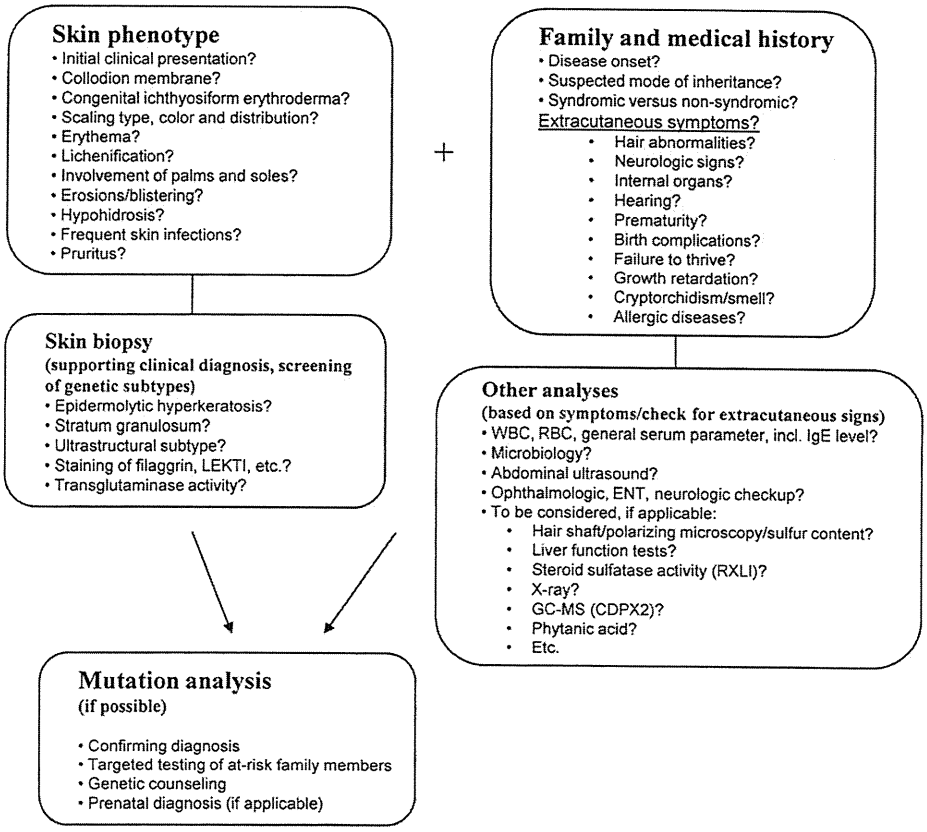

Fig 6. Concept for diagnostic approach. Diagnosis is based on dermatologic evaluation, careful family and medical history, and can be strongly supported by directed morphologic examinations and other special analyses. If available, molecular analyses are suggested to confirm diagnosis, allow for testing of family members, and prenatal diagnosis.

feature of TGase-1 deficiency,[211] aberrant vesicular structures may indicate *NIPAL4* (~*ICHTHYIN*) mutations in ARCI,[33] and trilamellar membrane aggregations in the SC and SG (EM type IV) are pathognomonic for ichthyosis prematurity syndrome.[89] Detachment of the SC from the SG with asymmetric cleavage of corneodesmosomes is a specific feature of NS.[165,212]

The image of the SC as viewed by conventional EM is still artifactual. In frozen sections, where lipid extraction is avoided, eg, by hydrophilic staining procedures, the compact structure of the SC can be appreciated. Similarly, the recent development of both osmium tetroxide and ruthenium tetroxide postfixation enables improved visualization of extracellular lipids, postsecretory changes in LB contents, and alterations of the lamellar bilayers in the SC, eg, lamellar/nonlamellar phase separation.[7] The combination of all alterations observed with this technique may be diagnostic for many forms of ichthyosis.[8] Most importantly, the ultrastructural demonstration of disturbances of lipid metabolism gives valuable insights into the pathophysiologic basis of many ichthyoses[11,60,159-164] and enables a function-driven approach.[7,8,11]

Histopathology, immunochemistry, and other nongenetic analyses

Routine histopathological findings in most ichthyoses are nondiagnostic, often demonstrating only epidermal hyperplasia and varying degrees of orthohyperkeratosis. In combination with characteristic features, routine histology can give an important clue for IV[213,214] or EI.[52,61,62,215,216] However, one should consider that a reduced or absent SG suggestive for IV can also be seen in acquired ichthyosis, NS, Refsum syndrome, TTDs, or Conradi-Hünermann-Happle syndrome. Hair mounts can demonstrate bamboo hairs (trichorrhexis invaginata) in NS[123]; although not invariably present, bamboo hairs are pathognomonic of this disorder. Parakeratosis and hypergranulosis is regarded a histopathological clue to loricrin keratoderma.[96,205] Polarization microscopy can demonstrate the tiger-tail pattern of TTD,[217,218] which

