Defective Laminin 5 Processing in Cylindroma Cells

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Cylindromas are benign skin tumors occurring as multiple nodules characteristically well circumscribed by an excess of basement membrane-like material. To determine the molecular defects leading to extracellular matrix accumulation, the ultrastructural, immunological, and biochemical properties of cylindroma tissue and isolated cells were analyzed. In cylindromas, hemidesmosomes are reduced in number, heterogeneous and immature compared to the normal dermal-epidermal junction. Expression of the $\alpha 6 \beta 4$ integrin in tumor cells is weaker than in basal keratinocytes of the epidermis. Moreover, although in the epidermis $\alpha 2 \beta 1$ -integrin expression is restricted to the basal cell layer, it is found in all neoplastic cells within the nodules. Laminin 5 is present throughout the whole thickness of the basement membrane-like zone whereas laminin 10 is restricted to the interface adjacent to the tumor cells. Furthermore, laminin 5 is not properly processed and most of the $\alpha 3 A$ and $\gamma 2$ laminin chains remain as 165-kd and 155-kd polypeptides, respectively. Mature laminin 5 is thought to be necessary for correct hemidesmosome and basement membrane formation and its abnormal processing, as well as the low expression of $\alpha 6 \beta 4$ integrins, could explain the lack of mature hemidesmosomes. Together, the results show that multiple molecular defects, including alteration of laminin 5 and its integrin receptors, contribute to structural aberrations of the basement membrane and associated structures in cylindromas. (*Am J Pathol* 2002, 160:459–468)

Cylindromatosis is a rare disease characterized by the occurrence of multiple benign tumors, most often on the face and the scalp.¹ Sporadic as well as autosomal-dominant inherited cases have been reported^{2,3} and the susceptibility gene, *CYLD*, was identified and mapped to chromosome 16q12-q13.^{4–6} The pathogenesis of these

tumors is controversial, they might be derived from the epithelial ducts of apocrine or eccrine glands and may represent a neoplastic proliferation of epithelial stem cells.^{1,7} Each nodule is surrounded by a hyaline layer of extracellular material closely resembling a basement membrane, although thickened when compared to that of the dermal-epidermal junction. The presence of molecules specific of the dermal-epidermal junction has been demonstrated, including integrin $\alpha 6 \beta 4$, laminin 5, and collagen VII.^{8–11} These represent important constituents of specialized anchoring devices, the hemidesmosomes, which provide a physical link between the epidermal and dermal compartments.¹²

Laminins are a family of multifunctional molecules with a central role in the organization and the physiology of basement membranes.^{13,14} By interacting with cell surface receptors of the integrin family, laminins are responsible for the anchorage of cells and for the initiation of specific intracellular signals. Through other interactions with extracellular matrix proteins, laminins are involved in the architecture, the organization, and the stability of basement membranes. At least three laminin isoforms are present at the dermal-epidermal junction, the most abundant laminin 5 ($\alpha 3 A \beta 3 \gamma 2$), laminin 6 ($\alpha 3 \beta 1 \gamma 1$), and laminin 10 ($\alpha 5 \beta 1 \gamma 1$). Like other laminins, their carboxy-terminal region is formed by the folding of the α chain into five globular domains, LG1 to LG5,¹³ that contain the binding sites for the $\alpha 3 \beta 1$, $\alpha 6 \beta 1$, and $\alpha 6 \beta 4$ epithelial integrins.^{15–17} Because of variations in their amino-terminal parts, these laminins differ in the binding to other extracellular matrix proteins and in their integration into architectural scaffolds. For instance, laminin 10 is thought to polymerize whereas laminin 5 alone does not.¹⁴ Instead, laminin 5 forms a dimer with laminin 6 or 7¹⁸ and binds to collagen VII.^{19,20} In addition, laminin 5 is synthesized as a 460-kd precursor that is extracellularly converted into tissular forms of 440- and 400-kd by enzymatic processing.²¹ A first cleavage of the $\alpha 3 A$ chain occurs in front of the carboxy-terminal domain LG4²² and a second within the amino-terminal domain IIIa,²³ converting the 200-kd precursor chain into 165- and 145-kd polypeptides, respectively. Processing of the 155-kd $\gamma 2$

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chain involves a cleavage within its domain III leading to a 105-kd polypeptide by the removal of the amino-terminal domains L4m and LE1 to LE3.^{23,24} Although the structural and biological consequences of the processing are not known, it can be intuitively hypothesized that it may impinge on the function of laminin 5. In particular, removal of the carboxy-terminal LG4 and LG5 domains may regulate the interaction between the $\alpha 6 \beta 4$ integrin and laminin 5. Similarly, the amino-terminal processing of laminin 5 may regulate the interactions with laminin 6 and collagen VII.

