# Culturing Keratinocytes and Fibroblasts in a Three-Dimensional Mesh Results in Epidermal Differentiation and Formation of a Basal Lamina–Anchoring Zone

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The purpose of this study was to characterize an *in vitro* co-culture model in which fibroblasts grown in a three-dimensional nylon mesh were recombined with human keratinocytes. The cultures were kept for 3 and 5 weeks and then processed for electron microscopy. Keratinocytes showed reconstruction of an epidermis consisting of a basal layer with hemidesmosomes, a stratified epithelium with tonofilaments and desmosomes, a granular layer with keratinosomes and keratohyaline granules, and a transitional stratum corneum. Anchoring filaments, lamina densa, anchoring fibrils, bundles of elastin-associated microfibrils (diameters 10 nm) and fine collagen fibrils were formed. Collagen fibrils near the epidermis were much thinner than those in the lower levels.

The present study shows that the dermal model containing metabolically active fibroblasts in their natural environment will support epidermal morphogenesis and differentiation including the formation of a basal lamina and anchoring zone. *J Invest Dermatol 100:35–39, 1993*

The study of epithelial tissue specificity in *"in vitro"* conditions requires a rather complex microenvironment where crucial epithelial-mesenchymal interactions can be preserved. Rheinwald and Green [1] grew human keratinocytes on a feeder layer of lethally irradiated 3T3 fibroblasts and noted proliferation and keratinizing colonies containing tonofilaments, desmosomes, and keratohyalin granules. Freeman *et al* [2] cultured human keratinocytes on dead porcine dermis and obtained an epidermal layer that closely resembled the *"in vivo"* situation. Similar studies were performed by seeding keratinocytes on a dead human dermis that retained basal lamina components [3]. Bell *et al* [4] cultured fibroblasts in a type I collagen gel. After contraction of the gel, a tissue-like fabric was created that supported proliferation and differentiation of epidermal cells. More recently, epidermal cells were also grown in a collagen-fibroblast matrix at an air-liquid interface [5]. This model revealed epidermal differentiation including the presence of a 67-kD keratin. Although the above-described systems demonstrated varying degrees of epidermal differentiation, data on the *"in vitro"* induction of a basal lamina and anchoring zone is less defined. Hirone and Taniguchi [6] grew epidermal cells on collagen gels and plastic, and noted development of hemidesmosomes and a less-defined basal lamina only in those cultures on collagen gels. David *et al* [7] conducted a similar experiment with mammary epithelial cells grown either on plastic or a type I collagen substrate. Although both cultures produced type IV collagen, laminin, and heparan sulfate, these were more abundant in the collagen gel cultures that also revealed a basal lamina ultrastructurally. Similar results were obtained by Chamson *et al* [8] after recombining keratinocytes with collagen gels populated by fibroblasts. Tinois *et al* [9] reported keratinocyte differentiation and basal lamina formation when cells were cultured on a substrate consisting of type I and type III collagens coated with type IV collagen.

Naughton *et al* [10] have described a three-dimensional culture of fibroblasts grown on a nylon mesh [10]. In this dermal model, fibroblasts attached to the mesh, proliferated, and created an ECM that was similar to human dermis [10,11]*. Preliminary experiments of a co-culture of keratinocytes and this dermal model suggested epidermal differentiation and formation of a basal lamina [10]. In the present study we present ultrastructural evidence that this keratinocyte dermal model co-culture results in epidermal differentiation, formation of a basal lamina, anchoring zone, and a dermis that closely resembles the *"in vivo"* situation.

## MATERIALS AND METHODS

**Keratinocyte/Dermal Model Co-Cultures** All cultures were grown at Advanced Tissue Sciences and shipped on nutrient agarose to Mount Sinai Medical Center for evaluation. The culture condi-

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Abbreviations:
ECM: extracellular matrix
EGF: epidermal growth factor
FGF: fibroblast growth factor
PBS: phosphate-buffered saline

* Contard P, Jacobs L, Perlish JS, Fleischmajer R: Fibrillogenesis in a new three dimensional fibroblast culture system (abstr). J Invest Dermat 96:605, 1991.

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tions have been previously reported [10,12].† Briefly, human neonatal foreskin fibroblasts and keratinocytes are isolated by sequential trypsin and collagenase digestion and expanded in monolayer culture using serum-free keratinocyte medium (Gibco). The fibroblasts are seeded onto a nylon mesh (8 × 8 cm) and allowed to grow for 26 d in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum and 100 μg/ml ascorbate. The keratinocytes in third passage are then seeded onto the dermal model, and the co-culture is grown submerged for 1 week and then at the air-liquid interface in DMEM containing 5% fetal calf serum, 100 μg/ml ascorbate, and 0.5 μg/ml hydrocortisone. At the end of the growth period (3–5 weeks), the mesh were laser cut into 11 × 11 mm squares, placed on agarose containing growth medium and shipped at room temperature for overnight delivery. Upon receipt, the cultures were removed from the agarose and fixed for electron microscopy.

