Initiation of skin basement membrane formation at the epidermo-dermal interface involves assembly of laminins through binding to cell membrane receptors

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SUMMARY

To study the mechanism of basement membrane formation, we determined by immunochemistry temporal and spatial expression of laminin-5 (Ln-5), laminin-1 (Ln-1) and their integrin receptors during early skin morphogenesis. A 3-dimensional skin culture was used that allows the study of the sequential molecular events of basement membrane formation at the epidermodermal interface. During early anchorage of keratinocytes to the extracellular matrix there is expression of Ln-5, BP-230 antigen and $\alpha 3$, $\beta 1$ integrin subunits. During epidermal stratification and prior to the formation of the lamina densa there is assembly of Ln-5, Ln-1, collagen IV and nidogen accompanied by keratinocyte basal clustering of $\alpha 2$, $\alpha 3$, $\alpha 6$, $\beta 1$, and $\beta 4$ integrin subunits. The assembly pattern of Ln-1 and Ln-5 can be disturbed with functional antibodies against the $\beta 1$ (AIIB2) and $\alpha 6$ (GoH3) integrin subunits. Ln-1 assembly can also be disturbed with antibodies against its E8 domain and by competitive inhibition with a synthetic peptide (AG-73) derived from its G-4 domain. Quantitative RT-PCR showed that the dermis contributes about 80% of the laminin $\gamma 1$ chain mRNA while 20% is produced by the epidermis which emphasizes its dual tissue origin and the major contribution of the mesenchyma in laminin production. The laminin $\gamma 2$ chain mRNA, present in Ln-5, was mostly of epidermal origin. This study presents evidence that during the initiation of basement membrane formation, laminins bind to keratinocyte plasma membrane receptors and thus may serve as nucleation sites for further polymerization of these compounds by a self-assembly process.

Key words: Basement membrane, Laminin, Integrin

INTRODUCTION

The basement membrane (BM) is a ubiquitous structure that establishes the boundary between epithelia and their surrounding mesenchyma and plays a significant role in morphogenetic events. The major components of the BM are laminins, collagen IV, entactin/nidogen and perlecan. Collagen IV and laminins form homotypic polymers which are stabilized by binding interactions with nidogen and heparan sulfate proteoglycans (Mayer et al., 1993; Battaglia et al., 1992). Laminins are proteins involved in important biological functions, including cellular anchorage, polarization, division and differentiation. Laminins are cross- or T-shaped hetero-trimeric proteins consisting of three chains designated α, β, and γ, which are assembled in an α-helical coiled-coil structure. Laminin (laminin-1) was first isolated from the mouse

Engelbreth-Holm-Swarm (EHS) tumor (Timpl et al., 1979) and revealed an $\alpha 1$, $\beta 1$, $\gamma 1$ chain composition (Beck et al., 1990). Although antibodies prepared from EHS tumors recognize laminin-1 they may also cross-react with other laminins. In this regard 11 different laminin isoforms have been discovered all of which show sequence homology to EHS laminin or laminin-1 (Timpl, 1996). The N-terminal short arms of laminin-1 participate in network formation (Yurchenco et al., 1992) while the carboxyl-terminal domain of the α chains (G-domain) and the center of the cross (P-1 domain) bind to certain integrins and probably play a role in transmitting signals from the extracellular matrix. It has been suggested that laminin-1 and collagen IV are incorporated into the basement membrane by a self-assembly process (Yurchenco and Furthmayr, 1984; Yurchenco et al., 1992). This hypothesis proposes that laminin-1 and collagen IV can self-assemble through a mass action-

driven process whereby protomers are secreted into a diffusion-limited space. Thus, laminin-1 solubilized into a buffer at physiological pH and ionic strength, at a critical concentration of 0.1 mM, in the presence of divalent cations, will polymerize into a macromolecular structure that closely resembles the 'in vivo' situation (Yurchenco et al., 1992). In a recent study using a 3-dimensional skin culture we showed during early BM formation that, prior to the development of the lamina densa, there is scaffold formation of α1 (IV) and α2 (IV) collagen chains at the epidermo-dermal interface through binding to β1 integrins (Fleischmajer et al., 1997). This scaffold can be disrupted with anti-β1 integrin antibodies and by competitive inhibition with CB-3 (IV) collagen derived peptide. Thus, the above study suggested that cell membrane receptors may play a role in BM assembly and raised the question as to whether similar mechanisms may apply to laminins.

The best characterized laminins in skin are laminin-5, -6, and -7, (Burgeson, 1996) although there is considerable evidence that laminin-1 is also present. Laminin-5, with the chain composition α3β3γ2, is a product of keratinocytes and has been localized in the hemidesmosome and anchoring fibrils (Rousselle et al., 1991). Two other isoforms, laminin-6 (α3β1γ1) restricted to anchoring filaments and laminin-7 (α3β2γ1) present in fetal skin are covalently associated with laminin-5 (Marinkovich et al., 1992b; Champliaud et al., 1996). Laminin-1 was demonstrated in human skin by immunochemistry but has not as yet been isolated by immunoprecipitation. Adult human skin expresses the α1, β1, and γ1 chains, thus suggesting the presence of laminin-1 (Sollberg et al., 1992).

Integrins are heterodimeric transmembrane glycoprotein receptors that play a role in cell-cell and cell-matrix interactions. The major integrins in human skin are α2β1, α3β1 and α6β4 (Hertle et al., 1991). α2β1 is a receptor for collagen IV (Vanderberg et al., 1991; Kern et al., 1993) although it also binds to laminins and probably to fibronectin (Kirchhofer et al., 1990). α3β1 is a rather promiscuous receptor and can bind to collagen IV, laminins, and fibronectin (Wayner and Carter, 1987; Gehlsen et al., 1989). More recently it has been shown that α3β1 is a major receptor for laminin-5 (Carter et al., 1991; Rousselle and Aumailley, 1994). α6β4 integrin binds to both laminin-5 and laminin-1 although it has more affinity for the former (Niessen et al., 1994). α1β1 is minimally expressed only during early skin embryogenesis while α5β1 and αvβ5 are expressed at later stages (Hertle et al., 1991).

In order to understand the origins of the basement membrane and the mechanism of its assembly, we have studied temporal and spatial expression of BM components and cell surface receptors in a 3-dimensional 'in vitro' skin model. In this model human skin fibroblasts seeded in a 3-dimensional mesh, produce a rich extracellular matrix (Naughton et al., 1989). When the above fibroblast culture system is recombined with human keratinocytes, an epidermis develops that closely resembles the 'in vivo' situation including the formation of tonofilaments, desmosomes, hemidesmosomes, anchoring filaments, a lamina densa and anchoring fibrils (Slivka et al., 1993; Contard et al., 1993). Various markers of keratinocyte differentiation are expressed including keratin-10, filaggrin and trichohyalin (Fleischmajer et al., 1997). In addition, temporal and spatial expression of epidermal integrin subunits in this

culture system are very similar to that described in embryonic human skin (Fleischmajer et al., 1993; Hertle et al., 1991). The main advantage of this culture system is that since the development of the lamina densa is predictable and occurs between days 21 through 28, earlier cultures (7 day through 14 day) can be used to study the initial events in BM formation.

Our results suggest that the initiation of BM formation involves cell surface assembly or scaffold formation of laminin-1 and laminin-5, accompanied by keratinocyte basal clustering of β1 and β4 integrins. This mechanism can be disturbed by functional antibodies and by competitive peptide inhibition, thus suggesting that cell surface assembly of laminin-1 and laminin-5 may serve as nucleation sites for further polymerization of these compounds by a self-assembly process.

