

# Epithelial Origin of Cutaneous Anchoring Fibrils

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**Abstract.** Anchoring fibrils are essential structural elements of the dermoepidermal junction and are crucial to its functional integrity. They are composed largely of type VII collagen, but their cellular origin has not yet been confirmed. In this study, we demonstrate that the anchoring fibrils are primarily a product of epidermal keratinocytes.

Human keratinocyte sheets were transplanted to a nondermal connective tissue graft bed in athymic mice. De novo anchoring fibril formation was studied ultrastructurally by immunogold techniques using an antiserum specific for human type VII procollagen. At 2 d after grafting, type VII procollagen/collagen was localized both intracellularly within basal keratinocytes and extracellularly beneath the discontinuous basal lamina. Within 6 d, a subconfluent basal lamina had developed, and newly formed anchoring fibrils and an-

choring plaques subjacent to the xenografts were labeled. Throughout the observation period of the experiment, the maturity, population density, and architectural complexity of anchoring fibrils beneath the human epidermal graft continuously increased. Identical findings were obtained using xenografts cultivated from cloned human keratinocytes, eliminating the possibility of contributions to anchoring fibril regeneration from residual human fibroblasts. Immunolabeling was not observed at the mouse dermoepidermal junction at any time.

These results demonstrate that the type VII collagen of human cutaneous anchoring fibrils and plaques is secreted by keratinocytes and can traverse the epidermal basal lamina and that the fibril formation can occur in the absence of cells of human dermal origin.

THE dermoepidermal junction of mammalian skin is defined by a group of discrete but intimately interrelated ultrastructural elements including a basal lamina (laminae lucida and densa), hemidesmosomes with their associated anchoring filaments and subbasal dense plaques, dermal microfibrillar bundles (terminal arborizations of the dermal elastic system), and anchoring fibrils (Briggaman and Wheeler, 1975; Tidman and Eady, 1984). These components form the architectural basis of the membrane zone between the epidermis and the dermis. The basal lamina is a continuous layer of extracellular matrix molecules including type IV collagen, laminin, and entactin. Most basal lamina components have been shown to be epithelial cell products (Briggaman, 1981; Hay, 1981, 1982; Kefalides et al., 1979; Kubo et al., 1984; Martin and Timpl, 1987; Roberts and Jenner, 1983) and are involved in the regulation of epithelial growth and differentiation (Foellmer et al., 1983). The basal keratinocytes adhere to their basal lamina via fine filaments known as anchoring filaments or microfilaments which extend from the hemidesmosomes through the lamina lucida into the lamina densa (see Fig. 1 a) (Hay, 1982; Kanwar and Farquhar, 1980). The lamina densa, in turn, is secured to the

underlying dermal connective tissue matrix by an interconnecting network of specialized fibrous structures called anchoring fibrils (Palade and Farquhar, 1965; Keene et al., 1987). Immediately beneath the epithelium, anchoring fibrils extend from the lamina densa to subjacent amorphous deposits of type IV and VII collagen known as anchoring plaques (Burgeson et al., 1985). Anchoring fibrils also span adjacent anchoring plaques forming a complex dermal network that enmeshes dermal collagen and other extracellular matrix components (Sakai et al., 1986). The importance of anchoring fibrils to the structural integrity of the skin and the stability of the dermoepidermal junction is epitomized by the dystrophic recessive form of epidermolysis bullosa, a disease in which anchoring fibrils are lacking and the skin blisters excessively (Heagerty et al., 1986; Leigh et al., 1988).

The diameter of the cross-banded central region of anchoring fibrils varies from 20 to 60 nm. However, the precise length of anchoring fibrils has been difficult to determine because these structures tend to undulate in and out of the section plane. Previously reported measurements of human anchoring fibril length vary from 200 to 800 nm. The primary structural component of anchoring fibrils is type VII collagen. During anchoring fibril formation, type VII procollagen molecules dimerize in an antiparallel fashion overlap-

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ping at their amino-terminal globular domains. Dimers, in turn, form parallel aggregates that insert into the lamina densa and the anchoring plaques at their carboxy termini (Bentz et al., 1983; Burgeson et al., 1985; Burgeson, 1987; Gipson et al., 1987; Keene et al., 1987; Morris et al., 1986; Sakai et al., 1986; Smith et al., 1988).