**Electron Microscopy** Co-cultures were removed from medium, and approximately 1 mm of the periphery of the mesh was trimmed, leaving only the central portion. Samples were fixed in Karnovsky's solution for 4 h at room temperature, post-fixed in ferrocyanide osmium tetroxide for 1 h then stained *en block* for 1 h each in an aqueous solution of 1% phosphotungstic acid followed by 2% uranyl acetate [13]. Samples were dehydrated in graded ethanols and embedded in Spurr's resin in a flat embedding mold. Upon embedding, meshes were rotated at a 45° angle with the point of each square-shaped opening aligned toward the knife edge. An opening within the mesh was selected and the block was rough trimmed with a razor blade. While holding the block with one hand, with a light source from below, we carefully trimmed away the nylon from the sides of the chosen opening. This left only the embedded cells and their matrices. Ultra thin sections were cut with a diamond knife on a Sorval MT-2b ultramicrotome and examined in a JEOL EM 100 electron microscope.

## RESULTS

Electron microscopic analysis of cultures 3 weeks after seeding of keratinocytes revealed a well-developed epidermis and underlying dermal equivalent (Fig 1*a*). Basal cells were cuboidal in shape and contained large nuclei and multiple tonofilaments. Several supra-basal layers were present that consisted of flattened cells containing macrofilaments and intercellular desmosomes. A granular layer was observed containing well-developed intracellular keratohyalin and membrane coating granules (Fig 1*b*). Although some of the cornified material was lost in preparation, signs of keratinization were evident in the upper-most layers of the epidermis (Fig 1*a*).

By week three after seeding of keratinocytes, hemidesmosomes were present along the cytoplasmic membrane of the basal cells (Fig 2*a*). Just below the cytoplasmic membranes were electron lucent and electron dense bands believed to be early lamina lucida and lamina densa, respectively. Anchoring filaments were noted extending from the hemidesmosomes to the lamina densa.

At 5 weeks after seeding the basal lamina is well developed and attached to the extracellular matrix (Fig 2*b*). Also present by week five after seeding was a lamina lucida with anchoring filaments coursing through it (Fig. 3*b,c*). The lamina densa was also well developed, and large bundles of 10-nm microfibrils were evident in proximity (Fig 3*a,b*). Anchoring fibrils showing typical banding were seen originating at the lamina densa (Fig 3*c,d*). Some of these fibers terminated in anchoring plaques (Fig 3*d*).

Collagen fiber diameter in the upper and lower dermis were compared at 5 weeks after seeding. Fibers in the upper dermis showed typical 67-nm periodicity and appeared to be thin ranging from 24 to 48 nm in diameter, whereas those of the lower levels ranged from 45 to 76 nm in diameter.

