

FORMATION AND ORIGIN OF BASAL LAMINA AND ANCHORING FIBRILS IN ADULT HUMAN SKIN

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ABSTRACT

The purpose of this investigation was to study the formation and origin of basal lamina and anchoring fibrils in adult human skin. Epidermis and dermis were separated by "cold trypsinization." Viable epidermis and viable, inverted dermis were recombined and grafted to the chorioallantoic membrane of embryonated chicken eggs for varying periods up to 10 days. Basal lamina and anchoring fibrils were absent from the freshly trypsinized epidermis before grafting although hemidesmosomes and tonofilaments of the basal cells remained intact. Basal lamina and anchoring fibrils were absent from freshly cut, inverted surface of the dermis. Beginning 3 days after grafting, basal lamina was noted to form immediately subjacent to hemidesmosomes of epidermal basal cells at the epidermal-dermal interface. From the fifth to the seventh day after grafting, basal lamina became progressively more dense and extended to become continuous in many areas at the epidermal-dermal interface. Anchoring fibrils appeared first in grafts consisting of epidermis and viable dermis at five day cultivation and became progressively more numerous thereafter. In order to determine the epidermal *versus* dermal origin of basal lamina and anchoring fibrils, dermis was rendered nonviable by repeated freezing and thawing 10 times followed by recombination with viable epidermis. Formation of basal lamina occurred as readily in these recombinants of epidermis with freeze-thawed, nonviable dermis as with viable dermis, indicating that dermal viability was not essential for synthesis of basal lamina. This observation supports the concept of epidermal origin for basal lamina. Anchoring fibrils did not form in recombinants containing freeze-thawed dermis, indicating that dermal viability was required for anchoring fibrils formation. This observation supports the concept of dermal origin of anchoring fibrils.

INTRODUCTION

Until recently, the belief was generally held that basal lamina (basement membrane) between epithelium and its underlying connective tissue arose exclusively from connective tissue (1, 2, 3). Although others have questioned this concept (4, 5, 6) Hay and Revel (7, 8) and Pierce and his associates (9, 10) provided the first direct evidence that basal lamina arose, in part or exclusively,

from epithelium. Since then, evidence in support of the epithelial origin of basal lamina has advanced along two lines: (a) basal lamina forms in the absence of connective tissue elements and (b) precursors of basal lamina are found in epithelial cells before incorporation into basal lamina.

Pierce et al. (9, 11), using light microscopy, showed that basement membrane formed around

isolated neoplastic cells which were derived from several different organs of mice grown *in vitro* in the absence of connective tissue elements. Pierce (12) further showed that basement membrane developed between embryonic mouse ectoderm and endoderm at a stage before the differentiation of mesenchymal cells, indicating that mesenchyme played no role in the development of basement membrane. By light microscopy, Dodson (13) demonstrated the formation of basement membrane underneath isolated chick embryo epidermis grown on collagen gel and on freeze-thawed dermis in the absence of viable fibroblasts or other connective tissue cells.

Using electron microscope radioautography, Hay and Revel (7) demonstrated proline-rich precursors of basal lamina in amphibian epidermis and associated fibroblasts and followed their sequential incorporation into basal lamina. Pierce et al. (14) performed similar experiments, using immunohistochemical methods, to show that isolated neoplastic mouse cells synthesize precursors of basal lamina and excrete the material extracellularly where it accumulates around the cells.

Formation of basal lamina in adult mammalian tissue has been demonstrated electron microscopically by Odland and Ross (15) and Croft and Tarin (16). These authors showed that basal lamina formed under epidermal basal cells in healing cutaneous wounds. The newly formed basal lamina was thought by these authors to arise from epidermis, although this issue could not be settled conclusively because viable dermal cells which might have contributed to basal lamina formation were present in the vicinity of the healing wound.

The purpose of our investigation was to study the formation and origin of basal lamina in adult human skin. A model was used in which basal

lamina forms at the interface between epidermis and dermis when recombinations of separated epidermis and dermis are cultured on the chick chorioallantoic membrane (CAM). The special advantage of this system is that the dermal component can be rendered nonviable by repeated freezing and thawing, thereby eliminating an active dermal contribution to basal lamina formation. Other advantages of this system are that normal, nonneoplastic adult tissue can be used as the starting material and that both light and electron microscopy can be performed on the tissue during the course of the experiment.

Using this model, we have obtained evidence that the basal lamina is of epidermal origin in adult human skin. In addition, we have obtained evidence consistent with the view that anchoring fibrils (special fibrils of the dermis) are of dermal origin. We believe that this is the first study dealing with the origin of anchoring fibrils.