The cellular origin of anchoring fibrils has not yet been determined and is the subject of some debate (Burgeson et al., 1990; Bruckner-Tuderman et al., 1987; Ghadially, 1988). Type VII procollagen is secreted in varying amounts by both keratinocytes (Lunstrum et al., 1986; Sakai et al., 1986) and dermal fibroblasts in culture (Bruckner-Tuderman et al., 1989; Sakai et al., 1986; Stanley et al., 1985), but it is still unclear which of these two cell types is the major contributor to anchoring fibril formation *in vivo* or to what extent they share in this process.

Preliminary studies on dermoepidermal junction formation in skin regenerated from cultured epithelial autografts have suggested that keratinocytes might be the major contributors to *de novo* anchoring fibril formation. When cultured keratinocyte sheets are transplanted to fascial wound beds in massively burned patients, hemidesmosomes, basal lamina and anchoring fibrils reform together in discrete foci along the attachment face of the regenerated epidermis within 1 wk after transplantation (Compton et al., 1989).

In the present study, cultured human keratinocyte sheets were transplanted onto a nondermal graft bed in athymic mice, the connective tissue subjacent to the panniculus carnosus of a dorsal skin flap (Barrandon et al., 1988), and *de novo* regeneration of anchoring fibrils and plaques was observed for 46 d. Some grafts were generated from a small pool of cloned human keratinocytes to eliminate the possible presence of residual human dermal fibroblasts in transplanted xenografts. Light and electron microscopic immunolabeling studies using an antiserum that is specific for human type VII procollagen and does not cross-react with mouse type VII collagen (Lunstrum et al., 1986) were performed to determine the species origin of the type VII collagen of the newly formed anchoring fibrils and plaques. The questions addressed were whether keratinocytes *in vivo* synthesize and secrete type VII procollagen, whether the large type VII procollagen/collagen molecules are able to traverse the basal lamina to form the anchoring fibrils, and whether dermal fibroblasts are necessary for anchoring fibril formation. Our results indicate that the type VII collagen of human cutaneous anchoring fibrils and plaques is derived from keratinocytes and that human dermal fibroblasts are not required for normal anchoring fibril synthesis.

## Materials and Methods

### Skin and Tissue Samples

Human keratinocytes were isolated from skin biopsies obtained from newborn foreskin (strain SR1), pediatric (6-yr-old male) sole skin (strain SH-BR), and fetal (17-19 wk estimated gestational age) sole skin (strain HFS2 and HFS3).

### Cell Culture

Keratinocytes were plated onto feeder layers of lethally irradiated 3T3-J2 cells (Rheinwald and Green, 1975) in a 3:1 mixture of Dulbecco-Vogt modified Eagle's medium and Ham's F12 medium (Hazelton Biologicals, Lenexa, KS) supplemented as previously described (Barrandon et al.,

1988). The cells were fed every 3-4 d and recombinant human epidermal growth factor was added to a concentration of 10 ng/ml beginning at the first feeding.

### Clone Isolation

Single cells were isolated from primary or secondary keratinocyte cultures of each selected strain as previously described (Barrandon and Green, 1985). After 7-8 d in culture, several well-growing clones of keratinocytes were trypsinized, and the cells were pooled and then cultivated onto a new feeder layer of 3T3 cells in a 60-mm petri dish. The cells were allowed to grow until the cultures reached a subconfluent state. The cloned keratinocytes were then plated at a density of  $0.5-1.0 \times 10^5$  cells/35-mm Petri dish to initiate the cultures that were then used for grafting.

### Grafting Procedure

At 2-3 d after confluence, keratinocyte cultures were released from the culture dishes with a 0.25% solution of Dispase II (Boehringer Mannheim Biochemicals, Indianapolis, IN), and the released cell sheets were transplanted to the inner surface of dorsal skin flaps in female athymic mice (NIH Swiss nu/nu; Taconic Farms [Germantown, NY] or Swiss nu/nu, Charles River Breeding Laboratories [Boston, MA]) as described by Barrandon et al. (1988). The basal surface of the keratinocyte sheets was placed onto the connective tissue subjacent to the panniculus carnosus, the subcutaneous muscle layer of rodent skin. Two sheets of silicone rubber (0.005 in. thick) (Dow Corning, Midland, MI) were interposed between the graft and the trunk of the animal, and the flap was closed with clips.

### Graft Harvest

Animals with newborn foreskin keratinocyte grafts were killed at 24-h intervals from day 1 to 14 after grafting, and then at 19 and 21 d after grafting. Grafts grown from cloned sole keratinocytes were harvested at days 19, 37, and 46. The entire skin flap was harvested and immediately trisected. One-third of each graft was fixed in formalin, one-third was processed for EM, and one-third was snap-frozen by immersion in isopentane/dry ice slush with subsequent storage in a  $-80^\circ\text{C}$  freezer.

