Table 1
Results of the 11q13.5 SNP and the prevalent *FLG* mutations in 194 Japanese eczema cases and 113 individuals from Japanese control population.

	Eczema cases			Control		
	Total	*FLG* (+)	*FLG* (−)	Total	*FLG* (+)	*FLG* (−)
C/C	147 (75.8%)	35 (70.0%)	112 (77.8%)	87 (77.0%)	2 (50.0%)	85 (78.0%)
C/T	44 (22.7%)	13 (26.0%)	31 (21.5%)	26 (23.0%)	2 (50.0%)	24 (22.0%)
T/T	3 (1.5%)	2 (4.0%)	1 (0.7%)	0 (0%)	0 (0%)	0 (0%)
Total	194	50	144	113	4	109

FLG (+), with *FLG* mutation(s); *FLG* (−), without any *FLG* mutation. Combined rs7927894[T] allele frequency, 0.129 (AE patients, $n = 388$); 0.115 (control individuals, $n = 226$); 0.17 (AE patients with *FLG* mutation(s), $n = 100$); 0.115 (AE patients without *FLG* mutation(s), $n = 288$).

Table 2
Cross-classification of genotypes of rs7927894 and *FLG* used for the interaction analysis.

Genotype		Cases			Controls		
		rs7927894			rs7927894		
		AA	AA	Aa	AA	Aa	aa
R501X	AA	147	44	3	87	26	0
	Aa	0	0	0	0	0	0
	aa	0	0	0	0	0	0
3321delA	AA	141	42	2	87	25	0
	Aa	6	2	1	0	1	0
	aa	0	0	0	0	0	0
S1695X	AA	147	44	3	86	26	0
	Aa	0	0	0	1	0	0
	aa	0	0	0	0	0	0
Q1701X	AA	144	44	3	87	26	0
	Aa	3	0	0	0	0	0
	aa	0	0	0	0	0	0
S2554X	AA	141	41	3	87	26	0
	Aa	6	3	0	0	0	0
	aa	0	0	0	0	0	0
S2889X	AA	133	36	2	86	25	0
	Aa	14	8	1	1	1	0
	Aa	0	0	0	0	0	0
S3296X	AA	141	43	3	87	26	0
	Aa	6	1	0	0	0	0
	Aa	0	0	0	0	0	0
K4022X	AA	144	43	3	87	26	0
	Aa	3	1	0	0	0	0
	Aa	0	0	0	0	0	0
Combined *FLG* null genotype	AA	109	31	1	85	24	0
	Aa	35	13	2	2	2	0
	Aa	0	0	0	0	0	0

AA, wild-type homozygous individuals for each genetic variant; Aa, wild-type/mutant heterozygous individuals; aa, individuals who are homozygous for each of the genetic variants tested.

between each of the *FLG* and rs7927894 risk alleles based on the cross-classification of genotypes in Table 2 showed no apparent epistatic effects.

rs7927894 is located in an intergenic region 38 kb downstream of *C11orf30* (chromosome 11 open reading frame 30) and 68 kb upstream of *LRRC322* (leucine rich repeat containing 32). Both *C11orf30* and *LRRC322* are ubiquitously expressed including skin and peripheral blood lymphocytes [1]. By genome-wide association study for global mRNA expression in lymphoblastoid cell lines from asthmatic children, there was no evidence for a *cis*-regulatory effect of rs7927894 [6]. Thus, regulatory role of rs7927894 on *C11orf30* and *LRRC322* gene expression is questionable. However, we cannot exclude the possibility of a pathogenetic link of rs7927894 to atopic eczema via *C11orf30* and *LRRC322* gene expression in the skin.

Our case–control study in the Japanese population did not confirm the result of Esparza-Gordillo et al. [1] or O'Regan et al. [2] that rs7927894 is at increased risk for AE. The association of rs7927894 with AE was reported in the European population, i.e. in the German population by Esparza-Gordillo et al. [1] and in the Irish population by O'Regan et al. [2]. The present data suggest that the association of rs7927894 with AE established in the European populations is not in the Asian populations, at least in the Japanese population.

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References

[1] Esparza-Gordillo J, Weidinger S, Fölster-Holst R, Bauerfeind A, Ruschendorf F, Patone G, et al. A common variant on chromosome 11q13 is associated with atopic dermatitis. Nat Genet 2009;41:596–601.
[2] O'Regan GM, Campbell LE, Cordell HJ, Irvine AD, McLean WHI, Brown SJ. Chromosome 11q13.5 variant associated with childhood eczema: an effect supplementary to filaggrin mutations. J Allergy Clin Immunol 2010;125: 170–4.
[3] Hannifin JM, Rajka G. Diagnostic features of atopic dermatitis. Acta Derm Venereol 1980;92:44–7.
[4] Nemoto-Hasebe I, Akiyama M, Nomura T, Sandilands A, McLean WHI, Shimizu H. FLG mutation p.Lys4021X in the C-terminal imperfect filaggrin repeat in Japanese atopic eczema patients. Br J Dermatol 2009;161:1387–90.
[5] Nomura T, Sandilands A, Akiyama M, Sakai K, Ota M, Sugiura H, et al. Unique mutations in the filaggrin gene in Japanese patients with ichthyosis vulgaris and atopic dermatitis. J Allergy Clin Immunol 2007;119:434–40.
[6] Dixon AL, Liang L, Moffatt MF, Chen W, Heath S, Wong KC, et al. A genome-wide association study of global gene expression. Nat Genet 2007;39:1202–7.

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CLINICAL REPORT

Response of Intractable Skin Ulcers in Recessive Dystrophic Epidermolysis Bullosa Patients to an Allogeneic Cultured Dermal Substitute

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Recessive dystrophic epidermolysis bullosa (RDEB) is an inherited skin disorder caused by mutations in the *COL7A1* gene, which encodes collagen VII (COL7). Skin ulcers in RDEB patients are sometimes slow to heal. We describe here the therapeutic response of intractable skin ulcers in two patients with generalized RDEB to treatment with an allogeneic cultured dermal substitute (CDS). Skin ulcers in both patients epithelialized by 3–4 weeks after this treatment. Immunohistochemical studies demonstrated that the COL7 expression level remained reduced with respect to the control skin and that it did not differ significantly between graft-treated and untreated areas. Electron microscopy showed aberrant anchoring fibrils beneath the lamina densa of both specimens. In conclusion, CDS is a promising modality for treatment of intractable skin ulcers in patients with RDEB, even though it does not appear to increase COL7 expression. *Key words: epidermolysis bullosa; collagen VII; cultured dermal substitute; fibroblast; growth factor.*

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Epidermolysis bullosa (EB) comprises a group of inherited bullous disorders that can be divided into three main phenotypes – epidermolysis bullosa simplex (EBS), junctional epidermolysis bullosa (JEB), and dystrophic epidermolysis bullosa (DEB) – depending on the level of skin cleavage (1). DEB is caused by mutations in the collagen VII gene (*COL7A1*), which encodes the main protein that forms anchoring fibrils beneath the dermal-epidermal junction (DEJ) (2). DEB is inherited as either autosomal dominant (DDEB) or recessive (RDEB) disease, each form having a different clinical presentation (2). Severe generalized RDEB (RDEB-sev gen) is characterized by a complete absence of collagen VII protein (COL7) from the DEJ and a total loss of anchoring fibrils ultrastructurally. A milder form of RDEB, generalized other RDEB (RDEB-O), always shows detectable but decreased COL7 expression at the DEJ. Patients with RDEB easily develop skin erosions at sites of trauma. These usually resolve spontaneously within several weeks, but occasionally lead to more persistent skin lesions or intractable ulcers.

Allogeneic tissues have been used to develop several therapeutic approaches for skin ulcers. Apligraf® (Organogenesis, Canton, MA, USA) is an allogeneic cultured skin substitute that consists of keratinocytes and fibroblasts supported on a scaffold (3). It is indicated for the treatment of venous ulcers (4). The application of Apligraf® to EB skin ulcers has been reported in approximately 30 cases thus far, with favourable results (5–7).

In parallel, Kubo & Kuroyanagi (8–11) have developed an allogeneic cultured dermal substitute (CDS) comprising a two-layered spongy matrix of hyaluronic acid and atelo-collagen containing fibroblasts. The efficacy of this CDS has been shown in animal models and some clinical trials (11–16). Recently, three patients with RDEB-sev gen were reported to have been treated successfully with CDS, although details regarding COL7 expression were not mentioned (17). Here, we confirm the efficacy of this CDS in the treatment of intractable skin ulcers in two RDEB-O patients, and we conducted immunohistochemical and ultrastructural investigation into whether the expression of COL7 is altered after this CDS treatment.