The basal lamina outlining the cylindroma nodules is known to be altered at the ultrastructural level although it contains the same components as the basement membrane of the dermal-epidermal junction. To unravel the underlying molecular defects, we have examined in detail the laminin and integrin repertoire of cylindroma cells and tissues. Ultrastructural, biochemical, and immunological studies indicate an entanglement of multiple defects. In particular, hemidesmosomes are not properly formed in cylindroma cells, laminin 5 is insufficiently processed and accumulates around the neoplastic nodules, and the integrin expression pattern of cylindroma cells resembles that of cells with high proliferation potential such as epidermal stem cells.

Figure 1. Structural organization of laminin 5 and epitope mapping of the antibodies used in the study. During the processing of the laminin $\alpha 3A$ chain, domains LG4 and LG5 are first removed (165-kd form), followed by removal of most of domain IIIa (145-kd form). Conversion of the 155-kd $\gamma 2$ chain into a 105-kd polypeptide involves removal of domains LE1-3 and L4m. The domains removed during laminin 5 processing are indicated by **stripped lines**.

Materials and Methods

Skin Biopsies

Skin biopsies from a patient affected with a sporadic form of cylindromatosis were obtained from surgical excisions of the affected areas at the Department of Dermatology. Some of the samples were immediately frozen in liquid nitrogen and stored at -80°C until used for embedding in tissue-freezing medium. Others were immersed in phosphate-buffered saline (PBS), pH 7.2, for cell cultures. Other samples were placed in a 3% solution of glutaraldehyde in PBS for further processing for histological examination and transmission electron microscopy. The patient signed the informed consent from the Department of Dermatology, University of Cologne, approved by the Institutional Commission of Ethics (Az. 9645/96).

Histology and Transmission Electron Microscopy

A portion of the tumor specimen was postfixed in Bouin fixative containing 75% (v:v) aqueous-saturated picric acid solution, 20% (v:v) formalin, and 5% (v:v) acetic acid for 24 hours and paraffin wax-embedded. Sections ($5\ \mu\text{m}$) were prepared with an ultramicrotome (Reichert-Jung) and further processed for trichromatic Masson staining with hematoxylin, fuchsin-ponceau, molybdc orange-G, and light green successively. Another portion of the sample was washed with 0.1 mol/L Na-cacodylate/HCl, pH 7.4, post-

Figure 2. Ultrastructure of the basement membrane-like zone surrounding cylindroma nodules in comparison to the epidermal basement membrane. **A:** Basement membrane-like zone bordering tumor cells. The thickness of the basement membrane (BM) is indicated by a **line with arrow** at both ends. Note the presence of electron-dense fibrils and filaments (**white arrowheads**) within the basement membrane-like zone. **B:** The basement membrane (BM) at the dermal-epidermal junction is indicated by an **arrowhead**. **Small arrows** point to the invaginations formed by hemidesmosomes at the ventral surface of basal keratinocytes of the normal epidermis. Note that such invaginations are not seen in **A**.

Figure 3. Ultrastructural alterations and heterogeneity of hemidesmosomes in cylindroma cells (**B–E**) compared to normal hemidesmosomes of basal keratinocytes of the epithelium (**A**). Hemidesmosomes (HD), lamina densa (LD), and anchoring fibrils (AF) are marked. Note in **B** to **E** the heterogeneous appearance of tumor cell hemidesmosomes and absence of a distinct lamina densa.

fixed (45 minutes) with 1% OsO₄ in 0.15 mol/L Na-cacodylate/HCl, pH 7.4, dehydrated in graded ethanol and propylene oxide solutions, and embedded in Araldite resin. Ultrathin (60 to 80 nm) sections were prepared with an Ultracut (Reichert-Jung), contrasted with uranyl acetate and lead citrate, and observed with a Philips 201 transmission electron microscope operating at 80 kV.