### Antiserum

Polyclonal antiserum generated in a New Zealand White rabbit against whole human type VII procollagen was a generous gift of Dr. R. E. Burgeson (Department of Biochemistry, Oregon Health Sciences University, Portland, OR). The antiserum has been characterized in detail and is reported to be monospecific (Lunstrum et al., 1986). It recognizes both the triple-helical and nonhelical determinants at the carboxy terminus of human type VII procollagen. Most epitopes, however, are located in the large ( $M_r$  150,000), collagenase-resistant, non-triple-helical domain. Since the antiserum recognizes both the pepsin-digested form of native type VII procollagen and the tissue form of type VII collagen, we have referred to labeled structures as type VII procollagen/collagen. This antiserum has been shown to have immunoblotting and immunostaining characteristics identical to those of a murine mAb directed against the extreme carboxy terminus of pepsin-treated human type VII procollagen (Sakai et al., 1986). For this study, the rabbit antiserum rather than the murine monoclonal antibody was used to avoid nonspecific cross-reactivity with murine antigens in the graft bed.

### Histological and Ultrastructural Analysis

Immunoperoxidase analyses were performed on 4- $\mu\text{m}$  frozen sections cut on a cryostat (Frigocut 2800E; Reichert-Jung, Nussloch, FRG) using 3-amino-9-ethylcarbazole (Aldrich Chemical Co., Milwaukee, WI) as the peroxidase substrate.

### Transmission Electron Microscopy

Specimens for EM were fixed in 2.5% glutaraldehyde, postfixed in 1%  $\text{OsO}_4$ , and stained en bloc with 2% uranyl acetate. Ultrathin sections were counterstained with uranyl acetate and lead citrate.

### Immunoelectron Microscopy

Frozen tissue sections (5-10  $\mu\text{m}$ ) obtained with a cryostat (Frigocut 2800E) were collected on coverslips, acetone-fixed, and incubated in primary anti-

body for 15 h at room temperature. Coverslips were washed in PBS for 2–4 h and incubated in 5–10 nm colloidal gold-conjugated secondary goat-anti-rabbit IgG (Janssen Pharmaceuticals, Piscataway, NJ) for 4 h at 4°C. Specimens were then fixed in 2.5% buffered glutaraldehyde for 1 h, post-fixed in 1% OsO<sub>4</sub> for 30 min, and stained with 2% uranyl acetate for 2 h. Sections were dehydrated in graded alcohols and embedded in epoxy resin and the coverslips were removed with liquid nitrogen. Ultrathin sections were counterstained with uranyl acetate and lead citrate.

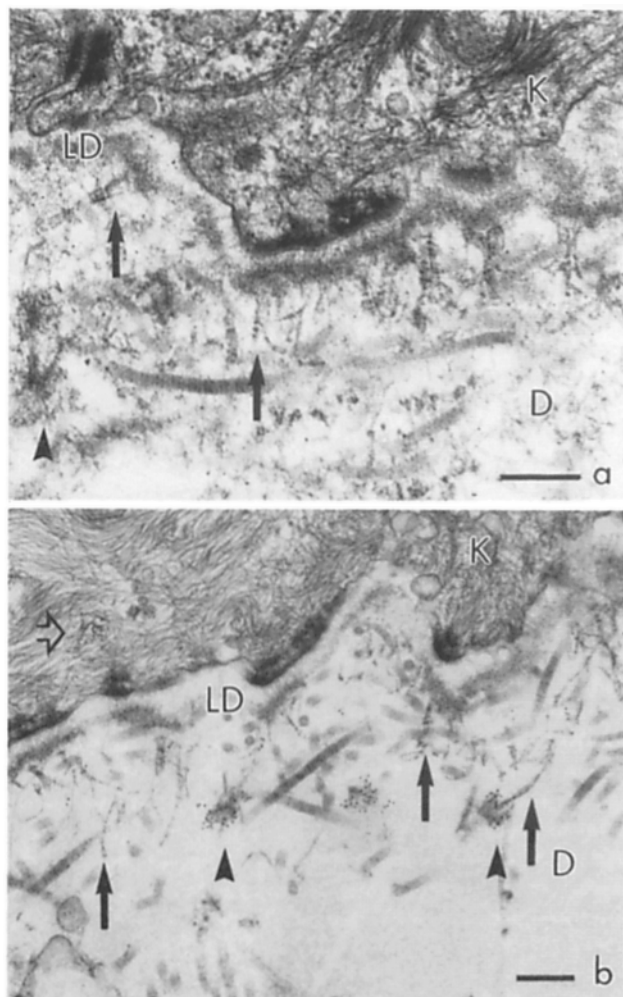
### Morphometry

Anchoring fibril density (number per unit length of basal lamina) was determined by counting only those fibrils which could be seen to insert into the lamina densa of the basal lamina.

