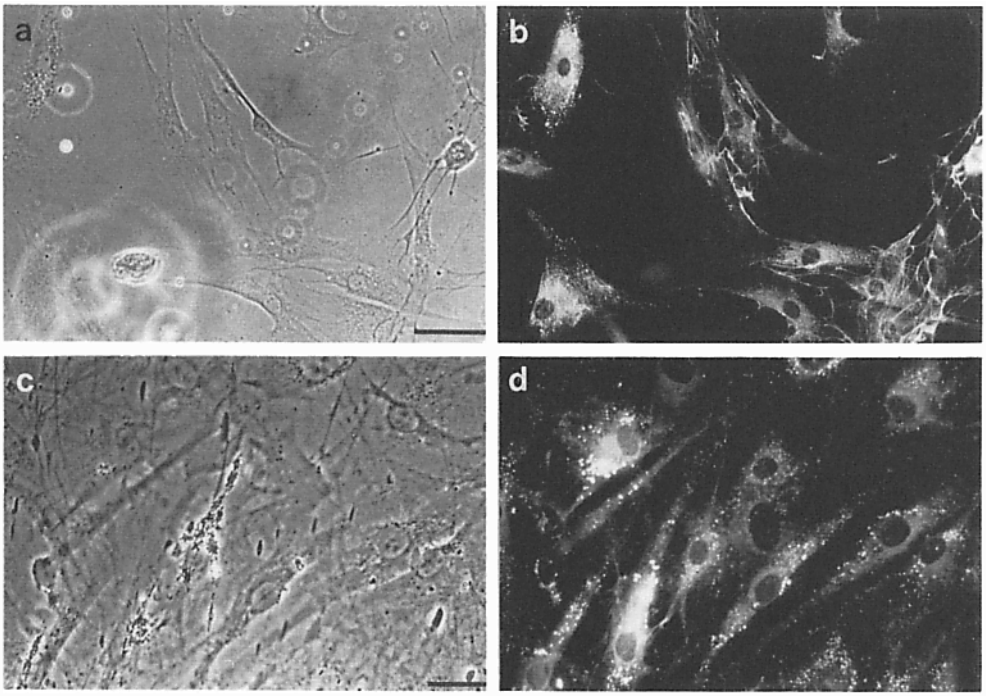

Figure 1. Characterization of microvascular endothelial cells: localization of Factor VIIIr:Ag in cultured human dermal microvascular endothelial cells (*a*, *b*) and uptake of Dil-Ac-LDL by cultured DMEC (*c*, *d*); *a* and *c* show phase contrast micrographs corresponding to *b* and *d*, respectively. Bars: (*a*, *b*) 50 μm; (*c*, *d*) 25 μm.

tum reticulare. Thus, the small cell clusters and single endothelial cells released by gentle squeezing originated from uppermost dermal microvessels (see also reference 25). After settling and spreading on fibronectin-coated culture dishes, small colonies of endothelial cells with typical cobblestone morphology and a distinct pericellular halo formed. With expansion endothelial cells gradually acquired a more elongated form, but remained positive for specific endothelial cell markers, usually tested at second or third passage. Antibodies to factor VIII related antigen (VIIIr:Ag), strongly labeled blood vessels in the dermis (not shown) and revealed the typical perinuclear granular staining by IIF irrespective of the actual cell shape (Fig. 1, *a* and *b*). As a further endothelial cell marker uptake of acetylated low density lipoprotein (fluorescent labeled Dil-Ac-LDL) was analyzed, which was internalized by DMEC and intracellularly deposited in vesicles (Fig. 1, *c* and *d*). Cells were also positive in IIF for collagen type IV and laminin while being negative for smooth muscle actin (not shown), thus, demonstrating absence of smooth muscle cells and pericytes. Throughout the whole culture period DMEC appeared as a rather homogenous cell population although minor contamination by fibroblasts or other DMEC-marker negative cells cannot be excluded. Also, cells derived and expanded from frozen stocks were essentially unchanged in their reactivity for factor VIIIr:Ag. Cells could be propagated up to passage 10 consistently maintaining their elongated shape (with a split ratio of 1:3). Then a marked reduction of growth rate was noted.

Typical fibroblast cultures on plastic were negative for factor VIIIr:Ag, Dil-Ac-LDL uptake and mostly though not always for collagen type IV and laminin, whereas ~10% were positive for smooth muscle actin. Due to the absence of any positive and unique fibroblast marker, these cells were defined by the absence of DMEC-markers and presence of vimentin cytoskeleton.

Growth Stimulation of Keratinocytes by DMEC and Fibroblasts in Monolayer Cultures

The positive impact on growth of normal human keratinocytes was demonstrated by cloning experiments on feeder layers. As seen with dermal fibroblasts, irradiation with 70 Gy completely inhibited growth of both dermal cells. While keratinocytes when seeded at clonal densities (up to 10^4 cells/60-mm dish) were unable to grow to colonies (not shown), these postmitotic mesenchymal cells actively supported their clonal growth (Fig. 2). A mixture (1:1) of irradiated DMEC and fibroblasts (with the same total number of feeder cells) did not demonstrate additional cooperative effects of both types of dermal cells on keratinocyte proliferation (Table II). In general, colony size and morphology were not grossly influenced by the type of feeder cells used. Keratinocyte colonies appeared mitotically active, were composed of small polygonal cells and showed virtually no signs of cornification after 10 d. These feeder effects of DMEC and fibroblasts, respectively, were consistently observed when the cells were tested between passage 3 and 7. On the other hand and in agreement with earlier literature data (42, 54), conditioned media of mesenchymal feeder cells and keratinocyte-fibroblast cocultures, respectively, were unable to induce clonal growth of keratinocytes (data not shown).

Regulation of KGF, IL 3, GM-CSF, IL 6 and Collagenase mRNA Levels in Epidermal-Dermal Cocultures

We further analyzed possible mechanisms which might be involved in the stimulation of keratinocyte proliferation by feeder layer cells and studied the regulation of known growth stimulating factors in epidermal-dermal cocultures versus dermal cells alone. For this purpose, mRNA levels for KGF, IL 3, GM-CSF, IL 6 and interstitial collagenase were ana-