Antibodies

For indirect immunofluorescence staining primary mouse monoclonal antibodies against laminin chains included BM165 against $\alpha 3$, GB3 against $\gamma 2$ (Seralab), and 4C7 against native $\alpha 5$ chain (Life Technologies). Affinity-purified rabbit antibodies were generated against recombi-

nant fragments of mouse laminin 5 produced in human 293-EBNA cells following established protocols.²⁵ The recombinant polypeptides (Figure 1) included the amino-terminal $\alpha 3$ AIIIa domain (position 1 to 212) and carboxy-terminal modules LG4 and LG5 of the $\alpha 3$ A chain, the amino-terminal LN and LE portion of the $\beta 3$ chain (posi-

Table 1. Morphometric Analysis of Hemidesmosomes

	Epidermis	Cylindroma
Length*	135 $\pm$ 22	197 $\pm$ 60
Number/10 μ m	27	15

*The length was measured for 60 and 34 hemidesmosomes of the epidermis and of the cylindroma cells, respectively. The results are expressed in nm $\pm$ SD.

Figure 4. Indirect immunofluorescence staining of laminins and nidogen in cylindromas (A–C) and at the dermal-epidermal junction (D–F). Cryosections (5 μm) of tissues were incubated with mouse monoclonal antibodies BM165 (A and D) or 4C7 (B and E) against the human laminin α3 and α5 chains, respectively, or with a polyclonal serum against nidogen 1 (C and F). Mouse or rabbit immunoglobulins were detected with Cy3-conjugated secondary antibodies. For each different staining, the pictures corresponding to the cylindroma islands (A–C) or to the dermal-epidermal junction are from the same tissue section. Immunoreactivity corresponding to the laminin α3 chain is observed in the whole thickness of the material surrounding the tumor islets (A). By contrast, immunoreactivity corresponding to the laminin α5 chain (B) or to nidogen (C) is mainly restricted to the inner face of the basement membrane outlining the cylindromas, and for α5 to some intranodular deposits.

tion 17 to 577), and the γ2 chain modules L4m (position 185 to 461) and γ2 LE4–6 (position 460 to 606). The characterization of fragments and antibody specificity after affinity purification will be described elsewhere.²⁶ Integrin subunits were detected with mouse monoclonal antibodies P1E6 against α2, P1B5 against α3, P4C10 against β1, and 3E1 against β4 and rat monoclonal antibody GoH3 against α6 (all from Chemicon, Hofheim, Germany). Rabbit polyclonal antibodies²⁷ were used for immunofluorescence detection of nidogen.

Immunoblotting detection was performed with mouse monoclonal antibodies against laminin α3 (BM 165) or γ2 (D4B5) chains (Chemicon) and with a goat antibody against a carboxy-terminal peptide of the human β3 (sc7651) chain (Santa Cruz, Heidelberg, Germany).

Indirect Immunofluorescence Staining

The biopsied tumor tissue was placed in Tissue Tek OCT Compound (Sakura distributed by Vogel, Giessen, Ger-

Figure 5. Unprocessed laminin 5 is present in cylindroma tissue. Cryosections of biopsied tissue were stained by indirect immunofluorescence with antibodies specific for different domains of the laminin α3A and γ2 chains as indicated on the figure. Staining with γ2 chain-specific antibodies indicate that domain L4m is retained in the laminin 5 deposited around cylindroma (A) but that it has been removed at the dermal-epidermal junction (E) whereas domains LE4–6 are present at both locations (B and F). Anti-α3AIIIa stains the basement membrane area around cylindromas (D) and at the dermal-epidermal junction (H). Anti-α3LG4–5 does not decorate specific structures at the dermal-epidermal junction (G), which indicates that the carboxy-terminal end of the α3A chain has been processed at this location. By contrast, it decorates with a thin line the periphery of the tumor nests and stains intranodular deposits reflecting the presence of unprocessed α3A chain within the tumor islands (C).

