A LI patients with *TGM1* mutations included in the present study

Patient No.	Age	Sex	*TGM1* mutations	Phenotype	Skin hyperkeratosis		References
					severity	localization	
1	0	M	p.[Arg106X]+[Gln234X]	LI (severe)	severe	generalized	this study
2	33	F	c.[371delA]+[=]	LI (severe)	severe	generalized	Ref. No. 24
3	0	M	p.[Arg307Trp]+[=]	LI (mild)	mild	localized (trunk)	Ref. No. 6
4	56	F	p.[Leu205Gln]+[Arg307Trp]	LI (mild)	mild	localized (trunk)	Ref. No. 20

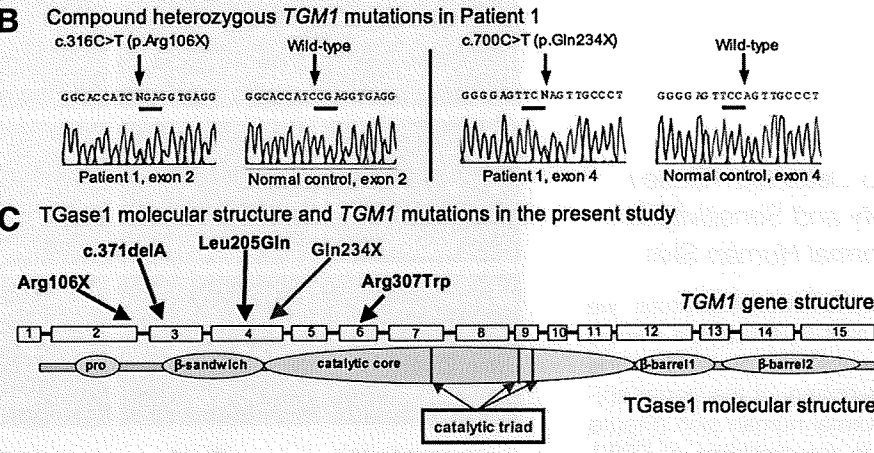

Figure 2. *TGM1* mutations and clinical features of LI patients in the present study. **A:** Summary of the *TGM1* mutations and phenotypes of the LI patients included in the present study. Note Patients 1 and 2 harbored truncation mutations in both alleles and exhibited a severe phenotype, and Patients 3 and 4 carried missense mutations in both alleles exhibiting a milder phenotype. An underlined mutation was a novel mutation. **B:** Direct sequence analysis of exons 2 and 4 of Patient 1 revealed heterozygous nonsense mutations, c.316C>T (p.Arg106X) and c.700C>T (p.Gln234X). **C:** Schematic sequential arrangement of the domain structure of the TGase1 polypeptide. Mutations in the present LI patients are marked by **arrows**. Red characters and **arrows** indicate novel mutations and black ones are previously reported mutations. Note that three truncation mutations are located upstream to the catalytic core domain. Two missense mutations are in the β-sandwich domain and the catalytic core domain, which are important for enzyme activity.

TGase1 activity and loricrin in the cell periphery of the upper spinous and granular layer cells (Figure 1, J–L).

PepK5 Detected in Situ *TGase1 Activity Efficiently Compared with Cadaverine*

We also compared the reactivity of FITC-pepK5 and FITC-cadaverine, which has been previously used for detection of *in situ* TGase activity in normal human skin at various concentrations, 10, 5, 1, and 0.1 μmol/L (Figure 1, M–R). At 10 μmol/L and 5 μmol/L concentrations, intense FITC-pepK5 labeling was observed mainly in the cell periphery of the upper spinous and granular layer keratinocytes in the normal human epidermis. At 1 μmol/L concentration, FITC-pepK5 labeled only the granular layer keratinocytes, and at 0.1 μmol/L concentration (data not shown) no FITC-pepK5 labeling was seen in the normal human epidermis. In contrast, using FITC-cadaverine at 10 μmol/L concentration, the entire epidermis was labeled, and at 5 μmol/L concentration only faint FITC-cadaverine labeling was seen in all of the layers of normal human epidermis. At 1 μmol/L or 0.1 μmol/L (data not shown) concentration, no FITC-cadaverine labeling was obtained in the epidermis. These results suggest that FITC-pepK5 detects endogenous TGase1 activity with greater sensitivity, at least more than ten times higher than FITC-cadaverine in human epidermis. In addition, considering the labeling patterns in the epidermis by the two substrates, specificity of pepK5 to TGase1 seemed to be much higher than that of cadaverine.

TGM1 Mutations and Clinical Features of LI Patients Involved in the Present Study

TGM1 mutations and clinical features of the patients included in the present study are summarized in Figure 2, A–C. Patients 1 and 2 showed a typical, classic LI phenotype. Patients 3 and 4 had a mild LI phenotype with mild hyperkeratosis mainly on the trunk. Patient 4 had a LI phenotype termed as "bathing suit ichthyosis"[26] with restricted affected regions on the trunk.

Patient 1 was a newly examined LI case. Patient 1 was compound heterozygous for the two *TGM1* nonsense mutations, p.Arg106X and p.Gln234X (c.[316C>T]+[700C>T]; p.[Arg106X]+[Gln234X]; Figure 2B) and showed a typical classic form of LI. One mutation p.Gln234X was a novel mutation and the other mutation p.Arg106X was previously reported.[27] These mutations were not found in 100 normal control alleles (50 unrelated, healthy Japanese individuals) and were not thought to be polymorphisms. The three other patients included in the present study had been reported previously to have a total of three *TGM1* mutations including p.Arg307Trp, a prevalent TGM1 mutation in the Japanese population.[6,20,24]

PepK5 Labeling Clearly Detected Defective TGase1 Activity in the Skin of LI Patients

In Patients 1 and 2, membranous TGase 1 activity detected by FITC-pepK5 in the upper spinous and granular layers of the patients' epidermis was completely lost (Figure 3, A and B). In Patient 3, membranous TGase 1 activity detected by FITC-pepK5 in the upper spinous and granular layers of the patient's epidermis was observed, but remarkably weaker (Figure 3C) than that of normal control human epidermis (Figure 3E). In Patient 4, membranous TGase1 activity demonstrated by FITC-pepK5 in the upper spinous and granular layers of the patient's epidermis was present, but restricted solely to the granular layer cells and cells just below the granular layer and was significantly weaker (Figure 3D) than that of normal control human epidermis (Figure 3E). In the