METHODS AND MATERIALS

Preparation and Cultivation of Recombinant Grafts

The techniques used in this study have been described in detail previously (17). Normal adult human skin was used in all studies. The procedure for the preparation and cultivation of recombinant grafts is diagrammed in Fig. 1. Skin was cut into thin sheets with a Castroviejo keratome set at a depth of 0.4 mm. The sheets of skin were then placed in 0.4% trypsin (Difco Laboratories, Inc., Detroit, Mich.; 1:250) solution at 4°C for 1-2 hr until the skin could be easily separated into isolated epidermis and dermis. Epidermal-dermal recombinants were prepared by inverting the separated dermis from its normal position and applying isolated epidermis to the inverted, freshly cut surface of the dermis. The dermis was inverted in order to eliminate contamination of the new epidermal-dermal interface of the

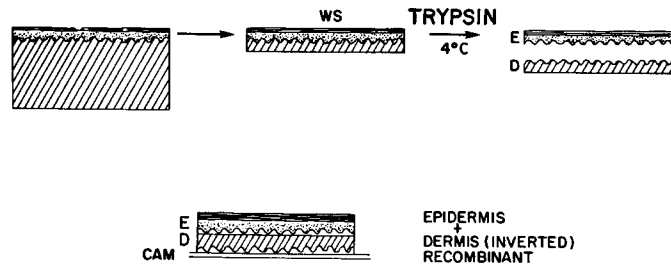


FIGURE 1 Procedure for the preparation and cultivation of recombinant grafts. Whole skin (WS), epidermis (E), dermis (D), and chorioallantoic membrane (CAM).

recombinant with residual basal lamina and anchoring fibrils from the previous epidermal-dermal interface. Recombinants were then grafted on the chorioallantoic membrane (CAM) of seven- to nine-day embryonated chicken eggs.

Recombinants of Epidermis and Viable Dermis

In one series of grafts, recombinants consisting of isolated epidermis and dermis, which had been subjected to no other manipulations than the separation and recombination procedure, were constructed as indicated above.

Recombinants of Epidermis and Nonviable (Freeze-Thawed) Dermis

In another series of grafts, recombinants were made using epidermis and nonviable dermis. In order to render the dermis nonviable, isolated dermis was alternately frozen at -40°C and thawed at 36°C 10 times. As a check on dermal viability, cultivation of fibroblasts was done on freeze-thawed dermis and

control pieces of untreated dermis. For each culture, six pieces of dermis measuring 0.5 mm in diameter were placed under a glass cover slip in a Leighton tube, and medium 199 with 33% fetal calf serum and antibiotics (penicillin 100 units/ml and streptomycin 100 $\mu\text{g}/\text{ml}$) was added. Tubes were gassed with 5% CO_2 in air, incubated at 36°C , and examined for fibroblastic outgrowth over a 4 wk period. Six fibroblast cultures of freeze-thawed dermis were compared with six control untreated dermal cultures during five separate experiments involving freeze-thawed dermis. Fibroblast outgrowth occurred in all 30 control cultures but in none of the 30 freeze-thawed dermal cultures. This supports the nonviability of freeze-thawed dermis.

18 samples of freeze-thawed dermis obtained from five separate experiments were examined by electron microscopy in order to determine the degree of cell damage produced by the freeze-thawing procedure. In all of these freeze-thawed dermal samples, severe cell degeneration of all dermal cells was noted (Fig. 2). Rupture of plasma membranes was manifest in most dermal cells, and nuclear and cytoplasmic degeneration was evident in all dermal cells. Col-

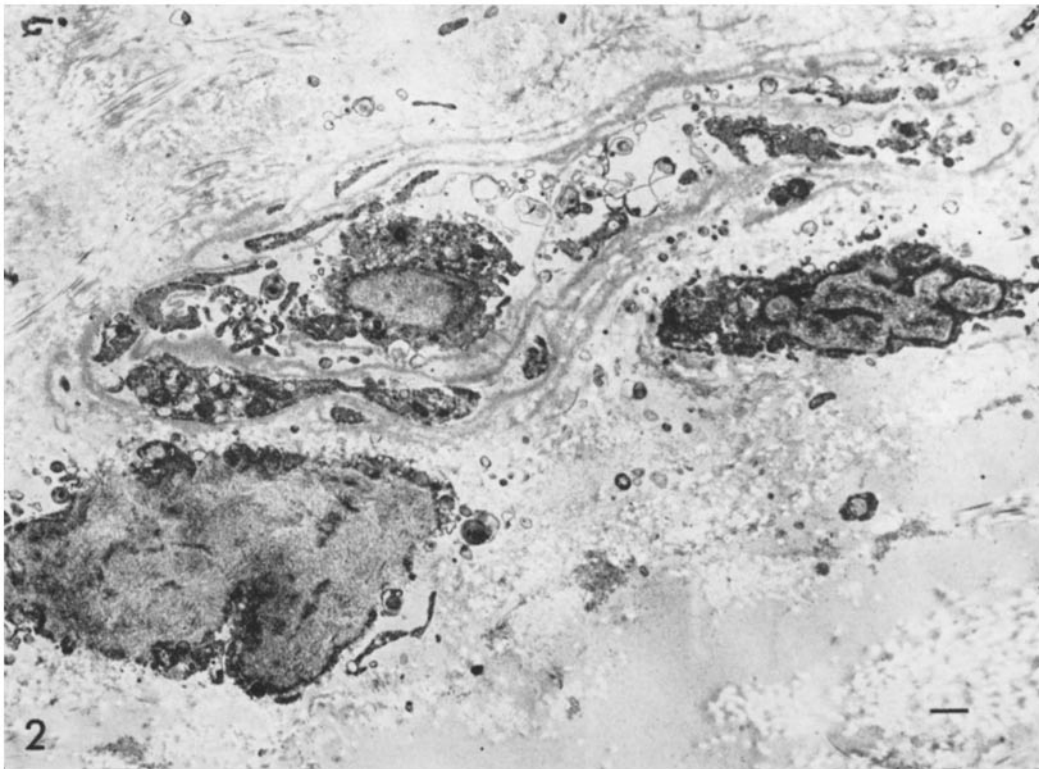


FIGURE 2 Freeze-thawed dermis showing severe cell degeneration of the dermal cells. Paraformaldehyde fixation. Calibration bar = 1μ . $\times 5400$.

lagen fibers appeared to be unaltered by the freeze-thawing procedure.