Figure 6. Altered processing of laminin 5 in primary cultures of cylindroma cells. The culture medium of cylindroma cells (Cy) and of primary human keratinocytes (Ke) was mixed 1:1 with Laemmli buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions on 4 to 10% acrylamide gels (50 μ l/lane). After transfer to nitrocellulose membranes, the blots were incubated with mouse monoclonal antibodies BM165 (A) or D4B5 (B) against the human laminin α 3 or γ 2 chains, respectively. The blot shown in B was extensively washed and reblotted with a goat antibody against a carboxy-terminal peptide of the laminin β 3 chain (C). Bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence. Molecular weight markers are indicated at the right of the blots. Arrowheads at the left of the blots indicate the migration mobility of the different laminin chains. The relative optical density of the bands corresponding to differently processed forms of the laminin α 3A and γ 2 chains was measured by scanning densitometry. Each column represents the relative percentage of the processed (dots) and unprocessed (stripes) forms of the laminin α 3A (D) and γ 2 (E) chains shown in blots A and B, respectively.

many) and snap-frozen in liquid nitrogen. Cryosections (5 μ m) (cryostat CM3050; Leica Instruments, Nussloch, Germany) were blocked with 1% bovine serum albumin (Fraction V; Serva, Boehringer-Ingelheim, Germany) and incubated with primary antibodies against mouse, rabbit, or rat immunoglobulins (Jackson, distributed through Dianova, Hamburg, Germany). The histoslides were mounted in DAKO fluorescent mounting medium (DAKO, distributed by Dianova) and were observed with a laser confocal microscope (Leica).

Cell Cultures

Cylindroma cell cultures were initiated from excised tumor material. Tumors were dissected from overlying epidermis, minced, and dissociated by collagenase treatment (1 mg/ml; Sigma, Deisenhofen, Germany) in Dulbecco's modified Eagle's medium (DMEM) containing 10 mg/ml of bovine serum albumin (BSA) overnight at 37°C. The enzymatic digestion was stopped by diluting the suspension in DMEM and washing twice in PBS. The cell aggregates were further dissociated in 0.1% trypsin, 0.05% ethylenediaminetetraacetic acid in PBS for 15 minutes at 37°C. Trypsin was blocked by addition of fetal calf serum and the cell suspension was washed in DMEM. Normal human keratinocytes were isolated from post mortem skin as described previously.²⁸ Briefly, after ster-

ilization in betadine solution (10%) for 30 minutes and several washes in PBS, 0.3-mm thick split thickness skin was prepared and incubated in 0.6% trypsin, 0.05% ethylenediaminetetraacetic acid in PBS for 30 minutes at 37°C. The epidermis was separated from the underlying dermis with fine forceps and transferred to DMEM containing 10% fetal calf serum. Epidermal cells were released by gentle aspiration from keratinocyte sheets and a single cell suspension was obtained by filtration through nylon gauze. Dissociated cylindroma cells or normal keratinocytes were seeded at a density of 1 to 2.5×10^4 cells/cm² on feeder layers of irradiated mouse 3T3 cells in culture medium consisting of DMEM and Ham's F12 (3:1, v:v) supplemented with 5% fetal calf serum (Boehringer Mannheim, Mannheim, Germany), 5 μ g/ml insulin, 1.8×10^{-4} mol/L adenine, 10^{-10} mol/L cholera toxin, 0.4 μ g/ml hydrocortisone, 50 μ g/ml ascorbic acid (all from Sigma Chemicals), and 10 ng/ml epidermal growth factor (Collaborative Research, Waltham, MA). Unlike normal human keratinocytes cylindroma cells grew first as tightly packed cell clumps eventually forming colonies. The medium was changed every 3 to 4 days until cells reached confluency. Conditioned medium was prepared by incubating confluent cylindroma cell and normal human keratinocyte cultures (second passage) with DMEM containing 50 μ g/ml ascorbic acid for 48 hours. Centrifugation and sterile filtration removed cellular debris, NaN₃ was added to 0.05% and supernatants were stored at -20°C.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immunoblotting

Equal volumes of cell culture medium were mixed 1:1 (v/v) with Laemmli buffer and submitted to sodium dodecyl sulfate-polyacrylamide (4 to 10%) gel electrophoresis in the absence or presence of 5% β -mercaptoethanol. The polypeptides were electrotransferred overnight (200 mA) to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) in 50 mmol/L of borate buffer, pH 8.5, containing 5% methanol. For immunodetection, the nitrocellulose membranes were saturated with 5% milk powder and 0.1% Tween-20, and incubated with primary antibodies followed by peroxidase-conjugated secondary antibodies (DAKO). Bound immunoglobulins were revealed using an enhanced chemiluminescence kit (Amersham, Braunschweig, Germany). Measurement of band intensity by densitometry was performed with FastScan (Molecular Dynamics, Amersham Pharmacia Biotech) and calculations were made using ImageQuant software.

