Inhibition of basement membrane formation by a nidogen-binding laminin γ1-chain fragment in human skin-organotypic cocultures

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This article is dedicated to Rupert Timpl who died in 2003

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Summary

Basement membranes generally determine different tissue compartments in complex organs, such as skin, playing not only an important structural but also a regulatory role. We have previously demonstrated the formation of a regular basement membrane in organotypic three-dimensional (3D)-cocultures of human skin keratinocytes and fibroblasts by indirect immunofluorescence and transmission electron microscopy. In this assembly process, cross-linking of type IV collagen and the laminin γ1 chain by nidogen is considered a crucial step. For a functional proof, we have now competitively inhibited nidogen binding to laminin in 3D-cocultures with a recombinant laminin γ1 fragment (γ1III3-5 module) spanning this binding site. Repeated treatment abolished the deposition of nidogen at the epithelial-matrix interface but also greatly perturbed the presence of other matrix constituents such as laminin and perlecan. This effect persisted over the entire observation period of 10 to 21 days. In contrast, some components of the basement membrane zone were only moderately affected, with the laminin-5 isoform (γ2 chain), type IV collagen and integrin α6ß4 still showing a distinct staining at their regular position, when seen by light microscopy. Furthermore, epidermal morphology and differentiation remained largely normal as indicated by the regular location of keratins K1/K10 and also of late differentiation markers. Ultrastructural examination demonstrated that the γ1 fragment completely suppressed any formation of basement membrane structures (lamina densa) and also of hemidesmosomal adhesion complexes. As a consequence of hemidesmosome deficiency, keratin filament bundles were not attached to the ventral basal cell aspect. These findings were further substantiated by immuno-electron microscopy, revealing either loss or drastic reduction and dislocation of basement membrane and hemidesmosomal components. Taken together, in this simplified human skin model (representing a 'closed system') a functional link has been demonstrated between compound structures of the extra- and intracellular space at the junctional zone providing a basis to interfere at distinct points and in a controlled fashion.

Key words: Nidogen, Basement membrane, Human skin-organotypic coculture

Introduction

In skin, as in other organs and complex tissues, the basement membrane (BM) serves as a selective barrier between different tissue compartments, and it also provides a firm support for the germinative basal cell layer. In addition, evidence is accumulating that BM has functions in maintaining of the regulation of epithelial physiology, differentiation and tissue balance (homeostasis). Consequently, injury-induced changes in the BM status or composition are involved in the triggering of wound responses by several mechanisms. Mediators of the respective signalling events are cell surface adhesion receptors, such as integrins, which can also function as modulators of, or in concert with, several growth factors. Despite specific tissue variations, common to all BM structures are type IV collagen, BM laminin (several isoforms with a γ1 chain), nidogen and the heparansulfate proteoglycan perlecan. In contrast to most extracellular matrix molecules nidogen shows no self-assembly, however, it harbours numerous potential binding sites to the other BM components (Aumailley et al., 1989; Fox et al., 1991; Mayer et al., 1993; Timpl, 1996). For these properties nidogen has been considered the classic cross-link, stabilising the hetero-polymeric texture of the BM. Apparently it connects the integrated meshworks of laminin and type IV collagen (Mayer et al., 1995; Timpl, 1996), which are believed to form independent entities (Yurchenco et al., 1992). In addition, nidogen contributes to the integration of the proteoglycan perlecan into the BM, which by its heparansulfate side chains implements a distinct zone of negatively charged residues, able to bind a variety of growth factors (Aviezer et al., 1994). The identification of the nidogen binding site within the short arm of the laminin γ1 chain in the III4 module (Mayer et al., 1993; Pöschl et al., 1994; Pöschl et al., 1996) has allowed

the assignment of specific biological functions to that interaction in transgenic mice (Willem et al., 2002). The most severe defects in animals lacking that site (deletion in γ1) were observed in kidney and lung development, leading to perinatal death. Other tissues, in particular skin, seemed to be less affected or normal (Willem et al., 2002), although detailed ultrastructural analysis may reveal subtle changes compromising function. By and large the laminin defect can be apparently be compensated for in those tissues, at least temporarily, while the early lethality allows no judgement on functional defects that would arise later in adult life. Certainly, this also does not exclude additional functions of nidogen other than providing intermolecular links. Nidogen is constitutively located in areas of epithelial-mesenchymal interactions where it is secreted by the resident fibroblasts (Fox et al., 1991; Marinkovich et al., 1993; Ekblom et al., 1994), which has been confirmed in experimental systems in vitro (Fleischmajer et al., 1995; Smola et al., 1998). In mammals two isoforms have been discovered, nidogen-1 which is the major form, and nidogen-2 (Kohfeldt et al., 1998), and knockout mice have been generated (Murshed et al., 2000; Dong et al., 2002; Schymeinsky et al., 2002) providing a more specific functional proof. The complete lack of either nidogen-1 or -2 did not produce an obvious phenotype in basement membrane structures or related functions. This may be explained by the widespread co-expression of both nidogens and functional compensation for the loss of either one by the other form. Thus, when nidogen-1 was missing for example, there was a modest increase and redistribution of nidogen-2 in areas with normally marginal expression, such as striated muscle (Miosge et al., 2002; Salmivirta et al., 2002).

Obviously mimicking the in vivo situation, normal epidermal keratinocytes (NEK) depend in vitro on the interaction with fibroblasts for continuous growth and complete epidermal organisation which, in the long run, should also require the presence of an intact BM. Formation of a regular epidermal tissue architecture had been achieved in vitro in an organotypic three-dimensional coculture system (3D-coculture) by growing NEK at the air-liquid interface on top of type I collagen gels populated with skin fibroblasts (Asselineau et al., 1985; Kopan et al., 1989; Smola et al., 1994; Limat et al., 1996; Ponec et al., 1997). These results corresponded well with those of other reports on similar models, but using fibroblast-produced extracellular matrices instead of collagen gels (Bouvard et al., 1992; Contard et al., 1993). However, in the latter cases these processes occurred in a wider time frame, which severely restricts the choice of external manipulations. It was also confirmed in such coculture systems that the BM constituents were produced in a cooperative manner (Contard et al., 1993; Limat et al., 1996; Smola et al., 1998; Fleischmajer et al., 2000), nidogen being exclusively expressed by fibroblasts and laminin-5 by keratinocytes, presumably providing a ‘nucleation site’ for BM assembly. With expression of the BM components in the right sequence an ultrastructurally defined BM zone was also developed (Contard et al., 1993; Tsunenaga et al., 1998; Stark et al., 1999), which corresponded to the findings in surface transplants of NEK on athymic nude mice (Breitkreutz et al., 1997).

We have used our established 3D-coculture system (Stark et al., 1999) to investigate the consequences of interfering with nidogen-laminin binding on the assembly and stability of BM as well as for epidermal tissue integrity. For this purpose a recombinant fragment of the laminin γ1 chain was applied (γ1III3-5, referred to here as Lγf), which harbours the binding site for nidogen (γ1III4-module). This efficiently inhibited the linear alignment of several BM components, particularly of nidogen, laminin and perlecan, and at the ultrastructural level the development of a defined BM zone including hemidesmosomes, the stable epidermal attachment sites.