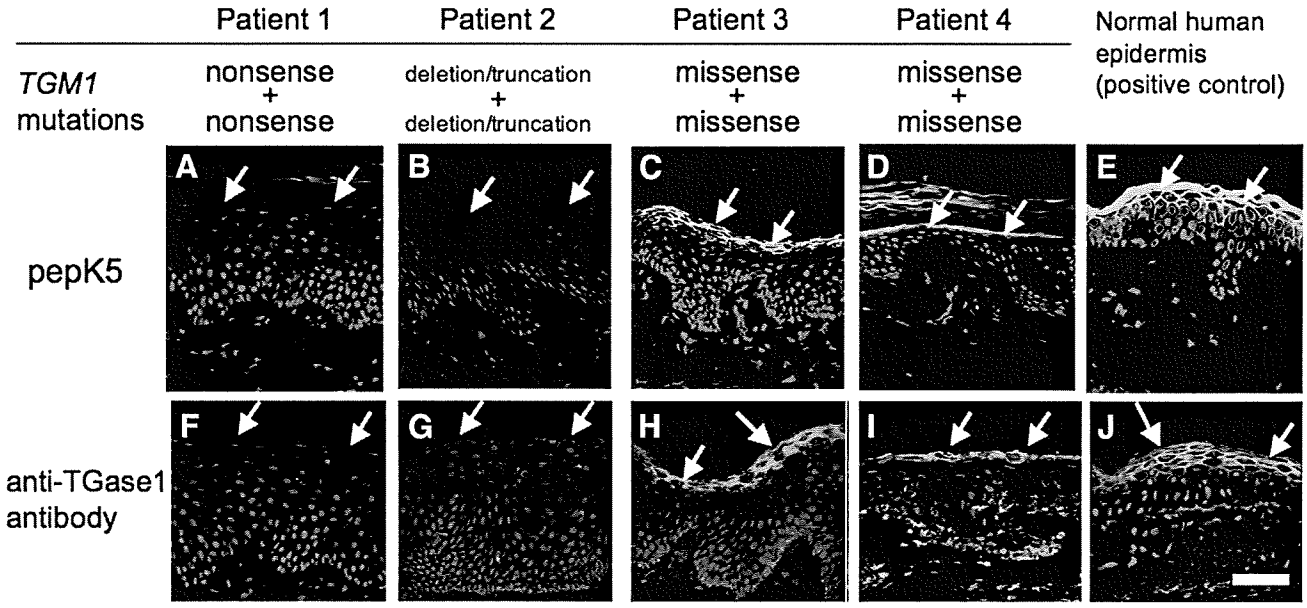

Figure 3. TGase1 deficiency detected by pepK5 labeling in the LI patients. **A and F:** Patient 1, a compound heterozygote for two *TGM1* nonsense mutations: FITC-pepK5 labeling (green) shows complete absence of TGase1 activity in the upper epidermis (**white arrows**; **A**), and TGase1 immunostaining (green) is also negative in the upper epidermis (**white arrows**; **F**). **B and G:** Patient 2, a homozygote for a *TGM1* deletion mutation causing truncation of the peptide: FITC-pepK5 labeling (green) reveals completely abolished TGase1 activity in the upper epidermis (**white arrows**; **B**) and no TGase1 immunolabeling (in green) is seen in the upper epidermis (**white arrows**; **G**). **C** and **H:** Patient 3, a homozygote for a *TGM1* missense mutation: detectable, but reduced membranous TGase1 activity is seen in the upper epidermis (**white arrows**) by FITC-pepK5 labeling (green; **C**). TGase1 immunostaining (green) in the upper epidermis (**white arrows**) confirms expression of TGase1 molecule (**H**). **D** and **I:** Patient 4, a compound heterozygote for two *TGM1* missense mutations: FITC-pepK5 labeling (green) shows faint TGase1 activity restricted to the granular layers (**white arrows**; **D**). Immunofluorescence labeling for TGase1 (green) reveals a positive staining in the granular layer (**white arrows**) in the patient's epidermis (**I**). **E and J:** In a normal human skin without any *TGM1* mutations, intense TGase1 activity is seen in the upper epidermis (**white arrows**) using FITC-pepK5 labeling (green; **E**). TGase1 immunolabeling (green) is also positive in the upper epidermis (**white arrows**; **J**). **A–E:** FITC-pepK5 labeling, green; **F–J:** rabbit polyclonal anti-TGase1 antibody staining, green (FITC); **A–J:** nuclear stain, red (propidium iodide). Scale bar = 50 μm.

epidermis of the two patients with ichthyosis caused by *ABCA12* mutations, other than *TGM1* mutations, intense membrane TGase1 activity was normally observed in the upper spinous and the granular layers by pepK5 labeling (data not shown).

Immunofluorescent labeling using rabbit polyclonal anti-TGase1 antibody revealed that TGase1 immunostaining was not seen in the epidermis of Patients 1 and 2 (Figure 3, F and G). In the epidermis of Patients 3 and 4, positive immunostaining for TGase1 molecule was observed mainly in the granular layer (Figure 3, H, I, and J). From the results of pepK5 labeling and immunostaining for the TGase1 molecule, in Patients 1 and 2, it was thought that immunoreactive, intact TGase1 molecule was absent from the epidermis, resulting in the absence of FITC-pepK5 labeling. In Patients 3 and 4, although immunoreactivity for TGase1 was detected in the epidermis, FITC-pepK5 labeling was remarkably weak, suggesting reduced enzyme activity of TGase1 molecules expressed in the epidermis of these patients.

In the epidermis of any LI patient, no significant difference in pepK5 labeling pattern and intensity was seen under various experimental conditions, pH 7.4, 8.0, and 8.4, temperature 25°C, 33°C, and 37°C (data not shown).

Using FITC-conjugated pepT26 (FITC-pepT26), a preferable substrate for TGase2, only faint labeling was obtained around the granular layer cells in all of the skin samples from the patients (data not shown).

Discussion

In the first half of the present study, we examined the ability of pepK5 to detect endogenous TGase1 activity in normal human skin sections. Ca^{2+}-dependent incorporation of FITC-pepK5 into glutamine acceptor substrates was clearly seen in human epidermal keratinocytes, mainly in the upper spinous and granular layers. To date, detection of cross-linked TGase products using tissue sections has used an FITC-labeled primary amine (FITC-cadaverine) or FITC-labeled substrate peptides.[28,29] The pattern of TGase activity that we observed was consistent with that seen in the skin using FITC-cadaverine.[29] In addition, the staining sensitivity of pepK5 was remarkably higher than that of cadaverine in normal human epidermis.

As observed in immunostaining analysis, TGase1 protein localizes to the peripheral regions of the keratinocytes in the granular and upper spinous layers, consistent with previous reports.[30,31] Double fluorescence staining clearly indicated that TGase1 activity labeled with pepK5 precisely colocalized with TGase1 immunolabeling at these sites. In addition, TGase1 activity demonstrated with pepK5 overlapped with the major CCE precursor proteins, loricrin and involucrin. These findings confirm that pepK5 labeling specifically demonstrates TGase1 activity at sites of CCE formation. In the *in vitro* assay with TGase2, pepK5 reacted to a small extent at

high peptide concentration.[21] Thus, in the present study, it was necessary to check endogenous TGase2 activity in the skin samples and we confirmed that there was no significant TGase2 activity in the skin sections by FITC-labeled pepT26 labeling. From these results, we conclude that pepK5 can act as a highly sensitive and specific probe to detect *in situ* endogenous TGase1 activity in the human epidermis.

In the last half of the present study, to assess the efficacy and usefulness of pepK5 as a preferred substrate for TGase1 in evaluating TGase1 activity in LI patients, we performed *in situ* TGase1 activity assays using pepK5 as a substrate in four LI patients with *TGM1* mutations.

From the nature and sites of *TGM1* mutations in each patient and their effect on TGase1 activity, according to the protein modeling of TGase1 based on the structure of the human factor XIIIa subunit,[32] a level of remnant TGase1 activity was theoretically speculated in each case as follows.

Patient 1 is a compound heterozygote for *TGM1* nonsense mutations (Figure 2). Both nonsense mutations led to truncation of the catalytic core domain and are expected to result in a complete loss of function of TGase1 activity. Patient 2 is a homozygote for a *TGM1* deletion mutation resulting in a frameshift and premature termination in an upstream of the catalytic core domain (Figure 2). Thus, TGase1 activity is also expected to be completely abolished in the epidermis of Patient 2. In addition, all of the three truncation mutations in Patients 1 and 2 led to early termination codons. This would probably lead to complete lack of the polypeptide in the present Patients 1 and 2. Furthermore, genomic premature termination codon mutations are subject to nonsense-mediated mRNA decay resulting in mRNA degradation in some instances, depending on the mutation site.[33,34]

Patient 3 is a homozygote of a missense mutation in the center of catalytic core domain of TGase1 peptide (Figure 2). Homozygosity of this mutation is expected to result in a significant, but not complete loss of TGase1 function. Patient 4 is a compound heterozygote harboring a missense mutation in the β-sandwich domain, and the missense mutation in the center of catalytic core domain, identical to the mutation harbored by Patient 3 (Figure 2). As described above, the latter mutation in the catalytic core domain is expected to lead to a significant but only partial loss of activity of TGase1. The former mutation p.Leu204Gln in the β-sandwich domain is considered to alter protein folding, which in turn affects the protein stability of TGase 1, as suggested in other missense mutations in the β-sandwich domain.[12] This instability may result in rapid degradation of the TGase1 polypeptide and reduce TGase1 activity in the patient's epidermis, although the reduction in activity might not be as serious compared with truncation mutations in Patients 1 and 2. In addition to this simplistic view based on the position of missense mutations in the primary structure, it has been demonstrated that *TGM1* mutations in specific residues have their specific effects on the TGase1 activity, leading to specific phenotypes. For example, the distinct phenotype of self-healing collodion baby can be caused by compound heterozygous *TGM1* mutations p.Gly278Arg and p.Asp490Gly.[35] Molecular modeling and biochemical assays suggested that the high hydrostatic pressure *in utero* significantly inhibit the mutant TGase1 activity. After birth, the mutant TGase1 molecules become partially active under ordinary hydrostatic pressure, resulting in the dramatic improvement of skin symptoms in a self-healing collodion baby.[35] In addition, several *TGM1* missense mutations in specific residues were reported to cause another specific phenotype, bathing suit ichthyosis, characterized by pronounced scaling restricted to the bathing suit areas.[26,36] The affected sites are warmer body areas, and bathing suit ichthyosis is thought to be a temperature-sensitive phenotype.[26] A marked decrease of *in situ* TGase1 activity was revealed at high temperature (37°C) in the patients with bathing suit ichthyosis.[26] Recent findings have shown that wild-type TGase1 activity is clearly reduced at 25°C compared with 37°C by *in vivo* activity analysis with cadaverine as a substrate. On the other hand, in case of reconstituted mutant TGase1 molecules with the specific mutations in bathing suit ichthyosis, such as p.Arg307Gly, the TGase1 activity is increased at 33°C (and even higher at 31°C) compared with 37°C.[37] In the present study, under various temperature incubation conditions, 25°C, 33°C, and 37°C, no significant difference in the pepK5 labeling intensity was observed in normal human epidermis or in the epidermis of any LI patient, although Patient 4 had a missense mutation in Arg307 (p.Arg307Trp) in which another mutation p.Arg307Gly causing bathing suit ichthyosis phenotype was previously reported.[26] We think these discrepancies on temperature sensitivity between previous reports[26,37] and our present results may be attributable to the fact that fluorescence labeling is not completely a quantitative method. In addition, we incubated tissue sections with a substrate solution for 90 minutes in our *in situ* TGase1 activity assay. Thus, we cannot exclude the possibility that the long-time incubation might make the enzymatic reaction almost saturated and make it difficult to detect fine difference in TGase1 activity.

