Materials and Methods

The EBS-PA family

We previously reported this family with EBS-PA, in which the first and second newborns exhibited the clinical features of blistering and PA and died shortly after birth.[7] We then identified the precise genetic abnormality in the family through immunohistochemical analysis and genetic screening using the candidate gene approach. *PLEC* mutation analysis of genomic DNA from the parents and the proband demonstrated a paternal c.1350G>A splice-site mutation and a maternal p.Q305X nonsense mutation.[7] c.1350G>A was originally described as c.1344G>A and corrected according to the latest sequence information (GeneBank Accession No. NM_000445), plectin isoform 1c.[10] The parents were found to be heterozygous carriers, and the proband was compound-heterozygous (Fig. 1). The parents sought PND for a subsequent pregnancy.

PND

Amniocentesis was performed at 16 weeks gestation. Genomic DNA isolated from one-week-cultured amniocytes maintained in Amniomax medium (Invitrogen, Carlsbad, CA, USA) was subjected to polymerase chain reaction (PCR) amplification, followed by direct automated sequencing using an ABI Prism 3100 genetic analyser (Advanced Biotechnologies, Foster City, CA, USA). PCR amplification of the *PLEC* gene exons 9 and 12 was performed using the following primers. Primers 5′-GTCGCT GTATGACGCCATGC-3′ and 5′-TGGCTGGTAGCTCCATC TCC-3′ were used for amplification of exon 9, producing a 387-bp fragment. Primers 5′-CCCACTCGCCTTAGGACAGT-3′ and 5′-AAACCAACTCTGCCCAAAGC-3′ were used for amplification of exon 12, synthesizing a 428-bp fragment. PCR conditions were five minutes at 94 °C for one cycle, followed by 38 cycles of 45 seconds at 94 °C, 30 seconds at 57 °C or 60 °C, and one minute at 72 °C. The genomic DNA nucleotides, the cDNA nucleotides, and the amino acids of the protein were numbered based on the latest sequence information (GeneBank Accession No. NM_000445).

Written informed consent was obtained from the parents. PND was approved by the Institutional Ethical Committee of Hokkaido University Graduate School of Medicine. This study was conducted according to the Declaration of Helsinki Principles.

Immunofluorescence analysis

Immunofluorescence analysis using a series of antibodies against basement-membrane-associated molecules on cryostat skin sections was performed as previously described.[11] Skin biopsy was performed for the aborted fetus and a healthy volunteer as the normal control. The following monoclonal antibodies (mAbs) were used: mAb HD1–121 (a gift from Dr K. Owaribe of Nagoya University) against plectin; mAb GoH3 (a gift from Dr A. Sonnenberg of the Netherlands Cancer Institute) against α6 integrin; and mAb 3E1 (Chemicon, CA, USA) against β4 integrin.

Results

Mutation analysis of genomic DNA from amniocytes showed both paternal c.1350G>A splice-site mutation and maternal p.Q305X nonsense mutation (Fig. 2a). These mutation data were briefly mentioned in our recent paper on plectin expression patterns in patients with EBS.[12] Each mutation was confirmed by restriction enzyme digestion of PCR products. The c.1350G>A and p.Q305X mutations resulted in the loss of a restriction site for *Hph* I and *Pst* I, respectively (Fig. 2b). The prenatal molecular genetic diagnosis suggested that the fetus

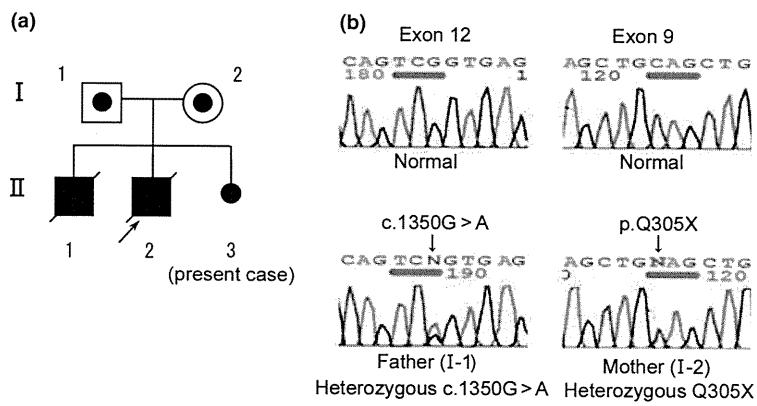

Figure 1 Family tree of the present case and the causative *PLEC* mutations. (a) The first and second newborns exhibited clinical features typical of EBS-PA and died shortly after birth. The proband (the second newborn) is indicated by an arrow. (b) The paternal splice-site mutation was a c.1350G>A transition at the end of exon 12. The maternal nonsense mutation was a c.913C>T transition in exon 9, leading to the substitution of glutamine 305 with a nonsense codon (p.Q305X)

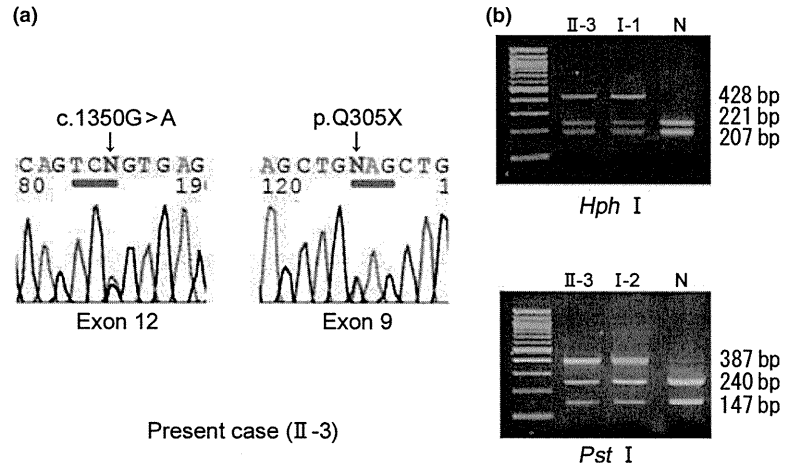

Figure 2 Analysis of the plectin gene mutations in genomic DNA from amniocytes of a fetus at risk. (a) Mutation analysis of genomic DNA from amniocytes shows both the c.1350G>A mutation in exon 12 and p.Q305X mutations in exon 9. (b) The presence of the mutations was verified by restriction enzyme digestion. The paternal mutation abolished a recognition site for the *Hph*I restriction enzyme. In the case of the normal allele, the 428-bp fragment was digested to 221 bp and 207 bp (lane N), whereas in the case of the mutant allele, a 428-bp fragment resisted digestion in the PCR product (father: lane I-1; present fetus: lane II-3). The maternal mutation also abolished a recognition site for the *Pst*I restriction enzyme. In the case of the normal allele, the 387-bp fragment was digested to 240 bp and 147 bp (lane N), whereas in the case of the mutant allele, a 387-bp fragment resisted digestion in the PCR product (mother: lane I-2; present fetus: lane II-3)

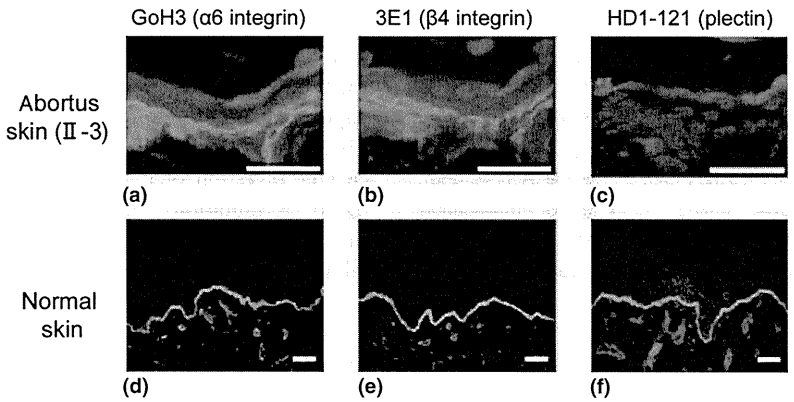

Figure 3 Absence of plectin expression in the abortus. α6 integrin (mAb GoH3) and β4 integrin (mAb 3E1) are expressed in the abortus skin (a, b) and the control skin (d, e). Staining with monoclonal antibody for plectin (mAb HD1–121) shows positive in the control skin (f) but negative in the skin of the abortus (c: blue frame). Note that the skin tissue from the abortus was subject to degeneration before skin sampling. Thus, protein localization cannot be evaluated in the degenerated tissue. Scale bar: 50 μm

was a compound-heterozygote and affected by JEB-PA. The parents elected for the fetus to be terminated at 20 weeks gestation.

Immunofluorescence analysis showed that immunoreactivity using the mAbs HD1–121 (plectin), GoH3 (α6 integrin), and 3E1 (β4 integrin) was positive in the normal control skin (Fig. 3d–f). The skin sample obtained from the abortus tested positive for α6 integrin and β4 integrin (Fig. 3a,b) but negative for plectin (Fig. 3c).

Discussion

This is the first successful PND of plectin-deficient EBS-PA, and the correct diagnosis was reconfirmed in the skin of the abortus. Given the universal mortality of EBS-PA due to *PLEC* mutations, there might be unreported PND cases for this form of EB. The prognosis of plectin-deficient EBS-PA is poor, and most patients commonly die within the first year of life,[13] as happened in the first- and

second-born progeny in the present family. Fetuses at risk of this condition are frequently terminated during pregnancy, and DNA-based PND plays an important role in prohibiting unnecessary termination of healthy fetuses at risk. Due to the recent elucidation of the causative genetic defects for genetic skin disorders, it has become possible to make DNA-based PND for severe genodermatoses by sampling of the chorionic villus or amniotic fluid in the earlier stages of pregnancy with a lower risk to fetal health and with a reduced burden on the mothers.