Materials and Methods

Organotypic three-dimensional coculture and treatment (3D-coculture)

The organotypic 3D-coculture system used in this study has previously been described in detail (Smola et al., 1994; Stark et al., 1999). In brief, normal epidermal keratinocytes (NEK, 10^6 cells/cm^2) from human trunk skin were grown submersed on type I collagen gels containing skin fibroblasts (2×10^5 cells/ml) using large (25 mm) or small (12 mm) filter inserts. They were placed in six- or twelve-well plates, respectively, and the cocultures were exposed to the air-liquid interface after 1-2 days. The treatment was started 3 days later, adding the laminin γ1 fragment (Lγf) three times a week either together with medium or on top of the epithelium (in 100 μl total volume). The recombinant laminin γ1 fragment was purified from the culture supernatant of transfected human embryonic kidney cells [293 cells; generously provided by Rupert Timpl, Martinsried, Germany (Mayer et al., 1993)]. In binding assays an IC_{50} (concentration for half-maximal inhibition) of 0.1 nM was determined for Lγf, while for the S-carboxymethylated Lγf, used as a ‘negative’ control, the IC_{50} was in the range of 500-1000 nM (possibly because of some variation in the chemical reaction) which is in the order of magnitude of a heptapeptide harbouring the binding motif (Pöschl et al., 1994).

Histological analysis

The 3D-cocultures, still attached to the filter inserts, were briefly rinsed with PBS and pre-fixed in 2% formaldehyde for 60-90 minutes. They were placed in 3.5% sucrose for at least 30 minutes and dissected for further processing. For routine histology, samples were then incubated in 4% formaldehyde overnight and treated accordingly for paraffin embedding; alternatively frozen sections were stained with Haematoxylin and Eosin.

Indirect immunofluorescence (IIF)

Pre-fixed samples were incubated in blocking solution (50 mM NH_4Cl), followed by 3.5% sucrose (2 hours or overnight), embedded in Tissue-Tec, and frozen in liquid nitrogen vapour. Cryostat sections (8 μm thick) were air-dried, incubated with primary antibodies overnight at 4°C and with secondary antibodies for 30 minutes at room temperature (RT) as described previously (Breitkreutz et al., 1998; Tomakidi et al., 1999). We used the following antibodies specific for BM components produced in rabbit and directed against: bullous pemphigoid antigen 1 (BPAG-1, BP230), generously provided by John Stanley (Bethesda, Maryland, USA) (Stanley et al., 1988), BPAG-2 (BP180/type XVII collagen; extracellular domain) provided by Leena Bruckner-Tuderman (Freiburg, Germany) (Schumann et al., 2000), laminin-5 provided by Peter Marinkovich (Stanford, California, USA) (Marinkovich et al., 1993) and laminin γ2 chain provided by Guerrino Meneguzzi (Nice, France) (Gagnoux-Palacios et al., 2001). Monoclonal antibodies against integrin α2, α3 and αv chain were donated by Eberhard Klein (Eva Bröcker, Würzburg, Germany) (Klein et al., 1991), rat monoclonal antibodies against integrin α6 (G_0H3) donated by Arnoud Sonnenberg (Amsterdam, The Netherlands) (Sonnenberg et al., 1991), integrin β4 (439/9B;

extracellular domain) donated by Rita Falcioni (CRS, Rome, Italy) (Falcioni et al., 1988), goat and rabbit antibodies against BM laminin (isolated from EHS tumour) (Paulsson et al., 1987) and type IV collagen donated by Jean-Michel Foidart (Liege, Belgium) (Foidart et al., 1980), rabbit anti-nidogen-1 and -2 donated by Roswitha Nischt and Rupert Timpl (Cologne and Martinsried, Germany) and rabbit anti-loricrin donated by Daniel Hohl (Lausanne, Switzerland) (Hohl et al., 1991). Mouse monoclonal antibodies specific for laminin α5 were a generous gift from Ismo Virtanen (Helsinki, Finland) (Virtanen et al., 2000), those against laminin γ1 chain modules were obtained after immunisation with human laminin fragment P1. The particular site specificity was determined by ELISA using the recombinant modules γIII3, γIII3/4, γIII4 and γIII3-5 (provided by Ulrike Mayer, Manchester, UK). Clone A6/2/4 specifically recognised module γIII4 and clone A52/2/4 module γIII5 (unpublished data, Martin Gerl, Aventis, Germany). All primary antibodies were adjusted with 1% BSA in PBS to the final working dilutions. Secondary antibodies were purchased from Biotrend (Cologne, Germany), Dianova (Hamburg, Germany) and Molecular Probes (Leiden, The Netherlands; Mobitec, Göttingen, Germany).

Electron microscopy (EM)

Specimens were fixed in 2.5% glutaraldehyde (in cacodylate buffer pH 7.2), followed by 2% OsO_4, stained en bloc with uranyl acetate, and processed for embedding in Epon 812-equivalent (Serva, Bioproducts, Heidelberg, Germany) according to our previous protocol (Breitkreutz et al., 1998; Tomakidi et al., 1999). Ultrathin sections were counterstained with uranyl acetate and lead citrate.

Immuno-electron microscopy (ImEM)

Samples were lightly fixed by placing them in 2% formaldehyde, 0.05% glutaraldehyde (in PBS) for 1 hour and, after blocking by 50 mM NH_4Cl, incubating them in 3.4% sucrose (30 minutes). The specimens were then dehydrated and embedded in Unicryl resin (British BioCell, Cardiff, UK). Ultrathin sections on nickel grids were incubated with primary antibodies (same as listed above for IIF), followed by secondary antibodies conjugated with 5, 10 or 15 nm gold particles (Aurion/BioTrend, Cologne, Germany) at RT for 2-5 hours, and counterstained as for EM. In some cases, for increasing sensitivity, 1 nm gold conjugates were also used, followed by silver enhancement according to the manufacturer's protocol (Aurion).

Analysis of BM proteins in 3D-cocultures (western blotting)

Fresh 3D-cocultures were immersed in 10 mM dithiothreitol (in PBS) for 1 hour at 4°C, following a procedure reported for skin separation (Epstein et al., 1979; Breitkreutz et al., 1984). The epithelium was carefully pealed off the collagen gel and both tissue parts were transferred to separate Eppendorf tubes containing 200 μl RIPA extraction buffer (150 mM NaCl, 0.1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 5 mM EDTA, 10 mM Tris, pH 7.2, with protease inhibitors leupeptin, pepstatin, aprotinin, 1 μg/ml each, and 10 mM PEFA block; from Biomol, Hamburg, Germany). After incubation on ice for 1 hour samples were sonicated, heated at 95°C for 5 minutes, cleared by centrifugation and aliquots (30 μg protein) separated on 6% SDS-polyacrylamide gels. After transfer to nitrocellulose membranes the banding patterns were stained with Ponceau Red to control sample loading and visualise marker bands. Membranes were blocked with 10% low-fat milk and incubated with primary antibodies for 16 hours at 4°C. Rabbit antibodies against nidogen (preferentially recognising nidogen-1) and type IV collagen are listed above, those against laminin α5 and mouse monoclonal antibodies against laminin γ1 were purchased from Santa Cruz (Santa Cruz, CA, USA). Reactive bands were detected with horseradish peroxidase-coupled secondary antibodies and chemiluminescence (Amersham-Pharmacia Biotech, Freiburg, Germany) following the supplier's instructions.