MATERIALS AND METHODS

Culture systems

Human keratinocytes and fibroblasts were obtained from preputial circumsized skin. Keratinocyte monolayers were grown in keratinocyte serum free medium (Gibco, Long Island, NY) while fibroblast monolayers were grown in DMEM supplemented with 10% fetal calf serum and non-essential amino acids. Skin equivalents (Advanced Tissue Science, La Jolla, CA) were prepared by growing fibroblasts in a 3-dimensional nylon mesh for 4 weeks. Keratinocytes were then seeded (keratinocyte-dermal model or K-D-M) and the culture submerged in a medium consisting of DMEM supplemented with 5% fetal calf serum, 100 μg/ml of ascorbic acid, 0.5 μg/ml hydrocortisone and a cholesterol rich lipid supplement (Sigma, St Louis) (Naughton et al., 1989; Slivka et al., 1993). After 7 days the K-D-M cultures were raised to an air liquid interface (Asselineau et al., 1989) for periods of 7, 8, 10, 14 and 28 days. This results in a stratified epidermis and a dermis containing numerous fibroblasts surrounded by a rich extracellular matrix (Contard et al., 1993).

Source of antibodies

Antibodies were generous gifts or purchased commercially as indicated: AIIB2 mAb against the β1 integrin subunit (Werb et al., 1989) (C. Damsky, University of California, San Francisco); rabbit polyclonal anti-β4 integrin subunit (F. G. Giancotti, New York University, New York); anti-α1 integrin subunit polyclonal (AB, 1934), anti-α2 integrin subunit (mAb P1E6) and anti-α3 integrin subunit (mAb P1B5) (Chemicon, San Diego, CA); GoH3 mAb against the α6 integrin subunit (A. Sonnenberg, Netherlands Cancer Institute, Amsterdam); BIIG2 mAb against the α5 integrin subunit (C. Damsky); affinity purified rabbit polyclonal anti-collagen IV (Davis et al., 1990) (H. Kleinmann, National Institutes of Health, Bethesda); mAb against α1 (IV) and α2 (IV) were prepared from synthetic peptides (Ninomiya et al., 1995); affinity purified rabbit polyclonal antibodies against laminin-1 and its P I fragment (Mann et al., 1988) (R. Timpl, Max Planck Institute fur Biochemie, Munich); affinity purified rabbit polyclonal antibodies against laminin-1 (Sigma Chemical Co., St Louis, MO). Although antibodies to laminin-1 recognize α1, β1, and γ1 chains they may cross-react with other laminins (Paulsson, 1994). Rabbit polyclonal antibodies against fibronectin (Rhode et al., 1979); mAb against the G domain of the laminin α3 chain (BM-165) (Rouselle et al., 1991) and a rabbit polyclonal antibody against laminin-5 (Marinkovich et al., 1992a) (R. E. Burgeson, Harvard University, Boston). The BM-165 mAb also recognizes laminin-6 which covalently associates with laminin-5 (Marinkovich et al., 1992b). Affinity purified and cross-absorbed rabbit polyclonal antibody against fragment E8 of laminin-1 (Sung et al., 1993) (P. Yurchenko, Rutgers University, NJ); rabbit polyclonal antibody against BP 230 (#1142) (Tanaka et al., 1990) (J. R. Stanley,

University Pennsylvania, Philadelphia); rabbit polyclonal antibody against tenascin (Lightner et al., 1989) (H. Erickson, Duke University Medical Center, Durham, NC).

Electron microscopy

Samples from the K-D-M cultures were fixed in Karnovsky's solution for 4 hours at room temperature, post fixed for 1 hour in ferrocyanide osmium tetroxide and stained 'en bloc' for 1 hour in 1% phosphotungstic acid followed by 1 hour in 3% uranyl acetate (Hulmes et al., 1981). Semi-thin and ultra-thin sections were obtained in a Sorval MT-2b ultramicrotome and examined in a Joel EM 100 electron microscope.

Immunofluorescence microscopy

K-D-M specimens were cut into 3-4 mm squares, frozen in Tissue Tech O.C.T. embedding compound (Miles Labs, Elkhart, IN), and processed for indirect immunofluorescence microscopy as previously described (Fleischmajer et al., 1993). Double immunofluorescence microscopy was carried out using rhodamine and fluorescein dyes. Specimens were examined with a microscope equipped with epifluorescence illumination, or by laser confocal scanning microscopy. Antibodies were used in concentrations of 0.2 to 0.5 mg/ml. Controls consisted of pure mouse IgG or rabbit or mouse serums from non-immunized animals.

Northern blotting

Total RNAs were isolated from fibroblasts and keratinocyte monolayers and from 24 meshes of 14-day K-D-M cultures. The K-D-M cultures were treated with thermolysin to separate the epidermis from the dermis. Thermolysin (0.2 mg/ml) in PBS containg calcium and magnesium was placed (4 ml per well) into a sterile 6-well plate, and placed on a rotary shaker (150 rpm) at room temperature. After 60 minutes the epidermis was removed from the dermis and total RNAs isolated using acid guanidine thiocyanate/phenol/chloroform (Chomczynski and Sacchi, 1987). RNA samples were electrophoresed on a 1% agarose gel containing 6% formaldehyde, transferred to hybond N membrane (Amersham, Chicago, IL), and hybridized to the laminin γ1 chain cDNA probe (see above) labeled with [^{32}P]dCTP by nick-translation.

Quantitative reverse transcription-PCR method

Total RNA was isolated from 14-day K-D-M as previously described (see above). cDNA was synthesized from 5 μg of total RNA with oligo dT as a primer using a kit for RT-PCR (Life Technologies, Geittersburgh, MD). Quantitative RT-PCR for human laminin γ1 and γ2 chains was performed using the PCR MIMIC construction kit (Clontech, Palo Alto, CA). A 2 μl aliquot of the oligo dT-primed cDNA reaction which had been diluted 5-fold and known amounts of the mimic, were co-amplified by PCR in a 50 μl reaction mixture containing 0.7 mM of primers for either γ1 or γ2 chains. The primers used for amplifying a 780 bp segment of human laminin γ1 mRNA (residues 4,300-5,090) were AAGGCCCTCGCTGAAGAAGCT and CTAGGGCTTTTCAATGGACGG. The two composite primers for the laminin γ1 mimic DNA were AAGGCCCTCGCTGAAGAAGCT caagtttcgtgagctgattg and CTAGGGCTTTTCAATGGACGGatttgatt-ctggaccatggc in which the γ1 primer sequences are in upper case and the v-erbB sequences for the neutral DNA fragment are in lower case. The primers used for amplifying a 646 bp segment of human laminin γ2 mRNA were GTTTCAGATGCCAGTGACAAG and TCACTGTT-GCTCAAGAGCCCT. The two composite primers for the γ2 mimic DNA were GTTTCAGATGCCAGTGACAAGcgcccaagtgaaatct-cctccg and TCACTGTTGCTCAAGAGCCCTatttgattctggaccatggc in which the γ2 primer sequences are in upper case and the v-erbB sequences for the neutral DNA fragment are in lower case. After 25 cycles of reaction (94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 60 seconds), one tenth of reaction products were electrophoresed on a 1.5% agarose gel.

Preparation of synthetic peptide AG-73

We have previously identified several cell binding sites from the C-terminal globular G domain of the mouse laminin α1 chain by systematic screening of synthetic peptides. AG-73, one of these peptides, is from the G4 subdomain of the G domain and its location is within the proteolytic fragment E3, from the C terminus of the laminin α1 chain. The sequence of AG73 is highly conserved between mouse and human and has been shown to be active for cell adhesion with human fibrosarcoma cells, HT1080 (Nomizu et al., 1995). AG-73 peptide (RKRLQVQLSIRT) (residues 2,719-2,730) and scrambled peptide AG73T (LQQRRSVLRTKI) were manually synthesized by the 9-fluorenyl-methoxycarbonyl (Fmoc) based solid-phase strategy and prepared as the C-terminal amide form as described previously (Nomizu et al., 1995). They were purified by reverse phase high performance liquid chromatography (HPLC). The purity and identity of the synthetic peptides were confirmed by analytical reverse phase HPLC and amino acid analysis.