METHODS

Patients

Two patients with RDEB-O whose diagnosis was made by *COL7A1* mutation analysis and electron microscopy had persistent skin ulcers on their feet that failed to respond to supportive care for more than 6 months.

Preparation of allogeneic CDS

The CDS was prepared as described previously (9, 11). Briefly, an aqueous solution of hyaluronic acid (HA) with a cross-linking agent was frozen to –85°C in a dish and then lyophilized to obtain an HA sponge. The sponge was thoroughly rinsed with distilled water to remove free cross-linking agent,

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then the hydrated HA sponge was frozen and lyophilized to obtain a purified HA sponge, which was immersed in a dish of atelo-collagen (AC) solution. Medical-grade AC was prepared by enzymatic cleavage of telopeptides on both ends of type I collagen molecules derived from porcine dermis. The hydrated HA sponge with AC was frozen and lyophilized to obtain a two-layered sponge of HA and AC. Both surfaces of the two-layered sponge were irradiated with an ultraviolet lamp to induce intermolecular cross-linking between AC molecules.

Cell banking was established as described previously (9, 11). The piece of skin used in this study was derived from a young donor who was free from infectious viruses such as hepatitis B and C (HBV and HCV), human immunodeficiency virus (HIV) and human T-lymphotropic virus (HTLV), and who tested negative in the treponema pallidum hemagglutination test (TPHA), in compliance with the ethical guidelines of St. Marianna University Graduate School of Medicine (Kanagawa, japan). Fibroblasts were isolated by enzymatic treatment. Cultivation of fibroblasts was initiated in culture medium to establish cell banking, as described (18). Viral infection of the cells, including HBV, HCV, HIV, HTLV and parvovirus, was excluded.

The fibroblasts cryopreserved in cell banking were thawed and cultured to obtain an adequate number of cells. These fibroblasts were seeded on a two-layered spongy matrix and cultured for one week. The number of fibroblasts seeded on the two-layered sponge was adjusted to 1.0×10^5 cells/cm^2. The resulting CDS was cryopreserved according to a previously described method (8, 19). Prior to clinical application, a polystyrene dish containing the CDS was placed in a foam polystyrene box at room temperature for 30 min and then floated in a water bath at 37°C.

Treatment regimens

After giving their informed consent, the patients received this CDS therapy. The surface of the designated skin ulcer was rinsed with saline solution. After thawing, then rinsing in lactated Ringer's solution, the CDS was applied to the wound surface, together with a gauze dressing to protect the CDS. The CDS was fixed with the bandage, and there were no restrictions on patient activity at any time after the CDS was in place. A new CDS was applied twice a week for the first 2 weeks and then once a week afterwards.

Immunofluorescence

Skin biopsies were taken from both patients under local anaesthesia from non-blistered and grafted skin areas after re-epithelialization. Follow-up biopsies were at 4 weeks (Patient 1) and 3 weeks (Patient 2) after the first CDS treatment, respectively, and one week after the last CDS application. The specimens were embedded in optimum cutting temperature (OCT) compound (Miles Scientific, Naperville, IL, USA). Immunofluorescence staining was performed on 5-micron cryosections of skin with the monoclonal antibody LH7:2 (recognizing the NC-1 domain of COL7) (20). To estimate the amount of COL7, serial dilution of LH7:2 was performed to 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640 and 1:1280. Labelling was visualized using fluorescein isothiocyanate (*FITC*)-conjugated goat anti-mouse immunoglobulin (Ig)G.

Electron microscopy

Skin biopsies were taken from Patient 2 under local anaesthesia from the intact and grafted skin areas after complete epithelialization. Skin biopsy samples were fixed in 2% glutaraldehyde solution, post-fixed in 1% OsO_4, dehydrated, and embedded in Epon 812 (TAAB Laboratories Ltd, Aldermaston, Berkshire, UK). The samples were sectioned at 1 μm thickness for light microscopy and ultrathin sectioned for electron microscopy (at 70 nm thickness). The thin sections were stained with uranyl acetate and lead citrate and examined by transmission electron microscopy (Hitachi H7100, Hitachi, Tokyo, Japan).

CASE REPORTS

Patient 1

A 51-year-old female with RDEB-O had a history of three cutaneous squamous cell carcinomas (SCC), the details of which have been described elsewhere (21). *COL7A1* gene mutation analysis revealed that the patient was a compound heterozygote for c.5443G >A (p.G1815R) and c.5818delC (22, 23). She presented with an intractable ulcer, measuring 30 × 11 mm, on the back of her right foot, which had failed to respond to conservative, supportive therapy for 10 months (Fig. 1A). A skin biopsy specimen from the ulcer showed no findings suggestive of SCC. The CDS treatment was performed at site of the ulcer, and epithelialization of the lesion was observed within 4 weeks after the onset of treatment (Fig. 1B). Labelling of the DEJ in the patient's non-grafted and grafted skin samples with anti-COL7 antibody LH7:2 revealed no significant difference in the intensity of COL7 staining (Figs 2A, B). Both samples showed positive up to 1:160 dilution of the antibody as compared to 1:640 in normal skin (data not shown).

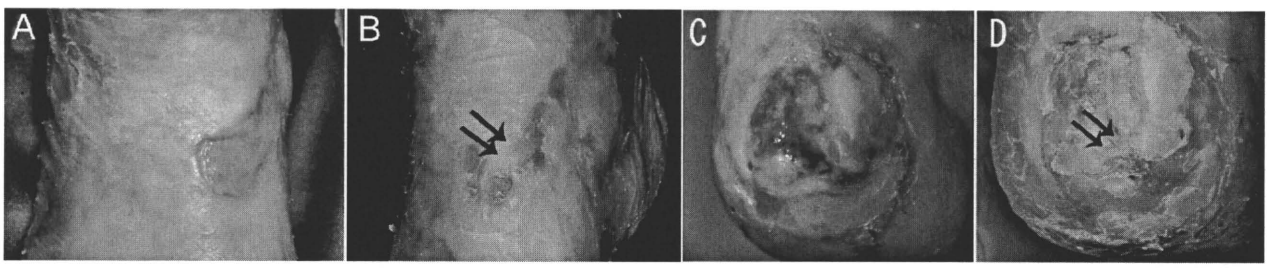

Fig. 1. Clinical response to allogeneic cultured dermal substitute (CDS) treatment (A). A skin ulcer measuring 30 × 11 mm on the back of the right foot in Patient 1. The ulcer had not healed for 10 months. (B) Re-epithelialization at 4 weeks after CDS treatment, although small erosions persist. (C) A skin ulcer measuring 21 × 20 mm on the right heel of Patient 2. The ulcer had persisted despite conservative treatment for 6 months. (D) Complete re-epithelialization 3 weeks after CDS treatment. The biopsy sites are indicated by arrows.

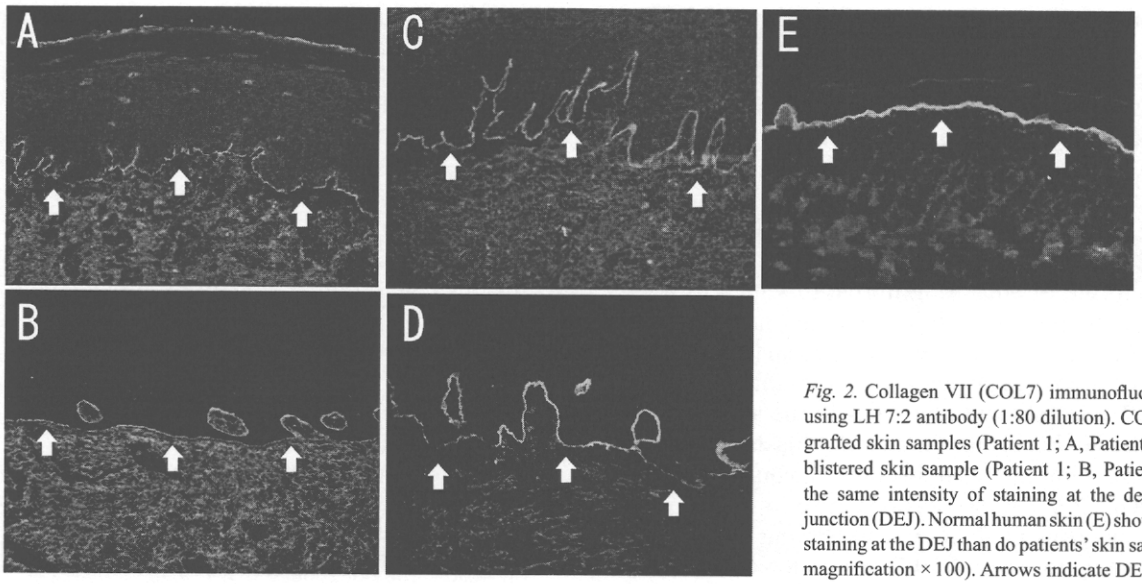

Fig. 2. Collagen VII (COL7) immunofluorescence study using LH 7:2 antibody (1:80 dilution). COL7 labelling in grafted skin samples (Patient 1; A, Patient 2; C) and non-blistered skin sample (Patient 1; B, Patient 2; D) shows the same intensity of staining at the dermal-epidermal junction (DEJ). Normal human skin (E) shows more intense staining at the DEJ than do patients' skin samples (original magnification × 100). Arrows indicate DEJ.