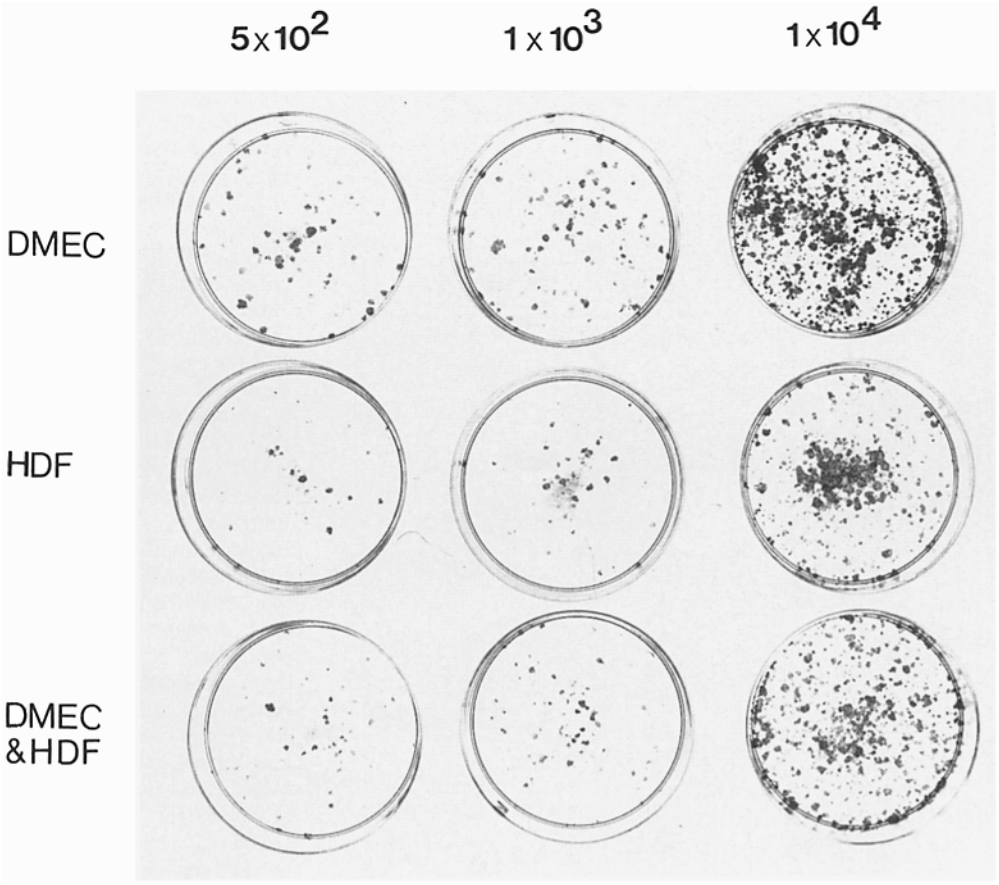

Figure 2. Colony formation of keratinocytes (third passage) seeded at indicated densities (5×10^2, 1×10^3, 1×10^4 cells) on 60 mm Petri dishes containing different dermal feeder cells: DMEC (1×10^5 cells), HDF (1×10^5 cells), and a mixture of DMEC and HDF (1:1, total of 1×10^5 cells) after 10 d in culture. Dishes were fixed and stained with hematoxylin. Control keratinocyte cultures (without feeder cells) did not form colonies in complete (FAD) growth medium up to 10^4 cells/60 mm dish. Few colonies were detected at 5×10^4 plated cells.

lyzed in cocultures of keratinocytes with irradiated DMEC and fibroblasts versus irradiated dermal cells (DMEC and fibroblasts) as well as nonirradiated fibroblasts. Dermal cells were selectively removed from the culture dish leaving keratinocytes still attached. From each cell compartment total RNA was prepared, blotted and hybridized with the indicated probes (Fig. 3). In controls, i.e., proliferating fibroblast and keratinocyte cultures (*c*), basal levels of KGF mRNA transcripts were faintly detected in fibroblasts whereas keratinocytes were negative as previously described (17). Irradiation of DMEC and fibroblasts with 70 Gy did not substantially change the level of KGF message in dermal cells. However, basal KGF mRNA levels were significantly stimulated when keratinocytes were cocultured with dermal feeder cells and grown to ~50% confluence. A marked and similar induction of KGF mRNA levels could be demonstrated in all dermal feeder cells (DMEC, a 1:1 mixture of DMEC and HDF). In contrast, dermal feeder cells cultured without keratinocytes expressed only low amounts of KGF mRNA, whereas keratinocytes cultured with and without dermal feeder cells were always negative for KGF message.

Table II. Colony Forming Efficiency of Keratinocytes on Irradiated Feeder Layers

Keratinocytes	DMEC	HDF	DMEC + HDF inoculated
5×10^2	89,2 ± 2,8 (209%)	42,6 ± 2,7 (100%)	65 ± 2,7 (152%)
1×10^3	128 ± 5,0 (192%)	66,4 ± 3,1 (100%)	102 ± 6,2 (153%)
1×10^4	NC	NC	NC

Keratinocyte numbers as indicated were inoculated onto x-ray-irradiated feeder layers of either 1×10^5 DMEC, or 1×10^5 fibroblasts (HDF), or a mixture of 5×10^4 DMEC and 5×10^4 HDF/6 cm dish and cultivated for 10 d. Stained colonies were counted from five replicate dishes and standard deviations were calculated. In parentheses, percentages of colony forming efficiences are given relative to colony numbers on fibroblast feeder layer (100%); (NC, not counted due to high colony density). Control keratinocyte cultures (without feeder cells) did not form colonies when plated at 1×10^3 and 1×10^4/60 mm dish (not listed).

For IL-3 no signals could be detected in total RNA of either keratinocytes or dermal feeder cells (not shown). However, GM-CSF transcripts (although at a low level) were induced in HDF feeder cells by keratinocytes but not in DMEC (Fig. 3). Hybridization signals for IL-6 mRNA were noticed in all feeder cell populations (DMEC, HDF and the 1:1 DMEC/HDF mixture) cocultured with keratinocytes. This was particularly remarkable, because cultures were grown in the presence of hydrocortisone (0.4 μg/ml), a known inhibitor of interleukin expression (56). In dermal feeder cells irradiation alone was sufficient to cause a detectable IL-6 mRNA expression in HDF but not DMEC. Interstitial collagenase was used as a positive control for epidermal/dermal cell interactions (55). Collagenase mRNA expression was strongly induced in dermal cells by keratinocytes, obviously more in DMEC than in HDF. In comparison, irradiated and nonirradiated dermal cells cultured without keratinocytes as well as keratinoytes themselves expressed no visible collagenase transcripts under these conditions.