Plectin, a component of the hemidesmosome inner plaque, is involved in the attachment and crosslinking of the cytoskeleton and intermediate filaments to specific membrane complexes.[10] It has been described that EBS associated with muscular dystrophy (EBS-MD) results from *PLEC* mutations.[14,15] Mutations in the rod domain of *PLEC* are known to cause EBS-MD.[9,14,15] In addition, recent reports have confirmed that some *PLEC* mutations also lead to EBS-PA.[7–9,13] One alternative splice *PLEC* mRNA transcript that lacks exon 31 encoding the central core rod domain was identified in rat tissues.[16] By plectin-domain-specific reverse transcriptase-PCR, expression of this rodless alternative spliced form was confirmed in human keratinocytes.[17] Recently, our group demonstrated that loss of the full-length plectin with maintenance of the rodless plectin leads to EBS-MD, whereas complete loss or marked attenuation of full-length and rodless plectin expression underlies the EBS-PA phenotype.[12] The present family further supports the hypothesis that homozygotes or compound-heterozygotes for mutations that cause plectin truncation outside the rod domain show the EBS-PA phenotype.

In summary, this is the first report of DNA-based PND of EBS-PA. EBS-PA has now been added to the list of severe genodermatosis for which DNA-based PND is feasible.

Acknowledgments

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Letter to the Editor

Altered lipid profiles in the stratum corneum of Sjögren-Larsson syndrome

Sjögren-Larsson syndrome (SLS) is a rare, autosomal recessive neurocutaneous disorder characterized by clinical triads, congenital ichthyoids, spasticity and mental retardation [1]. SLS is caused by mutations in fatty aldehyde dehydrogenase (*FALDH*) (or *ALDH3A2*) gene [1]. FALDH is a microtonal NAD-dependent enzyme, which oxidizes medium- to long-chain aliphatic aldehydes to fatty acids. Accumulation of fatty alcohol has been shown in cultured fibroblasts and in plasma from SLS patients [1]. Numbers of mutations of *FALDH* gene have been shown, although only three mutations have been identified in Japanese SLS patients [2–4]. We here report a SLS patient who is a homozygote for one of the known mutations. In addition to assessing skin phenotype, permeability barrier function and cutaneous morphology, biochemical analysis revealed novel alterations in lipid profiles in the stratum corneum associated with barrier function.

A 57-year-old Japanese woman complaining of slightly pruritic and dry skin with scaling visited our hospital. The patient has been suffering from scaly skin lesions over the entire body since her early childhood. She presented generalized dryness, widespread itchy hyperkeratosis scaly lesions with brown scaling plaques, and slight erythema on the trunk and extremities (Fig. 1a). The neurologic examination revealed severe spastic paraplegia in the lower limbs with an increased muscle tone, hyperreflexia in all limbs, and positive Babinski reflexes bilaterally. She also showed mental retardation (IQ 39). A skin biopsy specimen from the right arm revealed orthohyperkeratosis with thin granular layers and mild acanthosis with papillomatosis (Fig. 1b). Electron microscopic examination showed several lipid droplets without surrounding membrane in the cornified cells (Fig. 1c). Moreover, abnormal lamellar granules, which lacked lamellar contents, were present in the granular cells (Fig. 1d). From these clinical features and cutaneous morphology, this patient was diagnosed as SLS. Mutation analysis using a cDNA sample from the patient's peripheral white blood cells showed a homozygous point mutation c.1157A>G which results in alteration from asparagine to serine at cordon 386 (p.Asn386Ser) in the β-9 chains containing active domain of FALDH (Fig. 2a).

Transepidermal water loss (TEWL) of the ichthyosiform lesion on the extensor and flexor sides of the forearm and back (6.3, 12.2, 10.2 g h^{-1} m^{-2}, respectively) was within the normal range (0–10, very good; 10–15, good; 15–20, fair; 25–30, poor; more than 30, very poor). On the other hand, water retention capability was impaired in the lesion (25.5, normal > 60).

Major barrier lipid content of involved skin was assessed in comparison to non-ichthyotic scaly lesions from sunburn dermatitis as a control subject (note: we and others found that there is no significant difference in lipid content of sunburn scale and of non-sunburn scales from normal donors [5]). Although there was no difference in the quantity of cholesterol between the patient and control, free fatty acid (FFA) was increased by about two-fold over control (Fig. 2b). In contrast, ceramide (Cer) 1, 6, 7 were decreased in the patient's scales compared with those in control samples, while membrane-bound Cer species, Cer A, which are constituent of the corneocyte lipid envelope (CLE), were increased. We recently demonstrated that linoleate required for acylceramide synthesis is primarily derived from triglyceride (TG) [6]. However, TG content was not changed in SLS compared with that in control scales (Fig. 2b).

The identical mutation in our case was described in another Japanese patient with SLS [2]. The other mutations reported in the

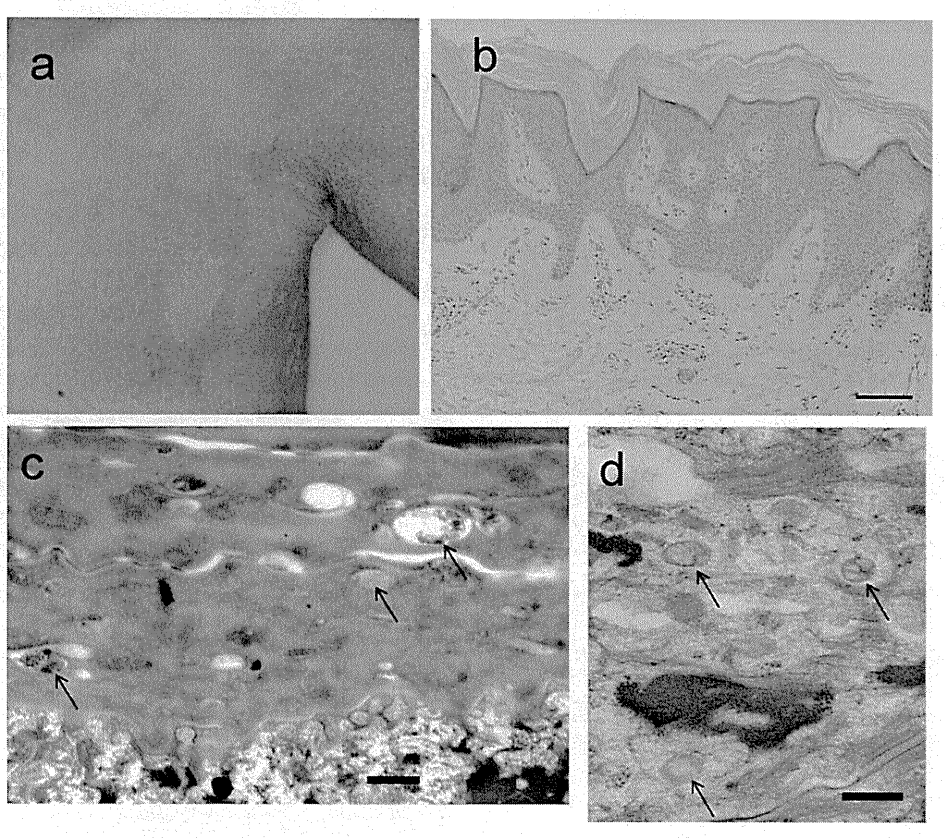

Fig. 1. Clinical appearance. (a) Scaly ichthyosiform erythema was apparent over the trunk. Morphological features of the patient's epidermis. (b) H&E staining of lesional skin from the patient's forearm. Orthohyperkeratosis, slightly thin granular layers and mild acanthosis with papillomatosis are noted, scale bar, 50 μm. (c) Ultrastructually, electron-lucent vacuoles are present within corneocytes (arrows) scale bar, 2 μm. (d) The presence of abnormal lamellar bodies lacking lamellar contents are evident in the cytoplasm of the granular cell (arrows) scale bar, 2 μm.

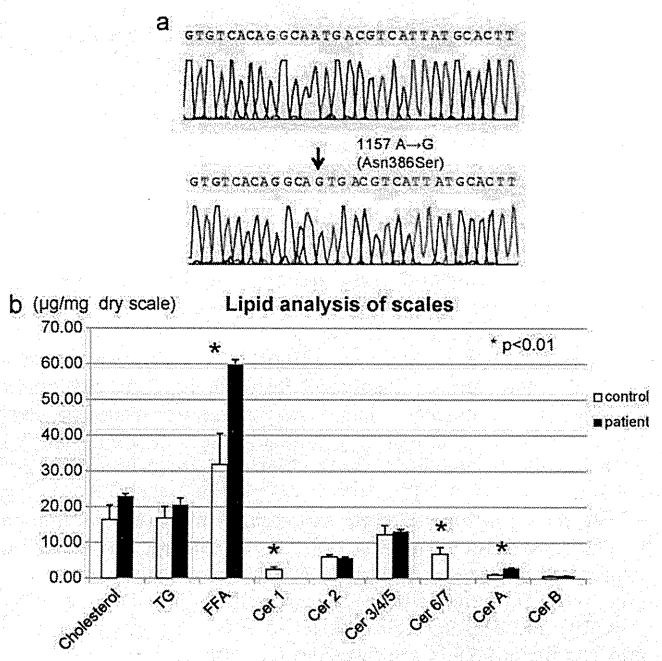

Fig. 2. (a) Sequencing analysis of FALDH gene. A homozygous point mutation (c.1157A>G) in the exon 8 that substitutes serine for asparagine at position 386 (p.Asn386Ser). (b) Lipid analysis of scales taken from sunburn lesions of a normal control individual (white bar) and from the patient's lesions (black bar) show increased FFA and Cer A level and decreased ceramide 1, 6, 7 levels in the patient's scale compared with control samples. Scales were taken from the upper back skin of the patient or control subjects. Gene and lipid analysis were performed as we described previously [4,6].