Results

Histological examination of the tumor specimen confirmed the diagnosis of cylindromatosis. The lesions, located in the upper dermis and with no connection with the overlying epidermis, were composed of clusters of multiple irregular nodules arranged in a jig-saw or mosaic

pattern (not shown). As shown by classical Masson staining, each tumor nodule was well individualized and enclosed in a mantle of hyaline material (not shown). Deposits of hyaline material were also seen within the tumor islets.

Abnormalities of Hemidesmosomes and Enlargement of the Basal Lamina Surrounding Cyldindromas

Observation of tissue sections by transmission electron microscopy indicated that the extracellular material surrounding the cyldindroma nodules resembled a highly enlarged basement membrane, which agrees with previous reports.⁸⁻¹⁰ At the ultrastructural level, this basement membrane-like zone deviates, however, from a normal basal lamina in several aspects. There was no clear distinction of a lamina densa and most of the material appeared like a lamina lucida containing numerous and abnormal inclusions of fibrillar material (Figure 2A, white arrowheads). The most striking hallmark of this basement membrane-like structure was its massive enlargement when compared to the basement membrane of the dermal-epidermal junction (Figure 2, compare A and B). Precise morphometric measurements indicated that the basement membrane-like zone around cyldindromas has a width ranging between 2.7 and 4.3 μm , whereas that of the basement membrane at the dermal-epidermal junction has an average thickness of 60 to 90 nm. Further, at a low-power magnification hemidesmosomes were not distinctly seen in the tumor cells abutting the basement membrane-like zone, whereas they were clearly observed in basal keratinocytes of the epithelium (Figure 2B). The plasma membrane of cyldindroma cells facing the basal lamina was rather linear (Figure 2A) and did not present the typical invaginations as those seen associated with the hemidesmosomes of basal keratinocytes (Figure 2B). Examination at higher power magnification revealed that hemidesmosomes with a heterogeneous morphology were present in cells located at the periphery of the nodules but a lamina densa was not clearly visible (Figure 3; B to E). The hemidesmosomes were irregularly spaced and displayed larger size variation than those of the basal keratinocytes (Table 1). In addition, the number of hemidesmosomes present along the surface of tumor cells facing the basement membrane-like zone was approximately half that observed for normal basal keratinocytes (Table 1).

The Distribution of Laminin 5 and 10 in Cyldindroma Tissue Is Not Similar

The basement membrane at the dermal-epidermal junction contains several laminin isoforms, in particular lami-

nins 5 and 10.¹⁵ Indirect immunofluorescence staining of tissue sections with monoclonal antibodies BM165 against the laminin $\alpha 3$ (Figure 4A) or GB3 against native $\gamma 2$ (not shown) revealed an intense and thick labeling circumscribing the cyldindromas, indicating that the entire thickness of the basement membrane-zone delimiting the tumoral nests was heavily loaded with laminin 5. By contrast, a monoclonal antibody against the laminin $\alpha 5$ chain stained the aspect of the basement membrane facing the tumor cells only and not its whole thickness (Figure 4B). In this case, the thin and linear staining observed was similar to that underneath the epidermis on the same tissue section (Figure 4E). Immunofluorescence detection of another basement membrane marker, nidogen, showed also a thin and linear decoration of mainly the inner face of the basement membrane surrounding the tumoral islands (Figure 4C), a staining comparable to that of the laminin $\alpha 5$ chain. These observations indicate that while in the basement membrane below the epidermis laminin 5, laminin 10, and nidogen are strictly co-distributed, this is not the case in the material surrounding the tumor nodules. Therein, the three proteins are co-localized only in the region immediately adjacent to the cells, whereas laminin 5 only is present throughout the whole thickness of the basement membrane-like zone.