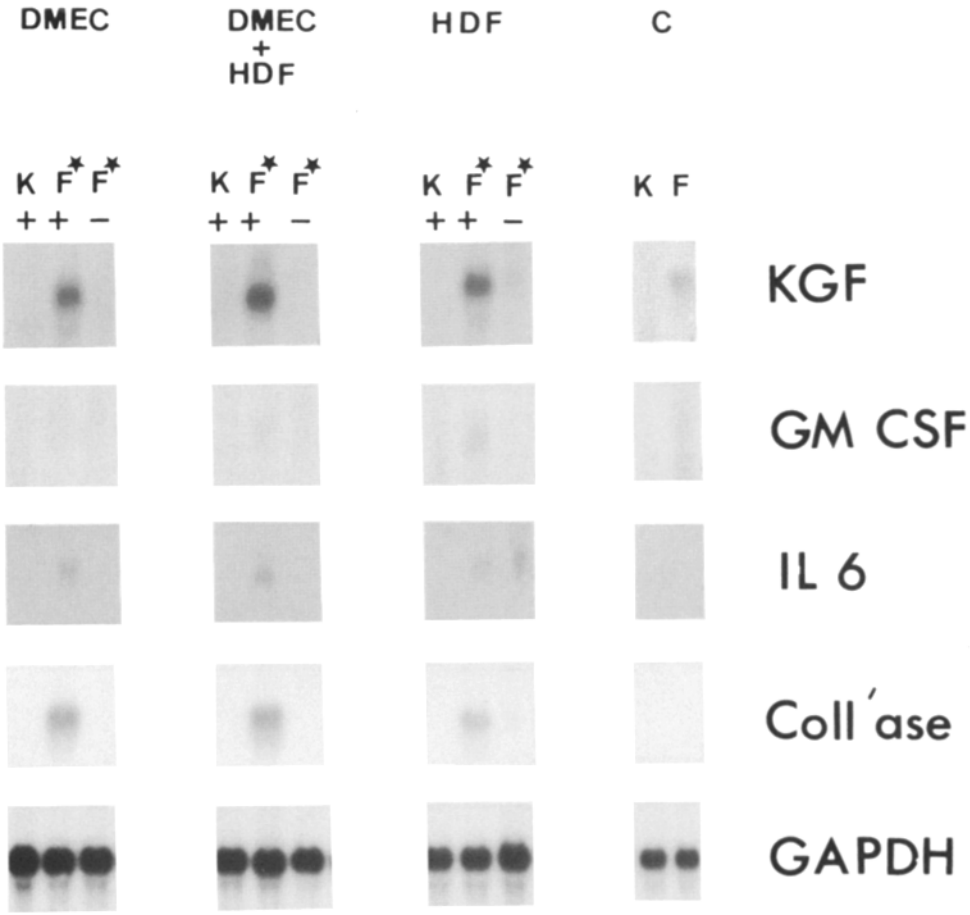

Figure 3. Northern blots of different dermal feeder cell populations and cocultured keratinocytes. DMEC, DMEC/HDF and HDF were irradiated with 70 Gy. These feeder cells (*F**) were either cocultured (*F**+) with keratinocytes or maintained without epidermal cells (*F**−). Keratinocytes cultured on these feeders (K+) were analyzed separately. As control (*C*) proliferating keratinocytes (*K*) grown without feeder cells and proliferating fibroblast cultures (*F*) were included. Total RNA was prepared of epidermal and dermal cells of cultures when at 50% confluence. The first set of lanes depicts keratinocytes cocultured with DMEC feeder cells; the second with a 1:1 mixture of DMEC+HDF and the third for HDF. cDNA hybridization probes are listed at the right margin with GADPH as a standard to demonstrate equal RNA loading of the gels.

Mesenchymal Cell Induced Epidermal Proliferation and Morphogenesis in Organotypic Cocultures

Comparable growth stimulatory effects on keratinocytes by cocultured dermal cells were observed in the organotypic culture assay. In contrast to control cultures of keratinocytes on lifted cell-free collagen gels (Fig. 4 *a*), epithelial growth and morphogenesis improved dramatically, when keratinocytes were cocultured with DMEC and fibroblasts, respectively, in such organotypic cultures (dermal equivalent type). Within 7 d, DMEC incorporated in the collagen gel induced a multilayered well structured epithelium. Cells in the lower layers appeared more cuboidal, and a typical stratum spinosum and granulosum were discernible after 10 to 14 d covered by several layers of cornified cells resembling a typical orthokeratotic stratum corneum (Fig. 4 *b*). The effects of DMEC in this system were generally comparable to those of dermal fibroblasts (Fig. 4 *c*). Further, the combination of both dermal cell types did not cause consistent or pronounced morphological changes in the developing epithelium (Fig. 4 *d*). In contrast, keratinocytes without DMEC or fibroblasts ceased to proliferate after 2–3 d, started terminal differentiation and were mostly atrophic after one week (Fig. 4 *a*). While this restricted proliferative activity of keratinocytes on lifted cell-free collagen gels was clearly evident by morphological observation, it could be further substantiated by detailed analysis. BrdU labeling (for 24 h) revealed only a few labeled cells at day 2 (maximum 5 per section with ~350 basal cells), a number which dropped within the following days to zero (not shown). However, when cocultured with dermal cells, labeling was maximal at 7 d with up to 50% positive basal and, to a lesser extent, immediate suprabasal cells (Fig. 5, *a* and *b*). At 10 d, the labeling index had dropped but still 10% of the basal cells were labeled. Although there was inhomogeneity in the distribution of labeled cells, with distinct clustering, both the number of labeled cells and the length of the proliferative period were always drastically higher in cocultures than in control (mesenchyme-cell free) organotypic cultures.

The analysis of histone 3 mRNA levels (as marker for cell replication) further underlined the effect of dermal cells to stimulate keratinocyte proliferation under these organotypic culture conditions (Fig. 6). Thus, in controls (organotypic cultures without dermal cells)—both at early (2 d, lane *1*) and late (10 d, lane *5*) stages—there was virtually no histone 3 expression by keratinocytes indicating absence of DNA synthesis. In contrast, keratinocytes in cocultures (here exemplified with fibroblasts) continued to proliferate even after 10 d (lane *6*), although there was a decrease in the histone 3 mRNA levels as compared to day 2 (lane *2*). This decrease reflects at least in part an increase of terminally differentiating cells at later stages, leading to a relative dilution of histone 3 transcripts in the RNA of the whole tissue.