Japanese cases were c.481delA, c.1087_1089delGTA, c.332G>A (p.Trp111X) and c.636T>G (p.Ser212Arg) [3,4]. All the mutations found in Japanese families were distinct from one another and no founder effect was suggested in *ALDH3A2* mutations underlying Japanese SLS cases.

Recent studies by lanthanium perfusion assay, which is more sensitive for assessing permeability barrier function *in vitro* using skin sections than TEWL measurements employed in our study, reveals abnormal permeability barrier formation, structures, and function in SLS patients [7], while our present study is the first time for assessing both TEWL and hydration of SLS patient *in vivo*. Consistent with this prior study abnormal epidermal barrier structures [7] are evident in our patient, but alterations of TEWL were not observed. We assume that hyperkeratosis could attempt to compensate barrier dysfunction as previously suggested [8] and result in attempting to minimize barrier abnormality. Yet, decreased SC hydration in a SLS patient could alter normal SC environment, leading to abnormal epidermal homeostasis.

It remains to be resolved, however, why FFA level was high in spite of the deficient activity of FALDH, which was the enzyme catalyzing the sequential oxidation of fatty alcohol to fatty acid. It is likely that increased levels of wax esters and alkyl-diacylglycerol in scales and keratinocytes of SLS [9] derived from fatty alcohol may contribute to FFA production via hydrolysis with lipase, because the levels of these lipids were high.

Consistent with a prior study showing a deficiency of Cer 1, 6 in SLS patients' skin [10], Cer 1, 6, 7 were decreased in the epidermis of our case. We further demonstrated that the levels of CLE-bound ceramides, Cer A, which are produced from acylglucosylceramide, elevated in the scale from the patient, although Cer 1 (EOS) generated from the same precursors decreased. Therefore,

acylglucosylceramides appear to be preferentially utilized for CLE-bound ceramide production rather than free (CLE-unbound) lipid production in the SC. Exact mechanisms for CLE formation have not been elucidated yet and it remains to be resolved whether preferential utilization of acylglucosylceramide for CLE formation occurs only in the present case or also in other SLS patients. Moreover, it is unknown how decrease in Cer 1, 6, 7 occur and whether barrier lipid abnormality in the patient was a primary event or a secondary phenomenon in the pathogenesis of SLS skin lesions. Cer 1 is essential lipid species to form epidermal permeability barrier formation. Thus, not only accumulation of free fatty acids, but also deficiency of specific ceramide species might contribute to formation of ichthyotic phenotype in SLS.

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Letter to the Editor

BMP-4 down-regulates the expression of Ret in murine melanocyte precursors

Bone morphogenetic proteins (BMPs) have been implicated in a diverse array of biological processes including development and apoptosis [1]. Ret is involved in the physiological mechanisms of melanocyte activation and melanin production [2]. Ret expression in enteric neural precursors is initiated shortly after they emigrate from the neural plate.

We established three distinct cell populations of mouse neural crest (NC) cells, NCCmelb4, NCCmelb4M5 and NCCmelan5. NCCmelb4 cells have the potential to differentiate into mature melanocytes, but since they express melanocyte markers such as tyrosinase-related protein 1, DOPAchrome tautomerase and Kit, we consider them to be immature melanocytes, not multipotent precursors that can differentiate into neurons, as well as glia [3]. NCCmelb4M5 cells belong to the melanocyte lineage, but are less differentiated than NCCmelb4 cells [4]. NCCmelb4M5 cells do not express Kit and grow independently of the Kit ligand; these cells have the potential to differentiate into NCCmelb4 cells, which are Kit-positive melanocyte precursors. NCCmelan5 cells demonstrate the characteristics of differentiated melanocytes. We have also established an oncogene Ret-transgenic mouse line, line 304/B6, in which skin melanosis, benign melanocytic tumors and malignant melanomas develop in a stepwise fashion [2]. A malignant melanoma cell line, Mel-Ret, was established from the Ret-transgenic mouse. We found that all four cell lines express BMP receptors using Western blotting analysis (data not shown).

Western blotting revealed expression of the Ret protein in NCCmelb4M5 and in Mel-Ret cells, but in contrast, there was no expression of the Ret protein in NCCmelb4 or NCCmelan5 cells (Fig. 1A). Immunostaining also revealed that NCCmelb4M5 (Fig. 1B) and Mel-Ret cells are positive for Ret, but NCCmelb4 and NCCmelan5 cells are negative for Ret. Thus, Ret protein is expressed in most immature melanoblasts, while melanocytes are negative for Ret. We then analyzed Ret protein expression in BMP-4-treated NCCmelb4M5 cells by Western blotting (Fig. 1C–F). BMP-4 was added to the medium and incubated for 3 days at varying concentrations. After incubation with 10 ng/ml BMP-4 for 3 days, Ret protein expression was decreased, and disappeared completely

REVIEW

Definitions and outcome measures for bullous pemphigoid: Recommendations by an international panel of experts

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Our scientific knowledge of bullous pemphigoid (BP) has dramatically progressed in recent years. However, despite the availability of various therapeutic options for the treatment of inflammatory diseases, only a few multicenter controlled trials have helped to define effective therapies in BP. A major obstacle in sharing multicenter-based evidences for therapeutic efforts is the lack of generally accepted definitions for the clinical evaluation of patients with BP. Common terms and end points of BP are needed so that experts in the field can accurately measure and assess disease extent, activity, severity, and therapeutic response, and thus facilitate and advance clinical trials. These recommendations from the International Pemphigoid

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Committee represent 2 years of collaborative efforts to attain mutually acceptable common definitions for BP and proposes a disease extent score, the BP Disease Area Index. These items should assist in the development of consistent reporting of outcomes in future BP reports and studies. (J Am Acad Dermatol 10.1016/j.jaad.2011.06.032.)

Key words: bullous pemphigoid; consensus; definitions; outcome measures; severity score.

CAPSULE SUMMARY

- It is impossible to compare the therapeutic outcomes from the majority of bullous pemphigoid studies using meta-analysis, as they have varying definitions and outcome measures.
- These recommendations, developed over the last 3 years by experts, provide appropriate definitions for the various stages of disease activity and therapeutic end points in bullous pemphigoid.
- These definitions can be used in case series and clinical trials to compare the efficacy of treatments for bullous pemphigoid.

Bullous pemphigoid (BP) is a common autoimmune bullous disease typically affecting the elderly. There have been only a handful of well-designed randomized controlled trials assessing the effectiveness of therapies for BP.[1] In relatively rare diseases where it is difficult to include enough patients to have sufficient power to compare different treatments, meta-analysis is a powerful tool that is used to pool data across trials. However, it is impossible to compare the therapeutic outcomes from the majority of these BP studies using meta-analysis, as they have varying definitions and outcome measures.

PURPOSE

The purpose of this statement is to provide appropriate definitions for the various stages of disease activity, define therapeutic end points in BP, and to propose an objective disease extent measure that can be used in clinical trials. The use of the same definitions and outcome measures makes the results of trials more comparable. Since definitions and outcome measures for pemphigus[2-4] have been published, most trials in pemphigus and reports have begun adopting these systems or referring to them when their existing trials using other measures were unable to show a difference.[5]

METHODS

An international BP definitions committee was organized in 2008, at the point when the international pemphigus definitions committee completed its similar work on pemphigus.[2] The committee was an expansion of the first committee and convened 7 times over 2 years to discuss the appropriate definitions. These meetings were held at the American Academy of Dermatology (AAD) annual meeting in San Antonio, TX, in 2009 (D. F. M. and V. P. W.); European Society for Dermatologic Research in Budapest, Hungary, in 2009 (D. F. M. and P. J.); the European Academy of Dermatovenereology in Berlin, Germany, in 2009 (D. F. M. and L. B.); the AAD in Miami, FL, in 2010 (D.F.M. and V. P. W.); the Pemphigus 2010 Meeting in Bern, Switzerland (V. P. W. and D. F. M.); and the International Pemphigus and Pemphigoid Meeting at the National Institutes of Health in November 2010 (V. P. W. and D. F. M.), in Bethesda, MD. The final meeting was held at the AAD in 2011 in New Orleans, LA (D. F. M. and V. P. W.). Meetings were supported in part by local dermatology societies. The draft definitions and end points were electronically mailed to the larger group, allowing for comments between meetings.

THE RECOMMENDATIONS

Observation points

The end points are illustrated and summarized (Fig 1 and Table I).

Early end points

"Baseline" is the point at which a physician starts treatment for BP.

"Control of disease activity" (disease control; beginning of consolidation phase) is defined as the point at which new lesions or pruritic symptoms cease to form and established lesions begin to heal. The time to disease control is the time between baseline and this control point.

"End of the consolidation phase" is defined as the time at which no new lesions or pruritic symptoms have developed for a minimum of 2 weeks and the majority (approximately 80%) of established lesions has healed. At this point tapering of corticosteroids often occurs. The length of the consolidation phase is the time between disease control and the end of consolidation phase.

Abbreviations used:

AAD: American Academy of Dermatology
BP: bullous pemphigoid
BPDAI: Bullous Pemphigoid Disease Area Index
DAI: Disease Area Index
PDAI: Pemphigus Disease Area Index

"Transient lesions" are new lesions that heal within 1 week or pruritus lasting less than a week and clearing without treatment.

"Nontransient lesions" are new lesions that do not heal within 1 week or pruritus continuing more than a week with or without treatment.

Intermediate end points

During this period, the corticosteroids and other treatments are usually being tapered, but for some patients medication doses do not change because of flaring with attempts to taper treatment. "Complete remission during tapering" is the absence of nontransient lesions while the patient is receiving more than minimal therapy. There is no minimum time point here as the patient is under control but has not yet reached the desired outcome of disease remission on minimal or no therapy.