Selection of Recombinant Grafts for Electron Microscope Examination

The recombinant grafts were first examined by light microscopy after 1–10 days of cultivation on the CAM. Only recombinants which were well maintained were selected for electron microscope evaluation. The light microscopic criteria for epidermal maintenance during cultivation were the presence of intact basal, Malpighian, and granular cell layers, and an acellular, compact stratum corneum. 80 recombinants of epidermis and viable dermis and 30 recombinants of epidermis and freeze-thawed dermis meet these criteria and serve as the basis for this ultrastructural study.

Basal lamina was noted subjacent to the CAM epithelium during the course of early observations. In order to avoid confusing basal lamina from CAM epithelium with basal lamina from human epidermis, only sections with stratum corneum and keratohyalin granules as seen by electron microscopy were considered in this study. The epithelium of the CAM lacks these structures which are characteristic for human epidermis.

Electron Microscopy

Specimens for electron microscopy were fixed immediately in either 2% solution of acetate/Veronal-buffered osmium tetroxide (18) for 60 min or in *s*-collidine-buffered paraformaldehyde solution (19) for 4–24 hr followed by postfixation in 2% osmium solution for 60 min. Tissues were dehydrated in graded alcohols and embedded in Epon 812. Sections were cut in a Porter-Blum ultramicrotome with a diamond knife, stained with uranyl acetate and lead hydroxide, and examined with a JEM T7 electron microscope.

OBSERVATIONS

Epidermal-Dermal Junction in Normal Human Skin

The ultrastructure of the epidermal-dermal junction has been described previously (20–24). The epidermal-dermal junction in adult human skin before any manipulations or cultivation is shown in Fig. 3. The basal portion of the epidermal basal cell is convoluted in most areas. The plasma membrane forms the more superficial portion of the epidermal-dermal junction. Along the plasma membrane are seen focal electron-opaque thickenings termed hemidesmosomes which consist of

intracellular and extracellular portions. Tonofilaments of the basal cells converge into the cellular portion of the hemidesmosomes. On the dermal side of the plasma membrane is the electron-lucent intermembranous space of uniform thickness. The intermembranous space separates the plasma membrane from the basal lamina. The basal lamina is a continuous, electron-opaque layer approximately 300–350 Å in thickness. Subjacent to the basal lamina, a new system of fibrils has recently been described in the dermis (24–29). These fibrils have been termed anchoring fibrils (25) (special fibrils of the dermis and anchoring filament bundles). Anchoring fibrils have a characteristic morphology consisting of an asymmetric, transverse-banded central area with filamentous or branched portions at either end extending superficially to the basal lamina and deep into the dermis.

Epidermal-Dermal Junction after Trypsinization-Isolated Epidermis and Isolated Dermis

Separation of epidermis and dermis occurred sharply and uniformly at the intermembranous space between the plasma membrane and basal lamina. The basal surface of the isolated epidermis was composed of the plasma membrane along which were seen intact hemidesmosomes with their accompanying tonofilaments (Fig. 4). Basal lamina and anchoring fibrils were absent from the basal surface of all isolated epidermal specimens. Neither collagen fibrils nor contaminating dermis was noted. Membrane-bounded blebs were seen projecting from the basal surface of some of the epidermal basal cells. The cytoplasm of the bleb was finely granular and lacked cellular organelles and tonofilaments.

Basal lamina comprised the adepidermal surface of the isolated dermis (Fig. 5). Anchoring fibrils were noted subjacent to the basal lamina. Structural alterations of the basal lamina and anchoring fibrils were not seen.

Epidermal-Dermal Junctions after Recombining Viable Epidermis and Dermis

The following description is based on the electron microscope observation of 80 recombinants of epidermis and viable dermis. Attention will be directed to the epidermal-dermal interface of the recombinants. At the time of grafting, the epi-

