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INVESTIGATIVE REPORT

Pathogenic Mechanisms in Epidermolysis Bullosa Naevi

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Epidermolysis bullosa naevi are large, eruptive melanocytic naevi which frequently arise in areas of former blisters in patients suffering from inherited epidermolysis bullosa. Morphologically, these naevi are similar to malignant melanoma, although so far no malignant transformation has been observed. To investigate the pathogenesis of these moles we documented their clinical evolution and their histopathological and immunocytological characteristics in three patients with epidermolysis bullosa. Clinically, we observed signs of malignant transformation, such as explosive growth and the occurrence of satellite lesions of epidermolysis bullosa naevi. However, malignant melanoma was excluded by histopathological evaluation. In addition, we evaluated the concentrations of various factors known to stimulate melanocyte growth in blister fluid. Human interleukin 8, basic fibroblast growth factor, human hepatocyte growth factor, GM-CSF, leukotriene B4 and prostaglandin E2 revealed concentrations comparable with the levels in inflammatory blisters. We were able to detect individual melanocytes/naevus cells in blister fluid from a blister over an epidermolysis bullosa naevus. The factors detected in the blister fluid might therefore promote the proliferation, migration and melanogenesis of disconnected melanocytes/naevus cells representing the basis of the highly dynamic appearance of epidermolysis bullosa naevi. ***Key words: blister fluid; cytokines; epidermolysis bullosa; growth factors; naevi.***

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Epidermolysis bullosa (EB) hereditaria comprises a group of mechano-bullous diseases characterized clinically by blister formation of the skin and mucous membranes following minor trauma; they are caused by mutations in the genes of various structural proteins of the dermo-epidermal basement membrane zone (1).

We have recently described a new entity of naevi which predominantly occur in recessively inherited forms of EB, i.e. EB simplex (EBS), non-Herlitz junctional EB and dystrophic EB (DEB): ''EB naevi'' are eruptive, large, asymmetrical, irregularly pigmented, highly dynamic melanocytic lesions with sharply demarcated borders outlining former blisters (2, 3). These moles are highly suspect if one applies the ABCD rules, which have been elaborated for clinical recognition of early malignant melanoma (4, 5). Microscopically, they either show aspects of common naevus cell naevi or, quite frequently, reveal criteria mimicking malignant transformation similar to those seen in persisting naevi/pseudomelanoma (6). Despite the clinically and sometimes also histopathologically highly suspect features of EB naevi, we have not noticed malignant transformation of these moles in over 20 years of clinical surveillance (3). Recent studies on EB naevi have been carried out by Annicchiarico et al. (7) and Bichel et al. (8).

The pathogenesis of common acquired naevi has not yet been elucidated. Cramer (9) speculated that these and congenital melanocytic naevi derive from melanocyte precursors located in nerve sheaths, which undergo a four-step differentiation pathway (nerve sheath precursor stage, dermal migratory stage, junctional migratory stage and dendritic stage). With regard to the pathogenesis of EB naevi, it has been shown histopathologically that melanocytes probably deriving from incipient naevi or subclinical nests of naevus cells are found in the blister cavity (2). This has led to the assumption that single melanocytes/naevus cells, after disconnecting and settling down, proliferate excessively in the microenvironment of epidermal regeneration, thus constituting the malignant aspect with alarming size and satellite lesions. So far, however, there has been neither formal proof of single melanocytes in the blister fluid nor data on growth factors/cytokines promoting the proliferation of melanocytes (10, 11) in EB blisters. We therefore investigated the clinical evolution, histopathology and immunocytology of EB naevi in three children with recessive EBS, EBS with muscular dystrophy and recessive DEB, respectively.

CASE REPORTS

Patient 1

An 8-year-old boy, the only child of healthy parents of Austrian origin without apparent consanguinity and with a negative family history of blistering skin diseases. A homozygous nonsense mutation in the KRT14 gene (744delC/insAG; Y248X) leading to a

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premature termination codon was shown to have caused complete loss of keratin 14 protein expression in basal keratinocytes of the patient. The clinical, histological and genetic findings in this patient have been reported elsewhere (12). The boy has been carefully examined at least once a year with regard to the status of his skin and secondary extracutaneous complications of EB. Multiple, rapidly enlarging, asymmetrical, irregularly pigmented melanocytic naevi, some of them with poorly defined borders, i.e. EB naevi, have been developing since the age of three (in 1998). A large EB naevus on the left buttock with an alarming clinical aspect has been reported previously (Patient l in Ref. 3). Initially, the patient presented with a newly developed approximately 4.5×4 cm wide, sharply demarcated naevus extending along the outlines of a former blister on the left buttock (Fig. 1a). One year later, a protrusion approximately 3 cm in length appeared at the cranial portion of the naevus (Fig. 1b) and more EB naevi elsewhere. The latero-basal border became lighter and blurred and speckled satellite lesions turned more prominent in colour. After a further year, the naevus has lost parts of the protrusion but has gained new satellite lesions, appears lighter and is more raised (Fig. 1c).