Proliferation of Mesenchymal Cells in Collagen Gels

Similar to keratinocytes growing at the surface of collagen gels, the mesenchymal cells, both fibroblasts and DMEC, incorporated into the gel continued to proliferate and this even in the absence of cocultured keratinocytes. This is in contrast

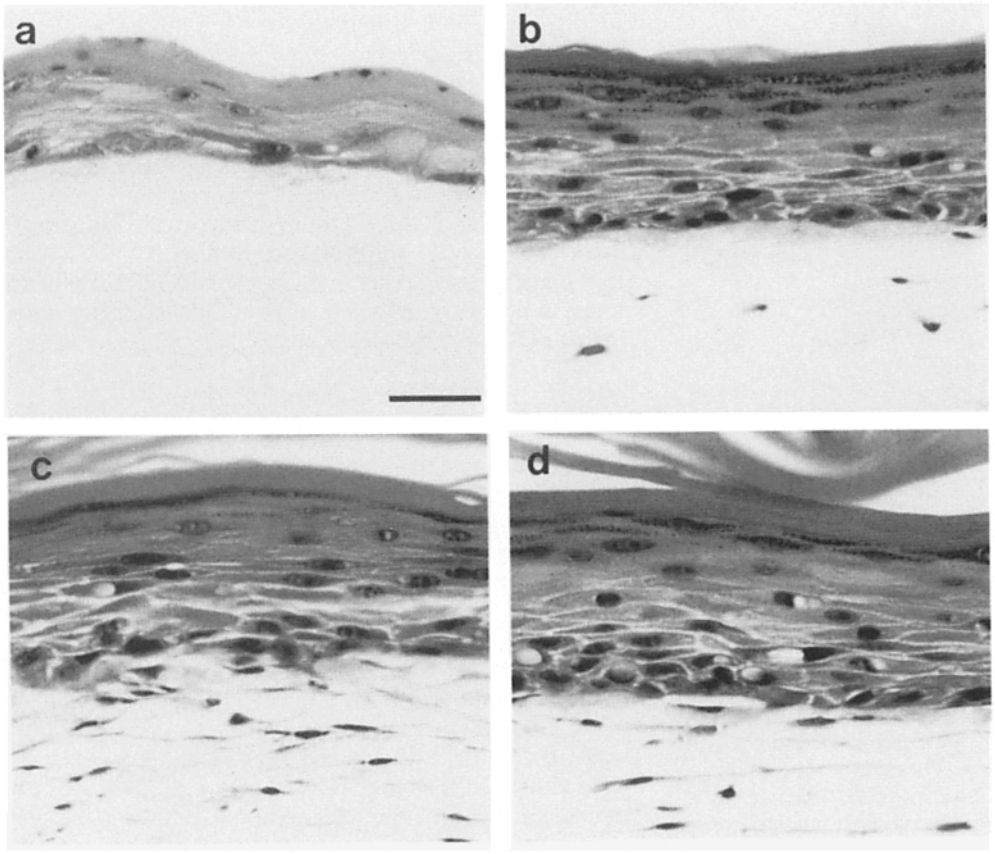

Figure 4. Effects of DMEC and fibroblasts on growth and differentiation of keratinocytes. Growth in organotypic culture (10 d) on collagen gels without mesenchymal cells (control) (*a*). Note the poor histological appearance of dystrophic epithelia in contrast to organotypic cocultures with DMEC (*b*), fibroblasts (*c*) and a mixture of DMEC and HDF (*d*) H.a.E.staining. Bar, 25 μm.

to what had been reported for contracting collagen gels (34, 37, 48) but could be clearly demonstrated by different methods.

Histone 3 mRNA expression revealed already rather high basic levels in fibroblasts in noncontracted collagen gels in the absence of keratinocytes both at 2 and 10 d after embedding (Fig. 6). This was even further stimulated by cocultured keratinocytes (Fig. 6, lanes *3* and *7* vs. lanes *4* and *8*). In BrdU labeling studies, labeled nuclei were observed in both control dermal equivalents (without cocultured keratinocytes) and cocultures with keratinocytes (see Fig. 5*a*) although quantitation was difficult due to uneven distribution of cells in the gel.

For DMEC, cell counting studies clearly proved both the continued proliferative activity of DMEC in noncontracted gels as well as a smaller increase in coculture with keratinocytes (Fig. 7). Both in control dermal equivalents and in cocultures with keratinocytes, the number of endothelial cells in the gel increases steadily with a steeper increase in controls as compared to cocultures. The reduction in relative DMEC number in cocultures with keratinocytes coincided with the onset of gel contraction and partial digestion, changes which were always less or absent in control cultures without keratinocytes. Whether the slower increase in DMEC in contracting cocultures is only due to the contraction-induced inhibition of cell proliferation as demon-

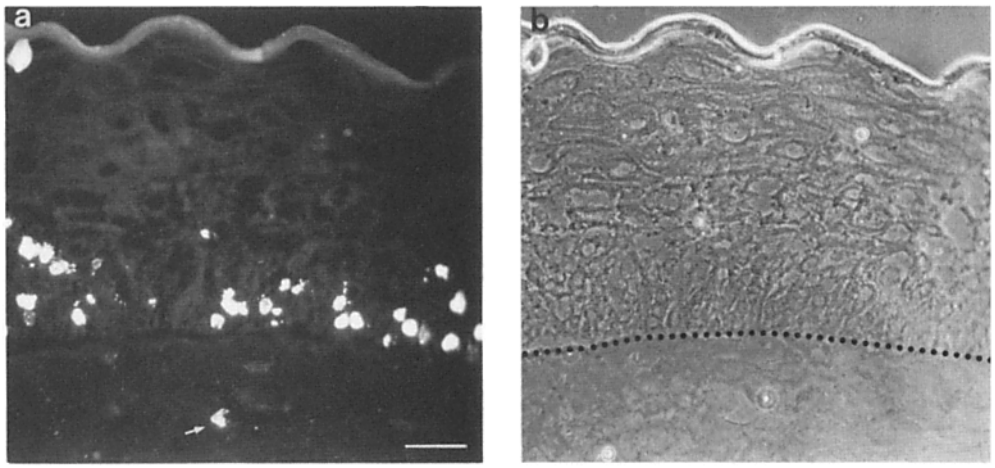

Figure 5. BrdU labeling studies reveal maximal number of labeled basal keratinocytes after 6 d in coculture with dermal cells (*a*) in IIF with BrdU mAbs; arrow indicates proliferating fibroblast in the noncontracting gel; dotted line in the corresponding phase contrast micrograph indicates the epidermal matrix border zone (*b*). Bar, 25 μm.