Table XIV. Examples of foundations, patient organizations, and useful Internet links

Foundations and registries	
United States: Foundation for Ichthyosis and Related Skin Types (www.scalyskin.org), Registry for Ichthyosis and Related Disorders (www.skinregistry.org)	
Germany (Europe): Network for Ichthyoses and Related Keratinization Disorders (www.netzwerk-ichthyose.de/)	
Japan: Registry for Autosomal Recessive Congenital Ichthyosis and Keratinopathic Ichthyosis supported by Health and Labor Science Research Grants, Research on Intractable Diseases, Ministry of Health, Labor, and Welfare	
Austria: National Registry for Genodermatoses Including Ichthyoses	
Patient organizations for ichthyosis	
Austria	www.selbsthilfe-tirol.at/Selbsthilfegruppen/Gruppen/Ichthyose.htm
Belgium	www.devidts.com/ichthyosis
Denmark	www.iktyosis.dk
Finland	www.iholiitto.fi/
France	www.anips.net/
Germany	www.ichthyose.de
Italy	www.ittiosi.it/
Japan	www.gyorinsen.com
Monaco	www.aaimonaco.org
Spain	www.ictiosis.org
Sweden	www.iktyos.nu//
Switzerland	www.ichthyose.ch
United Kingdom	www.ichthyosis.org.uk/
United States	www.scalyskin.org
Other databases and Internet links	
World Wide Web site hosted at National Center for Biotechnology Information (NCBI):	www.genetests.org
Portal for rare diseases and orphan drugs:	www.orpha.net
Human intermediated filament database:	www.interfil.org
German guidelines for diagnosis and treatment of ichthyoses:	www.uni-duesseldorf.de/AWMF/ll/013-043.htm

corresponds to the diagnostic low-sulfur protein content of the hair.[219,220] Special immunohistochemical procedures can be combined, eg, to confirm filaggrin deficiency in IV,[202,221] or demonstrate absent or reduced expression of LEKTI that supports the diagnosis of NS.[222-224] To screen for TGase-1 deficiency in ARCI unfixed cryostat sections are used for the enzyme activity assay.[225,226] Alternatively, superficial SC material can be subjected to a SDS heating test that visualizes absent cross-linked envelopes in TGase-1 deficiency.[227]

There are special useful analyses given in Tables IV to XII. For instance, steroid sulfatase deficiency underlying RXLI can be demonstrated by reduced arylsulfatase-C activity of leukocytes, or can readily be diagnosed by the widely available fluorescent in situ hybridization test for the STS gene region, because more than 90% of the cases are caused by a gene deletion. Gas chromatography-mass spectrometry reveals elevated serum levels of 8-dehydrocholesterol and cholesterol in Conradi-Hünermann-Happle syndrome and can identify a somatic *EBP* gene mosaicism in unaffected individuals.[228]

RESOURCES FOR CLINICIANS AND PATIENTS

Currently, therapy of most ichthyoses is neither type-specific nor corrective, but rather its goal is to relieve symptoms.[6,35,46,229-232] Importantly, clinicians have to consider the functional consequences of the epidermal barrier defect, such as increased risk of systemic absorption and toxicity, especially in infants.[231-233] Neonates with severe congenital phenotypes may require intensive care using humidified isolettes (incubators) to avoid temperature instability and hypernatremic dehydration, and observation for signs of cutaneous infection and septicemia. Caloric insufficiency as a result of evaporative energy losses places infants with severe phenotypes at risk for growth failure and requires early intervention.[234,235]

Affected individuals and/or their families should be offered genetic counseling to explain the nature of the disorder, its mode of inheritance, and the probability of future disease manifestations in the family.[1,3] They should be offered psychologic support and be informed of patient organizations or foundations (Table XIV).

We would like to dedicate this classification to all our patients and their families, and thank all colleagues and friends, who are helping to achieve optimal clinical care for affected individuals and/or promote through their research our knowledge about the disorders of cornification. We are deeply grateful for the generous financial support of the Laboratories Pierre Fabre, and would like to say "grand merci" to Anita Couteau, Didier Coustou, and Pascal Lefrancois—and to Brigitte Willis from the Network for Ichthyoses and Related Keratinization Disorders Center in Münster, who together perfectly organized the wonderful, unforgettable conference in Sorèze. Moreover, we would like to acknowledge the help of Dr Dan Ben Amitai and Dr Hagen Ott for providing photographs, and Jutta Bückmann for the help with the slides from the Department of Dermatology, Münster (head Thomas A. Luger). We also express gratitude to Meral Arin, Steffen Emmert, Rudolf Happle, Peter Höger, and Dieter Metze for their support and helpful comments. The first author wants to thank his wonderful family, namely Melody, Alanna, and Amechi.