Late observation end points

Late observation end points of disease activity are identified as: (1) complete remission off therapy; and (2) complete remission on therapy, both of which only apply to patients who have had no new or established lesions for at least 2 months. "Complete remission off therapy" is defined as an absence of new or established lesions or pruritic symptoms while the patient is off all BP therapy for at least 2 months.

"Complete remission on therapy" is defined as the absence of new or established lesions or pruritus while the patient is receiving *minimal* therapy for at least 2 months. "Minimal therapy" is defined as less than or equal to 0.1 mg/kg/d of prednisone (or the equivalent) or 20 g/wk of clobetasol propionate and/or minimal adjuvant or maintenance therapy for at least 2 months, as shown in Fig 1 and discussed further below.

Minimal adjuvant therapy in BP corresponds to the following doses or less: methotrexate 5 mg/wk; azathioprine 0.7 mg/kg/d (with normal thiopurine s-methyltransferase level); mycophenolate mofetil 500 mg/d; mycophenolic acid 360 mg/d; or dapsone 50 mg/d. There has only been one small randomized controlled trial on tetracycline and niacinamide,[6] which was underpowered because of low numbers and was unable to demonstrate a difference. Nevertheless, the committee's expert opinion is that full therapeutic doses of the tetracyclines may work in localized forms of BP. As the tetracycline class of drugs is relatively nontoxic, the full therapeutic dose was listed among minimal therapies for BP.

"Partial remission off therapy" is defined as the presence of transient new lesions that heal within 1 week without treatment and while the patient is off all BP therapy for at least 2 months.

"Partial remission on minimal therapy" is defined as the presence of transient new lesions that heal within 1 week while the patient is receiving minimal therapy.

A newer term, "mild new activity," refers to fewer than 3 lesions a month (blisters, eczematous lesions, or urticarial plaques) that do not heal within 1 week, or the extension of established lesions or pruritus once per week but less than

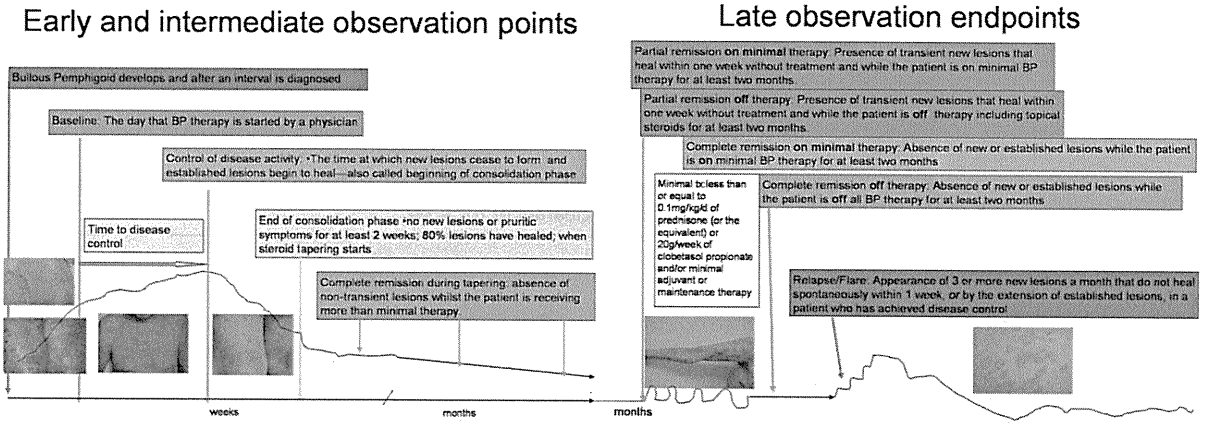

Fig 1. Pictorial depiction of end points in bullous pemphigoid.

Table I. Definitions for bullous pemphigoid

Early observation points	
Baseline	Day that BP therapy is started by physician
Control of disease activity	Time at which new lesions cease to form and established lesions begin to heal or pruritic symptoms start to abate
Time to control of disease activity (disease control; beginning of consolidation phase)	Time interval from baseline to control of disease activity
End of consolidation phase	Time at which no new lesions have developed for minimum of 2 wk and approximately 80% of lesions have healed and pruritic symptoms are minimal
Intermediate observation end points	
Transient lesions	New lesions that heal within 1 wk or pruritus lasting <1 wk and clearing without treatment
Nontransient lesions	New lesions that do not heal within 1 wk or pruritus continuing >1 wk with or without treatment
Complete remission during tapering	Absence of nontransient lesions while patient is receiving more than minimal therapy
Late observation end points	
Minimal therapy	≤0.1 mg/kg/d Of prednisone (or equivalent) or 20 g/wk of clobetasol propionate and/or minimal adjuvant or maintenance therapy
Minimal adjuvant therapy and/or maintenance therapy	Following doses or less: methotrexate 5 mg/wk; azathioprine 0.7 mg/kg/d (with normal thiopurine s-methyltransferase level); mycophenolate mofetil 500 mg/d; mycophenolic acid 360 mg/d; or dapsone 50 mg/d
Partial remission on minimal therapy	Presence of transient new lesions that heal within 1 wk while patient is receiving minimal therapy for at least 2 mo
Complete remission on minimal therapy	Absence of new or established lesions or pruritus while patient is receiving minimal therapy for at least 2 mo
Partial remission off therapy	Presence of transient new lesions that heal within 1 wk without treatment while patient is off all BP therapy for at least 2 mo
Complete remission off therapy	Absence of new or established lesions or pruritus while patient is off all BP therapy for at least 2 mo
Mild new activity	<3 Lesions/mo (blisters, eczematous lesions, or urticarial plaques) that do not heal within 1 wk, or extension of established lesions or pruritus once/wk but less than daily in patient who has achieved disease control; these lesions have to heal within 2 wk
Relapse/flare	Appearance of ≥3 new lesions/mo (blisters, eczematous lesions, or urticarial plaques) or at least one large (>10 cm diameter) eczematous lesion or urticarial plaques that do not heal within 1 wk, or extension of established lesions or daily pruritus in patient who has achieved disease control
Failure of therapy for initial control	Development of new nontransient lesions or continued extension of old lesions, or failure of established lesions to begin to heal or continued pruritus despite: Clobetasol propionate 40 g/d for 4 wk; or Prednisone 0.75 mg/kg/d equivalent for minimum of 3 wk with or without drugs used for maintenance therapy; or A tetracycline on full dosing for 4 wk; or Dapsone 1.5 mg/kg/d for 4 wk; or Methotrexate 15 mg/wk (if >60 kg and no major renal impairment) for 4 wk; or Azathioprine 2.5 mg/kg/d for 4 wk (if thiopurine s-methyltransferase level is normal); or Mycophenolate mofetil 40 mg/kg/d (if normal renal function, otherwise according to age/creatinine clearance) for 4 wk

BP, Bullous pemphigoid.

daily, in a patient who has achieved disease control. This term was not included in the pemphigus definitions but the committee thought that it might be important to capture this phase during studies to determine if some patients with BP and certain characteristics or treatments experienced new mild activity not significant enough to constitute a flare. In this way, it could be determined in the future if these patients with BP might benefit from a change of treatment plan or not.

Relapse/flare

The terms "relapse" and "flare" are used interchangeably and are defined as the appearance of 3 or more new lesions a month (blisters, eczematous lesions, or urticarial plaques) or at least one large (>10 cm diameter) eczematous lesion or urticarial plaque that does not heal within 1 week, or the extension of established lesions or daily pruritus in a patient who has achieved disease control.

Treatment failure

"Failure of therapy for initial control" is defined as the development of new nontransient lesions or continued extension of old lesions, or failure of established lesions to begin to heal or daily pruritus despite certain strengths of corticosteroids with or without higher doses of adjuvants. The dose of prednisone defined as treatment failure is 0.75 mg/kg/d equivalent for minimum of 3 weeks. This dose was selected because the Cochrane review of interventions for BP[1,7] determined that in acute BP there was no purpose in using prednisone at a higher dose than this. Topical clobetasol propionate at 40 g/d for 4 weeks was selected on the basis of the randomized controlled trials conducted by the French group.[8,9] Other therapies include tetracycline at full doses for 4 weeks; dapsone 1.5 mg/kg/d for 4 weeks; methotrexate 15 mg/wk (if >60 kg and no major renal impairment) for 4 weeks; azathioprine 2.5 mg/kg/d for 4 weeks (if thiopurine s-methyltransferase level is normal); or mycophenolate mofetil 40 mg/kg/d (if normal renal function, otherwise according to age/creatinine clearance) for 4 weeks. The definition does not imply these drugs and their respective doses are equivalent in therapeutic efficacy. Rather it provides a standardized agreement as to what can be defined as a failure of therapy.

BP disease activity index

Like the Pemphigus Disease Area Index (PDAI),[3] the BP Disease Area Index (BPDAI) measure has separate scores for skin and mucous membrane activity. Damage scores are separate as well and are included to remind physicians that not all visible lesions in BP represent active disease. Areas of the skin predominantly affected in BP[10] were taken into account when selecting the skin sites so that trials would better differentiate clinical response in BP. Hence, additional weighting was given to the arms and legs and less emphasis to the face and scalp, slightly different from the PDAI. The mucous membrane areas were retained from the PDAI even though it is relatively rare to see mucous membrane involvement in BP, so that the activity could be compared with extent of mucous membrane involvement in different autoimmune bullous diseases. There are separate columns for the extent of blistering and for the urticarial/eczematous lesions that may be more extensive in BP.

BPDAI PRURITUS COMPONENT - VAS

DATE: .....