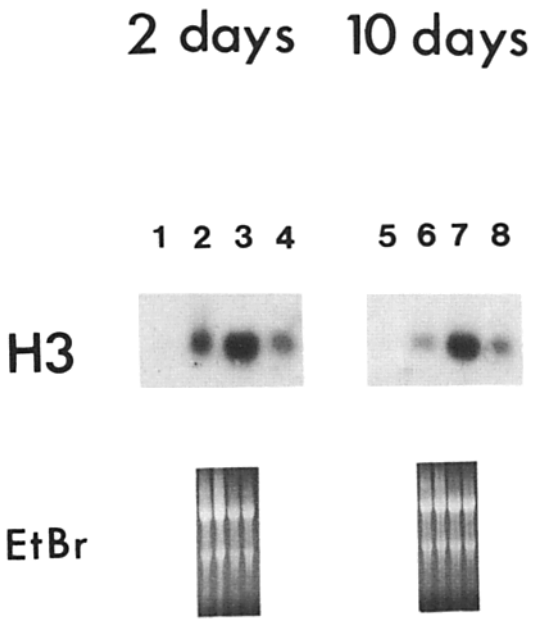

Figure 6. Detection of histone 3 (H3) specific mRNA transcripts in total RNA preparations of keratinocytes cultured on cell-free collagen gels as control cultures (lanes *1* and *5*), keratinocytes grown in organotypic cocultures (lanes *2* and *6*), dermal equivalents of cocultures with keratinocytes (lanes *3* and *7*) and dermal equivalents without cocultured keratinocytes (lanes *4* and *8*). RNA was isolated 2 d (lanes *1–4*) and 10 d (lanes *5–8*) after plating of cells. Ethidiumbromide (EtBr) stained agarose gels are shown below as an indication of equal loading of the gels.

strated by others (39, 47, 48) or additionally brought about by technical problems encountered in complete dissociation of these condensed lattices (and thus, incomplete cell recovery) cannot be answered at present.

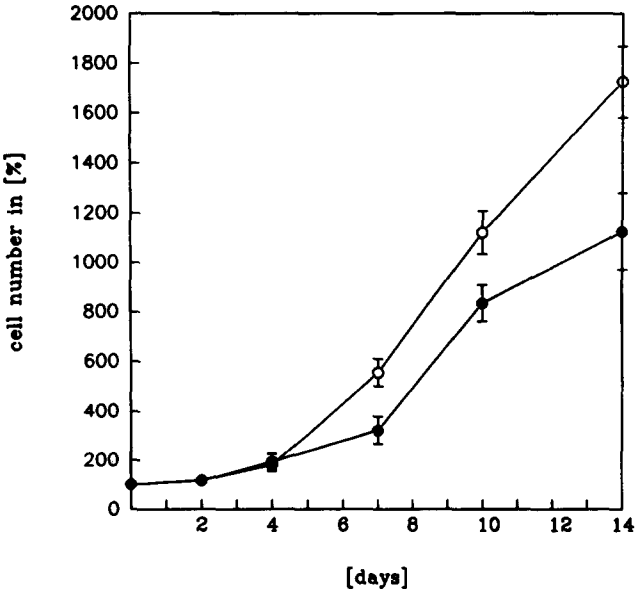

Figure 7. Relative increase in cell number (percent of control) of DMEC embedded in collagen gels and cultured with (●) or without (○) keratinocytes on the air-exposed surface of the gel. Values are means of triplicate cultures with SD. 100% represent cell number of incorporated cells at day 0.

While the relative increase in DMEC numbers after 2 wk was 10- and 17-fold, respectively (data of two experiments), the respective increase of fibroblasts was less (~twofold) with similar decrease in cocultures with keratinocytes after contraction of gels (data not shown). Comparable to feeder layer cultures, proliferation of dermal cells was not a prerequisite for their growth stimulatory influence on keratinocytes, since postmitotic (x-ray irradiated) cells had similar effects (not shown). Nevertheless, histone 3 mRNA data clearly demonstrate under organotypic culture conditions a mutual stimulatory influence of the epithelial and mesenchymal cell compartments.

Expression and Localization of Epidermal Differentiation Markers

Differentiation and functional organization of the epithelium formed under the influence of DMEC and HDF, were analyzed by IIF to identify and localize typical differentiation markers (Fig. 8). While keratin 14 (K14) is expressed and synthesized exclusively in basal cells in normal epidermis the protein persists in upper epidermal layers. Certain conformation specific antibodies (as LH8 used here [40]) exclusively decorate the basal layer of epidermis in situ (Fig. 8 *a*) and in keratinocyte surface transplants (Fig. 9 *g*). In contrast, in organotypic cocultures, all cell layers were uniformly and consistently stained for K14 by LH8 mAb.

The tissue distribution of involucrin is a very sensitive parameter for normal keratinization in vivo (localized in the apical parts of the stratum spinosum by IIF, Fig. 8 *b* [51]). In organotypic cocultures of keratinocytes and DMEC or HDF, the staining pattern indicated an aberrant differentiation because immunolabeling started already in cell layers immediately suprabasal (Fig. 8, *e* and *h*) as known for conventional submerged cultures. However, coculture with a mixture of DMEC and fibroblasts resulted in an upward shift of involucrin staining (Fig. 8 *k*).

As an additional marker of normalized basal keratinocyte function collagen type IV synthesis and linear deposition were investigated in the basement membrane zone. Collagen type IV staining was observed only in the presence of dermal cells in organotypic cocultures, again irrespective of whether DMEC, HDF or both in combination were used (Fig. 8, *f*, *i*, and *l*). At day 4, a punctate staining pattern was visible in IIF at the epithelial/matrix (collagen) boundary (not shown here). After 10–14 d, it always appeared as a continuous linear deposition. Preliminary data on the induction of mRNA of basement membrane components in keratinocytes in organotypic cocultures with HDF support these observations also at the transcriptional level (data not shown here), the precise mechanism of regulation, however, is at present still unclear.

Keratins 1 and 10 (K1 and K10) are expressed in vivo when cells leave the basal layer being present in all suprabasal layers and thus serve as early markers of normal epidermal differentiation. In the dystrophic epithelial sheets of organotypic control cultures (without dermal cells) the few flattened upper cell layers are usually stained for K1 and K10 (as shown by double IIF) sparing a single cell layer at the basal interface (Fig. 9, *a* and *b*). In the well-structured epithelia formed under the influence of DMEC, HDF, or a mixture of both (not shown here), there was extensive staining for K1 and K10, but the onset of their synthesis was considerably

