Mouse Epidermal Keratinocytes in Three-Dimensional Organotypic Coculture with Dermal Fibroblasts Form a Stratified Sheet Resembling Skin

Shuzo I KUTA,1 Nobufumi S EKINO,2 Takeshi H ARA,2 Yuriko S AITO,2 and Kazuhiro C HIDA2‡

1Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan
2Department of Animal Resource Sciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan

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We describe an organotypic model of mouse skin consisting of a stratified sheet of epidermal keratinocytes and dermal fibroblasts within a contracted collagen gel. The model was designed to maintain the polarity of stratified keratinocytes and permit their long-term culture at an air-liquid interface. After air exposure, the thickness of the keratinocyte sheet transiently increased and then decreased to two cell layers at 2 weeks. The two-cell-layer structure is similar to that of the adult mouse epidermis. Cytokeratin 5 was localized in the lowest cell layer in the epithelial sheet, but cytokeratin 1 and loricrin were localized in the outer cell layers, resembling mouse skin. The expressions of interleukin 1α/C11 and 1β/C12 in the keratinocytes and of keratinocyte growth factor 1 and 2 in the fibroblasts correlated with keratinocyte stratification. The mouse organotypic coculture is useful in studying epithelial cell-mesenchymal cell interactions in vitro.

Key words: organotypic coculture; epithelial cell-mesenchymal cell interactions; mouse skin model; epidermis equivalent; keratinocyte

The skin is the frontline barrier that prevents the entry of microbes and chemical substances. The homeostasis of the skin depends on interactions between epidermal keratinocytes and dermal fibroblasts. Paracrine factors secreted by keratinocytes or fibroblasts mutually regulate these cells.1–6 Although in vivo studies provide valuable insight into the formation and regeneration of the skin structure, the complex regulation of these phenomena is difficult to analyze in intact organisms because of the extensively accumulating effects of cell-cell and cell-matrix interactions. Cell culture studies indicate that epithelial and mesenchymal cells mutually regulate their proliferation, differentiation, and functions.7,8 Most of these studies were performed using monolayer cultures on a plastic substrate where cells could not fully express their differentiated phenotypes or adequately interact with their extracellular matrix and connective tissues. Three-dimensional skin-equivalent models, in which cells retain their differentiated cellular phenotypes, have an in vivo-like architecture.1−10 In organotypic coculture, the stratification and differentiation of keratinocytes are induced by exposure of the culture surface to air.8,11 Cocultures of epidermal keratinocytes on collagen gels containing dermal fibroblasts have been established using human2,8−14 and rat15,16 cells, but mouse keratinocytes are not used for stable cocultures because of the difficulty of isolating intact cells and of maintaining their potential for proliferation and differentiation over a long period.17 The greatest difficulty in such cocultures is obtaining a uniform epithelial thickness because the epidermis of an adult mouse has only two cell layers, which makes it much thinner than those of humans or rats. Recent advances in the generation of transgenic mice have greatly increased the need for mouse keratinocytes in three-dimensional-culture studies.

The goal of this study was to establish a mouse skin model in vitro to facilitate study of the mechanisms underlying the interactions between epithelial and mesenchymal cells under in vivo-like conditions. We developed a stable organotypic coculture using mouse primary keratinocytes and fibroblasts that closely resembles the skin in structure and function in vivo.

Materials and Methods

Chemicals and antibodies. The following chemicals...
and antibodies were purchased from the respective manufacturers: MCDB153, insulin, hydrocortisone, and ascorbic acid from Sigma (Saint Louis, MO); Dulbecco’s modified Eagle’s medium (DMEM) from Nissui (Tokyo), type-I collagen from Nitta Gelatin (Osaka); fetal calf serum (FCS), Ham’s F12 medium, and human epidermal growth factor (EGF) from Gibco (San Diego, CA); collagenase type 1 from Worthington (Lakewood, NJ); cell culture inserts containing a polyethylene terephthalate membrane (23 mm diameter) from Becton Dickinson (Franklin Lakes, NJ); rabbit antibodies against mouse cytokeratin 1 (K1), cytokeratin 5 (K5), and loricrin from BabCo (Richmond, CA); goat anti-rabbit IgG antibody conjugated with tetramethylrhodamine isothiocyanate from Jackson (West Grove, PA); and anti-5-bromo-deoxyuridine (BrdU) antibody conjugated with peroxidase from Roche (Mannheim, Germany).