☐Baseline
☐Consolidation phase
☐Tapering phase
☐Complete remission on minimal therapy
☐Complete remission off therapy
☐ Beginning Consolidation
☐End of Consolidation
☐Partial remission on minimal therapy
☐Partial remission off therapy
☐Flare

A. How severe has your itching been over the last 24 hours?

0 1 2 3 4 5 6 7 8 9 10
None Severe

Score out of 10 =

B. How severe has your itching been the past week?

0 1 2 3 4 5 6 7 8 9 10
None Severe

Score out of 10 =

C. How severe has your itching been in the past month?

0 1 2 3 4 5 6 7 8 9 10
None Severe

Score out of 10 =

Average INTENSITY SCORE FOR PAST MONTH = (A+B+C) = /30

OR

For BP patients with impaired mental functioning:

	No evidence of itch (no excoriations)	0
	Mild itch (isolated excoriations up to two body sites)	10
	Moderate itch (excoriations on ≥ 3 body sites, impairment of daily activity	20
	Severe itch (generalized excoriation, sleep impairment)	30
	TOTAL SCORE	/30

Fig 2. Subjective Bullous Pemphigoid (*BP*) Disease Area Index (*BPDAI*) pruritus score. *VAS*, Visual analog scale.

As a major symptom that may herald the onset and recurrence of BP is pruritus, a separate subjective component of the BPDAI is proposed to measure the severity of this (Fig 2). Naturally, other causes of pruritus in the elderly must be excluded, such as xerosis, dermatitis, renal impairment, liver impairment, and scabies. Providing that only pruritus related to BP is considered in the definitions and scored, this system can be used to subjectively grade the intensity of pruritus using a visual analog scale to answer the question, "How severe is your itching today?" and the patient marks an "x" on the 0- to 10-cm line where 0 is no itch and 10 is maximal itching. The degree of itching is measured as the distance in centimeters from 0, out of 10. This is repeated for the severity overall of itching in the past week and month. A total score is calculated from this out of 30. If the patient with BP is incapable of completing a reliable visual analog scale rating, for example, as a result of dementia, then the degree of pruritus is inferred, based on the extent of excoriations alone, also scored

BPDAI					
SKIN	ACTIVITY		ACTIVITY		DAMAGE
Anatomical location	Erosions/Blisters	Number of Lesions if <3	Urticaria/ Erythema / Other	Number of Lesions if <3	Pigmentation / Other
	0 absent		0 absent		Absent 0, present 1
	1 1-3 lesions, none > 1 cm diameter		1 1-3 lesions, none >6 cm diameter		
	2 1-3 lesions, at least one > 1 cm diameter		2 1-3 lesions, at least one lesion > 6 cm diameter		
	3 >3 lesions, none > 2 cm diameter		3 >3 lesions, or at least one lesion > 10 cm		
	5 >3 lesions, and at least one >2 cm		5 >3 lesions and at least one lesion > 25 cm		
	10 >3 lesions, and at least one lesion >5 cm diameter or entire area		10 >3 lesions and at least one lesion > 50 cm diameter or entire area		
Head					
Neck					
Chest					
Left arm					
Right arm					
Hands					
Abdomen					
Genitals					
Back/Buttocks					
Left leg					
Right leg					
Feet					
Total skin	/120		/120		
MUCOSA	Erosions/Blisters				
	1 1 lesion				
	2 2-3 lesions				
	5 >3 lesions, or 2 lesions >2cm				
	10 entire area				
Eyes					
Nose					
Buccal mucosa					
Hard palate					
Soft palate					
Upper gingiva					
Lower gingiva					
Tongue					
Floor of Mouth					
Labial Mucosa					
Posterior Pharynx					
Anogenital					
Total Mucosa	/120				

Fig 3. Objective bullous pemphigoid disease area index

out of 30 (Fig 2). This subjective itch score will not be combined with the objective part of the BPDAI (Fig 3). Eventually, a quality-of-life tool for BP will be necessary as well. The BPDAI will be undergoing validation studies, similar to the partial validation done thus far with the PDAI.[3]

DISCUSSION AND CONCLUSION

Despite many trials evaluating therapeutic options for BP, it has been difficult to compare the results from these trials because of the large number of end points and definitions of disease. The formation of an international committee of bullous

disease experts able to meet face to face on a regular basis has provided a mechanism for developing agreement on these issues for BP. This statement with agreed-upon common definitions, and the ongoing discussion and refinement of proposed common measurements for patients with BP, are the initial and necessary steps toward progress in the clinical evaluation and therapy of BP. Further progress and advancement will require a continued unified effort.

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Childhood subepidermal blistering disease with autoantibodies to type VII collagen and laminin-332

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MADAM, Autoimmune subepidermal blistering diseases include bullous pemphigoid, pemphigoid gestationis, linear IgA bullous dermatosis, mucous membrane pemphigoid (MMP), anti-p200 pemphigoid, epidermolysis bullosa acquisita (EBA) and bullous systemic lupus erythematosus.[1] Patients with EBA have IgG autoantibodies to type VII collagen while some patients with MMP have autoantibodies to laminin-332.[2,3] We describe a juvenile case of subepidermal blistering disease with autoantibodies to both type VII collagen and laminin-332. The present case is unique because of its childhood onset and successful remission following only topical steroid therapy.

A 12-year-old Japanese girl presented with pruritic eruptions on her scalp. A few weeks later, widespread pruritic vesicles gradually developed over her whole body. The vesicles were seen both on erythematous and normal skin (Fig. 1a, b). Blisters and erosions also appeared in her oral mucosa, but there was no involvement of genital or ocular mucous membranes (Fig. 1c). Neither nail changes nor alopecia were observed. She had no family history of any blistering disorders or autoimmune disease. There was no preceding illness or history of medication/vaccination that might have triggered her disease.

General laboratory examinations revealed no apparent abnormalities except for an increased serum IgE level (668·8 IU mL^{-1}; normal < 100 for age 7–14 years). A skin biopsy was taken from the edge of one blister on her right forearm. Light microscopy showed a subepidermal blister with an inflammatory cell infiltrate consisting of mainly neutrophils in the upper dermis (Fig. 2a). Direct immunofluorescence of the patient's lesional skin showed *in vivo* linear deposits of IgG and C3 at the epidermal basement membrane zone (Fig. 2b). On the blistered area, deposition of IgG and C3 was demonstrated on the dermal side of the separated skin (arrows, Fig. 2b). Indirect immunofluorescence with the patient's serum on 1 mol L^{-1} NaCl-split normal human skin showed IgG antibodies bound to the dermal side of the blister (Fig. 2c). Immunoblot analysis revealed that the patient's serum reacted with a 290-kDa protein in dermal extracts, and further with purified laminin-332 α3 protein (145, 165 kDa) (Fig. 2d, e). Laminin-332 was obtained from human keratinocytes and was purified using an antilaminin-332 affinity column as

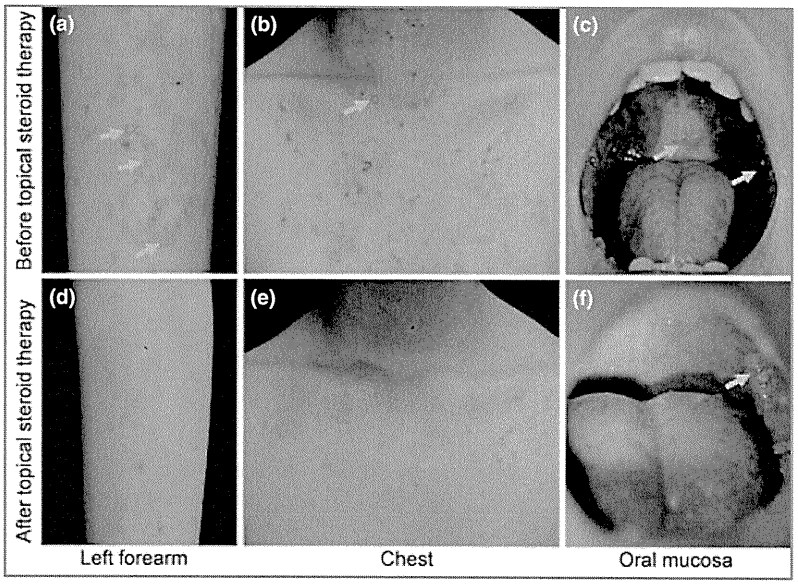

Fig 1. Clinical manifestations of the skin and oral mucosa. (a–c) Before topical steroid therapy. Erythema and tense vesicles on the left forearm and chest (a and b, arrows). Blisters and erosions over the oral mucosa (c, arrows). (d–f) After topical steroid therapy. Skin lesions healed within 9 days of the beginning of treatment, leaving residual pigmentation, scars and milia (d and e). Blisters and erosions on the oral mucosa subsided (f, arrow).