Patient 2

A 38-year-old female had been diagnosed with RDEB-O. She also had IgA nephropathy and was being treated with corticosteroids. DNA analysis revealed a recurrent *COL7A1* mutation c.5932C>T (p.R1978X) (23) and a novel mutation c.8029G>A (p.G2677S). She presented with a recalcitrant ulcer, measuring 21 × 20 mm, on her right heel, which had failed to respond to conservative therapy for the previous 6 months (Fig. 1C). Complete epithelialization of the lesion was observed 3 weeks after the beginning of CDS treatment (Fig. 1D). Labelling of the DEJ in the patient's non-blistered and grafted skin with LH7:2 revealed the same intensity of COL7 staining (Figs 2C, D). Both of the samples showed positive at the DEJ up to 1:320 dilution of the antibody (data not shown). Ultrastructurally, the anchoring fibrils from the patient's grafted skin samples were short, thin sub-lamina-densa structures (Fig. 3A) with the same features as those observed in the non-grafted skin samples (Fig. 3B).

DISCUSSION

Patients with EB have severe skin fragility and chronic wounding, which affect them physically and emotionally. Various controlled trials have been attempted with EB patients, including administration of phenytoin, topical bufexamac, aluminium chloride hexahydrate and oxytetracycline, although none of these has been uneqivocally successful (24). Experimental models of EB treatment have shown some promising results, but there are tremendous difficulties in translating such therapies into practical treatments for human patients (25). *Ex vivo* gene therapy for one patient with JEB

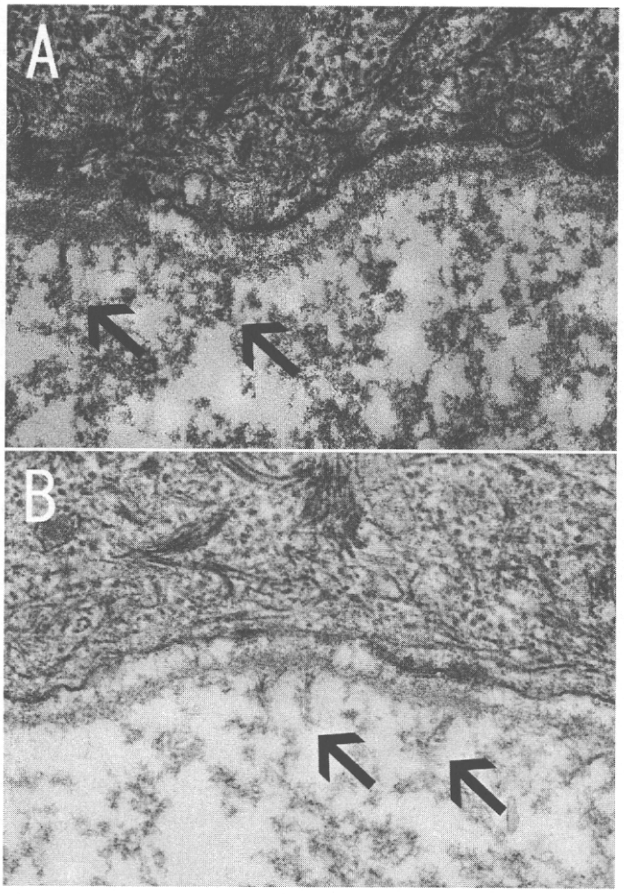

Fig. 3. Ultrastructural features of the sub-lamina densa region and basement membrane zone in the grafted and non-grafted skin of Patient 2. Discernible anchoring fibril-like structures (*arrows*) are observed beneath the lamina densa at the grafted skin site (A), as well as at the non-grafted skin site (B) (original magnification × 30,000).

(26) and allogeneic cell therapy for patients with RDEB (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.

REFERENCES

1. Fine JD, Eady RA, Bauer EA, Bauer JW, Bruckner-Tuderman L, Heagerty A, et al. The classification of inherited epidermolysis bullosa (EB): report of the Third International Consensus Meeting on Diagnosis and Classification of EB. J Am Acad Dermatol 2008; 58: 931–950.
2. Varki R, Sadowski S, Uitto J, Pfendner E. Epidermolysis bullosa. II. Type VII collagen mutations and phenotype-genotype correlations in the dystrophic subtypes. J Med Genet 2007; 44: 181–192.
3. Eaglstein WH, Falanga V. Tissue engineering and the development of Apligraf a human skin equivalent. Adv Wound Care 1998; 11: 1–8.
4. Falanga V, Margolis D, Alvarez O, Auletta M, Maggiacomo F, Altman M, et al. Rapid healing of venous ulcers and lack of clinical rejection with an allogeneic cultured human skin equivalent. Human Skin Equivalent Investigators Group. Arch Dermatol 1998; 134: 293–300.
5. Fivenson DP, Scherschun L, Cohen LV. Apligraf in the treatment of severe mitten deformity associated with recessive dystrophic epidermolysis bullosa. Plast Reconstr Surg 2003; 112: 584–588.
6. Falabella AF, Valencia IC, Eaglstein WH, Schachner LA. Tissue-engineered skin (Apligraf) in the healing of patients with epidermolysis bullosa wounds. Arch Dermatol 2000; 136: 1225–1230.
7. Falabella AF, Schachner LA, Valencia IC, Eaglstein WH. The use of tissue-engineered skin (Apligraf) to treat a newborn with epidermolysis bullosa. Arch Dermatol 1999; 135: 1219–1222.
8. Kubo K, Kuroyanagi Y. Development of a cultured dermal substitute composed of a spongy matrix of hyaluronic acid and atelo-collagen combined with fibroblasts: cryopreservation. Artif Organs 2004; 28: 182–188.
9. Kubo K, Kuroyanagi Y. Development of a cultured dermal substitute composed of a spongy matrix of hyaluronic acid and atelo-collagen combined with fibroblasts: fundamental evaluation. J Biomater Sci Polym Ed 2003; 14: 625–641.
10. Kubo K, Kuroyanagi Y. Characterization of a cultured dermal substitute composed of a spongy matrix of hyaluronic acid and collagen combined with fibroblasts. J Artif Organs 2003; 6: 138–144.
11. Kubo K, Kuroyanagi Y. Spongy matrix of hyaluronic acid and collagen as a cultured dermal substitute: evaluation in an animal test. J Artif Organs 2003; 6: 64–70.
12. Hasegawa T, Suga Y, Mizoguchi M, Muramatsu S, Mizuno Y, Ogawa H, et al. An allogeneic cultured dermal substitute suitable for treating intractable skin ulcers and large skin defects prior to autologous skin grafting: three case reports.

