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Deficiency of the integrin β4 subunit in junctional epidermolysis bullosa with pyloric atresia: consequences for hemidesmosome formation and adhesion properties

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SUMMARY

Junctional epidermolysis bullosa (JEB) comprises a group of inherited autosomal recessive blistering disorders characterized by dermo-epidermal separation through the lamina lucida of the basement membrane. We identified a patient with JEB associated with pyloric atresia (PA), in whom the integrin β4 subunit was completely absent. At the ultrastructural level, the hemidesmosomes were reduced in number, appeared rudimentary and lacked a subbasal dense plate and frequently an inner attachment plaque. However, keratin filaments were still anchored to the cytoplasmic plaque of the hemidesmosome. Immunofluorescence analysis showed that the β4 subunit was absent in the skin of the PA-JEB patient, whereas the α6 subunit appeared to be normally distributed along the basement membrane zone, as were the other hemidesmosomal components BP230, BP180 and HD1. Furthermore, the α3 and β1 subunits were not only detected at the lateral membranes of basal cells in PA-JEB skin, as in normal skin, but also along the basement membrane zone. The few hemidesmosome-like structures found in cultured keratinocytes from the PA-JEB patient contained the hemidesmosomal components BP230, BP180 and HD1, but not the integrin α6 subunit. Like α3, this subunit was colocalized with vinculin in focal contacts at the ends of actin stress fibers. Immunoprecipitation analysis revealed that α6 was associated with β1 on PA-JEB keratinocytes, whereas normal human keratinocytes (NHKs) exclusively express α6β4 on their cell surface. The initial adhesion of PA-JEB and normal keratinocytes to laminin-1 and laminin-5, both ligands for α6β1 and α6β4, was similar. In migration assays, the PA-JEB keratinocytes were more motile on laminin-5 than normal keratinocytes. Our observations indicate that the integrin α6β4 plays a crucial role in the proper assembly of hemidesmosomes and in the stabilization of the dermal-epidermal junction. The fragility of the skin and the blistering in this patient appear to have been due to the deficiency of the integrin β4 subunit, which results in the formation of too few and structurally abnormal hemidesmosomes.

Key words: Hereditary, Blistering, Integrin, Keratinocyte, Migration

INTRODUCTION

Hemidesmosomes are multi-protein complexes that provide firm adhesion of basal epithelial cells to the underlying basement membrane in stratified and pseudostratified epithelia, and which anchor keratin filaments at their cytoplasmic side. At the ultrastructural level, they consist of an intracellular plaque and a subbasal dense plate, which is thought to correspond to the external surface of the basal cell membrane. Hemidesmosomes are connected to the lamina densa by anchoring filaments (Garrod, 1993; Jones et al., 1994).

Two transmembrane molecules have been identified in the hemidesmosome, the integrin α6β4 (Stepp et al., 1990; Sonnenberg et al., 1991; Jones et al., 1991) and the BP180 molecule (Diaz et al., 1990; Ishiko et al., 1993), both of which are thought to mediate adhesion. The ligands for the α6β4 integrin are laminin-1 and laminin-5 (Lee et al., 1992; Niessen et al., 1994), both present in the basement membrane underlying the epidermis, whereas the ligand of BP180 has not yet been identified. The β4 subunit is unique among the integrin β subunits because of its very long cytoplasmic domain (Hogervorst et al., 1990; Suzuki and Naitoh, 1990) and its association with keratin filaments (Stepp et al., 1990; Sonnenberg et al., 1991). Most other integrins are associated with the actin cytoskeleton. The β1 and β3 cytoplasmic domains are essential for linking actin filaments to focal contacts (Sastry and Horwitz, 1993). This interaction is mediated via associated proteins, like talin and α-actinin, which link the integrin β subunit to F-actin (Horwitz et al., 1986; Otey et al., 1990). Similarly, in hemidesmosomes, cytoplasmic proteins such as

BP230 (Tanaka et al., 1990), plectin (Wiche et al., 1984), IFAP300 (Skalli et al., 1994) and HD1 (Hieda et al., 1992) may link the keratin filaments to the cytoplasmic domain of either the β4 subunit or BP180 or both. Hemidesmosomes in mice that are deficient in BP230 are not associated with keratin filaments (Guo et al., 1995), showing that this hemidesmosomal plaque protein is involved in the anchoring of keratins to the hemidesmosome. The nature of the interactions of BP230 with other hemidesmosomal components that establish the association of keratins with the plasma membrane is not yet known.

Several reports have suggested that α6β4 is essential for the assembly of hemidesmosomes. Antibodies to α6β4 do not only prevent hemidesmosome formation (Jones et al., 1991) but they can also disrupt existing hemidesmosomes and induce detachment of the epidermis (Kurpakus et al., 1991). During epithelial wound healing, the BP230 and BP180 proteins are internalized by keratinocytes, whereas α6β4 remains dispersed over the cell surface (Gipson et al., 1993). Dispase treatment of skin biopsies results in the internalization of hemidesmosomes, after which α6β4 is redistributed to the cell surface, before BP180 and BP230 (Poumay et al., 1994). Thus, α6β4 is one of the first hemidesmosomal components to appear at the basement membrane zone, the site where hemidesmosome formation occurs. Moreover, it has been shown that the cytoplasmic domain of β4 is essential for the association with the hemidesmosomal cytoskeleton (Spinardi et al., 1993) and that the localization of β4 is regulated by tyrosine phosphorylation (Mainiero et al., 1995). Finally, a β4 subunit that lacks the cytoplasmic domain, has a dominant-negative effect on hemidesmosome formation after overexpression in hemidesmosome forming 804G cells (Spinardi et al., 1995). Together, these observations indicate an essential role for α6β4 in the assembly and for the stability of hemidesmosomes.

Junctional epidermolysis bullosa (JEB) is a group of hereditary autosomal recessive disorders characterized by marked skin fragility and blistering in response to minor trauma (Fine et al., 1991). Ultrastructurally, dermo-epidermal separation consistently occurs through the lamina lucida (Eady et al., 1994). The most severe form of JEB is the Herlitz variant, which manifests itself by widespread mucocutaneous blistering and extracutaneous involvement with a poor prognosis. Recently, in some families with the Herlitz variant of JEB, mutations in the genes encoding the laminin-5 subunits have been identified (Pulkkinen et al., 1994a,b; Aberdam et al., 1994; Kivirikko et al., 1995). This laminin isoform is localized to anchoring filaments in stratified squamous epithelia (Rousselle et al., 1991; Carter et al., 1991) and has been shown to serve as a ligand for the integrins α3β1 (Carter et al., 1991; Delwel et al., 1994), α6β1 (Delwel et al., 1993) and α6β4 (Niessen et al., 1994). General atrophic benign epidermolysis bullosa (GABEB) represents another subtype of JEB and is characterized by a relatively benign course (Hashimoto et al., 1976; Hintner and Wolf, 1992). Defective protein expression of BP180 in the epidermal basement membrane has been reported in patients with GABEB (Jonkman et al., 1995, 1996). Moreover, a recent study (McGrath et al., 1995) has identified mutations in the BP180 gene in one GABEB patient. A subgroup of JEB patients is associated with pyloric atresia (PA-JEB). Both lethal and non-lethal cases have been reported for PA-JEB patients (Weber, 1987; Hayashi et al., 1991; Lacour et al., 1992). This disease is characterized by localized blistering of the skin and occlusion of the pylorus. Other clinical features often observed in this subgroup are congenital localized absence of skin and recurrent involvement of the gastrointestinal, respiratory and urinary tracts (Hayashi et al., 1991; Lestrigant et al., 1992). A substantial reduced expression of α6β4 has been shown in several PA-JEB patients (Philips et al., 1994) whereas in one patient no β4 was detectable at all (Gil et al., 1994). In support of this finding, mutations in the β4 gene in PA-JEB have recently been identified that result in a reduced expression of the α6β4 integrin (Vidal et al., 1995). At the ultrastructural level, hemidesmosomes are absent or their number is largely reduced in the lethal variants, whereas in non-lethal JEB, hemidesmosomes are present, although they often are incomplete (Eady et al., 1994).

In this report we describe a patient with PA-JEB, who completely lacked the integrin β4 subunit protein. At the ultrastructural level, hemidesmosomes were observed although they were rudimentary and reduced in number. The integrin α6 subunit was expressed in both skin and cultured keratinocytes, but in association with the β1 subunit, and was localized in focal adhesions. Functional analysis revealed that initial adhesion is comparable between PA-JEB and normal human keratinocytes but that their migration on laminin-5 is different.