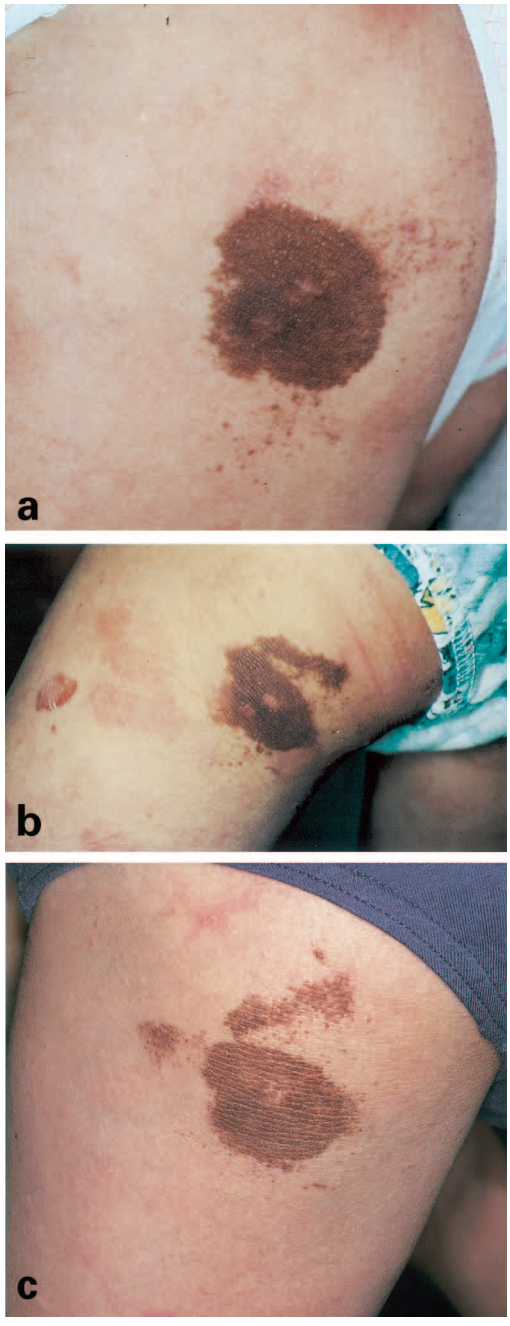

Fig. 1. Epidermolysis bullosa naevus on the left buttock of Patient l followed-up over 3 years: (a) In 1998 the mole showed typical criteria of an EB naevus, i.e. rapid growth, large diameter, irregular borders and satellite lesions (reprinted from Bauer JW, et al. Large melanocytic naevi in hereditary epidermolysis bullosa. J Am Acad Dermatol 2001; 44: 577–584; with the permission of Elsevier Science). (b) In 1999 a 3-cm long protrusion on the cranial aspect of the initial naevus was noticed. (c) In 2000 the naevus has lost a distal portion and new satellite lesions have developed on its lateral border.

Patient 2

A 5-year-old boy, the son of unaffected, not consanguineous parents of Turkish origin. The diagnosis "EBS with muscle dystrophy" was established by mutation analysis, which revealed compound heterozygosity for a 3-bp insertion at position 1287 in the PLEC 1 gene, that encodes for the linker protein plectin, leading to the insertion of leucine as well as the nonsense mutation Q1518X leading to a stop codon (13). The boy developed a large EB naevus at the dorsum of his right thumb/thenar at the age of 3 years. The lesion showed stippled pigmentation ranging from dark to light-brown and coalesced in a prominent centre. The irregular border outlines the shape of preceding blisters, giving this mole its bizarre configuration (Fig. 2).