## Results

### Anchoring Fibrils in Normal Skin Controls

The dermoepidermal junction of the control skin had a continuous basal lamina with mature hemidesmosomes and an-



**Figure 1.** Control specimens of newborn foreskin were processed for both transmission EM or immunoelectron microscopy on frozen 4- $\mu$ m sections. (a) Electron micrograph of the dermoepidermal junction of newborn foreskin. (b) Immunolocalization with polyclonal antiserum to type VII procollagen labels anchoring fibrils (arrows) that insert at the lamina densa and the anchoring plaques (arrowheads). LD, lamina densa; D, dermis; K, keratinocyte. Bars, 200 nm.

choring fibrils on standard transmission electron microscopic examination (Fig. 1 a). In the frozen tissue sections processed for immunoelectron microscopy, the basal lamina components were not optimally preserved compared with conventional electron microscopic techniques. Focal discontinuities of the basal lamina were regarded as technical artifacts in frozen sections processed for immunoelectron microscopy when the basal lamina was continuous in identical specimens fixed immediately in glutaraldehyde for standard transmission EM.

In control specimens of fetal sole and newborn foreskin, the dermis contained a network of interconnecting anchoring fibrils and anchoring plaques which extended from the lamina densa over an average distance of  $\sim$ 500 nm. Individual anchoring fibrils averaged  $\sim$ 300–700 nm long. Type VII procollagen/collagen was demonstrated both within the lamina densa and the subjacent anchoring plaques with immunogold localization (Fig. 1 b). In fetal sole skin, the density of anchoring fibrils was comparable to that in newborn foreskin, but gold label was seen only within anchoring plaques. Furthermore, the anchoring fibrils in fetal sole skin were smaller in mean diameter compared with those in newborn foreskin, and their characteristic banded pattern was less pronounced. Intracellular labeling for type VII procollagen/collagen, however, was apparent in both control tissues (Fig. 2).

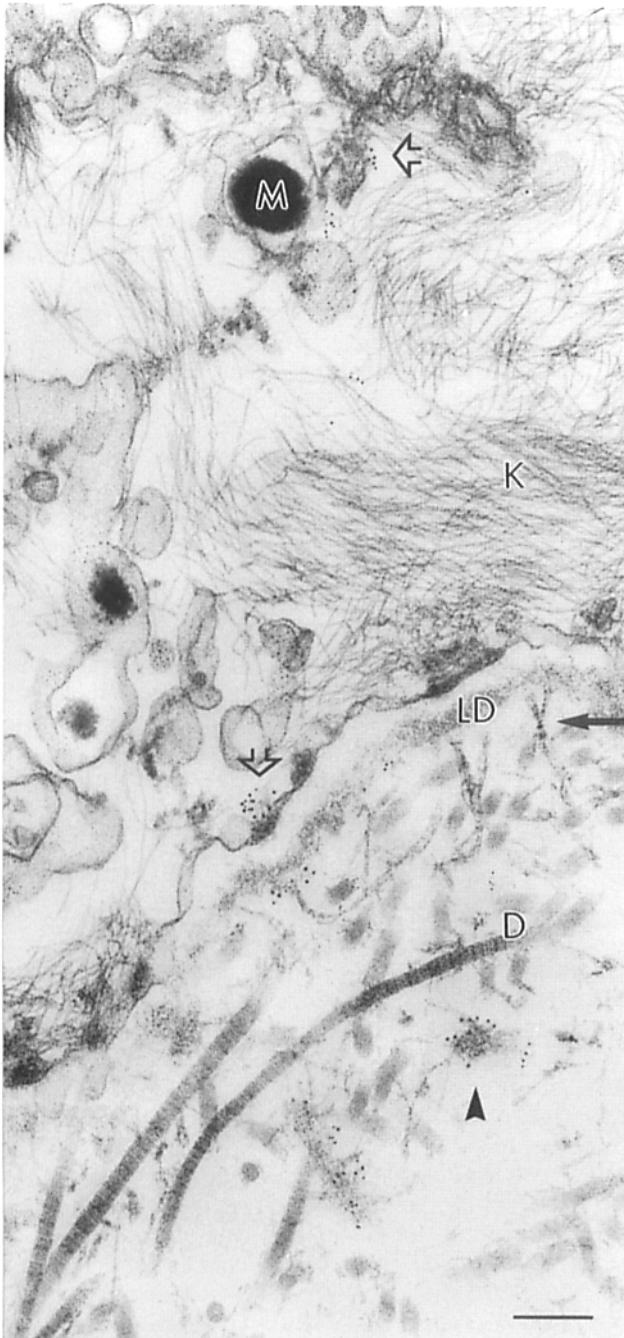
### Type VII Procollagen/Collagen Expression in Cultured Keratinocytes