MATERIALS AND METHODS

Cells

The squamous cell carcinoma line UMSCC-22B was cultured in DMEM containing 10% FCS, supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Paisley, UK). Normal human keratinocytes were obtained from human foreskin and the keratinocytes of the PA-JEB patient from a skin biopsy. From both tissue samples, keratinocytes were isolated according to the following procedure: the upper dermal and epidermal part, cleared of fibrous tissue and fat, were stretched on a sterile filter paper in a Petri dish with thermolysin (0.5 mg/ml, Sigma Chemical Co., St Louis, MO) and incubated overnight at 4°C. The epidermis was gently stripped from the dermis, collected and incubated with 0.025% trypsin/10 mM EDTA/PBS (Gibco BRL) for maximal 10 minutes at 37°C. The cell suspension was filtered through nylon gauze. Cells were seeded on collagen-coated (Vitrogen 100, Collagen Corp., Palo Alto, CA) 6-well tissue culture plates and grown in keratinocyte-SFM (Gibco BRL), supplemented with bovine pituitary extract (50 μg/ml), epidermal growth factor (5 ng/ml), penicillin (100 i.u./ml), streptomycin (100 μg/ml) and L-glutamine (2 mM). Alternatively, keratinocytes were grown by plating them on irradiated 3T3 cells in HAMF12:DMEM (1:3) containing 10% FCS, penicillin, streptomycin, L-glutamine, hydrocortisone (0.4 μg/ml, Sigma Chemical Co.) and isoproterenol (10^{-6} M). After three days of culture, EGF (10 ng/ml) was added to the medium. The 3T3 cells were plated one day earlier in HAMF12:DMEM (1:3) containing 10% FCS, penicillin, streptomycin and L-glutamine.

Antibodies

The following antibodies directed against human integrin subunits were used: the mouse mAbs P1E6 and P1H5 (anti-α2; Wayner and Carter, 1987), J143 (anti-α3; Kantor et al., 1987), P1B5 (anti-α3; Wayner and Carter, 1987), Sam-1 (anti-α5; Keizer et al., 1987), J8H (anti-α6; Delwel et al., 1994), NKI-M9 (anti-αv; Von dem Borne et al., 1989), TS2/16 (anti-β1; ATCC, Rockville, MD), 450-9D and 450-10D (anti-β4; Kennel et al., 1990) and the rat mAbs AIIB2 (anti-β1; Werb et al., 1989), GoH3 (anti-α6; Sonnenberg et al., 1988), and 439-

9B (anti-β4; Kennel et al., 1989). The mouse mAbs R815, 1D1 and 121 were used to detect BP230, BP180 and HD1, respectively (Owaribe et al., 1991). Vinculin was detected with a polyclonal rabbit serum (Geiger, 1979).

Flow cytometry analysis

Cultured keratinocytes were detached with trypsin and washed with PBS containing 4% FCS. The cell suspension was then incubated with the appropriate dilution of murine anti-integrin subunit antibodies (P1E6, J143, Sam-1, J8H, TS2/16 or 450-9D) for 30 minutes at 4°C. Then they were washed twice and incubated with FITC-conjugated goat anti-mouse IgG (Zymed Lab. Inc., San Francisco, CA). Following a final washing, the cells were resuspended in PBS and analyzed using a FACScan (Becton Dickinson, Mountain View, CA).

Immunoprecipitation

Cells were surface labeled with ^{125}I using the lactoperoxidase method (Sonnenberg et al., 1993) and subsequently lysed in buffer (1% NP-40, 25 mM Tris-HCl, pH 7. 6, 100 mM NaCl and 5 mM EDTA). After centrifugation for 10 minutes at 4°C, the lysate was precleared for 2 hours with Protein A-Sepharose beads. Subsequently, lysates were incubated with the appropriate antibodies, which had previously been coupled to Protein A-Sepharose. When mouse or rat antibodies were used, the Protein A-Sepharose beads were first incubated with rabbit anti-mouse or rabbit anti-rat after which the antibodies were bound. The Protein A beads were washed three times with lysis buffer and two times with PBS after which they were resuspended in SDS-sample buffer. Samples were analyzed on a 5% SDS-polyacrylamide gel under non-reducing conditions.

Immunofluorescence microscopy

Cryostat sections (4 μm) of skin specimens were processed and fluorescence antigen mapping was performed as previously described (Jonkman et al., 1992). Digital video microscopic images of tissue sections were obtained with an imaging system with long exposure times designed for the detection of low levels of fluorescence (Bruins et al., 1995).

For immunofluorescence studies on cultured keratinocytes, glass coverslips were coated with collagen (Vitrogen 100) and keratinocytes were plated in HAMF12:DMEM (1:3) medium containing 10% FCS, penicillin, streptomycin, L-glutamine, hydrocortisone and isoproterenol. After cells had been grown to near confluency, they were fixed with 1% paraformaldehyde in PBS for 10 minutes and permeabilized with 0.5% Triton X-100 for 5 minutes at room temperature. Non specific staining was blocked by incubating the coverslips with 2% BSA in PBS for 30 minutes. After incubations with primary antibodies for 30 minutes at 37°C the coverslips were washed three times with PBS and then incubated for 30 minutes with secondary antibodies conjugated with fluorescein (FITC). For double staining of actin filaments and integrin subunits, the cells were incubated with rhodamine-conjugated phalloidin (Sigma Chemical Co., St Louis, MO), anti-integrin α3 or α6 subunits mAbs and FITC-conjugated goat anti-mouse IgG. Bound rabbit anti-vinculin and murine anti-α3 or α6 mAbs were detected in double labeling experiments with FITC-conjugated goat anti-mouse and Texas red-conjugated anti-rabbit IgG (Amersham International, Buckinghamshire, UK). For double staining of BP180 and the α6 integrin subunit, the cells were first incubated with the murine mAb (1D1) to BP180, followed by FITC-conjugated goat anti-mouse IgG and subsequently with the rat mAb (GoH3) to α6, followed by rabbit anti-rat specific IgGs and Texas red-conjugated donkey anti-rabbit IgG. Coverslips were mounted in Vectashield (Vector Laboratories, Burlingham, CA), and viewed under a Bio-Rad MRC-600 confocal laser scanning microscope (Bio-Rad Laboratories, UK).

Cell adhesion and antibody inhibition assay

Microtiter plates (Greiner GmbH, Frickenhausen, Germany) were incubated with 100 μl of 20 μg/ml EHS tumor laminin-1 (Collaborative Biomedical Products, Bedford, MA) or a laminin-5 matrix deposited by RAC-11P cells as described (Sonnenberg et al., 1993). Cell adhesion assays were performed as previously described (Delwel et al., 1993). In antibody inhibition assays, ascitic fluid at a 1:100 dilution or hybridoma culture medium at 1:2 were added to the cell suspension, 10 minutes before plating the cells onto the substrate.

Cell migration assay

Coverslips were coated either with fibronectin (20 μg/ml) or BSA (100 μg/ml) and placed in 24-well polystyrene flat bottom plates. Laminin-5 matrix was deposited on the glass coverslips by the murine RAC-11P cell line (Sonnenberg et al., 1993). A colloidal gold salt suspension was added onto the matrix coated coverslips and incubated overnight at 4°C. After removal of unbound gold particles, cells were plated at a density of 2×10^3 cells/ml in keratinocyte-SFM medium containing 1% BSA. Cells were allowed to migrate for 15 hours after which they were fixed in 2% formaldehyde. Migration was quantified by computer-assisted image analysis of independent, randomly chosen fields. Results were expressed either as the percentage area of each field occupied by phagokinetic tracks, the so-called migration index (Woodley et al., 1988) or as the mean phagokinetic track left by one cell, with $n\geq40$. Results of these two quantification methods were comparable.

RESULTS

Clinical description

The junctional epidermolysis bullosa patient with pyloric atresia (PA-JEB) was born from clinically unaffected and unrelated parents. Congenital localized absence of skin was observed from toes to the knees, around the umbilicus and near the neck and left ear. Blisters were present on the lower arms and hands. Erosions were observed on the tongue and around the jaw. The patient died 5 days after birth.

