(27) have been described in the literature. Allogeneic tissue-engineered skin grafts have also been used for patients with RDEB. McGrath et al. (28) reported that cultured keratinocyte allografts did not significantly improve wound healing in 10 patients with RDEB. Apligraf®, a composite of cultured fibroblasts and keratinocytes, showed favourable results in 12 patients with RDEB (5, 6).

The main role of anchoring fibrils, of which COL7 is the main component, is in maintaining normal epidermal-dermal adherence between the basal lamina and the underlying superficial dermis. Besides this adhesive role, COL7 also aids and facilitates in the attachment and migration of keratinocytes and fibroblasts (29), and COL7 dysfunction can result in delayed wound healing (30). Indeed, COL7 staining is observed in the wound bed and neodermis in acute wound healing (31). This is the first study to assess COL7 expression of patients with RDEB after CDS treatment by immunofluorescence and electron-microscopy. In both of our cases, increased expression of COL7 after this CDS treatment could not be confirmed. Some technical difficulties in detecting a small increase in the protein could explain this result, because patients with RDEB-O who participated in this study expressed reduced but detectable amounts of COL7 in the baseline. It is also possible COL7 released from allogeneic fibroblasts could have been degraded in the wound bed instead of depositing at the DEJ.

The fibroblasts contained in the CDS release various cytokines and growth factors that play major roles in modulating wound healing. These cytokines and growth factors include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF)-beta1, and interleukins (IL)-6 and IL-8 (32, 33). These cytokines and growth factors may have contributed to accelerated wound healing in our patients, although the cytokine levels in the skin samples were not assessed.

This study demonstrated that CDS treatment potentially benefits patients with RDEB-O. Former studies also demonstrated that intractable ulcers of patients with RDEB-sev gen improved after CDS treatment (17). An application of CDS without fibroblasts could be used as a negative control and would have improved our study. We have reported previously a comparative study of CDS with and without cultured fibroblasts on animal models (34). However, it is not always ethically easy to design a control study in human clinical trials. Furthermore, in our study, we selected two RDEB patients whose persistent foot ulcers failed to respond to supportive care for more than 6 months and there were no other similar foot ulcer for a comparative study.

The clinical improvement observed after CDS treatment is promising, and no restrictions on patient activity are needed. However, it is not practical to apply CDS to all ulcers of RDEB patients, because multiple ulcers are typically found on the whole body of RDEB. Intractable ulcers in RDEB patients, which do not respond to supportive care for several weeks, should be the main target of CDS treatment.

In conclusion, our study clearly demonstrates the efficacy of this CDS in the treatment of intractable skin ulcers in RDEB patients. Further examination to elucidate the mechanism of this treatment is required.

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Circulating IgA and IgE autoantibodies in antilaminin-332 mucous membrane pemphigoid

K. Natsuga, W. Nishie, S. Shinkuma, R. Moriuchi, M. Shibata, M. Nishimura, T. Hashimoto* and H. Shimizu

Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15 West 7, Sapporo 060-8638, Japan
**Department of Dermatology, Kurume University School of Medicine, Kurume, Japan*

Correspondence
Ken Natsuga.
E-mail: natsuga@med.hokudai.ac.jp

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Summary

Background Antilaminin-332 mucous membrane pemphigoid (MMP) is a chronic autoimmune bullous disease that is often associated with internal malignancy. IgG autoantibodies against laminin-332 in patients with MMP are well documented; however, IgA and IgE autoantibodies against laminin-332 have not yet been described.

Objectives To characterize IgA and IgE autoantibodies binding to laminin-332 in sera from patients with antilaminin-332 MMP.

Methods Sera and skin samples from four patients who met the following criteria were used: (i) subepidermal blistering lesions present on the mucous membranes; (ii) *in vivo* deposition of IgG along the epidermal basement membrane zone of sampled skin; (iii) circulating IgG antibasement membrane zone antibodies that react with the dermal side of salt-split normal human skin; and (iv) circulating IgG autoantibodies that do not show positivity against type VII collagen or 200-kDa protein (p200 antigen) in immunoblot analysis using dermal extracts. Circulating IgG/IgA/IgE class autoantibodies against laminin-332 were determined by immunoblotting.

Results Circulating IgG autoantibodies against the γ2, α3/γ2, α3 and α3/β3/γ2 subunits of laminin-332 were demonstrated in sera from four patients, respectively. Serum from one of the four patients showed IgA reactivity with the α3/β3/γ2 subunits of laminin-332. Serum from one of the four patients showed IgE reactivity with the γ2 subunit of laminin-332. The control sera failed to display IgG/IgA/IgE reactivity to laminin-332.

Conclusions In addition to IgG autoantibodies, circulating IgA and IgE autoantibodies against laminin-332 are detectable in a subset of patients with antilaminin-332 MMP.

Mucous membrane pemphigoid (MMP) is a heterogeneous group of autoimmune subepidermal blistering disorders that are characterized by circulating autoantibodies against epidermal basement membrane zone (BMZ) components and mucous membrane involvement.[1] To date, several epithelial components in the BMZ have been identified as autoantigens recognized by autoantibodies in patients with MMP. These include laminin-332 (α3, β3 and γ2 subunits), laminin-311 (α3 subunit), BP230 (BPAG1), type XVII collagen (COL17), type VII collagen (COL7) and the β4 integrin subunit.[1] Among these, laminin-332, previously called laminin-5 or epiligrin, is a major autoantigen in patients with MMP.[2–7]

Clinical manifestations of patients with antilaminin-332 MMP (L332-MMP) are severe and often include blistering and erosions of the conjunctivae, oral mucosa, laryngeal tract and oesophagus.[6] Recent studies showed that patients with L332-MMP have an increased relative risk of solid cancer.[8,9] IgG autoantibodies against laminin-332 in patients with MMP are well documented. In addition, the pathogenicity of IgG antibodies against laminin-332 has been clarified using *in vivo* mouse models.[10,11] In contrast to IgG, other immunoglobulin subtypes, such as IgA and IgE, have not been described as autoantibodies in patients with L332-MMP.

This study aims to characterize the immunoglobulin subtypes of circulating autoantibodies in sera from patients with L332-MMP. Our data demonstrate that IgA and IgE autoantibodies are present in a subset of patients with L332-MMP.

Materials and methods

Antibodies

Affinity-purified fluorescein isothiocyanate-conjugated goat antihuman IgG, horseradish peroxidase (HRP)-conjugated goat $F(ab')_2$ antimouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, U.S.A.), HRP-conjugated rabbit antihuman IgG, HRP-conjugated rabbit antihuman IgA (Dakocytomation, Glostrup, Denmark) and monoclonal mouse antihuman IgE (GE-1) (Sigma Aldrich, St Louis, MO, U.S.A.) were used in this study.

Immunofluorescence analysis

Direct immunofluorescence was performed on perilesional skin biopsy specimens from patients. Indirect immunofluorescence was performed on 1 mol L^{-1} NaCl-split normal human skin as described previously.[12]

Immunoblot analysis

Normal human dermal extracts were derived as described previously.[13] Briefly, fresh normal human skin was incubated in phosphate-buffered saline containing 2 mmol L^{-1} ethylenediaminetetraacetic acid and 1 mmol L^{-1} phenylmethylsulphonyl fluoride (PMSF) for 48 h at 4 °C. After dermal–epidermal separation, the dermis was extracted by treatment with urea-containing buffer (25 mmol L^{-1} Tris–HCl, pH 7·0, 8 mol L^{-1} urea and 1 mmol L^{-1} PMSF) for 2 h at room temperature. After centrifugation, supernatants were dialysed against distilled water for 48 h at 4 °C and lyophilized. Purified laminin-332 was a courtesy gift from Dr S. Amano, Shiseido Life Science Research Center, Yokohama, Japan.[14,15]

For immunoblotting of normal human dermal extracts and purified laminin-332, each sample was solubilized in Laemmli's sample buffer and applied on sodium dodecyl sulphate–polyacrylamide gels, and transferred on to nitrocellulose membrane. A Ponceau S stain was performed for total protein staining and visualized on a digital camera. The membrane was blocked for 1 h at room temperature in 3% skimmed milk in Tris-buffered saline. For IgG detection, blots were incubated with 1 : 20 diluted serum overnight at 4 °C. Bound antibodies were visualized enzymatically using 1 : 100 diluted HRP-conjugated rabbit antihuman IgG. For IgA detection, membranes were incubated with 1 : 20 diluted serum overnight at 37 °C, and then incubated in 1 : 50 diluted HRP-conjugated rabbit antihuman IgA for 3 h at room temperature. For IgE detection, membranes were incubated with 1 : 3 diluted serum overnight at 4 °C followed by 1 : 1000 diluted mouse antihuman IgE for 3 h at room temperature, and finally 1 : 500 diluted HRP-conjugated antimouse IgG for 3 h at room temperature. Colour was developed with 4-choro-1-naphthol in the presence of H_2O_2.

Patients

Sera and skin samples from four patients with L332-MMP were used in this study. These patients met the following criteria: (i) subepidermal blistering lesions present on mucosal surfaces; (ii) *in vivo* deposition of IgG along the BMZ in skin samples from patients; (iii) circulating IgG anti-BMZ antibodies that react with the dermal side of 1 mol L^{-1} NaCl-split skin; and (iv) circulating IgG autoantibodies that do not show positivity against type VII collagen or 200-kDa protein (p200 antigen) by immunoblot analysis using dermal extracts as described above. Direct and indirect immunofluorescence on perilesional skin samples and sera showed no IgA or IgE deposition at the BMZ for any of the four patients.