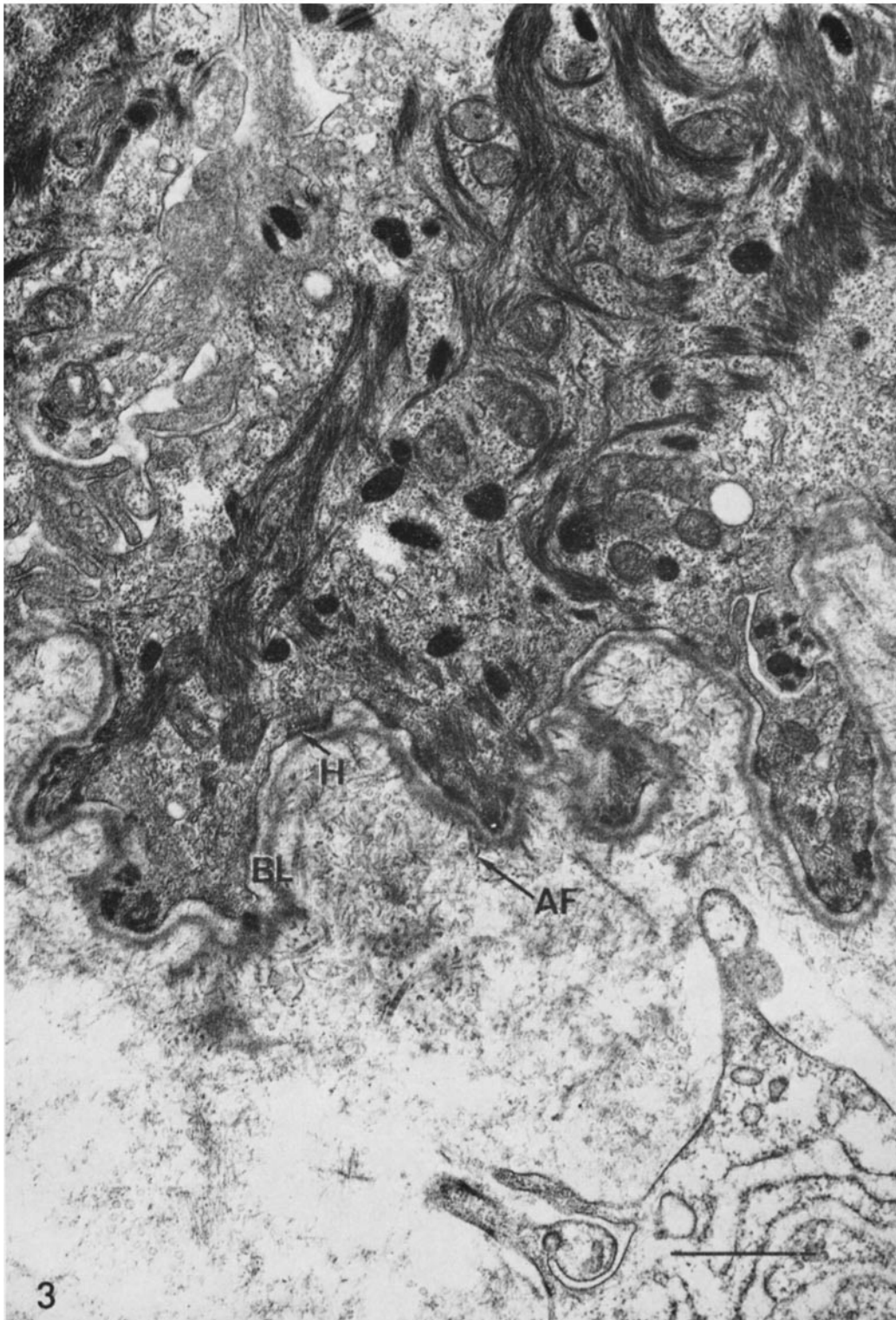


FIGURE 3 Epidermal-dermal junction in normal human skin. Hemidesmosomes (*H*) are present along the plasma membrane which is separated from the basal lamina (*BL*) by the intermembranous space. Anchoring fibrils (*AF*) and collagen fibers are seen in the dermis below the basal lamina. Paraformaldehyde fixation. Calibration bar = 1 μ . $\times 22,000$.