As the results of the present study, *in situ* TGase1 activity assays using pepK5 demonstrated a remarkably reduced or a complete lack of membrane-associated labeling in the epidermis in all patients with *TGM1* mutations compared with normal human epidermis and ichthyosis patients with *TGM1*-unrelated genetic defects. The present results indicate that pepK5 labeling can distinguish LI patients with *TGM1* mutations from normal healthy individuals and from ichthyosis patients with other causative gene mutations. In this context, specific and sensitive detection of TGase1 activity using pepK5 is thought to be a powerful tool for screening TGase1 deficiency in LI patients. Furthermore, in the present LI patients, we demonstrated that the TGase1 molecule was missing in a compound heterozygote and a homozygote for *TGM1* nonsense/truncation mutations and was present in a compound heterozygote and a homozygote for missense mutations. Accordingly, pepK5 labeling was missing in the patients with nonsense/truncation mutations, although there were weaker pepK5 signals in the

patients with missense mutations. In this context, it might be possible to differentiate LI patients with nonsense/truncation mutations and those with missense mutations, and to predict patients' clinical severity and courses from pepK5 labeling results. However, pepK5 fluorescence labeling is not a completely quantitative method and further accumulation of the pepK5 labeling data in LI cases with *TGM1* mutations is needed for its diagnostic application, especially for the prediction of clinical severity in patients.

Acknowledgments

We thank Akari Nagasaki, M.S. for her technical assistance and Associate Professor James R. McMillan for proofreading this manuscript.

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REVIEW ARTICLE

BJD
British Journal of Dermatology

FLG mutations in ichthyosis vulgaris and atopic eczema: spectrum of mutations and population genetics

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Summary

Filaggrin is a key protein involved in skin barrier function. Mutations in the gene encoding filaggrin (*FLG*) have been identified as the cause of ichthyosis vulgaris and have been shown to be major predisposing factors for atopic eczema (AE), initially in European populations. Subsequently, *FLG* mutations were identified in Japanese, Chinese, Taiwanese and Korean populations. It was demonstrated that *FLG* mutations are closely associated with AE in the Japanese population. Notably, the same *FLG* mutations identified in the European population were rarely found in Asians. These results exemplify differences in filaggrin population genetics between Europe and Asia. For mutation screening, background information needs to be obtained on prevalent *FLG* mutations for each geographical population. It is therefore important to establish the global population genetics maps for *FLG* mutations. Mutations at any site within *FLG*, even mutations in C-terminal imperfect filaggrin repeats, cause significant reductions in amounts of profilaggrin/filaggrin peptide in patient epidermis as the C-terminal region is essential for proper processing of profilaggrin into filaggrin. Thus, no genotype–phenotype correlation has been observed in patients with *FLG* mutations. A restoration of the barrier function seems a feasible and promising strategy for treatment and prevention in individuals with filaggrin deficiency.

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Accepted for publication
2 October 2009

Key words
atopic eczema, filaggrin, FLG, ichthyosis, population genetics

Conflicts of interest
None declared.

DOI 10.1111/j.1365-2133.2009.09582.x

Mutations in *FLG*, the gene encoding profilaggrin/filaggrin, have been identified as the underlying cause of ichthyosis vulgaris (IV; OMIM 146700)[1] and have also been shown to predispose patients to atopic eczema (AE).[2] Although *FLG* is very difficult to analyse because of its large size (> 12 kb) and highly repetitive nature, a polymerase chain reaction (PCR) strategy that permits routine and comprehensive sequencing of the entire coding region has recently been developed.[3] Using this method and the information from other identified mutant alleles, filaggrin mutation searches have been carried out in a variety of geographical populations including European and Asian populations. Based on the information of population-specific *FLG* mutations, many cohort studies of AE for *FLG* mutations have been performed and approximately 25–50% of patients with AE were revealed to harbour *FLG* mutations as a predisposing factor.

Skin barrier defects caused by *FLG* mutations are thought to play a crucial role in the pathogenesis of atopic disorders including AE, asthma and allergic rhinitis. Recently, it was demonstrated that mice deficient in filaggrin expression show enhanced transfer of antigens through the epidermis, thus providing compelling experimental proof for the barrier hypothesis in AE pathogenesis.[4] This review provides an overview of *FLG* population genetics because the information is essential for global *FLG* mutation screening in patients with AE.

Filaggrin is an indispensable component for the skin barrier

Filaggrin is initially synthesized as profilaggrin, a > 400-kDa, highly phosphorylated, histidine-rich polypeptide, which comprises a S100 calcium-binding domain, a B-domain and two imperfect filaggrin-repeat domains flanking 10–12 essentially identical filaggrin repeats, as well as a C-terminal domain.[3,5] During the keratinization of epidermal keratinocytes, keratohyaline granule degradation products subsequently occupy the cytoplasm of keratinized cells in the stratum corneum and play important roles in skin barrier function.[6] Keratohyaline granules in the granular layer of the epidermis are predominantly composed of profilaggrin polyproteins.[7–9]

Upon keratinocyte terminal differentiation, profilaggrin is dephosphorylated and cleaved into 10–12 essentially identical 37-kDa filaggrin peptide units. The liberated filaggrin subsequently and highly efficiently aggregates the keratin filament cytoskeleton,[7] causing the collapse of the granular cells into

flattened squames. The collapsed cytoskeleton is crosslinked by transglutaminases to bind it to the cornified cell envelope. Filaggrin degradation products also contribute to moisture retention in the cornified layers as a natural moisturizing factor.[6,10] Thus, filaggrin is a key epidermal protein essential for the formation of a normal, intact, protective and correctly moisturized skin barrier.[6,11]

Filaggrin deficiency caused by *FLG* mutations results in ichthyosis vulgaris

IV is a common inherited skin disorder exhibiting scaling and dry skin typically on the flexor limbs and lower abdomen, associated with palmoplantar hyperlinearity.[1,11,12] Histologically, IV is characterized by a decrease in the size and number or complete absence of keratohyaline granules in the upper epidermis.[12] Marked reduction in epidermal keratinocyte filaggrin due to *FLG* loss-of-function mutations was identified as the cause of IV.[1] Loss or reduction of filaggrin expression correlates with excessively dry skin and impaired barrier function, which variously manifests as IV.

Filaggrin mutations are a major predisposing factor for atopic eczema in Europe

AE is among the most common diseases in children from developed countries. Despite considerable efforts to elucidate AE susceptibility genes and to clarify the genetic background of atopic disorders, until recently no strong and reproducible genetic factor has been identified.[13] It has long been proposed that a permeability barrier abnormality in AE is not just an epiphenomenon but is rather an important driver of disease activity[14] because the level of the permeability barrier abnormality precisely parallels AE severity[15,16] and both clinically uninvolved skin regions and skin sites cleared of inflammation for as long as 5 years continue to show significant barrier abnormalities.[17]

As mentioned above, filaggrin is a major epidermal moisturizing factor and significantly contributes to the skin barrier function. For a long time, we as dermatologists have realized that AE often occurs in patients with IV,[18–20] although the pathophysiological mechanisms of this co-occurrence have not been fully clarified. Linkage of AE to the chromosome locus 1q21, containing the epidermal differentiation complex where *FLG* resides, has also been reported.[21] In addition, decreased filaggrin expression has been reported in the skin of patients with AE at both mRNA and protein levels.[22,23] Palmer *et al.*[2] initially reported that decreased or absent *FLG* expression due to loss-of-function mutations leads to impaired barrier function which manifests as AE.

Subsequently, it was confirmed that the strong effect of *FLG* mutations on AE risk exceeds that of any other candidate predisposing gene for AE identified so far.[24] A correlation between *FLG* mutations and eczema is one of the most robust genotype–phenotype linkages in complex trait genetics and several case–control association studies have been reported to date.[3,24–30] These studies have established *FLG* as a major genetic factor predisposing for AE, although they showed considerable differences in study design and strength of the genetic effect.

Henderson *et al.*[31] sought to determine the natural history and course of atopic diseases conferred by the two most common *FLG* mutations in a large, population-based birth cohort study in the U.K. and reported that eczema associated with these *FLG* mutations presents in early life and is more persistent. The risk of asthma was remarkably high in the context of eczema and firm associations were confirmed with sensitization to multiple allergens including grass, house dust mite and cat dander.

Prevalent filaggrin mutations are distinct in each population

To date, it has generally been considered that *FLG* mutations are a significant predisposing factor for AE in Europeans, Asians and quite possibly most other races worldwide to differing degrees.