Functional antibody perturbation studies

To perform antibody perturbation studies, 7-day K-D-M cultures were kept for an additional week in a culture containing 50 μl/ml of either the AIIB2 or GoH3 mAbs from hybridoma serum in a 1:5 dilution. Controls consisted of mouse IgG (50 μg/ml). The medium containing the mAbs and mouse IgG was changed at days 10 and 12. At day 14 the specimens were frozen and subjected to immunofluorescence microscopy using rabbit polyclonal antibodies against laminin-1 or laminin-5. To perform blocking experiments with the rabbit polyclonal anti-E8 fragment antibodies, 50 μl of serum (concentration 0.5 mg/ml) was used as described above.

Competitive inhibition studies with AG-73 peptide

K-D-M cultures at stage 7 days were cultured in medium containing 50 μg/ml of AG-73 peptide and kept for an additional 7 days. The medium plus AG-73 peptide was changed every 3 days. A control scrambled peptide AG-73T (50 μg/ml) was used in a similar fashion as described above. By day 14 the specimens were frozen and used for indirect immunofluorescence microscopy with polyclonal antibodies against laminin-1.

RESULTS

Epidermal morphogenesis is accompanied by three distinct temporal-spatial patterns of β1 integrin expression

A time sequence study of the K-D-M model allows the identification of 3 distinct stages in epidermal morphogenesis (Fig. 1a,b,c). The first stage (7 day) shows the development of a non-stratified epidermis and its early anchorage to the ECM. At this stage, there is a single layer of keratinocytes and an upper layer of cornified cells. Ultrastructurally, keratinocytes are poorly differentiated except for the presence of tonofilaments (Fig. 1d; Table 1). At this stage keratinocytes show β1 integrins in a pericellular distribution (Fig. 1g). Fourteen day K-D-M represent a proliferative stage characterized by a stratified epithelium where keratinocytes are randomly arranged, poorly differentiated and show areas with basal villous projections (Fig. 1b,e). There is clustering of β1 integrins at the matrix site of basal keratinocytes with mild expression at the apico-lateral sites (Fig. 1h). The third stage (28d) revealed numerous features of epidermal differentiation including a well organized basal layer of distinct cylindrical cells, a malpighian, granular and a horny layer (Fig. 1c). There are numerous desmosomes and the BM zone reveals immature
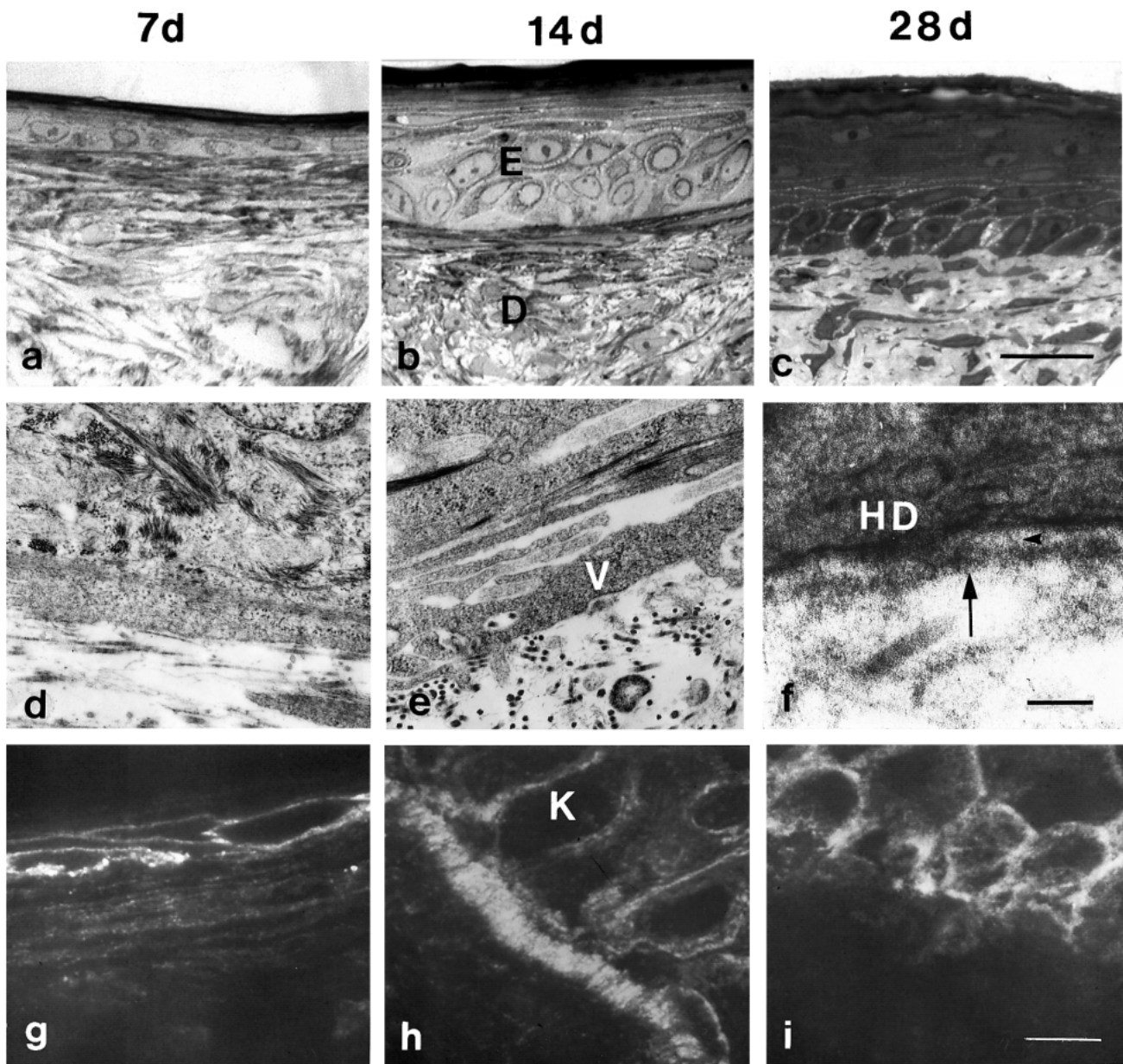
7d   14d   28d

Fig. 1. K-D-M cultures at different stages of development. (a) Non-stratified epidermis, (b) proliferative stage, and (c) differentiation stage. Semithin sections (1 μm thick) stained with Toluidine Blue. Bar, 20 μm. Electron microscopy of the epidermo-dermal interface (d,e,f) Bar, 100 nm. Distribution of β1 integrin subunit (AIIB2 mAb) (g,h,i). Bar, 10 μm. Note redistribution of β1 integrins from pericellular (g) to basal (h) to apico-lateral surfaces (i). E, epidermis; D, dermis; HD, hemidesmosome; K, keratinocyte; V, villous projections. Arrowhead, anchoring filaments; arrow, lamina densa.

hemidesmosomes, anchoring filaments, a lamina lucida, a lamina densa and anchoring fibrils (Fig. 1f; Table 1). This stage of differentiation is accompanied by a remarkable redistribution of β1 integrins to apico-lateral surfaces in basal keratinocytes while adjacent suprabasal keratinocytes show a pericellular pattern (Fig. 1i).

During early anchorage of keratinocytes to the ECM there is expression of laminin-5, BP-230 antigen and the α3, β1 integrin subunits

Non-stratified epidermis (7 day culture) revealed laminin-5 in a linear-interrupted pattern which co-localized with the BP-230

antigen (Fig. 2). This antigen was used as a marker for hemidesmosomes although these structures were not detected ultrastructurally (Table 1). Both laminin-5 and BP-230 were noted at the basal site of keratinocytes and represent early markers of cell polarity. The α3 integrin subunit was strongly expressed while the α1 (not shown), α2 and α6 subunits were absent (Fig. 2). The β4 integrin subunit was also absent (not shown). However, 8d K-D-M showed the α6 and β4 integrin subunits in a pericellular distribution (not shown) similar to that noted during early skin embryogenesis (Hertle et al., 1991). The above findings suggest that the earliest anchorage of epidermal cells to the ECM involve laminin-5, the BP-230

Table 1. Ultrastructure of keratinocyte-dermal-model cultures

Time period	Basal keratinocyte	Desmosomes	Anchoring filaments	Hemi-desmosomes	Lamina densa	Anchoring fibrils
7 day	Round or oval	Absent	Absent	Absent	Absent	Absent
14 day	Round or oval	Few	Absent	Immature	Absent	Absent
28 day	Cylindrical	Many	Present	Present	Present	Present

antigen and the α3, β1 integrin subunits. It has been shown that in skin the α3 integrin subunit dimerizes with the β1 subunit and is an excellent receptor for laminin-5 (Carter et al., 1991). Furthermore, the α3β1 integrin is expressed very early during skin embryogenesis (Hertle et al., 1991). Seven day K-D-M cultures also showed that the α1(IV) and α2(IV) collagen chains were expressed in basal keratinocytes while laminin-1 was absent (Fig. 2). The intracellular presence of collagen IV in basal keratinocytes was also demonstrated by superimposing phase contrast microscopy with immunochemistry (not shown). Nidogen and fibronectin were present throughout the dermis in a random distribution (not shown).