Results

Morphology and differentiation of Lγf-treated 3D-cocultures

Concerning the bio-compatibility of Lγf, this γ1 fragment did not cause any obvious morphological changes or reduction of the mitotic rate when tested in conventional two-dimensional cultures of both normal epidermal keratinocytes (NEK) and skin fibroblasts. Similar observations were made in organotypic 3D-cocultures of keratinocytes and fibroblasts, also developing in the presence of Lγf and producing morphologically normal skin equivalents (Fig. 1). The only clear difference was an increased tendency for splitting at the epidermal-collagen interface which, while it could have occurred during sample processing, still indicated weakening

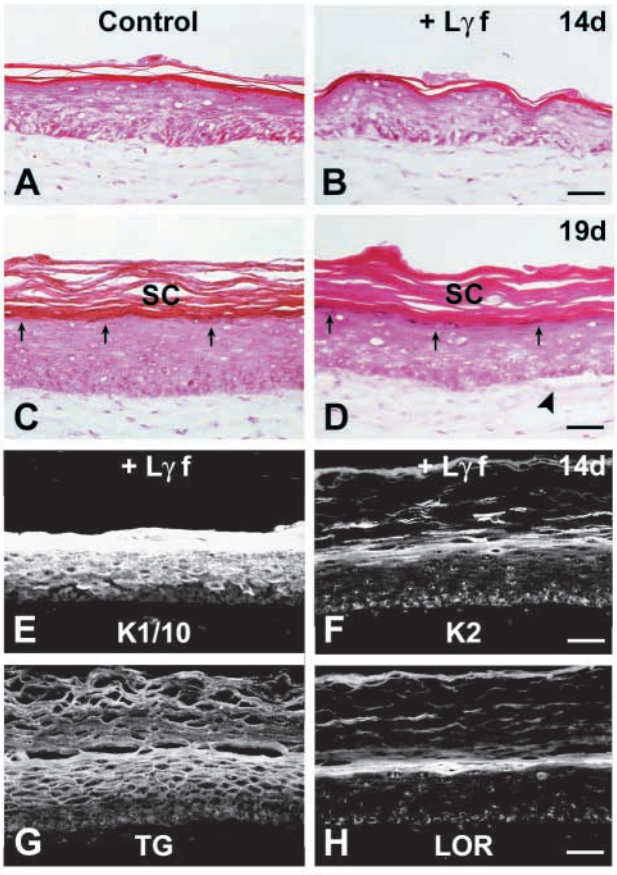

Fig. 1. Maintenance of morphology and differentiation in organotypic 3D-cocultures in the presence of laminin γ1-fragment (Lγf). Histology of epithelia at 14 (A,B) and 19 days (C,D), grown without (A,C) and with Lγf (B,D) reveals comparable stratification including defined granular (arrows) and cornified layers (SC), but an increased tendency for splitting at the tissue interface with Lγf (D; arrowhead). (E-H), Analysis of Lγf-treated tissue by indirect immunofluorescence (IIF) indicates a largely regular expression of the early epidermal differentiation markers keratin K1 and K10 (E) as well as the intermediate and late markers K2e (F), epidermal transglutaminase (G), and loricrin (H). Scale bars: 50 μm.

of the epithelial-matrix junction (Fig. 1D). However, epithelial stratification was not impaired and the presence of regular granular and cornified layers implied a normal differentiation (Fig. 1C,D). This was further confirmed by indirect immunofluorescence (IIF) using antibodies against keratins K1/K10 as early markers (Fig. 1E) and the intermediate or late differentiation products K2e (Fig. 1F), epidermal transglutaminase (Fig. 1G), loricrin (Fig. 1H) as well as involucrin, filaggrin and repetin (those not shown). Apart from some reduction of K2e in the treated 3D-cocultures, there was no substantial difference in location and intensity in comparison to untreated controls (see also Stark et al., 1999).

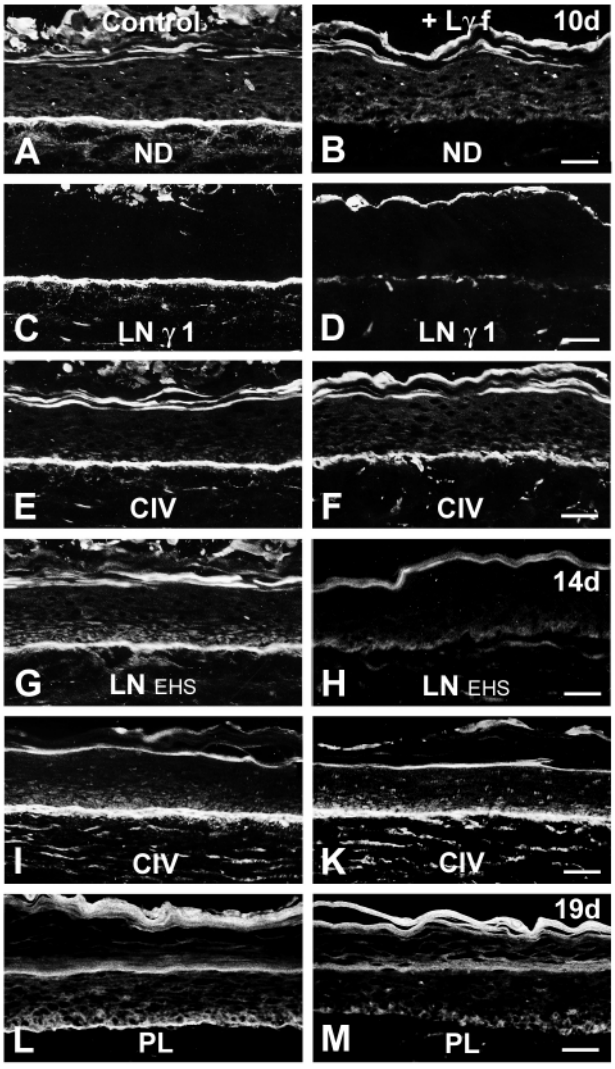

Fig. 2. Influence of Lγf on deposition of basement membrane (BM) components shown by indirect immunofluorescence (IIF) at days 10 (A-F), 14 (G-K) and 19 (L,M). Compared to controls (left column) treated epithelia (right column) have lost or dramatically reduced nidogen (A,B), BM laminin (C,D and G,H) and perlecan (L,M). For laminin detection either antibodies against the laminin γ1 chain (C,D) or laminin from EHS tumours (G,H) were employed. By contrast, type IV collagen (E,F and I,K) and laminin-5 (see Figs 3, 4) are only mildly affected by the treatment. Scale bars: 50 μm.

Effects of Lgf on basement membrane components

To define a suitable time window for intermittent Lγf treatment, the early stages of BM formation were monitored by IIF in control 3D-cocultures (without Lγf). While deposition of BM components was generally still marginal or low at day 7 (after air exposure), nearly maximal levels were reached around day 10, and persisting throughout the whole observation period (up to day 21). Therefore the initial concept was to start Lγf treatment before the onset of BM formation, but also to avoid interference with early steps of epithelialisation and stratification. When Lγf was added from day 3 on, a maximal response was observed by day 10 (Fig. 2A-F) when compared with 14 or 19 days (Fig. 2G-M). Whereas deposition of nidogen (Fig. 2A,B), 'classic' BM laminin (Fig. 2C,D and G,H), and perlecan (Fig. 2L,M) was virtually abolished, type IV collagen (Fig. 2 E,F,I,K) and laminin-5 (shown later) were only mildly affected. Nevertheless, the outcome of the laminin reaction was dependent on the type of antibodies used. With monoclonal antibodies against laminin γ1, usually a fine, although broken line was visible at the epithelial-matrix interface (Fig. 2D), while with the common antisera (rabbit or goat) against laminin from EHS tumours (Paulsson et al., 1987), sharing the β1 and particularly the γ1 chain with several or most other laminins, a very weak but diffuse reaction was seen (Fig. 2H). In addition we could demonstrate that BM laminin was essentially laminin-10, the isoform in BM of adult human and mouse skin. This was achieved by detection of the α5 chain in control but not in treated samples (laminin-10: α5β1γ1; α5 not shown here, but see Fig. 8 for western blots) (Miner et al., 1997; Fleischmajer et al., 2000; Virtanen et al., 2000). In contrast, labelling of the distinct isoform laminin-5 (α3β3γ2) was also very strong in the presence of Lγf throughout the observation period, as shown below (Figs 3, 4). The epidermal product laminin-5 (Rousselle et al., 1991; Marinkovich et al., 1993) is actually the first matrix component made by NEK in vivo, for example in wound regeneration (Larjava et al., 1993) or cell transplants (Breitkreutz et al., 1997) as well as in vitro (Marinkovich et al., 1993; Smola et al., 1998). Thus, one may speculate that the marginal, but distinct reaction for laminin γ1 indicates that low levels of laminin-6 (α3β1γ1) were present in complexes with laminin-5 as claimed for the epidermal junction (Champliaud et al., 1996; Timpl, 1996). These data were confirmed by double labelling and also examination by confocal laser scan microscopy, which allowed the scanning of the BM zone for the relative staining intensities (not shown).