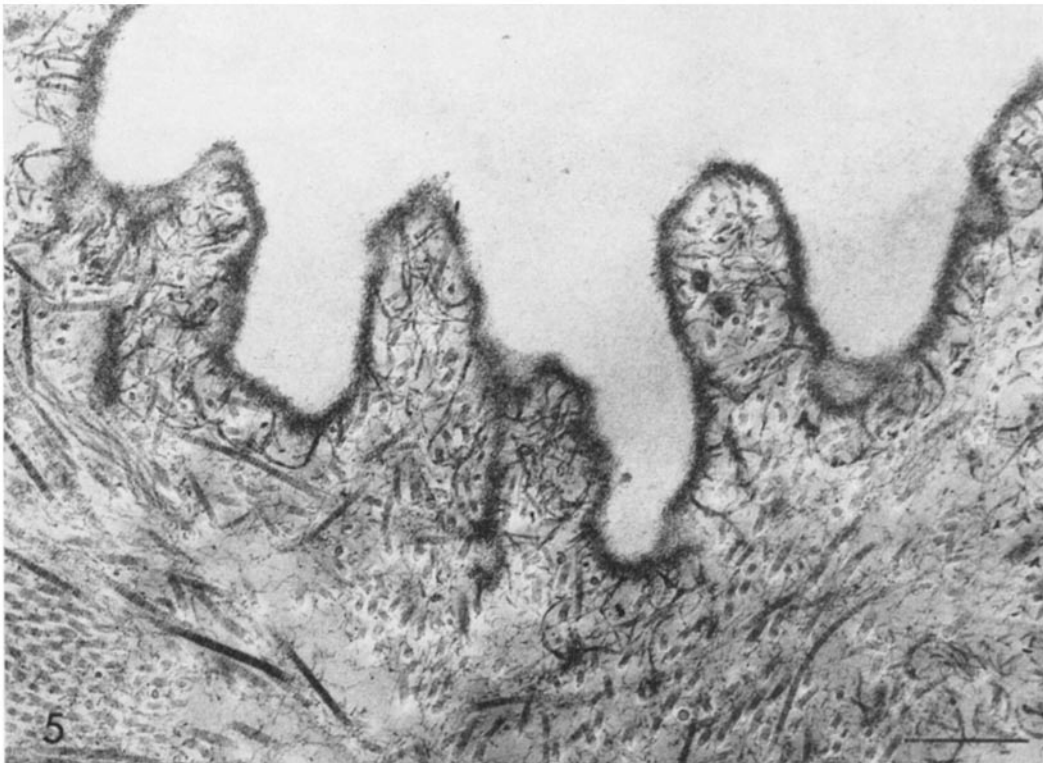


FIGURE 4 Isolated epidermis showing the former epidermal-dermal junction. Hemidesmosomes with accompanying tonofilaments are seen along the plasma membrane. No basal lamina or anchoring fibrils are seen. Membrane-bounded blebs are present (arrow). Osmium fixation. Calibration bar = 1μ . \times 21,000.

FIGURE 5 Isolated dermis showing intact basal lamina and anchoring fibrils at the former epidermal-dermal junction. Paraformaldehyde fixation. Calibration bar = 0.5μ . \times 33,000.

ermal component of the interface was composed of the plasma membrane of epidermal basal cells, intact hemidesmosomes along the plasma membrane, and tonofilaments radiating from the hemidesmosomes. The epidermal component was apposed to inverted dermis which was completely devoid of basal lamina or anchoring fibrils.

No basal lamina was noted during the first 2 days of cultivation. Beginning on the third day, focal areas of faint electron-opaque material were noted to accumulate immediately subjacent to hemidesmosomes (Fig. 6). Subsequent developments lead us to interpret this as the earliest indication of basal lamina formation. From the third to fifth day of cultivation, the focal areas of basal lamina became progressively more dense and extended laterally beyond the area subjacent to the hemidesmosomes (Fig. 7). The basal lamina was separated from the hemidesmosomes and the plasma membrane by a characteristic electron-lucent intermembranous space. By the sixth to the seventh days, focal areas of basal lamina fused to become continuous in some areas although the basal lamina was still most dense subjacent to hemidesmosomes (Fig. 8). Formation of basal lamina progressed through the ninth to 10th day of cultivation.

The previously described membrane-bounded blebs remained prominent during the first several days of cultivation and gradually involuted thereafter. Hemidesmosomes were absent on the plasma membrane of the blebs. Basal lamina failed to reform in the region of the blebs.

Anchoring fibrils first appeared during the fifth day of cultivation. Newly formed fibrils were seen underlying the basal lamina in the region of hemidesmosomes (Fig. 7-9). On longitudinal section, the fibrils were composed of an asymmetrical, transverse-banded central region with filamentous or branched regions radiating from either end (Fig. 10). Although initially sparse,

their numbers increased during cultivation until, by the ninth day, anchoring fibrils were numerous in many recombinants. Occasionally, the number appeared to be greater than seen in normal skin, in which case they were present not only attached to the basal lamina but randomly distributed in the dermis immediately under the basal lamina. Anchoring fibril formation was always preceded by basal lamina formation. Anchoring fibrils were not found in the absence of basal lamina.

Epidermal-Dermal Junctions after Recombining Viable Epidermis and Nonviable Freeze-Thawed Dermis

30 recombinants of viable epidermis and dermis rendered nonviable by repeated freezing and thawing were examined electron microscopically for basal lamina and anchoring fibril formation after varying periods of cultivation on the CAM. Formation of basal lamina followed the same time sequence as in recombinant grafts of epidermis and viable dermis. Basal lamina appeared first at three day cultivation subjacent to hemidesmosomes, after which it became progressively more dense and extended laterally to become continuous in some areas (Fig. 11). No differences were noted in the density or abundance of basal lamina between recombinants containing viable and nonviable dermis.

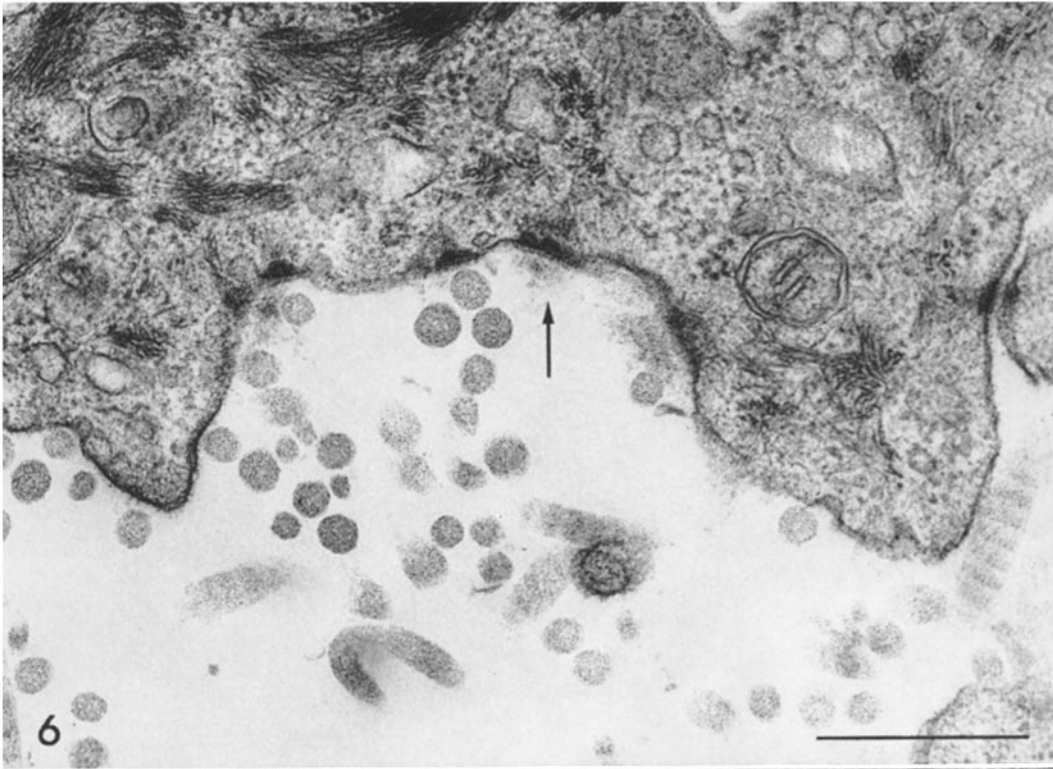
Anchoring fibrils were not found in any of the 30 recombinants of epidermis and freeze-thawed, nonviable dermis examined for periods up to 10 day cultivation on the CAM.