At transplantation, keratinocyte sheets were three to four cell layers thick and had a uniform basal layer, but suprabasal layers were poorly organized (Fig. 3 a). Terminal differentiation was incomplete, as evidenced by the lack of granular and cornified layers as is common in submerged cultures. Intracellular and extracellular labeling were detected along the attachment face of basal keratinocytes in subconfluent colonies growing on plastic slides and glass coverslips on immunoelectron microscopic examination with the antiserum to type VII procollagen.

Released confluent keratinocyte sheets were stained with the antiserum to type VII procollagen using immunoperoxidase techniques and immunoelectron microscopic techniques. No reaction product was found at the light microscopic level (Fig. 3 a). However, sparse intracellular gold was found in basal keratinocytes on immunoelectron microscopic analysis. Gold labeling of adherent extracellular plaque-like material was also seen along the plasma membrane of the attachment face of basal cells.

### Anchoring Fibrils in Xenografts

All transplanted keratinocyte sheets gave rise to a normally stratified differentiated epithelium resembling epidermis. They readily underwent differentiation and formed a dermoepidermal junction-like structure after transplantation to the murine graft bed. By the second day after grafting, the epithelium was stratified and showed a distinct basal layer, but organized granular and cornified layers were still lacking. The epithelium was loosely attached to the underlying stroma, and immunohistochemical reactions for type VII procollagen/collagen showed faint discontinuous staining at the attachment face of the basal keratinocytes. No basal lam-



**Figure 2.** Immunolocalization with antiserum to type VII procollagen of normal newborn foreskin. Note the intracellular gold (open arrows) and labeling of type VII procollagen/collagen at the lamina densa and the anchoring plaques (arrowheads). Arrow, anchoring fibril; LD, lamina densa; D, dermis; K, keratinocyte; M, melanosome. Bars, 200 nm.

ina or fibrous structures were detected on ultrastructural examination; both intra- and extracellular immunogold labeling was seen in close vicinity to the basement membrane with antiserum to type VII procollagen. No type VII procollagen/collagen was detected in suprabasal keratinocytes.

Within 6 d after grafting, epithelial differentiation of the cultured graft was histologically complete, and fully developed spinous and granular layers were present. Immunolocalization of human type VII procollagen/collagen yielded