Concerning the effectiveness of Lγf-application, at early stages there was apparently no difference if Lγf was added to the culture medium or placed directly on top of the epithelial cells. Some variations were observed with topical administration later on, presumably because it hindered penetration of Lγf with the increasing epithelial keratinisation. The dose of 200 μg Lγf/ml used initially (shown in Fig. 2) could be reduced to 40 μg/ml; regularly 10-20 μg/ml (555-1110 nM) still gave virtually complete inhibition. Application of 1-2 μg/ml (55-110 nM) resulted in half-maximal inhibition, while the effect became negligible below 0.5 μg/ml (data not shown). These values should be considered a coarse estimate, largely depending on culture conditions (here application in medium), but they clearly indicate a specific dose-dependent

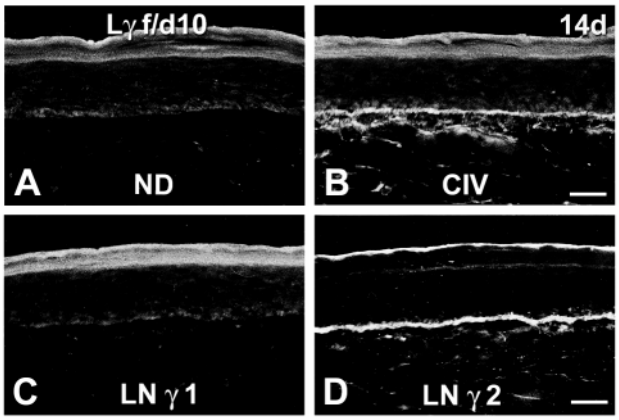

Fig. 3. Dissociation of assembled BM components by delayed Lγf treatment (starting at day 10) and analysed at day 14. Nidogen (A) and BM laminin (C; detection of γ1 chain) are almost absent after this short treatment, whereas the linear deposition of both type IV collagen (B; but some diffuse dislocation into gel matrix) and laminin-5 (D; γ2 chain) persists. Scale bars: 50 μm.

effect. Furthermore, an S-carboxy-methylated Lγf fragment, having the identical amino acid sequence but a tertiary structure different from 'native' Lγf, revealed only a very low affinity to nidogen (see Materials and Methods) and had no visible effect at 10 μg/ml. A slight inhibition, seen at higher doses was presumably in part due to contamination by incompletely modified Lγf (below 1%). Considering the sequence-specific (conformation-independent) binding affinity, determined by in vitro assays, a much lower biological activity would have been expected in this in vivo-like situation. In contrast, other recombinant 'native' laminin short arm fragments (with different EGF repeats) exhibited no inhibitory activity in this dose range (data not shown).

Reversible disassembly of basement membrane components

Starting Lγf treatment later, at day 10 instead of day 3, caused a progressive loss of BM material from the interface, which became apparent at day 14 (Fig. 3; 4 days of treatment). Again nidogen (Fig. 3A), BM laminin (Fig. 3C, γ1) and perlecan (not shown) vanished, while type IV collagen (Fig. 3B) and laminin-5 (Fig. 3D, γ2) were not severely affected. Accordingly, BM assembly was still reversible at day 10 indicating that it is a rather dynamic process.

In contrast, halting Lγf treatment at day 10 (after 7 days of application) allowed visible recovery by day 14 and a substantial improvement of BM staining at day 19 (Fig. 4). This clearly indicated that the blockade could be reversed (Fig. 4, left side) and that there were no subtle toxic effects, which should accumulate with longer treatment (Fig. 4, right). In this respect the 3D-cocultures represented undoubtedly a far more sensitive system to test the bio-compatibility of Lγf than conventional cultures on plastic dishes. However, there was an increased tendency for splitting at the matrix-epithelial junction and presumably extensive membrane shedding (Fig. 4A,C,G) which could mean that a full, functional recovery was not yet accomplished in this time frame. However, the faint pericellular staining for nidogen and laminin γ1 which was

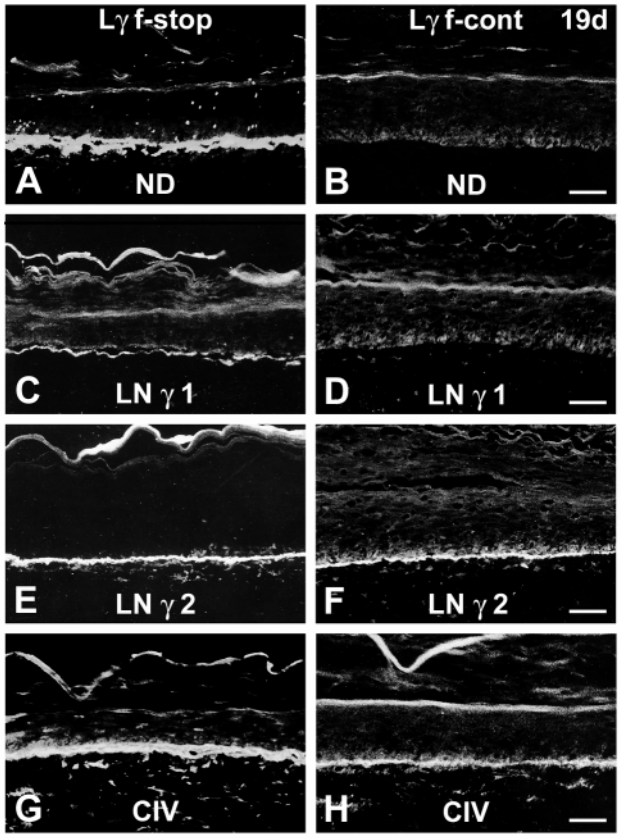

Fig. 4. Reversibility of the Lγf-effect on the deposition of BM components. Upon withdrawal of Lγf at day 10 the deposition of nidogen (A) and BM laminin (C; γ1 chain) is largely or partially recovered, whereas continuous treatment (B,D) reveals, at best, only marginal binding to the surface of basal cells. By contrast, there is only little change, at most a moderate reduction in staining for laminin-5 (E,F; γ2 chain) and type IV collagen (G,H) between these pairs (control/treated). All shown at day 19. Scale bars: 50 μm.

largely corresponding (Fig. 4B,D; continuous Lγf treatment) may indicate some binding of respective complexes to the basal cell surface via integrins (see below).

Ultrastructural changes at the epidermal-matrix junction

Examination by EM clearly demonstrated the complete lack of an ultrastructurally defined basal lamina at day 14 in the 3D-cocultures treated with Lγf (Fig. 5B,D). Furthermore, stable epidermal attachment complexes, the hemidesmosomes seen in controls (Fig. 5A,C), were totally absent. Consequently, keratin filaments became sparse at the basal regions of the cell upon Lγf treatment, lacking the specific intracellular attachment sites, i.e. the inner/outer dense plaques of the hemidesmosomes. In this context it should be noted that in contrast to our observations in grafts of malignant cells or experimental tumours (Tomakidi et al., 1999) there was no sign of so-called type II hemidesmosomes. These are considered to represent immature or intermediate forms that occur, for example, during development and in healing skin wounds (Green and Jones, 1996; Nievers et al., 1999**)**. This demonstrated that the blockade of BM assembly was causing a profound functional collapse within the entire BM zone.