J Dermatol 2005; 32: 715–720.
13. Kashiwa N, Ito O, Ueda T, Kubo K, Matsui H, Kuroyanagi Y. Treatment of full-thickness skin defect with concomitant grafting of 6-fold extended mesh auto-skin and allogeneic cultured dermal substitute. Artif Organs 2004; 28: 444–450.
14. Moroi Y, Fujita S, Fukagawa S, Mashino T, Goto T, Masuda T, et al. Clinical evaluation of allogeneic cultured dermal substitutes for intractable skin ulcers after tumor resection. Eur J Dermatol 2004; 14: 172–176.
15. Yamada N, Uchinuma E, Kuroyanagi Y. Clinical trial of allogeneic cultured dermal substitutes for intractable skin ulcers of the lower leg. J Artif Organs 2008; 11: 100–103.
16. Yonezawa M, Tanizaki H, Inoguchi N, Ishida M, Katoh M, Tachibana T, et al. Clinical study with allogeneic cultured dermal substitutes for chronic leg ulcers. Int J Dermatol 2007; 46: 36–42.
17. Hasegawa T, Suga Y, Mizoguchi M, Ikeda S, Ogawa H, Kubo K, et al. Clinical trial of allogeneic cultured dermal substitute for the treatment of intractable skin ulcers in 3 patients with recessive dystrophic epidermolysis bullosa. J Am Acad Dermatol 2004; 50: 803–804.
18. Hashimoto A, Kuroyanagi Y. Standardization for mass production of allogeneic cultured dermal substitute by measuring the amount of VEGF, bFGF, HGF, TGF-beta, and IL-8. J Artif Organs 2008; 11: 225–231.
19. Kubo K, Kuroyanagi Y. The possibility of long-term cryopreservation of cultured dermal substitute. Artif Organs 2005; 29: 800–805.
20. Leigh IM, Eady RA, Heagerty AH, Purkis PE, Whitehead PA, Burgeson RE. Type VII collagen is a normal component of epidermal basement membrane, which shows altered expression in recessive dystrophic epidermolysis bullosa. J Invest Dermatol 1988; 90: 639–642.
21. Tomita Y, Sato-Matsumura KC, Sawamura D, Matsumura T, Shimizu H. Simultaneous occurrence of three squamous cell carcinomas in a recessive dystrophic epidermolysis bullosa patient. Acta Derm Venereol 2003; 83: 225–226.
22. Sato-Matsumura KC, Yasukawa K, Tomita Y, Shimizu H. Toenail dystrophy with COL7A1 glycine substitution mutations segregates as an autosomal dominant trait in 2 families with dystrophic epidermolysis bullosa. Arch Dermatol 2002; 138: 269–271.
23. Sawamura D, Goto M, Yasukawa K, Sato-Matsumura K, Nakamura H, Ito K, et al. Genetic studies of 20 Japanese families of dystrophic epidermolysis bullosa. J Hum Genet 2005; 50: 543–546.
24. Langan SM, Williams HC. A systematic review of randomized controlled trials of treatments for inherited forms of epidermolysis bullosa. Clin Exp Dermatol 2009; 34: 20–25.
25. Aumailley M, Has C, Tunggal L, Bruckner-Tuderman L. Molecular basis of inherited skin-blistering disorders, and therapeutic implications. Expert Rev Mol Med 2006; 8: 1–21.
26. Mavilio F, Pellegrini G, Ferrari S, Di Nunzio F, Di Iorio E, Recchia A, et al. Correction of junctional epidermolysis bullosa by transplantation of genetically modified epidermal stem cells. Nat Med 2006; 12: 1397–402.
27. Wong T, Gammon L, Liu L, Mellerio JE, Dopping-Hepenstal PJ, Pacy J, et al. Potential of fibroblast cell therapy for recessive dystrophic epidermolysis bullosa. J Invest Dermatol 2008; 128: 2179–2189.
28. McGrath JA, Schofield OM, Ishida-Yamamoto A, O'Grady A, Mayou BJ, Navsaria H, et al. Cultured keratinocyte allografts and wound healing in severe recessive dystrophic epidermolysis bullosa. J Am Acad Dermatol 1993; 29: 407–419.
29. Goto M, Sawamura D, Nishie W, Sakai K, McMillan JR, Akiyama M, et al. Targeted skipping of a single exon harboring a premature termination codon mutation: implications and potential for gene correction therapy for selective dystrophic epidermolysis bullosa patients. J Invest Dermatol. 2006; 126: 2614–2620.
30. Chen M, Kasahara N, Keene DR, Chan L, Hoeffler WK, Finlay D, et al. Restoration of type VII collagen expression and function in dystrophic epidermolysis bullosa. Nat Genet 2002; 32: 670–675.
31. Haapasalmi K, Makela M, Oksala O, Heino J, Yamada KM, Uitto VJ, et al. Expression of epithelial adhesion proteins and integrins in chronic inflammation. Am J Pathol 1995; 147: 193–206.
32. Kubo K, Kuroyanagi Y. Effects of vascular endothelial growth factor released from cultured dermal substitute on proliferation of vascular endothelial cells in vitro. J Artif Organs 2003; 6: 267–272.
33. Kubo K, Kuroyanagi Y. A study of cytokines released from fibroblasts in cultured dermal substitute. Artif Organs 2005; 29: 845–849.
34. Tanaka M, Nakakita N, Kuroyanagi Y. Allogeneic cultured dermal substitute composed of spongy collagen containing fibroblasts: evaluation in animal test. J Biomater Sci Polym Ed 1999; 10: 433-453.

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Letter to the Editor

A founder effect of c.1938delC in *ITGB4* underlies junctional epidermolysis bullosa and its application for prenatal testing

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Abstract: Junctional epidermolysis bullosa associated with pyloric atresia (JEB-PA) is one of the most severe inherited skin diseases, characterized by generalized blister formation and occlusion of the pylorus at birth. Most JEB-PA patients have mutations in the gene encoding β4 integrin (*ITGB4*). No recurrent mutations in *ITGB4* have been described as having founder effects. We collected three JEB-PA families with c.1938delC in *ITGB4*. Haplotype analysis using single nucleotide polymorphism markers throughout *ITGB4* suggested one rare haplotype (2.8% of the Han Chinese and ethnic Japanese populations) in all alleles with c.1938delC. The parents of one of the three families sought prenatal diagnosis for a subsequent pregnancy. We succeeded in performing prenatal exclusion of JEB-PA using the foetal genomic DNA. Our study clearly demonstrated that recurrent c.1938delC in *ITGB4* is a founder mutation in JEB-PA patients, and that genotyping of the mutation can be utilized for prenatal diagnosis of JEB-PA.

Key words: basement membrane zone – haplotype analysis – single nucleotide polymorphism

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Background

Recurrent mutations in a population might be explained by founder effects, in which the mutant alleles of a few ancestors spread in the population because of limited gene pool, genetic drift and healthy carrier migration (1).

Epidermolysis bullosa (EB) comprises a group of disorders characterized by congenital skin fragility. EB has been classified into EB simplex, junctional epidermolysis bullosa (JEB), dystrophic EB and Kindler syndrome (2–4). JEB is subclassified into three clinical subtypes: Herlitz JEB, non-Herlitz JEB and JEB with pyloric atresia (JEB-PA). JEB-PA is characterized by generalized blistering and occlusion of the pylorus at birth, which usually leads to early demise (5). Mutations in the gene encoding α6 (*ITGA6*) or the β4 integrin subunit (*ITGB4*) are responsible for JEB-PA (6,7). Most patients with JEB-PA have mutations in *ITGB4* (8). No frequent prevalent mutations have been noted, except in the Hispanic population, where c.1802G>A (p.Cys601Tyr) is present on five of 10 alleles of JEB-PA patients (9).

Here, we have collected three JEB-PA families, in which c.1938delC in *ITGB4* is present. Haplotype analysis revealed c.1938delC as a founder mutation in JEB patients. Based on these data, we successfully performed prenatal exclusion of JEB-PA with this mutation.

Experimental design

Patients

Three unrelated non-consanguineous Japanese families (A, B and C) with JEB-PA in this study are summarized in Fig. S1a. Family A and B originate from Shikoku Island in Japan and family C is from other part of the country. A-1 and B-1 are newly identified JEB-PA patients. They died of disseminated intravascular coagulation 1 and 2 months after birth, respectively. Immunofluorescence study of skin specimens from both of the patients showed the absence of β4 integrin and weak expression of α6 integrin subunits (data not shown). Immunostaining for laminin 332, type IV collagen, type VII collagen, type XVII collagen, plectin and BP230 revealed normal linear labelling patterns (data not shown). C-2 is a patient with non-lethal variant of JEB-PA. The case description and mutational data of C-2 have been reported previously (10).

Mutation detection

Genomic DNA (gDNA) was extracted from blood cells of the probands and their parents. Mutation detection was performed after polymerase chain reaction (PCR) amplification of all exons and intron–exon borders of *ITGB4*, followed by direct sequencing using an ABI Prism 3100 genetic analyzer (Advanced Biotechnologies Inc., Columbia, MD, USA) (11–13). The genomic DNA nucleotides, the complementary DNA nucleotides and the amino

acids of the protein were numbered based on the following sequence information (GenBank accession No. NM_000213).

Haplotype analysis

To determine whether c.1938delC is a founder mutation, we performed haplotype analysis of three JEB-PA families. We constructed linkage disequilibrium (LD) blocks containing *ITGB4* using genotype data from the HapMap database (International HapMap Consortium, 2005). The haplotype structure with its tag-single nucleotide polymorphisms (SNPs) was determined using Haploview (14). We genotyped 15 tag-SNPs (Fig. S1b) using the ABI Prism 3100 genetic analyzer (Advanced Biotechnologies Inc.).

Prenatal diagnosis

We performed prenatal diagnosis of a foetus (A-2) at risk for JEB-PA from family A. A total of 30 ml of amniotic fluid was obtained under ultrasound guidance at 16 weeks' gestation. Foetal DNA was extracted from fresh cells from 10 ml of amniotic fluid. Genomic DNA isolated from amniotic fluid cells was subjected to polymerase chain reaction (PCR) amplification, followed by direct automated sequencing as described. The mutation site was sequenced using both forward and reverse strands and verified by *PmlI* (New England Biolabs Inc., Beverly, MA, USA) enzyme digestion of the PCR products.