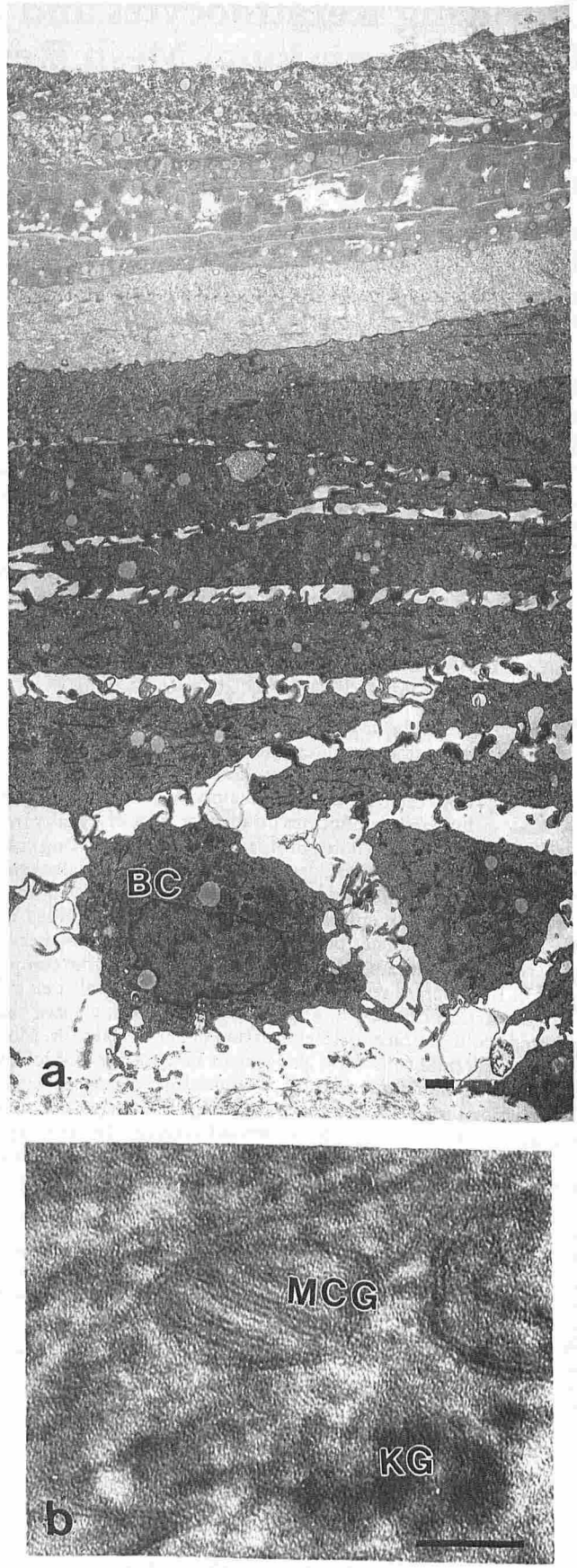


**Figure 1.** Epidermis of keratinocyte/dermal model co-culture at 3 weeks. Epidermis consists of a basal cell layer (BC) and several flattened supra basal layers (*a*). *Bar*, 1 μm. Membrane coating granules (MCG) with keratohyalin granules (KG) were present in the granular layer (*b*). *Bar*, 50 nm.

† Slivka SR, Laudeen L, Zimber MP, Bartel RL: Biochemical characterization, barrier function and drug metabolism in an in vitro skin model (abstr). J Cell Biol 115:236a, 1991.