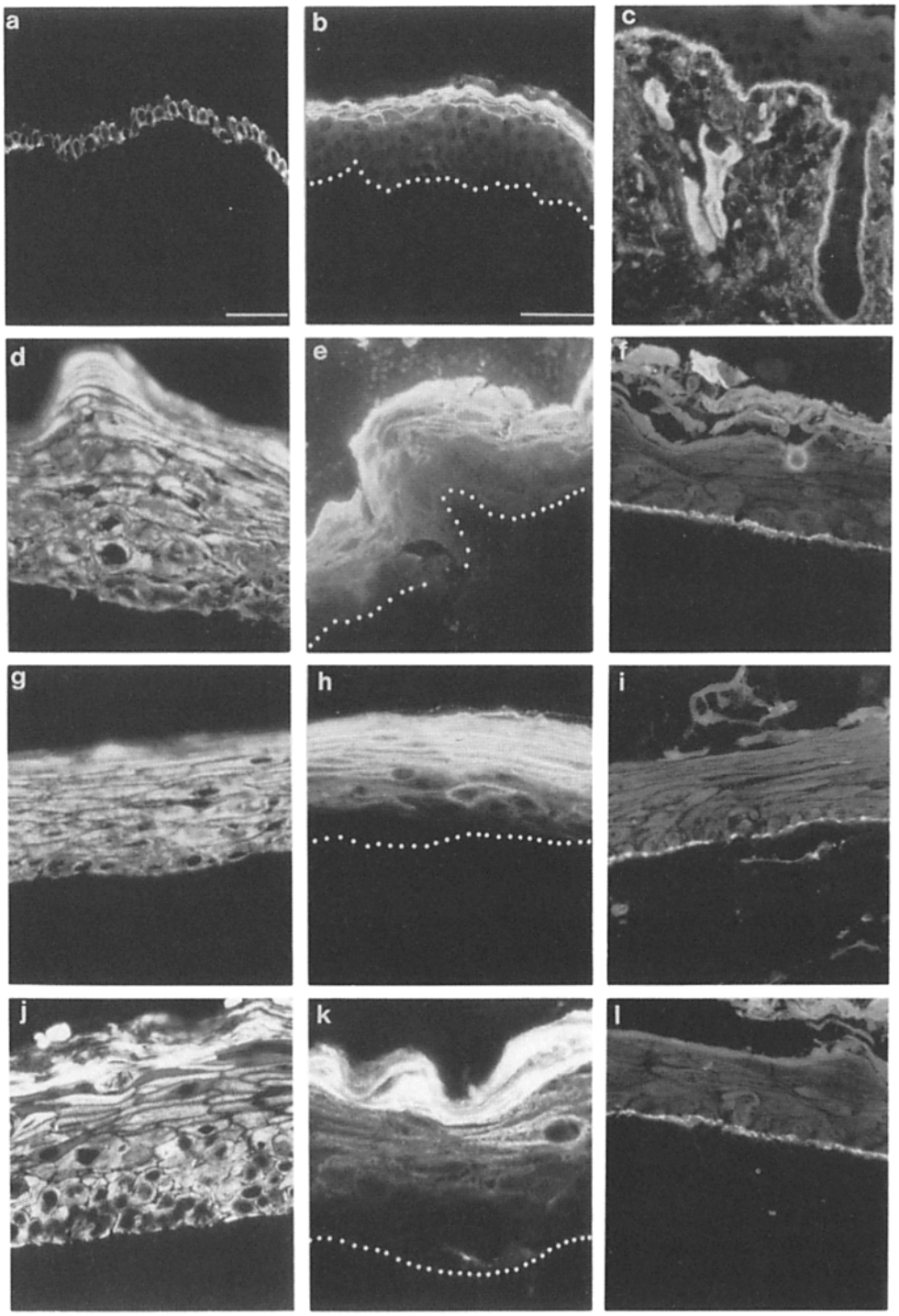

Figure 8. IIF staining of unfixed frozen sections of skin and 10-d old organotypic cocultures for differentiation markers. Distribution of K14, left column (*a, d, g, j*), involucrin, middle column (*b, e, h, k*) and collagen type IV, right column (*c, f, i, l*) in normal human skin (*a–c*), in epithelium formed by keratinocytes grown on collagen gels containing either microvascular endothelial cells (*d–f*), fibroblasts (*g–i*) or a 1:1 mixture of both (*j–l*). Dotted lines indicate borderline of epidermis and dermis or collagen gel, respectively. Bars: (*a, d, e, g, h, j, k*) 25 μm; (*b, c, f, i, l*) 50 μm.

delayed (Fig. 9, *c–f*). Usually 5–8 lower cell layers remained unstained irrespective of the cocultured dermal cell type. There was only a very minor delay in the appearance of K10 as compared to K1 (occurring also in normal epidermis) indicating a rather regular coregulation of both keratins (although starting late in this reconstructed epidermis). The somewhat earlier disappearance of K10 staining towards horny layers reflects a rather normal modification or loss of epitopes (8, 9).

This retardation in the onset of K1 and K10 synthesis was rapidly reversed to normality when such cocultures were transplanted onto nude mice using our surface transplantation assay (Fig. 9 *g*; see reference 7). Within 7–10 d after transplantation both staining for keratin 14 was confined to the basal layer and keratin 10 (as well as K1, not shown here) localization started in the immediate suprabasal layer. This normalization was achieved long before the collagen gel (with cultured dermal cells) was dissolved and keratinocytes got in contact with the mouse mesenchyme.

Discussion

Extensive studies have documented the importance of dermal or mesenchymal elements for keratinocyte growth and

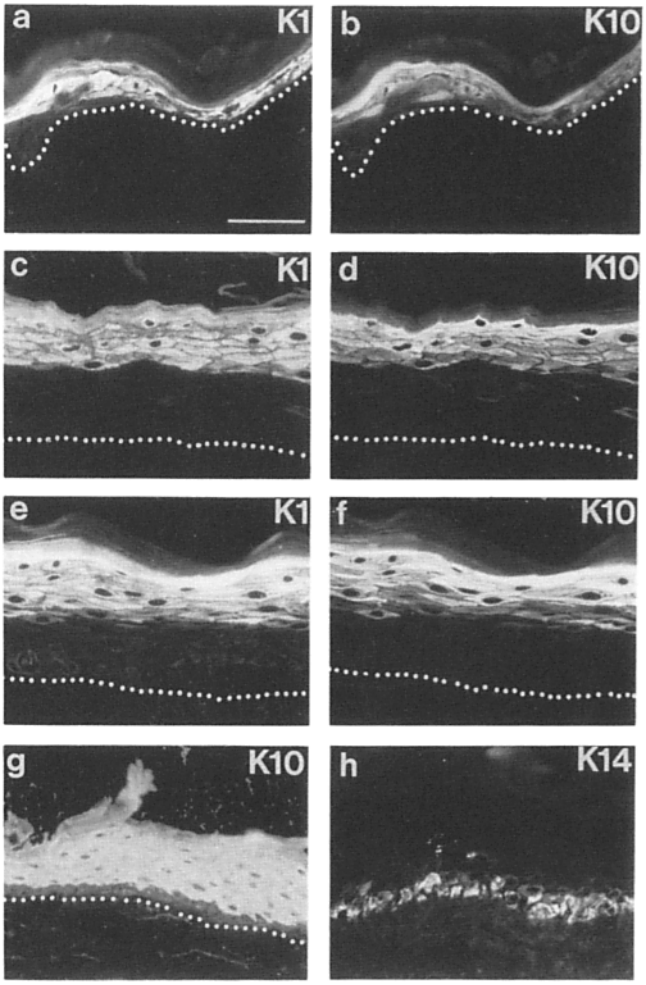

Figure 9. Double-IIF staining for K1 and K10 using sequence specific polyclonal antibodies directed against K1, left column (*a*, *c*, *e*) and K10, right column (*b*, *d*, *f*). Coexpression of K1 and K10 is seen in thin suprabasal sheets of 10-d old organotypic control cultures (without mesenchymal cells) (*a*, *b*). In cocultures with DMEC (*c*, *d*) and fibroblasts (*e*, *f*) several lower cell layers remain unstained. In double IIF on 10-d old transplants onto nude mice with antibodies against K10 (*g*) and K14 (*h*) both keratins are regularly localized. Dotted lines indicate borderline of epithelium and collagen gel. Bar, 50 μm.