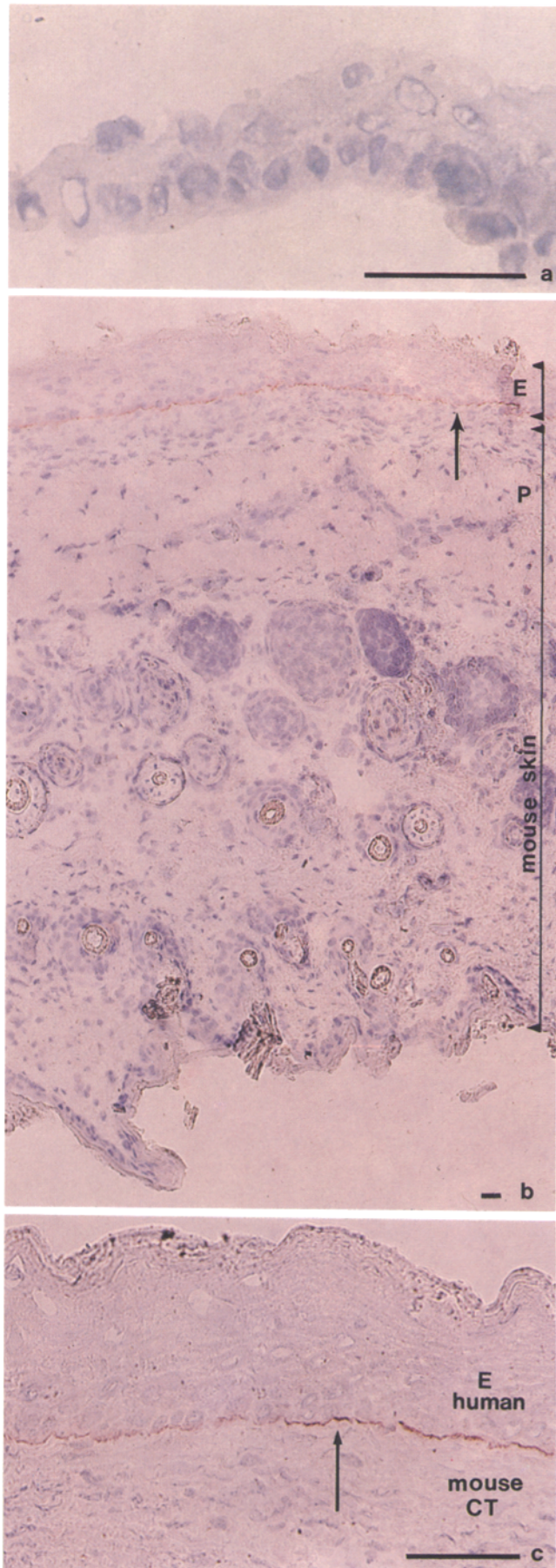
a continuous positive staining of the basement membrane zone beneath the human graft (Fig. 3, *b* and *c*) but an unstained dermoepidermal junction of the murine skin flap (Fig. 3). On electron microscopic examination, a discontinuous basal lamina appeared in association with immature hemidesmosomes in discrete foci along the attachment face of the epithelium. Anchoring fibrils were first seen at 5 d beneath the immature hemidesmosomes as sparse, wispy fibers lacking any appreciable periodicity. By day 6, a periodic cross-banded pattern typical of triple-helical collagen became evident, and small anchoring plaques were seen in association with the newly formed anchoring fibrils. At this stage, type VII procollagen/collagen could be localized to the subjacent anchoring plaques but not to the insertion site of anchoring fibrils in the lamina densa (Fig. 4, *a* and *b*). Intracellular immunogold labeling for type VII procollagen/collagen was seen exclusively in basal keratinocytes and was never observed in suprabasal keratinocytes. Within basal cells, labeling was limited to the basal region of the cytoplasm and was absent in the cell apices.

At 8–10 d postgrafting, the basement membrane zone showed strong, continuous positivity at the light microscopic level and, when viewed ultrastructurally, the basal lamina was confluent. The ultrastructural appearance and immunostaining characteristics of the xenografts produced from cloned sole-skin keratinocytes were identical to those generated from uncloned foreskin keratinocytes. Numerous anchoring fibrils projected from the lamina densa into the underlying connective tissue and inserted into regularly arranged anchoring plaques beneath a 37-d-old cloned keratinocyte graft. Intracytoplasmic type VII procollagen/collagen was also detected in the basal but not suprabasal keratinocytes (Fig. 4 *c*). The longest anchoring fibril measured ~750 nm (Fig. 4 *d*), but in longitudinal sections, anchoring fibrils undulating in and out of the section plane were rarely seen at full length. The average length of the anchoring fibrils present was comparable to that of native sole skin or newborn foreskin. The cross-banded pattern with its characteristic periodicity of the helical region of anchoring fibrils was distinct (Fig. 4 *c*). The anchoring fibrils not only extended between the lamina densa and the anchoring plaques but also interconnected individual plaques forming a structural complex network of human anchoring fibrils with the mouse connective tissue.

During the first week after grafting, the anchoring fibrils were irregularly distributed and less numerous (up to 15 anchoring fibrils/10  $\mu$ m basal lamina) than in normal site- and age-matched control skin (33/10  $\mu$ m). By 46 d, the number of anchoring fibrils reached a density of 30/10  $\mu$ m, close to that of the donor skin. At this time, however, the mean diameter of the regenerating anchoring fibrils (range 10–18 nm) was still less than that of the normal skin specimen (range 30–40 nm).