There is scaffold formation of laminin-5, laminin-1, nidogen and collagen IV and basal clustering of β1 and β4 integrins at the epidermo-dermal interface prior to the formation of the lamina densa

The most prominent feature of 14-day cultures is marked cellular proliferation resulting in a stratified epidermis. Immunochemistry of areas where basal keratinocytes show a flat matrix surface revealed assembly of laminin-1 and collagen IV just within or beneath laminin-5 (Fig. 3). Laminin-5 co-

localized with the BP-230 antigen which can be regarded as an intracellular hemidesmosomal marker (not shown). It is noteworthy, that anchoring filaments are not present despite strong expression of laminin-5. In other sections, laminin-1, collagen IV and nidogen stained as a broad band about 5 to 10 μm thick, which represents keratinocytes with numerous basal villous projections (Fig. 4). However, in these areas laminin-5 showed a linear-interrupted pattern, suggesting that this laminin is not expressed in the villous projections but only at their bases. In these areas β1 integrin subunits co-localize with laminin-1 (Fig. 5), and with collagen IV as previously described (Fleischmajer et al., 1997). Laminin-1, collagen IV and nidogen were also noted at lower levels of the dermis in a random pattern. Although laminin-1 was identified with antibodies derived from an EHS-tumor antigen and from the P-1 domain of laminin-1 it is conceivable that these antibodies not only recognize laminin-1 but may also cross-react with other laminins. However, the presence of laminin-1 was further supported by the expression of the laminin α1 chain mRNA in the epidermis and dermis of K-D-M cultures (R. Fleischmajer et al., unpublished data). At this stage there is clustering of β1 (Fig. 1h), α2, α3, β4, α6 integrin subunits mostly at the matrix
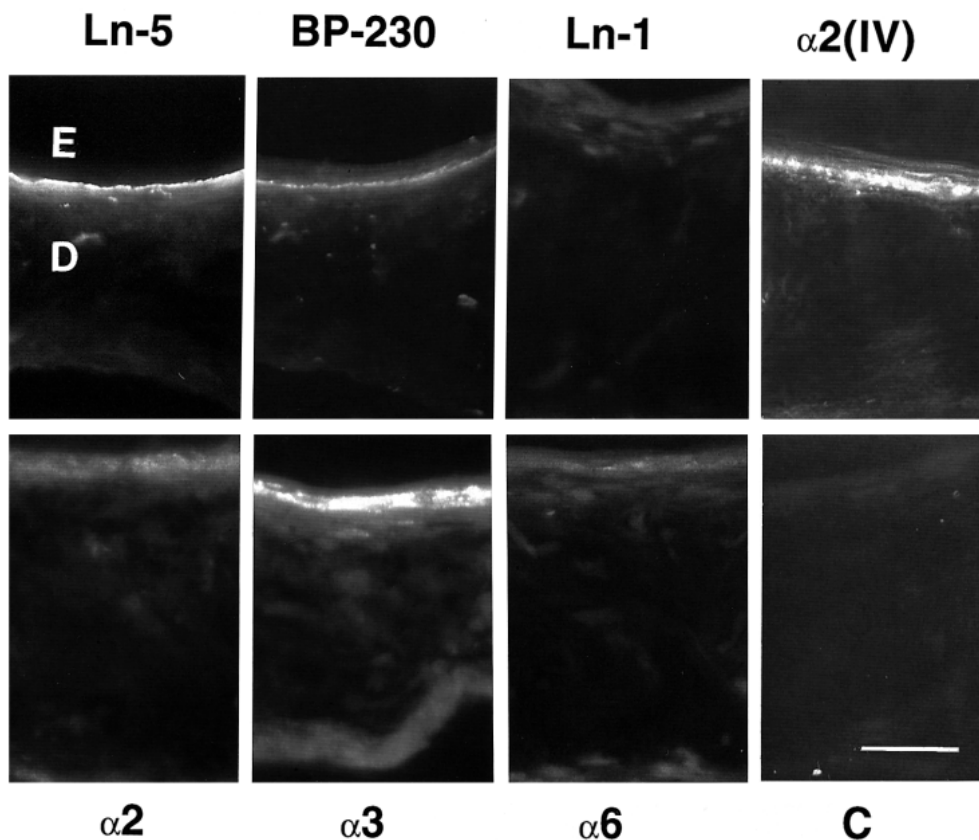

Fig. 2. Indirect immunofluorescence microscopy of the epidermo-dermal interface of 7 day K-D-M cultures. Note co-localization of laminin-5 (Ln-5) and BP-230; laminin-1 (Ln-1) is absent while the α2 (IV) collagen chain is mostly in the basal cell layer. Note strong expression of α3 integrin subunit while α2 and α6 subunits are almost absent. C, non-immunized mouse serum; E, epidermis; D, dermis. Bar, 2 μm.