DISCUSSION

Basal lamina formed in recombinants of viable epidermis with both viable and nonviable dermis, indicating that basal lamina formation is not dependent upon the viability of dermis. Since epidermis is the only tissue capable of active synthesis in both situations, we conclude that

FIGURE 6 Epidermal-dermal junction in recombinant of viable epidermis and dermis at 3 days of cultivation on CAM. Focal areas of electron-opaque material are seen subjacent to hemidesmosomes (arrow). Osmium fixation. Calibration bar = 0.5μ . $\times 57,000$.

FIGURE 7 Epidermal-dermal junction in recombinant of viable epidermis and dermis at 5 days of cultivation on CAM. Basal lamina has become progressively more dense and spread out from the area subjacent to hemidesmosomes. Anchoring fibrils are seen under the basal lamina. Osmium fixation. Calibration bar = 0.5μ . $\times 25,500$.



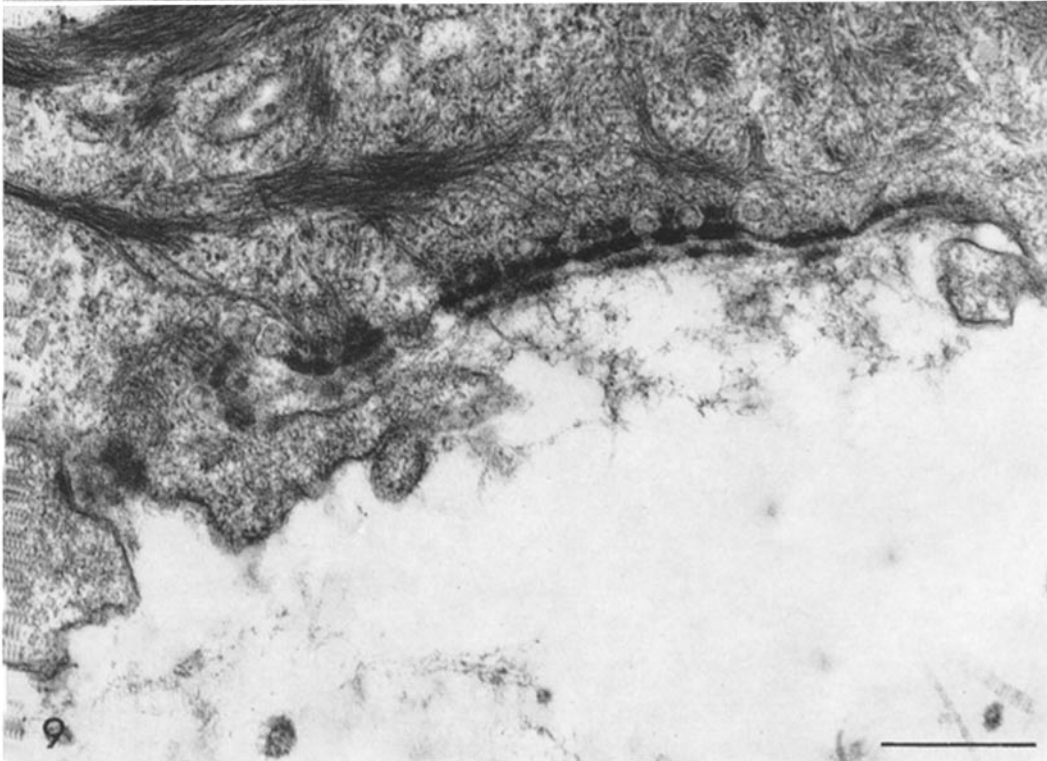
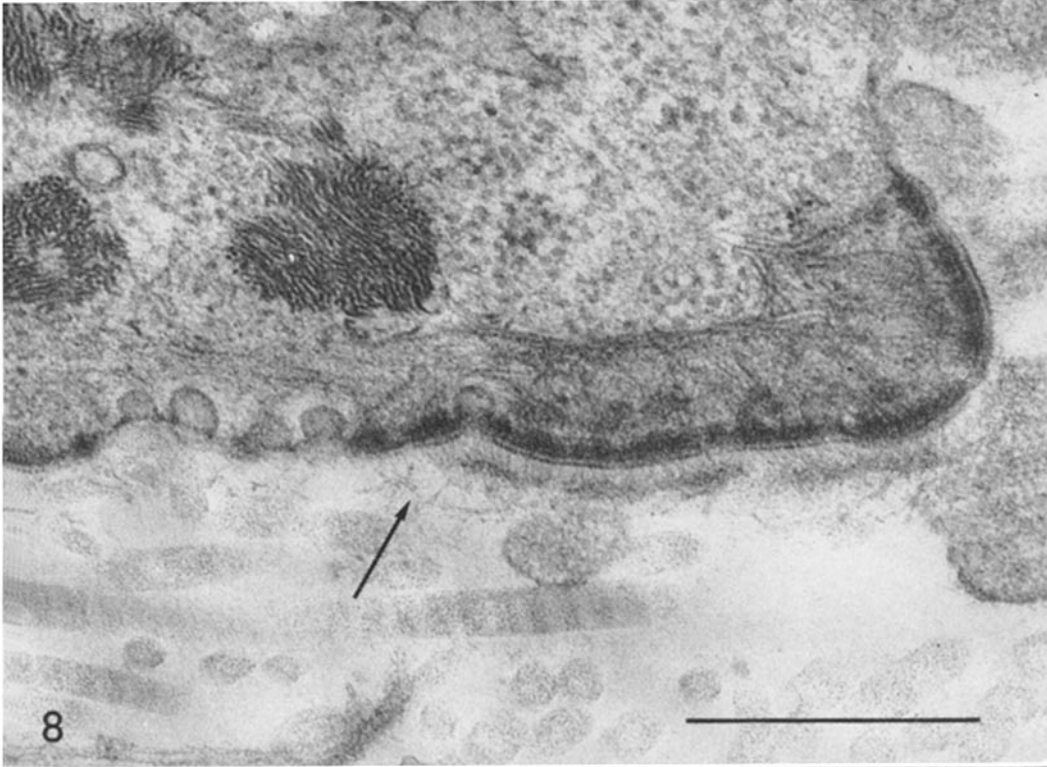