## Discussion

This study examines the epithelial contribution to de novo anchoring fibril formation after transplantation of cultured human keratinocyte grafts onto athymic mice. The newly developed anchoring fibrils and plaques beneath the human keratinocyte grafts consisted primarily of human type VII procollagen/collagen, a finding that is consistent with deriva-

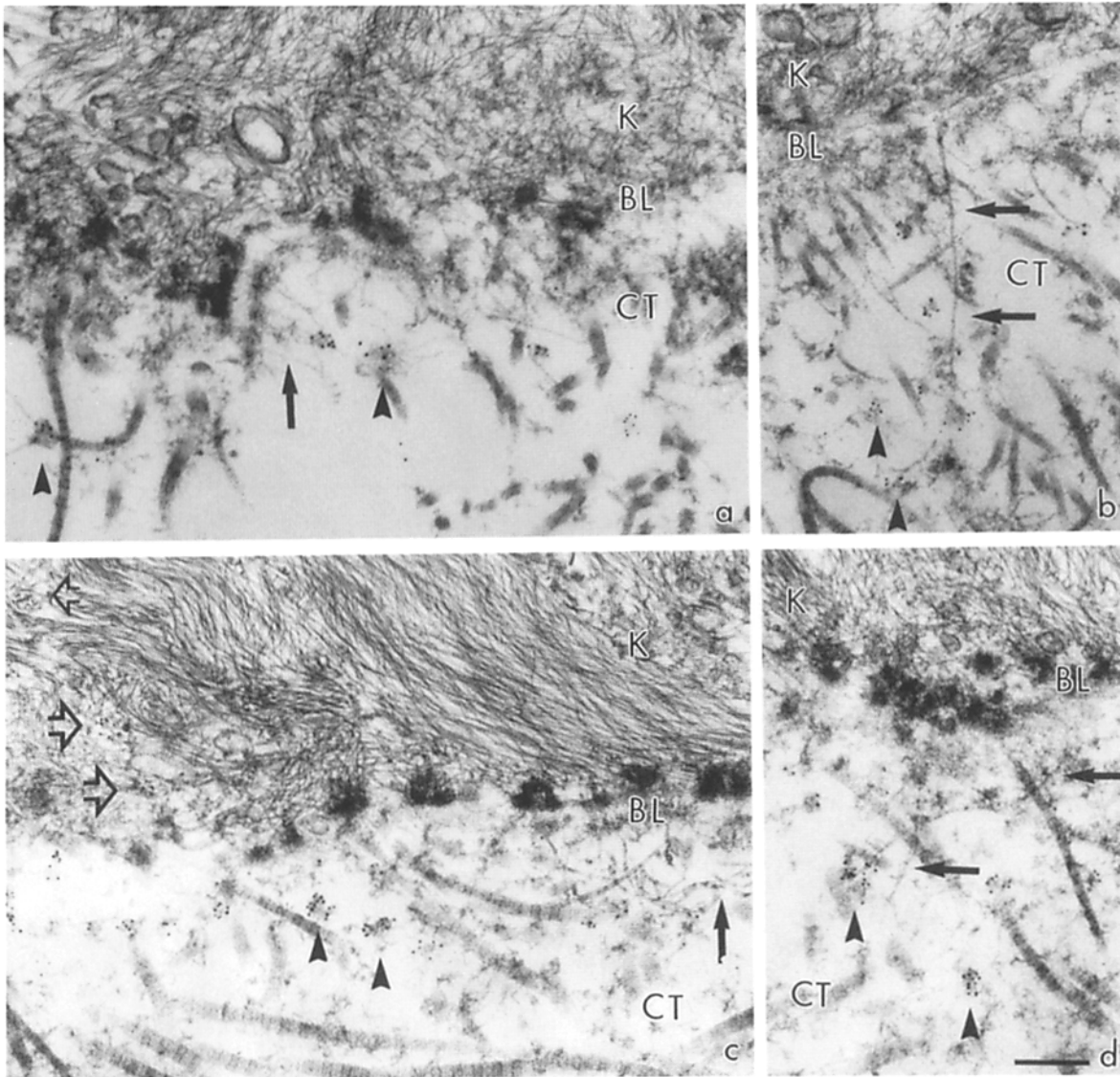


tion from the overlying epithelium. The possibility that murine fibroblasts may have contributed to the anchoring fibril biosynthesis, however, has not been strictly ruled out, since murine type VII procollagen/collagen is undetected in this system. Neither do these findings exclude the possibility of a regulatory or permissive role for mouse connective tissue cells in keratinocyte expression of type VII collagen or in anchoring fibril assembly. Nevertheless, our findings demonstrate that human cutaneous anchoring fibrils are derived primarily from epidermis and contrast with previous assertions that anchoring fibrils are dermal in origin, presumably produced by fibroblasts (Bruckner-Tuderman et al., 1987; Ghadially, 1988).