Case reports

Patient 1

A 77-year-old man with a 3-year history of rheumatoid arthritis noticed erosions on his oral mucosa 2 months before he was referred to our hospital. He had not taken any medication for his arthritis. Upon physical examination, multiple blisters and erosions were observed on his trunk, extremities and oral mucosa. Systemic corticosteroids gradually alleviated his skin and mucosal condition.

Patient 2

The patient was a 63-year-old man who had had rheumatoid arthritis for 5 years and was being treated with bucillamine. He noticed multiple bullae on his extremities and erosions on the oral mucosa and both conjunctivae 6 months before referral to our hospital. His symptoms showed no improvement at 2 months after discontinuation of the bucillamine. Physical examination revealed erosions on the oral mucosa and the whole body, and scarring on the conjunctivae. He refused further investigation and treatment.

Patient 3

A 62-year-old man with bronchial asthma and diabetes mellitus had complained of conjunctival congestion 5 years before referral. The diagnosis of ocular pemphigoid was made by ophthalmologists, and he was treated with systemic corticosteroids. He was referred to our hospital after his condition worsened with a tapering of the corticosteroids. Multiple bullae on his extremities, erosions on the oral mucosa and scarring of both conjunctivae were observed. Oesophageal involvement was noted. Cyclophosphamide in combination with prednisolone ameliorated his skin and mucosal condition, although the conjunctival scarring remained.

Patient 4

The patient was an 85-year-old man with end-stage carcinoma of the lung. Blisters and erosions appeared on his extremities,

trunk and oral mucosa. After systemic corticosteroid treatment was started, his skin symptoms improved.

Histopathology

Histopathological findings of perilesional skin samples from all patients revealed subepidermal blister formation with infiltration of inflammatory cells, including a few eosinophils. There were no notable differences in histopathological features between samples.

Results

IgG autoantibodies against purified laminin-332 in sera from the four patients

Ponceau S and control L332-MMP serum revealed four distinctive proteins that characterize laminin-332: 165-kDa processed α3 subunit, 145-kDa degraded α3 subunit, 140-kDa β3 subunit and 105-kDa γ2 subunit (Fig. 1a). Serum from patient 1 had circulating IgG autoantibodies against the γ2 subunit of laminin-332. Serum from patient 2 had circulating IgG autoantibodies against the α3 and γ2 subunits of laminin-332. Serum from patient 3 had circulating IgG autoantibodies against the α3 subunit of laminin-332. Serum from patient 4 had circulating IgG autoantibodies against all three subunits (α3, β3 and γ2) of laminin-332 (Fig. 1a).

IgA autoantibodies against purified laminin-332 were found in a subset of the patients with antilaminin-332 mucous membrane pemphigoid

Immunoblot analysis using purified laminin-332 showed that IgA autoantibodies from patient 3 showed reactivity against all three subunits (α3, β3 and γ2) (Fig. 1b).

Circulating IgE autoantibodies against purified laminin-332 were present in one of four patients

IgE autoantibodies from patient 1 tested positive for the γ2 subunit (Fig. 1c).

Healthy control sera failed to display any IgG/IgA/IgE reactivity to purified laminin-332 (Fig. 1a–c). Table 1 summarizes the four patients with L332-MMP, the immunoglobulin subtypes demonstrated to be autoantibodies and the antigenic subunits of laminin-332.

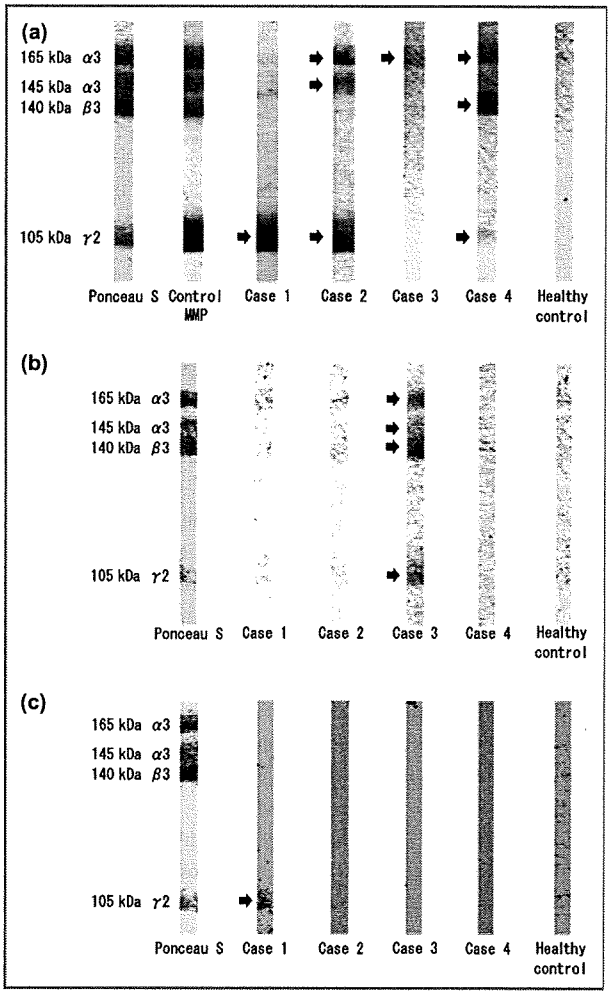

Fig 1. IgG, IgA and IgE autoantibodies against purified laminin-332. (a) Immunoblot analysis using purified laminin-332 revealed circulating IgG autoantibodies against the γ2 subunit, 105 kDa (arrow, case 1), the α3 and γ2 subunits, 165 kDa, 145 kDa and 105 kDa (arrows, case 2), the α3 subunit, 165 kDa (arrow, case 3), and all the α3/β3/γ2 subunits, 165 kDa, 140 kDa, 105 kDa (arrows, case 4) in sera from patients with mucous membrane pemphigoid. (b) IgA from case 3 serum reacted with all the α3/β3/γ2 subunits, 165 kDa, 145 kDa, 140 kDa, 105 kDa (arrows). (c) Case 1 serum had circulating IgE autoantibodies against the γ2 subunit, 105 kDa (arrow).

Discussion

IgG is the main immunoglobulin subtype that has been confirmed as an autoantibody against BMZ components in sera from patients with MMP. In sera from patients with L332-MMP, only IgG autoantibodies have been described so far. Previous studies revealed that passive transfer of rabbit antilaminin-332 IgG induces subepidermal blisters in neonatal mice.[10] Furthermore, antilaminin-332 IgG antibodies purified from human patients are known to induce subepidermal blistering in human skin grafts on SCID mice.[11] These *in vivo* experiments suggest that IgG antibodies against laminin-332 play a pathogenic role in MMP.

IgA autoantibodies are another major immunoglobulin subtype found in sera from patients with MMP, and these autoantibodies specifically recognize COL17 (anti-COL17 MMP).[16–21] Recent studies have revealed that passive transfer of monoclonal mouse IgA against the linear IgA dermatosis antigen, which is the shed ectodomain of COL17, into human skin grafts transplanted on SCID mice produces subepidermal separation and neutrophil infiltration.[22] This

Table 1 Summary of patients with antilaminin-332 mucous membrane pemphigoid, autoantibody immunoglobulin subtypes, and antigenic subunits of laminin-332

Patient	Sex/age (years)	Concurrent illness	Treatment	Autoantibody subclass			Antigenic subunits of laminin-332
				IgG	IgA	IgE	
1	M/77	Rheumatoid arthritis	PSL	+	–	+	γ2 (IgG), γ2 (IgE)
2	M/63	Rheumatoid arthritis	PSL	+	–	–	α3/γ2 (IgG)
3	M/62	Bronchial asthma, diabetes mellitus	PSL + CPM	+	+	–	α3 (IgG), α3/β3/γ2 (IgA)
4	M/85	Lung carcinoma	PSL	+	–	–	α3/β3/γ2 (IgG)

PSL, prednisolone; CPM, cyclophosphamide.

supports the theory that IgA autoantibodies also play a pathogenic role in IgA-related autoimmune bullous diseases. It was recently argued that IgE autoantibodies play a pathogenic role in autoimmune blistering diseases. Some patients with bullous pemphigoid (BP) have IgE autoantibodies against COL17[18,23–26] and BP230,[23,26,27] and injection of purified IgE against COL17 produced subepidermal blistering of normal human skin grafts in immunodeficient mice.[28,29] Therefore, IgE might also play an important role in the pathogenesis of certain autoimmune blistering diseases. However, IgA and IgE autoantibodies against laminin-332 in MMP sera have not been described.

The correlation between clinical manifestations and the immunoglobulin subtypes in autoantibodies is difficult to define. This is because of the limited number of patients included in our study, although patient 3 in this study, with IgA autoantibodies against laminin-332, had severe conjunctival involvement. Previous studies showed IgE autoantibodies in cases of severe BP.[23,24] In our study, patient 1, with IgE autoantibodies against laminin-332, showed a good response to systemic corticosteroid treatment without sequelae.