Mutations in *FLG* were initially identified in European families. After the establishment of sequencing methods for the entire *FLG* coding region,[1–3] to date approximately 40 loss-of-function *FLG* mutations have been identified in IV and/or AE.[32,33]

Major differences exist in the spectra of *FLG* mutations observed between certain globally distinct ancestral groups. In the European population, the genetic spectrum of *FLG* mutations is complicated, with up to six recurrent mutations and several other family-specific mutations, and the two mutations R501X and 2282del4 are the most prevalent in the U.K. population (Fig. 1).[3]

From 2006 to date, to establish baseline *FLG* mutation data in the Japanese population, we performed *FLG* mutation searches in more than 30 Japanese families with IV. We carried out comprehensive sequencing of the entire *FLG* coding region using an overlapping PCR strategy and identified four Japanese population-specific mutations in *FLG*, c.3321delA, p.Ser2554X, p.Ser2889X and p.Ser3296X.[34,35] Two *FLG* mutations among them, p.Ser2889X and p.Ser3296X, were reported later by another Japanese group independently using shotgun methods.[36] In 2009, we reported two additional novel *FLG* mutations, p.Ser1695X and p.Gln1701X, in the Japanese population.[37] Furthermore, we studied 19 newly recruited Japanese patients with AE and identified a novel *FLG* nonsense mutation c.12069A>T (p.Lys4021X) in one patient with AE without any other known Japanese *FLG* mutation (Fig. 1).[33] In addition, one of the common European mutations p.Arg501X was reported in a Japanese family, although the mutant allele with p.Arg501X reported in the Japanese family was shown to be on a different haplotype from the common European variant of the same residue.[38] Thus, the Japanese p.Arg501X mutation was thought to arise separately.[38] This p.Arg501X mutation is a CpG mutation and can arise commonly as well as being present in Europeans as an

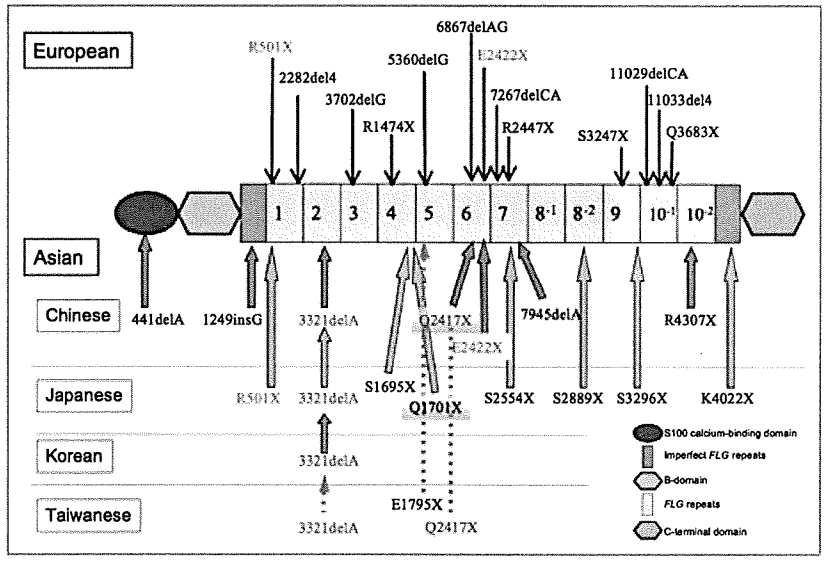

Fig 1. Reported *FLG* mutations shown in a scheme of profilaggrin peptide. Profilaggrin contains 10–12 highly homologous filaggrin-repeat domains. Note that *FLG* mutations in the European and the Asian populations appear to be unique in each population. Only two mutations shown in green (R501X and E2422X) were reported in both European and Asian populations. 3321delA, shown in red, was found in all the four East Asian populations. Q2417X, shown in blue, was reported in both Chinese and Taiwanese populations. Mutations are distributed widely in the profilaggrin sequence and the mutation p.Lys4022X (K4022X) we reported recently[29] is the most distal mutation located in the C-terminal incomplete filaggrin repeat. Some individuals have duplication of the 8th and/or 10th filaggrin repeat(s). Duplicated filaggrin repeats are represented as 8^{-1}, 8^{-2}, 10^{-1} and 10^{-2}.

ancient, ancestral mutation. In total, there are at least eight *FLG* variants in the Japanese population.

A Japanese AE case–control study for the eight *FLG* mutations demonstrated that about 27% of the patients in our Japanese AE case series carry one or more of the eight *FLG* mutations and that these variants are also carried by 3·7% of Japanese control individuals.[33] It was thus confirmed that *FLG* mutations are significantly associated with AE in the Japanese population (Fig. 2).

In other Asian populations, for example the Singaporean Chinese population, it was reported that *FLG* mutations are again different from those found in Europeans and Japanese.[39] In total, six *FLG* mutations, five previously unreported mutations and one known mutation, were found in eight Singaporean Chinese patients with IV.[39] The known mutation was previously identified in a single patient with IV from the Netherlands[3] and, in fact, the patient had Chinese ancestry.[39]

Examining the Taiwanese population, we examined 12 individuals from four unrelated Taiwanese families with IV and identified three *FLG* mutations.[40] One mutation, E1795X, was a previously unidentified *FLG* mutation which might be Taiwanese specific. Interestingly, another *FLG* mutation, 3321delA, is prevalent both in the Japanese population[34] and the Chinese population.[3] This mutation 3321delA was also reported in a Korean patient with IV.[41] The other mutation, Q2417X, was found in the Singaporean Chinese population. No *FLG* mutation identified in the European population was found in the Taiwanese population. The present findings suggest that the Taiwanese population, as an East Asian group, shares *FLG* mutations with both the Japanese and the Chinese populations. These results exemplify differences in filaggrin population genetics between Europe and Asia (Fig. 1).

As mentioned above, most *FLG* mutations are specific to each population, such as European,[3] Japanese,[33,35,37] Singaporean Chinese[39] and Taiwanese.[40] Major differences exist in the spectra of *FLG* mutations observed between different ancestral groups. Prevalent *FLG* mutations are distinct in both the European and the Asian populations. In addition, there is a need for assessing the ancestral admixture in geographical regions in order to know precisely the spectrum and preferential occurrence of *FLG* mutations in different populations.

Every population is likely to have a unique set of *FLG* mutations. Population differences highlighted by *FLG* mutations make it difficult to perform world-wide screening for *FLG* mutations in patients with AE. We cannot perform *FLG* mutation screening in one population using the *FLG* mutations reported in other populations. For example, we cannot use the prevalent European *FLG* mutations when we perform screening of Asian patients with AE. For the mutation screening, we have to obtain information on prevalent *FLG* mutations in each population. It is therefore important to establish global population genetic maps for patient *FLG* mutations.

No genotype–phenotype correlation in *FLG* mutations so far

Genotype–phenotype correlation in *FLG* mutations is lacking. *FLG* truncation mutations at any site within the profilaggrin

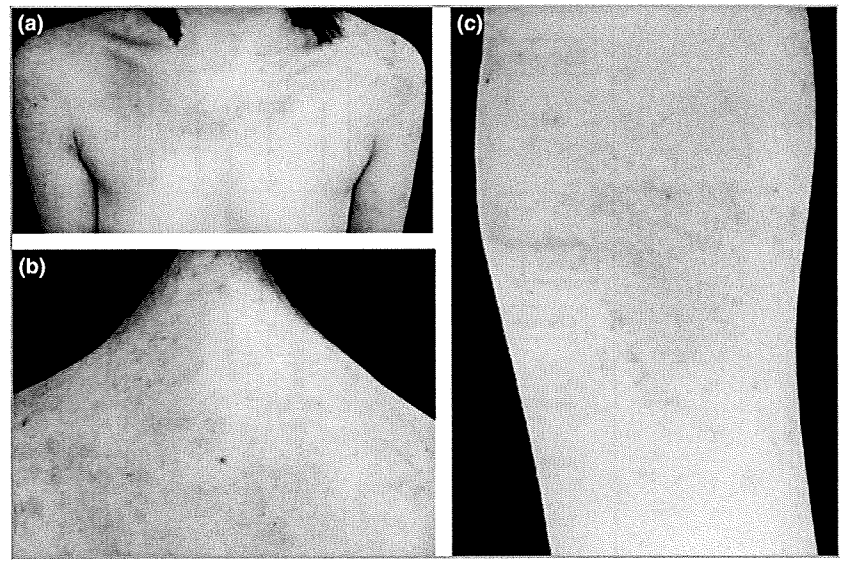

Fig 2. Clinical features of a patient with atopic eczema with compound heterozygous *FLG* mutations. (a) Erythematous lesions and reddish papules with scratch marks and lichenification are seen on the chest (a), back (b) and arm (c). Mutation screening revealed that the patient is a compound heterozygote for *FLG* mutations c.3321delA and p.Ser2554X.

peptide were reported uniformly to result in severe deficiency of profilaggrin/filaggrin processing.[3] It has been hypothesized that the C-terminal region of profilaggrin is essential for proper processing of profilaggrin to filaggrin and, in due course, truncation at any site of profilaggrin results in abolishment of filaggrin/profilaggrin peptides.[3] The nonsense mutation p.Lys4022X that we identified most recently[33] is in the C-terminal incomplete filaggrin repeat and is the most distal mutation among those previously reported. In the epidermis of the patients carrying this mutation, *FLG* mRNA expression including messages derived from the mutant alleles was not significantly reduced. However, histopathologically the size of keratohyaline granules in the granular layers decreased and immunohistochemically profilaggrin/filaggrin peptides were remarkably reduced in the patients' epidermis.[33] These observations further support the hypothesis that the profilaggrin C-terminal region is essential for proper profilaggrin processing. In this context, it is now generally considered that all the truncation mutations lead to serious loss of profilaggrin/filaggrin peptides, resulting in a lack of genotype–phenotype correlations as regards *FLG* mutations in IV or AE.