Ln-1   Ln-5   Ln-1;Ln-5

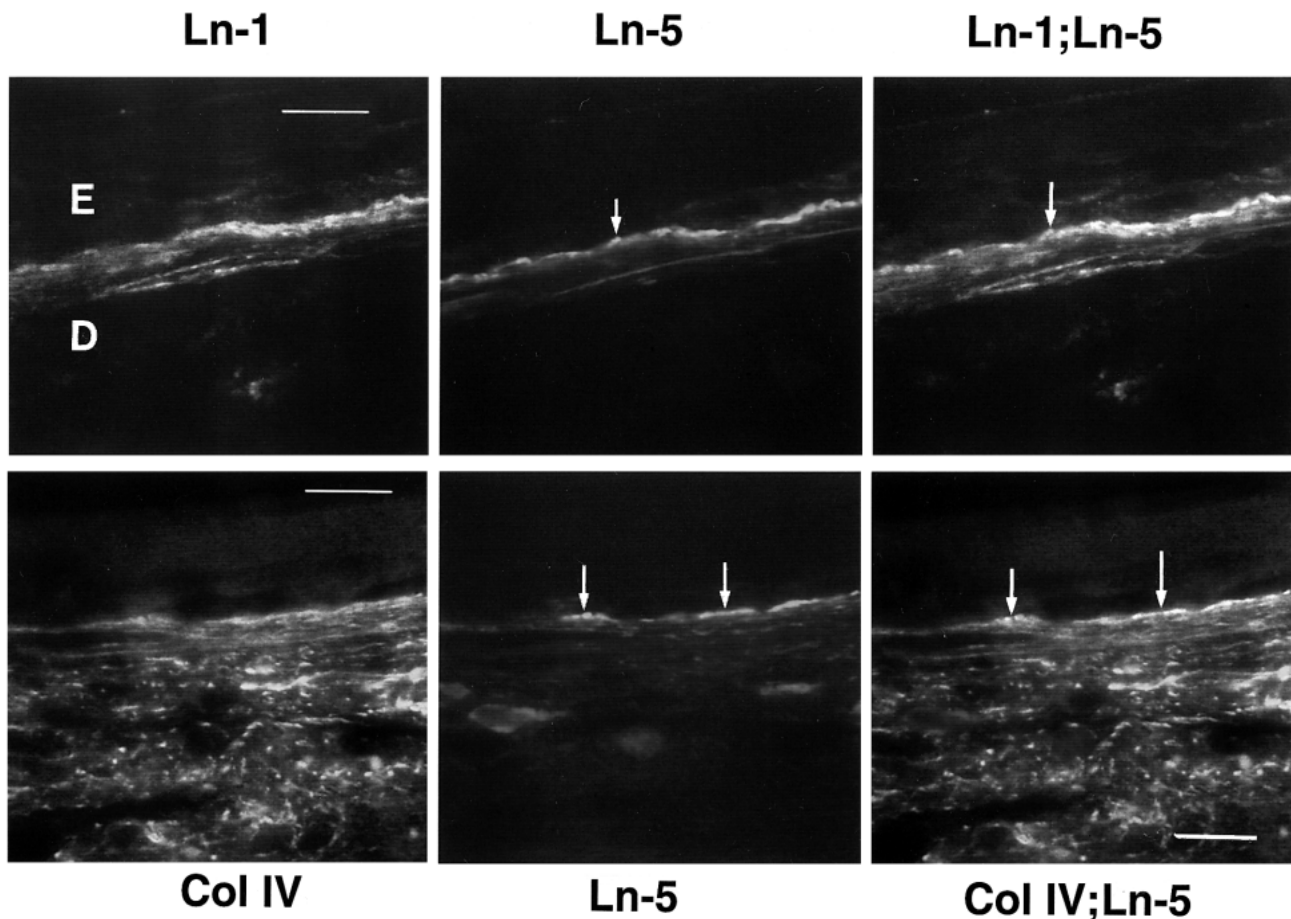## Col IV   Ln-5   Col IV;Ln-5

Fig. 3. Double immunostaining of 14-day K-D-M cultures with laminin-5 (Ln-5)-laminin-1 (Ln-1), and Ln-5-collagen IV (col IV) by laser scanning confocal microscopy. Note co-localization of Ln-1-Ln-5 and Col IV-Ln-5 (arrows) at the epidermo-dermal interface. E, epidermis; D, dermis. Bar, 10 μm.

site of basal keratinocytes. Fibronectin revealed a diffuse pattern of fluorescence throughout the dermis (Fig. 4). Although at this stage the α5 integrin subunit and the αvβ5 integrin were not expressed, fibronectin could conceivably bind to α2β1 and α3β1 integrins. Tenascin also showed a diffuse pattern of deposition throughout the dermis (not shown). The nidogen scaffold is most likely the result of its linkage to collagen IV and laminins (Mayer et al., 1993). However, there is some evidence that nidogen shows weak cell adhesion properties through recognition of the α3β1 integrin (Dedhar et al., 1992).

AIIB2 mAb (anti-β1), and GoH3 mAb (anti-α6) alter deposition patterns of laminin-1 and laminin-5 at the epidermo-dermal interface

We next tested the hypothesis that laminin-1 and laminin-5 form a scaffold at the epidermo-dermal junction presumably by binding to integrin receptors. Since laminin-1 has been shown to bind to the α1β1, α2β1 and α3β1 integrins, functional perturbation was performed with the AIIB2 mAb (anti-β1). In addition, since laminin-1 also binds to the α6β4 integrin (Niessen et al., 1994) blocking experiments were also performed with the GoH3 mAb (anti-α6). As shown in Fig. 6, both antibodies disturbed the deposition of laminin-1 at the epidermo-dermal interface. Similar functional perturbation

was also noted with an antibody against the E8 domain of laminin-1which interacts mostly with domain I and the G1, G2, G3 domains of the laminin α1 chain (Sung et al., 1993) (Fig. 6). Since laminin-5 binds to α3β1 and α6β4 integrins, similar blocking experiments were carried out with the AII2B2 and GoH3 mAb. Although both antibodies disturbed the fluorescence pattern of laminin-5 the most striking effect was noted with the GoH3 mAb (Fig. 7).

Interestingly enough the GoH3 mAb did not disturb the assembly of collagen IV which does not bind to the α6β4 integrin (not shown). The question could be raised as to whether the above blocking experiments primarily affected keratinocyte adhesion to the ECM and thus, the interference in BM assembly regarded as a secondary event. However, histology by semithin-sections and electron microscopy at no time revealed detachment of the epidermis from the adjacent dermis. The above data suggest that basal clustering of β1 and β4 integrins accompanied by cell surface assembly of laminin-5 and laminin-1 may be operative at the epidermo-dermal interface prior to the development of the lamina densa.

Competitive inhibition by peptide AG-73 on laminin-1 deposition at the BM zone

The AG-73 peptide derived from the G-4 domain of the carboxyl-terminal domain of the laminin α1 chain revealed
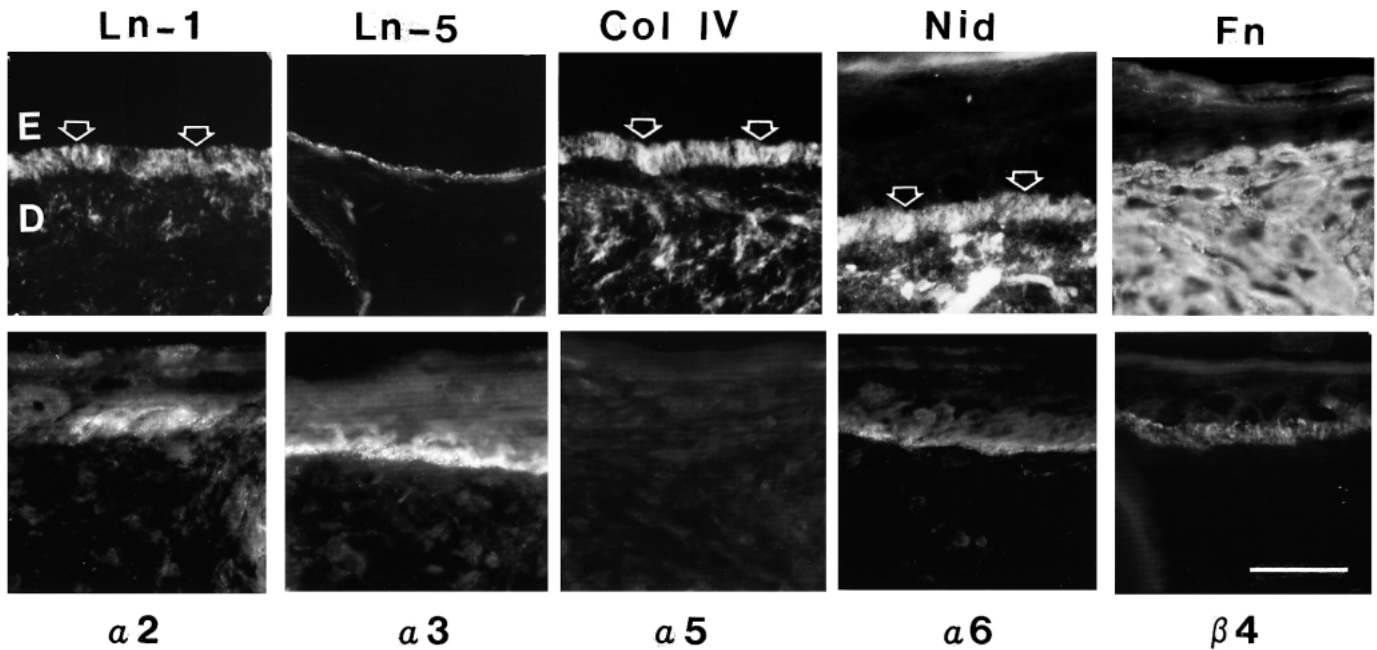
Fig. 4. Indirect immunofluorescence microscopy of the epidermo-dermal interface of 14-day K-D-M cultures. Note assembly (arrows) of laminin-1 (Ln-1), collagen IV and nidogen (Nid). Laminin-5 (Ln-5) shows a linear-segmented pattern. Fibronectin (FN) is diffusely expressed throughout the dermis. Note expression of α2, α3, α6 and β4 integrin subunits, mostly at the basal site. The α5 integrin subunit is absent. E, epidermis; D, dermis. Bar, 20 μm.

cell attachment and spreading properties with human rhabdomycosarcoma cells (Nomizu, 1995). AG-73 also promotes malignant behavior of melanoma cells 'in vitro' and tumor growth 'in vivo' (Song et al., 1997; W. H. Kim et al., unpublished data). However, cell adhesion and spreading on peptide AG-73 was not disturbed with antibodies against the β1, α2 or α6 integrin subunits suggesting that this peptide may bind to a different integrin or to a non-integrin receptor (Nomizu et al., 1995). We performed a competitive inhibition study with peptide AG-73 during the transition period between 7-day and 14-day K-D-M cultures and assessed for possible perturbation of laminin-1 deposition at the epidermo-dermal interface. The results in Fig. 8 showed that peptide AG-73 alters the assembly pattern of laminin-1 at the epidermo-dermal interface and, thus, suggests that its G-4 domain binds to a cell surface receptor. The data further suggest that multiple

interactions with cells at different sites on laminin occur at this stage.