The concentration of IgA/IgE is much lower than that of IgG, which may explain the difficulty of detecting circulating IgA/IgE antibodies. Immunofluorescence analysis of the patients with MMP in our study showed no detectable deposition of IgA or IgE at the BMZ, although IgE and IgA autoantibodies against laminin-332 were detected by immunoblot in patients 1 and 3, respectively. In previous studies, immunoblot analysis also detected anti-COL17 IgA or IgE autoantibodies in sera from patients whose skin specimens and sera showed no deposition of IgA or IgE at the BMZ.[18] This phenomenon can be explained by the difference in sensitivity between immunofluorescence and immunoblot.

IgG is still the main immunoglobulin subtype of autoantibodies against laminin-332. Nevertheless, IgA and IgE autoantibodies against laminin-332 were detectable in a small subset of patients with MMP. In summary, this study is the first report to describe IgA and IgE autoantibodies against laminin-332 in patients with MMP. Further study is needed to elucidate the frequency and pathogenicity of IgA/IgE antibodies in patients with L332-MMP.

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RESEARCH ARTICLE

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Plectin Expression Patterns Determine Two Distinct Subtypes of Epidermolysis Bullosa Simplex

Ken Natsuga,[1] Wataru Nishie,[1] Masashi Akiyama,[1] Hideki Nakamura,[1] Satoru Shinkuma,[1] James R. McMillan,[1] Akari Nagasaki,[1] Cristina Has,[1] Takeshi Ouchi,[3] Akira Ishiko,[3] Yoshiaki Hirako,[4] Katsushi Owaribe,[4] Daisuke Sawamura,[1] Leena Bruckner-Tuderman,[2,5] and Hiroshi Shimizu[1]*

[1]*Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan;* [2]*Department of Dermatology, University Medical Center Freiburg, Germany;* [3]*Department of Dermatology, Keio University School of Medicine, Tokyo, Japan;* [4]*Division of Biological Science, Graduate School of Science, Nagoya University, Nagoya, Japan;* [5]*Freiburg Institute of Advanced Studies, School of Life Sciences, Freiburg, Germany*

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ABSTRACT: Plectin is a cytoskeletal linker protein that has a dumbbell-like structure with a long central rod and N- and C-terminal globular domains. Mutations in the gene encoding plectin (*PLEC1*) cause two distinct autosomal recessive subtypes of epidermolysis bullosa (EB): EB simplex with muscular dystrophy (EBS-MD), and EB simplex with pyloric atresia (EBS-PA). Here, we demonstrate that normal human fibroblasts express two different plectin isoforms including full-length and rodless forms of plectin. We performed detailed analysis of plectin expression patterns in six EBS-MD and three EBS-PA patients. In EBS-PA, expression of all plectin domains was found to be markedly attenuated or completely lost; in EBS-MD, the expression of the N- and C-terminal domains of plectin remained detectable, although the expression of rod domains was absent or markedly reduced. Our data suggest that loss of the full-length plectin isoform with residual expression of the rodless plectin isoform leads to EBS-MD, and that complete loss or marked attenuation of full-length and rodless plectin expression underlies the more severe EBS-PA phenotype. These results also clearly account for the majority of EBS-MD *PLEC1* mutation restriction within the large exon 31 that encodes the plectin rod domain, whereas EBS-PA *PLEC1* mutations are generally outside exon 31.
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KEY WORDS: *PLEC1*; basement membrane zone; skeletal muscle; mRNA decay; truncation

Introduction

Plectin is a 500-kDa intermediate filament-binding protein that serves as a crosslinking element of the cytoskeleton to supply mechanical strength to cells and tissues [Wiche, 1998]. Plectin is expressed in a wide variety of tissues, including skin, striated muscle, and gastrointestinal tract epithelia. Within the cutaneous epithelium, plectin is especially concentrated along the basal pole of basal keratinocytes, where it functions as a linker between the keratin intermediate filament cytoskeleton, hemidesmosomes, and the underlying basement membrane zone (BMZ) [Borradori and Sonnenberg, 1999]. Plectin interacts with α6 and β4 integrins [Geerts et al., 1999; Litjens et al., 2003, 2005; Niessen et al., 1997a,b; Rezniczek et al., 1998; Schaapveld et al., 1998], BPAG2 [Koster et al., 2003], and periplakin [Boczonadi et al., 2007].

Epidermolysis bullosa (EB) comprises a group of heterogeneous disorders in which congenital skin fragility leads to dermal–epidermal junction separation. EB is subdivided into the three major groups of EB simplex, junctional EB, dystrophic EB, and the one minor group of Kindler syndrome, based on the level of blister formation [Fine et al., 2008]. So far, mutations in 13 different genes have been identified as underlying EB subtypes [Fine et al., 2000, 2008]. Among them, mutations in the gene encoding plectin, *PLEC1* (MIM# 601282), are responsible for two distinct types of autosomal recessive EBS (EBS with muscular dystrophy [EBS-MD] and EBS with pyloric atresia [EBS-PA]) and one subtype of autosomal dominant EBS (EBS-Ogna) [Fine et al., 2008]. Patients with EBS-Ogna are heterozygous for one amino acid substitution in the rod domain of plectin [Koss-Harnes et al., 2002]. EBS-Ogna is thought to be caused by plectin perturbation that results from dominant negative interference [Pfendner et al., 2005]. In contrast, homozygous or compound heterozygous loss-of-function mutations in *PLEC1* lead to EBS-MD or EBS-PA.

EBS-MD is characterized by generalized blistering and delayed onset of muscular dystrophy. Defective expression of plectin was found in patients with EBS-MD [Gache et al., 1996] and mutations in *PLEC1* were found to be responsible for the EBS-MD phenotype [McLean et al., 1996; Smith et al., 1996]. To date, more than 30 EBS-MD patients have been reported to have *PLEC1* mutations [Bauer et al., 2001; Chavanas et al., 1996; Dang et al., 1998; Koss-Harnes et al., 2004; Kunz et al., 2000; McMillan et al., 2007; Mellerio et al., 1997; Pfendner et al., 2005; Pulkkinen et al., 1996; Rouan et al., 2000; Sawamura et al., 2007; Takahashi et al., 2005; Takizawa et al., 1999]. Most reported *PLEC1* mutations in EBS-MD patients are located within exon 31 encoding the large rod domain of plectin [Pfendner et al., 2005; Sawamura et al., 2007]. In contrast to the phenotype seen in EBS-MD, clinical manifestations of EBS-PA are more severe and are characterized by more generalized blistering and pyloric atresia,

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: Hiroshi Shimizu, Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15 West 7, Sapporo 060-8638, Japan. E-mail: shimizu@med.hokudai.ac.jp

which frequently leads to early death in affected patients. Similarly, junctional EB with pyloric atresia (JEB-PA) has been known to be caused by the mutation in the gene encoding α6/β4 integrin (*ITGA6*; MIM# 147556; *ITGB4*; MIM# 147557), and about 60 *ITGA6* or *ITGB4* mutations have been described [Fine et al., 2008; Varki et al., 2006]. Recently, our group and others identified *PLEC1* mutations in eight patients with EBS-PA [Nakamura et al., 2005; Pfendner et al., 2005; Pfendner and Uitto, 2005; Sawamura et al., 2007]. EBS-MD and EBS-PA represent distinct clinical phenotypes, although both are caused by *PLEC1* mutations. The exact mechanisms that produce the clinical differences between EBS-MD and EBS-PA subtypes have not been elucidated, although it has been postulated that the severity of EBS patients with *PLEC1* mutations could be associated with alternative splicing of plectin [Sawamura et al., 2007; Sonnenberg and Liem, 2007].

The present study demonstrates that normal human fibroblasts express two different plectin isoforms: full-length plectin, and a shorter rodless plectin. In light of this finding, we collected skin samples and cultured cells from patients with EBS-MD and EBS-PA in which we precisely analyzed their expression levels of plectin using immunoblotting, immunofluorescence, and semiquantitative RT-PCR to determine the different pathogenic mechanisms underlying *PLEC1* mutations. Our data suggest that EBS-MD and EBS-PA exhibit different plectin expression patterns, and this study gives further insight toward improving our understanding of genotype-phenotype correlation in EBS patients with *PLEC1* mutations.

Materials and Methods

Patients and Mutation Detection

Nine EBS patients in whom *PLEC1* mutations had been confirmed were analyzed: six EBS-MD and three EBS-PA unrelated individuals (Table 1). *PLEC1* mutations in four EBS-MD and three EBS-PA cases were previously described in the literature [Kunz et al., 2000; Nakamura et al., 2005; Pulkkinen et al., 1996; Sawamura et al., 2007; Takizawa et al., 1999]. Patients EBS-MD1 and EBS-MD5 were newly identified cases in the present study.

EBS-MD1 was a 24-year-old Japanese female. She was the first child of nonconsanguineous, healthy parents. Generalized blistering and erosions of the skin were noted at birth, together with nail dystrophy. She had no history of pyloric atresia. At the age of 10, she developed muscular dystrophy. EBS-MD5 was a 7-year-old Croatian male. He was the second child of nonconsanguineous, healthy parents. His elder brother was healthy. He developed generalized blistering, including of the oral mucosal, and laryngeal stridor, immediately after birth. Pyloric atresia was not observed. To date, he has not developed muscular dystrophy.