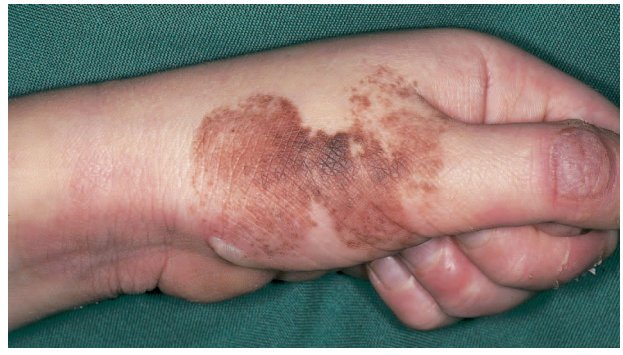

Fig. 2. The epidermolysis bullosa naevus on the thumb/thenar of Patient 2 appears clinically highly suspect because of its size, polycyclic irregular configuration, mottled pigmentation and satellite lesions. Note the tense blister on the thenar and the older flaccid blister over the interphalangeal joint.

Patient 3

A 9-year-old Austrian girl with a history of blister formation following minimal trauma since birth and a negative family history with regard to bullous skin diseases. Erosions healed with milia formation and atrophic scarring. Mucosal involvement, i.e. erosions in the oral cavity and oropharynx, varied in intensity. Toenails were completely lost in early childhood, whereas hair and teeth were unaffected. The girl was identified to be heterozygous for a mutation at position 425 (425A→G) in the COL7A1 gene, which encodes for type VII collagen (14). A second mutation has not yet been revealed in this girl with presumed recessively inherited DEB. A large EB naevus with satellite lesions developed at the dorsal aspect of the right heel within a few months (Fig. 3).

Histopathology

In Patient 1 the naevus on the right buttock was biopsied when he was 5 years of age. In Patient 2 the biopsy was taken from the naevus on the right thenar at the age of 5 years, while he underwent circumcision in general anaesthesia. Because of the highly suspect clinical aspect of Patient 3's EB naevus we took two punch biopsies, one from the centre the other from a satellite lesion, to exclude malignant melanoma. Histological evaluation of the naevus of Patient 1 revealed nests of melanocytes localized along the dermoepidermal junction, particularly in partly confluent rete ridges. Between the nests, single melanocytes predominated, a few of which were also located in upper Malphigian layers (often described as pagetoid spreading). Melanocytes were also located in the follicular epithelium. In addition, there was a discrete lymphocytic infiltrate in the papillary dermis (Fig. 4a). The melanocytic lesion in Patient 2 consisted of slightly unequal melanocytic nests in epidermal and dermal location. Single melanocytes were rarely seen

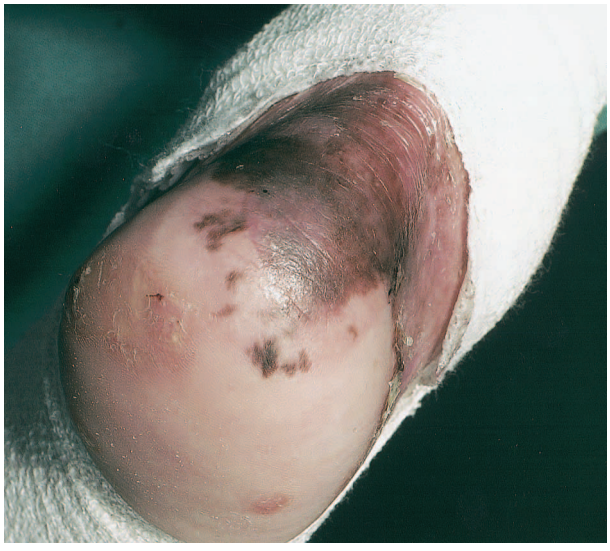

Fig. 3. The polycyclic margin of a large epidermolyis bullosa naevus on the heel of Patient 3 outlines preceding blisters. A flaccid, partially torn bulla causes the blurred, opaque aspect of this dark-brown naevus with satellite lesions.