The medical ethical committee of Hokkaido University and National Center for Child Health and Development approved all described studies. The study was conducted according to Declaration of Helsinki Principles. Participants gave their written informed consent.

Results

Recurrent c.1938delC in *ITGB4*

ITGB4 mutation analysis revealed that A-1 was homozygous for c.1938delC (Fig. 1c). The father and mother of A-1 were heterozygous for c.1938delC (Fig. 1a, b). B-1 was heterozygous for paternal c.1938delC and maternal c.4050_4057del (data not shown). c.1938delC was previously described in a patient with non-lethal variant of JEB-PA who is compound heterozygous for c.1938delC and c.2168C>G (p.Pro723Arg) (C-2) (10). c.4050_4057del was also reported in a JEB-PA patient who is compound heterozygous for c.4050_4057del and c.3434delT (12).

Founder effects of c.1938delC

The haplotype structure containing *ITGB4* was constructed using genotype data from the HapMap database (Fig. S1b, c). The haplotype block was represented by 16 haplotypes with >2% frequency (Fig. S1b, c). The chromosome containing c.1938delC in A-1 and B-1 had haplotype XI (GGGACGGGCGTCACC), which is seen in 2.8% of the Han Chinese and ethnic Japanese populations. The chromosome containing c.1938delC in C-2 might have had this haplotype although the phase was not determined.

Prenatal exclusion of JEB-PA

Direct sequencing of PCR products from the foetal gDNA (A-2) revealed the presence of c.1938delC in one allele and wild-type sequence in another allele (Fig. 1d). To confirm the results of direct sequencing, we performed restriction enzyme analysis. c.1938delC was found to result in the loss of a restriction enzyme for *PmlI*. The PCR product from the proband (A-1) after *PmlI* digestion revealed a 285-bp band, which indicated that she was homozygous for c.1938delC (Fig. 1f). In contrast, the PCR product from the parents and the foetus (A-2) after *PmlI* digestion showed 285-, 195- and 90-bp bands, which indicated that they were heterozygous for c.1938delC (Fig. 1f). Haplotype analysis of this family using microsatellite markers excluded maternal contamination of foetal cells (data not shown). These results predicted that the foetus would not be affected, and the pregnancy was continued. A neonate was born at full term in good health with completely normal skin.

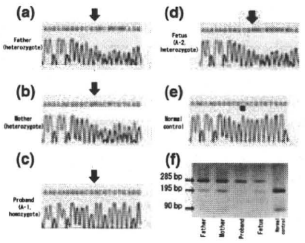

Figure 1. Prenatal diagnosis of junctional epidermolysis bullosa with pyloric atresia (family A). (a–e) Direct sequencing of *ITGB4*. The parents were heterozygous for c.1938delC in *ITGB4* (a, b). A-1, the proband, was homozygous for that mutation (c). A-2, the foetus, was found to be a heterozygous carrier (d). A cytosine at cDNA position 1938 in normal control is underlined (e). Arrows indicate a deleted cytosine in *ITGB4* sequence. (f) *PmlI* restriction enzyme digestion of the PCR products from the family members' genomic DNA. c.1938delC results in the loss of a site for *PmlI*. *PmlI* restriction enzyme digestion of the PCR products from normal control reveals 195- and 90-bp bands. Only a 285-bp band is observed in A-1 (the proband), who is homozygous for c.1938delC. In contrast, 285-, 195- and 90-bp bands are detected in the father, mother and A-2, suggesting that they are heterozygous for c.1938delC.

Conclusions

There are no recurrent *ITGB4* mutations that have been demonstrated to have founder effects in JEB-PA patients. Our study detected recurrent c.1938delC in *ITGB4* and revealed this to be a founder mutation in JEB-PA patients.

DNA-based prenatal testing of JEB-PA has been described (15–18). Our study has demonstrated the successful prenatal exclusion of JEB-PA with c.1938delC through mutation analysis of the foetal genomic DNA.

In summary, our study identified a founder c.1938delC in *ITGB4* and showed that this mutation can be applied for prenatal diagnosis of JEB-PA.

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Conflicts of interest

The authors declare no conflicts of interest.

References

1 Castiglia D, Zambruno G. Dermatol Clin 2010: **28**: 17–22.
2 Bowden P E, Knight A G, Liovic M. Exp Dermatol 2009: **18**: 650–652.
3 Dang N, Murrell D F. Exp Dermatol 2008: **17**: 553–568.
4 Fine J D, Eady R A, Bauer E A *et al.* J Am Acad Dermatol 2008: **58**: 931–950.
5 Chung H J, Uitto J. Dermatol Clin 2010: **28**: 43–54.
6 Ruzzi L, Gagnoux-Palacios L, Pinola M *et al.* J Clin Invest 1997: **99**: 2826–2831.

7 Vidal F, Aberdam D, Miquel C *et al.* Nat Genet 1995: **10**: 229–234.
8 Dang N, Klingberg S, Rubin A I *et al.* Acta Derm Venereol 2008: **88**: 438–448.
9 Varki R, Sadowski S, Pfendner E *et al.* J Med Genet 2006: **43**: 641–652.
10 Abe M, Sawamura D, Goto M *et al.* J Dermatol Sci 2007: **47**: 165–167.
11 Natsuga K, Nishie W, Arita K *et al.* J Invest Dermatol 2010: **130**: 2671–2674.
12 Takizawa Y, Shimizu H, Nishikawa T *et al.* J Invest Dermatol 1997: **108**: 943–946.
13 Nakano A, Pulkkinen L, Murrell D *et al.* Pediatr Res 2001: **49**: 618–626.
14 Barrett J C, Fry B, Maller J *et al.* Bioinformatics 2005: **21**: 263–265.
15 Shimizu H. Prenat Diagn 2006: **26**: 1260–1261.
16 Ashton G H, Sorelli P, Mellerio J E *et al.* Br J Dermatol 2001: **144**: 408–414.
17 Gache Y, Romero-Graillet C, Spadafora A *et al.* J Invest Dermatol 1998: **111**: 914–916.
18 Pfendner E G, Nakano A, Pulkkinen L *et al.* Prenat Diagn 2003: **23**: 447–456.

Supporting Information

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

Figure S1. Haplotype analysis of the junctional epidermolysis bullosa families.

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Letter to the Editor

IL-1 signalling is dispensable for protective immunity in *Leishmania*-resistant mice

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Abstract: Leishmaniasis is a parasitic disease affecting ~12 million people. Control of infection (e.g. in C57BL/6 mice) results from IL-12-dependent production of IFNγ by Th1/Tc1 cells. In contrast, BALB/c mice succumb to infection because of preferential Th2-type cytokine induction. Infected dendritic cells (DC) represent important sources of IL-12. Genetically determined differences in DC IL-1α/β production contribute to disease outcome. Whereas the course of disease was not dramatically altered in IL-1RI$^{-/-}$ mice, local administration of IL-1α to infected C57BL/6 mice improved disease outcome. To definitively elucidate the involvement of IL-1 in immunity against leishmaniasis, we now utilized IL-1α/β-double-deficient C57BL/6 mice. C57BL/6 mice are believed to be a good surrogate model for human, self limited cutaneous leishmaniasis (CL). *Leishmania major*-infected IL-1α/β$^{-/-}$ mice were resistant to experimental CL comparable to controls. In addition, DC-based vaccination against leishmaniasis in C57BL/6 mice was independent of IL-1. Thus, in *Leishmania*-resistant C57BL/6 mice, IL-1 signalling is dispensable for protection.

Key words: IL-1 – dendritic cells – *L. major*

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Background

Leishmaniasis is a parasitic disease transmitted by the bite of a sand fly. The disease ranges from cutaneous leishmaniasis (CL) to visceral leishmaniasis and ~12 million people are affected worldwide (1). In murine experimental leishmaniasis, control of infection results from IL-12-dependent production of Th1/Tc1-derived IFNγ that activates infected macrophages (MΦ) to eliminate parasites (2–5). In disease-resistant C57BL/6 mice, skin DC infected with *Leishmania major* represent important sources of IL-12 (6). In contrast, BALB/c mice respond to infection with preferential Th2-type cytokine production, which is associated with disease progression. Genetically determined DC-derived factors that influence disease susceptibility of BALB/c mice include elevated levels of inhibitory IL-12p80 (7) and decreased release of IL-1α/β (8,9). Previously, we demonstrated that IL-1α/β facilitates Th1 induction in several inflammatory disease models (9–11). Treatment of BALB/c mice with IL-1 during T cell priming inhibited progressive disease by shifting the immune response towards Th1 (9). However, prolonged administration of IL-1α promoted Th2 expansion in already established infections and worsened disease outcome (11).