Genomic DNA (gDNA) was isolated from peripheral blood leukocytes (EBS-MD1 and her parents) or cultured fibroblasts (EBS-MD5). The mutation detection was performed after polymerase chain reaction (PCR) amplification of all *PLEC1* exons and intron–exon borders, followed by direct automated sequencing using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Oligonucleotide primers and PCR conditions used in this study were derived from a previous report [Nakamura et al., 2005]. The gDNA nucleotides, the complementary DNA (cDNA) nucleotides, and the amino acids of the protein, were numbered based on the previous sequence information (GenBank accession no. AH003623) [McLean et al., 1996].

The medical ethical committees of Hokkaido University, Keio University, and University Medical Center Freiburg approved all described studies. The study was conducted according to The Declaration of Helsinki Principles. Participants gave their written informed consent.

A schematic of plectin structure and *PLEC1* mutations detected in EBS patients in this study is shown in Figure 1A.

Antibodies

The plectin domains where the antibodies used in this study react are summarized in Figure 1B. Mouse monoclonal antibodies (mAbs), PN643 against the actin-binding domain of plectin and PC815 against the C-terminal plectin repeats were prepared by immunizing mice with recombinant His-tagged fusion proteins. To produce recombinant proteins, the cDNAs that encode the actin-binding domain of plectin and C-terminal plectin repeats comprising amino acids 171–595 and 2,930–3,153 (GenBank accession no. AAB05428.1), respectively, were cloned into a pET32c vector. The resultant recombinant proteins were expressed in the *Escherichia coli* expression host BL21(DE3)pLysS and purified using a His-Bind column (Novagen, Madison, WI). Spleen cells derived from immunized mice were fused with mouse myeloma cells. Hybridomas producing antibodies against plectin were selected by immunofluorescent microscopy screening using normal human skin. Immunoblotting using cytoplasmic extracts from DJM-1 cells confirmed that both of the antibodies reacted with a 500-kDa protein.

In addition to PN643 and PC815, the following mAbs against BMZ components were used: mAbs HD1-121 [Hieda et al., 1992; Okumura et al., 1999], 10F6 [Foisner et al., 1991], and 5B3 [Foisner et al., 1994] against the rod domain of plectin; mAb LH7.2 (Sigma, St. Louis, MO) against type VII collagen. mAb 10F6 and 5B3 were kind gifts from Dr. G. Wiche of the University of Vienna. C20, a goat polyclonal antibody against the C-terminus

Table 1. EBS Patients and *PLEC1* Mutations

Patient	Mutation 1 (predicted consequence)	Exon	Mutation 2 (predicted consequence)	Exon	Reference
EBS-MD1	**c.6549_6582del (p. Ala2183fs)**	31	**c.13040dup (p.Gly4347fs)**	32	Present case
EBS-MD2	c.4348C>T (p.Gln1450X)	31	c.4348C>T (p.Gln1450X)	31	Sawamura et al., 2007
EBS-MD3	c.3157C>T (p.Gln1053X)	24	c.5806C>T (p.Gln1936X)	31	Takizawa et al., 1999
EBS-MD4	c.5866del (p.Leu1956fs)	31	c.5866del (p.Leu1956fs)	31	Pulkkinen et al., 1996
EBS-MD5	**c.4643_4667dup (p.Arg1556fs)**	31	**c.7120C>T (p.Gln2374X)**	31	Present case
EBS-MD6	c.5188C>T (p.Gln1713X)	31	c.7102C>T (p.Arg2351X)	31	Kunz et al., 2000
EBS-PA1	c.7396C>T (p.Gln2466X)	31	c.7633C>T (p.Gln2545X)	32	Sawamura et al., 2007
EBS-PA2	c.3565C>T (p.Arg1189X)	27	c.7612C>T (p.Gln2538X)	32	Nakamura et al., 2005
EBS-PA3	c.913C>T (p.Gln305X)	9	c.1344G>A (p.Gln447fs; =)[a]	12	Nakamura et al., 2005

The novel *PLEC1* mutations detected in this study are in bold. MD, Muscular dystrophy; del, deletion; dup, duplication; fs, frameshift.
[a]The mutation c.1344G>A is at the 3′ end of exon12. Predicted consequences resulting from this mutation are discussed in the Results.

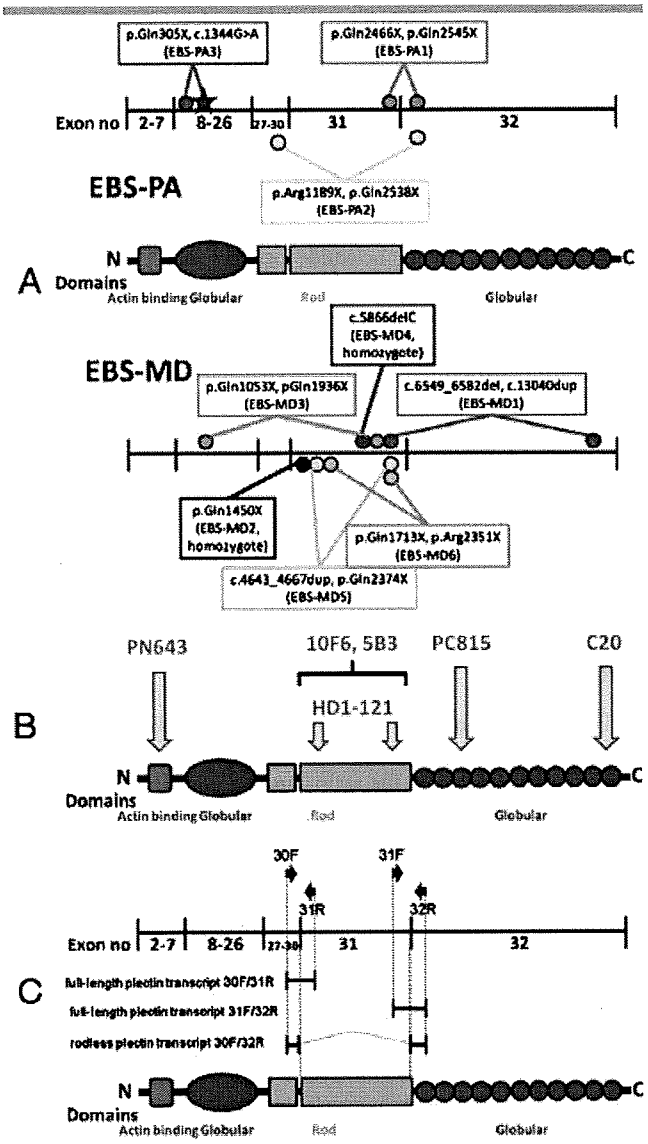

Figure 1. Scheme of plectin structure, PLEC1 mutations, antibodies against plectin and specific primers to detect the rodless transcript form of plectin. **A:** Plectin molecules consist of a central rod flanked by amino-terminal and carboxy-terminal globular domains. PLEC1 mutations observed in EBS patients of this study are indicated. Dots represent truncated mutations. The star indicates a splice-site mutation (c.1344G>A). **B:** PN643 is a monoclonal antibody (mAb) against the N-terminal actin-binding domain of plectin. HD1-121, 10F6, and 5B3 are mAbs against the rod domain of plectin. PC815 is a mAb and C20 is a polyclonal antibody against the C-terminal globular domain of plectin. **C:** Specific primers used to detect the presence of transcripts for full-length (30F/31R and 31F/32R) and rodless plectin (30F/32R) on cDNA synthesized from the mRNA of normal human, EBS-MD5 and EBS-PA3F cells.

of plectin, was purchased from Santa Cruz (Santa Cruz, CA). Anti-beta-actin mAb (AC15, Sigma) was used to confirm equal protein loading.

Immunofluorescence Studies

Immunofluorescence analysis was performed using skin specimens from the patients (Table 1). Fresh-frozen skin specimens were embedded in optimal cutting temperature (OCT) compound and quickly frozen in isopentane cooled over liquid nitrogen. 5-μm cryostat-cut sections were incubated overnight at 4°C with primary antibodies including the following mAbs: PN643 (working dilution of 1:160), HD1-121 (1:100), 10F6 (1:10), 5B3 (1:20), PC815 (1:20), and LH7.2 (1:10). After washing in phosphate-buffered saline, the sections were incubated with secondary antibodies conjugated with fluorescein-isothiocyanate.