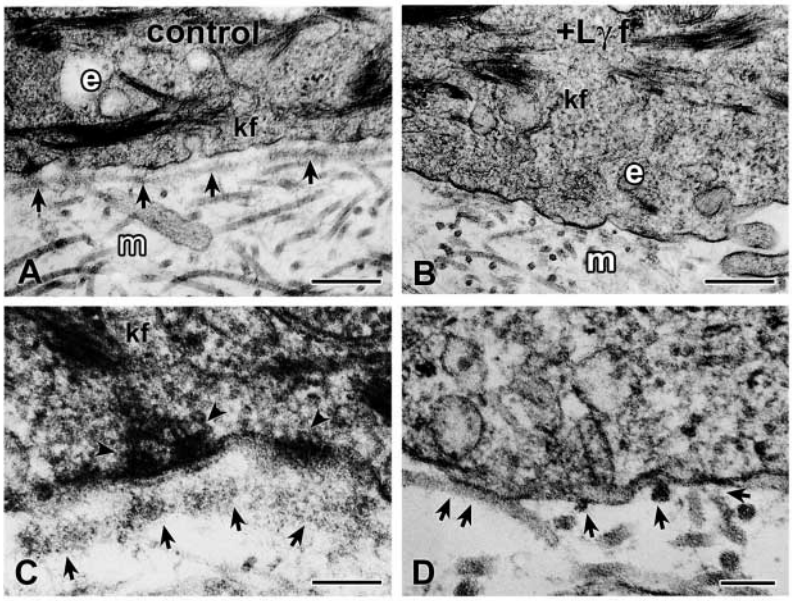

Fig. 5. Loss of ultrastructural elements of the BM zone caused by Lγf. At 14 days untreated 3D-cocultures (A,C) reveal a mostly continuous BM (arrows indicate the lamina densa) and regularly structured hemidesmosomes (arrowheads in C; detailed view) linked to prominent keratin filament bundles (kf). Treatment with Lγf not only prevents assembly of BM, but also of hemidesmosomes (B,D) and the association of keratin filaments (kf). Basal cells seem to attach frequently to type I collagen fibrils which can be seen in cross and longitudinal section (D; some contacts are indicated by short arrows). e, epithelium; m, matrix. Scale bars: 250 (A,B) and 100 nm (C,D).

Instead, membranes of basal cells seemed to have a close contact to type I collagen fibrils, seen in longitudinal and cross-sections (Fig. 5B,D; Lγf-treated).

Consequences on epithelial adhesion

In the absence of BM structures basal cells frequently seemed to adhere directly to type I collagen fibrils when seen by electron microscopy (Fig. 5D). Since integrins are considered to be the main cell-matrix receptors, we examined the patterns of the common epidermal integrins (α2β1, α3β1, α6β4) (Carter et al., 1990; De Luca et al., 1990; Hertle et al., 1991) and some of the inducible wound-type integrins (such as αv, combined with various β-chains (Zambruno et al., 1995) (for a review, see Watt, 2002). Comparable to wound healing, in early growth phases of cell transplants the integrin profiles changed with matrix composition (Larjava et al., 1993; Breitkreutz et al., 1997; Breitkreutz et al., 1998). By contrast, in the 3D-cocultures (with or without Lγf) normalisation of the integrin patterns, which reflects epithelial polarisation, occurred more slowly (Fig. 6). Upon Lγf treatment only marginal differences, if any, were observed. Whereas the distribution of α2 appeared slightly more polarised with Lγf (Fig. 6A,B), basal linear staining of α3 became less pronounced (Fig. 6C,D), resembling most closely β1 (not shown), and the αv pattern was more irregular (Fig. 6E,F). Finally, the α6 chain strongly accumulated at the basal surface, but we also observed an increased tendency of shedding into the collagen matrix in treated cocultures (Fig. 6G,H). This staining pattern corresponded to that of its partner β4 (not shown). Interestingly, another hemidesmosomal component, bullous pemphigoid antigen 2 (BPAG2/ BP180) revealed a regular pericellular decoration, clearly restricted to the basal cell layer in both controls and treated epithelia, whereas its preferential localisation at the basal surface was largely lost upon Lγf treatment (Fig. 6I,K). In ‘blistering’ areas usually some staining for these transmembrane proteins was seen on top of

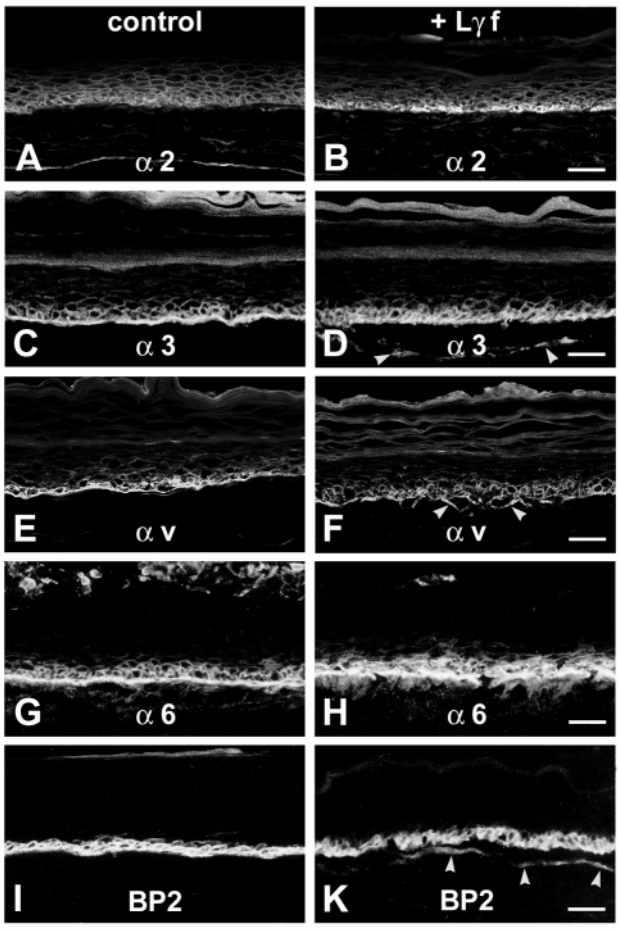

Fig. 6. Persistence of adhesion molecules in 3D-cocultures at 14 (A-D), 19 (E,F) and 21 days (G-K). The patterns of controls (left column) generally undergo only minor changes in the presence of Lγf (right column) as demonstrated for the integrin chains α2 (A,B), α3 (C,D), αv (E,F), α6 (G,H), and bullous pemphigoid antigen 2 (I,K; BPAG2/BP180). In treated samples, α2 (B) is slightly stronger in basal cells, while the distribution of α3 (D) appears slightly more pericellular and αv (F) is less polarised. Further the prominent co-localisation of α6 and BPAG2 in controls (G,I) is not maintained upon treatment with Lγf (H,K), indicating extensive shedding of α6 (H) and a mostly pericellular label of basal cells with BPAG2 (K). Arrowheads in D,F,K indicate the partially labelled surface of the collagen matrix in split areas. Scale bars: 50 μm.

the collagen matrix (Fig. 6D,F,K; arrowheads). While we regularly saw co-alignment of integrins (e.g. α3β1, α6β4) with matrix at the lower epithelial surface, the presumptive ligands (such as laminin-5) appeared weaker or were missing at the blister floor. This may imply that with membrane shedding integrins are being deactivated.

