other hand vacuolar structure can be seen in basement membrane on normal skin plantation stimulated with PEDF. We previously reported cytotoxic effect of PEDF. PEDF directly induce tumor cell apoptosis via Fas–FasL interaction [10]. Therefore PEDF affects epidermis resulting vacuolization of dermal–epidermal junction. Normal skin underwent more hyperproliferative response after transplantation compared to psoriatic skin as previous reports [18,19]. The mechanism underlying the hyperplastic response in normal skin after transplantation is unknown at present. Some degree of epidermal hyperplasia is often seen as part of the wound-healing response in the skin. Perhaps one or more growth factors present in the healing murine skin is responsible for triggering proliferation of epidermal keratinocytes in the transplanted human tissue [19].

To evaluate the effects of PEDF on angiogenesis and epidermal proliferation in this *in vivo* model, we enumerated the CD31+ capillary endothelial cells in the superficial dermis and the Ki-67+ proliferating keratinocytes by immunofluorescence. The number of CD31 positive capillary endothelial cells in the papillary dermis was significantly reduced after PEDF treatment (Fig. 3) in both psoriasis and normal skin grafts. The frequency of proliferating Ki-67-positive cells in the basal cell layer also was significantly reduced after PEDF treatment (Fig. 4).

Since inflammatory cell infiltration is considered important in the pathogenesis of psoriasis (refs), it is possible that the reduction of epidermal thickening or acanthosis is due to the inhibition of inflammatory cell infiltration. However, the number of T cells (CD3+), neutrophils (Gr-1+) and monocytes (Cd11b+) in the superficial dermis were not statistically different between the treated and un-treated group (*data not shown*).

3.5. Improvement of clinical and histologic features of psoriasis by topical application of PEDF peptide

Although intact skin is impermeable to many bio-molecules such as proteins, compounds less than 1 kDa in mass may pass transcutaneously. Moreover, inflammatory changes in the skin, as occur in psoriasis, frequently lead to reduced barrier function due to aberrant epidermal cell differentiation and alterations in ceramide content [20,21]. We thus considered that psoriasis skin lesions may be amenable to topical application of low molecular weight, PEDF-derived peptides [22].

To identify potential PEDF peptides that might exhibit anti-psoriatic properties, we screened peptides derived from the proteolytic fragmentation of PEDF for their anti-proliferative action on MG63 cells, which previously have been shown to be sensitive to the growth inhibitory action of PEDF [14]. As shown in Fig. 5A and B, PEDF fragment F3, but not F1 or F2, significantly inhibited the growth of MG63 cells at concentrations comparable to intact PEDF protein (Fig. 5B). The PEDF-derived peptide, P5 also exhibited an anti-proliferative properties on MG63 cells (Fig. 5C). P5–1 (381–387, MW: 841), P5–2 (388–393, MW: 770) or P5–3 (388–393, MW: 770) peptides had similar growth-inhibitory activity when compared with the P5 peptide (Fig. 5D).

To investigate the inhibitory activity of PEDF peptides on angiogenesis, we first assessed endothelial tube formation *in vitro*. P5–2 and P5–3 but not P5–1 showed significant inhibitory effects on tube formation (Fig. 6A). The active PEDF peptides inhibited endothelial tube formation concentrations of 100 and 500 nM. To investigate the cooperative effects of VEGF and PEDF, we added PEDF peptide and VEGF simultaneously in endothelial tube formation assay. P5–2 and P5–3 but not P5–1 normalized VEGF-induced tube formation (Fig. 6A). All of these peptides also inhibited the proliferation of endothelial cells (Fig. 6B), however this suppressive effect was in concentrations that were in the μM range. We and others have previously reported that PEDF inhibits VEGF-stimulated endothelial cell proliferation, whereas only PEDF has only a minimal effect on endothelial cell proliferation in the absence of VEGF stimulation [9,14]. Therefore PEDF peptides might be required to be present in high concentration to inhibit endothelial cell proliferation. A peptide control with the same amino acid content and a randomized sequence did not show any effect. PEDF has been reported to suppress VEGF-stimulated endothelial proliferation via cell cycle inhibition [23]. We therefore

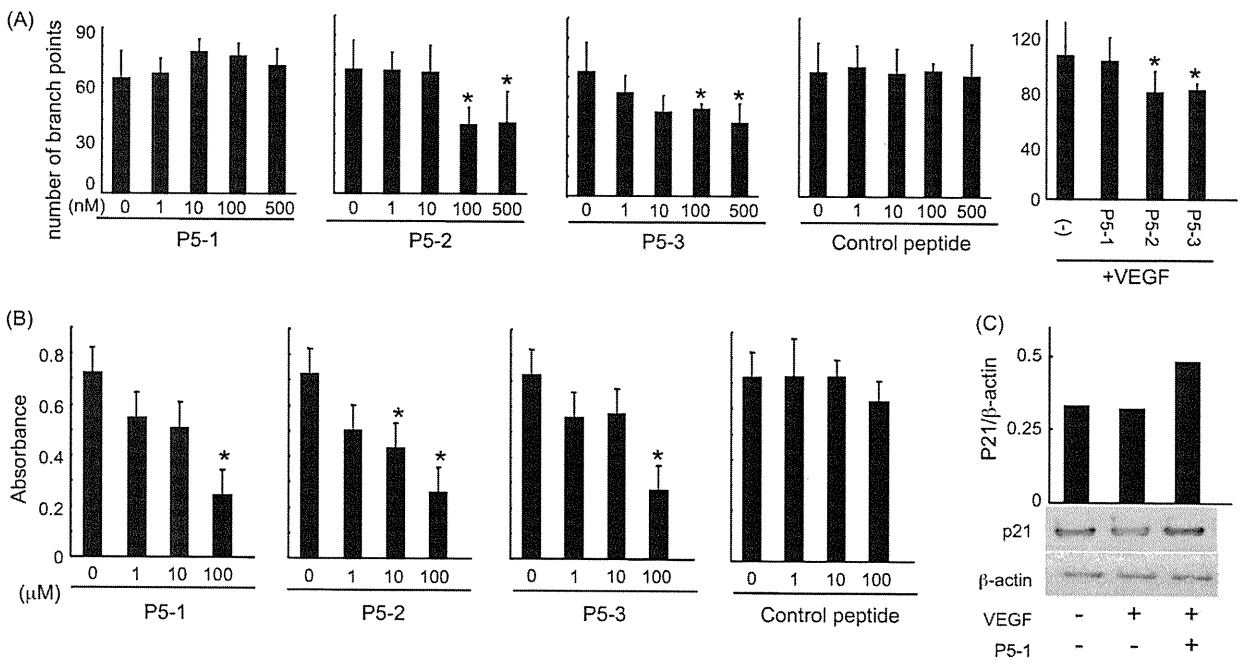

Fig. 6. (A) The endothelial tube formation assay. PEDF 1–3 showed a level of anti-angiogenic activity comparable to recombinant PEDF (*$p < 0.05$). (B) Diagram of the PEDF peptides studied for their effect on the growth of HUVEC. HUVEC were treated with or without 100 nM PEDF, fragments or peptides and then BrdU incorporation into the cells was measured. The percentage of BrdU incorporation is indicated on the ordinate and related to the value of the control. *$p < 0.01$ compared to the value with no addition.

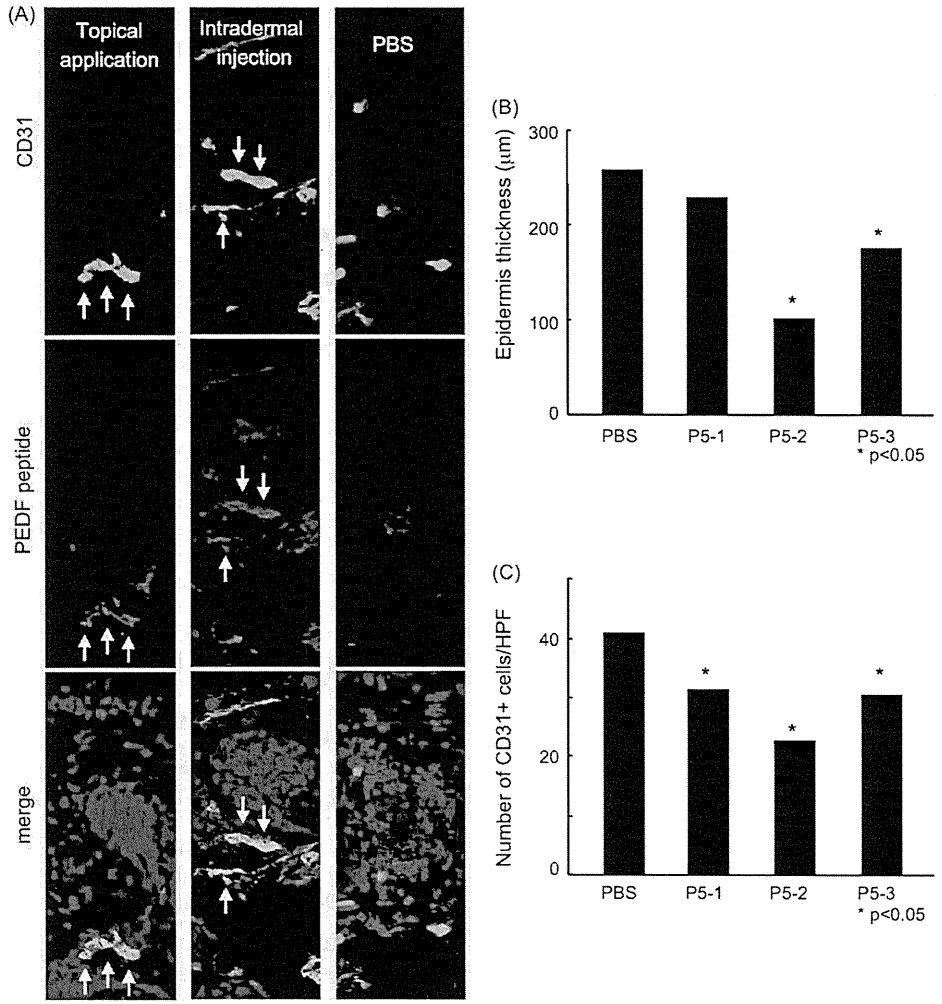

Fig. 7. Topically applied PEDF peptides penetrate into skin and reduce epidermal thickness and angiogenesis. (A) Biotin-labeled PEDF peptide (P5–1) was applied to the skin and its localization studied 2 h later using rhodamine–avidin staining as described in Section 2. Co-localization with PEDF peptide and endothelial cells (CD31+) is indicated by the arrows. The P5–2 and P5–3 also penetrated into the skin (*data not shown*). (B) Local application of PEDF peptide reduced thickness of grafted epidermis in xenotransplanted SCID mice. (*$p < 0.05$). (C) CD31-positive cells (capillary endothelial cells) were enumerated by immunofluorescence. The PEDF-treated group showed significantly reduced number of CD31+ cells (*$p < 0.05$).

analyzed the expression of the p21, cyclin-dependent kinase inhibitor [16]. PEDF peptide increased the expression of p21, suggesting that its inhibitory effect is mediated at least in part via p21 induction (Fig. 6C).