Cell Culture and Immunoblot Analysis

Cultured fibroblasts were obtained from skin biopsies of a normal human volunteer and of patient EBS-MD5. Cultured amniocytes were derived from an aborted fetus who was a sibling of EBS-PA3 (EBS-PA3F). Prenatal diagnosis of EBS-PA3F revealed that the fetus had the same *PLEC1* mutations as were detected in EBS-PA3 (data not shown). Cultured fibroblasts and amniocytes were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. Cultured oral keratinocytes were obtained from biopsies of a normal human volunteer and were maintained in CnT-57 medium (CELLnTEC). Whole-cell lysates of human skeletal muscle were purchased from Abcam (ab82589). For sample preparation, cultured cells were lysed in Nonidet-40 (NP-40) containing buffer (1% NP-40, 25 mM Tris-HCl [pH 7.6], 4 mM EDTA, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride [PMSF], and proteinase inhibitor cocktail [Sigma]) on ice for 30 min; cell debris was removed by centrifugation at 14,000 rpm for 15 min; and supernatant was collected. Supernatants were solubilized in Laemmli's sample buffer [Laemmli, 1970], applied to SDS-polyacrylamide gels, and transferred to a PVDF membrane. The membrane was incubated with PN643, HD1-121, C20, and AC15 overnight at 4°C followed by incubation with horseradish peroxidase (HRP) conjugated anti-mouse IgG (for PN643, HD1-121, and AC15) and HRP-conjugated antigout IgG (for C20) for 1 hr at room temperature. The blots were detected using ECL Plus Detection Kit (GE Healthcare, Fairfield, CT). The images were obtained with LAS-4000 mini (Fujifilm, Tokyo, Japan). To elucidate the quantitative ratio of full-length/rodless plectin, immunoblotting of lysates from normal human fibroblasts, keratinocytes, and skeletal muscle was performed in triplicate. Band intensities were analyzed by densitometry (ImageJ).

Semiquantitative RT-PCR Analysis

Total RNA was isolated from cultured fibroblasts (normal human volunteers and EBS-MD5) or amniocytes (EBS-PA3F) using RNeasy kit (Qiagen, Valencia, CA), and first-strand cDNA was made using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). First-strand cDNA was then amplified by PCR with primers specific for the exon boundaries flanking the rod domain of plectin as described previously [Koster et al., 2004] (Fig. 1C). The following primers were used in this study: 30F, 5′-CATCAGCGAGACTCTGCGGC-3′; 31R, 5′-TGCGCCTGTCG-CTTTTGTGC-3′; 31F, 5′-AGCTGGAGATGAGCGCTGA-3′; 32R, 5′-TGCTGCAGCTCCTCCTGC-3′. PCR conditions were as follows: 94°C for 5 min, followed by 30 cycles (31F/32R, 30F/32R) and 35 cycles (30F/31R) at 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, and extension at 72°C for 7 min. To ensure equal loading, a housekeeping gene (GAPDH) was simultaneously amplified. The PCR products were assessed on a 2% agarose gel. The images were obtained with LAS-4000 mini (Fujifilm). To confirm the skipping of exon 31 in rodless transcript, direct sequencing was performed for the PCR products (30F/32R). To analyze transcripts derived from the c.1344G>A mutant allele,

PCR amplification of synthesized EBS-PA3F cDNA from exon 9 to exon 14 was performed using the following primers: 5′-GATTGAGATCCTGTGGTCTC-3′ and 5′-CTCTGCACACTCT-GCAGAGT-3′. PCR products were cloned in the TA cloning vector pCRII (Invitrogen) and then sequenced.

Results

PLEC1 Mutation Detection

PLEC1 mutational analysis in case EBS-MD1 demonstrated that the affected patient was a compound heterozygote for the maternal c.13040dup mutation in exon 32 and the paternal c.6549_6582del mutation in exon 31 (Supp. Fig. 1A). Both of the mutations resulted in a frameshift that caused 8- and 21-amino-acid missense sequences, respectively, followed by a premature termination codon (PTC). These mutations were novel, and they were confirmed by *Mwo*I restriction enzyme digestion and TA-cloning, respectively (data not shown). In addition, the c.10453C>T (p.Arg3485Trp) transition in exon 32 was also detected in one allele of the patient and her mother. This c.10453C>T transition was not found by sequence analysis in 100 normal unrelated Japanese alleles (50 healthy unrelated Japanese individuals), and it was unlikely to be polymorphism, although the contribution of this missense mutation to the EB phenotype remains unclear.

EBS-MD5 was a compound heterozygote for the c.4643_4667dup and c.7120C>T (p.Gln2374X) mutations in exon 31 (Supp. Fig. 1B). The c.4643_4667dup resulted in a frameshift that caused a 90-amino-acid missense sequence, followed by a PTC. These mutations were also novel and were confirmed by TA-cloning and *Pst*I restriction enzyme digestion respectively (data not shown).

Differential plectin isoform expression by immunoblotting in normal human fibroblasts

Immunoblot analysis of lysates from normal human cultured fibroblasts revealed that two closely spaced bands, putatively corresponding to two forms of plectin (500 kDa full-length and 390 kDa rodless form) reacted with PN643 and C20, antibodies recognizing the N- and C-termini of plectin (Fig. 2). Using HD1-121, an antibody against the rod domain of plectin, lysates from normal human fibroblasts reacted only with full-length plectin (Fig. 2). These results showed that normal human fibroblasts expressed two different *PLEC1* isoforms: full length and a shorter rodless plectin isoforms.

The Quantitative Ratio of Full-length/Rodless Plectin in Normal Human Fibroblasts, Keratinocytes, and Skeletal Muscle

To elucidate the relative amount of full-length and rodless plectin in normal human fibroblasts, keratinocytes, and skeletal muscle, we performed immunoblot analysis of lysates from each sample using PN643, an antibody against the N-terminus of plectin. Both full-length and rodless plectin were detected in each sample (Fig. 3). Band intensities were measured in triplicate ±SD. The quantitative ratio of full-length/rodless plectin was 14.2±4.2 in fibroblasts, 21.3±6.4 in keratinocytes, and 1.37±0.23 in skeletal muscle.

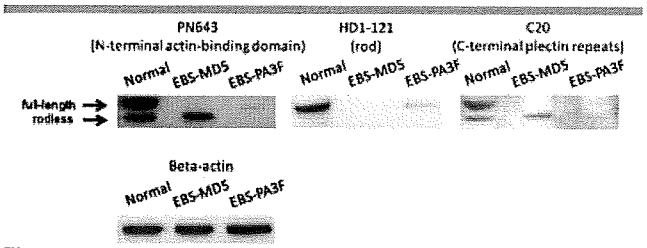

Figure 2. Immunoblot analysis of cultured fibroblasts from normal human control and EBS-MD and amniocytes from EBS-PA. Immunoblot analysis of extracts from cells of normal control, EBS-MD5, and an aborted sibling of EBS-PA3 (EBS-PA3F). Analysis used PN643 against the N-terminal actin-binding domain, HD1-121 against the rod domain and C20 against the C-terminal plectin repeats. Rodless plectin, detected with PN643 and C20, migrates just below full-length plectin in normal human fibroblasts. Using HD1-121, only full-length plectin was demonstrated in the normal control. In contrast, EBS-MD5 fibroblasts contained only rodless plectin, which was detected with PN643 and C20. Full-length plectin did not appear in EBS-MD5 using any antibody. EBS-PA3F amniocytes contained a greatly reduced amount of full-length plectin. Equal protein loading was confirmed by reprobing with AC15 (anti-beta-actin antibody). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

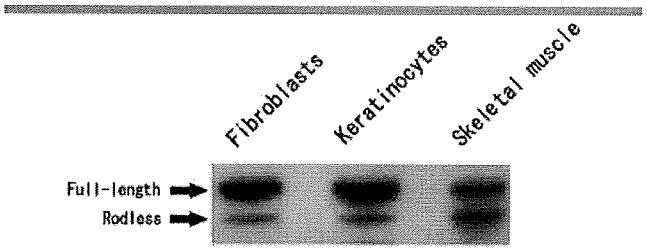

Figure 3. Relative amounts of full-length and rodless plectin in normal human fibroblasts, keratinocytes and skeletal muscle. Immunoblot analysis of lysates from normal human fibroblasts, oral keratinocytes, and skeletal muscle using PN643 against the N-terminal actin-binding domain of plectin was performed. Both full-length and rodless plectin were detected in each sample. The quantitative ratio of the two isoforms was calculated, using Image J software, as follows: 14.2±4.2 in fibroblasts, 21.3±6.4 in keratinocytes, and 1.37±0.23 in skeletal muscle. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Characterization of cutaneous plectin expression patterns in EBS-MD and EBS-PA patients by immunofluorescence analysis

To assess whether expression patterns of plectin in the skin differ between EBS-MD and EBS-PA, we performed immunofluorescence analysis using five different antibodies (Fig. 1B). PN643 weakly reacted with skin specimens from all EBS-MD patients and two out of three EBS-PA patients (EBS-PA1, 3), but failed to react with specimens from EBS-PA2 (Fig. 4A–J). HD1-121 showed weakly reactivity in three EBS-MD patients (EBS-MD1, 4, 6) and one EBS-PA patient (EBS-PA3), but was negative in the other patients (EBS-MD2, 3, 5, EBS-PA1, 2) (data not shown). 5B3, the mAb against the rod domain of plectin, was faint but identifiable in two EBS-MD patients (EBS-MD1, 6) and one EBS-PA patient (EBS-PA3), but was negative in the other patients (EBS-MD2-5, EBS-PA1, 2) (Fig. 4L–T). No skin specimens reacted with 10F6, a monoclonal antibody against the rod domain, except EBS-PA3 (data not shown). PC815 recognized the C-terminus of plectin weakly but detectably in all EBS-MD patients and EBS-PA3, but not in EBS-PA patients 1 and 2 (Fig. 4V–AD). These results