Novel skin barrier-oriented care and prevention approach to atopic eczema

The concept of epidermal barrier dysfunction caused by *FLG* mutations as a major contributor to the pathogenesis of AE has opened up a new era over the past few years. It is now believed that, at least in a subset of patients with AE, the skin barrier defect is the primary event that initiates disease pathogenesis, allowing the entrance of numerous antigens into the epidermis.[42] Thus, restoration of barrier function seems a feasible and promising strategy for prophylactic treatment of AE in an individual with a filaggrin deficiency.

The range of clinically valuable methods to restore skin barrier function in individuals harbouring *FLG* mutations includes general moisturization measures, or specific lipid replacement therapy. Moisturizers have already been widely used in AE[43] and have been shown to reduce topical steroid use by a specialist dermatology nurse.[44] Lipid replacement therapy is well under development as a triple-lipid, ceramide-dominant, barrier repair therapy for AE, that is provided in an acidic formulation.[43]

One clinical study supports the efficacy of targeted, ceramide-dominant lipid replacement therapy in AE.[15] In the study, topical application of a ceramide-dominant, physiological lipid-based emollient improved skin barrier defects and reduced AE severity significantly in the majority of patients.

Regarding the association between filaggrin deficiency and sensitization to specific antigens: during early life allergen exposure may increase the risks of AE, but the protective effect of reduction in allergen exposure remains uncertain. According to the population-based, longitudinal birth cohort study by Henderson *et al.*,[31] eczema associated with *FLG* mutations presents in early life and is more persistent. In addition, a strong association of *FLG* mutations was identified with sensitization to grass, house dust mite and cat dander. Our study revealed that AE disease severity and specific IgE for house dust, mite allergen and cat dander were significantly correlated in *FLG* mutation-related patients with AE.[45]

In this context, if we select patients with *FLG* mutations and perform early intervention to reinforce/improve their skin barrier function and reduce sensitization to allergens, we may achieve a significant prophylactic effect against AE development. Further studies are required to clarify the preventive effect of early intervention to AE in filaggrin-deficient, high-risk children.

Acknowledgments

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan to M.A. (Kiban B 20390304).

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Journal of Dermatological Science 57 (2010) 183–191

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.elsevier.com/jds

Topical application of anti-angiogenic peptides based on pigment epithelium-derived factor can improve psoriasis

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ARTICLE INFO

Article history:
Received 5 August 2009
Received in revised form 17 December 2009
Accepted 17 December 2009

Keywords:
Angiogenesis
Keratinocyte
Psoriasis
Pigment epithelium-derived factor
Vascular endothelial growth factor

ABSTRACT

Background: Psoriasis is a common chronic inflammatory skin disorder with a high prevalence (3–5%) in the Caucasian population. Although the number of capillary vessels increases in psoriatic lesions, there have been few reports that have specifically examined the role of angiogenesis in psoriasis. Angiogenic factors, such as vascular endothelial growth factor (VEGF), may dominate the activity of anti-angiogenic factors and accelerate angiogenesis in psoriatic skin.
Objective: We investigated to identify small peptide mimetics of PEDF that might show anti-angiogenic potential for the topical treatment for psoriasis.
Methods: We examined the expression of PEDF in skin by immunohistochemical staining, immunoblotting, and RT-PCR. To identify potential PEDF peptides, we screened peptides derived from the proteolytic fragmentation of PEDF for their anti-proliferative action. Anti-psoriatic functions of these peptides were analyzed using a mouse graft model of psoriasis.
Results: The specific low-molecular weight peptides (MW < 850 Da) penetrated the skin and showed significant anti-angiogenic activity in vitro. Topical application of these peptides in a severe combined immunodeficient mouse model of psoriatic disease led to reduced angiogenesis and epidermal thickness.
Conclusions: These data suggest that low-molecular PEDF peptides with anti-angiogenic activity may be a novel therapeutic strategy for psoriasis.

1. Introduction

Psoriasis is a common skin disease affecting 0.5–3% of the Caucasian population [1]. Histopathologically, this disorder is characterized by accelerated epidermal proliferation, by the infiltration of inflammatory cells into the epidermis and upper dermis, and by telangiectasia in the superficial dermis. Although the molecular pathogenesis of psoriasis remains unclear, several hypotheses have been proposed. Activated T lymphocytes infiltrate into the lesional skin areas where they secrete a variety of cytokines such as tumor necrosis factor (TNF)-α, interferon-γ, IL-2 and IL-12, and thus play an important role in psoriatic inflammatory changes [2]. In addition, epidermal proliferation is influenced by inappropriate vascular expansion in the superficial dermis [3]. Furthermore, these microvascular changes in psoriatic skin lesions include pronounced capillary dilatation, increased vessel permeability and endothelial cell proliferation and protrusion into the dermal papillae capillaries. Therefore inappropriate angiogenic growth has been proposed to contribute to the pathogenesis of psoriasis [4,5]. The overexpression of angiogenic factors also occurs; for instance, vascular endothelial growth factor (VEGF) is strongly up-regulated in psoriatic skin lesions [6].

There have been a number of therapeutic strategies devised for psoriasis. Topical steroids, topical vitamin D3 analogs, oral retinoids, UV irradiation such as PUVA and narrow-band UVB, cyclosporine and other immunosuppressants have been widely used. In addition, biological agents that target cytokines such as TNF-α have recently been developed [7]. Most strategies are aimed at reducing the inflammatory reaction and epidermal proliferation, but yet there have been few agents targeting angiogenesis in psoriasis.

Pigment epithelium-derived factor (PEDF) is a glycoprotein that belongs to the superfamily of serine protease inhibitors, and it was

Abbreviations: PEDF, pigmented epithelium-derived factor; VEGF, vascular endothelial growth factor.

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0923-1811/$36.00
doi:10.1016/j.jdermsci.2009.12.010

first identified as a retinal pigment epithelium-derived protein with neuronal differentiating activity [8]. Recently, PEDF has been shown to have potent anti-angiogenic activity in cell culture and in animal models. PEDF inhibits retinal endothelial cell growth and migration, and it suppresses ischemia-induced retinal neovascularization [9]. In addition, we reported that PEDF inhibits malignant melanoma growth by suppressing tumor angiogenesis [10]. These observations led us to hypothesize that an imbalance in anti-angiogenic factors potentially involving PEDF may contribute to the pathogenesis of psoriasis. PEDF also shows anti-inflammatory activity, suggesting an additional, ameliorative role in the control of inflammation and keratinocyte proliferation.

In this study, we examined PEDF protein production in psoriasis lesions and in normal skin, and we investigated the effect of PEDF on keratinocyte proliferation in vitro and on psoriatic skin in a murine xenograft model. We also report the identification of low molecular weight PEDF peptides that show anti-angiogenic activity after topical application.

2. Experimental procedures

2.1. Patients

Sera were obtained from 21 psoriasis patients (13 males and 8 females, and mean age 46.9 years) and 14 healthy volunteers (males 7 and females 7, and mean age 42.2 years) from the Department of Dermatology, Hokkaido University Hospital. The diagnosis of psoriasis was made on the basis of clinical images and histopathological findings from skin biopsies. The enrolled patients had generalized plaque psoriasis, which were evaluated by a single qualified dermatologist. Three skin tissue specimens were obtained from each psoriatic lesion. Normal skin tissues also were obtained from healthy volunteers. Informed consent was obtained from each volunteer according to the Declaration of Helsinki Principles. All the experiments using human samples were performed under the approval of the ethical committee of Hokkaido University.

2.2. Experimental mice

The C.B-17/Icr-scid/scidJcl SCID mouse (Clea, Tokyo, Japan) was used for xenotransplantation experiments. All the animal experiments were performed under the approval of the ethical committee for animal studies in Hokkaido University.

2.3. Immunohistochemistry

The paraffin-embedded skin tissues from psoriasis patients were cut into 4 μm-thick sections. The sections were deparaffinized, incubated with 0.1% trypsin at 37 °C for 15 min. Endogenous peroxidase activity was inhibited by pretreatment with 3% hydrogen peroxide. The sections were then treated with 10% normal goat serum at room temperature for 30 min, followed by incubation with the anti-PEDF antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight. After washing, the sections were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG at room temperature for 30 min and the PEDF-positive staining visualized with diaminobenzidine (Dojin, Kumamoto, Japan) as a chromogen and hematoxylin as a counterstain.