We next examined whether PEDF-derived peptides penetrate into the skin. Biotin-labeled PEDF peptide was applied to murine skin and its localization analyzed 2 h later using rhodamine–avidin staining. The PEDF peptide was detected in the dermis and co-localized with endothelial cells (Fig. 7A); the staining pattern was similar to that observed after the intradermal injection of the peptide. In addition, endothelial cells express PEDF receptor [24]. Therefore PEDF peptides might colocalize with endothelial cells.

Finally, we assessed the therapeutic potential of PEDF peptides after their topical application to human psoriatic skin grafted onto SCID mouse. PEDF peptides were dissolved in PBS (1 mM) and 70 μl of peptide applied to the grafted site each day for 10 days. Mice in the control group received the same volume of PBS. After two weeks of treatment, the epidermal thickness of the grafted area was significantly reduced in the P5–2 and P5–3-treated group (Fig. 7B). The number of CD31-positive capillary endothelial cells in the papillary dermis was significantly reduced in the all PEDF peptide-treated groups (Fig. 7C).

4. Discussion

In this study, we demonstrate that PEDF is produced both within the human epidermis and dermis, and that significantly higher levels are present in the psoriatic epidermis. Cultured keratinocytes and fibroblasts constitutively secrete PEDF; however, incubation with the model inflammatory stimulus LPS increases PEDF production only by keratinocytes. In addition, the local administration of PEDF reduces both acanthosis in psoriasis lesions and the hyperplasia of normal skin in a xenograft transplant model. This effect appeared to due to the inhibition of dermal capillary angiogenesis and epidermal proliferation. Finally, we identified a low-molecular weight, anti-angiogenetic PEDF peptide showed that its topical application reduced the proliferative and inflammatory features of psoriatic lesions.

Inappropriate angiogenesis has been proposed to contribute to the pathogenesis of psoriasis [4,5], although the precise cellular and molecular basis for this response remains unclear. Angiogenic processes are regulated by a delicate balance of pro-angiogenic and anti-angiogenic factors [25]. Under conditions such as tumor formation, wound healing, and possibly psoriasis, the positive regulators of angiogenesis predominate and vascular endothelial

cells become activated. In psoriasis, angiogenic factors such as VEGF are up-regulated and anti-angiogenic factors such as PEDF are simultaneously up-regulated to maintain a homeostatic balance. However, the overexpression of angiogenic factors may overcome and surmount this balance in psoriasis, resulting an acceleration of angiogenesis [4,5].

Interestingly, the level of PEDF protein in uninvolved lesions was observed to be much higher than that in psoriatic lesions. These data suggest that the angiogenic balance is maintained by an up-regulated expression of PEDF in uninvolved lesions, whereas insufficient up-regulation of PEDF may contribute to the psoriatic phenotype. The regulation of PEDF may be an innate feature of psoriasis rather than a consequence of inflammation.

We found no significant differences in the serum levels of PEDF between psoriatic patients and normal controls. A previous report has suggested that circulating PEDF has the capacity to inhibit angiogenesis at the systemic level [23]. Our investigations showed that PEDF is up-regulated in the psoriatic epidermis, which likely affects the local microenvironment; however, local PEDF production by keratinocytes was not sufficient to lead to an increase in the serum concentration of this mediator. We hypothesize that VEGF levels in psoriatic skin may overcome the inhibitory action of PEDF on angiogenesis, resulting in a pro-angiogenic switch in the microenvironment around psoriasis lesions. In cultured keratinocytes, PEDF is up-regulated by LPS stimulation, suggesting that PEDF production by keratinocytes might occur in response to inflammatory activation.

We showed herein that PEDF was detected in both the epidermis and dermis, which contrasts with a previous paper that reported that PEDF was only detected in dermal layers, and not in normal epidermis [17]. In our immunohistochemical studies, PEDF was highly expressed in psoriatic keratinocytes, although the normal, steady-state epidermis showed only weak staining. We confirmed that PEDF is secreted by cultured keratinocytes and induced LPS stimulation, suggesting that PEDF production by keratinocytes might depend on inflammatory activation. By contrast, cultured fibroblasts constitutively secrete PEDF regardless of LPS activation. Accordingly, we hypothesize that fibroblasts are major contributors to PEDF production under normal conditions, and that keratinocytes contribute to PEDF production in certain inflammatory conditions.

A receptor for PEDF has been recently reported [24], however we could not detect the expression of this protein in keratinocytes using RT-PCR (*data not shown*). Because PEDF has not only anti-angiogenic but also anti-proliferative effects on many cell types, we speculate that PEDF may interact with multiple receptors in addition to the one previously reported. It has been reported that the anti-angiogenic effects of PEDF reside in the N-terminus within residues 24–57, which is distinct from the sequence we report herein [26]. Residues 24–57 are included in the F1 fragment of PEDF in the present study, however F1 did not show the inhibitory activity of PEDF (Fig. 5). We nevertheless were successful in identifying low-molecular weight peptides (MW < 850 Da) that penetrate the skin and show a significant anti-angiogenic effect in vitro and in vivo.

We demonstrated that acanthosis of human psoriatic skin was significantly reduced by local administration of PEDF in a mouse xenograft model, and that this effect appeared due to reduced angiogenesis and basal cell proliferation. These results suggest that a PEDF-based, topical therapeutic might be an effective therapy for psoriasis. We identified PEDF peptides with molecular weights <850 Da that penetrate the skin and showed these peptides to have anti-angiogenic activity and to reduce psoriatic epidermal hyperplasia. In addition, drug delivery system is one of the most critical issue about clinical application. Small peptide is rapidly degraded in vivo, so we have to develop a slow-release system such as biodegradable gelatin microspheres.

In conclusion, these studies provide the first report of a role for PEDF in the pathogenesis of psoriasis. Furthermore, low molecular weight peptides derived from PEDF show anti-angiogenic activity in psoriatic skin in an *in vivo* model of disease, and may offer a novel therapeutic approach for the treatment of psoriasis.

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ABCA12 Mutations and Autosomal Recessive Congenital Ichthyosis: A Review of Genotype/Phenotype Correlations and of Pathogenetic Concepts

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ABSTRACT: Mutations in *ABCA12* have been described in autosomal recessive congenital ichthyoses (ARCI) including harlequin ichthyosis (HI), congenital ichthyosiform erythroderma (CIE), and lamellar ichthyosis (LI). HI shows the most severe phenotype. CIE and LI are clinically characterized by fine, whitish scales on a background of erythematous skin, and large, thick, dark scales over the entire body without serious background erythroderma, respectively. To date, a total of 56 *ABCA12* mutations have been reported in 66 ARCI families including 48 HI, 10 LI, and 8 CIE families of African, European, Pakistani/Indian, and Japanese origin (online database: http://www.derm-hokudai.jp/ABCA12/). A total of 62.5% of reported *ABCA12* mutations are expected to lead to truncated proteins. Most mutations in HI are truncation mutations and homozygous or compound heterozygous truncation mutations always results in HI phenotype. In CIE families, at least one mutation on each allele is typically a missense mutation. Combinations of missense mutations in the first ATP-binding cassette of ABCA12 underlie the LI phenotype. ABCA12 is a keratinocyte lipid transporter associated with lipid transport in lamellar granules, and loss of ABCA12 function leads to a defective lipid barrier in the stratum corneum, resulting in an ichthyotic phenotype. Recent work using mouse models confirmed ABCA12 roles in skin barrier formation.
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KEY WORDS: ABCA12; congenital ichthyosiform erythroderma; harlequin ichthyosis; lamellar ichthyosis

Introduction

Severe autosomal recessive congenital ichthyoses (ARCI) can be devastating to patients' quality of life in those seriously affected, even though other organs are uninvolved. ARCI comprises three major subtypes, harlequin ichthyosis (HI; MIM# 242500), congenital ichthyosiform erythroderma (CIE; MIM# 242100), and lamellar ichthyosis (LI; MIM#s 242300, 604777, 601277, 606545) [Akiyama and Shimizu, 2008]. HI is the most devastating congenital ichthyosis, and affected newborns show large, thick, plate-like scales over the whole body with severe ectropion, eclabium, and flattened ears [Akiyama, 2006a]. Patients with CIE demonstrate fine, whitish scales on a background of erythematous skin over the whole body. Conversely, LI is clinically characterized by large, thick, dark scales over the entire body surface without a serious background erythroderma [Akiyama et al., 2003].

Because transglutaminase 1 gene (*TGM1*; MIM# 190195) mutations were identified as the cause in LI in 1995 [Huber et al., 1995; Russell et al., 1995], significant progress has recently been made in the understanding of the molecular basis of the human epidermal keratinization processes, and mutations in several other genes have also been identified in ARCI. In HI cases, only ABCA12 mutations have been reported as underlying genetic defects [Akiyama and Shimizu, 2008]. In contrast, CIE and LI are both heterogeneous genetic disorders and several causative or underlying molecules including ABCA12 have been identified [Jobard et al., 2002; Lefèvre et al., 2003, 2004; Lefèvre, 2006]. Mutations in six genes have been described in non-HI ARCI to date, including *TGM1* [Huber et al., 1995; Russell et al., 1995], *ABCA12* [Lefèvre et al., 2003; Natsuga et al., 2007], *NIPAL4* (also known as *ICHTHYIN*) [Lefèvre et al., 2004], *CYP4F22* [Lefèvre, 2006], *ALOX12B* and *ALOXE3* [Jobard et al., 2002]. Among them, *TGM1* is thought to be the most prevalent causative gene [Fischer, 2009; Herman et al., 2009]. *TGM1* encodes transglutaminase 1, which is expressed in the upper epidermis and catalyzes crosslinking of cornified cell envelope precursor proteins to form a cornified cell envelope in the stratum corneum [Herman et al., 2009]. *NIPAL4* (or *ICHTHYIN*) encodes a protein of unknown function. ALOX12B and ALOXE3 encode for arachidonate 12(R)-lipoxygenase and arachidonate lipoxygenase-3, respectively. The protein product of CYP4F22, a cytochrome P450 protein, and the two lipoxygenases arachidonate 12(R)-lipoxygenase and arachidonate lipoxygenase-3 are part of the lipid metabolism pathway involved in the formation of ω-hydroxyceramides from arachidonic acid [Brash et al., 2007]. ABCA12 (MIM# 607800) missense mutations leading to defects in the ATP-binding cassette were reported in LI cases (type 2 LI [MIM# 601277]) [Lefèvre et al., 2003] and ABCA12 truncation mutations were reported underlying HI patients [Akiyama et al., 2005; Kelsell et al., 2005]. Recently, we reported that *ABCA12* missense mutations are a major cause of CIE cases in the Japanese population [Akiyama et al., 2008; Natsuga et al., 2007; Sakai et al., 2009]. Thus, *ABCA12* mutations lead to all three ARCI clinical phenotypes including HI, LI and CIE and *ABCA12* mutations are highlighted as one of the major causes of ARCI.

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

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ABCA12 is a member of the large superfamily of the ATP-binding cassette (ABC) transporters [Annilo et al., 2002], which bind and hydrolyze ATP to transport various molecules across a limiting membrane or into a vesicle [Borst and Elferink, 2002]. The ABCA subfamily members are thought to be lipid transporters [Peelman et al., 2003]. ABCA12 was recognized as a key molecule in keratinocyte lipid transport [Akiyama et al., 2005; Sakai et al., 2007]. ABCA12 is a keratinocyte transmembrane lipid transporter protein associated with lipid transport in lamellar granules to the apical surface of granular layer keratinocytes [Akiyama et al., 2005]. In this article, the importance of *ABCA12* mutations as a cause for ARCI is reviewed and a genotype/phenotype correlation of ARCI with *ABCA12* mutations is discussed.