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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,

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).

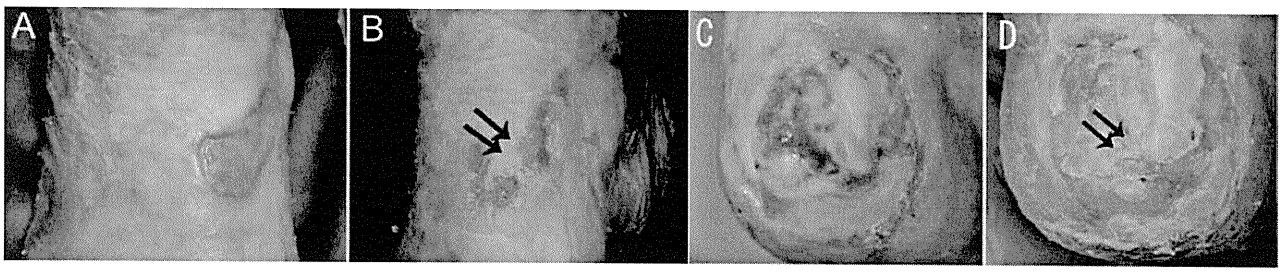

Fig. 1. Clinical response to allogeneic cultured dermal substitute (CDS) treatment (A). A skin ulcer measuring 30 × 11 mm on the back of the right foot in Patient 1. The ulcer had not healed for 10 months. (B) Re-epithelialization at 4 weeks after CDS treatment, although small erosions persist. (C) A 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.

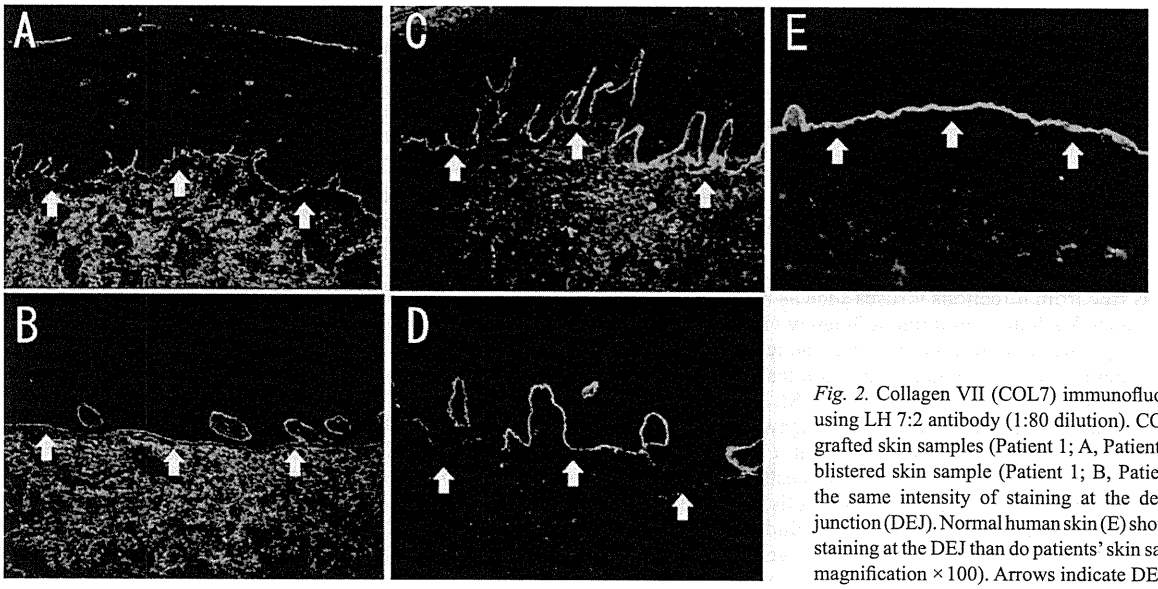

Fig. 2. Collagen VII (COL7) immunofluorescence study using LH 7:2 antibody (1:80 dilution). COL7 labelling in grafted skin samples (Patient 1; A, Patient 2; C) and non-blistered skin sample (Patient 1; B, Patient 2; D) shows the same intensity of staining at the dermal-epidermal junction (DEJ). Normal 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