Unprocessed Laminin 5 Is Present in Cyldindroma Tissue

Maturation of laminin 5 has been reported to require processing of its $\alpha 3A$ and $\gamma 2$ chains.²³ To determine whether these chains were processed in the basal lamina surrounding the cyldindroma nodules, domain-specific affinity-purified antibodies were used for immunofluorescence staining of the tissue. Antibodies recognizing the L4m (Figure 5A) or the LG4-LG5 (Figure 5C) domains present in unprocessed $\gamma 2$ or $\alpha 3A$ chain, respectively, decorated the periphery of the neoplastic lesions by a thin labeling restricted to the interface where the tumor cell plasma membranes abut the basal lamina, but they did not stain the dermal-epidermal junction (Figure 5, E and G). This indicated that these domains are removed in the laminin 5 deposited underneath the epidermis, but they are present in the laminin 5 associated with the tumor cells. Antibodies against domain LE4-6, which is retained in the processed form of the $\gamma 2$ chain, intensively stained the whole thickness of the basement membrane-like zone delimiting the nodules (Figure 5B) as well as a thick line underneath the epidermis (Figure 5F). Finally an antibody raised against domain IIIa of the $\alpha 3A$ chain stained the whole thickness of the zone surrounding the nodules (Figure 5D), while a thin linear labeling of the dermal-epidermal junction was observed (Figure 5H). A similar staining was observed with an antibody specifi-

Figure 7. Integrin expression in cyldindroma cells and basal keratinocytes of the epidermis. Tissue cryosections were processed for indirect immunofluorescence staining with antibodies against the integrin $\beta 1$ (A-C), $\beta 4$ (D-F), $\alpha 2$ (G-I), $\alpha 3$ (J-L), and $\alpha 6$ (M-O) chains. **Left:** Overviews of the staining of cyldindroma nodules (low magnification) (A, D, G, J, M). With each antibody, higher magnification of the stainings observed for tumor cells (B, E, H, K, N) and basal keratinocytes (C, F, I, L, O) were recorded on the same tissue section.

cally recognizing the amino-terminal portion of the $\beta 3$ chain (not shown).

A Fraction of the $\alpha 3A$ and the $\gamma 2$ Chain of Laminin 5 Is Not Processed in the Culture Medium of Cylindroma Cells

To test the ability of cylindroma cells to process laminin 5, primary cultures of cylindroma cells and of human basal keratinocytes were established. The presence of laminin 5 chains in the culture media was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting with specific antibodies. Both type of cell culture media contained the 165- and 145-kd forms of the laminin $\alpha 3A$ chain (Figure 6A), the 140-kd $\beta 3$ chain (Figure 6C), and the 155- and 105-kd forms of the $\gamma 2$ chain (Figure 6, B and C). However, the amounts of fully processed $\alpha 3A$ (145 kd) and $\gamma 2$ (105 kd) chains were consistently lower in the medium of cylindroma cells than in the medium conditioned by basal keratinocytes (Figure 6, A and B). Measurement of band intensity by densitometry of the blots shown in Figure 6, A and B, indicated that the 145-kd form of the laminin $\alpha 3A$ chain in the culture medium of cylindroma cells or keratinocytes represented 17% and 39%, respectively, of the total amount of laminin $\alpha 3A$ chain (Figure 6D). Similarly the 105-kd form of the $\gamma 2$ chain represented 43% and 80% of the total amount of $\gamma 2$ polypeptides present in the medium of cylindroma cells and keratinocytes, respectively (Figure 6E). These results indicate that cylindroma cells have the capability to synthesize and secrete laminin 5, but that the extent of processing of both the $\alpha 3A$ and $\gamma 2$ chains is decreased when compared to that occurring in normal basal keratinocyte cultures.