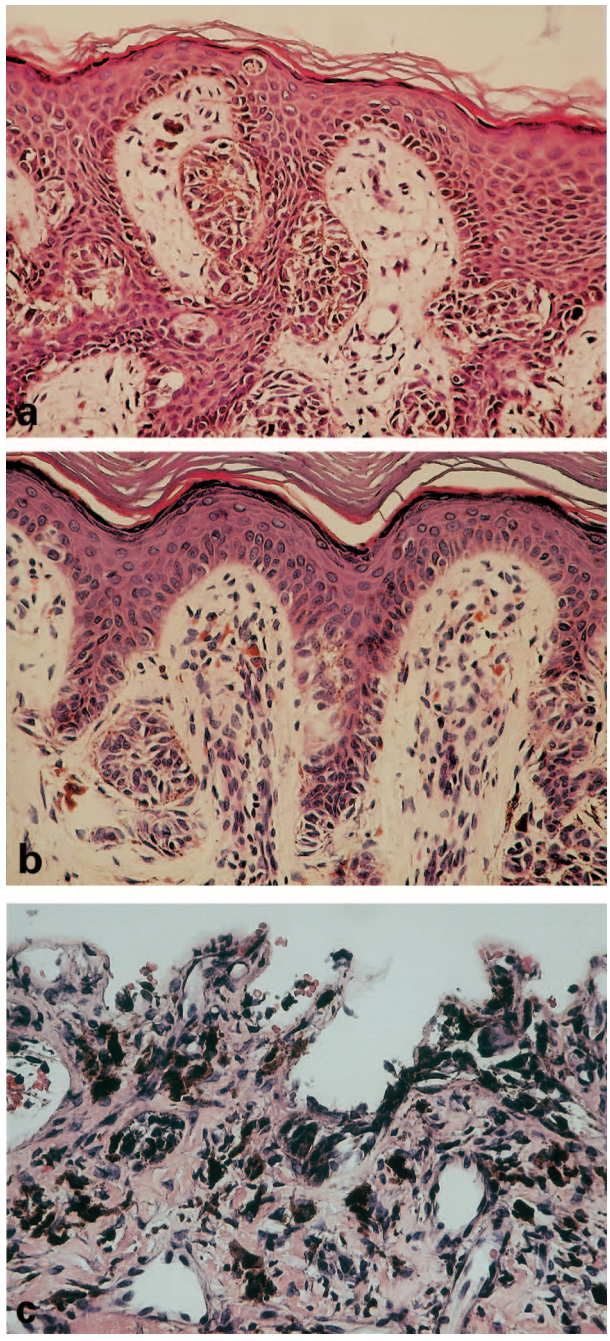

Fig. 4. Histopathology of epidermolysis bullosa naevi. (a) Patient 1. Melanocytes arranged in intraepidermal nests, particularly in partly confluent rete ridges. Single melanocytes are found in upper Malphigian layers (×200). (b) Patient 2. Uneven nests of melanocytes in epidermal and dermal location. Slight fibroplasia around rete ridges. Dermal melanophages are in vicinity to melanocytes (×400). (c) Patient 3. The dermal part of the biopsy showed melanocytes in nests in the papillary dermis and a lymphocytic infiltrate with melanophages. There are no cytological atypia or mitoses present (×400).

in suprabasal epidermal layers. Besides slight focal fibroplasia, there were only a few scattered lymphocytes and melanophages in the dermis (Fig. 4b). In conclusion, the histological features of these two EB naevi were consistent with a melanocytic naevus of the compound type with some features of a Clark's naevus. Because of dermoepidermal separation of the biopsy of Patient 3's naevus during the surgical procedure, the epidermis and the dermal part were embedded separately. The epidermal part of the biopsy showed acral epidermis with single melanocytes in the basal layer, but only one melanocytic nest located directly beneath the dermoepidermal junction (subepidermally). No melanocytes were found in the upper Malphigian layers (not shown). In contrast, the dermal component of the biopsy showed melanocytes in nests in the papillary dermis surrounded by a lymphocytic infiltrate mixed with melanophages (Fig. 4c). There were neither cytological atypia nor mitoses. This melanocytic lesion was therefore an acral naevus of compound type. In contrast to other EB naevi that we have investigated previously, there were no histological changes of a persistent naevus and/or a pseudomelanoma in any of the biopsies.

Immunohistochemistry

Sections of specimens from all three patients were stained with antibodies against the S-100 protein (Z 0311, Dako, Glostrup, Denmark), HMB-45 (M0634, Dako) and the Ki-67 (M 7240, Dako) proliferation marker-protein. S-100 stains showed the expected reactivity of melanocytic cells (also in the upper epidermis) and of Langerhans' cells, dermal dendritic cells and neuronal structures (not shown). HMB-45 as a marker for premelanosomes, stained positive in melanocytes along the basal layer and in dermal nests, but also in melanophages. Interestingly, Ki-67 was preferentially expressed in epidermal and adnexal keratinocytes, while expression was low in melanocytes of all three naevi (Fig. 5). In the naevus of Patient 3, staining was completely negative for Ki-67 in the dermal part of the biopsy (not shown).