ABCA12 Mutations

A review of the literature was performed to identify all of the known *ABCA12* mutations. To date, 56 *ABCA12* mutations have been described (online database: http://www.derm-hokudai.jp/ABCA12/) in 66 unrelated families including 48 HI, 10 LI and 8 CIE families (Supp. Table S1 and Fig. 1). Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (GenBank NM_173076.2), according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1. Mutations have been reported among ARCI patients with African, European, Pakistani/Indian, and Japanese backgrounds, from almost all over the world. Of the 56 mutations, 36% (20) are nonsense, 25% (14) are missense, 20% (11) comprise small deletions, 11% (6) are splice site, 5% (3) are large deletions, and 4% (2) are insertion mutations. At least, 62.5% (35) of the total reported mutations are predicted to result in truncated proteins. There is no apparent mutation hot spot in *ABCA12*, although mutations underlying LI phenotype are clustered in the region of the first ATP-binding cassette [Lefèvre et al., 2003].

The most common reported mutation in *ABCA12* is c.7322delC (p.Val2442SerfsTer28) in exon 49, which has been reported in seven HI families with Pakistani background [Kelsell et al., 2005; Thomas et al., 2006, 2008]. This mutation has been identified only in the Pakistani population. Thomas et al. [2008] reported that 80% of HI patients and parents (10 screened) originated from the Pakistani/Indian area had the mutation 7322delC. Microsatellite-based haplotype analysis of the genomic region harboring *ABCA12* in three patients homozygous for the mutation c.7322delC suggested that c.7322delC is a possible founder mutation in the Pakistani population [Thomas et al., 2008]. The second most common reported *ABCA12* mutation is a missense mutation p.Asn1380Ser in Walker A motif of the first ATP-binding cassette, which is essential for the transporter function of ABCA12 [Lefèvre et al., 2003]. This missense mutation p.Asn1380Ser has been identified in five LI families from Africa (three families from Morocco and two families from Algeria) [Lefèvre et al., 2003]. Haplotype analysis confirmed that p.Asn1380Ser is a founder mutation in the patients from Morocco/Algeria region [Lefèvre et al., 2003]. Out of further 10 different *ABCA12* mutations, each mutation has been identified in two unrelated families from certain geographic regions. Among these 10 mutations, 5 *ABCA12* mutations, c.2021_2022del2, c.3295−2A>G, p.Thr1387del, p.Arg1950Ter, and p.Arg2482Ter, were found in two independent patients from Japan [Akiyama et al., 2005, 2006a, 2007a; Sakai et al., 2009]. As for the other five mutations, p.Trp1294Ter, p.Gly1651Ser, p.Tyr1090Ter, c.2025delG, and p.Trp1744Ter were found in two independent families with Pakistani [Rajpar et al., 2006; Thomas et al., 2006], Algeria [Lefèvre et al., 2003], Albanian/Bosnian [Thomas et al., 2008], Anglo-Saxon [Thomas et al., 2006], and native American [Kelsell et al., 2005] origins, respectively. These data suggest the presence of founder mutations in patients in Pakistani/Indian, African, European, and Japanese origins.

Clinical Significance; Prevalence of *ABCA12* Mutations as a Causative Gene for ARCI Patients

ARCI is a basket diagnosis, and HI, CIE, and LI are the major subtypes comprising the ARCI group. Among the 48 HI families in whom *ABCA12* mutation analysis has been reported (Supp. Table S1), ABCA12 mutations have been identified in all HI families; the *ABCA12* mutation detection rate is 100% (48/48) in the HI families. Kelsell et al. [2005] reported one HI patient in whom *ABCA12* mutation was not detected by direct sequencing analysis. However, multiplex PCR and oligonucleotide array analysis subsequently revealed deletion of exon 8 in this patient [Thomas et al., 2006]. In this context, HI is thought to be genetically homogeneous for causal *ABCA12* mutations.

In contrast, CIE and LI, the other two ARCI subtypes, to date, six genes, *ABCA12* [Lefèvre et al., 2003], *TGM1* [Huber et al., 1995; Russell et al., 1995], *ALOX12B* (MIM# 603741) [Jobard et al., 2002], *ALOXE3* (MIM# 607206) [Jobard et al., 2002], *ICHTHYIN* (MIM# 609383) [Lefèvre et al., 2004] and *FLJ39501* (MIM# 611495) [Lefèvre, 2006], have been reported to cause CIE; and four out of these six, *ABCA12* [Lefèvre et al., 2003], *TGM1* [Huber et al., 1995; Russell et al., 1995], *ALOX12B* [Jobard et al., 2002], and *ICHTHYIN* [Lefèvre et al., 2004], are also known to underlie LI. Recently, Fischer [2009] reported that in her cohort of 520 patients from 520 independent families with LI and CIE, causative mutations were detected by direct sequencing analysis in 78% of the patients. Only 5% of the patients were harbored *ABCA12* mutations although only exons 28–32 of *ABCA12* were sequenced for the majority of the patients in this study [Fischer, 2009]. The results suggest that ABCA12 is rather a minor cause for ARCI probably in the European and African populations, although the exact ethnic background of the 520 families was not provided in the report. Different from the situation in Europe, from the results of our mutation search, *ABCA12* mutations were frequently found in CIE families, but not in LI families, at least in the Japanese population [Sakai et al., 2009]. Thus, there might be a difference in the prevalence of causative genes for CIE and LI between the global subpopulations.

Genotype–Phenotype Correlation in *ABCA12* Mutations

Several genotype/phenotype correlations with *ABCA12* mutations have now come to light.

In HI (Supp. Fig. S1A), 44 *ABCA12* mutations were reported to date. Among them, most mutations are truncation mutations including nonsense mutations, frameshift mutations (deletion/insertion mutations), and splice site mutations (Table 1). Other mutations reported in HI families are missense mutations, exon deletions, and single amino acid deletions.

Most truncation or deletion mutations underlying HI are thought to lead to severe loss of ABCA12 protein function affecting important nucleotide-binding fold domains and/or transmembrane domains. Thus far, in HI patients, at least one mutation on each allele must be a truncation or deletion mutation

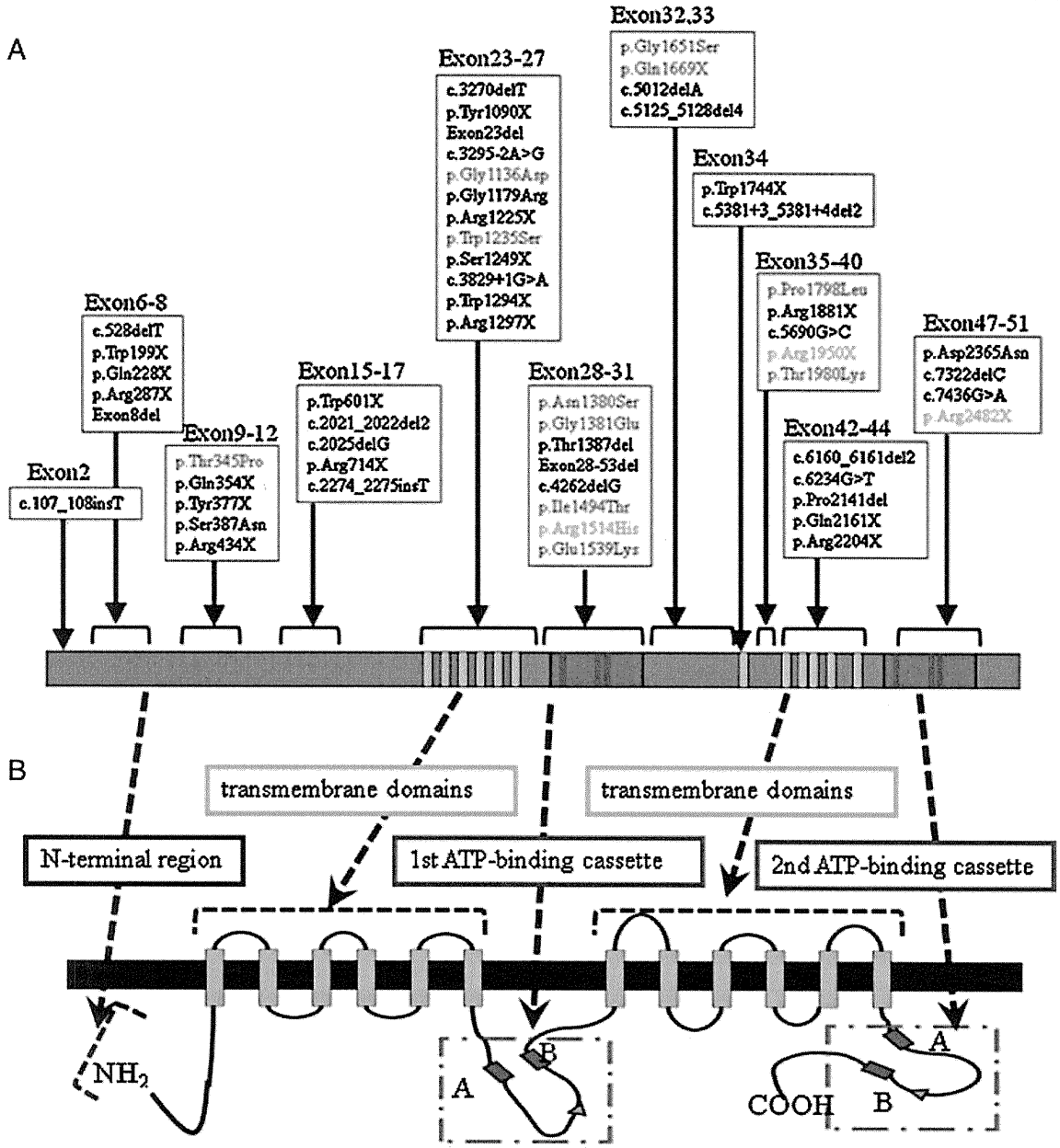

Figure 1. *ABCA12* mutations associated with autosomal recessive congenital ichthyosis. **A:** Reported *ABCA12* mutations and their localization within the *ABCA12* cDNA sequence. Mutations in black, red, and blue characters underlie HI, CIE, and LI, respectively. Mutations in green letters lead to two distinct phenotypes, p.Arg1950Ter and p.Arg2482Ter both result in CIE and HI phenotypes; p.Arg1514His underlies both CIE and LI phenotypes. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence (GenBank NM_173076.2), according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1. **B:** ABCA12 protein structure and domains. Analysis of the ABCA12 predicted protein disclosed features that are typical of ABCA transporters, and the position of the conserved ATP-binding cassettes as well as that of the two transmembrane domains, each composed of six well-defined hydrophobic helices [Annilo et al., 2002]. See Supp. Table S1 for a complete list of mutations with both DNA and protein names.

within a conserved region to cause serious loss of ABCA12 function [Akiyama et al., 2005, 2006a,b, 2007a, b; Castiglia et al., 2009; Kelsell et al., 2005; Rajpar et al., 2006; Thomas et al., 2006, 2008]. Complete loss of ABCA12 function due to homozygous or compound heterozygous truncation mutations always results in the HI patient phenotype (Table 1).

In contrast, most mutations underlying LI and CIE are missense mutations and are expected to affect ABCA12 function more modestly.