Integrin Expression in Cylindroma Cells

Integrins are involved in the deposition and organization of their extracellular matrix ligands, including laminin 5.¹⁵ In normal human epidermis, integrin expression is restricted to basal epithelial cells and includes the $\alpha 2\beta 1$ and the $\alpha 3\beta 1$ at the lateral cell plasma membranes and the $\alpha 6\beta 4$ integrin at the basal surface of keratinocytes.^{29,30} The presence and the distribution of these integrins was analyzed in cylindromas by indirect immunofluorescence staining of tissue sections. Antibodies against the $\alpha 6$ and $\beta 4$ integrin subunits gave a linear staining restricted to the periphery of all of the tumoral nodules (Figure 7, D and M). Comparison of integrin immunostaining in cylindroma and at the dermal-epidermal junction showed that for both integrin subunits the labeling was, however, weaker for the tumoral cells (Figure 7, E and N) than for the basal keratinocytes of the epithelium (Figure 7, F and O). It indicated that although expressed at a lower level in cylindromas than in the epidermis, the $\alpha 6$ and $\beta 4$ subunits were correctly polarized at the basal plasma membrane and likely to form a heterodimer associated with its ligands in cylindroma cells such as in normal epidermal cells. The integrin $\alpha 3$

chain was faintly stained in cylindroma cells in a manner similar to that observed for basal keratinocytes (Figure 7; J to L). The antibodies against the $\alpha 2$ and $\beta 1$ integrin chains intensively labeled all of the cylindroma nodules (Figure 7, A and G). Although the pericellular localization of the $\alpha 2$ and $\beta 1$ integrin was identical in cylindroma cells and keratinocytes, a major difference was that all of the cells forming the neoplastic lesions were decorated by both antibodies (Figure 7, B and H), whereas in the epidermis only the first and second layers of epidermal cells were stained (Figure 7, C and I). This suggests that all cylindroma cells display a basal cell phenotype in term of $\alpha 2\beta 1$ integrin expression.

Discussion

Contrary to most invasive or noninvasive tumors, a hallmark of cylindromas is that the neoplastic islands are surrounded by an excessive basement membrane zone that forms a barrier physically separating tumor cells from the dermal mesenchyme. Nevertheless, it does not prevent proliferation of the tumor cells and local growth in the upper dermis of the multiple benign lesions. This suggests that besides the ultrastructural abnormalities, the biological function of the tumor basal lamina might also be disturbed, because of defects in its molecular composition and/or in the interactions of the neoplastic cells with the adjacent extracellular matrix. To test this hypothesis, we have analyzed in detail the extracellular matrix surrounding the tumors and the integrins that interact with it. The results show that in comparison with normal keratinocytes, the cylindroma cells have abnormal hemidesmosomes, and that both laminin 5 and its integrin receptors are altered.

In addition to a massive enlargement, the basement membrane-like zone surrounding cylindromas is rather electron transparent with no clear distinction of a lamina densa in agreement with previous reports. In addition, we show here that, compared to the dermal-epidermal junction, hemidesmosomes in cylindromas have a heterogeneous and abnormal morphology and a twofold reduction in number. At the molecular level, immunofluorescence staining shows the presence of laminin 5 in the entire thickness of the basement membrane-like zone, whereas nidogen and laminin 10 are restricted to the part immediately adjacent to cylindroma cells. This indicates that the basal laminae around cylindromas and at the dermal-epidermal junction differ in terms of distribution of matrix molecules. Furthermore, a fraction of laminin 5 is not correctly processed into its mature forms in cylindroma tissue and cultured cells. Immunofluorescence staining reveals that the carboxy-terminal LG4-LG5 domains of the laminin $\alpha 3A$ chain are retained in the fraction of laminin 5 immediately associated with the tumor cells. By contrast, these domains are absent from the dermal-epidermal junction, which agrees with results showing that the precursor form of the laminin $\alpha 3A$ chain is processed into a 165-kd polypeptide in tissue and in cell cultures.^{21,23,31} The second processing of the $\alpha 3A$ chain, ie, removal of the amino-terminal domain IIIa, is also

defective in cylindromas as shown by the presence of this domain in the entire thickness of the basal lamina encircling cylindromas. This is confirmed by immunoblotting analysis of the laminin α 3A chain showing less of the 145-kd form in the culture medium of cylindroma cells compared to that of normal keratinocytes. Conversion of the laminin α 3A chain from a 165- to a 145-kd polypeptide has been infrequently seen in keratinocyte monocultures.²¹ This apparent discrepancy might be because of the fact that, in this report, normal keratinocytes and cylindroma cells were co-cultivated with fibroblasts, a culture condition that favors processing of the laminin α 3A chain (A El Khal, L Tunggal, N Knöss, M Aumailley, manuscript in preparation) and that reflects more closely the *in vivo* situation in which 50% of laminin 5 is fully processed.²¹