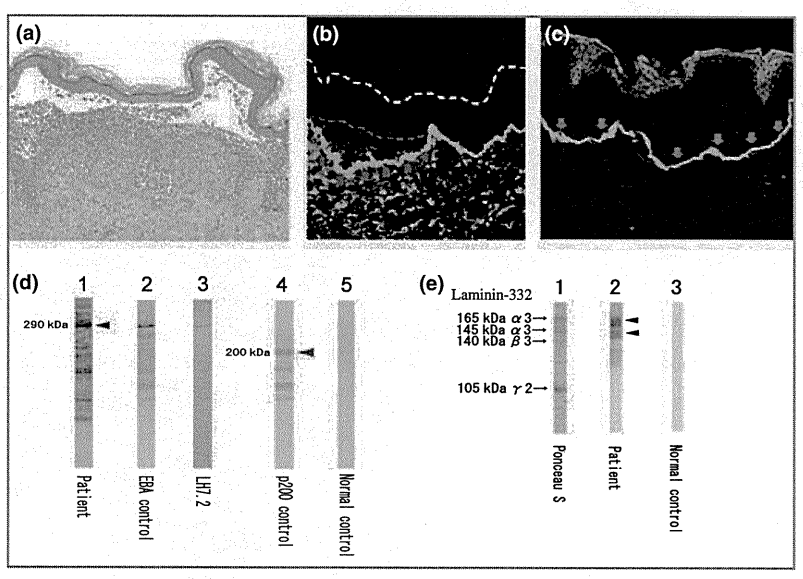

Fig 2. Histopathological findings, immunofluorescence staining and immunoblot analyses. (a) A subepidermal blister with an inflammatory cell infiltrate composed of mainly neutrophils in the upper dermis (haematoxylin and eosin; original magnification × 40). (b) Direct immunofluorescence showed *in vivo* linear deposits of IgG along the basement membrane zone. On the blister area, deposition of IgG was shown to be towards the dermal side of separated skin (arrows) (original magnification × 40; white dotted line is the skin surface and red dotted line is the roof side of separated skin). (c) Indirect immunofluorescence with the patient's serum on 1 mol L^{-1} NaCl-split normal human skin showed IgG antibodies bound to the dermal side (arrows) (original magnification × 40). (d) Immunoblot analysis revealed that the patient's serum (lane 1), like both serum from a reference patient with epidermolysis bullosa acquisita (EBA, lane 2) and monoclonal antibody LH7.2 to type VII collagen (lane 3), reacted with a 290-kDa protein in dermal extracts (arrowhead). Control anti-p200 serum did not react with the 290-kDa but with a 200-kDa protein (red arrowhead) (lane 4). Normal control serum (lane 5) showed reactivity with neither. (e) In immunoblotting of purified laminin-332, lane 1 shows Ponceau S stain (protein staining using amido black). Reactivity with 145-kDa and 165-kDa purified laminin-332 α3 protein (arrowheads) was indicated in the patient's serum (lane 2), but not in the normal control serum (lane 3).

previously described.[4,5] Purified laminin-332 was a generous gift from Dr S. Amano, Shiseido Life Science Research Centre, Yokohama, Japan. The patient was diagnosed as having an autoimmune subepidermal blistering disease with circulating autoantibodies to type VII collagen and laminin-332.

Treatment was initiated with 0·05% clobetasol propionate ointment 20 g daily to skin lesions, which healed within 9 days after the beginning of treatment, leaving residual pigmentation, scars and milia (Fig. 1d, e). Blisters and erosions on the oral mucosa subsided without any topical therapy (Fig. 1f). The dose of topical corticosteroids was progressively decreased, and no recurrence of skin lesions was observed. The titre of antibasement membrane zone antibodies in indirect immunofluorescence studies decreased from 1 : 320 to 1 : 40 over 2 months. We performed further immunoblot analyses on five serial serum samples obtained from the patient after her antibasement membrane zone antibodies decreased. All five samples showed similar reaction bands to both 290-kDa protein in dermal extracts and purified laminin-332 α3 protein (145, 165 kDa) (data not shown). Hence it is difficult to speculate the major target antigen in this patient from these results. No local or systemic side-effects of topical corticosteroids were noticed during the entire treatment duration.

EBA and MMP are distinct autoimmune bullous diseases that are both characterized by autoantibodies to dermoepidermal junction components.[1] Detection of autoantibodies to either type VII collagen or laminin-332 differentiates these two diseases.[1] Interestingly, besides antitype VII collagen antibodies, circulating antilaminin-332 α3 antibodies were also found in our patient's serum. According to our survey of the literature, three other previous cases of subepidermal blistering disease with circulating antibodies to both type VII collagen and antilaminin-332 have been reported (Table 1).[6–8] All of the reported cases are of adult onset, thus our report is the first juvenile case. Similar to our patient, these reported patients all presented with mucosal involvement.

Our case is unique in its course and prognosis as well as age at onset. All of the previously reported patients needed systemic corticosteroids or immunosuppressant agents for proper disease control. In the studies by Jonkman *et al.*[6] and Umemoto *et al.*,[7] the bullous lesions of the patients relapsed after systemic prednisolone was tapered. The skin lesions of the patient reported by Baican *et al.*[8] were refractory to systemic prednisolone, azathioprine and dapsone. However, our juvenile case was successfully treated with only topical steroids, and no recurrence was observed in the following 6 months. Our case suggests that the treatment outcome and prognosis of juvenile cases are better than those of adult-onset cases. Further accumulation of similar juvenile cases is needed to confirm this hypothesis. The differ-

Table 1 Comparison of four reported patients with circulating antitype VII collagen and antilaminin-332 antibodies

Patient	Age (years)/sex	Skin lesion	Mucosal involvement	Treatment	Outcome	Immunoblot analysis	Reference
1	64/F	Blisters and erythema on the hands and feet	Oral/genital	Oral prednisolone 80 mg daily	Lesions resolved without scars/milia. Mild relapse occurred when tapering to oral prednisolone 5 mg daily	Type VII collagen, laminin-332 α3	Jonkman *et al.*[6]
2	46/M	Erythematous plaque, blisters, erosions and crusts on the trunk and extensor aspects of extremities	Oral	Oral colchicine 1·5 mg daily (refractory to prednisolone, azathioprine and dapsone)	Previous lesions healed with milia and scars. Free of new blisters but erythematous plaque persisted with erosions and crusts	Type VII collagen, laminin-332 α3, γ2	Baican *et al.*[8]
3	35/F	Vesicular lesions on the face, neck and upper back	Oral/genital	Oral prednisolone 40 mg daily	Lesions resolved without scars/milia. Mild relapse occurred when tapering to oral prednisolone 25 mg daily	Type VII collagen, laminin-332 α3, β3	Umemoto *et al.*[7]
4	12/F	Blisters, erosions and erythema on the face, trunk, hands and feet	Oral	Topical clobetasol propionate ointment 20 g daily	Lesions resolved with scars and milia. No recurrence was found	Type VII collagen, laminin-332 α3	Our patient

ences between childhood-onset and adult-onset cases seem to mirror those of EBA at different ages. Compared with adult cases, childhood EBA cases respond relatively better to treatment, and usually low-dose oral prednisolone and dapsone are effective and sufficient.[1]

In conclusion, we report the first juvenile case with autoantibodies to both type VII collagen and laminin-332, successfully treated with only topical steroid therapy. Our case suggests that juvenile cases have different characteristics from those of adult-onset cases in their course, including treatment outcome and prognosis. As topical steroid therapy has several advantages over systemic corticosteroids due to less severe complications, we consider topical steroids as preferable to systemic steroids for childhood-onset autoimmune subepidermal bullous disease.

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RESEARCH ARTICLE

Abca12-mediated lipid transport and Snap29-dependent trafficking of lamellar granules are crucial for epidermal morphogenesis in a zebrafish model of ichthyosis

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SUMMARY

Zebrafish (*Danio rerio*) can serve as a model system to study heritable skin diseases. The skin is rapidly developed during the first 5-6 days of embryonic growth, accompanied by expression of skin-specific genes. Transmission electron microscopy (TEM) of wild-type zebrafish at day 5 reveals a two-cell-layer epidermis separated from the underlying collagenous stroma by a basement membrane with fully developed hemidesmosomes. Scanning electron microscopy (SEM) reveals an ordered surface contour of keratinocytes with discrete microridges. To gain insight into epidermal morphogenesis, we have employed morpholino-mediated knockdown of the *abca12* and *snap29* genes, which are crucial for secretion of lipids and intracellular trafficking of lamellar granules, respectively. Morpholinos, when placed on exon-intron junctions, were >90% effective in preventing the corresponding gene expression when injected into one- to four-cell-stage embryos. By day 3, TEM of *abca12* morphants showed accumulation of lipid-containing electron-dense lamellar granules, whereas *snap29* morphants showed the presence of apparently empty vesicles in the epidermis. Evaluation of epidermal morphogenesis by SEM revealed similar perturbations in both cases in the microridge architecture and the development of spicule-like protrusions on the surface of keratinocytes. These morphological findings are akin to epidermal changes in harlequin ichthyosis and CEDNIK syndrome, autosomal recessive keratinization disorders due to mutations in the *ABCA12* and *SNAP29* genes, respectively. The results indicate that interference of independent pathways involving lipid transport in the epidermis can result in phenotypically similar perturbations in epidermal morphogenesis, and that these fish mutants can serve as a model to study the pathomechanisms of these keratinization disorders.

INTRODUCTION

Clinical and genetic heterogeneity of ichthyosis

Ichthyosis comprises a group of both acquired and heritable keratinization disorders characterized by hyperkeratotic and scaly skin (Brown and Irvine, 2008). Although the phenotypic spectrum of ichthyosiform dermatoses is extremely broad, with either limited or extensive involvement of the skin, among the inherited forms, three clinically and genetically distinct subtypes have been identified: ichthyosis vulgaris, X-linked ichthyosis and lamellar ichthyosis (LI) (McGrath and Uitto, 2008; Brown and Irvine, 2008; Brown and McLean, 2008; Elias et al., 2004). LI in itself is a heterogeneous group of autosomal recessive disorders with large plaque-like brown scales over most of the body, associated with ectropion and alopecia.

Harlequin ichthyosis (HI) is a rare, extremely severe form of ichthyosis, most closely associated with the LI group of these disorders (Akiyama, 2006a). Neonates are born encased in a thick skin that not only restricts their movement, but also distorts their facial features, averting their lips and eyelids. Although newborns with HI frequently die within the first few days of life, a few of these affected individuals do survive, and their skin eventually resembles severe non-bullous congenital ichthyosiform erythroderma or LI.