For immunofluorescence, skin tissues were immediately embedded in optimal cutting temperature (OCT) reagent (Sakura Finetechnical, Tokyo, Japan) and snap-frozen in liquid nitrogen. Cryosections of 5 μm were prepared, washed with PBS, and then fixed in cold acetone for 10 min at −20 °C. Primary and secondary antibodies were applied at room temperature for 1 h. The sections were finally washed with PBS and mounted on microscope slides. The samples were analyzed using a Fluoview confocal laser scanning microscope (Olympus, Nagano, Japan). The following antibodies were used: rat anti-mouse CD31 antibody, anti-mouse CD3 antibody, anti-mouse Gr-1 antibody, and anti-mouse CD11b antibody (BD Biosciences, San Jose, CA), rabbit polyclonal anti-pankeratin antibody (PROGEN Biotechnik, Heidelberg, Germany), rabbit polyclonal anti-Ki67 antibody (Novocastra, Newcastle, UK), FITC-conjugated goat anti-rabbit antibody, FITC-conjugated goat anti-rat antibody (Jackson ImmunoResearch, West Grove, PA), TRITC-conjugated anti-rabbit antibody (Southern Biotechnology Associates, Birmingham, AL).

2.4. Immunoblots

Skin tissues of normal volunteers and psoriasis patients were treated with 1 M sodium hydroxide at 4 °C overnight, and the epidermal sheets easily removed from the dermal components. These tissues were frozen and then homogenized in PBS. Samples obtained from epidermis and dermis were electrophoresed on SDS–PAGE. Proteins on the gel were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) and the membranes probed with first antibody at 4 °C overnight, washed three times for 5 min, and then incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. Proteins were visualized with a Konica immunostaining kit (Konica, Tokyo, Japan). The following antibodies were used: anti-PEDF and anti-VEGF rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-α-tubulin mouse monoclonal antibody (Sigma, St. Louis, MO), HRP-conjugated goat anti-rabbit IgG, and HRP-conjugated goat anti-mouse IgG (Biosource, Camarillo, CA). We used anti-PEDF at 1:200, and the secondary antibodies at 1:1000 dilutions.

2.5. RT-PCR analysis

RNA (0.5 μg) was used to produce cDNA using a reverse transcription kit (Sigma, Poole, Dorset, United Kingdom). PCR was done using a 2400 thermocycler (Perkin-Elmer, Norwalk CT) with conditions set to 40 s at 94 °C, 60 s at 55 °C, and 60 s at 72 °C (30 cycles). The quality of DNA was verified by 0.59 kb β-actin PCR products using primers (forward 5′-ATGATATCGCCGCGCTCGTC-3′; reverse 5′-CGCTCGGTGAGGATCTTCA-3′). PEDF forward and reverse primers were 5′-GGTGCTACTCCTCTGCATT-3′ and 5′-ACTGAACCTGACCGTACAAGAAAGGATCCTCCTCCTC-3′. PCR products were separated by 2% agarose gel and visualized under UV light following ethidium bromide staining.

2.6. Preparations of PEDF proteins

The PEDF proteins were purified as described previously [9]. Briefly, 293T cells (ATCC, Rockville, MD, USA) were transfected with the recombinant vector pBK-CMV-C terminally hexahistidine-tagged PEDF using FuGENE® 6 transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The PEDF proteins were purified from conditioned media by a Ni-NTA spin kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of purified PEDF proteins revealed a single band with a molecular mass of about 50 kDa, which showed positive reactivity with monoclonal antibodies directed against human PEDF (Transgenic, Kumamoto, Japan).

2.7. PEDF enzyme-linked immunosorbent assay

A PEDF enzyme-linked immunosorbent assay was performed as previously reported [11]. Briefly, a 96-well microtiter plate (Nalge

Nunc International, Rochester, NY) was coated by overnight incubation with anti-PEDF monoclonal antibody (Transgenic, Kumamoto, Japan). Samples were diluted 50-fold in 10 mM PBS pH 7.4, 0.25% BSA and 0.05% Tween-20, and then incubated at room temperature for 2 h. After washing, a biotinylated anti-human PEDF polyclonal antibody (R&D Systems, Minneapolis, MN) was added and incubation continued for 2 h at room temperature. The plate was then incubated with HRP-conjugated streptavidine solution (Zymed, South San Francisco, CA) at room temperature for 30 min. After washing, the chromogenic substrate solution (Dako, Tokyo, Japan) was added and the plate was incubated at room temperature for 15 min. Optical densities were measured at 450 nm and protein concentrations calculated from a standard curve generated by a curve-fitting program (Berthold Technology, Bad Wildbad, Germany).

2.8. PEDF secretion from cultured keratinocytes and fibroblasts

Normal human epidermal keratinocytes (NHEKs) were purchased from Clontech (Mountain View, CA and cultured in KGM® medium (Cambrex, East Rutherford, NJ) until 70% confluence. Normal human fibroblasts were purchased from Dainippon Seiyaku (Osaka, Japan) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% FBS, 1% penicillin, 1% streptomycin and 1% amphotericin B until 70% confluence. The cells were expanded in 12 cm sterile culture dish with 10 ml of medium, and then stimulated with lipopolysaccharide (Sigma) at 37 °C for 72 h. Media was collected 1 day after stimulation. PEDF concentrations in collected medium were assessed by ELISA as described above.

2.9. Keratinocyte proliferation assay

NHEKs were seeded into 96-well plates at a concentration of 10^3 cells in 100 μl of medium per well. After cultivation with 1, 10, 100 nM recombinant PEDF [12] and/or 100 ng/ml recombinant VEGF (R&D systems) for 2 and 4 days, 10 μl of Cell Counting Kit (Dojin) was added to each well. After incubation for 2 h, the absorbance at 450 nm was measured on a microplate reader.

2.10. Treatment of the grafted skin lesions with recombinant PEDF

A graft bed of approximately 1 cm^2 was created on the shaved area of the back of a 7 to 8-week-old anesthetized SCID mouse by removing the full-thickness skin and keeping the vessel plexus intact on the fascia overlying back muscles. The human skin obtained by biopsy was washed in PBS containing 1% penicillin, 1% streptomycin and 1% amphotericin B, and fatty deposits were removed by gentle dissection. The full-thickness human skin graft was placed onto wound bed. The transplants were held in place using 5/0 silk suture material, and 1% gentamicin sulfate ointment was applied. The graft was covered with an adhesive wound