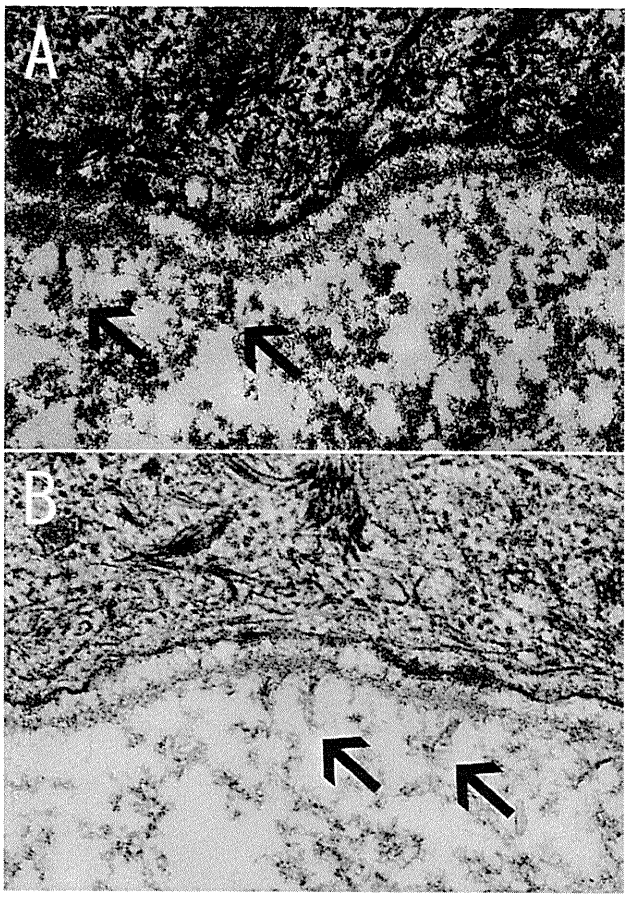

Fig. 3. Ultrastructural features of the sub-lamina densa region and basement membrane zone in the grafted and non-grafted skin of Patient 2. Discernible anchoring fibril-like structures (*arrows*) are observed beneath the lamina densa at the grafted skin site (A), as well as at the non-grafted skin site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

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

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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.

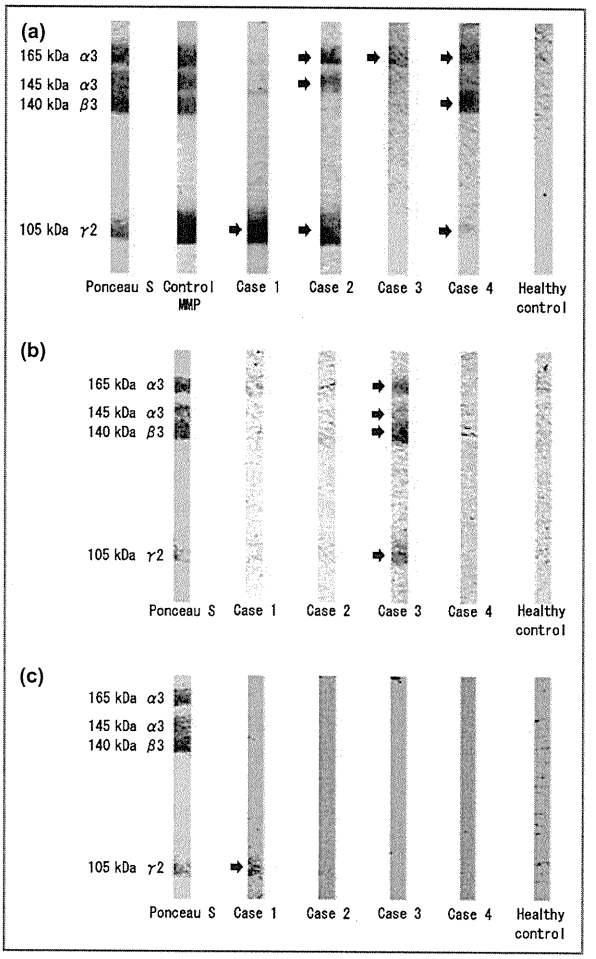

Fig 1. IgG, IgA and IgE autoantibodies against purified laminin-332. (a) Immunoblot analysis using purified laminin-332 revealed circulating IgG autoantibodies against the γ2 subunit, 105 kDa (arrow, case 1), the α3 and γ2 subunits, 165 kDa, 145 kDa and 105 kDa (arrows, case 2), the α3 subunit, 165 kDa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