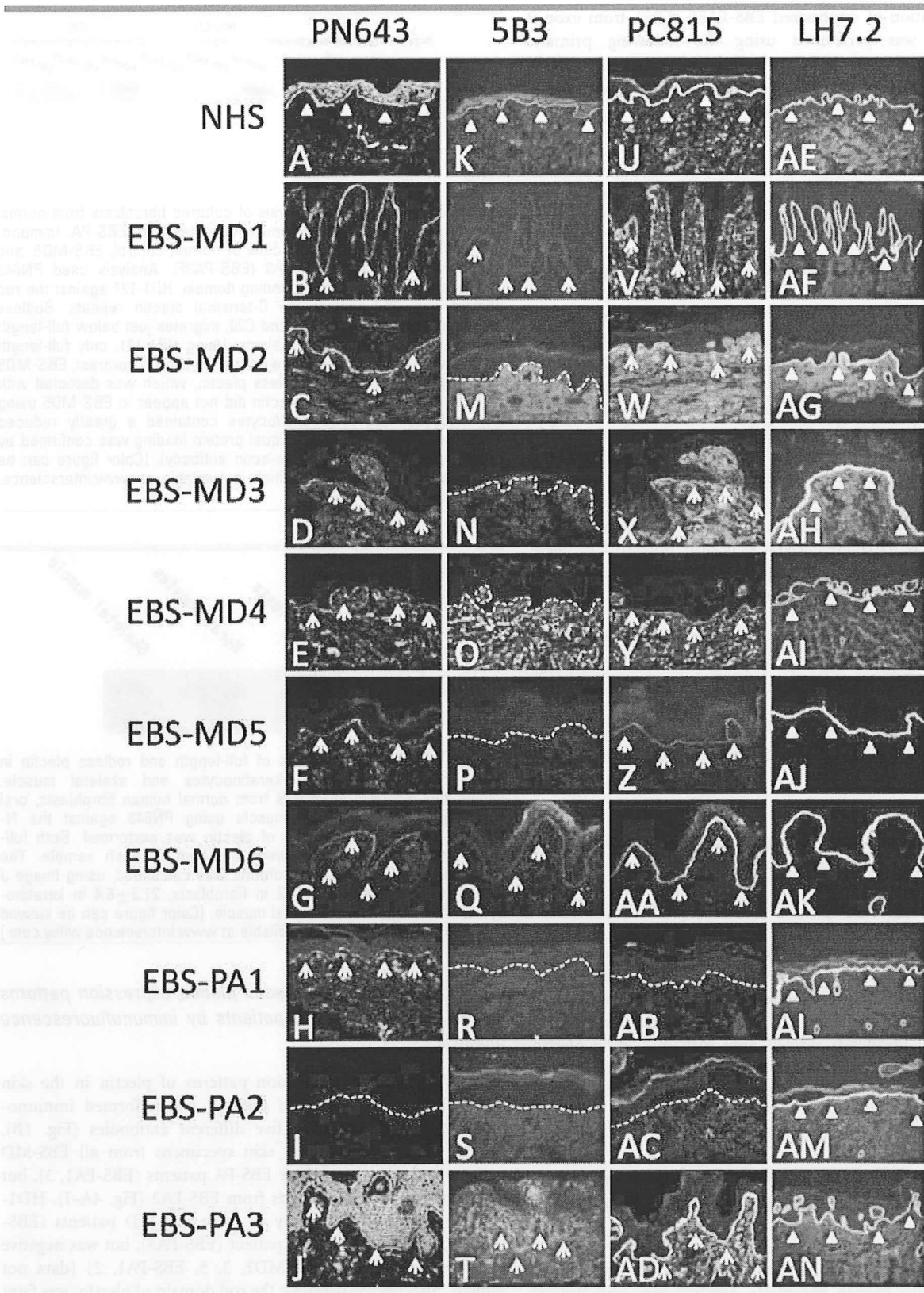

Figure 4. Immunofluorescence analysis of cutaneous plectin expression in EBS-MD and EBS-PA. In normal human skin (NHS), immunofluorescence shows that all mAbs against plectin (PN643, 5B3, and PC815) and type VII collagen (LH7.2) tested in the study bind to the dermal epidermal junction (DEJ) (**A, K, U, AE**). DEJ labeling of PN643 is weakly positive in all EBS-MD cases (**B–G**) and EBS-PA1, 3 (**H, J**), but negative in EBS-PA2 (**I**). DEJ labeling of 5B3 show faintly positive in EBS-MD1, 6 and EBS-PA3 (**L, Q, T**), and negative in EBS-MD2-5 and EBS1, 2 (**M–P, R, S**). DEJ labeling using PC815 is weakly positive in all EBS-MD cases and EBS-PA3 (**V-AA, AD**), but negative in EBS-PA1, 2 (**AB, AC**). Type VII collagen shows normal linear labeling in all EBS cases (**AF–AN**). Strong staining is indicated by arrowheads. Weak labeling is indicated by arrows. Negative labeling is indicated by dotted lines.

revealed loss of full-length plectin with the maintenance of a rodless plectin isoform in EBS-MD. EBS-PA skin specimens harbored greatly reduced amounts of both full-length and rodless plectin.

Protein and mRNA expression patterns of plectin in cultured cells from EBS-MD and EBS-PA patients

Plectin expression patterns of EBS-MD and EBS-PA cultured cells were assessed at both the protein and mRNA levels to confirm the comparative immunofluorescence analysis using skin biopsy specimens showing that the majority of EBS-MD patients expressed a rodless plectin variant, but not full-length plectin and that expression of both full-length and rodless-plectin variant peptides was remarkably reduced or completely abolished in EBS-PA patients. Immunoblot analysis of lysates from fibroblasts of patient EBS-MD5 failed to show any HD1-121 bands, although a band corresponding to rodless plectin was observed by using PN643 and C20 (Fig. 2). Lysates from cultured amniocytes from an aborted sibling of EBS-PA3 (EBS-PA3F) showed that a diminished amount of full-length plectin reacted with PN643, HD1-121, and C20 (Fig. 2).

Using RT-PCR, the presence of an RNA message that does not encode the rod domain was demonstrated in the normal human control as well as the EBS-MD5 and EBS-PA3F cells (Figs. 1C and 5A) (30F/32R). Direct sequencing confirmed the skipping of exon31 in the PCR products (30F/32R) (Fig. 5B). mRNA encoding full-length plectin containing the rod domain was also detected in normal human control, EBS-MD5, and EBS-PA3F cells (Figs. 1C and 5A) (30F/31R and 31F/32R). Judging from the PCR analysis results, the quantity of full-length plectin transcript was greatly reduced in EBS-MD5 and EBS-PA3F compared with those in the normal human controls. In addition, the rodless plectin transcripts were markedly diminished in quantity in EBS-PA3F compared with those of the normal human controls, although expression of the rodless plectin transcripts was maintained in EBS-MD5.

These data suggest that EBS-MD5 fibroblasts express only rodless truncated forms of plectin without the full-length isoform, presumably because of nonsense-mediated mRNA decay (NMD) of the full-length plectin transcript induced by the mutations within *PLEC1* exon 31 (Table 1 and Fig. 1A). Conversely, EBS-PA3F amniocytes expressed a much lower level of plectin than normal human fibroblasts due to NMD of both full-length and rodless plectin transcripts induced by mutations within exons encoding the N-terminal globular domain.

The expression of a small amount of plectin in EBS-PA3 and EBS-PA3F is explained by the splice donor site mutation, c.1344G>A (Table 1 and Fig. 1A). The *PLEC1* cDNA corresponding to exons 9–14 was amplified by PCR using synthesized first-strand cDNA from EBS-PA3F and was cloned into a TA vector. Sequence analysis of the cloned PCR products revealed three different splicing patterns, one of which was a normal transcript from the wild-type allele without c.1344G>A (Fig. 6A and B). In addition to the normal transcript, most of the transcripts derived from the c.1344G>A mutant allele exhibited a 4-bp deletion at nucleotide position 1341–1344 in cDNA (Fig. 6C). This led to a frameshift followed by a PTC at amino acid position 475 (Fig. 6D), whereas small amounts of mRNA exhibiting a