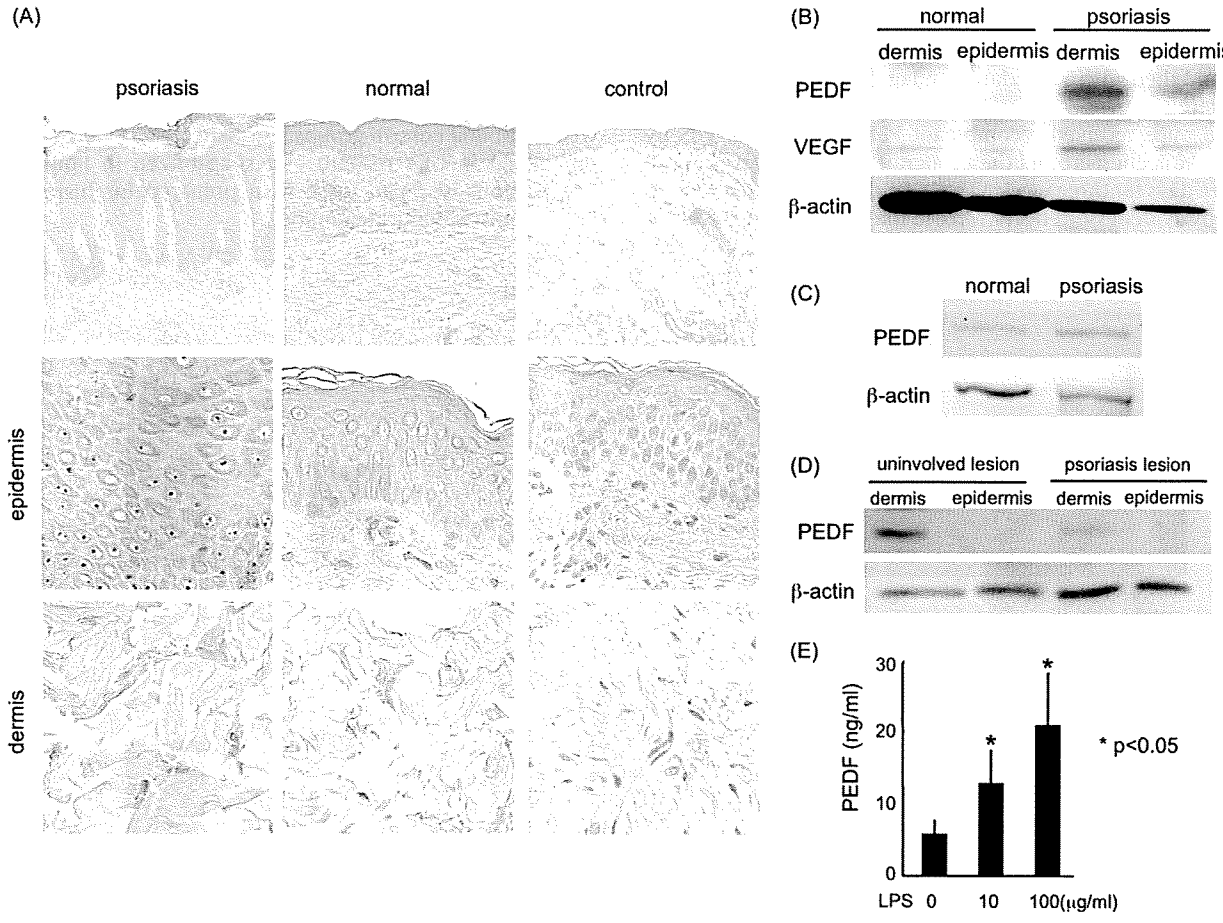

Fig. 1. PEDF is expressed in both the epidermis and dermis. (A) Immunohistochemistry of normal and psoriatic skin lesions. In normal skin, PEDF was detected in both the epidermis and the dermis. PEDF was significantly up-regulated in psoriatic epidermis in comparison with normal epidermis. (B) The expression of PEDF protein was up-regulated in psoriasis lesions. Positive bands were identified with a molecular weight of about 50 kDa from both the epidermis and dermis, which corresponds to the molecular weight of PEDF. (C) PEDF mRNA levels were analyzed using RT-PCR. The expression of PEDF mRNA was slightly up-regulated in psoriasis lesions compared to that of normal skin. (D) The expression of PEDF protein was up-regulated in uninvolved lesion of psoriasis patient compared to psoriasis lesion. (E) The levels of PEDF in the supernatants of cultured normal human keratinocytes were assessed by ELISA. LPS (10 or 100 μg/ml) was used to stimulate keratinocyte production of PEDF (*$p < 0.05$).

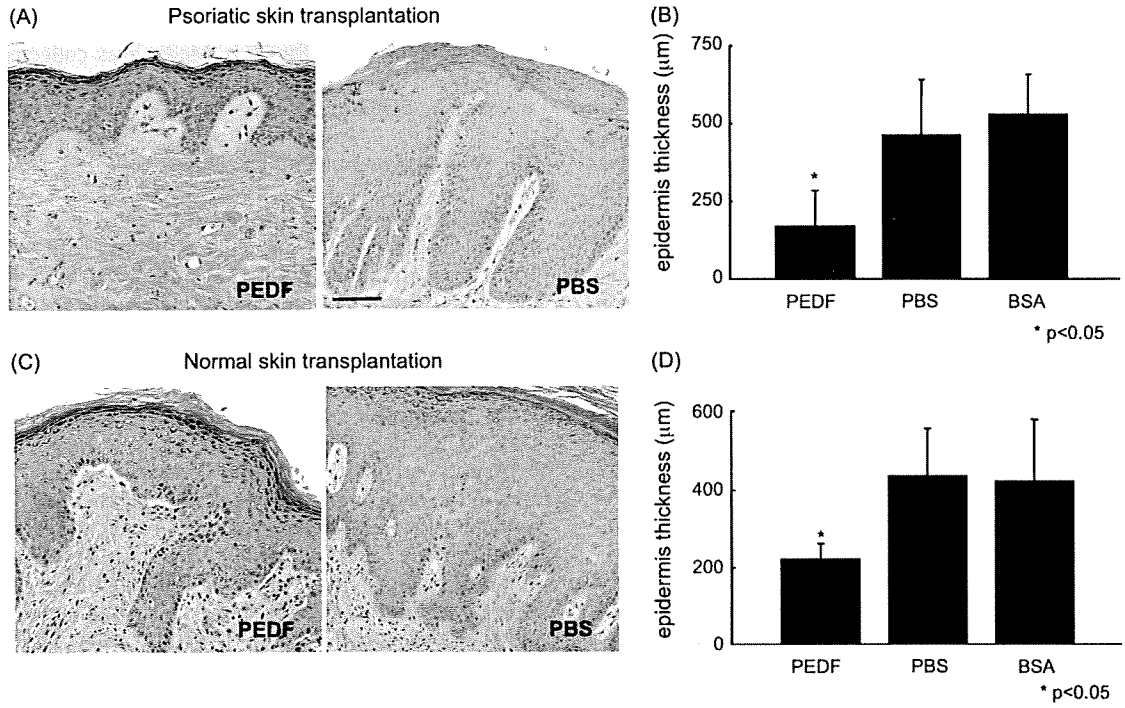

Fig. 2. Intradermal PEDF administration reduced the thickness of grafted epidermis in xenotransplanted SCID mice. Acanthosis was significantly reduced both in psoriatic (A, B) and normal (C, D) skin when compared to PBS or BSA injected groups. Scale bar, 50 μm. Values shown are means and SDs based on four to six measurements per histological section in four histological sections per mouse from duplicate mice transplanted with skin samples from four donors (*$p < 0.05$).

dressing and then with a standard bandage. Dressing material and sutures were removed 7 days after transplantation.

Grafted mice received recombinant PEDF in 50 μl of PBS by intradermal injection around the xenograft lesion at 30 μg/mouse every three days for three weeks. The PEDF dose was well tolerated without any evident side effects. Mice in the control group received the same volume of PBS or BSA (30 μg). The day after the last injection, biopsies were collected from the transplants from both treatment and control groups. The skin tissues were immediately embedded in OCT reagent and snap-frozen in liquid nitrogen. Cryosections of 5 μm were then prepared for histological and immunohistochemical staining.

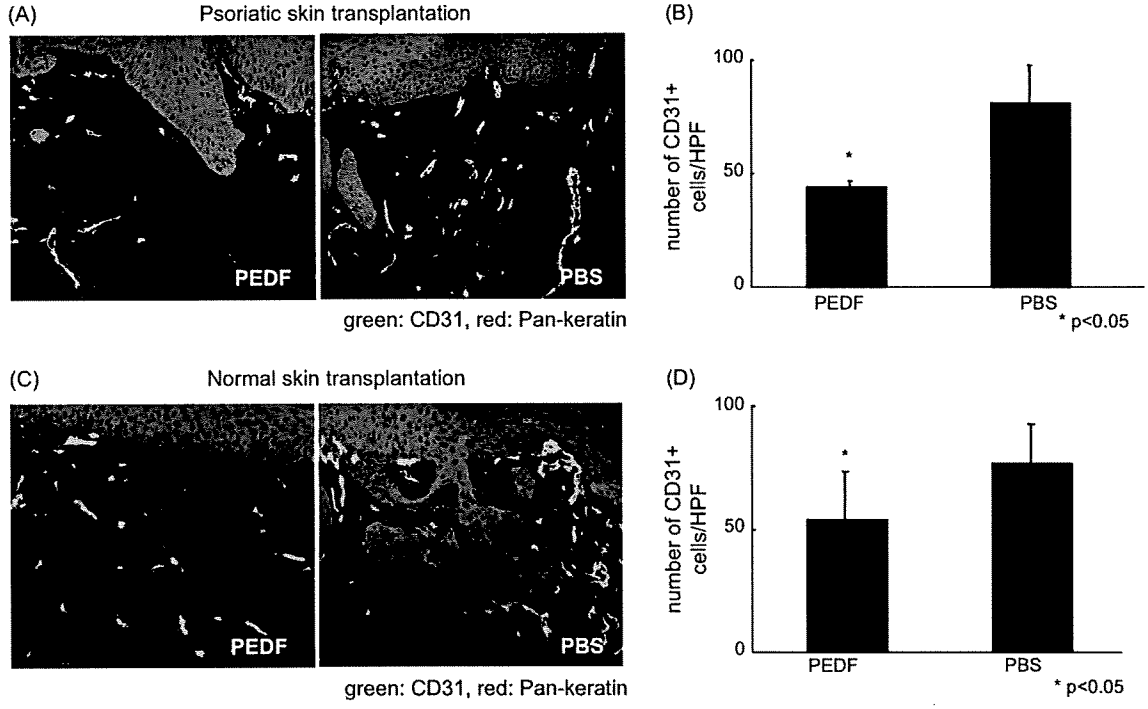

Fig. 3. Intradermal PEDF administration reduced angiogenesis of grafted epidermis. CD31-positive cells (capillary endothelial cells) were enumerated by immunofluorescence after the treatment of psoriatic (A, B) and normal skin (C, D) with PEDF or PBS. Quantification of CD31-positive blood vessels per 100× microscopic field in human skin grafted areas. The data are presented as mean CD31-positive blood vessel numbers per 100× microscopic field, ±SD (*$p < 0.05$).