differentiation in culture and transplantation models (for review see reference 19). It is well recognized that cellular as well as matrix constituents of the dermis contribute to the regulation of keratinocyte proliferation and differentiation (6, 7, 9, 13, 19, 20, 30–32). While the precise mechanisms are still mostly undefined diffusible factors must be considered as major regulatory factors, because the effects are equally well observed whether or not keratinocytes growing on extracellular matrix gels are in contact with mesenchymal cells (6, 7, 9). Among the various mesenchymal components the actual impact of capillary endothelium on keratinocytes is poorly understood. Comparably, the identity and regulatory mechanims of the factors mediating epithelial-mesenchymal interaction are largely unknown.

In this study, we investigated the effects of cultured human dermal microvascular endothelial cells on keratinocyte growth and differentiation in vitro in comparison to the effects of fibroblast/keratinocyte interactions. DMEC were isolated from stratum papillare of abdominal skin and characterized by specific functional markers. Even though the cells showed a variable morphology, it had been demonstrated that depending on vessel type and tissue, endothelial cells from different species and organs show variations in phenotype, growth requirements and functional properties (14, 46) as well as major immunological and metabolic differences (28). Thus, although variations in phenotype and a rather variable morphology were observed in vitro, this was not associated with the loss of functional endothelial characteristics.

To analyze whether DMEC support keratinocyte proliferation in vitro keratinocytes were grown on postmitotic DMEC as feeder cells. Dermal feeder cells allow growth of normal keratinocytes when inoculated at low clonal densities in serum-containing medium and the number and area of developing keratinocyte colonies represent a very sensitive assay for growth-modulatory effects. Originally, mouse 3T3 cells have been employed as feeder cells (42, 54), however, human dermal fibroblasts were demonstrated to function similarly well under these conditions (30). Irradiated, postmitotic DMEC reproducibly supported keratinocyte proliferation slightly better than fibroblasts in regard to colony numbers. Interestingly, no substantial cooperative effect was seen with a DMEC and HDF mixture as feeder cells (compared to HDF alone).

To analyze underlying mechanisms of this well-known "feeder effect" steady state levels of several growth factor mRNAs were examined which had previously been identified as keratinocyte growth stimulating cytokines in vitro. In irradiated DMEC, DMEC/HDF and HDF only low levels of KGF expression were detected. However, these mesenchymal cells could be substantially induced by coculture with keratinocytes to express abundant levels of KGF message. This finding of KGF stimulation in dermal cells by keratinocytes parallels recent results of wound healing studies (52) indicating that keratinocyte mediated induction of KGF and other members of the FGF family (36) in mesenchymal cells is a physiologic mechanism. In addition, other cytokines such as GM-CSF, IL-3 (23), and IL-6 (21) have been shown to support keratinocyte proliferation. In our experiments GM-CSF transcripts were induced at a low level in fibroblasts, cocultured with keratinocytes, but interestingly not in DMEC, whereas IL-3 transcripts could not be detected at all. IL-6 was already induced by irradiation alone in HDF but stronger by interaction with keratinocytes in DMEC and HDF (even in the presence of high levels of hydrocortisone in the culture medium known to suppress interleukin expression).

The feeder effect of 3T3 cells on keratinocytes could not or only to a small extent be replaced by conditioned medium of 3T3 or other fibroblast cultures (own results and reference 42). Here we present evidence that in cultures of irradiated DMEC and HDF production of specific cytokines has to be induced by the cocultured keratinocytes, which in turn will then stimulate keratinocyte proliferation in a paracrine fashion. As shown here for RNA and recently demonstrated at the protein level (50), the steady state levels of cytokine production for KGF and IL-6 are very low. In feeder layer cultures of keratinocytes on postmitotic fibroblasts large quantities of IL-6 have been recently determined (50) and

our data indicate that these have been produced by the feeder cells following induction by the cocultured keratinocytes. IL-6 increase was also reported in psoriatic plaques and in the plasma of psoriasis patients, and this cytokine may be associated with epidermal hyperplasia (21). As a possible mechanism for this induction, we could recently observe expression of IL-1α in keratinocytes and the induction of the 80-kD IL-1 (type I) receptor RNA in mesenchymal cells of feeder layers and organotypic cocultures (Smola et al., manuscript in preparation), which may in part explain keratinocyte/dermal cell cross-talk. In another coculture assay, the production of IL-1 by keratinocytes was demonstrated, which in turn stimulated expression and production of IL-8 in the cocultured fibroblasts (43). Taken together, these data indicate rather complex mechanisms of mutual paracrine growth regulation as a consequence of specific cell–cell interactions in epithelial-mesenchymal cocultures. Moreover, we could demonstrate that this interaction is not limited to dermal fibroblasts but functions similarly with dermal microvascular endothelial cells.

Further experiments indicated, however, that additional components are required for successful support of clonal keratinocyte growth. Conditioned media of feeder cell cultures (with or without keratinocytes) were not effective in stimulating and maintaining colony formation of keratinocytes seeded at low cell density. Preliminary experience further indicates that addition of KGF (with insulin) similarly failed to replace the feeder cells and addition of KGF neutralizing antibodies did not reduce the feeder effect in complete medium. Thus, more complex mechanisms have to be postulated to explain the coculture effects observed with irradiated feeder cells involving cell–cell contact and/or cell matrix interactions. The extracellular matrix may serve as an important reservoir or mediator of peptide growth factors, particularly for the heparin binding factors of the FGF family such as KGF (17, 45). Other studies have evidenced a keratinocyte growth-promoting activity on the surface of the feeder cells and demonstrated that cell–cell contact was essential to mediate the feeder effect, indicating a membrane-bound or matrix-associated factor (54).