Cell isolation. All experiments were carried out with strict adherence to guidelines for minimizing distress in experimental animals. Normal epidermal keratinocytes and dermal fibroblasts were isolated from the dorsal skin of newborn ICR mice (1 d old) as follows: The mice were killed by decapitation with scissors. After sterilization with 70% ethanol, the dorsal skin was cut and incubated in a solution of 0.1% collagenase in MCDB153 supplemented with 10% FCS for 16 h at 4°C. The skin was cut and incubated in a solution of 0.1% collagenase in MCDB153 supplemented with 10% FCS for 16 h at 4°C. The epidermis was peeled off the dermis with forceps and gently stirred in 0.05% trypsin for 15 min at room temperature. The cell suspension was filtered through a 70-μm mesh and centrifuged. The precipitated cells were resuspended in MCDB153 supplemented with 10% FCS and used as primary keratinocytes.

Fibroblasts were isolated from the remaining dermis by trypsinization and cultured in DMEM supplemented with 10% FCS for 5 d at 37°C under 5% CO₂ in air. The fibroblasts grown were trypsinized and used in organotypic cocultures.

Organotypic cocultures. For the preparation of a dermis equivalent, type-I collagen (2.4 mg/ml) was solubilized in F12 medium (pH 7.4) supplemented with 20 mM N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) and 2.2 mg/ml NaHCO₃ and kept on ice. A suspension of cultured fibroblasts in DMEM supplemented with 10% FCS was added to the collagen solution at a final concentration of 2.2 mg/ml type-I collagen and a density of 1 × 10⁵ cells/ml. Three milliliters of the mixture was added to a hanging-drop-culture insert in one well of a multiwell tray and allowed to gel at 37°C for 1 h (Fig. 1). The resulting gel was equilibrated with FAD medium consisting of a mixture of F12 medium and DMEM (1:3 volume), 7.5% FCS, 5 μg of insulin, 0.4 μg of hydrocortisone, 50 μg of ascorbic acid, and 1 ng of EGF per milliliter for 24 h at 37°C in an atmosphere of 5% CO₂. The isolated keratinocytes were gently overlaid onto the surface of the equilibrated gel and cultured in MCDB153 supplemented with 10% dFCS for 24 h. By adding the same volume of FAD medium, the culture was further continued for 24 h. The gel surface was raised to the air-liquid interface by lowering the medium level. One-half the volume of the culture medium was replaced with FAD medium daily.

Histochemistry. Paraffin cross sections of the organotypic cocultures were prepared by the PLP-AMeX method. Briefly, the gels were washed three times with phosphate-buffered saline (PBS) and immersed in a PLP fixative for 6 h at 4°C. They were then washed with PBS, dehydrated, and embedded in paraffin. The cross sections (4 μm thick) were deparaffinized with xylene and ethanol, and then stained with hematoxylin and eosin. The thickness of the keratinocyte sheet without a cornified layer was measured with an image processor (Olympus Optics, Tokyo).

For immunochemistry, the deparaffinized sections were blocked with 5% normal goat serum and then incubated with primary antibodies overnight at 4°C in a moist chamber. After washing with PBS three times, the sections were incubated for 1 h with secondary antibodies conjugated with fluorochrome.

To investigate the process of DNA synthesis, 65 μM BrdU was added to the cocultures 24 h before fixation.
Incorporated BrdU was detected using an anti-BrdU antibody conjugated with peroxidase and diaminobenzidine as chromogen.

**RT-PCR.** Total RNA was isolated from the cultured gel and reverse-transcribed using an oligo(dT) primer. The resulting cDNA was used as a template for PCR. The following primer sets were used (the sizes of each amplicon are also indicated): (a) glyceraldehyde-phosphate dehydrogenase (GAPDH), 5'-GTG AAG GTC GGT GTC GGT GCC AGT GAT GAT-3' (GenBank accession no. M32599, nucleotides 50–604), 555 bp; (b) interleukin 1α (IL-1α), 5'-TGG CCA AAG TTC CTG ACT TGT TT-3' and 5'-CAG GTC ATT TAA CCA AGT GGT GCT-3' (X01450, 62–549), 488 bp; (c) IL-1β, 5'-ATG GCA ACT GTT CCT GAA CTC AAC T-3' and 5'-CAG GAC AGG TAT AGA TTC TTT CCT TT-3' (BC011437, 69–631), 563 bp; (d) keratinocyte growth factor 1 (KGF1 or FGF7), 5'-ATG CGC AAA TGG ATA CTG ACA CGG-3' and 5'-CTT AGG TTA TTG CCA TAG GAA G-3' (BC052847, 348–932), 585 bp; (e) KGF2 (FGF10), 5'-ATG CGC AAA TGG ATA CTG ACA CGG-3' and 5'-CTT AGG TTA TTG CCA TAG GAA G-3' (BC052847, 348–932), 585 bp; (f) transforming growth factor β1 (TGFβ1), 5'-CGG GCC GAC CTG GCC ACC ATC CAT GAC-3' and 5'-CTG CTC CAC CTT GGG CTT GCG ACC CAC-3' (M13177, 1082–1486), 405 bp. The number of PCR cycles for cytokine and GAPDH were 30 and 20, respectively.