Changes in the molecular/ultrastructural distribution of basement membrane components

To obtain a clearer picture of possible changes in molecular interactions, the ultrastructural localisation of several components of the BM zone was examined by ImEM (Fig. 7). This confirmed that in Lγf-treated 3D-cocultures basal cells were not tightly associated with type IV collagen (as suggested already by IIF and confocal laser scanning microscopy, not shown) but formed loose aggregates (compare Fig. 7A,B). In addition, laminin-5 was not aligned at a defined distance from the cell surfaces (Fig. 7C,D) which was consistent with the total lack of anchoring filaments upon treatment (see Fig. 5). According to the previous observations it was not surprising that (the tested) hemidesmosomal components did not co-localise, as seen in the controls. Thus, the integrin α6β4 [here β4 chain, extracellular domain (Falcioni et al., 1988)] was mostly located close to the basal cell membrane (Fig. 7C,D) or shed into the matrix, while bullous pemphigoid antigen 1 (BPAG1/BP230) was irregularly distributed (Fig. 7E,F), consistent with the lack of distinct lining observed by IIF. Finally, the hemidesmosomal constituent HD1 (plectin or homologue) (Rezniczek et al., 1998) was not detectable at all (also positive control not shown). All three, the integrin α6β4 (interacting by the cytoplasmic domain of β4), BP230 and HD1 are supposed to form the inner and outer dense plaques of hemidesmosomes which are indispensable for the keratin filament anchorage (compare Fig. 7G and H) (Sonnenberg et al., 1991; Rezniczek et al., 1998; Borradori and Sonnenberg, 1999; Nievers et al., 1999).

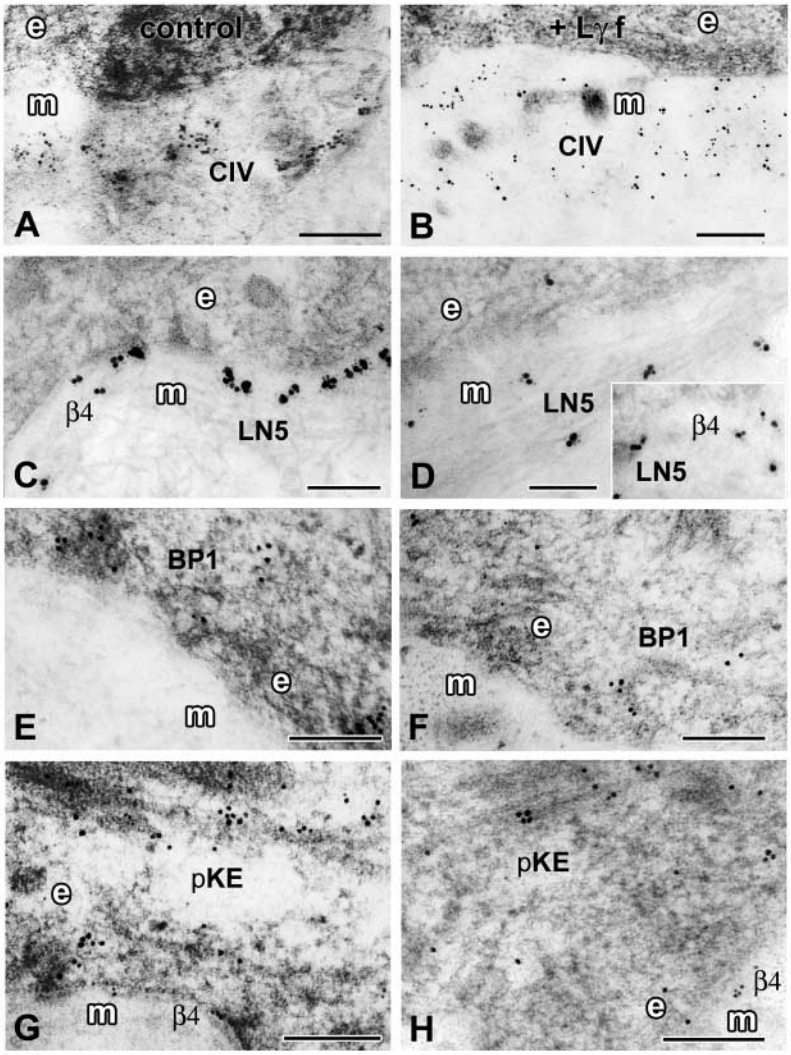

Fig. 7. Localisation of BM components and adhesion molecules in control (left column) and Lγf-treated epithelia (right column) at 14 days. The control shows a band of denser gold labelling for type IV collagen (A) which becomes extensively dispersed and dislocated in the presence of Lγf (B; larger area, about two-fold lower magnification). Similar effects are seen for laminin-5 (C, D; 10 nm gold) which by double labelling also reveals a looser association of the integrin β4 chain than in controls (C versus D; 5 nm gold). In addition, and in contrast to regular clusters of bullous pemphigoid antigen 1 (E; BPAG1/BP230; 10 nm), only a few single gold particles are seen in Lγf-treated specimens (F; outside the field, vast areas without any label). Consequently keratin-positive filaments ('pan'-keratin; 10 nm), normally attached to the hemidesmosomes (G), are, in treated samples (H), largely retracted from the basal cell pole, which is partially demarcated by β4 (5 nm). In A and B 1 nm gold particles were used together with silver enhancement. e, epithelium; m, extracellular matrix. Scale bars: 150 (A,C-H) and 300 nm (B).

Protein analysis in separated tissue compartments

To follow the fate or alterations of individual BM components, epithelia were separated from the collagen matrices after incubation with dithiothreitol. This method yields a viable epidermis with an intact BM zone (Epstein et al., 1979). Protein extracts of corresponding epithelial and 'dermal' compartments from 3D-cocultures, grown for 12 and 14 days, were analysed by SDS-gel electrophoresis and western blotting using antibodies against nidogen, type IV collagen, and the laminin α5 and γ1 chain (Fig. 8). In controls the presence of laminin α5 (major band >300 kDa) and γ1 (200 kDa) exclusively in the epithelial part indicated a clean separation from the collagen gels, while nidogen (160 kDa) and type IV collagen (180 kDa; not shown here) could be detected in both tissue compartments, nidogen appearing generally weaker in the epithelial part. This seemed to correlate with the strong mRNA expression of both BM components by the fibroblasts residing in the collagen matrix (Smola et al., 1998). However, no significant

change was seen in the treated samples, the efficacy of the blockade being controlled by IIF on a small piece of those samples being extracted. A plausible explanation for the discrepancy between these results would be a drastically altered, rather homogeneous distribution either in the extracellular matrix or within the epithelia, thus escaping visual detection.

Discussion

We have demonstrated that a laminin γ1 chain fragment (Lγf), binding nidogen with high affinity, entirely blocked the formation of a basement membrane (BM) zone in an organotypic 3D-coculture model mimicking human skin. Immunofluorescence (IF) revealed that the Lγf effect was dose dependent, largely reversible, but also somewhat selective. Thus, most, but not all, of the BM components were affected to varying extents. The ultrastructure of Lγf-treated epithelia indicated that, in addition to the complete absence of BM structures, any recognisable elements of hemidesmosomes or intermediate forms were abolished. Consequently, there was no linkage of strong keratin filament bundles to the ventral aspect of basal cells. Interestingly, overall epithelial integrity was maintained including epidermal stratification and the complete programme of differentiation.