Immunofluorescence analysis of skin biopsies

Neither antibodies against the extracellular domain nor antibodies against the cytoplasmic domain of β4 reacted with skin samples of the PA-JEB patient (Fig. 1F). In normal skin these antibodies reacted with the basal side of the basal keratinocytes (Fig. 1B). Both in normal skin and in skin of the patient, the α6 subunit was present at the basal side of the epidermal cell layer, but there was less α6 in the PA-JEB than in the normal skin (Fig. 1A,E). The hemidesmosomal components HD1 (Fig. 1C,G) and BP230 (Fig. 1D,H) were normally present at the basement membrane zone of the epidermis, although antibodies against both proteins appeared to react more weakly in the skin of the patient. In normal skin, β1 was present only at the lateral surfaces between the basal cells (Fig. 1I), but in PA-JEB skin also near the basement membrane zone of the basal cell layer (Fig. 1K). The distribution of α3 appeared to be similar to that of β1 (Fig. 1J,L).

Electron microscopic analysis of skin biopsies

Electron microscopy studies of PA-JEB skin revealed the presence of hemidesmosomes (Fig. 2A), although in much smaller numbers than in normal skin and they did not contain a subbasal dense plate. In most of them, the inner plaque was also strongly reduced or even absent, although a limited number of keratin filaments were still attached to the hemidesmosomes (Fig. 2B,C). This result indicates that in the

absence of α6β4 the formation of hemidesmosomes can still be initiated, although less efficiently, and that this integrin is important for their maturation and/or their maintenance.

Distribution of hemidesmosomal components in cultured keratinocytes

In cultured keratinocytes of the patient there was no reaction with anti-β4 (Fig. 3E), whereas this subunit was present in NHKs (Fig. 3A). As in NHKs, anti-BP180 antibodies produced a punctate pattern in the PA-JEB cells, representative of hemidesmosome-like structures (Fig. 3F,B). This confirms that hemidesmosome-like structures can be formed in the absence of the integrin β4 subunit. Reaction patterns of other hemidesmosomal components HD1 and BP230 were similar (Fig. 3C,G and Fig. 3D,H). Thus, in keratinocytes derived from the PA-JEB patient other hemidesmosomal components can be incorporated into hemidesmosome-like structures despite the absence of β4.

Association of α6 with β1 in the PA-JEB keratinocytes

Although the integrin α6 subunit can associate with both the β1 and the β4 subunit, in normal human keratinocytes it is almost exclusively associated with β4. In the skin of the β4 deficient patient, the α6 subunit was normally distributed at the epidermal basement membrane zone, although its level of expression appeared to be reduced. Flow cytometry analysis of cultured NHKs and PA-JEB keratinocytes revealed no obvious

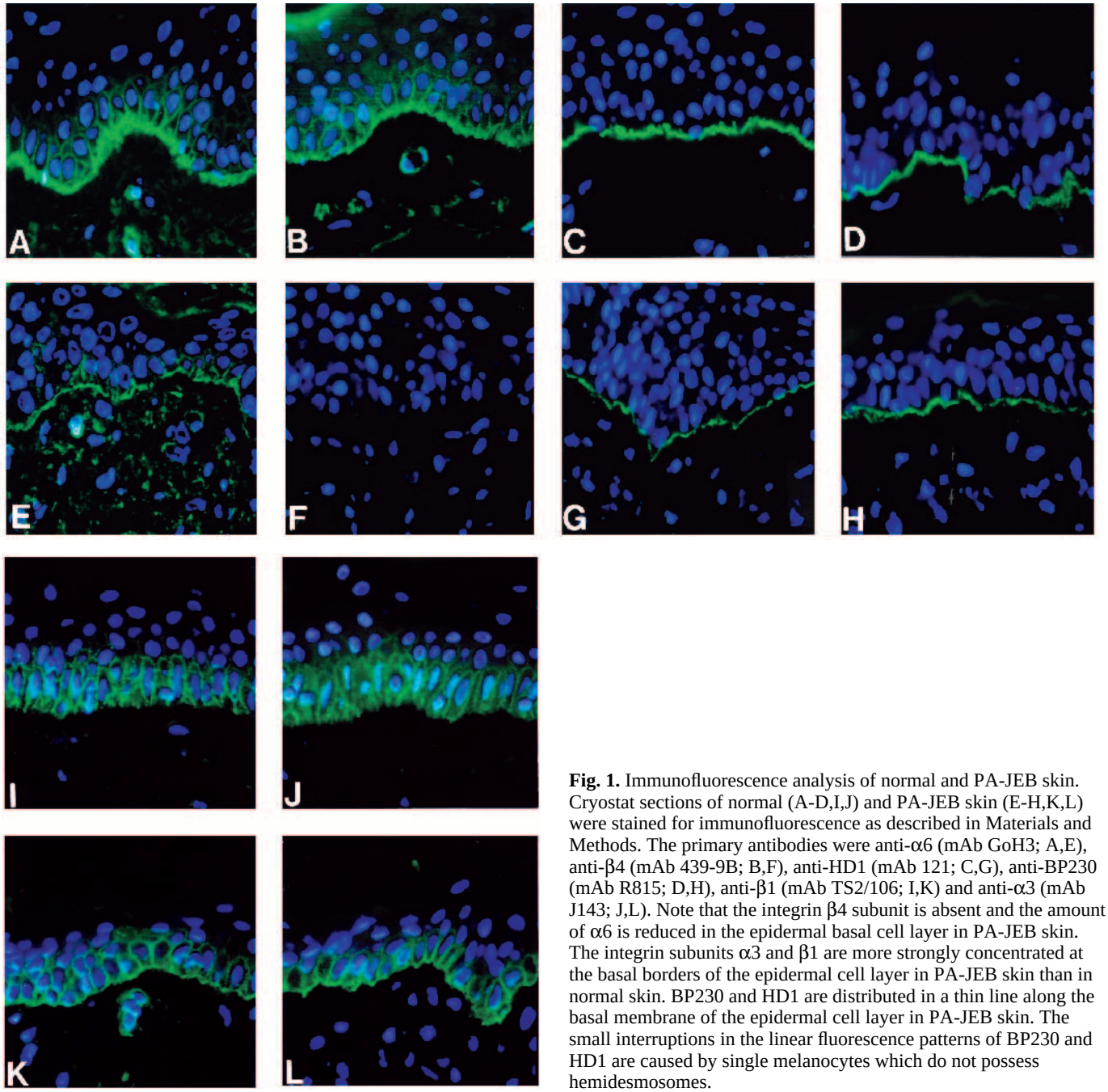

Fig. 1. Immunofluorescence analysis of normal and PA-JEB skin. Cryostat sections of normal (A-D,I,J) and PA-JEB skin (E-H,K,L) were stained for immunofluorescence as described in Materials and Methods. The primary antibodies were anti-α6 (mAb GoH3; A,E), anti-β4 (mAb 439-9B; B,F), anti-HD1 (mAb 121; C,G), anti-BP230 (mAb R815; D,H), anti-β1 (mAb TS2/106; I,K) and anti-α3 (mAb J143; J,L). Note that the integrin β4 subunit is absent and the amount of α6 is reduced in the epidermal basal cell layer in PA-JEB skin. The integrin subunits α3 and β1 are more strongly concentrated at the basal borders of the epidermal cell layer in PA-JEB skin than in normal skin. BP230 and HD1 are distributed in a thin line along the basal membrane of the epidermal cell layer in PA-JEB skin. The small interruptions in the linear fluorescence patterns of BP230 and HD1 are caused by single melanocytes which do not possess hemidesmosomes.

differences in the cell surface expression of α6 (Fig. 4). Furthermore, the α2, α3, α5 and β1 subunits seem to be equally expressed, but no β4 was detected on the PA-JEB keratinocytes, as expected. Since in addition to α6, β1 is also concentrated at the basement membrane zone of the skin of the PA-JEB patient, these two subunits are probably associated to form α6β1. This was confirmed by immunoprecipitation of lysates of ^{125}I surface labeled PA-JEB keratinocytes and NHKs (Fig. 5). Antibodies against α6 precipitated α6 and β4 from NHKs, whereas from the PA-JEB keratinocytes α6 and β1 were precipitated but not β4 (Fig. 4, lane 6). A mAb to β1 precipitated β1, together with the α2, α3, α5 and α6 subunits from the PA-JEB keratinocytes (Fig. 4, lane 5) but only α2, α3 and α5 were co-precipitated with β1 from NHKs (Fig. 4, lane 5). Immunoprecipitation by anti-α2, anti-α3, anti-α5 and anti-αv antibodies from NHKs and PA-JEB keratinocytes was the same (Fig. 4, lanes 1, 2, 3 and 4). BP180 was precipitated from both cell types by antibodies against it (Fig. 4, lane 8). Thus, α6 is associated with β1 on the cell surface of cultured keratinocytes of the patient.

