Heterogeneity of Severe Dystrophic Epidermolysis Bullosa: Overexpression of Collagen VII by Cutaneous Cells from a Patient with Mutilating Disease

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Severe mutilating recessive dystrophic epidermolysis bullosa presents with extensive blistering, scarring, and pseudosyndactylies. The skin of most affected individuals lacks normal anchoring fibrils and contains no, or drastically reduced amounts of, collagen VII, the major fibril component. Here we present evidence for molecular heterogeneity of the mutations underlying this phenotype. A patient with severe mutilating disease, but with apparently normal anchoring fibrils and abundant collagen VII, was defined. Indirect immunofluorescence examination of the patient’s skin exhibited a strong staining for collagen VII at the dermo-epidermal junction and at the roof of a natural blister, and immunoblotting of skin extracts revealed collagen VII of normal size. The patient’s keratinocytes expressed two- to threefold increased amounts of collagen VII at the mRNA and protein level compared to controls. Synthesis of matrix metalloproteases by the patient’s keratinocytes was comparable to normal cells, indicating that the overexpression of collagen VII did not affect the synthesis of these enzymes. We hypothesize that in this patient a mutation affecting interactions of the anchoring fibrils with other components of the basement membrane zone underlies the disease. Key words: anchoring fibrils/basement membrane/bullous disease/dermo-epidermal junction/matrix metalloproteases. J Invest Dermatol 102:155–159, 1994

Dystrophic epidermolysis bullosa (EBD) is a genetically and phenotypically heterogeneous group of skin disorders characterized by blistering and scarring after minor trauma [1–3]. At least six subgroups have been defined based on the mode of inheritance and distribution of the lesions, but in all of them the cleavage occurs below the basement membrane, at the level of the anchoring fibrils. Qualitative and quantitative abnormalities of the fibrils in patients’ skin have been revealed by ultrastructural analysis [4–6], and aberrant expression of collagen VII, the major anchoring fibril protein, has been detected in several affected individuals [7–11]. In line with these findings, genetic linkage was recently established between the collagen VII gene, and both dominant and recessive mutilating EBD [12,13], further implying that collagen VII mutations cause these diseases.

The most severe subtype of EBD is the recessively inherited generalized, mutilating EBD [1–3]. Extensive blistering leads to severe scarring and mutilation, often to secondary carcinomas and poor prognosis. Lack of intact anchoring fibrils and collagen VII from the skin has been described as a characteristic sign of the disease [4–9,14], although some individuals exhibit small amounts of immunoactive collagen VII at the dermo-epidermal junction [2,15]. Different pathways have been suggested to lead to lack of anchoring fibrils in this EBD subtype. Impaired collagen VII synthesis was observed in some patients [16] and elevated collagenolytic activity in fibroblasts of others [17,18].

To elucidate the mechanisms underlying the severe phenotype in mutilating EBD, we assessed collagen VII expression in skin and cells derived from several patients. The clinically uniform patients showed ultrastructural and molecular heterogeneity. Whereas most probands had no or drastically reduced amounts of collagen VII in skin, the present propositus, a severely affected patient, had apparently normal anchoring fibrils and exhibited strong collagen VII immunofluorescence staining. We have now extended the studies on this patient and show that cultured keratinocytes overproduce collagen VII whereas the synthesis of matrix metalloproteases (MMP) appears normal.

MATERIALS AND METHODS

Patient The 20-year-old male Norwegian proband (EB 79-1) is an adopted child, but extensive genealogy of the biologic ancestors ruled out consanguinity. He suffered from blistering of the skin since birth and a severe course of the disease ending in extensive synchiae that needed repeated plastic surgery and contractures that led to partial wheelchair dependency [19]. The patient thus presented with a characteristic phenotype of severe mutilating recessive EBD, with affection of the skin and mucous membranes but not of other organs. Diagnostic electron microscopy showed sublamina densa splitting in spite of the presence of numerous normal anchoring fibrils in intact skin, and antigen mapping revealed strong fluorescence with antibodies to collagen VII [5]. New biopsies were obtained from clinically uninvolved skin and roofs of fresh trauma-induced blisters were removed for keratinocyte cultures.

Cell Cultures Keratinocytes were obtained by trypsinization of control skin biopsies or fresh blister roofs and cultured in serum-free keratinocyte

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growth medium containing 0.09 mM calcium [20] (KGM; Gibco, Grand Island, NY), supplemented with bovine pituitary extract (BPE) and epidermal growth factor (EGF). EBD and normal keratinocytes from early passages were used for the experiments. Two days prior to indirect immunofluorescence (IF) staining or extraction of keratinocytes, BPE and EGF were omitted from the medium, and 50 µg/ml ascorbate was added daily.