Laminin fragment blocks BM formation – functional cooperation of nidogen with laminin

Our findings in these 3D-cocultures suggest that of all its bridging properties, the interaction of nidogen with BM laminin may be initially the predominant event. However, in spite of nidogen blockade and lack of laminin polymerisation in the presence of Lγf, deposition of type IV collagen was little different from controls when viewed by IIF. Presumably, type IV collagen was still regularly secreted and cross-linked under these conditions, forming an independent network as previously postulated (Yurchenco et al., 1992). Although this implied a rather selective action, there was an imperfect, less compact texture as indicated already by confocal laser scan microscopy and confirmed by ImEM (see also below). Direct binding of nidogen to epithelial cells may play a crucial role, presumably, as previously reported, via integrins such as α3β1 (Dedhar et al., 1992), αvβ3 (Salmivirta et al., 2002) and possibly αvβ5. This would be compatible with the appearance of nidogen in our transplantation model shortly after laminin-5, both far earlier than the deposition of BM laminin (in adult skin, laminin-10) and type IV collagen (Breitkreutz et al., 1997; Breitkreutz et al., 1998). The respective integrins are expressed or induced under those circumstances in vivo and also in the early phases of 3D-coculturers resembling regenerating tissue. As nidogen is protected from proteolysis when bound to BM laminin (Mayer et al., 1995), it could then act as a molecular adapter facilitating binding of laminin to cells and perhaps influence laminin polymerisation by helping to concentrate laminin close to the cell membrane (Yurchenco et al., 1992; Colognato and Yurchenco, 2000). Indeed, the signalling function of laminin for mammary gland differentiation is enhanced by nidogen in a cooperative manner (Streuli et al., 1995; Pujuguet et al., 2000), which also seems to prevent apoptosis of those cells (Alexander et al., 1996). Furthermore, nidogen is required in embryonic tissue models for the induction of branching morphology (Ekblom et al., 1994) where laminin-1 should be obligatory as well (Kadoya et al., 1997; Virtanen et al., 2000) (for a review, see Colognato and Yurchenco, 2000). Thus, besides competitive binding, it is also possible that the nidogen-Lγf complex has dominant negative effects on the cellular information transfer in our model. Finally, for ascertaining specificity a chemically modified Lγf (S-carboxymethylated; totally altered conformation), when tested in this system, produced a drastically lowered inhibition (about two orders of magnitude). This could have been due to some residual unmodified fragment and low binding activity as seen in short peptides just covering the binding site (Pöschl et al., 1994). Other laminin short-arm fragments (containing distinct domains) had no visible effects at comparable doses (data not shown).

Role of the isoforms nidogen-1 and nidogen-2

In mouse both isoforms of nidogen, 1 and 2, bind to the laminin γ1 chain at the same site within the γ1III4 module (Salmivirta et al., 2002), which readily explains the observed compensation by nidogen-2 in nidogen-1-deficient mice (Miosge et al., 2002). In contrast to the initial findings, the human nidogen-2 has a similar affinity to laminin-γ1 and displays comparable binding to other BM components (Salmivirta et al., 2002). Nevertheless, nidogen-2 deposition was virtually undetectable after Lγf treatment (data not shown). Preliminary data from 3D-cocultures with nidogen-1 lacking fibroblasts indicated a compensation for BM assembly by nidogen-2 and thus, a BM dependence on the general nidogen level (C.S., E. C. Wönne, A. Baranowsky, R. Nischt, N. Smyth, N.M., N.E.F. and D.B., unpublished data).

Epithelial adhesion is weakened upon functional nidogen depletion

In the Lγf-treated 3D-cocultures the matrix-epidermal junction appeared more fragile, but adhesion was obviously sufficient to allow regular growth and epithelial stratification. Although the frequently observed separation of the tissue compartments presumably occurred during sample preparation and processing, it reflects a weaker attachment of the epithelium to the extracellular matrix. This mimics blister formation under tension in skin of patients suffering from forms of epidermolysis bullosa (EB) with severe defects in specific BM molecules (for a review, see Pulkkinen and Uitto, 1999). By and large examination of the main matrix receptors, epidermis-typical integrins, revealed no major alterations. In spite of a somewhat less pronounced polarity in the treated epithelia (regarding α3, αv and β1 integrin subunits), the α2 chain was slightly more concentrated towards the basal aspect. This fits with a preferential binding to collagen fibrils, confirmed by our ultrastructural data (Fig. 5D), but α3 may contribute equally well in accordance with the observed normal phenotype of α2 knockout mice (Holtkötter et al., 2002). A second striking finding by IIF was the shedding of cell membrane material, being most pronounced for integrin α6β4, when treated with Lγf (Fig. 6; correlating also with EM, see below).

Ultrastructural changes in the epithelial junctional zone

As in regenerating epithelial wounds, the development of an ultrastructurally distinct basement membrane in control 3D-cocultures was correlated to a bright, linear staining for BM laminin, type IV collagen, nidogen and perlecan, observed by IIF. Although considered to be a major component of the BM lamina densa, the marked type IV collagen deposition seen in these Lγf-blocking experiments, was not sufficient for building up an ultrastructurally defined BM. However, the lack of other components was presumably also responsible for the loosely organised type IV collagen scaffold, detectable by ImEM (Fig. 7) and confocal laser scan microscopy (not shown). Furthermore, in contrast to the controls we could not detect any hemidesmosomes, regularly mediating epidermal attachment in skin. There were also no transitional forms or so-called type II hemidesmosomes (Green and Jones, 1996; Nievers et al., 1999), as were present in epithelial transplants of malignant HaCaT-ras cells with largely deficient BM (Tomakidi et al., 1999). Accordingly, upon Lγf treatment anchoring filaments, normally associated with hemidesmosomes, did not form despite the abundance of laminin-5. Instead, the ultrastructural distribution of laminin-5 was less ordered, revealing extensive displacement (Fig. 7D). Similar irregularities were also seen for the integrin β4 chain and BPAG1 (BP230; Fig. 7F), BPAG2 (BP180; not shown) and HD1 (plectin; not shown), all regular constituents of hemidesmosomes (Green and Jones, 1996; Rezniczek et al., 1998; Borradori and Sonnenberg, 1999; Nievers et al., 1999). Consequently, the keratin tonofilaments in the basal keratinocytes were not attached to the ventral cell borders (Fig. 7H) and there was a striking increase in cell protrusions and membrane blebs. Interestingly, this disturbance of the keratin cytoskeleton had no dramatic consequences for stratification and, apart from a slight delay in the onset, for the regular course of epidermal differentiation. This is in line with a largely normal differentiation pattern, despite epidermal blistering, in epidermolysis bullosa simplex (EBS) patients with defective keratin K5 or K14 (Pulkkinen and Uitto, 1999). Generally, cell-matrix interactions, including their modulation, are considered as crucial signalling events for tissue development (Streuli et al., 1995; Pujuguet et al., 2000) (for reviews, see Miranti and Brugge, 2002; Yamada and Even-Ram, 2002). From the data presented here it would appear that in the case of the epidermis its differentiation is at most only fine-tuned by the dermo-epidermal BM.

Possible fate of BM components upon treatment

Neither accumulations of nidogen nor the Lγf fragment (in treated samples, using domain-specific antibodies) were detectable in the matrix of the 3D-cocultures by IIF, employing various fixation modes (including formaldehyde and/or glutaraldehyde). One phenomenon to be considered was enhanced nidogen turnover upon treatment because of its high sensitivity to proteolysis, when not bound to matrix (Fox et al., 1991; Mayer et al., 1995). As relevant candidates, the matrix metallo-proteinases MMP-2 and MMP-9 are both expressed by epidermal cell lines growing within a type I collagen matrix (Baumann et al., 2000). Furthermore, the tissue collagenase MMP-1 can be activated in keratinocytes growing on collagen, in a complex with integrin α2β1 (Dumin et al., 2001), which may weaken the matrix scaffold and thus, trigger further proteolytic processes.