Blister fluid analysis

Blister fluids were collected in sterile syringes by puncturing skin blisters. "Control" samples included fluids from burn blisters, blisters of a patient with Stevens-Johnson syndrome and blisters of two patients with bullous pemphigoid. Samples were aliquoted and immediately stored at $-70^{\circ}C$ after the addition of aprotinin (500 Units ml^{-1}) to protect proteins from degradation.

For quantification of factors known to stimulate melanocyte growth, the following commercial ELISA kits (Quantikine, R&D Systems, Minneapolis, IL, USA) were used, all in accordance with the manufacturer's instructions: Interleukin 8 (IL-8, No. D8050); basic fibroblast growth factor (bFGF, No. DFB50); hepatocyte growth factor (HGF, No. DHG00); granulocyte monocyte colony stimulating factor (GM-CSF, No. DGM00); leukotriene B4 (LTB4, No. DE0275); and prostaglandin E2 immunoassay (PGE2, No. DE0100). For each compound, duplicate readings were averaged and concentrations (pg ml^{-1}) were calculated corresponding to the standard curve.

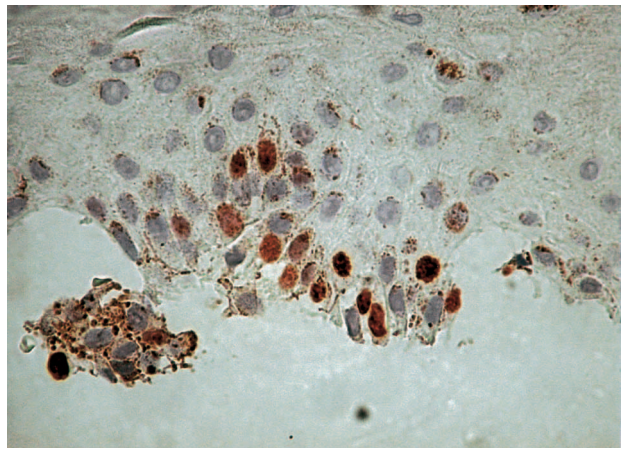

Fig. 5. Immunohistochemistry of an epidermolysis bullosa naevus in Patient 3. The Ki-67 score is low and preferentially present in keratinocytes. Melanin in nested melanocytes, which are loosely attached to the epidermis, is unspecifically stained, whereas the nuclei are negative for Ki-67 ($\times 400$).

The concentrations of IL-8, GM-CSF, LTB4, PGE2 and HGF in the blister fluid of the patients with EB showed no significant differences compared to the concentrations in the blister fluid from "control" patients. Interestingly, bFGF was barely detected in any of the blister fluids (Table I).

To demonstrate any free-floating melanocytes in the blister fluid, haemorrhagic fluid was obtained from a large blister over the EB naevus on the right heel of Patient 3 and was immediately centrifuged in a

Table I. *Quantity (in pgml^{-1}) of cytokines/growth factors in blister fluids from 3 patients with epidermolysis bullosa and in "control blister fluids" from patients with bullous pemphigoid (BP), Stevens-Johnson syndrome (SJS) and second-degree burns (BURN). Because of minute amounts of fluid punctured from blisters, not all cytokines/growth factor concentrations could be evaluated in all samples. Not done (nd).*

	Patient 1	Patient 2	Patient 3	BP	SJS	BURN
HGF	7900	9000	nd	nd	nd	4950
IL8	1200	1850	1780	1850	1600	1100
b-FGF	4	2	nd	0	nd	0
GM-CSF	15	80	25	31	2	4
PGE_2	420	nd	1000	nd	400	nd
LTB_4	100	nd	0	nd	200	nd

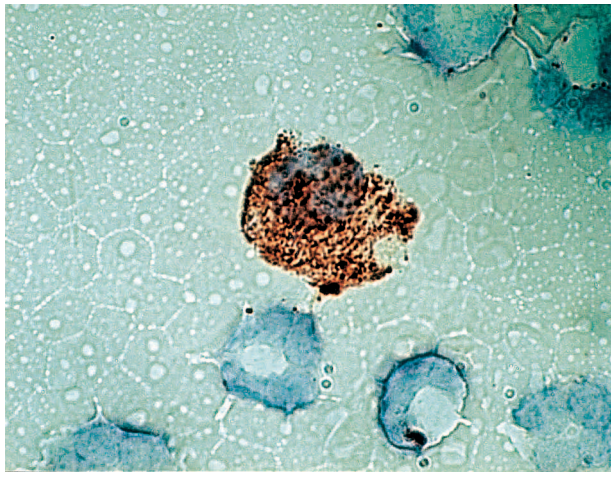

Fig. 6. HMB-45 stain of a cytospin specimen of blister fluid from Patient 3. A melanocyte was detected floating free in the blister fluid of a blister over an epidermolysis bullosa naevus (× 1000).