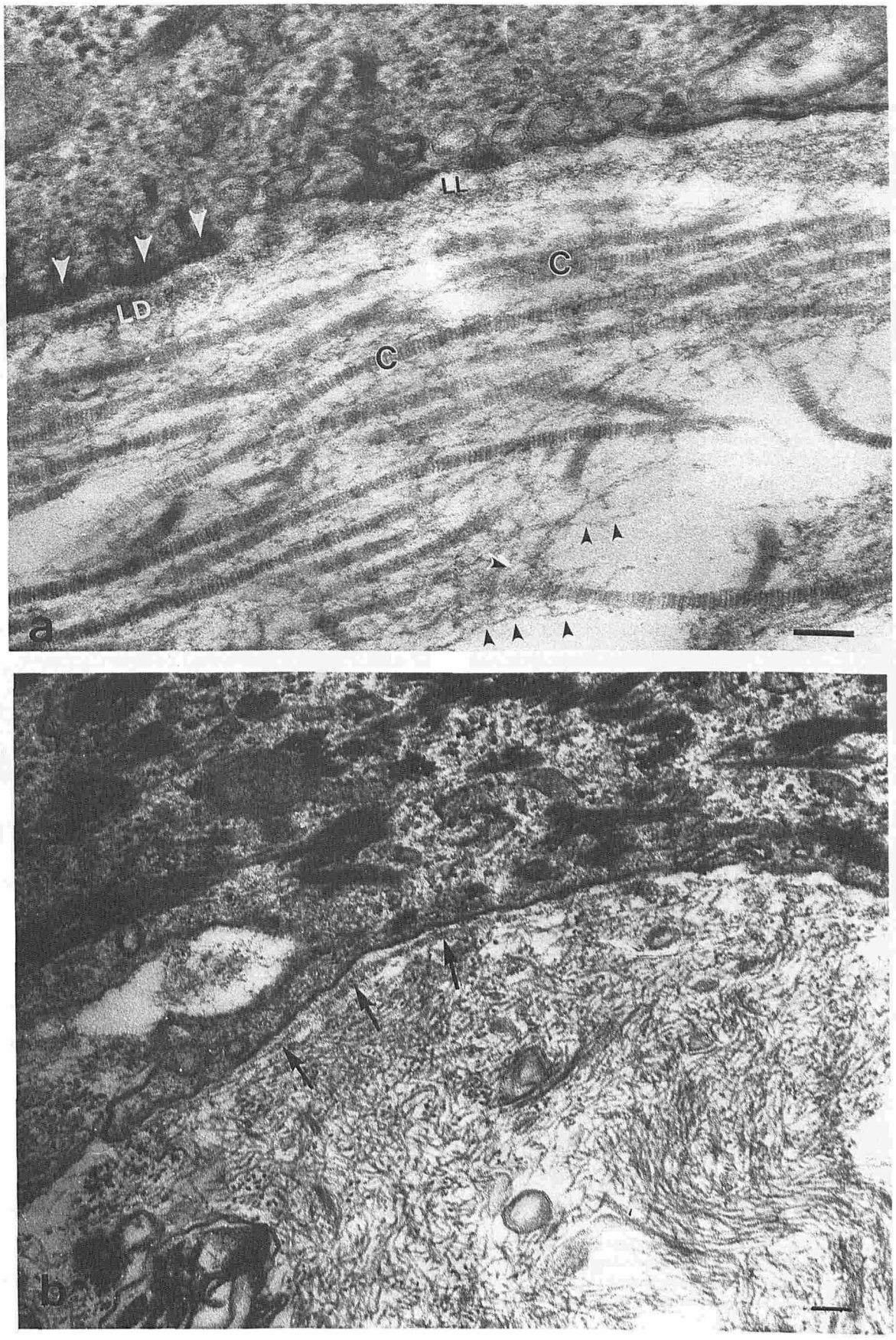


**Figure 2.** Dermal-epidermal junction in the co-culture at 3 (*a*) and 5 weeks (*b*). Three-week co-culture: hemidesmosomes along the basal cell membrane (*arrowheads*). A lamina lucida (LL) and lamina densa (LD) are also evident. Abundant thin collagen fibers (C) and microfibrils (*small arrowheads*) are seen in the upper dermis (*a*). Five week co-culture: note basal cells with large amounts of tonofilaments, a well-developed basal lamina (*arrows*), and an attached extracellular matrix (*b*). *Bar*, 100 nm.