Epidermal versus dermal mRNA expression of laminin γ1 and γ2 chains during the formation of a stratified epidermis

Since the laminin γ1 chain is a ubiquitous component of all laminins except for laminin-5, estimation of its mRNA expression will disclose information about the relative origin of laminins in the epidermis versus dermis during early skin morphogenesis. Northern blots showed two transcripts for the laminin γ1 chain mRNA at 8.2 and 5.6 kb and this is consistent with previous data (Mattei et al., 1988). As shown in Fig. 9, most of the γ1 mRNA chain was expressed in the dermis. Both fibroblasts and keratinocytes derived from monolayer cultures also expressed the laminin γ1 chain mRNA although the yield
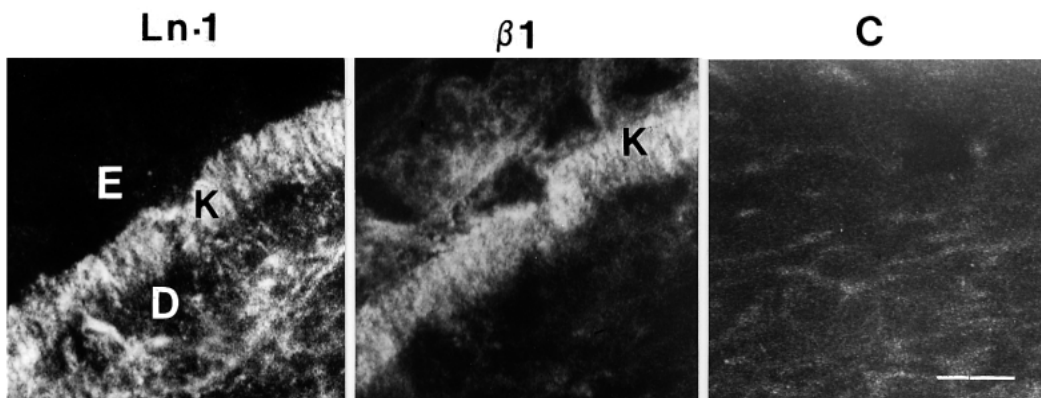

Fig. 5. Double immunostaining of 14-day K-D-M cultures with laminin-1 (Ln-1) and β1 (AIIB2) mAb integrin subunit. Note co-localization of Ln-1 and β1 integrins in villous projections at the basal site of keratinocytes. Also note marked expression of Ln-1 in the dermis. E, epidermis; D, dermis; K, keratinocyte; C, control consisting of non-immunized mouse serum. Bar, 10 μm.
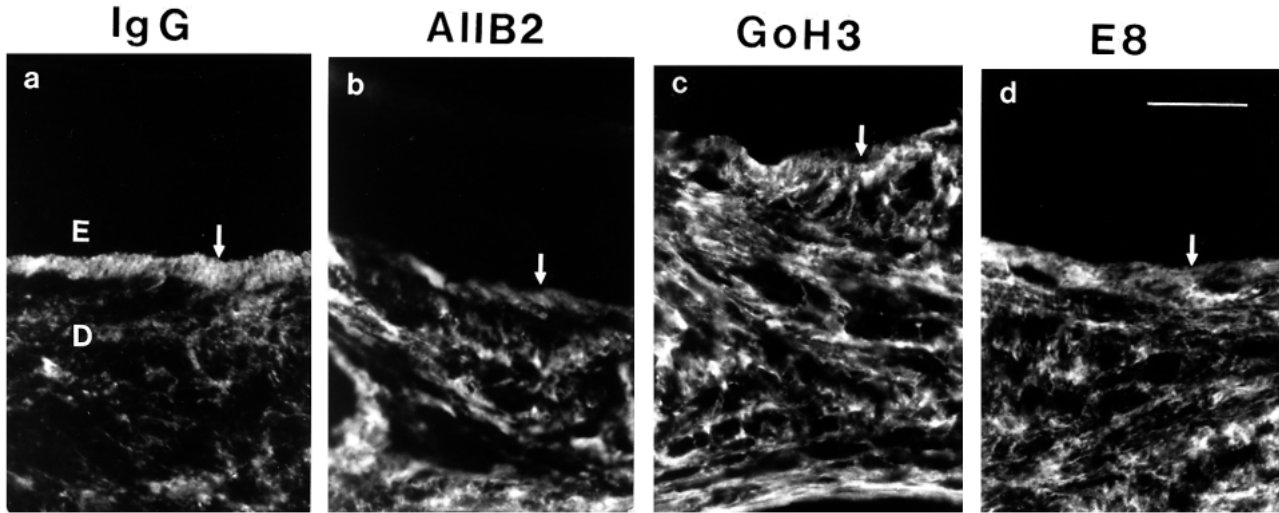
Fig. 6. Functional antibody perturbation studies of laminin-1 assembly at the epidermo-dermal interface of K-D-M cultures with (a) mouse IgG; (b) AIIB2 mAb (anti-β1 integrin subunit); (c) G0H3 mAb (anti-α6 integrin subunit); and (d) rabbit polyclonal antibody against the E-8 domain of laminin-1. Arrow, perturbation of laminin-1 assembly. E, epidermis; D, dermis. Bar, 20 μm.

was more intense in the former. Since the above results were unexpected we also performed quantitative RT-PCR for both laminin γ1 and γ2 chains. This study corroborated the northern blots. As shown in Figs 10 and 11, about 80% of the laminin γ1 mRNA derived from the dermis and about 20% from the epidermis. These data correlate well with the protein expression of laminin-1 (Figs 5, 6). On the other hand, the laminin γ2 mRNA, as expected, derived exclusively from the epidermis. Keratinocytes in culture expressed the laminin γ1

and γ2 chain mRNA while fibroblasts expressed only the γ1 chain (Fig. 11). In summary, this study shows that both keratinocytes and fibroblasts are contributors of the mRNA for the laminin γ1 chain during the initiation of BM formation, although most of it derives from fibroblasts. The laminin γ2 chain mRNA appears to be an exclusive product of keratinocytes as previously shown in other models (Burgeson, 1996).