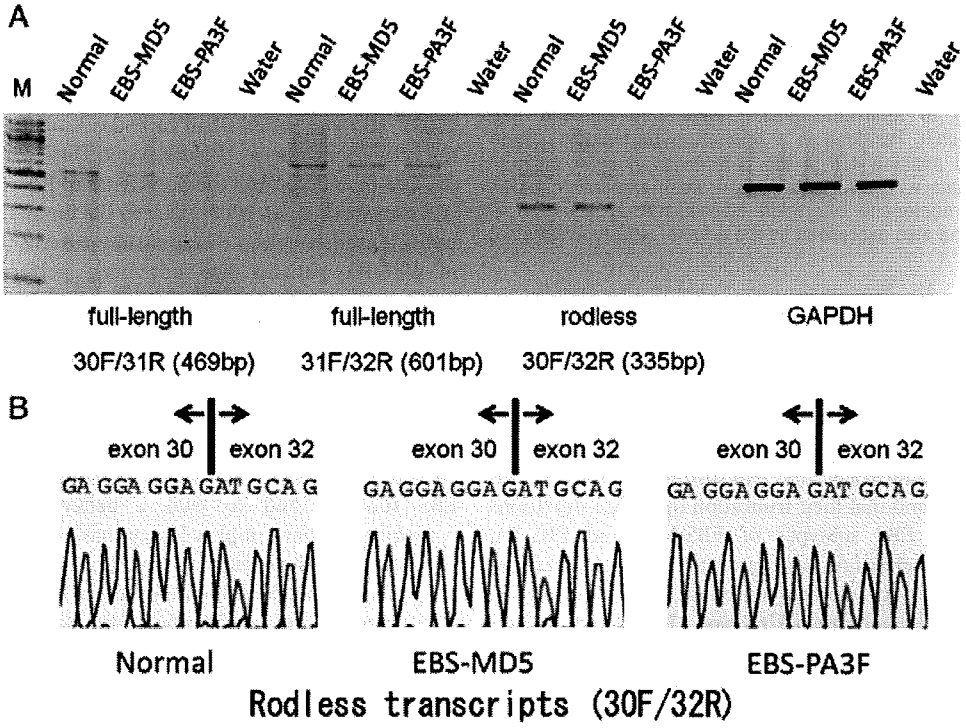

Figure 5. Semiquantitative RT-PCR on full-length and rodless plectin transcripts. **A:** Compared with the normal human control, the EBS-MD5 and EBS-PA3F cells revealed a reduced mRNA level of full-length plectin (30F/31R and 31F/32R). mRNA levels of rodless plectin in EBS-PA3F cells are reduced compared with EBS-MD5 and the normal human control (30F/32R). GAPDH mRNA expression was used as a loading control in these experiments. The negative control reaction (DNA-free water instead of cDNA) shows no PCR products. The molecular weight standard (lane M) is a 100-bp ladder. **B:** Direct sequencing demonstrates skipping of exon 31 in PCR products (30F/32R) from normal human, EBS-MD5, and EBS-PA3F.

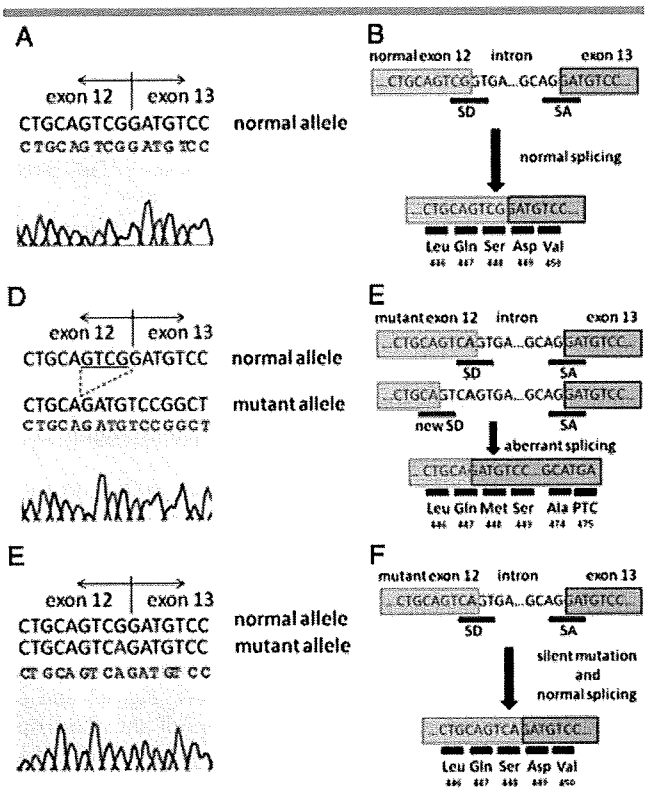

Figure 6. Abnormal splicing due to c.1344G>A mutation in EBS-PA3F, and its consequences **A:** Normal transcripts of the exon 12–exon 13 junction derived from EBS-PA3F cells. **B:** Normal splicing at the exon 12–exon 13 junction. Boxes represent exons, blue underlines are splice sites (SD: splice donor site; SA: splice acceptor site) and black underlined regions are amino acids. **C:** Mutant transcripts with deletion of four nucleotides from exon 12. Deleted nucleotides are underlined. **D:** c.1344G>A mutation altered the G nucleotide of the original splice donor site at the end of exon 12 and activated a cryptic splice donor site (red underline) four nucleotides upstream, leading to aberrant splicing with 4-bp deletion and subsequent frameshift, resulting in a premature termination codon at the amino acid position 475 in the N-terminal globular domain. **E:** Mutant transcripts with c.1344G>A. **F:** A small amount of mRNA carrying a silent nucleotide alteration c.1344G>A at amino acid position 448 Ser was also expressed by the original wild-type splicing.

normal splicing pattern with a silent mutation c.1344G>A at amino acid position 448 Ser were expressed (Fig. 6E and F).

Discussion

This study has demonstrated that two distinct plectin isoforms function in the skin, and that their truncation by *PLEC1* mutations causes the distinct EBS subtypes of EBS-MD and EBS-PA, depending on the pattern of remaining plectin peptide expression.

Plectin has a large rod domain encoded by *PLEC1*exon 31. Alternative splicing of transcripts lacking exon 31 results in a rodless plectin isoform, and it has been demonstrated that the rodless variant is expressed in various rat tissues, including skin, heart, brain, muscle, testis, and liver [Elliott et al., 1997; Fuchs et al., 2005; Steinboeck and Kristufek, 2005]. In addition, the rodless plectin isoform has been found in human muscle cells and keratinocytes [Koster et al., 2004; Schroder et al., 2000]. The significance of this rodless plectin splice variant in the skin remains unclear, but accumulation of *PLEC1* mutational data has revealed that most EBS-MD patients have mutations in exon 31 encoding the large rod domain of plectin, suggesting that conserved expression of the rodless variant plectin could be related to the pathogenesis of EBS-MD in patients with mutations in exon 31 [Pfendner et al., 2005; Sawamura et al., 2007]. However, little data that clarify this hypothesis has been reported, and only one report noted that cultured keratinocytes from one EBS-MD patient were able to express both N- and C-termini plectin epitopes without the expression of rod domain [Koster et al., 2004]. Our data including plectin isoform expression patterns in six EBS-MD patients clearly demonstrate that loss of full-length plectin with conserved rodless plectin isoform expression leads to an EBS-MD phenotype, which is consistent with accumulated clinical and genetic data. We also analyzed the relative amounts of two isoforms of plectin in normal human fibroblasts, keratinocytes, and skeletal muscle (Fig. 3). Our data revealed that the amount of full-length plectin is much greater than that of rodless plectin in fibroblasts and keratinocytes. In contrast, the full-length/rodless ratio in skeletal muscle is a little more than 1. These data are compatible with the fact that EBS-MD patients have skin fragility at birth and develop muscular dystrophy later in life. These data suggests that substantial amounts of rodless plectin in skeletal muscle might delay muscular symptoms while EBS-MD patients are in infancy.

In contrast to the EBS-MD patients, EBS-PA patients are significantly more likely to have mutations in domains outside exon 31 [Pfendner et al., 2005; Sawamura et al., 2007]. The majority of EBS-PA patients included in this study also exhibited *PLEC1* mutations in the gene outside exon 31(Table 1 and Fig. 1A). In the EBS-PA patients in this study, at least one allele is expected to have a stable product (the normal splicing variant from c.1344G>A in EBS-PA1; p.Gln2538X in EBS-PA2, and p.Gln2545X in EBS-PA3). There are three examples in which there are nulls in both alleles that have the PTC outside exon 31 but not in the terminal exon: (1) c.[2727_2740del]+c.[2727_2740del] (exon 22) [Charlesworth et al., 2003], (2) c.[1567_1570del]+c.[1567_1570del] (exon 14) [Pfendner and Uitto, 2005], and (3) p.[Gln305Term]+p.[Gln305Term] (exon 9) [Pfendner and Uitto, 2005]. All three patients had early deaths. Patients (2) and (3) had the EBS-PA phenotype [Pfendner and Uitto, 2005]. Patient (1) had the EBS phenotype, but the occurrence of PA was not substantiated [Charlesworth et al., 2003]. Due to the limited number of EBS-PA patients available, detailed expression patterns of plectin in the skin of EBS-PA patients has not been performed. In addition, comparative analysis of EBS-MD and EBS-PA skin specimens has not been performed. To our knowledge, the present report is the first to compare cutaneous plectin expression in EBS-MD and EBS-PA subtypes using multiple tissues and cells with antibodies that span a range of plectin domains including the N-terminus, rod domain, and C-terminus of plectin. This comparison between EBS-MD and EBS-PA enabled us to identify the differences in these EBS subtypes and to gain a better understanding of the consequences that complete loss or markedly attenuated expression of plectin has. These data are also consistent with the fact that EBS-PA patients generally show more severe skin symptoms than EBS-MD cases, in which expression of a rodless plectin isoform is maintained at least in the skin, although one EBS-PA patient (EBS-PA1) showed a relatively mild skin phenotype [Sawamura et al., 2007]. Also, in some cases of JEB-PA, another subtype of EB with pyloric atresia, the skin manifestations have been reported to be relatively mild and to improve with age, and surgical correction of the PA allowed growth of the patients [Pulkkinen et al., 1998]. It is possible that EBS-PA patients could develop muscular dystrophy if they survived longer. However, to our knowledge, such EBS-PA patients have not been reported in the literature. Figure 7A–C