In LI, five *ABCA12* mutations were reported in nine families and all the patients were homozygotes or compound heterozygotes for the mutations [Lefèvre et al., 2003]. None of the LI mutations was demonstrated to cause HI phenotype, although one mutation p.Arg1514His was identified to result in both LI and CIE phenotypes (Supp. Table S1). All the five mutations were missense mutations resulting in only one amino acid alteration in the first ATP-binding cassette of the ABCA12 peptide [Lefèvre et al., 2003] (Table 1). All the families were from Africa [Lefèvre

Table 1. Genotype/Phenotype Correlation in *ABCA12* Mutations in Harlequin Ichthyosis (HI), Congenital Ichthyosiform Erythroderma (CIE), and Lamellar Ichthyosis (LI)

Genotype →	Phenotype
[truncation]+[truncation]	HI
[truncation]+[exon or conserved amino acid deletion]	HI
[exon or conserved amino acid deletion]+[exon or conserved amino acid deletion]	HI
[truncation]+[missense]	HI, CIE
[exon or conserved amino acid deletion]+[missense mutation]	HI, CIE
[missense]+[missense]	LI, CIE
Phenotype →	**Genotype**
HI	[truncation]+[truncation]
	[truncation]+[exon or conserved amino acid deletion]
	[exon or conserved amino acid deletion]+[exon or conserved amino acid deletion]
	[truncation]+[missense mutation]
	[exon or conserved amino acid deletion]+[missense mutation]
LI	[missense]+[missense]
CIE	[missense]+[missense]
	[missense]+[truncation]
	[missense mutation]+[exon or conserved amino acid deletion]

et al., 2003]. These LI patients showed clinically generalized LI with large dark pigmented scales, ectropion, palmoplantar keratoderma and no erythema. They were born as collodion babies.

CIE patients (Supp. Fig. S1B) were also reported to harbor *ABCA12* mutations as the causative genetic defect [Akiyama et al., 2008; Natsuga et al., 2007; Sakai et al., 2009]. To date, 10 *ABCA12* mutations have been reported in eight CIE families. Two mutations, p.Arg1950Ter and p.Arg2482Ter, were identified to cause both CIE and HI disease phenotypes (Supp. Table S1). Only one mutation p.Arg1514His was reported to underlie both CIE and LI phenotypes (Supp. Table S1). In CIE, most underlying mutations are missense mutations. At least one mutation on each allele is a missense mutation in CIE (Table 1). In the CIE cases with *ABCA12* mutations, the scales are slightly larger than those in typical CIE cases and are classified as "medium sized" rather than "fine" scales.

Intrafamilial variation, for example, of CIE and HI cases from one family, has never as yet been reported. Thus, there is no evidence that any other gene(s) in these patients play a noticeable role affecting the phenotypes.

Further accumulation of patients and their *ABCA12* mutation data is needed to better elucidate genotype/phenotype correlations and will aid in predicting patients' prognosis.

Biological Significance; Pathomechanisms of Ichthyosis Involving *ABCA12* Mutations

In HI affected epidermis, several morphologic abnormalities including abnormal lamellar granules in the keratinocyte granular layer and a lack of extracellular lipid lamellae within the stratum corneum had been reported [Akiyama et al., 1994, 1998; Dale et al., 1990; Milner et al., 1992]. Lack of ABCA12 function subsequently leads to disruption of lamellar granule lipid transport in the upper keratinizing epidermal cells resulting in malformation of the intercellular lipid layers of the stratum corneum in HI [Akiyama et al., 2005] (Fig. 2). Cultured epidermal keratinocytes from an HI patient carrying *ABCA12* mutations demonstrated defective glucosylceramide transport, and this phenotype was recoverable by in vitro *ABCA12* corrective gene transfer [Akiyama et al., 2005]. To date, intracytoplasmic glucosylceramide transport has been studied using cultured keratinocytes from a total of three patients harboring *ABCA12* mutations. One patient was a homozygote for a splice site mutation c.3295−2A>G [Akiyama et al., 2005] and another patient was a compound heterozygote for p.Ser387Asn and p.Thr1387del [Akiyama et al., 2006a]. Only one heterozygous mutation p.Ile1494Thr was identified in the other patient [Natsuga et al., 2007]. Cultured keratinocytes from all the three patients showed apparently disturbed glucosylceramide transport, although this assay is not quantitative.

Interestingly, ABCA3, a member of the same protein superfamily as ABCA12, functions in pulmonary surfactant lipid secretion again via the production of similar lamellar-type granules within lung alveolar type II cells [Shulenin et al., 2004; Yamano et al., 2001].

In addition, defective lamellar granule formation was observed in the skin of two CIE patients with *ABCA12* mutations [Natsuga et al., 2007]. Electron microscopic observation revealed that, in the cytoplasm of granular layer keratinocytes, abnormal, defective lamellar granules were assembled together with some normal-appearing lamellar granules [Natsuga et al., 2007].

Formation of the intercellular lipid layers is essential for epidermal barrier function. In ichthyotic skin with ABCA12 deficiency, defective formation of the lipid layers is thought to result in a serious loss of barrier function and a likely extensive compensatory hyperkeratosis [Akiyama, 2006b].

A study in one *Abca12* disrupted *Abca12*$^{-/-}$ HI model mouse indicated that a lack of desquamation of skin cells, rather than enhanced proliferation of basal layer keratinocytes, accounts for the fivefold thickening of the *Abca12*$^{-/-}$ stratum corneum using in vivo skin proliferation measurements [Zuo et al., 2008]. It was suggested that this lack of desquamation was associated with a profound reduction in skin linoleic esters of long-chain ω-hydroxyceramides and a corresponding increase in their glucosylceramide precursors. ω-hydroxyceramides are required for correct skin barrier function, and these results from the HI model mice establish that ABCA12 activity is required for the generation of long-chain ceramide esters that are essential for the development of normal skin structure and function [Zuo et al., 2008].

One hypothetical pathomechanism for ABCA12 deficient in ARCI is the differentiation defect theory (Fig. 2), derived from the clinical features of HI patients. Fetuses affected with HI start developing their ichthyotic phenotype while they are in the

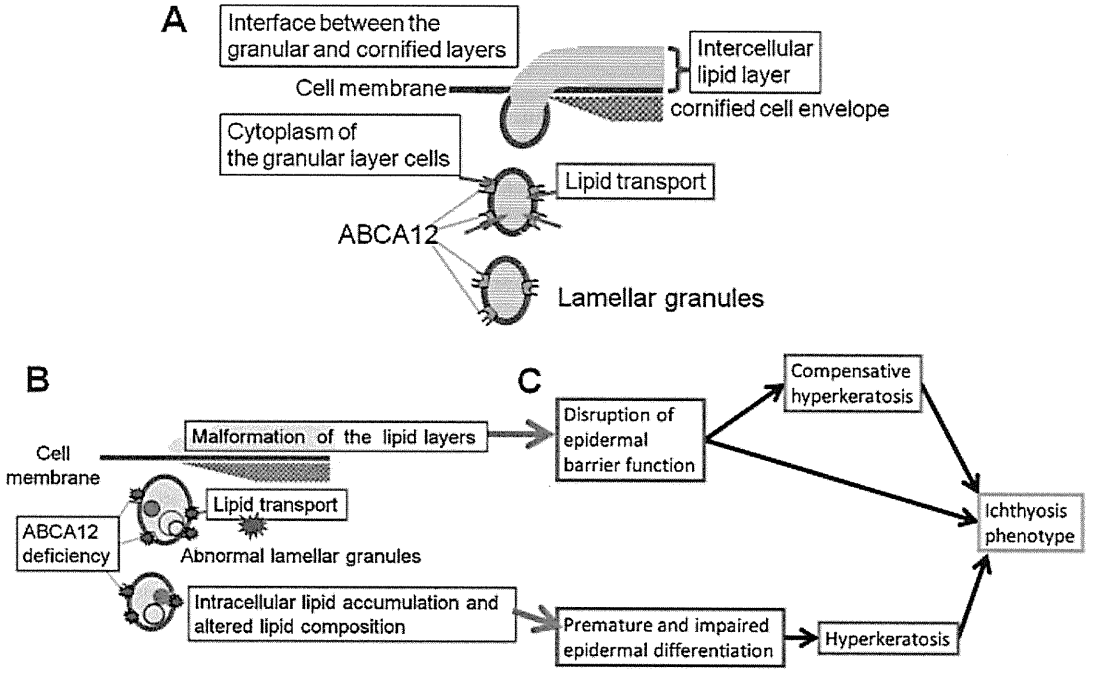

Figure 2. Physiological role(s) of ABCA12 in lipid trafficking of epidermal keratinocytes and the model of ichthyotic pathogenetic mechanisms underlying ABCA12 deficiency. **A:** Model of how ABCA12 transports lipids for keratinocyte differentiation and epidermal barrier function. ABCA12 in the limiting membrane of lamellar granules transports lipid into the lamellar granules. Accumulated lipid contents in the lamellar granules are secreted to the intercellular space forming the intercellular lipid layers, which are important for epidermal barrier function. **B:** Model of how loss of ABCA12 function leads to lipid abnormality and lipid barrier malformation in the upper epidermis. Loss-of-function mutations in ABCA12 disrupts lipid accumulation into the lamellar granules and normal lamellar granule formation, resulting in disturbed lipid transport and secretion to the extracellular space and abnormal lipid deposit in the cytoplasm. **C:** Disruption of epidermal barrier function and epidermal differentiation defects result from malformation of the stratum corneum lipid layers and abnormal intracellular lipid accumulation, respectively. It is hypothesized that lipid barrier defects and disturbed keratinocyte differentiation coordinately cause hyperkeratosis and the ichthyosis phenotype.

amniotic fluid where stratum corneum barrier function is not required. Thus, barrier defects cannot be involved directly in the pathogenesis of HI phenotype, at least during the in utero fetal period. In this context, disturbed keratinocyte differentiation is speculated to play an important role in the pathogenesis of HI phenotype. In fact, three dimensional culture studies revealed that HI keratinocytes differentiate poorly using morphologic criteria, and show reduced expression of keratin 1 and defective conversion from profilaggrin to filaggrin [Fleckman et al., 1997].

In an *ABCA12* ablated organotypic coculture system, an in vitro model of HI skin, expression of keratinocyte late differentiation-specific molecules was dysregulated [Thomas et al., 2009]. Expression of specific proteases associated with desquamation, kallikrein 5 and cathepsin D, was dramatically reduced in the ABCA12 ablated organotypic coculture system [Thomas et al., 2009]. In the model system, *ABCA12* ablation resulted in a premature terminal differentiation phenotype [Thomas et al., 2009]. Furthermore, in the mutant mice carrying a homozygous spontaneous missense mutation, loss of Abca12 function led to premature differentiation of basal keratinocytes [Smyth et al., 2008]. In contrast, in our *Abca12*$^{-/-}$ HI model mice, immunofluorescence and immunoblotting of *Abca12*$^{-/-}$ neonatal epidermis revealed defective profilaggrin/filaggrin conversion and reduced expression of the differentiation-specific molecules, loricrin, kallikrein 5, and transglutaminase 1, although their mRNA expression was upregulated [Yanagi et al., 2010]. These data suggest that ABCA12 deficiency may lead to disturbed keratinocyte differentiation during fetal development, resulting in an ichthyotic phenotype at birth. From these observations, ABCA12 deficiency might have global effects on keratinocyte differentiation, resulting in both impaired terminal differentiation and premature differentiation of the epidermis.