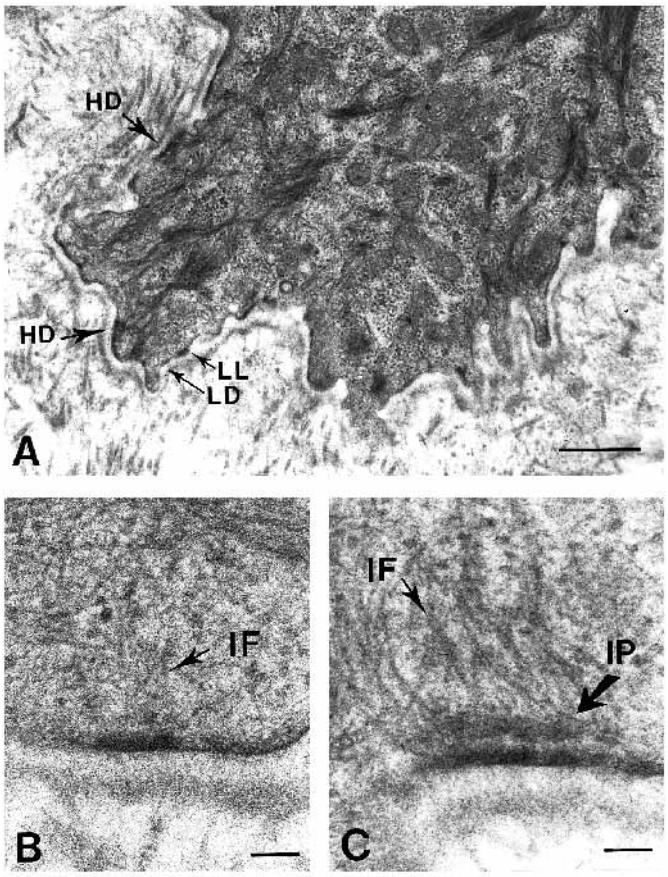

Fig. 2. Electron microscopy of clinically normal skin of the PA-JEB patient. Several hemidesmosomes (HD) with electron dense attachment plaques and associated intermediate filaments are present at the basal plasma membrane of keratinocytes. The basal lamina is continuous and composed of a lamina lucida (LL) and lamina densa (LD). (B and C) Details of two hemidesmosome-structures. The more complete hemidesmosome in C shows a tripartite plaque structure, membrane associated and inner plaque (IP), to which many intermediate filaments (IF) are attached. Fewer IF are associated with the hemidesmosomal structure shown in B. Bars: (A), 500 nm; (B and C), 100 nm.

Distribution of α6 and α3 integrin subunits in cultured keratinocytes

Recently, Hopkinson et al. (1995) showed that α6 interacts with BP180. The α6 subunit is associated with β1 in the PA-

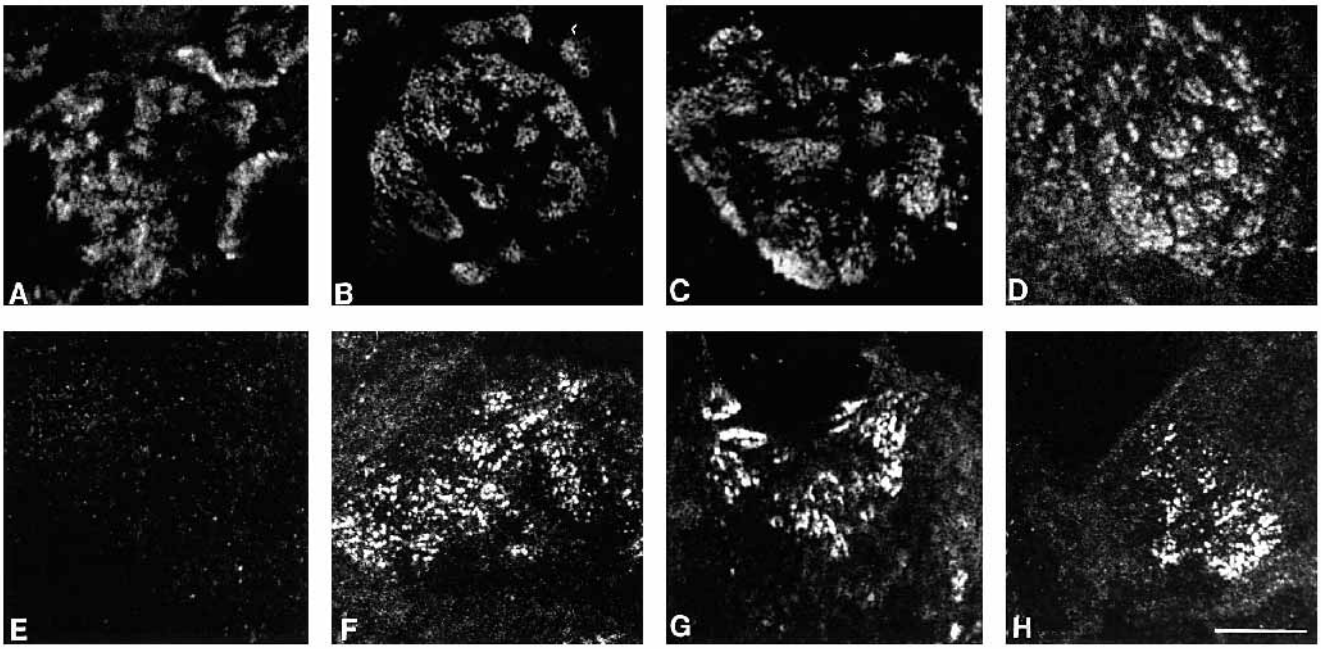

Fig. 3. Immunofluorescence analysis of normal and PA-JEB keratinocytes. NHKs and PA-JEB keratinocytes grown on glass coverslips were examined by immunofluorescence using anti-β4 (A,E), anti-BP180 (B,F), anti-HD1 (C,G) and anti-BP230 mAbs (D,H). No β4 reactivity is observed in cultured PA-JEB keratinocytes, but other hemidesmosomal components are distributed in a punctate staining pattern (E-H), comparable to that in NHKs (A-D). Bar, 25 μm.

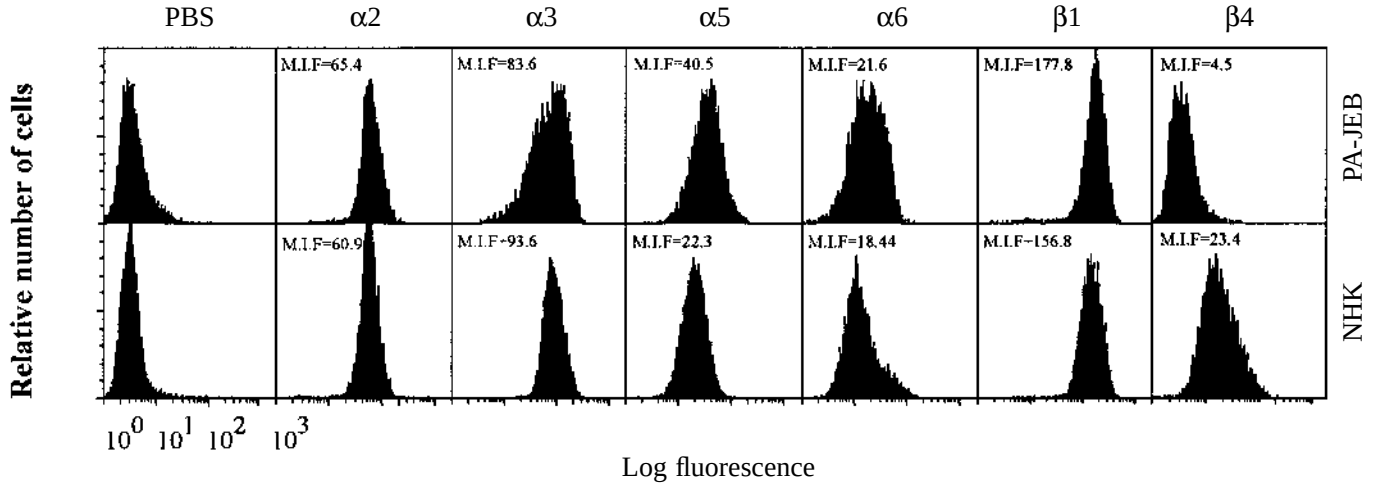

Fig. 4. Cell surface expression of integrin subunits on NHKs and PA-JEB keratinocytes. Keratinocytes were studied by flow cytometry using mAbs against α2, α3, α5, α6, β1 and β4, followed by incubation with FITC-labeled goat anti-mouse IgG. The levels of expression of integrin subunits, except β4, were similar on NHKs and PA-JEB keratinocytes. M.I.F., mean immunofluorecence intensity.