Abbreviations: CL, cutaneous leishmaniasis; DC, dendritic cells; MΦ, macrophages.

Question addressed

IL-1 is a key mediator of inflammation (12,13). IL-1α and IL-1β exert similar biological functions by binding to the IL-1 type I receptor (IL-1RI) (14). To definitively elucidate the involvement of IL-1 in immune responses in CL, we utilized IL-1α/β-double

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

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autoimmune blistering diseases, basement membrane zone, cicatricial pemphigoid, immunoglobulin subtypes, internal malignancy

Conflicts of interest
None declared.

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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.

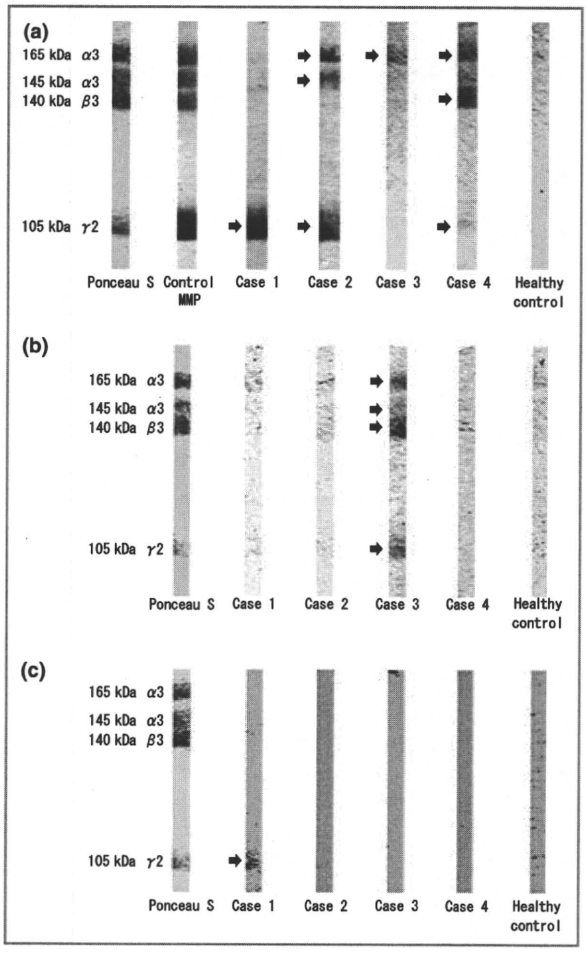

Fig 1. IgG, IgA and IgE autoantibodies against purified laminin-332. (a) Immunoblot analysis using purified laminin-332 revealed circulating IgG autoantibodies against the γ2 subunit, 105 kDa (arrow, case 1), the α3 and γ2 subunits, 165 kDa, 145 kDa and 105 kDa (arrows, case 2), the α3 subunit, 165 kDa (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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References

1 Chan LS, Ahmed AR, Anhalt GJ *et al.* The first international consensus on mucous membrane pemphigoid: definition, diagnostic criteria, pathogenic factors, medical treatment, and prognostic indicators. *Arch Dermatol* 2002; **138**:370–9.

2 Domloge-Hultsch N, Gammon WR, Briggaman RA *et al.* Epiligrin, the major human keratinocyte integrin ligand, is a target in both an acquired autoimmune and an inherited subepidermal blistering skin disease. *J Clin Invest* 1992; **90**:1628–33.

3 Kirtschig G, Marinkovich MP, Burgeson RE *et al.* Anti-basement membrane autoantibodies in patients with anti-epiligrin cicatricial pemphigoid bind the alpha subunit of laminin 5. *J Invest Dermatol* 1995; **105**:543–8.

4 Lazarova Z, Hsu R, Yee C *et al.* Antiepiligrin cicatricial pemphigoid represents an autoimmune response to subunits present in laminin 5 (alpha3beta3gamma2). *Br J Dermatol* 1998; **139**:791–7.

5 Leverkus M, Schmidt E, Lazarova Z *et al.* Antiepiligrin cicatricial pemphigoid: an underdiagnosed entity within the spectrum of scarring autoimmune subepidermal bullous diseases? *Arch Dermatol* 1999; **135**:1091–8.

6 Egan CA, Lazarova Z, Darling TN *et al.* Anti-epiligrin cicatricial pemphigoid: clinical findings, immunopathogenesis, and significant associations. *Medicine (Baltimore)* 2003; **82**:177–86.

7 Lazarova Z, Salato VK, Lanschuetzer CM *et al.* IgG anti-laminin-332 autoantibodies are present in a subset of patients with mucous membrane, but not bullous, pemphigoid. *J Am Acad Dermatol* 2008; **58**:951–8.

8 Egan CA, Lazarova Z, Darling TN *et al.* Anti-epiligrin cicatricial pemphigoid and relative risk for cancer. *Lancet* 2001; **357**:1850–1.

9 Sadler E, Lazarova Z, Sarasombath P *et al.* A widening perspective regarding the relationship between anti-epiligrin cicatricial pemphigoid and cancer. *J Dermatol Sci* 2007; **47**:1–7.

10 Lazarova Z, Yee C, Darling T *et al.* Passive transfer of anti-laminin 5 antibodies induces subepidermal blisters in neonatal mice. *J Clin Invest* 1996; **98**:1509–18.

11 Lazarova Z, Hsu R, Yee C *et al.* Human anti-laminin 5 autoantibodies induce subepidermal blisters in an experimental human skin graft model. *J Invest Dermatol* 2000; **114**:178–84.

12 Gammon WR, Briggaman RA, Inman AO 3rd *et al.* Differentiating anti-lamina lucida and anti-sublamina densa anti-BMZ antibodies by indirect immunofluorescence on 1.0 M sodium chloride-separated skin. *J Invest Dermatol* 1984; **82**:139–44.

13 Dmochowski M, Hashimoto T, Bhogal BS *et al.* Immunoblotting studies of linear IgA disease. *J Dermatol Sci* 1993; **6**:194–200.

14 Tsunenaga M, Adachi E, Amano S *et al.* Laminin 5 can promote assembly of the lamina densa in the skin equivalent model. *Matrix Biol* 1998; **17**:603–13.

15 Amano S, Nishiyama T, Burgeson RE. A specific and sensitive ELISA for laminin 5. *J Immunol Methods* 1999; **224**:161–9.

16 Murakami H, Nishioka S, Setterfield J *et al.* Analysis of antigens targeted by circulating IgG and IgA autoantibodies in 50 patients with cicatricial pemphigoid. *J Dermatol Sci* 1998; **17**:39–44.

17 Nie Z, Hashimoto T. IgA antibodies of cicatricial pemphigoid sera specifically react with C-terminus of BP180. *J Invest Dermatol* 1999; **112**:254–5.

18 Christophoridis S, Budinger L, Borradori L *et al.* IgG, IgA and IgE autoantibodies against the ectodomain of BP180 in patients with bullous and cicatricial pemphigoid and linear IgA bullous dermatosis. *Br J Dermatol* 2000; **143**:349–55.

19 Cozzani E, Drosera M, Parodi A *et al.* Frequency of IgA antibodies in pemphigus, bullous pemphigoid and mucous membrane pemphigoid. *Acta Derm Venereol (Stockh)* 2004; **84**:381–4.

20 Oyama N, Setterfield JF, Powell AM *et al.* Bullous pemphigoid antigen II (BP180) and its soluble extracellular domains are major autoantigens in mucous membrane pemphigoid: the pathogenic relevance to HLA class II alleles and disease severity. *Br J Dermatol* 2006; **154**:90–8.

21 Schmidt E, Skrobek C, Kromminga A *et al.* Cicatricial pemphigoid: IgA and IgG autoantibodies target epitopes on both intra- and extracellular domains of bullous pemphigoid antigen 180. *Br J Dermatol* 2001; **145**:778–83.

22 Zone JJ, Egan CA, Taylor TB *et al.* IgA autoimmune disorders: development of a passive transfer mouse model. *J Investig Dermatol Symp Proc* 2004; **9**:47–51.

23 Delaporte E, Dubost-Brama A, Ghohestani R *et al.* IgE autoantibodies directed against the major bullous pemphigoid antigen in patients with a severe form of pemphigoid. *J Immunol* 1996; **157**:3642–7.

24 Dopp R, Schmidt E, Chimanovitch I *et al.* IgG4 and IgE are the major immunoglobulins targeting the NC16A domain of BP180 in bullous pemphigoid: serum levels of these immunoglobulins reflect disease activity. *J Am Acad Dermatol* 2000; **42**:577–83.