Animal Models

Recently, bioengineered disease models were established to investigate ichthyotic pathomechanisms due to ABCA12 defective function and to aid development of innovative treatments for ichthyosis with ABCA12 deficiency.

We transplanted cultured keratinocytes from patients with HI and succeeded in reconstituting HI skin lesions in immunodeficient mice [Yamanaka et al., 2007]. These reconstructed HI lesions showed similar changes to those observed in HI patients' skin. In addition, we generated *Abca12* disrupted (*Abca12*$^{-/-}$) mice and our *Abca12*$^{-/-}$ mice closely reproduced the human HI phenotype, showing marked hyperkeratosis with eclabium and skin fissure [Yanagi et al., 2008a]. Lamellar granule abnormalities and defective ceramide distribution were remarkable in the epidermis. Skin permeability assays of *Abca12*$^{-/-}$ mouse fetuses revealed severe skin barrier dysfunction after the initiation of keratinization. Surprisingly, *Abca12*$^{-/-}$ mice also demonstrated lung alveolar collapse immediately after birth. Lamellar bodies in alveolar type II cells from *Abca12*$^{-/-}$ mice lacked normal lamellar structures [Yanagi et al., 2008a]. The level of surfactant protein B, an essential component of alveolar surfactant, was reduced in the *Abca12*$^{-/-}$ mice [Yanagi et al., 2008a]. Another group independently

developed *Abca12*$^{-/-}$ mice and the mice also confirmed the clinical features of HI [Zuo et al., 2008]. In addition, a mouse strain carrying a homozygous spontaneous missense mutation was reported to show skin manifestations similar to ichthyosis [Smyth et al., 2008]. Lipid analysis in *Abca12* mutant epidermis revealed defects in lipid homeostasis, suggesting that *Abca12* plays a crucial role in maintaining lipid balance in the skin [Smyth et al., 2008]. The cells from the *Abca12* mutant mouse have severely impaired lipid efflux and intracellular accumulation of neutral lipids [Smyth et al., 2008]. Abca12 was also demonstrated as a mediator of Abca1-regulated cellular cholesterol efflux [Smyth et al., 2008]. Injection of a morpholino designed to target a splice site at the exon 4/intron 4 junction to block *Abca12* pre-mRNA processing induced altered skin surface contours, disorganization of the melanophore distribution, pericardial edema and enlargement of the yolk sac at 3 days postfertilization in the larvae of the zebrafish. It was also associated with premature death at around 6 days postfertilization. These results suggest that *Abca12* is an essential gene for normal zebrafish skin development and provide novel insight into the function of ABCA12 [reported at the Annual Meeting of the Society for Investigative Dermatology 2010; Abstract, Frank et al. J Invest Dermatol 2010;130:S86].

HI patients often die in the first 1 or 2 weeks of life. However, once they survive beyond the neonatal period, HI survivors' phenotypes improve within several weeks after birth. In order to clarify mechanisms of the phenotype recovery, we studied grafted skin and keratinocytes from *Abca12*-disrupted (*Abca12*$^{-/-}$) mouse [Yanagi et al., 2010]. *Abca12*$^{-/-}$ skin grafts kept in a dry environment exhibited dramatic improvements in all the abnormalities seen in the model mice. Increased transepidermal water loss, a parameter of barrier defect, was remarkably decreased in grafted *Abca12*$^{-/-}$ skin. 10 passage-subcultured *Abca12*$^{-/-}$ keratinocytes showed restoration of intact ceramide distribution, differentiation-specific protein expression, and profilaggrin/filaggrin conversion, which were defective in the primary culture [Yanagi et al., 2010]. These observations suggested that, during maturation, *Abca12*$^{-/-}$ epidermal keratinocytes regain normal differentiation processes, although the exact mechanisms of this restoration is still unknown [Yanagi et al., 2010].

We tried fetal therapy with systemic administration of retinoid or dexamethasone, which are effective treatments for neonatal HI and neonatal respiratory distress, respectively, to the pregnant mother mice; however, neither improved the skin phenotype or extended the survival period [Yanagi et al., 2008a]. Retinoids were also ineffective in in vivo studies using cultured keratinocytes from the model mice [Yanagi et al., 2010].

Prenatal Diagnosis of Harlequin Ichthyosis

In families with a history of HI, the parents' request for prenatal diagnosis is not easily ignored.

Before the causative gene for HI was identified, prenatal diagnosis had been performed by fetal skin biopsy and electron microscopic observation during the later stages of pregnancies at 19–23 weeks estimated gestational age for more than 20 years [Akiyama et al., 1994, 1999; Blanchet-Bardon et al., 1983; Shimizu et al., 2005]. The late timing of prenatal testing was a heavy burden on the pregnant mothers. In addition, when a fetus was diagnosed as affected, it was a major problem to induce a therapeutic termination at that late stage of pregnancy. After identification of *ABCA12* as the causative gene for HI, it has become feasible to perform DNA-based prenatal diagnosis for HI by chorionic villus or amniotic fluid sampling at a much earlier stage of pregnancy, with a significantly lower risk to fetal health and a reduced burden on mothers [Akiyama et al., 2007b]. Indeed, prenatal diagnosis and exclusion of HI by DNA testing were performed in our laboratory [Akiyama et al., 2007b; Yanagi et al., 2008b].

In the near future, it is hoped that much earlier prenatal diagnosis by completely noninvasive analysis of DNA from fetal cells in maternal circulation [Uitto et al., 2003] and preimplantation genetic diagnosis [Fassihi et al., 2006; Wells and Delhantry, 2001] will be available for HI.

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Comparison of skin barrier function and sensory nerve electric current perception threshold between IgE-high extrinsic and IgE-normal intrinsic types of atopic dermatitis

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Summary

Background Two types of atopic dermatitis (AD) have been proposed, with different pathophysiological mechanisms underlying this seemingly heterogeneous disorder. The extrinsic type shows high IgE levels presumably as a consequence of skin barrier damage and feasible allergen permeation, whereas the intrinsic type exhibits normal IgE levels and is not mediated by allergen-specific IgE.

Objectives To investigate the relationship between pruritus perception threshold and skin barrier function of patients with AD in a comparison between the extrinsic and intrinsic types.

Methods Enrolled in this study were 32 patients with extrinsic AD, 17 with intrinsic AD and 24 healthy individuals. The barrier function of the stratum corneum was assessed by skin surface hydration and transepidermal water loss (TEWL), and pruritus perception was evaluated by the electric current perception threshold (CPT) of sensory nerves upon neuroselective transcutaneous electric stimulation.

Results Skin surface hydration was significantly lower and TEWL was significantly higher in extrinsic AD than intrinsic AD or normal controls. Although there was no statistically significant difference in CPT among extrinsic AD, intrinsic AD and normal controls, CPT was significantly correlated with skin surface hydration and inversely with TEWL in intrinsic AD and normal controls, but not extrinsic AD. Finally, CPT was correlated with the visual analogue scale of itch in the non-lesional skin of patients with extrinsic but not intrinsic AD.

Conclusions Patients with extrinsic AD have an impaired barrier, which increases the pre-existing pruritus but rather decreases sensitivity to external stimuli. In contrast, patients with intrinsic AD retain a normal barrier function and sensory reactivity to external pruritic stimuli.

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Conflicts of interest
None declared.

T.M. and K.I. contributed equally to this study.

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Atopic dermatitis (AD) is a chronic inflammatory skin disease with complicated pathophysiological mechanisms and causative agents. Two subtypes of AD have been proposed: extrinsic AD and intrinsic AD. The extrinsic type is the IgE-mediated common form of AD and is associated with respiratory allergies, such as rhinitis and asthma, and high serum levels of IgE.[1–3] In contrast, intrinsic AD is characterized by the absence of allergen-specific IgE and thus shows normal total IgE levels, although this newly introduced concept is still controversial among academic dermatologists.[1–3] Approximately 20%[4] or fewer[5] patients are estimated as having intrinsic AD. Its characteristics include female predominance, absence of atopic diseases, later onset of disease, and milder disease severity.[3–6] A history of atopy, recurrent conjunctivitis, palmar hyperlinearity, keratosis pilaris, pityriasis alba, and hand and/or foot eczema are significantly less present in the intrinsic type, but Dennie-Morgan fold is positively associated with intrinsic AD.[3]

Several studies have suggested differences in various aspects of pathophysiology between extrinsic and intrinsic AD.

Increased transepidermal water loss (TEWL) and reduced skin surface hydration are hallmarks of atopic skin, and there are some differences in these values between the two types of AD.[4] Immunologically, surface expression of the high- and low-affinity receptor for IgE and of the interleukin (IL)-4Rα chain is elevated in monocytes from patients with extrinsic AD, but serum levels of IL-13 are significantly increased in patients with intrinsic AD.[7] Skin lesions of extrinsic AD show high levels of chemokines such as CCL18.[8] Expression of neurotrophins is increased comparably in both types.[6]

The stratum corneum of the epidermis, consisting of more than 10 layers of corneocytes and intercellular lipids, serves as the skin barrier.[9] In extrinsic AD, impairment of the skin barrier may be the primary condition which facilitates permeation of environmental allergens and leads to immunological responses such as elevation of allergen-specific IgE.[1] A recent finding of filaggrin gene mutations in a high percentage of patients with AD,[10,11] together with an older finding of ceramide reduction in the stratum corneum,[12,13] have further suggested the presence of skin barrier damage in extrinsic AD. On the other hand, intrinsic AD shows normal or mildly elevated serum IgE, in striking contrast to extrinsic AD.[14] The mechanisms underlying intrinsic AD remain unclear and more speculative than those underlying extrinsic AD.[2,6,8,15]

Patients with AD are well known to be sensitive to irritation from the environment due to the impaired skin barrier function. Given that the extrinsic and intrinsic types are different from each other in the skin barrier condition, each type might respond to external stimuli in a different manner. However, little is known regarding the difference in sensitivity to irritants and in elicitability of pruritus between the two types. It appears that most previous studies on sensitivity were performed in patients with extrinsic AD because of its higher incidence.

There are several reported methods to assess the threshold for the itch sensation to various environmental stimuli.[16–18] Local administration of histamine, either by needle injection or by iontophoresis, is one of the most common procedures for this purpose.[19] Electrically evoked itch is another useful method with the use of a neuroselective transcutaneous electrical stimulator, Neurometer™ CPT/C (Neurotron Inc., Baltimore, MD, U.S.A.), in a noninvasive fashion. Evaluation of electric current perception threshold (CPT) quantifies the sensory threshold to electric stimulation of the sensory nerves.[20,21] This device has been used mainly by neurologists to demonstrate abnormalities in a variety of neuropathic conditions. It does not measure the sensation only to histamine, but the device directly excites large- and small-diameter sensory nerve fibres in a differentiating fashion, independent of local factors such as skin thickness, temperature and substances involved in the induction of pruritus.[20,22] The CPT for 250- and 5-Hz frequency current emitted by the Neurometer CPT/C has been reported to enable quantification of the sensory threshold of Aδ- and C-fibres, respectively, that are thought to transmit the itch sensation from the skin. Therefore, this instrument allows us to investigate the elicitability of pruritus in patients with AD. Kobayashi *et al.*[23] have reported that patients with AD showed lower CPT than healthy controls, and CPT was inversely correlated with TEWL after tape stripping in normal subjects.