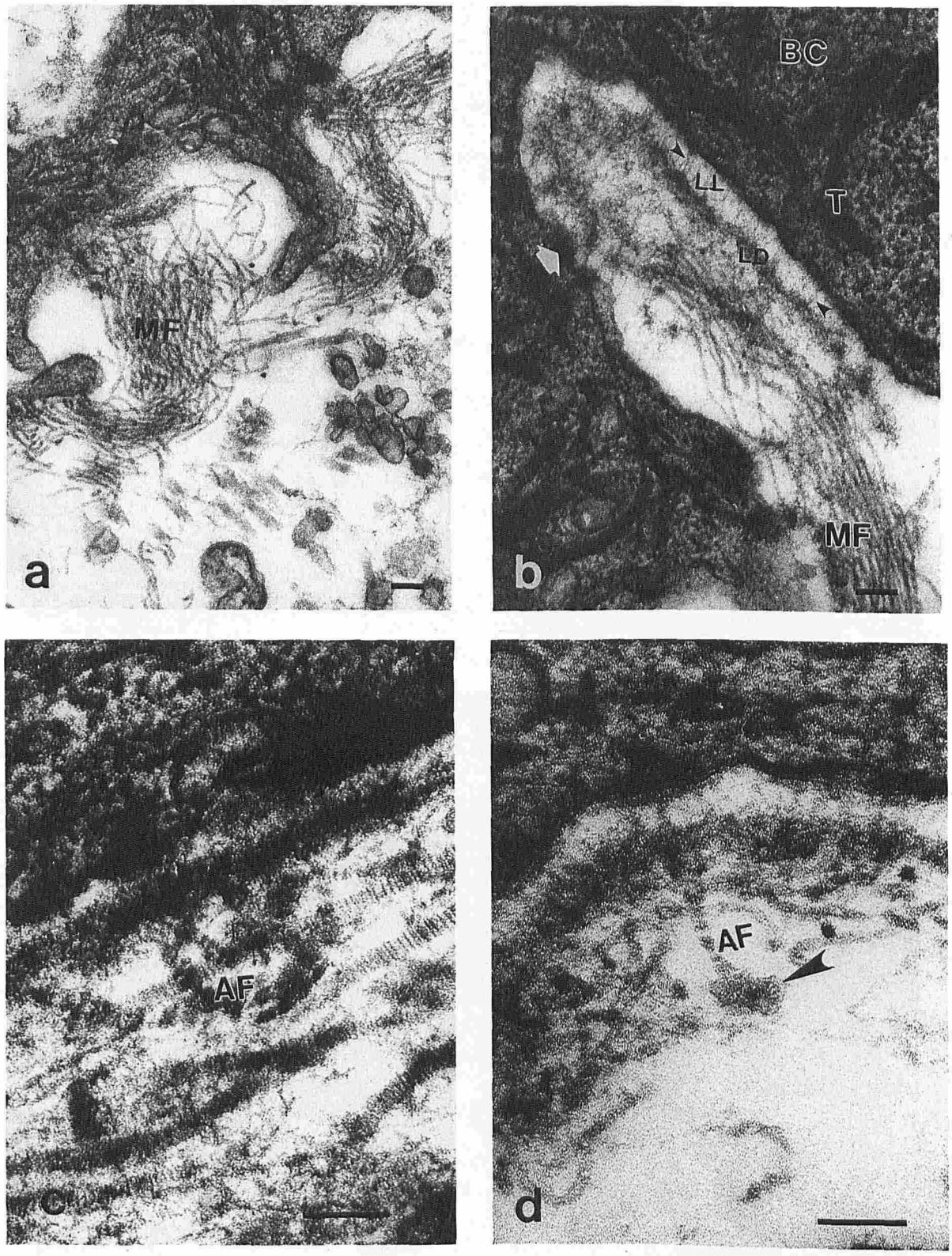


**Figure 3.** Basement membrane zone at 5–6 weeks post keratinocyte seeding. Large bundles of microfibrils (MF) at the basement membrane zone (*a*). All of the components of the basement membrane zone including a basal cell (BC) containing tonofilaments (T), hemidesmosomes (*arrow*), a lamina lucida (LL), thin anchoring filaments (*small arrowheads*) coursing through the lamina lucida, and a lamina densa (LD) (*b*). Anchoring fibrils (AF) originating at the basement membrane (*c*). Anchoring fibril (AF) terminating in an anchoring plaque (*arrowhead*) (*d*). *Bar*, 100 nm.

## DISCUSSION

The most striking achievement of this model was the development of a basal lamina and an anchoring zone. An incomplete, fragmentary lamina densa was noted using 3-week co-cultures. However, by 5 weeks, most areas showed a distinct lamina densa, separated from basal keratinocytes by a lamina lucida. Anchoring filaments extended from the hemidesmosomes to the lamina densa.

The anchoring zone in this skin-culture model was characterized by three supramolecular structures: anchoring fibrils, bundles of microfibrils, and a network of thin collagen fibers. These last structures appeared to be enlaced within anchoring fibrils. Anchoring fibrils extend from the lamina densa to anchoring plaques. Anchoring fibrils have never been seen either in *"in vitro"* co-culture experiments involving keratinocytes and fibroblasts in collagen gels or when only fibroblasts were seeded in a three-dimensional mesh. Thus, to the best of our knowledge, the model proposed in this study is the first *"in vitro"* system where anchoring fibrils and anchoring plaques have been reconstituted.

Elastin-associated microfibrils 10 nm in diameter are produced in large quantities when fibroblasts are grown in the absence of other cell types in a three-dimensional nylon mesh [11]. In these cultures microfibrils are seen as single units or in bundles frequently intermingled with collagen fibrils in a parallel fashion [11]. In the co-culture, the above pattern persisted throughout the dermal component. However, in the anchoring zone, microfibrils had a tendency to group into bundles arranged parallel or perpendicularly to the lamina densa, suggesting an epidermal morphogenetic effect.

Fibroblasts grown in a three-dimensional nylon mesh in the presence of ascorbic acid create an abundant extracellular matrix (ECM) with intense deposition of collagen fibrils (Contard P, Jacobs L, Perlish JS, Fleischmajer R: Fibrillogenesis in a new three-dimensional fibroblast culture system (abstr). J Invest Dermat 96:605, 1991). Furthermore, histograms of collagen fibril diameter in cultures 7 to 54 d old showed active fibril growth from about 15–20 nm to up to 80 nm. It is noteworthy that in this recombinant model collagen fibrils near the epidermal dermal junction were thinner than those present in the lower levels, suggesting an epidermal component in the regulation of fibril formation. This organization resembles the *"in vivo"* situation whereby reticulin fibrils are concentrated in the dermal epidermal junction [14].

This keratinocyte/dermal model co-culture corroborates the concept that promotion and maintenance of epidermal morphogenesis and differentiation is greatly dependent upon interactions with the microenvironment. Furthermore, it also suggests that the formation of a basal lamina–anchoring zone is the result of a cooperative process between epidermal cells and their surrounding ECM. We hope that the model characterized here will be suitable for the study of the basic mechanisms of how the ECM interacts with adjacent epithelia and thus regulates biologic events that control morphogenesis and cellular differentiation.

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