**Results**

**Organotypic coculture**

Mouse dermal fibroblasts were embedded in a gel of type-I collagen within a hanging-drop-culture insert, the bottom of which was a membrane filter (Fig. 1). Almost all the fibroblasts in the gel showed spindle-shaped morphology and ceased to grow, resembling those in the dermis in vivo. The fibroblasts slightly contracted the collagen gel, to about 90% of the initial volume after a 1-week culture. The collagen gel with fibroblasts was used as a dermis equivalent.

Almost all the cells isolated from the mouse epidermis expressed cytokeratin but not vimentin (data not shown). In the monolayer cultures on the plastic substratum, the epithelial cells grew and formed a cobblestone-like sheet in low-calcium medium, MCDB153 supplemented with 10% dFCS, and differentiated to cornified envelopes in high-calcium medium, DMEM supplemented with 10% FCS (data not shown), showing typical characteristics of keratinocytes.

In the three-dimensional cocultures, as shown in Fig. 2, the primary keratinocytes attached to the surface of the dermis-equivalent gel, spread homogenously, and formed a single-cell-layer sheet. With air exposure, the surface of the gel progressively changed its appearance from wet to dry. Keratinocytes grew, stratified, and formed six to eight cell layers 40 µm thick within 4 d (Fig. 3). Nuclei were clearly observed in keratinocytes of the lower cell layers. Upper-layer keratinocytes gradually flattened, granulated, enucleated, and cornified, eventually resembling the mouse epidermis under hyperplasia. Thereafter, the thickness of the stratified...
sheet decreased to two cell layers after 2 weeks (Figs. 2 and 3). The two-cell-layer epithelial structure, which is similar to the structure of the adult mouse epidermis, was observed until 6 weeks after air exposure, but keratinocytes on a fibroblast-free collagen gel formed no multiple cell layer with air exposure (data not shown), showing that the stratification of keratinocytes depends on fibroblasts. This indicates that the coculture of mouse epidermal keratinocytes and dermal fibroblasts has a skinlike architecture in vivo.

**Keratinocyte proliferation and differentiation**

We examined the proliferation of keratinocytes in the coculture. As shown in Fig. 4, BrdU-positive nuclei were detected in 90% of the lowest-layer keratinocytes within 1 week of air exposure. At 2 weeks, 8% of the keratinocytes incorporated BrdU. These data indicate that the proliferation of the lowest-layer keratinocytes was stable and was similar to that of basal cells in adult mouse epidermis in vivo.

The expression of some epidermal differentiation markers was examined. As shown in Fig. 5, K5 was detected in the lowest-layer keratinocytes 2 weeks after air exposure. On the other hand, K1 and loricrin were detected in the upper-layer keratinocytes but not in the lowest-layer cells on the dermis equivalent. In the normal epidermis of an adult mouse in vivo, K5 was expressed in the basal layer, and K1 and loricrin were exclusively expressed in suprabasal layers. These results indicate that the organotypic cocultures of mouse epidermal keratinocytes and dermal fibroblasts in air-exposed culture were stable and resembled the skin in vivo.

**Cytokine expression**

We examined the expression of IL-1α, IL-1β, TGFβ1, KGF1, and KGF2 in the cocultures, which cytokines are involved in inflammation and wound repair in the skin.

After air exposure, the IL-1α mRNA level increased, reached a peak on day 2, and then gradually decreased to 65% of the level of the peak on day 10 (Fig. 6A). In the fibroblast-free gel, IL-1α induction also occurred but was delayed for 2 d, and then finished quickly. No IL-1α mRNA was detected in the fibroblasts without upper-layer keratinocytes. These results indicate that cocultured fibroblasts enhanced and maintained IL-1α expression in the keratinocytes.

The IL-1β mRNA level in the coculture increased, reached a peak on day 1 or 2, decreased, and then almost disappeared on day 10 (Fig. 6B). In the keratinocytes without fibroblasts or in the fibroblasts without upper-layer keratinocytes, it was difficult to detect IL-1β mRNA for 2 weeks. This indicates that fibroblasts control IL-1β induction in keratinocytes.