To address the differences in the mechanisms between extrinsic and intrinsic AD, we measured CPT in patients with AD, together with measurements of stratum corneum function. Our results show that there are prominent differences in the relationship of CPT with the skin barrier function between the two types.

Materials and methods

Participants

Patients over 18 years of age from our department were included in this study. Forty-nine patients with AD (25 men and 24 women), diagnosed in accordance with the Hanifin and Rajka classification,[24] and 24 healthy controls were enrolled in this study. The distribution of skin symptoms in all patients was characterized for adult AD. The hands, shoulders, neck, flexures and face were the predilection sites, while the extremities were less involved. Patients who had total serum IgE levels < 220 U mL^{-1} (normal range for Japanese subjects) were classified as having intrinsic AD, and those with levels > 400 U mL^{-1} were classified as having extrinsic AD. There was no patient with IgE levels between 220 and 400 U mL^{-1}. IgE RAST for *Dermatophagoides pteronyssinus* was measured in 20 patients. The disease activity was assessed by SCORAD (severity scoring of AD). Patient details are listed in Table 1. All participants provided written informed consent, and the institutional review board approved this study.

Electric current perception threshold and stratum corneum function

C-fibres are sensory nerves conducting itch and pain. Transcutaneous electric current with 5-Hz sine wave stimulates C-fibres, as assessed by the active action potentials of rat dorsal root ganglia.[21,25] Depending on the body surface site, 5-Hz electric current induces itch and/or pain. In addition, transcutaneous 250-Hz current has also been known to stimulate Aδ-fibres, thus inducing itch.[25] In this direct stimulation of nerve fibres with transcutaneous electric current, the condition of the stratum corneum possibly modifies the perception by affecting the current or other factors.

We measured CPT, skin surface hydration and TEWL at the nonlesional flexor forearm, the nonlesional lower leg, and at lesional skin on the trunk or extremities. When the patients had skin lesions on the flexor forearms or lower legs we avoided these regions and chose clinically normal areas on the volar skin as the sites to perform measurements on nonlesional skin. The patients did not apply any ointment or cream to the examined sites for at least 2 days before the measurements. Concerning the inflammatory state at the clinically normal sites tested, we have previously demonstrated

Table 1 Patients and healthy individuals enrolled in this study

	Extrinsic AD	Intrinsic AD	Healthy controls
Number of subjects	32 (21 men and 11 women)	17 (four men and 13 women)	24 (nine men and 15 women)
Age (years), mean ± SD (range)	30·0 ± 8·1 (19–51)	33·0 ± 10·4 (18–57)	28·9 ± 3·8 (23–37)
IgE (U mL^{-1}), mean ± SD (range)	5034·8 ± 7538·0 (436–30 000)	110·5 ± 66·8 (11–219)	–
SCORAD, mean ± SD (range)	41·8 ± 19·0 (4·6–84·5)	27·1 ± 20·6 (3·5–73)	–
VAS (nonlesional forearm), mean ± SD	30·9 ± 20·6	15·8 ± 22·1	–
VAS (nonlesional lower leg), mean ± SD	36·0 ± 24·9	20·4 ± 28·5	–
VAS (lesional skin), mean ± SD	55·3 ± 28·3	47·3 ± 36·0	–

AD, atopic dermatitis; VAS, visual analogue scale.

that clinically normal-appearing skin of patients with AD has no histological evidence of inflammation.[26] As control, we measured CPT, skin surface hydration and TEWL on the mid-flexor forearm and lower leg in healthy individuals. CPT was measured by using the Neurometer CPT/C as described previously.[23] Skin surface hydration was evaluated by capacitance using the Corneometer CM825 (Courage & Khazaka Electronic GmbH, Cologne, Germany) and was expressed as arbitrary units.[27] TEWL was measured by detecting the evaporated water using the VapoMeter SWL-2 (Delfin Technologies Ltd, Kuopio, Finland).

Visual analogue scale

All patients rated current itching on a 100-mm visual analogue scale (VAS)[28] at the following sites: nonlesional forearm, nonlesional lower leg and lesional skin.

Statistical analyses

Data were expressed as mean ± SD and assessed for statistical significance. We used Student's t-test to compare skin surface hydration, TEWL and CPT. A linear regression analysis was performed for correlations between the skin surface hydration or VAS and CPT, using Peason's correlation coefficient. For all tests, $P < 0{\cdot}05$ was considered statistically significant.

Results

Impaired skin barrier function in extrinsic but not intrinsic atopic dermatitis

Patients were classified as having extrinsic or intrinsic AD by means of IgE level (> 400 and < 220 U mL^{-1}, respectively). IgE RAST was scored by index values 0–6 according to the manufacturer's criteria (BML, Tokyo, Japan). An index value > 3 to *D. pteronyssinus* was obtained in 11 of 12 (92%) patients with extrinsic AD, but in only one of eight (12·5%) patients with intrinsic AD. Moreover, 67% of the patients with extrinsic AD showed a RAST score index value of 6, and none of the patients with intrinsic AD showed this highest score. As summarized in Table 1, more patients had extrinsic AD than intrinsic AD, and women predominated in the intrinsic type, as previously reported.[3–5] No significant difference was noted in age between the two types. There was a tendency that SCORAD and VAS at the three test sites were higher in extrinsic than intrinsic AD, as reported previously.[3–5]

As extrinsic AD is caused by external allergens invading through the damaged skin barrier, we initially examined the skin surface hydration (capacitance) and TEWL at the nonlesional forearm and lower leg of patients and normal volunteers in a comparison between extrinsic and intrinsic AD. Skin surface hydration was significantly lower in extrinsic AD than in normal control subjects (Fig. 1). There was no significant difference in the hydration level between intrinsic AD and healthy controls. Extrinsic AD tended to show lower values than intrinsic AD at both sites. TEWL, another assessment of the barrier function, was statistically significantly higher in extrinsic AD than intrinsic AD and normal controls at the nonlesional forearm (Fig. 1). Thus, the skin barrier function was impaired in extrinsic AD and preserved in intrinsic AD, validating this clinical dichotomy.

Correlation between disease severity and pruritus in both extrinsic and intrinsic atopic dermatitis

In advance of analysing CPT, we also examined the correlation between the itch levels and SCORAD in the two types of AD. In both types, VAS scores on the lesional skin were correlated with SCORAD (Fig. 2), suggesting that both types of AD are associated with disease severity-dependent pruritus.

Significant correlation between electric current perception threshold (CPT) and skin surface hydration and between CPT and transepidermal water loss in intrinsic atopic dermatitis as well as in normal individuals

CPT for 5- and 250-Hz current stimuli was measured in patients with AD and normal volunteers. In all experiments, the results from 5- and 250-Hz current stimuli were virtually the same. Figure 3 shows the mean ± SD CPT in each group, and there was no significant difference between the groups in the nonlesional or lesional skin.

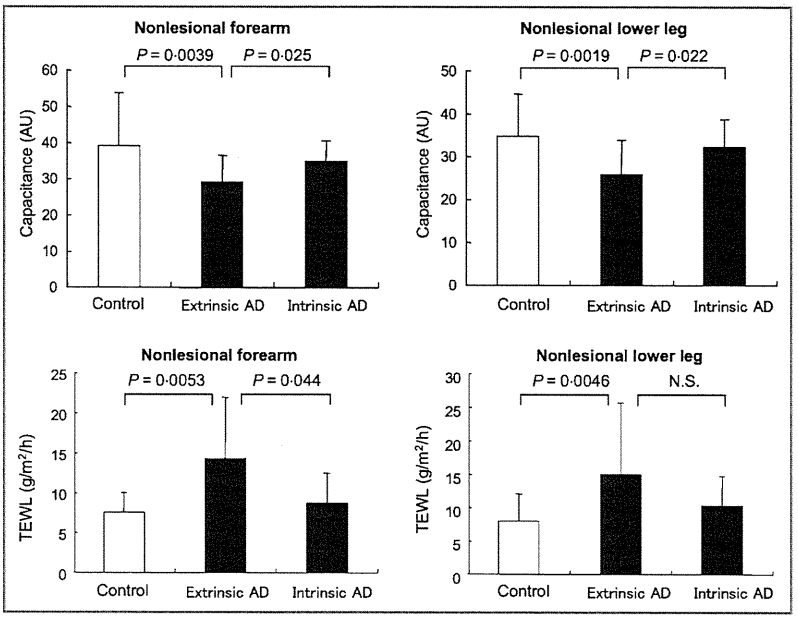

Fig 1. Skin surface hydration on nonlesional forearm and lower leg in extrinsic and intrinsic atopic dermatitis (AD) and healthy controls. Skin surface hydration is represented by capacitance in arbitrary units (AU). Results are shown as mean ± SD. N.S., not significant.

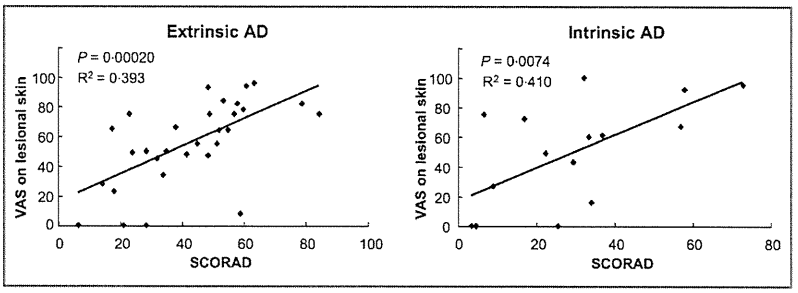

Fig 2. Relationship between SCORAD and visual analogue scale (VAS) score in extrinsic and intrinsic atopic dermatitis (AD).

When CPT was analysed in relation to skin surface hydration, an interesting finding was obtained. In normal subjects (control), CPT was significantly correlated with skin surface hydration (Fig. 4), suggesting that the water-poor cornified layer has a property to evoke pruritus in response to external stimuli. Similarly, the lesional skin of patients with intrinsic AD showed such a significant correlation between CPT and skin surface hydration. However, there was no correlation in extrinsic AD, as large individual variations of CPT were seen in the patients with extrinsic AD and low levels of skin surface hydration.

As to the relation of CPT to TEWL, there was no significant correlation between these two parameters in the lesional skin of patients with AD. However, CPT of nonlesional forearm, as assessed by 250-Hz sensitivity, was inversely correlated with TEWL in intrinsic AD as well as in controls (Fig. 5). The results suggest that intrinsic AD is associated with a normal stratum corneum and no excess elicitability of externally stimulated pruritus, while extrinsic AD does not show such a regular, surface hydration-related irritant perception.

Different electric current perception threshold levels in relation to pre-existing pruritus between extrinsic and intrinsic atopic dermatitis

It is possible that CPT is affected by the itch state in patients with AD. We therefore investigated the relationship between CPT and the pre-existing pruritus assessed by VAS. In the lesional skin of both types of AD there was no correlation between CPT and VAS (data not shown). In the nonlesional lower leg, however, CPT was significantly correlated with VAS in extrinsic but not intrinsic AD (Fig. 6), suggesting that the pre-existing pruritus rather downmodulates the sensitivity to external stimuli in extrinsic AD. The nonlesional forearm exhibited the same tendency but without statistical significance. Thus, the pruritic normal-appearing skin seems to be insensitive to further itchy stimuli in extrinsic AD.