HI is an autosomal recessive disorder caused by mutations in the ATP-binding cassette, sub-family A, member 12 (*ABCA12*) gene, which encodes a lipid transporter protein localized to lamellar granules in epidermal keratinocytes (Sakai et al., 2007). Mutations in the *ABCA12* gene result in congested lipid secretion and impaired barrier function of the stratum corneum (Kelsell et al., 2005). Thus, *ABCA12* is crucial to the development of the skin-lipid barrier in the stratum corneum.

An *Abca12*$^{-/-}$ mouse model has been vital in confirming the role of this transporter molecule in the skin abnormalities seen in HI, i.e. hyperkeratosis, impaired barrier function, abnormal lamellar bodies and the retention of lipid droplets in the epidermis (Yanagi et al., 2008; Smyth et al., 2008; Sundberg et al., 1997). The role of Abca12 in transporting lipids was confirmed by culturing keratinocytes from *Abca12*$^{-/-}$ mice and observing impaired lipid

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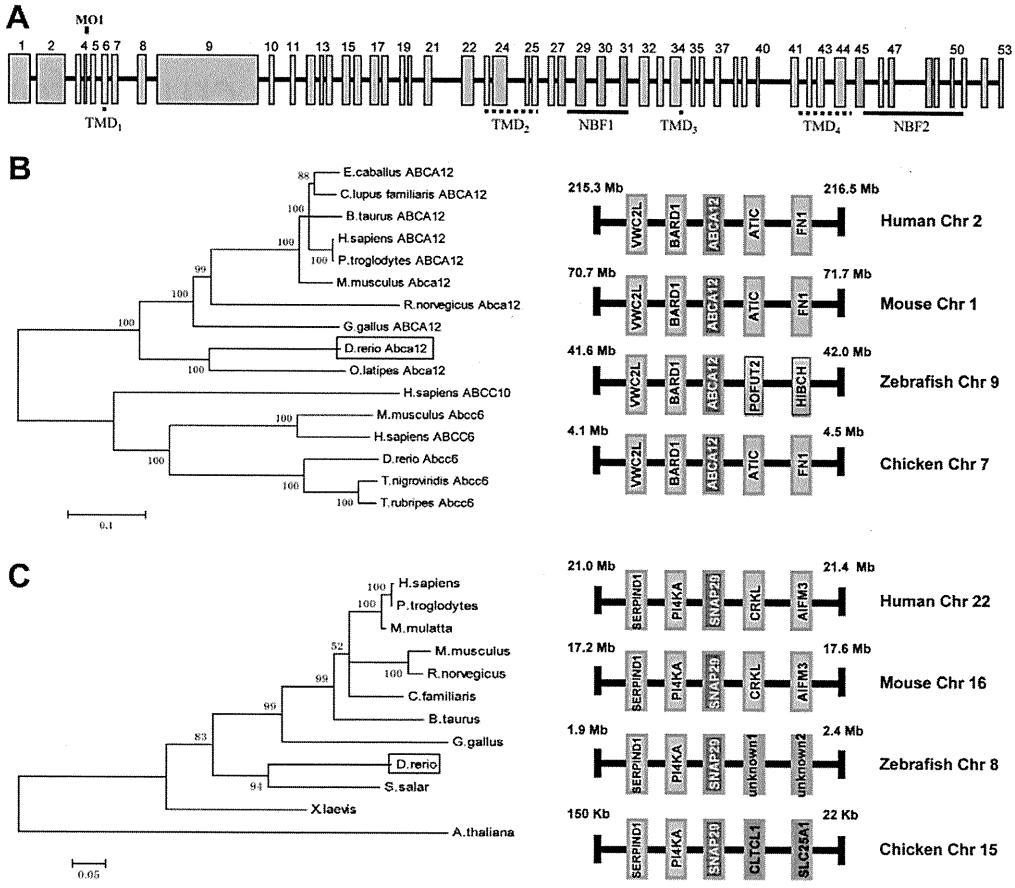

Fig. 1. Schematic representation of the zebrafish *abca12* gene, and the phylogenetic trees of the protein sequences of Abca12 and Snap29, together with syntenic analysis of the corresponding genes. (A) The *abca12* gene consists of 53 exons, which are numbered on the top, and the coding segments for transmembrane domains (TMDs) and nucleotide binding folds (NBFs; green) are underlined. Note the location corresponding to the morpholino (MO1) at the exon-4–intron-4 junction. (B) The phylogenetic relationship between zebrafish Abca12 and the other members of the ABC family of transporters estimated by the neighbor-joining method (left panel). The syntenic analysis of the *abca12* and flanking genes in human, mouse, zebrafish and chicken chromosomes is shown on right. (C) Cladogram and syntenic analysis of *snap29*. The unknown genes 1 and 2 in zebrafish chromosome 8 have been designated as si:dkey-178e17.1 and si:dkeyp-117b11.1, respectively.

efflux leading to intracellular accumulation of lipids, specifically ceramides (Akiyama et al., 2005). However, the drawback of the mouse model is the long gestation period and small number of offspring per litter.

In addition to nonsyndromic variants, ichthyosis can be associated with clinical manifestations in a number of organ systems besides the skin. An example of syndromic ichthyoses is the CEDNIK syndrome, a rare autosomal recessive disorder with cerebral dysgenesis, neuropathy, ichthyosis and keratoderma. This syndrome has been shown to be associated with mutations in the *SNAP29* gene, which encodes soluble n-ethylmaleimide sensitive factor (NSF) attachment protein (SNAP)29, a member of the SNAP receptor (SNARE) family of proteins (Sprecher et al., 2005; Fuchs-Telem et al., 2011). SNARE proteins are required for vesicle trafficking and they mediate the fusion between the vesicles and their target membranes. *SNAP29* deficiency has been suggested to result in impaired maturation and secretion of lamellar granules, particularly interfering with the transport of lipids to stratum corneum; however, no animal model for the CEDNIK syndrome exists.

In an attempt to create an alternative, and perhaps more expedient, model system to study ichthyosis, we have performed work on zebrafish (*Danio rerio*), which has nearly the same complement of genes as mammals. Some of the benefits to working with zebrafish include their rapid development and the ease with which one can manipulate their gene expression by morpholino-based antisense oligonucleotides (Kari et al., 2007; Li et al., 2011). Zebrafish develop rapidly, with all major organs, including the skin, having developed by 5-6 days post-fertilization (dpf). They also produce a large number of embryos per laying, approximately 50-100 per female. In this study, we performed experiments to show that *abca12* and *snap29* gene knockdown in zebrafish causes epidermal changes that are similar, attesting to the concept that diverse pathogenetic pathways, as a result of mutations in different genes, can result in phenotypes in the spectrum of ichthyotic diseases. Thus, zebrafish provide a novel and expedient model system to study this group of devastating, currently intractable, diseases.

RESULTS

Identification of an *ABCA12*-related gene in the zebrafish genome

Search of the online gene database (NCBI) identified one human *ABCA12*-related sequence, *abca12*, which mapped to zebrafish chromosome 9. This zebrafish *abca12* gene had an open reading frame, and all splice sites appeared intact, which allowed deduction of the intron-exon organization. The *abca12* gene consists of 53 exons, with sizes ranging from 55 to 2415 bp (Fig. 1A). The predicted primary sequence of the corresponding protein consists of 3634 amino acids, whereas the corresponding human primary sequence comprises 2595 amino acids. The overall conservation at the protein level was 49.3% and, consequently, the zebrafish *abca12* gene can be considered to be the human *ABCA12* homolog.

Alignment of human and zebrafish protein sequences revealed that zebrafish Abca12 has an extended 486 amino acid N-terminal sequence, as well as a number of insertions in the N-terminal half of the protein. However, alignment of zebrafish and human sequences identified conservation of domains that are characteristic of the ABC transporter proteins. Specifically, the zebrafish sequence, similar to the human sequence, was predicted to consist of four transmembrane domains (TMD1-4) and to contain two nucleotide binding fold domains (NBF1 and NBF2) (Tusnády et al., 2006) (Fig. 1A). The NBFs displayed characteristic sequences for Walker A and B motifs, as well as a highly conserved ABC signature sequence. Comparison of the deduced amino acid sequence within the NBF1 domain of zebrafish Abca12 showed 74% identity to the corresponding NBF1 domain in the human protein, whereas the NBF2 domain had 68% identity to human NBF2.

Evolutionary conservation of zebrafish *abca12*

Differences between the zebrafish *abca12* gene and homologous genes in other species were examined by phylogenetic analysis of the corresponding protein sequence by cladistic measurement (Fig. 1B). The cladogram suggested that the zebrafish gene is distant from most of the other *ABCA12*-related genes in a number of species, and, therefore, presumably diverged early. However, inclusion of other members of the ABC transporter family, such as ABCC10 and ABCC6, in different species, serving as an outgroup, indicated that the zebrafish Abca12 protein sequence is closer to human ABCA12 than it is to the sequences in the outgroup. To confirm that the zebrafish *abca12* is the correct ortholog of human *ABCA12*, syntenic analysis of *Abca12* in different species was performed (Fig. 1B). These analyses revealed that *ABCA12* and its flanking genes, *VWC2L* and *BARD1*, were located on the same chromosome in the same gene order in human, mouse, zebrafish and chicken genomes (Fig. 1B).

Expression of the zebrafish *abca12* gene during early embryonic development

The temporal expression profile of *abca12* was examined in embryos collected during the first 8 days of development, and the corresponding mRNA levels were determined by reverse transcriptase (RT)-PCR. An undetectable level of expression was noted in embryos at the time of fertilization [0 hours post-fertilization (hpf)], but detectable levels of mRNA transcripts were noted at 6 hpf, with a significant further increase by 1 dpf. During the subsequent days (2-8 dpf), the expression levels remained relatively constant in comparison with the control gene, *β-actin* (Fig. 2A).