JEB keratinocytes, and because β1 is normally linked to the actin cytoskeleton, we investigated whether α6 is present in focal contacts or in hemidesmosome-like structures, due to its interaction with BP180. Immunofluorescence staining of NHKs and PA-JEB keratinocytes with both anti-α6 and anti-vinculin showed that in NHKs, the α6 subunit is not co-distributed with vinculin; the α6 staining pattern was punctate (Fig. 6B), comparable to that of other hemidesmosomal components (Fig. 3), whereas vinculin was found in the focal contacts. In contrast, in the PA-JEB keratinocytes, the staining patterns of α6 and vinculin overlapped, indicating the presence of α6 in focal contacts (Fig. 6F). As a control, both kinds of keratinocytes were also double-stained with anti-α3 and anti-vinculin, and in both, α3 and vinculin were found together in focal contacts (Fig. 6A and E). Furthermore, we found that the distribution of F-actin and α6 does not overlap in the NHKs (Fig. 6D), as expected because hemidesmosomes are associated with the keratin filaments. In the PA-JEB keratinocytes, α6 was found at the ends of the actin stress fibers, and thus the α6 containing focal contacts are indeed attached to the actin filaments (Fig. 6H). Similarly, α3 was found in the focal contacts at the ends of the actin stress fibers in both the NHKs and the PA-JEB keratinocytes (Fig. 6C,G). Staining of the PA-JEB keratinocytes for both BP180 and α6 revealed no co-localization (Fig. 7), indicating that these molecules are located in different structures.

Adhesion to laminin-1 and laminin-5

The deficiency for β4 results in the absence of the α6β4 integrin and, as we show, in the expression of α6β1. Both α6β1 and α6β4 are receptors for laminin-1 and laminin-5 (Delwel and Sonnenberg, 1996), and they are both present in the basement membrane underlying the epidermis. The blisters in the PA-JEB patient may have been due to reduced attachment of the basal epidermal cells to the basement membrane. An adhesion assay was performed with the NHKs and the PA-JEB keratinocytes using laminin-1 and laminin-5 as substrates. The human squamous cell carcinoma line UMSCC-22B was used as a control. All three cell types adhered to laminin-1 (Fig. 8), although the percentage of binding was low (~10%) for both

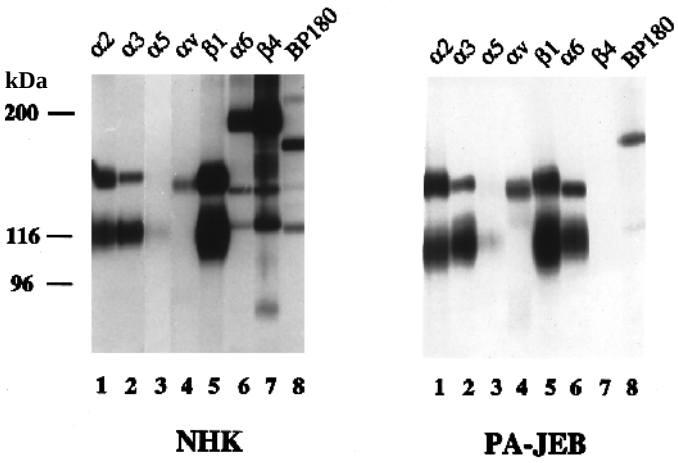

Fig. 5. Immunoprecipitation of integrin complexes and BP180 from NHKs and PA-JEB keratinocytes. Lysates of ^{125}I-labeled NHKs and PA-JEB keratinocytes were immunoprecipitated with mAbs against α2 (P1E6; lane 1), α3 (J143; lane 2), α5 (Sam-1, lane 3), αv (NKI-M9; lane 4), β1 (TS2/16; lane 5), α6 (GoH3; lane 6), β4 (439-9B; lane 7) and BP180 (1D1; lane 8). Samples were analysed on SDS-polyacrylamide (5%) gels under non-reducing conditions.

NHKs and PA-JEB keratinocytes, whereas the percentage of adhering UMSCC-22B cells was about 60%. All cell types adhered better to laminin-5 than to laminin-1 (Fig. 8). Binding to laminin-5 of both types of keratinocytes was comparable (~50%) whereas 90% of the UMSCC-22B cells bound to laminin-5. In conclusion, we found that the ability of the PA-JEB keratinocytes to adhere to laminin-1 and laminin-5 under static conditions was comparable to that of normal keratinocytes.

Integrin receptors involved in adhesion to laminin-1 and laminin-5

Keratinocytes express several integrins that can function as a receptor for laminin-1 (α2β1, α6β1 and α6β4) and/or laminin-5 (α3β1, α6β1 and α6β4). Antibody inhibition studies were

performed to determine the role of the α2β1, α3β1, α6β1 and α6β4 integrins in the adhesion to laminin-1 or laminin-5. Adhesion of the PA-JEB keratinocytes to laminin-1 was partially inhibited by antibodies to α2, α3 and α6 (Fig. 9A), although the extent of inhibition by the different antibodies varied; anti-α6 (mAb GoH3) almost completely blocked adhesion whereas anti-α2 (mAb P1H5) inhibited adhesion by only 30%. A reduction of adhesion to 60% was observed with the anti-α3 mAb (P1B5), suggesting that α3β1 is involved in the adhesion to laminin-1. As expected, the anti-β1 (AIIB2) antibody completely inhibited the adhesion of the PA-JEB keratinocytes to laminin-1. A similar effect was found using a combination of either mAb to α2 and α6 or to α3 and α6. The combination of the anti-α2 and -α3 mAbs blocked adhesion for 75%. The adhesion of the NHKs and the UMSCC-22B cells to laminin-1 was similarly inhibited (Fig. 9A). Again, anti-α2, -α3 or -α6 mAbs inhibited adhesion only partially although each to a different extent, whereas a combination of the mAbs to α2 or α3 with anti-α6 mAbs inhibited adhesion by more than 90%, and of anti-α2 and -α3 by more than 80%. The anti-β1 mAb also completely inhibited adhesion to laminin-1, which was unexpected because the α6 mAb alone inhibited adhesion by more than 50% and α6 is almost exclusively associated with β4 on NHKs and UMSCC-22B cells. The weak binding to laminin-1 of the PA-JEB keratinocytes was mediated by α2β1, α3β1 and α6β1, whereas the laminin-1 binding of NHKs and UMSCC-22B cells was mediated by α2β1, α3β1 and α6β4.

Adhesion of the PA-JEB keratinocytes to laminin-5 was only partially blocked by anti-α3 mAb whereas anti-α2 or anti-α6 mAbs did not inhibit binding at all (Fig. 9B). Inhibition by the combination of anti-α2 and -α3 was not stronger than by anti-α3 alone. A combination of mAbs to α3 and α6 completely inhibited the adhesion of the PA-JEB keratinocytes to laminin-5, as did the anti-β1 mAb. The adhesion of NHKs to laminin-5 was only partially inhibited by anti-α3 or anti-β1 antibodies (Fig. 9B). The anti-α2 or anti-α6 mAbs had no effect. Again, the combination of anti-α2 and anti-α3 mAbs inhibited adhesion to the same extent as the anti-α3 mAb alone. The combination of anti-α3 and anti-α6 mAbs completely abolished binding of NHKs to laminin-5, whereas adhesion of the UMSCC-22B cells to laminin-5 was inhibited by 25%. No or little inhibition of adhesion of UMSCC-22B to laminin-5 was observed with either the anti-α3 or -α6 alone or with the β1 mAb. Thus, adhesion to laminin-5 is mediated by both α3β1 and α6β1 on the PA-JEB keratinocytes whereas the NHKs use the α3β1 and α6β4 integrins for binding to this ligand.