Discussion

The precise concept of intrinsic AD in comparison with extrinsic AD has been a matter of controversy. Extrinsic AD seems

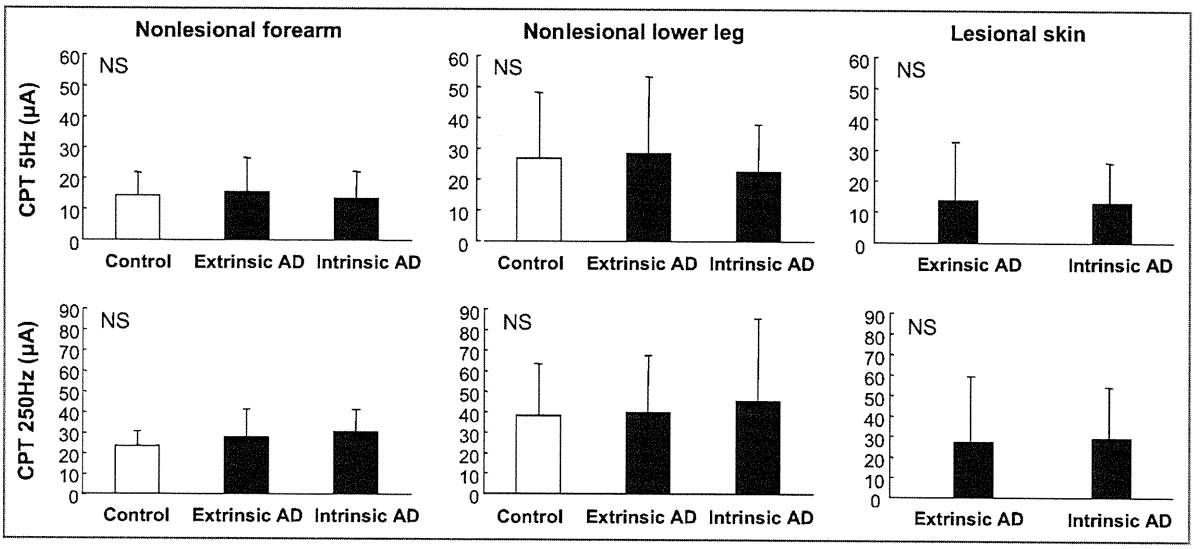

Fig 3. Electric current perception threshold (CPT) on nonlesional forearm or lower leg and lesional skin in extrinsic and intrinsic atopic dermatitis (AD). Results are shown as mean ± SD. N.S., not significant.

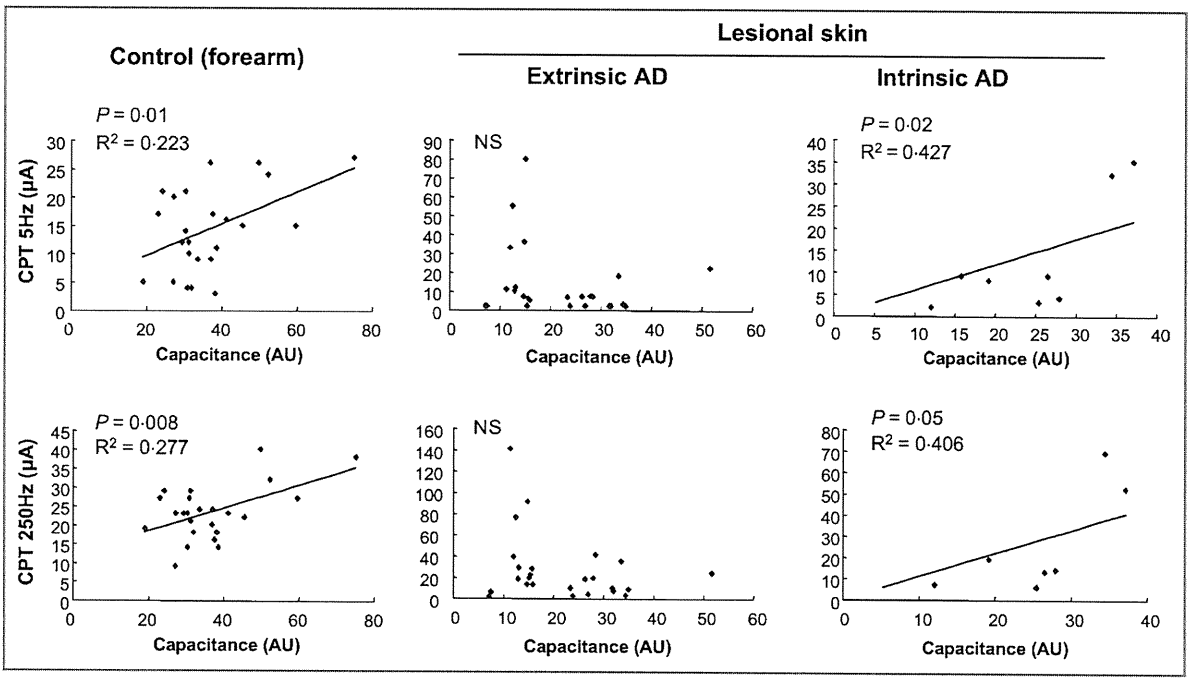

Fig 4. Relationship between skin surface hydration, represented by capacitance in arbitrary units (AU), and electric current perception threshold (CPT) in lesional skin of patients with extrinsic and intrinsic atopic dermatitis (AD) and in nonlesional forearm skin of healthy controls. NS, not significant.

to be induced by sequential events, including impairment of stratum corneum, permeation of external substances, exposure of immunocompetent cells to the allergens, and T cell and IgE responses to the antigenic determinants.[1] Growing evidence has supported this mechanism underlying the extrinsic type. The recent finding that filaggrin gene mutations are a predisposing factor for AD has clearly demonstrated the presence of barrier impairment in patients with AD.[10,11] On the other hand, the pathophysiology of intrinsic AD remains obscure, and it may be difficult for clinicians to differentiate the two

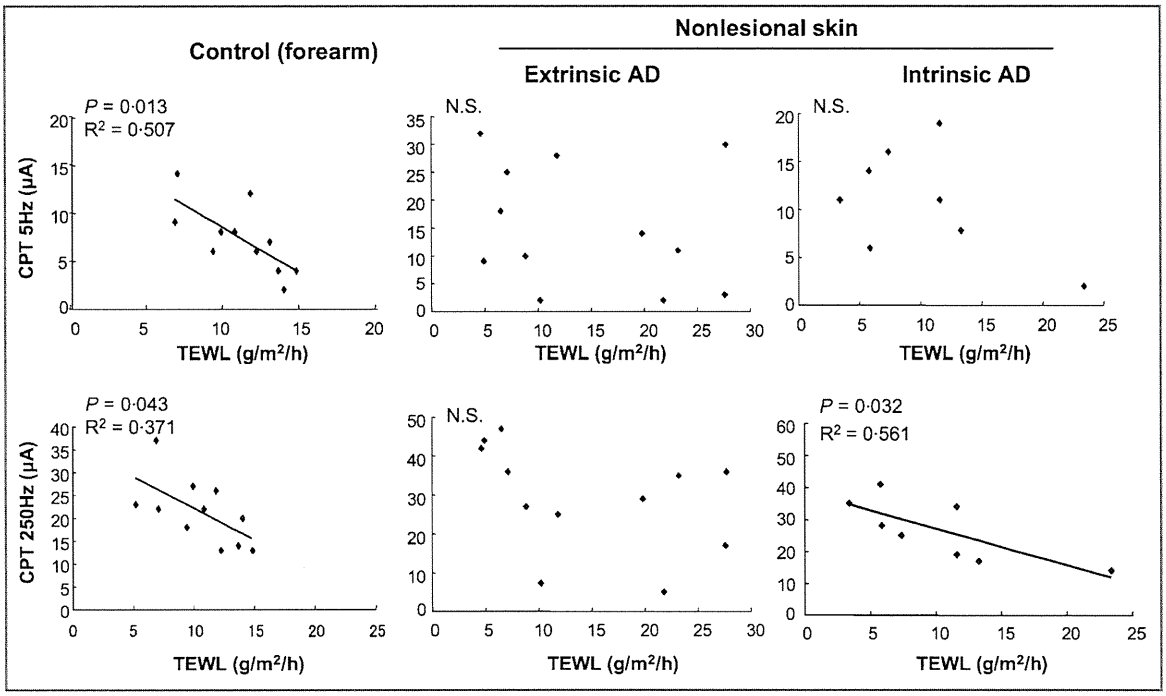

Fig 5. Relationship between transepidermal water loss (TEWL) and electric current perception threshold (CPT) in nonlesional forearm skin of patients with extrinsic and intrinsic atopic dermatitis (AD) and healthy control skin. NS, not significant.

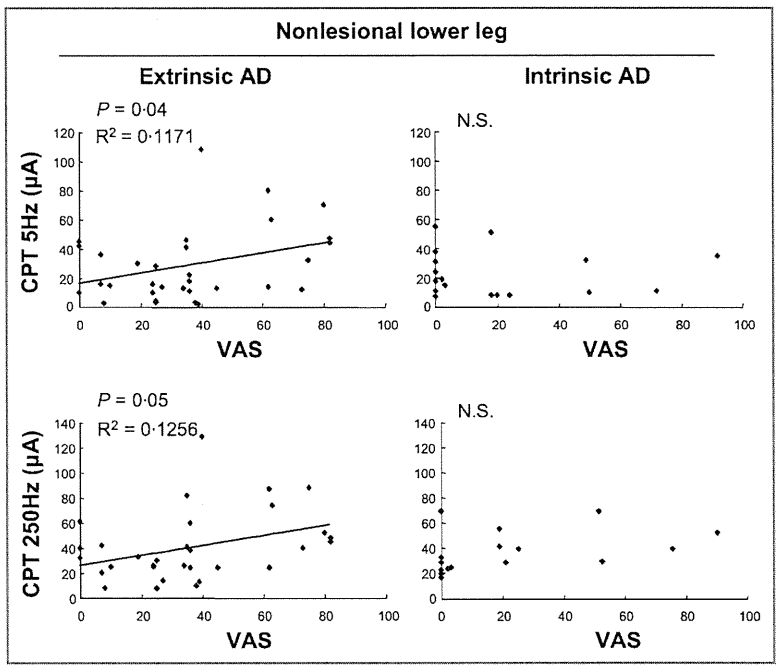

Fig 6. Relationship between visual analogue scale (VAS) score and electric current perception threshold (CPT) on nonlesional skin of patients with extrinsic and intrinsic atopic dermatitis (AD). NS, not significant.

types accurately. Only one clear way to discriminate the two types is the serum levels of IgE,[29] but its precise cutoff value has not been determined.

In this study, we tentatively divided the patients with AD into two groups by IgE levels of > 400 and < 220 U mL^{-1}, because the normal range in Japanese individuals is < 220 U mL^{-1}. This division was confirmed by a high percentage and high scores of positive RAST to *D. pteronyssinus* in extrinsic AD and a low percentage in intrinsic AD. We found that more of our patients had intrinsic than extrinsic AD, and that women were more likely than men to have intrinsic AD, as already reported in previous studies.[5] In contrast to

extrinsic AD, intrinsic AD is thought to show a normal skin barrier function. We validated this general concept by measuring skin surface hydration and TEWL, and found no significant difference in these values between patients with intrinsic AD and normal individuals, while the patients with extrinsic AD had lower surface hydration levels and higher TEWL levels than the normal subjects.