Anchoring fibrils of xenografts derived from fetal skin were identical in ultrastructural appearance and immunolabeling characteristics to those of xenografts derived from newborn skin during the observation period of the experiment. By 17 wk gestational age, fetal keratinocytes seem capable of regenerating anchoring fibrils that are morphologically comparable to those regenerated from postnatal keratinocytes.

Anchoring fibrils regenerated from uncloned foreskin keratinocyte grafts were comparable in length to those in the skin from which the keratinocytes were originally derived, and those from cloned fetal sole grafts were comparable to age- and site-matched control skin. Although they were sparse, thin and lacked appreciable periodicity in the first week after grafting, anchoring fibrils increased in number, and their characteristic cross-banding periodicity became more pronounced with increasing individual fibril diameter. However, the observation period of this study was short, and full maturation to normal fibril diameter was not observed during the time frame of the experiment. Previous studies of cultured autografts in burn patients have shown that full maturation of anchoring fibrils may take as long as 1 yr (Compton et al., 1989). The slowly increasing diameter of anchoring fibrils beneath the human xenografts supports the hypothesis that anchoring fibrils grow by continued unstag-gered lateral aggregation of newly synthesized type VII procollagen/collagen dimers (Burgeson et al., 1985) and that they are not inserted into the hemidesmosomes from the stromal side (Gipson and Trinkhaus-Randall, 1985) after complete assembly in the dermis. The findings suggest that the transport of type VII procollagen/collagen can occur across a confluent basal lamina. Early in basement membrane formation, type VII procollagen/collagen molecules may pass through the discontinuities in the lamina densa. Once the basal lamina had reached confluence (8–10 d after grafting), however, the anchoring fibrils continued to increase in density and diameter.

**Figure 3.** Immunoperoxidase staining using an antiserum to human type VII procollagen of (a) a confluent keratinocyte sheet, released with Dispase, and (b and c) a mouse skin flap bearing a human keratinocyte graft. (a) In the detached keratinocyte sheet, no staining is seen at the microscopic level. (b) In a transverse section through the entire mouse skin flap, positive staining (arrow) is seen along the basement membrane zone of a 6-d-old xenograft grown from human foreskin keratinocytes. (c) The junction of human epidermal and mouse connective tissue is shown at higher magnification. CT, connective tissue; E, epidermis; P, panniculus carnosus. Bars: (a and c) 100  $\mu$ m and (b) 10  $\mu$ m.



**Figure 4.** Immunogold labeling of the basement membrane zone of human keratinocyte grafts on mice. (a and b) The basal lamina of the 6-d-old cultured uncloned newborn foreskin keratinocyte graft is discontinuous, but anchoring fibrils (arrows) are extending from the lamina densa and inserting into the anchoring plaques (arrowheads). (c and d) Type VII procollagen is localized intracellularly (open arrows) in a 37-d-old cloned fetal sole keratinocyte graft. Arrows, anchoring fibrils; BL, basal lamina; CT, mouse connective tissue; K, human keratinocytes. Bar, 200 nm.

As early as 2 d after grafting, before anchoring fibrils or hemidesmosomes can be recognized ultrastructurally, intracellular as well as extracellular type VII procollagen/collagen can be demonstrated. Initiation of type VII procollagen/collagen production and secretion does not seem to depend on the prior presence of hemidesmosomes. However, anchoring fibril and hemidesmosome formation *in vivo* seem to occur synchronously as has been reported in fetal development (Smith et al., 1988) and under *in vitro* conditions (Gipson et al., 1988). Assembly of type VII procollagen/collagen to anchoring fibrils may thus depend on interaction with hemidesmosomes. Anchoring fibrils forming beneath cultured xenografts in this study could be identified ultrastructurally as slender fibrils beneath hemidesmosomes by 5 d after grafting. This finding contrasts with previous studies on

cultured keratinocyte grafts grown on collagen lattices and transplanted to nude mice in which sparse anchoring fibril formation was observed only at 30 d after grafting (Bosca et al., 1988).

This study has also shown that anchoring fibril formation is not dependent upon the presence of dermis, as postulated by Briggaman et al. (1971). The keratinocyte sheets transplanted to a nondermal murine connective tissue graft bed developed anchoring fibrils morphologically identical to those in normal skin. Furthermore, using grafts generated from cloned keratinocytes, we have demonstrated that human type VII procollagen/collagen produced by keratinocytes participates in the formation of anchoring fibrils and is not induced by the presence of human dermal fibroblasts within the graft. Therefore, it can be concluded that ker-

atinocytes (epidermal cells) can play a major role in the reconstruction of the basement membrane in the absence of dermal cells.

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