Migration of NHKs and PA-JEB keratinocytes on laminin-1 and laminin-5

It has been suggested that α6β4 mediates stable adhesion whereas α3β1 is involved in dynamic adhesion in spreading and migratory cells (Carter et al., 1990). Using the PA-JEB keratinocytes we were able to study the migratory properties of keratinocytes in the absence of α6β4. PA-JEB keratinocytes and NHKs were plated for 15 hours on laminin-5, fibronectin or BSA substrates. Both types of keratinocytes migrated only minimally on BSA, resulting in very small round tracks (Figs 10A,C, 11). The extent of migration was comparable for the two types of keratinocytes, when plated on a fibronectin matrix (Fig. 10B,D). Both cell types were motile when plated on a laminin-5 matrix (Fig. 10E,F), although to a different extent: the PA-JEB keratinocytes migrated over a two times longer distance than the NHKs (Fig. 11). This observation indicates that the absence of α6β4 results in a higher motility on a laminin-5 substrate and that α6β4 is not required for migration of keratinocytes.

DISCUSSION

In this paper we describe a patient with pyloric atresia-junctional epidermolysis bullosa (PA-JEB), who was deficient for the integrin β4 subunit. Deficiencies in the expression of β4 have previously been reported to be associated with JEB combined with PA, but in all these cases, except one, expression of β4 was only reduced (Philips et al., 1994; Gil et al., 1994). In one case, the molecular basis for this reduction in β4 expression has been defined (Vidal et al., 1995). This particular patient appeared to be a compound heterozygote, containing two affected β4 alleles. In one allele of the β4 gene a single base was deleted resulting in a premature stop codon, whereas in the other allele a mutation in a splice donor consensus sequence had occurred, resulting in the skipping of an exon encoding a 17 amino acid fragment in the cytoplasmic domain of the β4 subunit. The mutant β4 subunit translated from this allele was still expressed at the surface in combination with the α6 subunit.

The skin of the PA-JEB patient contained only a few complete hemidesmosomes. Most of the hemidesmosomes appeared hypoplastic and lacked an inner cytoplasmic plaque. Moreover, they all lacked a subbasal dense plate. Nevertheless, the fact that hemidesmosome-like structures can be recognized indicates that their formation can be initiated in the absence of α6β4. This finding was unexpected because of previous in vitro results suggesting that hemidesmosomes cannot be formed without α6β4 (Jones et al., 1991; Kurpakus et al., 1991). GABEB patients, who are deficient in the BP180 molecule, also have rudimentary hemidesmosomes (Jonkman et al., 1995, 1996). Apparently, the presence of one adhesion molecule, either α6β4 or BP180, is sufficient to initiate the formation of hemidesmosomes, but not for their normal maturation. The hemidesmosomes in PA-JEB are more severely affected than in GABEB patients, in whom the subbasal dense plate is still present. This suggests that for stable adhesion to the underlying basement membrane α6β4 is more important than BP180.

Hemidesmosomes are multi-protein complexes and the interactions between their different components have not yet been elucidated (Jones et al., 1994). In the skin of both PA-JEB and GABEB patients, other hemidesmosomal components, such as BP230 and HD1, are found at the basement membrane zone of the basal cell layer, and these molecules are localized in hemidesmosome-like structures in cultured keratinocytes of these patients (Jonkman et al., 1995, 1996; McGrath et al., 1995). Although from these results it seems fairly certain that these hemidesmosomal components are present in the rudimentary hemidesmosomes, it would have been worthwhile to confirm their presence by immunoelectronmicroscopy. Unfortunately, no further skin samples could be obtained from the PA-JEB patient to perform such an

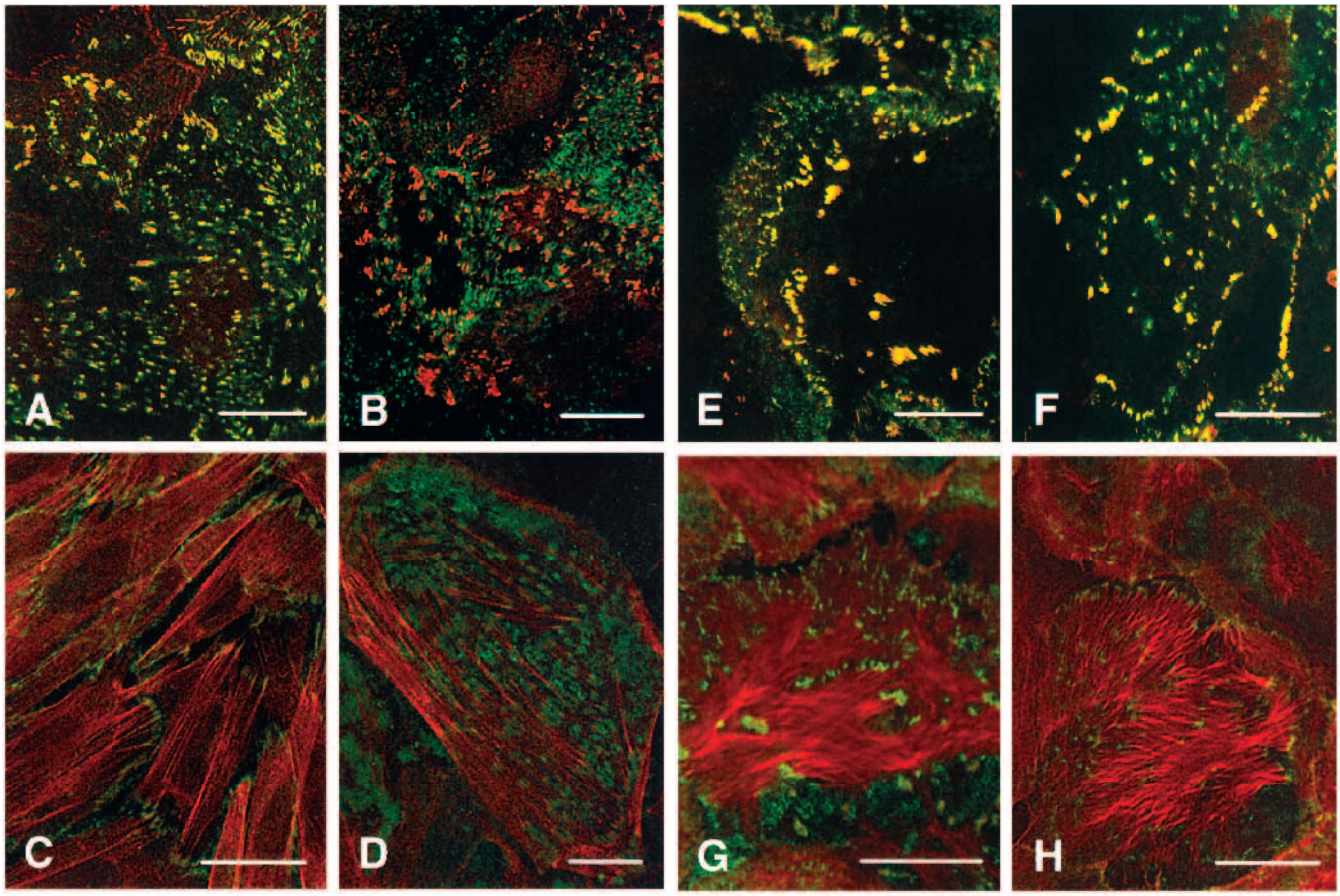

Fig. 6. Immunolocalization of the integrin α3 and α6 subunits in NHK and PA-JEB keratinocyte cultures. Cells were double-labeled by immunofluorescence for α3 (green) and vinculin (red) in A and E, α6 (green) and vinculin (red) in B and F, α3 (green) and actin (red) in C and G, and α6 (green) and actin (red) in D and H. Note that in NHKs the α3 subunit is codistributed with vinculin at the ends of actin filaments whereas α6 is not. This subunit is present in hemidesmosome-like structures. In contrast, in the PA-JEB keratinocytes both α3 and α6 are colocalized with vinculin at the ends of actin stress fibers. Bars, 25 μm.

analysis. In the skin of BP230 null mutant mice hemidesmosomes are present, although they lack an inner cytoplasmic plaque and keratin filaments are no longer anchored to their cytoplasmic face (Guo et al., 1995). Also in these mice, the other hemidesmosomal components are found at the basal side of the epidermis, as in normal skin. Thus, in the absence of either BP180, BP230 or α6β4, other hemidesmosomal components are localized together, suggesting multiple interactions between the various components of hemidesmosomes. Partial interruption of this network of interactions by the loss of one of these components may be the cause of rudimentary hemidesmosomes.