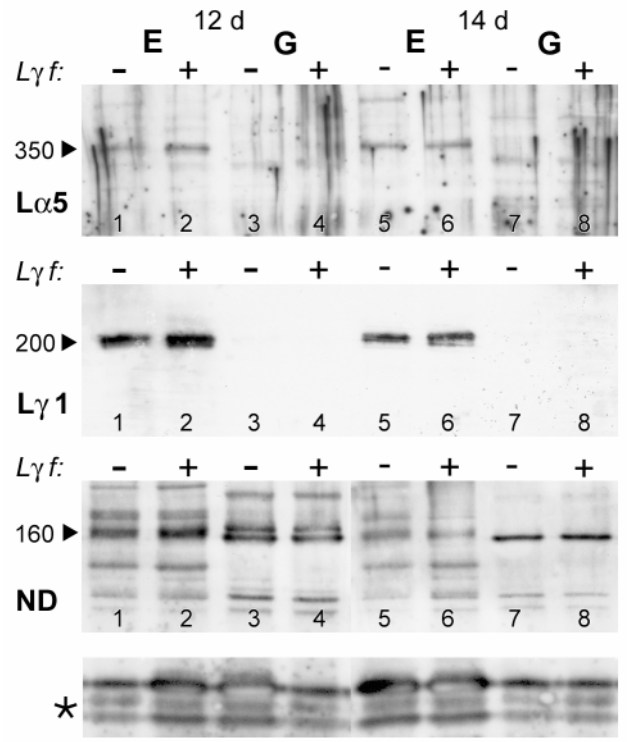

Fig. 8. Protein analysis by western blotting of the separated epithelial and 'dermal' compartments (collagen matrix with resident fibroblasts). Extracts were prepared from 3D-cocultures (–/+ Lγf treatment as indicated) grown for 12 (left panel) or 14 (right panel) days at the air-liquid interface. Reactions with antibodies against the laminin α5 (Lα5) and γ1 chain (Lγ1), and against nidogen (ND) are shown, the lower part of third blot (*****) demonstrates comparable sample loading. The size of the bands of presumptive BM components is indicated in kDa.

However, we could exclude major proteolytic cleavage by direct western blot analysis of the BM components. For the preparation of protein extracts from both tissue compartments a method of separating epidermis from dermis using dithiothreitol (Epstein et al., 1979) proved to be very efficient. Thus, on western blots of controls (Fig. 8) the presence of laminin α5 (major band at >300 kDa) and γ1 (200 kDa) in the epithelial and not in the collagen gel fraction indicated a clean separation. However, we detected nidogen-1 (160 kDa) in both fractions, although always weaker in epithelia, and type IV collagen (180 kDa), in good correlation to previous mRNA expression data (Smola et al., 1998). No significant protein changes were observed in Lγf-treated samples, controlling the efficacy of the blockade by IIF on small pieces of the samples. While the data do not rule out minor proteolytic modifications at the N- or C-terminus, with, for example, possible influence on laminin assembly, they also demonstrate that considerable changes in the overall production of BM components did not occur. This is in accordance with a very recent report on embryoid bodies where the presence of a corresponding binding fragment gave rise to complete redistribution of nidogen and laminin without significant changes in protein levels (Tunggal et al., 2003). In our case another explanation

may be, that upon treatment, nidogen and laminin-10 remain only weakly bound to the keratinocytes and become loosely redistributed, perhaps by being phagocytosed. It is also possible that the loss of initial BM structures affects further the polarised secretion of proteins. We saw the same discrepancy in detection (IF negative, western blots positive) we have observed for laminin-10 when nidogen was not present in this system (C.S., E. C. Wönne, A. Baranowsky, R. Nischt, N. Smyth, N.M., N.E.F. and D.B., unpublished data).

Correlation to models in vivo and further perspectives

In essence, our results would not have been predicted when extrapolating the data from transgenic mice [deleted nidogen-binding site within laminin γ1 (Willem et al., 2002)]. Despite severe organ failure due to malfunctioning BM structures, no dramatic changes had been reported to occur in the dermo-epidermal junction of these animals. Nevertheless, a detailed ultrastructural analysis, also from nidogen double-knockout animals (lacking nidogen-1 and -2) has not been presented yet. This may reveal more subtle irregularities of BM (shown by Willem et al., in other tissues only) or hemidesmosomes, which should be more apparent at higher resolution. From our findings, including localisation by ImEM, one should expect disturbances in the molecular alignment, which seems to be highly defined according to more recent studies on skin ultrastructure (McMillan et al., 2003). Apart from that, in vivo there are probably several stabilising factors or elements, which should largely explain the discrepancies to our results. For example, the high density of hair follicles in skin of new-born mice is strongly enforcing epidermal adhesion, reducing the tendency for blister formation. We had also noted this previously when separating epidermis from dermis for the analysis of keratins (Breitkreutz et al., 1984). Accordingly, problems could arise for example after the first hair cycle or during wound healing, if these animals lived long enough. Furthermore, compared to the ‘soft hydro-gel’ of type I collagen in vitro, the dermal connective tissue consists of a much denser meshwork of collagen fibrils. Together with other dermal constituents, such as microfibrils and in particular highly negatively charged proteoglycans (perlecan and others), the decreased pore size should increase molecular interactions and lower the diffusion rate of larger BM molecules secreted by the epidermis. Certainly, critical concentrations for initiation of the BM assembly process will be reached more readily in vivo. Work in progress suggests that mechanical enforcement of the supporting matrix will further promote BM formation in our in vitro and in vivo models (H.-J.S., M. Wilhauck, N.M., K. Boehnke, A. Pavesio and N.E.F., unpublished data). In general, the equilibrium of all components and their availability at distinct sites at certain times should be crucial, especially in reconstruction and remodelling of the BM. Thus, besides refining structural features nidogen may be a key regulator for the kinetics of BM formation and also critically involved in turnover. From more recent experiments we obtained strong evidence that, up to certain levels, nidogen could actually accelerate this process and may function as a pace maker. In reverse then, it should be rate limiting in terms of the kinetics, in other words lower levels preventing a premature stable assembly. This would also readily explain the more severe impact of nidogen (or binding) defects on epithelial remodelling/branching observed in developmental models (Ekblom et al., 1994; Kadoya et al., 1997). Finally, the initially weak connections between epidermis and dermis may be further strengthened by formation of covalent bonds, for example, catalysed by matrix-associated tissue transglutaminases (for a review, see Lorand and Graham, 2003), an issue which will be investigated in the near future. Nevertheless, our model seems to reveal molecular assembly defects, which obviously did not become apparent in all tissues of the deficient mice, perhaps because of the limited time frame.

Taken together, these results show that the laminin-nidogen complex represents an integrated functional unit, actually linking the internal keratin cytoskeleton with the dermal extracellular matrix, and is required for BM assembly in the coculture model. By avoiding compensatory mechanisms anticipated in vivo, this human organotypic model with a defined input and output allows a specific dissection of BM assembly, including the kinetics. Thus, skin-specific processes can be analysed by measures not compatible with foetal development or postnatal life. In turn, investigations on such compensatory mechanisms or additional elements acting in vivo may become possible by employing this skin-organotypic coculture system. Finally, the combination of both the in vivo and in vitro approach, using cells from transgenic or knockout animals in such a coculture system seems to be very promising according to our current work.

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