For determination of MMP synthesis, keratinocytes were seeded in 6-well plates in KGM. One batch of each cell line was used in a confluent and the other in a preconfluent state. Each culture was washed three times with Hank's balanced salt solution (Gibco) and incubated for 24 h in 1.5 ml of KGM without BPE and EGF. To one aliquot of the harvested medium, a gelatinase buffer concentrate was added to give a final concentration of 0.2% bovine serum albumin (BSA), 100 mM Hepes, pH 7.2, and 10 mM CaCl₂, and to another aliquot a collagenase buffer concentrate to give a final concentration of 100 mM Hepes, pH 7.2, and 10 mM CaCl₂. The buffered media were stored at -20°C until use. To correct for variation in the cell content, DNA was measured in each cell layer [21]. A minimum of three parallel measurements per strain were performed. The average variability was less than 5%.

Antibodies and IF Staining Polyclonal affinity-purified antibodies to the triple helical domain of human collagen VII were prepared as described [22]. The monoclonal antibody LH-7.2 [23] to the N-terminal globular domain of collagen VII was purchased from Chemicon International Inc. (Temecula, CA). Polyclonal antibodies to human laminin were a generous gift from Dr. M. Paulsson, M.E. Muller Institute for Biomechanics, University of Berne, a monoclonal antibody to collagen IV from Dr. B. Odermatt, Department of Pathology, University of Zürich, and a monoclonal antibody to kalinin from Dr. R. Burgeson, Harvard Medical School, Boston, MA. As a bollus pemphigoid antibodies, a high-titer serum from a patient was used. The fluorescein isothiocyanate (FITC)-labeled anti-rabbit, anti-mouse, and anti-human antibodies to collagen VII standards were separated and reacted with water-insoluble peroxidase-4-chloro-1-naphthol. The migration position of collagen VII on the non-reacted lanes was marked with help of these standards, and the corresponding bands were excised and transferred to Eppendorf tubes. They were incubated in 0.5 ml of a substrate solution containing 0.03% o-phenylenediamine, 25 mM citric acid, 50 mM Na₂HPO₄, and 0.01% hydrogen peroxide for 15 min. The reaction was stopped by adding 100 µl of 2 M sulfuric acid. The residual filter-bound color product was eluted with 200 µl of N,N-dimethyl formamide, and both chromophore solutions were combined prior to reading the absorbance at 492 nm. For calibration, 5–30 µl of collagen VII containing dermal extract were run on the same gel and measured in the same assay, parallel with the test samples. Due to the semiquantitative nature of this assay, arbitrary units for collagen VII were defined. For measurement of collagen VII present in 1 µl of dermis extract that was prepared by using 400 µl of extraction buffer per cm² of skin surface, as described above [22,24]. Application of 5–30 µl of skin extract as a standard resulted in a linear response in the assay. All cell culture samples were measured as duplicates in the linear range of the calibration curve.

Northern and Southern Blotting Poly A⁺ RNA was isolated from keratinocyte cultures using a Pharmacia QuickPrep mRNA Purification Kit (Pharmacia, Uppsala, Sweden) following the instructions of the manufacturer. mRNA (1–3 µg per well) was separated on a 1% agarose gel containing 2.2 M formaldehyde, vacuum transferred onto a Nytran nylon filter (Schleicher & Schuell, Dassel, Germany), and both chromophore solutions were combined prior to reading the absorbance at 492 nm. For calibration, 5–30 µl of collagen VII containing dermal extract were run on the same gel and measured in the same assay, parallel with the test samples. Due to the semiquantitative nature of this assay, arbitrary units for collagen VII were defined. For measurement of collagen VII present in 1 µl of dermis extract that was prepared by using 400 µl of extraction buffer per cm² of skin surface, as described above [22,24]. Application of 5–30 µl of skin extract as a standard resulted in a linear response in the assay. All cell culture samples were measured as duplicates in the linear range of the calibration curve.

Figure 1. Immunofluorescence staining of the patient’s skin with antibodies to collagen VII. A) Intact dermo-epidermal junction with strong linear fluorescence. B) Blister roof; note the fluorescence at the basal aspect of the epidermis. Asterisk, blister cavity.

Figure 2. EBD keratinocytes overexpress collagen VII. IF staining with antibodies to the triple-helical domain of collagen VII of normal (A) and EBD (B) keratinocytes grown on coverslips.
were extracted and immunoblotted. Both EBD keratinocytes and their media contained more collagen content in the cell cultures, confluent keratinocytes and their media contained more collagen than controls.

Antigen Mapping IF staining of clinically uninvolved skin with antigen showed a normal staining, and localized to the blister roof in separated areas. The diagnosis of EBD. Antibodies to other basement membrane zone (Fig VII).