BP180 was recently found to co-precipitate with α6β1 from cells that do not express other hemidesmosomal components, suggesting an interaction between α6 and BP180 (Hopkinson et al., 1995). However, in the PA-JEB keratinocytes α6β1 and BP180 were not colocalized: the α6β1 integrin was found in focal contacts, whereas BP180 was distributed in a hemidesmosome-like pattern. Since PA-JEB keratinocytes, unlike the cells used by Hopkinson et al. (1995) express not only α6β1 and BP180 but also other hemidesmosomal components, BP180 may have a higher affinity for the latter than for α6.

The expression of the α6β4 integrin is virtually restricted to cells that are in direct contact with a basement membrane (Sonnenberg et al., 1990a; Kennel et al., 1992). It is conceivable that it plays a role in the assembly of basal lamina. However, ultrastructural analysis of PA-JEB skin showed the presence of an apparently normally developed basement membrane underlying the epidermis. Our observation suggests that a basement membrane can be formed in the absence of α6β4, either because other receptors, e.g. α3β1 and/or α6β1, also take part in its formation and are sufficient for it or because the formation of basement membranes is independent of receptors.

The initial adhesion of NHKs and PA-JEB keratinocytes to laminin-1 and laminin-5, which are both ligands for the α6β1 and α6β4 integrins (Lee et al., 1992; Sonnenberg et al., 1993; Delwel et al., 1993; Niessen et al., 1994; Spinardi et al., 1995; Delwel and Sonnenberg, 1996), was only slightly different. The weak binding of the PA-JEB keratinocytes to laminin-1 was mediated by α2β1, α3β1 and α6β1 whereas the NHKs and the UMSCC-22B cells used α2β1, α3β1 and α6β4 as receptors. With the exception of α2β1, the same set of receptors were involved in the binding of PA-JEB keratinocytes (α3β1 and α6β1) and NHKs (α3β1 and α6β4) to laminin-5. The α2β1 integrin binds to domain VI on the laminin-1 molecule (Pfaff et al., 1994). There is no similar domain on the laminin-5 molecule, which explains why α2β1 does not mediate adhesion of NHKs and PA-JEB keratinocytes to this laminin isoform.

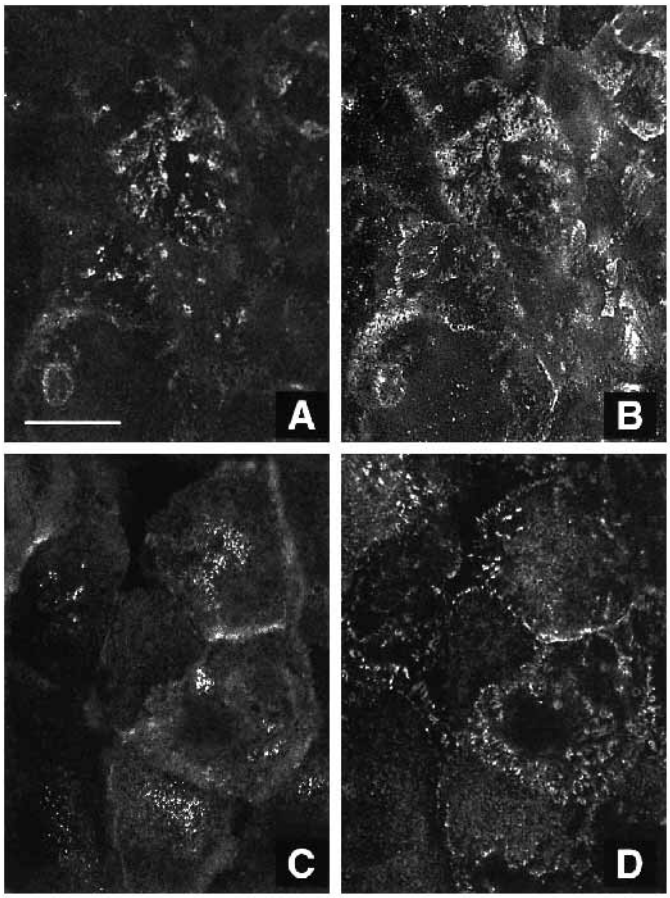

Fig. 7. Comparative localization of α6 and BP180 in NHKs and PA-JEB keratinocyte cultures. Cells were double-labeled with immunofluorescence for α6 (A and C) and BP180 (B and D). BP180 and α6 are colocalized in hemidesmosome-like structures in NHKs (A and B) but not in PA-JEB keratinocytes (C and D). In PA-JEB keratinocytes, the staining of α6 is typical for focal contacts whereas that of BP180 is the punctate pattern typical for hemidesmosomes. Bar, 25 μm.

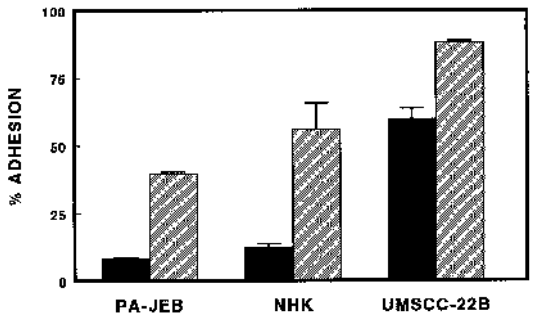

Fig. 8. Comparison of adhesion of cultured NHKS, PA-JEB keratinocytes and UMSCC-22B cells to laminin-1 (filled bars) and laminin-5 (striped bars). Cells were tested for adhesion to laminin-1 or to laminin-5 rich matrix deposited by RAC-11P/SD cells. Results are expressed as the percentage of the total number of cells added to the coated substrates and represent the averages ± s.d. of triplicate values within a representative of three experiments.

Surprisingly, the β1 mAb completely inhibited the binding of NHKs and UMSCC-22B cells to laminin-1, although the α6

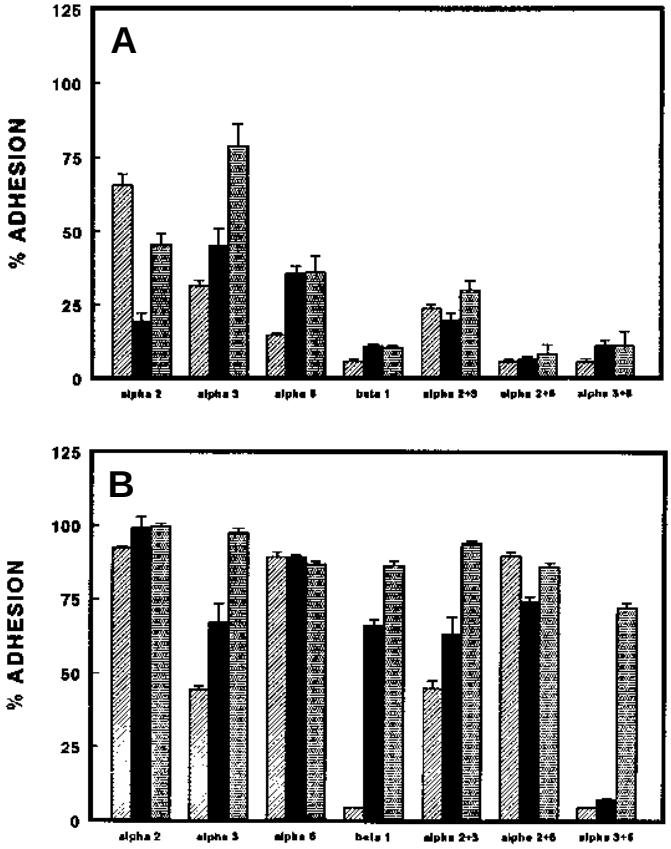

Fig. 9. Involvement of integrins in adhesion of NHKs and PA-JEB keratinocytes to laminin-1 and laminin-5. Cell adhesion experiments to laminin-1 (A) and laminin-5 (B) were performed in the presence of antibodies against integrin subunits. The antibodies were P1H5 (anti-α2), P1B5 (anti-α3), GoH3 (anti-α6) and AIIB2 (anti-β1), or combinations thereof. Binding in the absence of antibodies is indicated as 100%. Data are presented as the averages ± s.d. of triplicate values. This experiment was repeated twice and produced the same findings. PA-JEB keratinocytes (striped bars) adhere to laminin-1 by the α2β1, α3β1 and α6β1 integrins whereas α2β1, α3β1 and α6β4 mediate adhesion of NHKs (filled bars) and UMSCC-22B cells (stippled bars) to this substrate. Binding of PA-JEB keratinocytes to laminin-5 is mediated by α3β1 and α6β1 whereas NHKs use α3β1 and α6β4 for adhesion to this substrate.

subunit is exclusively associated with β4 on these cell types. Anti-α6 mAbs blocked adhesion to this substrate by more than 50%. Inhibition of binding of cells expressing α6β4 to laminin-1 by anti-β1 has also been found by others (Carter et al., 1990; DeLuca et al., 1990; Sonnenberg et al., 1990b). The results of our antibody inhibition studies demonstrated that a combination of antibodies directed against two of the three integrins involved in the adhesion to laminin-1 almost completely blocked binding, suggesting that these cells require at least two functional receptors for proper binding to laminin-1. The anti-β1 mAb interferes with the binding of both α2β1 and α3β1 to laminin-1 and this explains the inhibitory effect of this antibody.