25 Dimson OG, Giudice GJ, Fu CL *et al.* Identification of a potential effector function for IgE autoantibodies in the organ-specific autoimmune disease bullous pemphigoid. *J Invest Dermatol* 2003; **120**:784–8.

26 Ishiura N, Fujimoto M, Watanabe R *et al.* Serum levels of IgE anti-BP180 and anti-BP230 autoantibodies in patients with bullous pemphigoid. *J Dermatol Sci* 2008; **49**:153–61.

27 Ghohestani RF, Cozzani E, Delaporte E *et al.* IgE antibodies in sera from patients with bullous pemphigoid are autoantibodies preferentially directed against the 230-kDa epidermal antigen (BP230). *J Clin Immunol* 1998; **18**:202–9.

28 Fairley JA, Burnett CT, Fu CL *et al.* A pathogenic role for IgE in autoimmunity: bullous pemphigoid IgE reproduces the early phase of lesion development in human skin grafted to nu/nu mice. *J Invest Dermatol* 2007; **127**:2605–11.

29 Zone JJ, Taylor T, Hull C *et al.* IgE basement membrane zone antibodies induce eosinophil infiltration and histological blisters in engrafted human skin on SCID mice. *J Invest Dermatol* 2007; **127**:1167–74.

MUTATION IN BRIEF

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Plectin Deficiency Leads to Both Muscular Dystrophy and Pyloric Atresia in Epidermolysis Bullosa Simplex

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ABSTRACT: Plectin is a cytoskeletal linker protein which has a long central rod and N- and C-terminal globular domains. Mutations in the gene encoding plectin (*PLEC*) cause two distinct autosomal recessive subtypes of epidermolysis bullosa: EB simplex (EBS) with muscular dystrophy (EBS-MD), and EBS with pyloric atresia (EBS-PA). Previous studies have demonstrated that loss of full-length plectin with residual expression of the rodless isoform leads to EBS-MD, whereas complete loss or marked attenuation of expression of full-length and rodless plectin underlies the more severe EBS-PA phenotype. However, muscular dystrophy has never been identified in EBS-PA, not even in the severe form of the disease. Here, we report the first case of EBS associated with both pyloric atresia and muscular dystrophy. Both of the premature termination codon-causing mutations of the proband are located within exon 32, the last exon of *PLEC*. Immunofluorescence and immunoblot analysis of skin samples and cultured fibroblasts from the proband revealed truncated plectin protein expression in low amounts. This study demonstrates that plectin deficiency can indeed lead to both muscular dystrophy and pyloric atresia in an individual EBS patient. ©2010 Wiley-Liss, Inc.

KEY WORDS: basement membrane zone; skeletal muscle; mRNA decay; truncation

INTRODUCTION

Plectin is a 500-kDa protein of the plakin family, which interlinks different element of the cytoskeleton (Rezniczek, et al., 2010). Plectin is prominently expressed in muscle and in stratified and simple epithelia, including in the skin and gastrointestinal tract (Rezniczek, et al., 2010). In skin, plectin localizes to the inner plaque of the hemidesmosomes, at the site of interaction with intermediate filaments (Smith, et al., 1996). Plectin has a unique dumbbell-like structure with a central rod domain and N- and C-terminal globular domains (Wiche, et

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al., 1991). Various types of plectin transcripts, including rodless ones, that do not encode for a central rod domain have been reported (Elliott, et al., 1997).

Epidermolysis bullosa (EB) comprises a group of heterogeneous congenital disorders characterized by dermal-epidermal junction separation. EB is subdivided into the three major groups of EB simplex (EBS), junctional EB and 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 14 different genes have been identified as underlying the EB subtypes (Fine, et al., 2008; Groves, et al., 2010). Among them, mutations in the gene encoding plectin, *PLEC* (MIM# 601282), have been known to be causal for two subtypes of autosomal recessive EBS (EBS with muscular dystrophy (EBS-MD) and EBS with pyloric atresia (EBS-PA)) and for one subtype of autosomal dominant EBS (EBS-Ogna) (Fine, et al., 2008).

Characteristic manifestations of EBS-MD are generalized skin blistering and late onset muscle weakness. Previous studies revealed defective expression of plectin in EBS-MD skin samples (Gache, et al., 1996; Shimizu, et al., 1999a; Shimizu, et al., 1999b) and mutations in *PLEC* in EBS-MD patients (McLean, et al., 1996; Pulkkinen, et al., 1996; Smith, et al., 1996; Takizawa, et al., 1999). The *PLEC* mutations detected in EBS-MD patients are mainly within exon 31, which encodes the large-rod domain of plectin (Natsuga, et al., 2010; Pfendner, et al., 2005; Sawamura, et al., 2007).

In contrast to patients with EBS-MD, those with EBS-PA typically develop a more severe phenotype that includes more generalized blistering and pyloric atresia (PA) (Nakamura, et al., 2005). The prognosis of EBS-PA is very poor, and affected patients usually die within months after birth (Nakamura, et al., 2005; Pfendner, et al., 2005; Pfendner and Uitto, 2005). *PLEC* mutations of EBS-PA were mostly located outside of exon 31 (Natsuga, et al., 2010).

Although both EBS-MD and EBS-PA are autosomal recessive EBS caused by *PLEC* mutations, the pathomechanisms distinguishing two subtypes were unclear. Recently, our group and others demonstrated that EBS-MD patients typically express a rodless plectin isoform, although the full-length plectin is absent (Koster, et al., 2004; Natsuga, et al., 2010). In contrast, both full-length and rodless plectin isoforms are deficient in EBS-PA patients, leading to the more severe disease phenotype (Natsuga, et al., 2010). In light of these findings, it has been postulated that EBS-PA patients could develop muscular dystrophy (MD) if they survived longer (Natsuga, et al., 2010). However, to our knowledge, there have been no EBS patients who suffered from both MD and PA.

Here, we report the first patient with EBS who developed both PA and MD. Both of the mutations identified in the patient were within the last exon (exon 32) of *PLEC*. Immunofluorescence and immunoblot analysis confirmed diminished and truncated plectin expression, using several antibodies against different domains of plectin. This study gives further insight toward improving our understanding of the genotype-phenotype correlation in EBS patients with *PLEC* mutations.

MATERIALS AND METHODS

Electron Microscopy

Skin biopsy samples were fixed in 2% glutaraldehyde solution, post-fixed in 1% OsO_4, dehydrated, and embedded in Epon 812. The samples were sectioned at 1 μm thickness for light microscopy and thin-sectioned for electron microscopy (70 nm thick). The thin sections were stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy.

Mutation Detection

Genomic DNA (gDNA) was isolated from peripheral blood leukocytes of the proband and her parents. The mutation detection was performed after polymerase chain reaction (PCR) amplification of all *PLEC* exons and intron-exon borders, followed by direct automated sequencing using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA). The 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 GenBank sequence information (accession no. NM_000445.3). PCR amplification of two parts of exon 32 was performed using the following primers. Primers 5'-GTGGAGACCACGCAGGTGTAC-3' and 5'-GGAGCCCGTGCCATAGAGG-3' for a single part of exon 32 synthesized a 420-bp fragment including c.10735 to c.11154. Primers 5'-AGCGGCTGACTGTGGATGAGG-3' and 5'-TGCGTGTCCTTGTTGAGGT-3' for another single part of exon 32 synthesized a 283-bp fragment including c.11230 to c.11512. Both of the mutations in the proband were confirmed by restriction digestion of PCR products. c.10984C>T and c.11453_11462del caused the generation of new restriction enzyme sites for *BsrI* and *BbvCI*, respectively.

The mutation nomenclature follows the journal's guidelines (www.hgvs.org/mutnomen) according to the reference sequence NM_000445.3, with +1 as the A of the ATG initiation codon.

Haplotype analysis

Genotype analysis of this family to establish the *de novo* nature of c.11453_11463del in the proband was performed using three chromosome 8 markers (D8S272, D8S264, D8S270) and six non-chromosome 8 markers (D1S468, D1S252, D1S2842, D3S1297, D3S1566 and D3S1311). All microsatellite markers (ABI Prism Linkage Mapping Set Version 2.5; Applied Biosystems, Warrington, UK) were amplified with fluorescently labeled oligonucleotides and used under conditions recommended by the manufacturer. Electrophoretic analysis was performed on an ABI Prism 310 Genetic Analyzer with Performance Optimized Polymer 4 (POP4) using GeneScan software (Applied Biosystems). The allele sizes were analyzed using Genotyper software (Applied Biosystems).

Immunofluorescence Studies

Immunofluorescence analysis was performed using skin specimens from the proband as previously described (Natsuga, et al., 2010). Briefly, fresh-frozen skin specimens were embedded in optimal cutting temperature (OCT) compound and quickly frozen in isopentane cooled over liquid nitrogen. 5-μm cryostat sections were incubated with primary antibodies. After washing in phosphate-buffered saline, the sections were incubated with secondary antibodies conjugated with fluorescein-isothiocyanate.