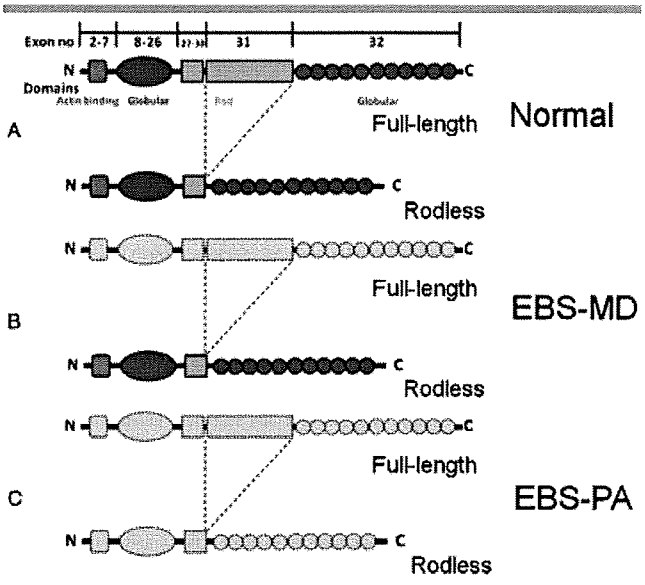

Figure 7. Schematic diagram of cutaneous plectin expression patterns in normal human skin and in skin from EBS-MD and EBS-PA patients. **A:** Two distinct isoforms of plectin—full-length and rodless—are expressed in the normal human control. **B:** Only rodless plectin is expressed in EBS-MD. **C:** Both the full-length and rodless plectin isoforms are greatly diminished or completely lost in EBS-PA. The peptides in light gray are not expressed or are markedly diminished in the patients.

depicts a schematic diagram of the predicted plectin expression pattern among the normal human control, EBS-MD, and EBS-PA.

As described above, almost all EBS-MD patients have one or two truncated mutations in exon 31 encoding the large rod domain of plectin, whereas most *PLEC1* mutations detected in EBS-PA are outside exon 31. To our knowledge, we have three cases of EBS-MD and one case of EBS-PA in the literature whose mutations are not explained by our data: (1) c.[2719_2727del] (exon 21)+c.[2719_2727del] (exon 21) (EBS-MD) [Pulkkinen et al., 1996], (2) c.[1541_1576del] (exon 14)+c.[2677_2685del] (exon 21) (EBS-MD) [Uitto and Pfendner, 2004], (3) c.[2769_2789del] (exon 21)+c.[2769_2789del] (exon 21) (EBS-PA) [Uitto and Pfender, 2005], and (4) c.[13803_13804ins16] (exon 32)+c.[13803_13804ins16] (exon 32) (EBS-MD) [Schroder et al., 2002]. The former three EBS patients had in-frame *PLEC1* deletion mutations outside exon 31 but not in the terminal exon. The last EBS-MD patient was homozygous for out-of-frame mutation in the terminal exon predicting a premature stop-codon within the exon. c.[2719_2727del] was in the nucleotide sequence where CAGGAGGCC was tandemly repeated. Therefore, this in-frame deletion was predicted to result in slipped misparing of DNA [Krawczack and Cooper, 1991; Pfendner and Uitto, 2005]. It is hard to figure out how altered plectin is synthesized from c.[1541_1576del]+c.[2677_2685del] and c.[2769_2789del]+c.[2769_2789del]. It is noteworthy that the phenotype of the EBS-MD patient with c.[1541_1576del]+c.[2677_2685del] was relatively mild, and that muscular dystrophy did not develop until the age of 42 [Uitto and Pfendner, 2004].

In previous studies, the expression of plectin was mainly evaluated by monoclonal antibodies raised against the rod domain. However, several splicing variants had previously prevented us from identifying whether plectin is completely lost or expressed in a truncated protein form in EBS patients with *PLEC1* mutations. Antibodies including those raised against both the plectin N- and C-termini are required to distinguish the expression of rodless splicing variants from a complete protein loss. Nevertheless, we have now elucidated how differences in plectin expression can lead to the two distinct skin blistering-associated phenotypes of muscular dystrophy and pyloric atresia.

Our former study on an EBS-PA3 patient [Nakamura et al., 2005] described different predicted transcripts of the c.1344G>A splice-site mutation from those of the present study. Our previous report employed an exon-trapping system, which is a tool to predict the transcripts that arise from a splice-site mutation when mRNA samples from patient tissues or cells are not available [Buckler et al., 1991]. In that system, the gDNA that is to be screened is subcloned into the exon trapping vector. The subcloned vector is transfected into cells, and mRNA is extracted from the cells to elucidate the splicing consequences. The system is useful, but it is such an artificial way of predicting the splicing products that the induced splicing patterns in the cell culture system are not necessarily correct nor are they the same as those in patient tissues or cultured cells [Schneider et al., 2007]. Because we used cultured amniocytes from EBS-PA3F in the present study, the results shown in Figure 6 supersede the results that were obtained by using an exon-trapping system in the previous report.

To summarize, EBS-MD patients typically express a rodless plectin isoform, although the full-length plectin is lost. In contrast, both full-length and rodless plectin isoforms are deficient in the EBS-PA patients, leading to a more severe disease phenotype. These findings demonstrate that deficiency of both plectin isoforms—full-length and rodless—leads to the severe phenotype of EBS-PA, and in contrast, conserved expression of the rodless isoform results in muscular dystrophy without pyloric atresia. The present results provide important insights toward further understanding the pathomechanisms of muscular dystrophy and pyloric atresia in plectin-deficient patients.

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Letters to the Editor

Title:
Two cases of cutaneous sporotrichosis in continental/ microthermal climate zone: global warming alert?

Authors:
Daisuke Inokuma, Akihiko Shibaki, Hiroshi Shimizu

Affiliation:
Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Correspondence to
Daisuke Inokuma, M.D., Ph.D.
Department of Dermatology: Hokkaido University Graduate School of Medicine
N15 W7, Sapporo 060-8638, Japan.
Tel: +81-11-716-1161 ex.5962 Fax: +81-11-706-7820
e-mail:inokuma@med.hokudai.ac.jp

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Case report

Sporotrichosis is commonly encountered in tropical and subtropical areas, but rarely in continental/ microthermal climate zone of the Köppen-Geiger Climate Classification[1]. Here, we report two cases of cutaneous sporotrichosis in Hokkaido, an island in the continental/ microthermal climate zone in Japan.

A 55-year-old Japanese female presented with a 6-month history of two dark-red-crusted infiltrated skin lesions measuring about 10 mm and 2 mm on the left upper eyelid on 2009 (Fig. 1a). She was working as a farmer in Hokkaido and had never lived in any other part of Japan. Histopathological examination of hematoxylin and eosin stained specimens revealed a prominent epidermal hyperplasia and abundant inflammatory infiltration in the dermis (Fig. 1b, c). Periodic acid Schiff stain (Fig. 1d) and Grocott's methenamine silver stain (Fig. 1e) showed a few round and budding yeast-like cells scattered in the dermis, occasionally within a giant cell. Cultures of the tissue sections of lesion on Sabouraud's dextrose agar and potato dextrose agar grew dark brown velvety colonies (Fig. 1f). Slide cultures from the colonies showed septate branching hyphae with slender, tapering conidiophores arising at right angles (Fig. 1g). A sporotrichin skin test showed positive reaction.

A 55-year-old male patient presented with a chronic erosive nodule measured 20 x 10 mm on the left mandible, which was present for over 1 year on 2002 (Fig. 2a). He was working as a carpenter in Hokkaido, and he had never lived in any other part out of Hokkaido. Histopathological analysis showed prominent epidermal hyperplasia and chronic granulomatous inflammatory cells infiltrate (Fig. 2b, c). The periodic acid Schiff stain and Grocott's staining sections revealed the presence of round yeast-like cells scattered in the dermis (Fig. 2d). Cultures of the skin biopsied tissue samples on Sabouraud's dextrose agar produced dark brown velvety colonies (Fig. 2e). Slide cultures from the colonies showed septate, branching hyphae (Fig. 2f). Sporotrichin skin test showed positive reaction.

Cutaneous sporotrichosis is a fungal infection commonly encountered in tropical and subtropical areas[1]. In Japan, more than 3,500 cases of cutaneous sporotrichosis had been reported as of 2001 on Honshu Island, which falls in temperate/ mesothermal climates zone[2]. In contrast, no case reports were available in Hokkaido in English journals as of 2004. Similarly, few cases had been reported in continental/ microthermal climate zone around the world[3], suggesting that cutaneous sporotrichosis is extremely rare in that zone. This

geographic difference in reported cases may be due to the fact that *Sporothrix schenckii,* the pathogenic fungus that causes sporotrichosis, prefers moderate temperatures (around 22 degrees Celsius) [1]. The yearly temperature in Hokkaido averaged for the years 2000 to 2008 was 9.1 degrees Celsius, so these results further suggest that *Sporothrix schenckii* rarely develops in Hokkaido. Interestingly, three cases of cutaneous sporotrichosis, including the two abovementioned cases, have been reported from Hokkaido in the Japanese literature since 2000, whereas only one case was recorded before 2000. It is suggested that the prevalence of cutaneous sporotrichosis in Hokkaido may be increasing as a result of recent global warming.

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