DISCUSSION

In this study we used a 3-dimensional skin culture system to determine spatial and temporal expression of BM components and integrin receptors during early skin morphogenesis. Our hypothesis suggests that during the development of the BM, prior to the formation of the lamina densa, there is cell surface assembly of collagen IV, laminin-5, laminin-1 and probably other laminins at the matrix site of basal keratinocytes through binding to integrin and probably non-integrin cell membrane receptors. This assembly may act as a nucleation site for further polymerization of the above BM components, most likely by a self-assembly process (Yurchenco and Furthmayr, 1984; Yurchenco et al., 1992). Self-assembly has been proposed as a mechanism for the spontaneous formation of supramolecular structures such as collagen fibrils, ribosomes, actin filaments, etc. (Engel, 1994). Although components of the BM such as collagen IV, laminins and proteoglycans have the potential for self-assembly, the role of cells and their surface receptors cannot be ruled out (Kalb and Engel, 1991). Previous studies suggested that the self-assembly of collagen IV and laminin-1 takes place in diffusion-limited spaces which allow for a critical concentration of secreted protomers and thus promote their polymerization (Yurchenco and Ruben, 1987; Yurchenco et al., 1992). Although a diffusion-limited chamber may be feasible between adjacent cells, such as is seen with smooth muscle cells or between epithelial and endothelial cells in
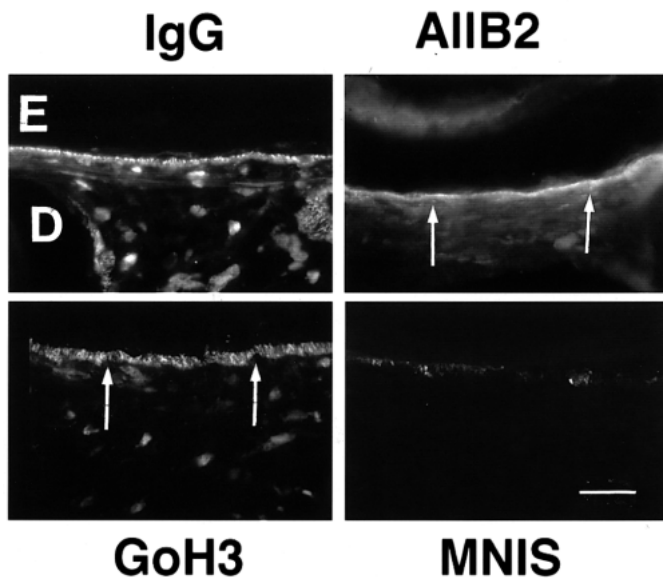

Fig. 7. Functional perturbation of laminin-5 assembly at the epidermo-dermal interface with AIIB2 mAb and GoH3 mAb. IgG (mouse origin) shows the linear-segmented pattern of laminin-5 that co-distributes with BP-230 (not shown). Note perturbation of laminin-5 assembly by AIIB2 and GoH3 (arrows). MNIS, mouse non-immunized serum. E, epidermis; D, dermis. Bar, 10 μm.
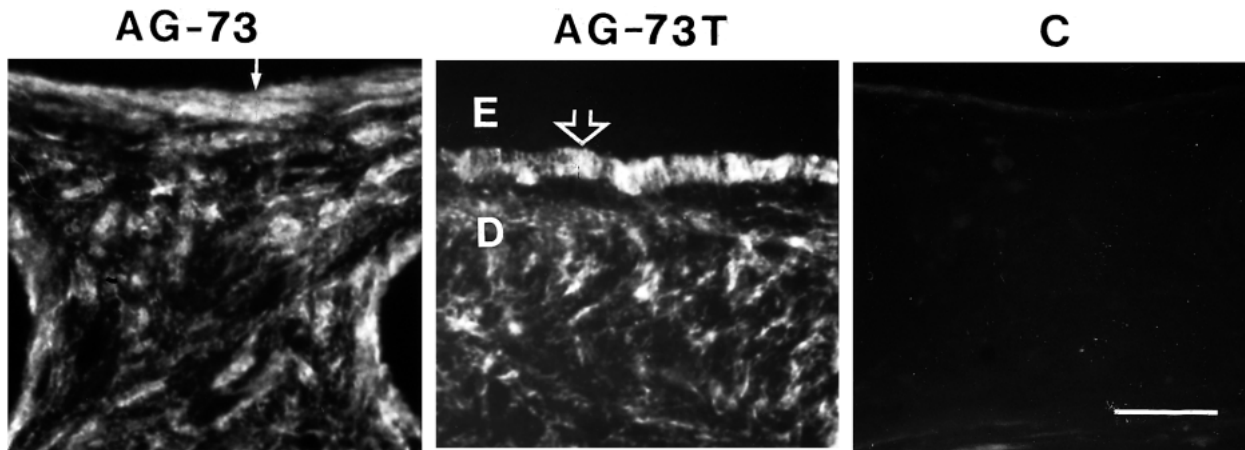
Fig. 8. Functional perturbation of laminin-1 assembly at the epidermo-dermal interface with synthetic peptide AG-73 derived from the G-4 domain of laminin-1. AG-73T, scrambled peptide of Ag-73; large arrow, assembly of laminin-1; small arrow, perturbation of laminin-1 assembly; C, rabbit non-immunized serum. E, epidermis; D, dermis. Bar, 20 μm.

kidney glomeruli, this is not the case in the epidermis where basal keratinocytes are in contact with the ECM. Thus, it stands to reason that the initiation of BM formation in the skin will be facilitated by cell surface assembly of BM components through binding to cell surface receptors. In this regard, keratinocytes express integrin receptors for collagen IV, laminin-1 and laminin-5 (see above). To test the above hypothesis, we used a skin reconstruction culture system which allowed us to examine the expression of integrins and their ligands during early stages of skin morphogenesis, prior to the development of the lamina densa and anchoring filaments. Seven day K-D-M cultures represent an anchorage stage whereby keratinocytes attach to the ECM and form a non-stratified epidermis. At this stage laminin-5 and BP-230 antigen co-localize at the basal site of keratinocytes in a linear segmented fashion which suggests early hemidesmosome assembly. The assembly of hemidesmosomes preceeds the development of the lamina densa as was also shown in wound healing (Kurpakus et al., 1990). At this stage keratinocytes express β1 and α3 integrin subunits in a pericellular pattern (Fig. 1). Since the α3β1 integrin is a receptor for laminin-5 (Carter et al., 1991), these findings suggest that the early anchorage of keratinocytes to the ECM involve binding of laminin-5 to α3β1 integrin. In this regard, the α3β1 integrin has been shown to mediate adhesion accompanied by immobilization of migrating keratinocytes (Kim et al., 1992). Furthermore, it is noteworthy that α3β1 defficient mice revealed a disorganized BM in skin (DiPersio et al., 1997) and in kidney glomeruli (Kreidberg et al., 1996). The expression of laminin-5 by keratinocytes and the absence of laminin-1 and collagen-IV in the dermis is similar to that described during keratinocyte migration in early wound healing (Larvaja et al., 1993). This early co-localization of laminin-5 and BP-230 is interesting since it has been suggested that laminin-5 plays a role in hemidesmosome assembly (Langhofer et al., 1993).

Fourteen day cultures showed a stratified epidermis in a rather poorly differentiated state. At this stage there is scaffolding of laminin-5 and laminin-1, at the epidermo-dermal interface, and this is accompanied by basal clustering of the α2, α3, α6, β1, and β4 integrin subunits. In a previous study we showed a similar assembly of collagen IV that could be disturbed by blocking β1 integrins with the AIIB2 mAb or by competitive inhibition with a cyanogen bromide digestion derived peptide CB3 (IV) which carries the ligand for the α2β1 and α1β1 integrins (Vanderberg et al., 1991; Fleischmajer et al., 1997). In the current study, similar functional perturbation studies with AIIB2 and GoH3 mAb, altered the cell surface assembly of laminin-1 and laminin-5 at the epidermo-dermal interface. In addition, competitive inhibition studies with the AG-73 peptide suggest that laminin-1, through its G-4 domain may also bind to a yet to be identified cell surface receptor. The AG-73 peptide has been shown to disrupt acinar formation of human submandibular gland cells by binding to syndecan-1
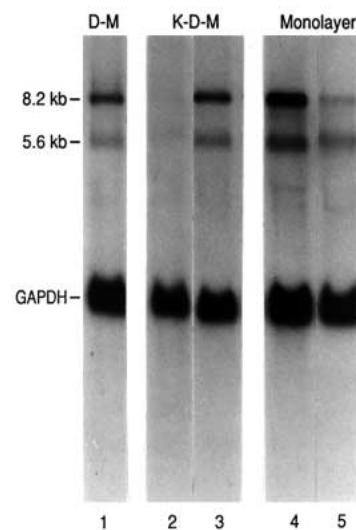