TGFβ1 mRNA was also detected in the coculture and the keratinocyte sheet on a fibroblast-free gel, but not in the fibroblasts without upper-layer keratinocytes (data not shown), but the TGFβ1 expression remained at almost the same level for 2 weeks, indicating that fibroblasts do not affect TGFβ1 expression in keratinocytes (data not shown).

The KGF1 mRNA level in the coculture quickly increased on day 1, remained at the same level until day 7, and then gradually decreased to the basal level on day 14 (Fig. 6C). In the fibroblasts without upper-layer keratinocytes, KGF1 mRNA was also detected, but the expression remained at almost the same level for 2 weeks. In the keratinocytes on fibroblast-free gel, no KGF1 mRNA was detected. These results indicate that keratinocytes induced KGF1 expression in fibroblasts.

The KGF2 mRNA level in the coculture increased,
reached a peak on day 4, and then decreased to the basal level within 10 d (Fig. 6D). Induction of KGF2 did not occur in the fibroblast-free gel. In the fibroblasts without upper-layer keratinocytes, the KGF2 mRNA level also increased on day 1, reached a peak on day 2, and decreased to the basal level on day 4. These results indicate that keratinocytes enhanced KGF2 induction in fibroblasts.

**Discussion**

Reconstituted mouse skin consists of two major parts. One is the dermis equivalent, a collagen gel containing fibroblasts. The other is an epidermis-like sheet of stratified keratinocytes that proliferate and differentiate in a controlled manner similar to an *in vivo* process. The architecture of the reconstituted skin might be formed by paracrine interaction between keratinocytes and fibroblasts. Indeed, the expression of IL-1β in keratinocytes and of KGF1 and KGF2 in fibroblasts correlated well with keratinocyte stratification.

Keratinocytes in monolayer cultures have specific characteristics depending on extracellular calcium concentration: proliferation and differentiation in low- and high-calcium concentrations, respectively. Increased extracellular calcium induces a differentiation program in keratinocytes in culture. In our organotypic coculture, mouse keratinocytes attached directly to the gel surface and proliferated stably, even under high-calcium conditions, suggesting that the polarity of keratinocytes is important in the response to extracellular calcium.

A three-dimensional culture of mouse keratinocytes on human dead epidermized dermis that has a thicker keratinocyte sheet than mouse skin *in vivo* has been developed. A three-dimensional culture containing keratinocytes derived from mouse embryonic stem cells grown on a matrix secreted from normal human fibroblasts also forms a thick epithelial sheet that resembles the epidermis of humans but not that of mice. Recently, Sadagurski et al. have demonstrated a mouse organotypic coculture using keratinocytes and fibroblasts, which is similar to our coculture, but the keratinocyte sheet remains thicker. The differences between our culture and theirs are the following: collagenase and trypsin for isolation of keratinocytes, FAD and Eagle’s medium for cocultures. The thickness of keratinocyte layers might depend on isolation of keratinocytes and culture conditions.

Inflammatory cytokines affect the proliferation and differentiation of keratinocytes in the skin. IL-1α, IL-1β, KGF1, and KGF2 stimulate the proliferation of keratinocytes and fibroblasts in monolayer cultures. On the other hand, TGFβ1 inhibits the proliferation and stimulates the differentiation of keratinocytes. In our
mouse organotypic coculture, the stratification of keratinocytes correlated with the expression patterns of IL-1α and IL-1β in keratinocytes and with those of KGF1 and KGF2 in fibroblasts, but did not correlate with that of TGFβ1. These expression patterns indicate possible mechanisms underlying mutual interactions between keratinocytes and fibroblasts. In the skin, IL-1α and IL-1β secreted from keratinocytes can pass through the basal membrane and reach fibroblasts in the dermis.13) As a possible response, the fibroblasts might produce and secrete KGFs, which probably pass through the basal membrane and act as mitogens for basal cells in the epidermis. As a possible response, the fibroblasts might produce and secrete KGFs, which probably pass through the basal membrane and act as mitogens for basal cells in the epidermis.14) but further studies are necessary to understand the mechanisms underlying cytokine expression and to elucidate the role of cytokines in the stratification of keratinocytes at the molecular and cellular levels.

Mouse skin organotypic cocultures have great advantages in stability, applicability, and similarity to tissues in vivo. Regeneration of skin and steady-state maintenance can be controlled by molecules secreted by epidermal keratinocytes and dermal fibroblasts, and this model is a tool for investigating such epithelial cell-mesenchymal cell interactions. One can use cells derived from transgenic mice in coculture and identify molecules that have important roles in epithelial cell-mesenchymal cell interactions. Furthermore, as cocultures advance, one can freely select species, cell types, molecules, chemicals, and even microbes that can be applied to organotypic cocultures.

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