Antibodies

The following antibodies against basement membrane zone (BMZ) components were used: monoclonal antibody (mAb) PN643 against the N-terminal actin-binding domain of plectin; mAb HD1-121 against the rod domain of plectin; C20 and mAb PC-815 against the C-terminal globular domain of plectin (Fig. 1A); mAbs GoH3 and 3E1 (Chemicon International, CA) against α6 and ß4 integrins, respectively; mAb GB3 (Sera-lab, Cambridge, UK) against laminin 332; mAb LH7.2 (Sigma, St. Louis, MO) against type VII collagen; mAb PHM-12^{+}CIV22 against type IV collagen (NeoMarkers, Fremont, CA); and S1193 and mAb HDD20 against BP230 and type XVII collagen, respectively. mAbs PN643, HD1-121 and PC815 were generously donated by Prof. K. Owaribe of Nagoya University, and antibody S1193 by Prof. J. R. Stanley of the University of Pennsylvania. C20, a goat polyclonal antibody against the C-terminus of plectin, was purchased from Santa Cruz. Anti-beta-actin mAb (AC15, Sigma, St. Louis, MO) was used to confirm equal protein loading.

Cell Culture and Immunoblot Analysis

Cell culture and immunoblot analysis was performed as previously described (Natsuga, et al., 2010). Cultured fibroblasts were obtained from skin biopsies of a normal human volunteer and the proband. Cultured fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. For sample preparation, cultured cells were lysed in Nonidet-40 (NP-40) containing buffer (1% NP-40, 25mM Tris-HCl (pH 7.6), 4mM EDTA, 100mM NaCl, 1mM phenylmethylsulfonyl fluoride (PMSF), and proteinase inhibitor

cocktail (Sigma, St. Louis, MO)); cell debris was removed by centrifugation; and the supernatant was collected. Supernatants were boiled in Laemmli's sample buffer (Laemmli, 1970), applied to a 4–12% gradient Bis-Tris gel (Invitrogen, Carlsbad, CA), and transferred to a PVDF membrane. The membrane was incubated with PN643, HD1-121, C20 and AC15 followed by incubation with horseradish peroxidase (HRP) conjugated anti-mouse IgG (for PN643, HD1-121 and AC15) and HRP-conjugated anti-gout IgG (for C20). The blots were detected using ECL Plus Detection Kit (GE Healthcare, Fairfield, CT).

Semi-quantitative RT-PCR Analysis

Semi-quantitative reverse transcription PCR (RT-PCR) analysis was performed as previously described (Natsuga, et al., 2010). Total RNA was isolated from cultured fibroblasts (from normal human volunteers and the proband, using RNeasy kit (Qiagen, Valencia, CA)), and first-strand cDNA was made using Superscript III 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; Natsuga, et al., 2010). The following primers were used (Fig. 1B): 30F, 5'-CATCAGCGAGACTCTGCGGC-3'; 31R, 5'-TGCGCCTGTCGCTTTTGTGC-3'; 31F, 5'-AGCTGGAGATGAGCGCTGA-3'; 32R, 5'-TGCTGCAGCTCCTCCTGC-3'. 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, Tokyo, Japan).

The medical ethics committee of Hokkaido University Graduate School of Medicine approved all of the described studies. The study was conducted according to The Declaration of Helsinki Principles. Participants gave their written informed consent.

RESULTS

Case Description

The proband was a first child of non-consanguineous Japanese parents. There was no family history of bullous diseases. He was born by cesarean section after a 39-week gestation because of non-reassuring fetal status. Clinically the proband showed extensive blistering and aplasia cutis on the extremities (Fig. 2A, B). Routine abdominal X-ray revealed a single bubble sign, suggesting the presence of PA (Fig. 2C). Generalized muscle hypotonia, dysphagia and difficulty in breathing were also observed from birth. Laboratory examination at birth revealed markedly elevated levels of creatine kinase (CK) (11,852U/L, normal value; 60-400U/L). The skeletal muscle isoform of CK (CK-MM) was 84% of total CK (CK, 2058U/L at age 12 days). Elevated levels of muscle enzymes including CK and aldolase (normal value; 1.7-5.7U/L) persisted over the course of his life (CK, 1924U/L; aldolase, 40.0U/L at age 25 days). Based on the clinical features and laboratory data, the presence of MD was confirmed. Muscle biopsy and reconstructive surgery for PA was not performed because the parents did not consent. The proband died 3 months after birth. Permission for autopsy was refused.

Skin Separation in Basal Keratinocytes

Electron microscopy of the skin samples from the proband showed that the skin separation localized to the base of the basal keratinocytes (Fig. 2D). Hemidesmosomes were hypoplastic and found at the base of the intraepidermal split (Fig. 2D). Keratin clumps were not observed.

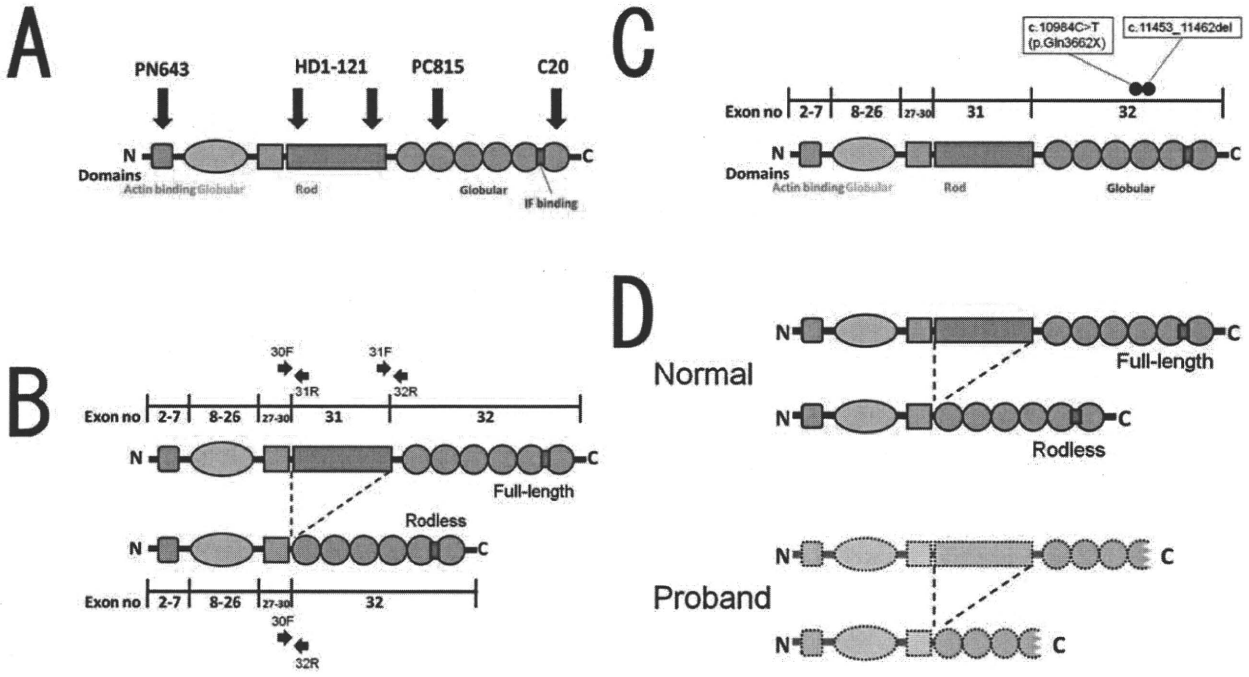

Figure 1. Plectin structure, antibodies against plectin, specific primers to amplify the full-length and the rodless plectin transcripts and *PLEC* mutations of the proband. (A) Plectin protein is composed of an actin-binding domain, N- and C-terminal globular domains, an intermediate filament (IF)-binding domain and a central rod domain. The C-terminal globular domain has 6 plectin repeat domains. The IF-binding domain is located between C-terminal repeats 5 and 6. PN643 is a monoclonal antibody (mAb) against the N-terminal actin-binding domain of plectin. HD1-121 is a mAb against the rod domain of plectin. PC815 is a mAb and C20 is a polyclonal antibody against the C-terminal globular domain of plectin. (B) The 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 mRNA of normal humans and the proband's fibroblasts. (C) c.10984C>T and c.11453_11462del are located in the *PLEC* encoding C-terminal plectin repeat 4. (D) Normal humans express both full-length and rodless plectin. In our case, the *PLEC* mutations produced diminished and truncated plectin protein without the IF-binding domain.