Fig. 9. Northern blot analysis of the laminin γ1 chain mRNA extracted from 14-day K-D-M cultures. Lane 1: D-M or dermal model containing only fibroblasts. Lane 2: epidermis of K-D-M. Lane 3: dermis of K-D-M. Lane 4: fibroblast monolayer. Lane 5: keratinocyte monolayer. Note that most laminin γ1 chain derives from the dermis or from fibroblast monolayers. GAPDH, glyceraldehyde-3 phosphate dehydrogenase.
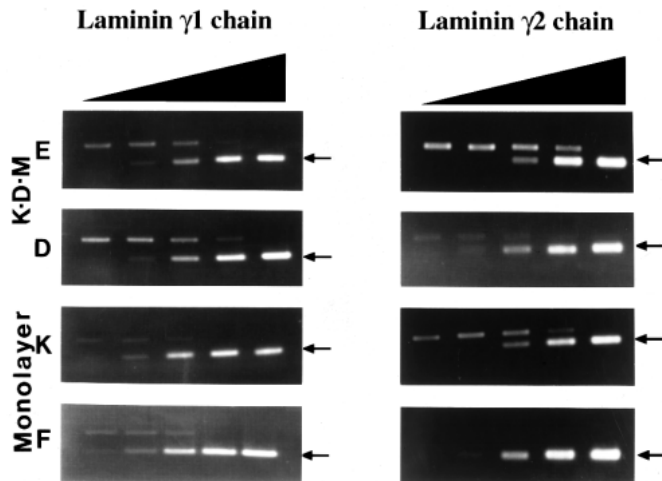
Laminin γ1 chain   **Laminin γ2 chain**

Fig. 10. Quantitative RT-PCR of human laminin γ1 and γ2 chain mRNAs in total RNAs extracted from epidermis and dermis of 14-day K-D-M cultures and from human fibroblast and keratinocyte monolayer cultures. A 2 μl aliquot of dt-primed cDNA (diluted 5-fold) and known amounts of the mimic (arrows) were amplified by PCR in a 50 μl reaction mixture containing 0.7 mM of primers of either γ1 or γ2 chains. E, epidermis; D, dermis; K, keratinocytes; F, fibroblasts.

Fig. 11. Quantitative RT-PCR of human laminin γ1 chain mRNA (a) and laminin γ2 chain mRNA (b) in 14-day K-D-M cultures and in keratinocyte and fibroblast monolayers, expressed as atto moles per 5 μg of RNA. Note that the laminin γ1 chain mRNA is mostly expressed in the dermis (80%) while 20% is seen in the epidermis. Small amounts are expressed equally in keratinocyte and fibroblast monolayer cultures (a). The laminin γ2 chain is mostly expressed in the epidermis and in keratinocyte monolayer cultures (b).

(M. P. Hoffman et al., unpublished). This suggests that the interference of laminin-1 assembly by the AG-73 peptide in our study could be due to its interaction with syndecan-1.

Previous studies with embryonic human skin showed β1 integrins in a pericellular distribution while in adult skin they are restricted to apico-lateral sites, thus suggesting that they are only involved in cell-cell interactions (DeLuca et al., 1990; Ryynanen et al., 1991). In our 'in vitro' skin model we could delineate 3 patterns of β1 integrin distribution which correlate well with 3 distinct stages in epidermal morphogenesis. The initial stage (7 day culture) represents the development of a non-stratified epidermis; its anchorage to the ECM and β1 integrins show a pericellular distribution. The second stage, characterized by the formation of a stratified epidermis, shows basal clustering of β1 integrins. Since at this stage there is no lamina densa, ligands like laminins and collagen IV, derived mostly from the dermis, could easily reach and bind to keratinocyte cell surface receptors. The third stage, characterized by numerous features of epidermal differentiation, shows a re-distribution of β1 integrins to apico-lateral surfaces, while α6, β4 integrin subunits are restricted to the basal site (Kurpakus et al., 1991). The clustering of β1 integrins at the basal site of keratinocytes is most likely promoted by ligand binding and correlates well with a stage of cell proliferation as has been described in other experimental models (Varner and Cheresh, 1996). Furthermore, it has also been shown that anchorage of keratinocytes to the ECM is a prerequisite for activation of cell division (Adams and Watt, 1990, 1993). There is evidence that ligation of integrins to ECM proteins regulates the mitotic cell cycle by inducing a cascade of intracellular signals including Na^{2+}/H^+ antiporter, tyrosine phosphorylation of focal adhesion kinase, increases in intracellular Ca^{2+} levels, inositol lipid synthesis, synthesis of cyclins and activation of early growth response genes (Varner and Cheresh, 1996). It has also been shown that cell anchorage
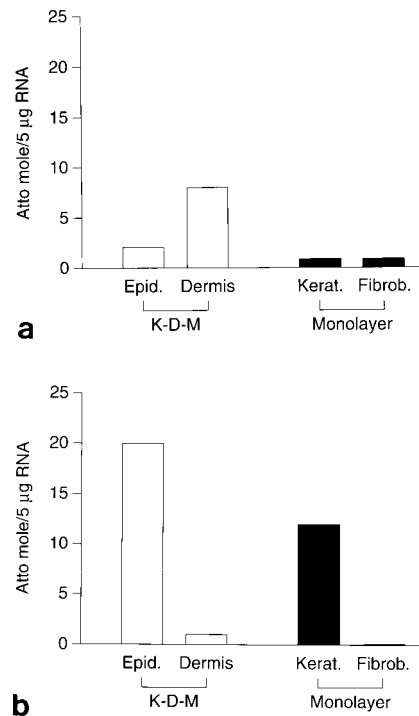
is necessary for growth factors to stimulate cell division (Guadagno et al., 1991). In this regard, it is noteworthy that 14-day K-D-M cultures expressed bFGF in the upper dermis (R. Fleischmajer and J. S. Perlish, unpublished observation) which is known to stimulate keratinocyte proliferation.

Although early studies suggested that laminins are of epithelial origin, there is current evidence that both epithelial and mesenchymal cells can synthesize laminins (Simo et al., 1992; Thomas and Dziadek, 1993; Marinkovich et al., 1993; Fleischmajer et al., 1993). Our 'in vitro' skin model showed that the laminin γ2 chain mRNA is expressed predominantly by keratinocytes, thus confirming other studies on the epidermal origin of laminin-5 (Rousselle et al., 1991). On the other hand, most of the mRNA for the laminin γ1 chain, about 80%, was of mesenchymal origin while only 20% derived from the epidermis. These findings correlate well with *Drosophila* embryogenesis where most of the laminin γ1 chain mRNA derives from the mesoderm while small amounts are expressed in the ectoderm (Montell and Goodman, 1989). Also, during intestinal embryogenesis the laminin γ1 chain appears to derive only from the mesenchyma (Senior et al., 1988). The prominent role of the mesenchyma during BM formation is further emphasized by other studies with the K-D-M culture, showing that nidogen mRNA is almost an exclusive product of fibroblasts (Fleischmajer et al., 1995) and that 80% of the α2 (IV) collagen chain mRNA is synthesized by fibroblasts while

20% derives from keratinocytes (Fleischmajer et al., 1997). The dual tissue origin of the laminin γ1 chain mRNA, raises the question as to whether each tissue plays a different role during BM formation. Thus, it is tempting to speculate that collagen IV and laminins of epidermal origin bind to integrins and are used as nucleation sites for further polymerization of protomers or oligomers derived from the adjacent dermis. This hypothesis is supported by the observation that α1 (IV) and α2 (IV) collagen chains in 7 day K-D-M cultures are first noted in the epidermis followed by their rapid expression in the adjacent dermis (Fleischmajer et al., 1997). Since the laminin γ1 chain mRNA is frequently expressed in epithelial and mesenchymal tissues during embryogenesis (Thomas and Dziack, 1993) it would be of interest to determine whether the initial deposition of laminins into the BM involves a co-operative temporal-spatial mechanism between epithelial and mesenchymal tissues.

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