C-fibres (unmyelinated fibres) are sensory nerves conducting pruritus. A transcutaneous electric current at 5 Hz can stimulate C-fibres.[25] Alternating current stimulus at 250 Hz activates Aδ-fibres (small myelinated fibres), which may also participate in itch. The condition of the stratum corneum may modify the perception by affecting the current or other factors. In our study, low levels of hydration of the stratum corneum reduced CPT. This suggests that the itch perception to external stimuli is promoted in skin with low hydration.

The difference in the barrier function between the extrinsic and intrinsic types raised the possibility that the elicitability of pruritus differs between them. In normal individuals, CPT and skin surface hydration or TEWL were correlated with each other, suggesting that the barrier-damaged skin is sensitive to external irritants. In normal individuals, Kobayashi *et al.*[23] have reported that CPT is inversely correlated with TEWL levels after tape stripping, providing further evidence that barrier damage leads to elicitability of sensation. In our study, the correlation between CPT and skin surface hydration and the inverse correlation between CPT and TEWL were also found in patients with intrinsic AD, suggesting that intrinsic AD shows a normal skin barrier and elicitability of sensation to external stimuli. In contrast, the patients with extrinsic AD showed different elicitability with individual variations presumably due to the low surface hydration. Kobayashi *et al.*[23] have also shown that the skin of patients with AD is not extremely sensitive as compared with that of normal individuals to the electric stimulation of their Aδ- and C-fibres. Their patients seem to include those with both extrinsic and intrinsic AD. Our results suggest that some patients with extrinsic AD have high CPT levels despite the impaired barrier function.

Pre-existing pruritus elevated CPT on the nonlesional skin of patients with extrinsic AD, as CPT and VAS were correlated with each other in the nonlesional sites of the extrinsic type. Accordingly, Ikoma *et al.*[30] found that when histamine prick tests are performed in nonlesional skin of patients with AD, itch rating increases more slowly and is significantly lower than in controls.[30] Our unexpected finding was not observed in intrinsic AD. It is possible that in the already itchy skin of extrinsic AD, Aδ- and C-fibres are in a stimulated state, resulting in insensitivity to external irritants, while the steady-state interaction between the barrier and sensory fibres might be kept in intrinsic AD. The end of sensory fibres in the skin of extrinsic AD seems to be continuously stimulated by the damaged stratum corneum, leading to the elevated CPT.

Our study suggests that the two types of AD are different from each other in the mode of elicitability of pruritus, because of the different skin barrier states between them. Furthermore, it was recently found that IgE autoantibodies can target keratinocytes in AD; this might promote barrier damage and modify resultant itch elicitability in extrinsic AD.[31] As the response of sensory nerves to irritants appears to be intact in intrinsic AD, the mechanisms of pruritus underlying this type of AD are an important issue to be elucidated.

Acknowledgments

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CD30-positive primary cutaneous anaplastic large-cell lymphoma and definite squamous cell carcinoma

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CD30-positive primary cutaneous anaplastic large cell lymphoma (PCALCL) is one of several primary cutaneous CD30-positive lymphoproliferative disorders. The overlying epidermis often shows epidermal hyperplasia with ulceration, and pseudocarcinomatous hyperplasia has been reported in a small number of CD30-positive PCALCL cases.[1–3] However, separate and distinct squamous cell carcinoma (SCC) has rarely been seen in CD30-positive PCALCL, and only one SCC (keratoacanthoma type) was reported by Cespedes *et al.*[2] We report a rare case of CD30-positive PCALCL with SCC that infiltrated the deep dermis.

A 20-year-old woman presented with a 3-month history of an enlarging hyperkeratotic tumour (50 mm in diameter) on the left thigh.

Histological examination of a biopsy from the tumour showed infiltrative proliferation of atypical keratinocytes (Figs 1 and 2a) as well as a diffuse and dense background infiltrate of large atypical lymphoid cells mixed with many eosinophils in the dermis (Fig. 2c). The atypical keratinocytes were well differentiated (Fig. 2b). Large atypical lymphoid cells strongly expressed CD30 (Fig. 2d) and CD3, but were negative for ALK-1. There was no evidence of extracutaneous involvement of the tumour based on the findings from chest and abdominal computed tomography and 67-Ga scintigraphy.

The diagnosis of PCALCL with well-differentiated SCC was made. The lesion was completely excised and has not recurred in > 11 years.

Some reports have described epidermal hyperplasia together with CD30-positive PCALCL. The pathogenesis of prominent epidermal hyperplasia in association with CD30-positive PCALCL has been attributed to a variety of mediators including epidermal growth factor (EGF), transforming growth factor (TGF)-α and epidermal growth factor receptor (EGFR).[1] Courville *et al.* reported stronger expression of EGF and TGF-α in T cells and stronger epidermal expression of EGFR in cutaneous T-cell lymphoma (CTCL) with pseudocarcinomatous hyperplasia than in control cases of CTCL without pseudocarcinomatous hyperplasia.[1] EGF causes epidermal proliferation and TGF-α is an important factor in wound healing and carcinogenesis. EGFR is the receptor for both EGF and TGF-α, and amplification or overexpression of EGFR has been seen in SCC of the skin.[4] It is currently thought that EGFR is important in squamous cell carcinogenesis, and anti-EGFR antibody (cetuximab) has been used to treat cutaneous SCC.[5] From the common association of CD30-positive PCALCL with epidermal hyperplasia and less commonly, with SCC, we speculate that these mediators may play a role in epidermal proliferation and tumorigenesis.

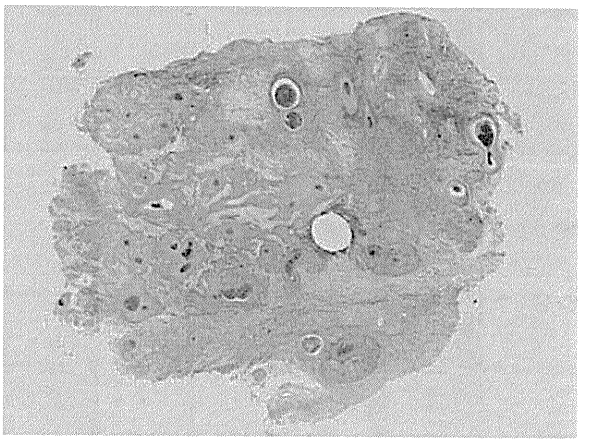

Figure 1 A skin biopsy taken from the hyperkeratotic plaque on the left thigh showed deep infiltrative proliferation of atypical keratinocytes in the reticular dermis near the subcutaneous tissue (haematoxylin and eosin, original magnification × 10).

Treatment for SCC or epidermal hyperplasia overlying CD30-positive PCALC should be carefully planned. Scarisbrick *et al.*[3] reported that epidermal hyperplasia in CD30-positive PCALCL may be mistakenly diagnosed as SCC, thereby leading to inappropriate overtreatment. We agree with this assessment and that epidermal hyperplasia with CD30-positive PCALCL need not to be treated with wide local excision, but definite SCC such as in our case does need wide local excision.

In conclusion, our case of CD30-positive PCALCL coexisting with SCC is very rare. It suggests that CD30-positive PCALCL may induce not only epidermal hyperplasia but also SCC in specific cases.

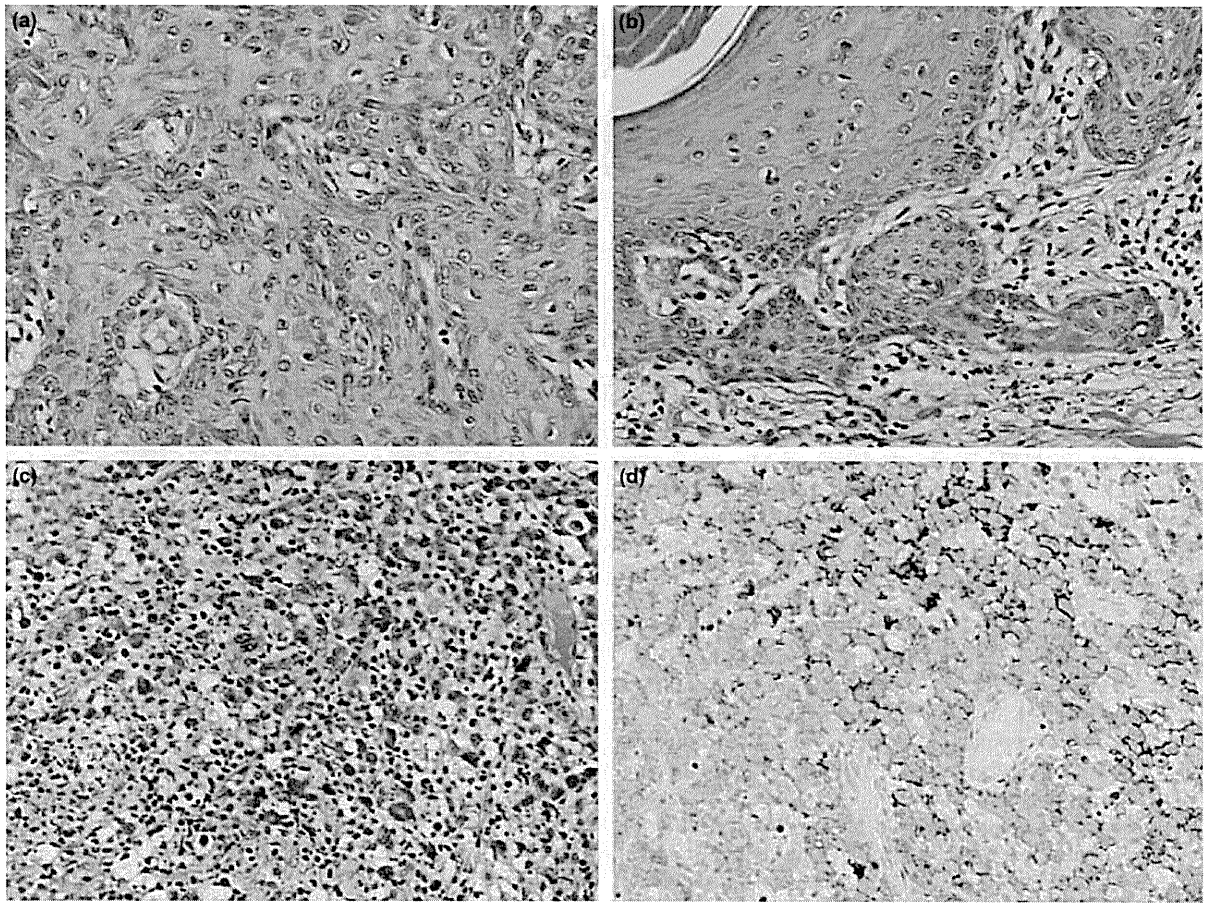

Figure 2 (a) Loss of the normal orderly stratified arrangement of the epidermis; (b) central keratinization and horn pearl formation, which suggested well-differentiated squamous cell carcinoma; (c) the typical keratinocyte infiltrates were associated with both diffuse and dense infiltration of large atypical lymphoid cells mixed with many dermal eosinophils. Haematoxylin and eosin, original magnification (a–c) × 100. (d) Large atypical lymphoid cells strongly expressed CD30 (original magnification × 100).

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