As pointed out, coculture with dermal feeder cells under standard submerged culture conditions effectively favors keratinocyte growth, while regular epidermal differentiation is not observed under these conditions (8, 9, 24). In organotypic coculture systems, however, DMEC and fibroblasts supported morphological organization into a rather normal epidermal structure in addition to induction of keratinocyte proliferation. Incorporation of both, DMEC and HDF, in the same collagen gel did not improve morphology further, making cooperative effects on epidermal morphogenesis unlikely under these conditions. On the other hand, DMEC and HDF together consistently shifted involucrin staining, a very sensitive indicator of orthokeratinization and tissue homeostasis, to more suprabasal layers, thus resembling more closely normal epidermis than described for most other coculture systems (2).

Earlier studies had reported that improved epidermal morphogenesis in organotypic cultures recombined with dermal tissue or fibroblasts, was paralleled by induction of various differentiation parameters, although there were some discrepancies to normal epidermis as far as spatial distribution was concerned (2, 7, 27). Keratin 14 (K 14) is mainly synthesized in the basal layer of the epidermis in situ, but detectable throughout all epidermal cell layers (49). However, certain K14 antibodies are conformation-dependent (40) and stain only basal cells in normal skin (compare also reference 53). Despite the nearly normal tissue architecture in our organotypic cocultures, all cell layers were positive indicating either different processing of K14 or loss of masking of the epitope in suprabasal layers.

At variance with some of the earlier reports (1, 7, 27), the onset of synthesis of the suprabasal keratins K1 and K10 was not strictly suprabasal, but significantly delayed and shifted five to eight cell layers above the basal layer irrespective of cocultured dermal cell types (DMEC, HDF or both). Because this observation was consistent throughout different experiments with interfollicular and outer root sheath keratinocytes (31), this finding may be due to different experimental procedures in this report as compared to earlier publications. A common variable comparing different systems might be the effective dose of retinoids, well known modulators of epithelial differentiation. Retinoic acid has been shown to repress K1 and K10 expression in organotypic cocultures in a dose dependent fashion (2, 27). While a direct influence of the medium level of retinoids seems quite unlikely (controls readily expressed suprabasal keratins), the effective dose in cocultures might be different, possibly due to differences in retinoid metabolism. Alternatively, an enlargement of the proliferative epithelial compartment might cause an upward shift of the onset of suprabasal keratin synthesis (comparable, e.g., to psoriasis [4, 5]).

In most of the previous studies, keratinocytes have been seeded onto contracted dermal equivalents, cultured submerged for 7 d and then lifted to the air medium interface (e.g., 1, 3). In contrast, we have plated keratinocytes on non-contracted lattices (prevented by CRD and the high collagen concentration) to produce a confluent monolayer after 24 h, when keratinocytes were air-exposed. On lifted cell-free collagen gels, keratinocyte proliferation rapidly decreased within 2–3 d to undetectable levels, but was drastically stimulated in cocultures with mesenchymal cells. This hyperstimulation of keratinocyte proliferation is most probably due to the relatively high number of the mesenchymal cells.

While it was shown that fibroblasts in contracting collagen gels ceased to proliferate and decrease in cell number, while collagen type I synthesis was repressed (34, 37, 48), our experiments demonstrated continued DNA replication of both dermal cell types within the gel, even in the absence of cocultured keratinocytes. This was evident by detection of histone 3 mRNA, BrdU incorporation and by cell counting. These data correspond well with results by Lambert et al. (29) demonstrating continued thymidine incorporation in lattices kept under tension as compared to a rapid decline in proliferation in free floating and contracting collagen gels. Thus, the continued proliferation of the dermal cells, which results in a higher number with increased experimental period, is a major characteristic of our dermal equivalent. This may as a consequence lead to increased levels of (keratinocyte-induced) growth factors and thus, to an overstimulation of keratinocyte proliferation. BrdU labeling studies, however, demonstrated that the proliferating cells were essentially restricted to the basal layer. The few cells located suprabasally may have moved upwards during the 24-h labeling period.

Epidermal hyperproliferation may therefore be a major cause for the delayed onset of K1 and K10 expression, though this delay may not simply be due to an enlargement of the proliferative compartment. A shortened transit-time of keratinocytes from the basal to the keratinized layers due to increased turnover rates may contribute to the observed retardation in keratinization. Nevertheless, it cannot be excluded that the delayed onset of keratinization is, in addition to the hyperproliferative stimulus, due to differentiation-inhibitory signals produced by cocultured dermal cells.

In addition to these commonly studied differentiation markers, we could demonstrate that coculture with DMEC or HDF resulted in a time-dependent expression and linear deposition of collagen type IV immunoreactive material at the epidermal-matrix interface. The same applies for other basement membrane constituents, both at the protein and RNA level (manuscript in preparation). For these pronounced effects, the presence of DMEC or HDF seemed to be a necessary requirement in contrast to our previous results on mouse keratinocytes in vitro (6). However, in accord with these earlier studies, a structured basement membrane is apparently not formed in vitro (manuscript in preparation) and is thus not prerequisite for epidermal keratinization (22). Synthesis and linear deposition of basement membrane constituents which increased with improving epidermal differentiation may be considered another differentiation product of keratinocytes regulated by mesenchymal cell interactions.

These data demonstrate that in the organotypic coculture systems keratinocyte differentiation, although drastically improved, still remained incomplete. When such cultures, however, were brought under in vivo influence, an epithelium with all the characteristics of the original epidermal tissue including reestablishment of a typical basement membrane (6; and manuscript in preparation) was reformed. This was achieved one to 2 wk post-transfer of intact organotypic cocultures as surface transplants onto nude mice (7). In addition, as shown here, the keratin profiles were also changed towards the pattern characteristics of the epithelium in vivo, in particular as far as keratin K10, K1 and K14 are concerned. These transplantation studies have clearly demonstrated that tissue homeostasis is achieved in vivo while the transplanted keratinocytes are still separated from the underlying mouse mesenchyme by a collagen gel. Thus, it can be postulated that the additional regulatory effects of the mesenchyme are also mediated by diffusible factors, either originating from other mesenchymal cell types or the blood circulation.

From these data we conclude that keratinocytes in coculture with dermal fibroblasts and microvascular endothelial cells, respectively, induce (possibly via IL-1) in the mesenchymal cells the expression of growth factors such as IL-6, KGF, and GM-CSF, which then in turn (with other components) may act back on keratinocyte proliferation. Due to the increasing number of dermal cells in the (at least initially) noncontracted gels of organotypic cocultures used in this study, an overstimulation of keratinocyte proliferation leads to a hyperplastic epidermal phenotype and delayed onset of K1 and K10 expression. Thus, with this model, hyperproliferative or diseased states of skin (comparable to psoriasis [4, 5]) can be mimicked and possibly modulated. Moreover, pathophysiologic mechanisms of skin disorders can be studied in the context of altered epithelial-mesenchymal interactions. This may lead to a better understanding and pharmacologic treatment of related dermatologic diseases.

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