Cytospin® 3 Cytocentrifuge (Thermo Shandon, Pittsburgh, PA, USA) at 800 rpm for 5 min, fixed in acetone and immunohistochemically stained with anti-HMB-45 antibodies (M0634, Dako). Two melanocytic cells were detected in this sample, one of which is shown in Fig. 6.

DISCUSSION

EB naevi, which bear the exact outlines of the borders of preceding bullae as a morphological hallmark, i.e. "the imprint" of the underlying blistering skin disorder, undergo the same fate as most common acquired melanocytic naevi (15). They gradually appear in the first or second decade of life, begin as flat lesions that grow horizontally, and later while acquiring dermal components and losing pigment (16), develop papular areas resulting in the chagrin-like appearance of dermal naevi (see Fig. 3 in Ref. 3).

Sander et al. (17) describe 17% of 126 melanomas in Swedish persons below 20 years of age as having had an associated precursor lesion. In 17 out of 36 children with malignant melanoma, Schmid-Wendtner et al. (18) documented a precursor lesion at the site of the subsequent melanoma and emphasized that prophylactic excision of suspect pigmented lesions was mandatory. According to the ABCD rules (4, 5), the EB naevi in our three children with recessive EB clinically resembled malignant melanomas, including satellite lesions. However, we have not seen any malignant transformation of EB naevi in 19 patients during a follow-up period of more than 20 years in some cases. The benign nature of EB naevi has also been emphasized by Voglino & Voglino (19). However, one has to keep in mind that chronic injury, inflammation and wound healing provide optimal conditions for tumour promotion, as is shown by the high incidence of aggressively metastasing squamous cell carcinomas and the increased risk for the development of malignant melanomas in patients with recessive, dystrophic EB (20). There is also the occurrence of multiple keratoacanthomas (21) and squamous cell carcinomas in patients with junctional EB (22, 23). Nevertheless, after histopathological evaluation of the EB naevus (occasionally multiple biopsies) a "wait-and-see" strategy with regular (at least annual) clinical follow-up can be an alternative complete excision. This approach is especially suited for patients with EB with skin fragility and potentially impaired wound healing.

Kopf et al. (24) followed up two children with hundreds of benign, eruptive naevocytic naevi after a severe bullous disease over 4 and 6 years, respectively, and recorded the condition as having reached a point of stability in terms of the number of lesions with no tendency to involute spontaneously. Interestingly, such naevi have not been reported in autoimmune blistering diseases. EB naevi differ from these eruptive naevi in size and shape (i.e. the imprinted shape of the preceding blister) and, most notably, show a continuing dynamic growth pattern over years, as seen in Patient 1, until they finally disappear or turn into a chagrin-naevus.

To gain more insight into the pathogenesis of EB naevi, we compared cytokine/growth factor levels in the blister fluid of our patients with the levels from patients with second-degree burn-blisters, Stevens-Johnson syndrome and bullous pemphigoid. We were able to detect HGF, IL-8, GM-CSF, PG-E2 and LTB4, but virtually no bFGF in the blister fluids of our three patients with recessive EB or in other patients with junctional and dystrophic EB (25). The lack of bFGF is surprising, since Arbiser et al. (26) showed elevated urinary bFGF in patients with recessive dystrophic EB and hypothesized that this could contribute to the development of squamous cell carcinomas in these patients. There are two explanations for this discrepancy: on the one hand bFGF may rapidly degrade in blisters (27, 28), or, on the other, as bFGF is a marker of chronic activation of fibroblasts it may not be involved in the acute event of blister formation.

According to Riley (29) and Valyi-Nagy et al. (30), the loss of adhesion in the dermo-epidermal basement membrane zone alone could be an initial factor promoting the proliferation and migration of free-floating melanocytes. These cells have been shown to disperse like "flocking birds" in a histological section of a skin blister of a patient with junctional EB (2). In addition, we were able to demonstrate two melanocytic cells in a cytospin specimen made of fluid drawn from a blister located on top of a large EB naevus in Patient 3 by immunohistochemical HMB-45 staining. We assume that the cytokines/growth factors detected in acute blisters of patients with EB may be ancillary to enhance

the rapid proliferation and spreading of free floating melanocytes/naevus cells to form the typical, large, "blister-shaped" EB naevi.

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