All assays, in at least tripliates, were carried out for 24 h in Matrix Metalloprotease (MMP) Activities The MMPs are secreted into culture medium in a latent form, and were converted to active forms by limited proteolysis using trypsin [32–34]. Collagenase activity was determined with reconstituted 

Southern blotting [29] was carried out with the K131 cDNA [30] on genomic DNA after digestion with the restriction endonuclease Pvu II (Boehringer-Mannheim, Buchs, Switzerland).

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RESULTS

Antigen Mapping IF staining of clinically uninvolved skin with antibodies to both the triple-helical and the N-terminal domain of collagen VII revealed a strong fluorescence at the basement membrane zone (Fig 1A). In a blister roof, the linear fluorescence was localized to the basal border of the epidermis (Fig 1B), confirming the diagnosis of EBD. Antibodies to other basement membrane components, collagen IV, laminin, kalinin, and bullous pemphigoid antigen showed a normal staining, and localized to the blister roof in separated areas.

Collagen VII Expression in Skin and Cultured Keratinocytes IF staining of cultured keratinocytes revealed a stronger fluorescence of collagen VII in the patient’s cells than in controls (Fig 2). To allow for a better characterization of this increased collagen VII content in the cell cultures, confluent keratinocytes and their media were extracted and immunoblotted. Both EBD keratinocytes and their media contained more collagen VII than controls (Fig 3).
cultures. As shown in Table II, low-density cultures produced more MMP than high-density cultures, but EBD cells did not produce more MMP than controls. High-density, but not low-density, cultures of the patient showed an increased gelatinase activity.

**DISCUSSION**

Morphologically and immunohistochemically, the mutilating EBD has been defined by absence or dramatic reduction of anchoring fibrils and collagen VII from skin [1–9]. Our recent studies have demonstrated that at least in some individuals, impaired synthesis of collagen VII underlies this phenotype, as cultured EBD keratinocytes neither synthesized detectable amounts of collagen VII nor could they be stimulated with TGF-β to do so [16]. In concert with this finding, Hilal et al reported mutations in the collagen VII gene in four patients with recessive mutilating EBD. Three mutations led to a premature stop codon in the 5' gene region coding for the N-terminal globular domain of collagen VII, thus providing an explanation for lack of a protein product. Here we present evidence for extensive molecular heterogeneity of this disorder. A patient with a typical severe phenotype of mutilating EBD, but with overexpression of apparently normal collagen VII, was defined. Collagen VII production in EBD keratinocytes was two- to threefold higher, at both protein and mRNA level, than in controls. The constellation agrees with the presence of numerous anchoring fibrils in the patient's skin in situ.

It should be pointed out that the molecular type of EBD described here may only be present in a small minority of recessive EBD mutilans patients. A dominant mutation of some sort cannot be totally excluded in the present propositus, because these patients rarely reproduce to disclose the inheritance. However, on one side his ancestry is rural and closely associated with the inbred ancestry of another EBD patient with generalized non-mutilating recessive EBD [19]. This fact not only supports recessive inheritance for the present patient, but also leads to interesting predictions, which can be tested on the other prototype homozygous patient [19]. Given the ancestry of the present patient, the possibility also exists that he is a compound heterozygote carrying two different mutations.

The disturbed cohesion of the dermo-epidermal junction in the present case may be due to a mutation in the collagen VII gene but also may involve any abnormality of any basement membrane component. The dermo-epidermal junction consists of an integral network of structural macromolecules and depends strongly on physical and chemical interactions for both its architecture and its function. A structural defect of any component may perturb a specific interaction, and lead to a particular clinical phenotype. For instance, basement membrane or anchoring plaque proteins that bind collagen VII, such as collagen IV [35], can be considered as candidate molecules. A mutation in the collagen VII binding domains of these proteins may cause dysfunction at the dermo-epidermal junction and thus lead to EBD, but not affect other organs. Alternatively, a yet-unknown component of the basement membrane zone may be affected.

Excessive degradation of the anchoring fibrils by collagenolytic enzymes is not the primary event in this proband because both anchoring fibrils and intact collagen VII were present in ample amounts. In addition, collagenase activity was similar in both EBD and control cells. Further studies must show whether the increased collagenolytic activity in high-density cultures of the patient's cells is causally relevant, especially in regard to the apparently intact anchoring fibrils.

Interestingly, cultured EBD keratinocytes expressed more apparently normal collagen VII than controls. Analogous overexpression has been observed in other heritable connective tissue disorders [36], but the nature of the signals leading to this phenomenon have remained elusive and require further investigation of the regulation of gene expression of extracellular matrix components.

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OVEREXPRESSION OF COLLAGEN VII IN SEVERE EBD