FIGURE 8 Epidermal-dermal junction in recombinant of viable epidermis and dermis at 5 days of cultivation on CAM. Anchoring fibrils are seen under the basal lamina (arrow). Osmium fixation. Calibration bar = $0.5 \mu \times 78,000$.

FIGURE 9 Epidermal-dermal junction in recombinant of viable epidermis and dermis at 7 days of cultivation on CAM. Basal lamina. Osmium fixation. Calibration bar = $0.5 \mu \times 40,800$.

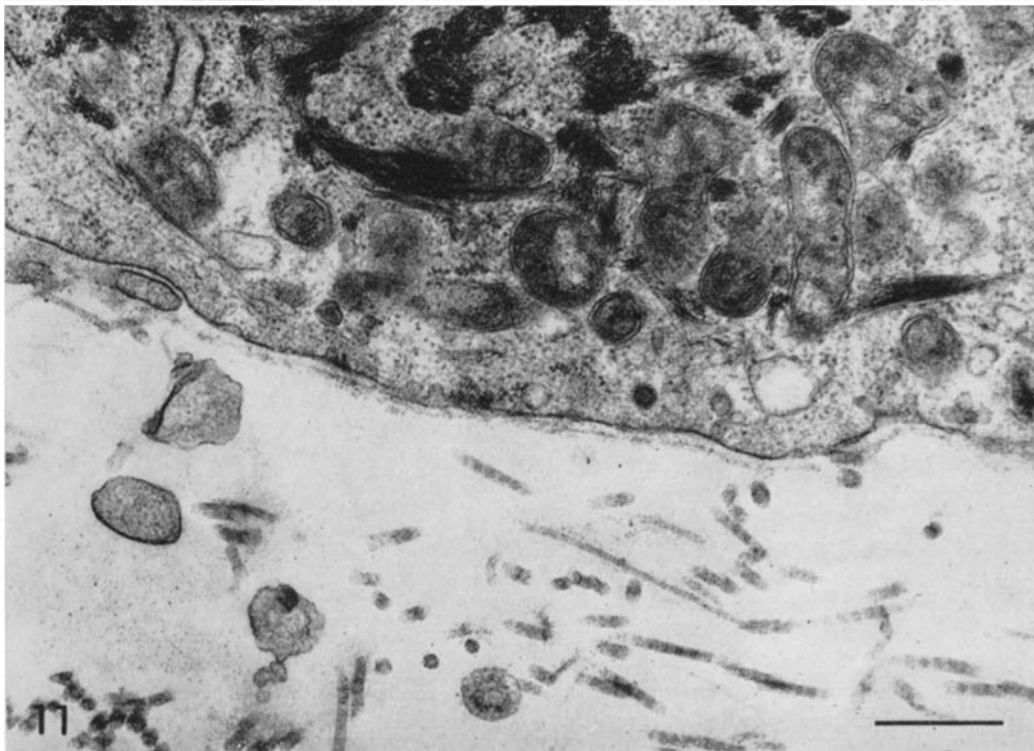
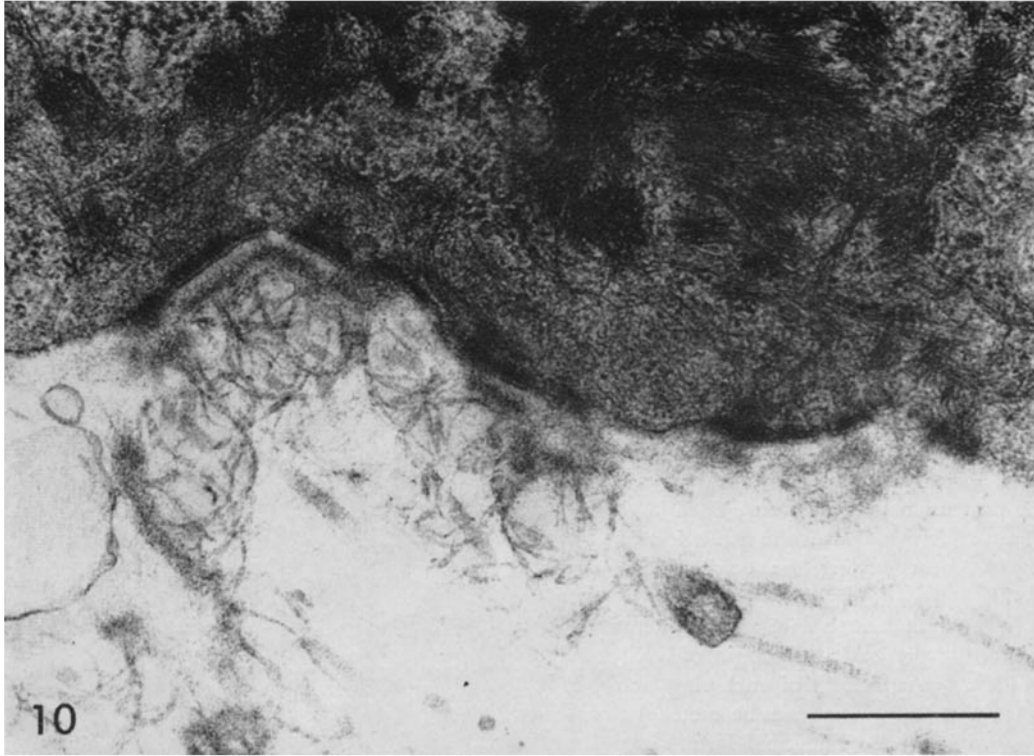