A lack of processing is also observed for the laminin γ 2 chain in cylindromas. Immunofluorescence labelings show the presence of the amino-terminal domains of this chain, including domain L4m, in the fraction of laminin 5 associated with cylindroma cells whereas they are absent underneath the epidermis. By contrast, a strong staining of the entire thickness of the basal lamina surrounding the neoplastic nests and of the dermal-epidermal junction is observed with antibodies against epitopes present in processed laminin γ 2 chain. It suggests that the γ 2 chain is processed under basal keratinocytes but not in cylindromas. This is confirmed by immunoblotting analysis showing that more than half of the γ 2 chain remains as a 155-kd polypeptide in the medium of cylindroma cells whereas a larger proportion is converted to 105-kd in the medium of keratinocytes. Together, these results demonstrate that the processing of the laminin α 3A and γ 2 chains is impaired in cylindromas which results in the abnormal presence of structural domains specific of immature laminin 5.

As emphasized by the phenotypes of patients affected with epidermolysis bullosa junctionalis or resulting from site-directed mutagenesis in mice, one of the most critical interactions required for the formation and the stability of the dermal-epidermal junction is that occurring between the α 6 β 4 integrin and the carboxy-terminus of laminin 5.³² In cylindroma cells, the expression of the α 6 β 4 integrin is slightly weaker than in basal keratinocytes (not shown), suggesting a reduced number or a diminished clustering of the integrin. Alternatively, the abnormal presence of domains LG4 and LG5 at the carboxy-terminus of laminin 5 may sterically alter the interaction. Furthermore, the amino-terminal region of laminin 5 develops interactions with collagen VII and laminin 6 that are likely to be important for the anchorage of laminin 5 to network-forming extracellular matrix molecules located deeper in the basal lamina and in the underlying dermis.^{19,20} The presence of the uncleaved amino-terminus both in the γ 2 and the α 3A chains may be a steric hindrance to these interactions. The ectopic localization of collagen VII within the basement membrane-like zone circumscribing the neoplastic nodules (Bruckner-Tudermann et al¹⁰; this report) is in favor of that hypothesis. It is tempting to speculate that the processing of the amino-terminal parts of the α 3A and γ 2 chains are critical for the different

interactions to occur and that lack in the processing might impair the whole architecture of the basal lamina and of the adhesion complexes including hemidesmosomes.

In addition to their role in the mechanical anchorage of cells to the underlying basal lamina, integrins are involved in the formation of basement membranes, including early embryonic and epidermal basal lamina.^{33–36} The abnormalities discussed above, in particular the presence of unprocessed laminin 5 directly at the contact with cylindroma cells, could therefore trigger an altered feed-back regulation loop leading to overproduction of laminin 5. Although overproduction of laminin 5 was not observed in cell cultures, it is obvious from the thickness and the immunofluorescence stainings that an excess of laminin 5 has been produced *in vivo* by cylindroma cells. Alternatively, the high expression of the α 2 β 1 integrins suggests that the differentiation stage of cylindroma cells is different from that of normal keratinocytes. Up-regulation of β 1 integrins in suprabasal epithelial cells has been associated with hyperproliferation³⁷ and with cells of high proliferative potential such as epidermal stem cells.^{38,39} Although the profile of such cells in terms of extracellular matrix synthesis and cell-matrix interactions is not known, it can be predicted that de-localization out of their niche will result in an alteration of their behavior. Finally, integrin function and cell-matrix interactions primarily depend on the integrity of the cytoskeleton and of the intracellular cell machinery. The gene mutated in cylindromatosis encodes for a protein predicted to contain cytoskeletal-associated protein-glycine-conserved (CAP-GLY) domains playing a role in the organization of cellular microtubules and organelles.⁶ Absence of the product of the *CYLD* gene may be deleterious for the intracellular architecture and machinery and consequently for the interactions of integrins with their extracellular matrix ligands.

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