Whole-mount in situ hybridization of *abca12* in zebrafish

To determine the spatial expression of *abca12* during different stages of zebrafish development, whole-mount in situ hybridization was performed using probes specific for the *abca12* gene (Fig. 2B). An antisense probe for *abca12* gave specific expression patterns. During the gastrula period, expression of *abca12* was observed in cells of the enveloping layer (EVL; Fig. 2B). Expression of the *abca12* gene in this tissue, which is named periderm after the end of gastrulation, is observed until the end of embryonic development. After 24 hpf, expression of *abca12* was also observed, although at lower levels, in the olfactory vesicle as well as in mucus-secreting cells (Fig. 2B). At the end of embryonic development, expression was observed mainly in olfactory vesicle, pharynx and mucus-secreting cells. A sense probe was used as a control and did not give a specific expression pattern.

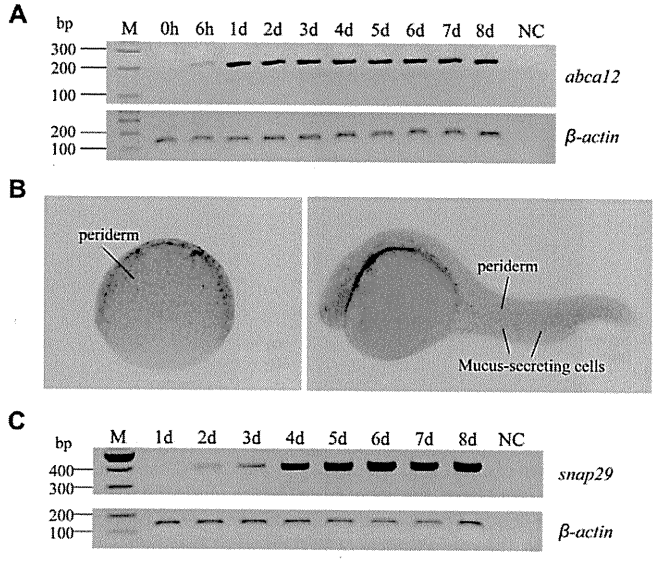

Fig. 2. *abca12* and *snap29* gene expression in normal zebrafish. (A-C) Zebrafish embryos were collected at 0 and 6 hpf and 1-8 dpf, and total RNA was isolated and cDNA prepared. The *abca12* (A) and *snap29* (C) mRNA expression levels were measured by RT-PCR and standardized against the mRNA expression level of the *β-actin* gene. (B) Whole-mount in situ hybridization of embryos at different stages of early development for *abca12* expression; gastrula period (left panel), 24 hpf (right panel).

Morpholino knockdown of *abca12* expression results in an altered skin phenotype

Morpholino antisense oligonucleotide (MO1) directed against a splice donor site in *abca12* was injected into one- to four-cell-stage embryos, and amplification of total RNA was performed by primers corresponding to exons 4 and 5. Using these primers, PCR amplification of *abca12* cDNA resulted in a 189 bp product, whereas amplification of genomic DNA generated a 356 bp product (Fig. 3A). RT-PCR of total RNA extracted from zebrafish 3 days after injection with MO1 revealed that essentially all (>90%) of the pre-mRNA remained unprocessed, attesting to the efficiency of the morpholino knockdown (Fig. 3A). Injection of control morpholinos, either a global standard control MO (scMO) or 5-bp mismatched control (cMO), had no effect on pre-mRNA processing (Fig. 3A).

The effect of the injection of morpholinos into one- to four-cell embryos was first examined by determining the survival of the embryos. Of the 180 embryos injected with *abca12* MO1, 76% survived at 3 dpf, a number that did not statistically differ from the survival of embryos injected with standard control morpholino (81%) (Table 1). At 5 dpf, the survival of embryos injected with MO1 was only 6%, a statistically significant reduction from the survival noted with scMO and uninjected controls (81% and 87%, respectively; $P<0.0001$) (Table 1).

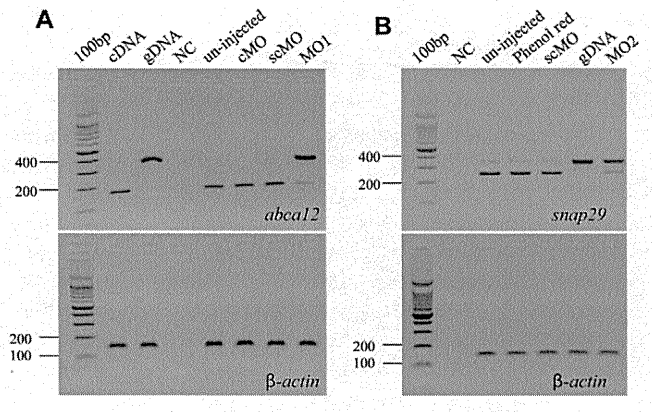

Fig. 3. Knockdown of *abca12* and *snap29* expression by morpholinos. (A) Knockdown of *abca12*. (B) Knockdown of *snap29*. MO1 and MO2 morpholinos (right lanes, the upper panel), which target the splice donor site at the exon-4–intron-4 border of the corresponding genes, prevents pre-mRNA splicing. The consequences of MO1 on *abca12* pre-mRNA splicing and MO2 on *snap29* mRNA splicing were determined by RT-PCR. The results showed the retention of intron 4 in the majority of mRNA transcripts (>90%) as compared with the normally transcribed control. The mRNA levels were normalized by the level of *β-actin* mRNA (lower panels). cDNA and gDNA represent amplification of the corresponding complementary DNA and genomic DNA, respectively. Injections with the 5-bp mismatched control morpholino for *abca12* (cMO) or global standard control morpholino (scMO) did not alter pre-mRNA processing, similar to the uninjected controls or those injected with phenol red.

Examination of the morphology of zebrafish larvae injected with MO1 (n=180) revealed profound changes during development. Although no differences were noted between the morphant and control larvae at 1 dpf, by 3 dpf the morphants had developed noticeable changes in the distribution of pigment along their trunk and tail, in addition to pericardial edema (Fig. 4A). Upon careful examination at 3 dpf, 92% of larvae displayed yolk sac enlargement and severe disruption of their chromatophore distribution, with 75% exhibiting concomitant pericardial edema (Table 1).

Altered epidermal morphology in the morphant larvae

To examine the consequences of the morpholino-mediated knockdown of *abca12* expression in the skin of zebrafish, we first used scanning electron microscopy (SEM) to examine the surface contour and cellular morphology of the epidermis. In 3-dpf controls (n=21), well-demarcated keratinocytes with distinct borders and characteristic microridges were observed (Fig. 5). Examination of the skin surface of the morphant larvae (n=4) revealed perturbations in the architecture of the microridges, with spicules protruding from the center of each keratinocyte. Thus, the development of the top layer of skin during the first 3 days of zebrafish development was perturbed in the absence of Abca12 activity.

Alterations in the epidermis at 3 dpf were further examined by transmission electron microscopy (TEM) both in control and morphant larvae (n=4 in each group). At this developmental stage, normal epidermis consists of two unicellular layers, the superficial layer and the basal layer. The contour of the outer surface of the superficial layer is studded with spicules that correspond to the microridges noted previously on SEM (Fig. 6A). The epidermis rests on a basement membrane, which separates the epidermis from the underlying developing dermis.

The epidermis of the morphant larvae similarly consisted of two cell layers resting on a basement membrane (Fig. 6B). However, in contrast to the control larvae, both layers of the morphant epidermis contained an abundance of electron-dense granules, approximately 440 nm in average diameter. Closer examination of these aggregates at higher magnification suggested the presence of lipid-like vesicles within the larger electron-dense granules (Fig. 6C,D). It should be noted that, although somewhat similar aggregates of electron-dense material were noted in the epidermis of the control specimens, they were localized only to the area of the superficial layer just below the microridges.

Co-injection of human *ABCA12* mRNA rescues the morpholino-mediated phenotype

To test the specificity of the phenotypic changes associated with MO1 injection, a rescue experiment with co-injection of in vitro transcribed human *ABCA12* mRNA was performed. The injection of MO1 alone caused characteristic phenotypic changes, whereas co-injection of human mRNA together with MO1 partially rescued the phenotype (Fig. 4A,B). Specifically, at 5 dpf, the survival of the co-injected larvae was 62%, which is statistically different from the 6% in those injected with MO1 alone (P<0.0001) (Table 1). Also, 27% of co-injected larvae (n=184) had a phenotype that was indistinguishable from the controls. In the remaining 73% of co-injected larvae, the degree of yolk sac enlargement and chromatophore disorganization was noticeably less than in the larvae injected with MO1 alone. Of this 73%, 70% also manifested

Table 1. Survival of and development of phenotype in zebrafish injected with *abca12* morpholino

Experimental group	No. of fish	3 dpf			5 dpf		
		Survival (%)	Skin phenotype (%)	Edema (%)	Survival (%)	Skin phenotype (%)	Edema (%)
Uninjected control	152	87	0	0	87	0	0
scMO	177	81	0	0	81	0	0
abca12 MO1	180	76	92[a]	75[a]	6[a]	0	0
abca12 MO1+ hABCA12 mRNA	184	87[b]	81[b] (milder)	74 (milder)	62[c]	73[c] (milder)	70[c] (milder)

This is a representative experiment in which all groups were followed in parallel. Similar results were obtained in >ten additional experiments with the same design. Skin phenotype refers to epidermal perturbations, disruption of the chromatophore distribution and yolk sac enlargement. Edema is pericardial edema.
scMO, standard control morpholino with no biological function and no target sequence in zebrafish genome (Robu et al., 2007).
[a]Statistical significance between the *abca12* MO1 group and scMO group (Fisher's exact test: P<0.0001).
[b,c]Statistical significance between the *abca12* MO1 group and the *abca12* MO1+hABCA12 mRNA group (Fisher's exact test: [b]P<0.05; [c]P<0.0001).