FIGURE 10 Epidermal-dermal junction in recombinant of viable epidermis and dermis at 9 days of cultivation. Anchoring fibrils are evident on the dermal side of the basal lamina and in the dermis. Paraformaldehyde fixation. Calibration bar = 0.5μ . $\times 51,200$.

FIGURE 11 Epidermal-dermal junction in recombinant of viable of epidermis and freeze-thawed (non-viable) dermis at 7 days of cultivation on CAM. Basal lamina is present although anchoring fibrils are absent. Paraformaldehyde fixation. Calibration bar = 0.5μ . $\times 33,700$.

basal lamina is primarily derived from the epidermis. The possibility that dermis makes a passive contribution to basal lamina formation is not excluded by this study. The finding that basal lamina formation is consistently initiated directly subjacent to hemidesmosomes further substantiates the intimate relationship of the epidermis to basal lamina formation. The conclusion reached in our study on adult human skin is in accord with previous studies which utilized neoplastic and embryonic tissues (7-16).

Anchoring fibrils form in recombinants of viable epidermis and viable dermis but not in recombinants containing nonviable dermis. Anchoring fibril formation, therefore, is dependent upon dermal viability. This observation supports the contention that anchoring fibrils are derived from the dermis. This is the first evidence, to our knowledge, regarding the origin of anchoring fibrils.

In order that these data can be interpreted as supporting the epidermal origin of basal lamina and the dermal origin of anchoring fibrils, it is essential that two conditions be met: (a) contamination of the new epidermal-dermal interface of the recombinant with residual basal lamina and anchoring fibrils from the previous epidermal-dermal interface must be excluded, and (b) freeze-thawed dermis must be nonviable and incapable of active synthesis.

Neither residual basal lamina nor anchoring fibrils were found in samples of isolated epidermis examined by electron microscopy. Separation induced by the "cold trypsinization" procedure used in this study occurred uniformly at the intermembranous space, leaving apparently undamaged basal lamina and anchoring fibrils attached to the dermis. If contamination of isolated epidermis with basal lamina or anchoring fibrils does occur in the separation procedure, it must be rare and does not account for the observations reported in this study. Contamination from the isolated dermis is excluded by inversion of the dermis so that a freshly cut dermal surface where basal lamina and anchoring fibrils are normally absent is presented to the new epidermal-dermal interface. The absence of basal lamina before the third day and anchoring fibrils before the fifth day of cultivation as well as the sequential formation of these structures provide further evidence for the new formation of basal lamina and anchor-

ing fibrils as opposed to residuals from epidermis or dermis before cultivation.

The observation that fibroblasts uniformly fail to grow out from the freeze-thawed dermis in tissue culture, whereas fibroblasts consistently grow out from viable dermis, provides evidence that the freeze-thawed dermis is nonviable. Further evidence of the nonviability of freeze-thawed dermis is the severe cellular degeneration noted on electron microscopy. It is inconceivable that such severe degeneration is compatible with cellular viability or active cellular synthetic activity.

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