Adhesion of both keratinocyte cell types to laminin-1 and laminin-5 was substantially weaker than that of the human squamous cell carcinoma cell line UMSCC-22B. This was especially striking for the weak binding of the PA-JEB keratinocytes to laminin-1 although these cells express α6β1 on

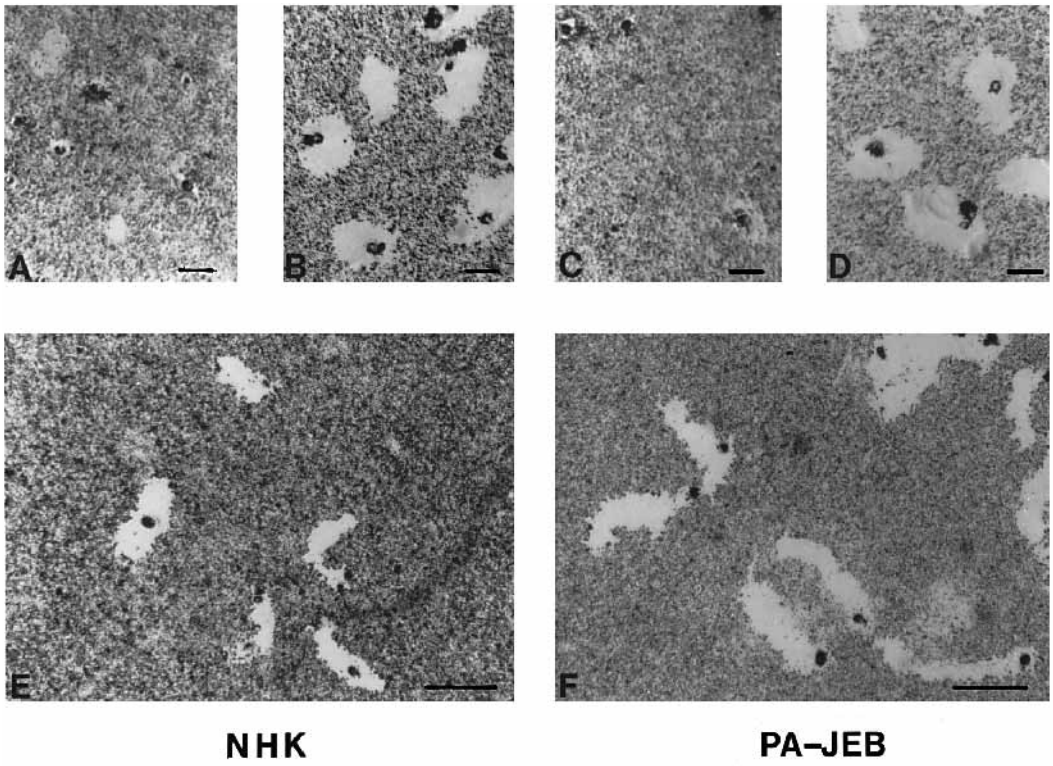

Fig. 10. Migration of NHKs and PA-JEB keratinocytes on different substrates. Cells were seeded on colloidal gold particles deposited onto glass coverslips coated with BSA (A and C), fibronectin (B and D) or laminin-5 (E and F) and allowed to migrate for 15 hours, after which the cells were fixed. Photographs were taken under bright field microscopy. Bars, 10 μm.

their cell surface, which in other cell systems has been shown to mediate efficient binding to laminin-1. Possibly, the integrins expressed on the keratinocytes are partially inactivated. Indeed, it has been shown that integrins are functionally inactivated upon differentiation of keratinocytes, before their expression is downregulated (Hotchin and Watt, 1992). Moreover, Jones and Watt (1993) showed that in a keratinocyte population only a small percentage of the cells, representing undifferentiated keratinocytes, adhered rapidly to extracellular matrix components. The keratinocytes we used were possibly in a differentiation state at which integrins are still expressed on the cell surface but have already been partially inactivated.

Both normal and patient keratinocytes bound more strongly to laminin-5 than to laminin-1, probably because these cells express two high affinity receptors for laminin-5 (α3β1 and α6β4 on NHKs and α3β1 and α6β1 on PA-JEB keratinocytes) and only one high affinity receptor for laminin-1 (either α6β4 or α6β1). In the skin of the PA-JEB patient both α3 and β1 were more concentrated at the basement membrane zone of the basal cell layer than in normal human skin. Because α3β1 as well as α6β1 contribute to the adhesion of the PA-JEB keratinocytes to laminin-1 and laminin-5 in vitro, it is likely that these integrins have a similar role in the epidermis of the patient. Their presence at the basal side may partially compensate for the loss of the α6β4 integrin by providing an alternative keratinocyte-basement membrane interaction, although it did not prevent the formation of blisters. Our failure to detect a clear difference in the adhesion of PA-JEB keratinocytes and NHKs to laminin-1 and laminin-5 may be due to the short incubation period in our assay in which only initial adhesion can be investigated but not its possible consolidation by the formation of hemidesmosomes. Because in the patient the formation of hemidesmosomes was impaired, the keratinocytes

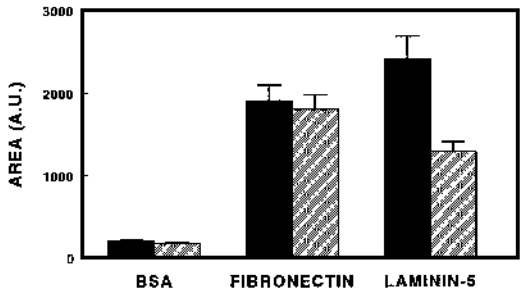

Fig. 11. Quantification of migration of NHKs (striped bars) and PA-JEB keratinocytes (filled bars) on different substrates. Migration was measured as the mean phagokinetic track made by one cell. The error bars represent s.e.m.s of a representative of four experiments, n=50 for BSA, n=40 for fibronectin and n=45 for laminin-5. The PA-JEB keratinocytes are more motile on a laminin-5 matrix than NHKs. No differences between the two cell types are found on fibronectin or BSA.

were likely to adhere less firmly to laminin substrates than the NHKs and this might be the basis for the formation of blisters.

We found that the PA-JEB keratinocytes were more motile on a laminin-5 substrate than the NHKs. Thus, the presence of α6β4 on keratinocytes is not required for migration. In contrast, our results rather suggest that α6β4 inhibits migration, in agreement with the suggestion that α6β4 provides stable adhesion to the extracellular matrix, whereas α3β1-mediated adhesion is dynamic (Carter et al., 1990; Gil et al., 1994).

Both lethal and non-lethal PA-JEB have been described (Weber, 1987; Hayashi et al., 1991; Lacour et al., 1992). It would be interesting to determine if this difference in severity is due to different mutations in β4 that result in either a slight

or severe disturbance of its function. Characterizing the mutations in these two kinds of patients may reveal the importance of certain domains in the β4 molecule. It cannot, however, be excluded that defects in other hemidesmosomal components are also responsible for the difference between PA-JEB subtypes. In conclusion, we describe a PA-JEB patient, in whom the β4 subunit was absent. The formation of hemidesmosomes was initiated in this patient but their number was reduced and they were rudimentary, indicating an important role for α6β4 in the maturation of these structures. The severity of the phenotype with respect to the formation of blisters and the congenital absence of skin suggests an important role for α6β4 in the stable adhesion of the epidermis to the underlying basement membrane.

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