2.11. Identification of functional PEDF peptides

Full-length human PEDF cDNA was divided into three parts. Polymerase chain reaction (PCR) products digested by NdeI and SalI were ligated into the multiple cloning site of expression vector pGEX-6P-1 (Amersham Biosciences, Buckinghamshire, United Kingdom). Sequences of the sense and antisense primers were: 5′-AAACATATGCAGGCCCTGGTGCTACTCCTCTGCAT-3′ and 5′-CCCGTCGACTTATGACTTTTCCAGAGGTGCCACAAA-3′ for amplifying F1 cDNA fragment, 5′-AAACATATGTATGGGACCAGGCCCAGAGTCCTGA-3′ and 5′-CCCGTCGACTTAGTCATGAATGAACTCGGAGGTGA-3′ for F2, and 5′-GGGCATATGATAGACCGAGAACTGAAGACCGTGCA-3′ and 5′-AAAGTCGACTTAGGGGCCCCTGGGGTCCAGAAT-3′ for F3. Each human PEDF fragment was purified according to the method of Walker et al. [13]. Human PEDF peptides (see Fig. 6) were synthesized (Sigma–Aldrich, Tokyo, Japan). MG63 human osteosarcoma cells (Health Science Research Resources Bank, Tokyo, Japan) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum (FBS) (ICN Biomedicals Inc., Aurora, OH, USA) and 100 units/ml penicillin/streptomycin. PEDF fragment or peptide treatment was carried out in a medium containing 0.1% of FBS. HUVECs and MG63 cells were treated with or without 100 nM PEDF protein, fragments (F1–F3) or peptides (P1–P6, P5–1, P5–2, and P5–3) or VEGF (25 ng/ml) for 24 h. HUVECs additionally were treated with 100 ng/ml recombinant VEGF (R&D systems) for 2 and 4 days. Cells were incubated with [^{3}H]thymidine (Amersham Bioscience) or 5-bromo-2′deoxyuridine (BrdU) (Roche, Basel, Switzerland) for the last 4 h of culture and proliferation assessed as described previously [14,15].

For the analysis of p21 production, 50 μg of whole cell lysates were prepared and assayed for the expression of p21 and β-actin by Western blotting. Reaction with antibodies and detection with an enhanced chemiluminescence detection system (Amersham Biosciences) were performed as described previously [16].

2.12. Skin penetration of topical applied PEDF peptide

Biotin-labeled PEDF peptide (Sigma–Aldrich) was dissolved in PBS (1 mM) and 70 μl applied to the mouse skin. After 2 h, the applied site was removed and localization in the skin was determined by rhodamine–avidin staining (BD Biosciences). CD31 staining (BD Biosciences) was performed simultaneously and the samples analyzed using a Fluoview confocal laser scanning microscope (Olympus). The experiments of peptide application were repeated 3 times, and 3 mice were used in each experiment.

2.13. Treatment of the grafted psoriatic lesions with PEDF peptide

PEDF peptide was dissolved in PBS (1 mM) and 70 μl of the solution was applied on the grafted site daily for 14 days. No side effects were apparent at the applied sites. Mice in the control group received the same volume of PBS. Biopsies were collected on the day following the last injections and analyzed as described above.

2.14. Statistical analysis

Data were analyzed using unpaired, 2-tailed Student's *t* test. A *p* value less than 0.05 was considered significant.

3. Results

3.1. PEDF is highly expressed in epidermal psoriasis lesions

Immunohistochemical analysis revealed that PEDF protein is present in the cytoplasm of keratinocytes of both psoriatic and normal skin (Fig. 1A). In the dermis, fibroblasts also were positive for PEDF, but the staining was less intense than in the epidermis. Western blotting analysis of human epidermal and dermal proteins revealed a single band with a molecular weight of about 50 kDa (Fig. 1B). PEDF protein and mRNA levels were significantly higher in psoriasis lesions when compared to normal skin (Fig. 1C).

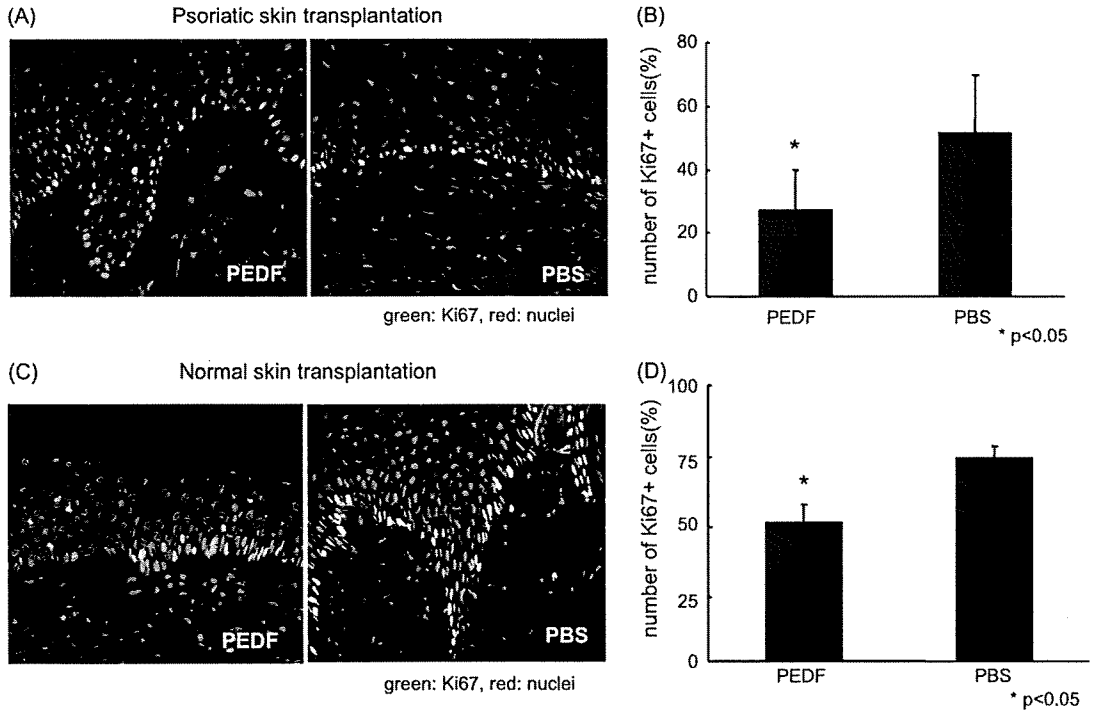

Fig. 4. Epidermal proliferation in basal keratinocytes is inhibited by PEDF. Ki-67-positive (proliferating) cells were stained and enumerated by immunofluorescence after the treatment of psoriatic (A, B) and normal skin (C, D) with PEDF or PBS. The Ki-67-positive keratinocytes in the basal layer were enumerated and the percentage of Ki-67-positive cells in basal layer calculated (*$p < 0.05$).

Interestingly, PEDF protein levels of uninvolved lesion of psoriasis patient were higher than that in psoriasis lesions (Fig. 1D). VEGF also was increased in psoriatic skin, which is consistent with prior reports [6].

3.2. PEDF secretion from cultured keratinocytes after lipopolysaccharide stimulation

PEDF was constitutively secreted by cultured keratinocytes (Fig. 1E) and after LPS stimulation, its secretion was significantly up-regulated in a dose-dependent manner ($p < 0.05$). Fibroblasts by contrast failed to show up-regulation of PEDF secretion after LPS or IL-1β stimulation (data not shown). These results imply that keratinocytes but not fibroblasts secrete PEDF in a regulated fashion in response to inflammatory stimulation. These data contrast with a prior report that PEDF is detected primarily in the dermis, with little protein evident in the epidermal layers [17].

3.3. PEDF levels in serum of psoriasis patients and normal controls

Serum VEGF levels have previously been reported to be significantly elevated in psoriasis patients [6]. If elevated serum VEGF values reflect cytokine overproduction in the skin that then enters the systemic circulation, the pathogenesis in psoriasis might relate not only to a disruption of local angiogenesis in the skin but also to angiogenesis at the systemic level. We next examined whether serum PEDF serum levels also were elevated in psoriasis patients, however we observed no significant difference in PEDF levels between psoriasis patients (14.9 ± 4.1 μg/ml) ($n = 21$) and normal controls (15.1 ± 2.9 μg/ml) ($n = 14$). Furthermore there is no correlation between psoriasis severity and serum PEDF concentration.

3.4. Intradermal injection of PEDF reduces acanthosis, dermal angiogenesis and keratinocyte proliferation of grafted skin by

We hypothesized that PEDF produced by keratinocytes not only regulates local angiogenesis, but also suppresses epidermal proliferation and the resulting acanthosis in psoriatic inflammatory lesions. To investigate this possibility *in vivo*, we studied a psoriasis graft model in which patient-derived skin is xenografted onto severe combined immunodeficient (SCID) mice. Recombinant PEDF (30 μg) was injected intradermally in the area of the graft for three weeks and epidermal thickness evaluated histopathologically. The epidermal thickness of the grafted area was significantly reduced after treatment with PEDF when compared to BSA or PBS treated controls (Fig. 2). Injections of equivalent amounts of BSA, as a non-specific protein control, did not reduce epidermal thickness. Normal human skin transplanted to SCID mice also showed a reduction of epidermal thickness after PEDF-treatment. On the

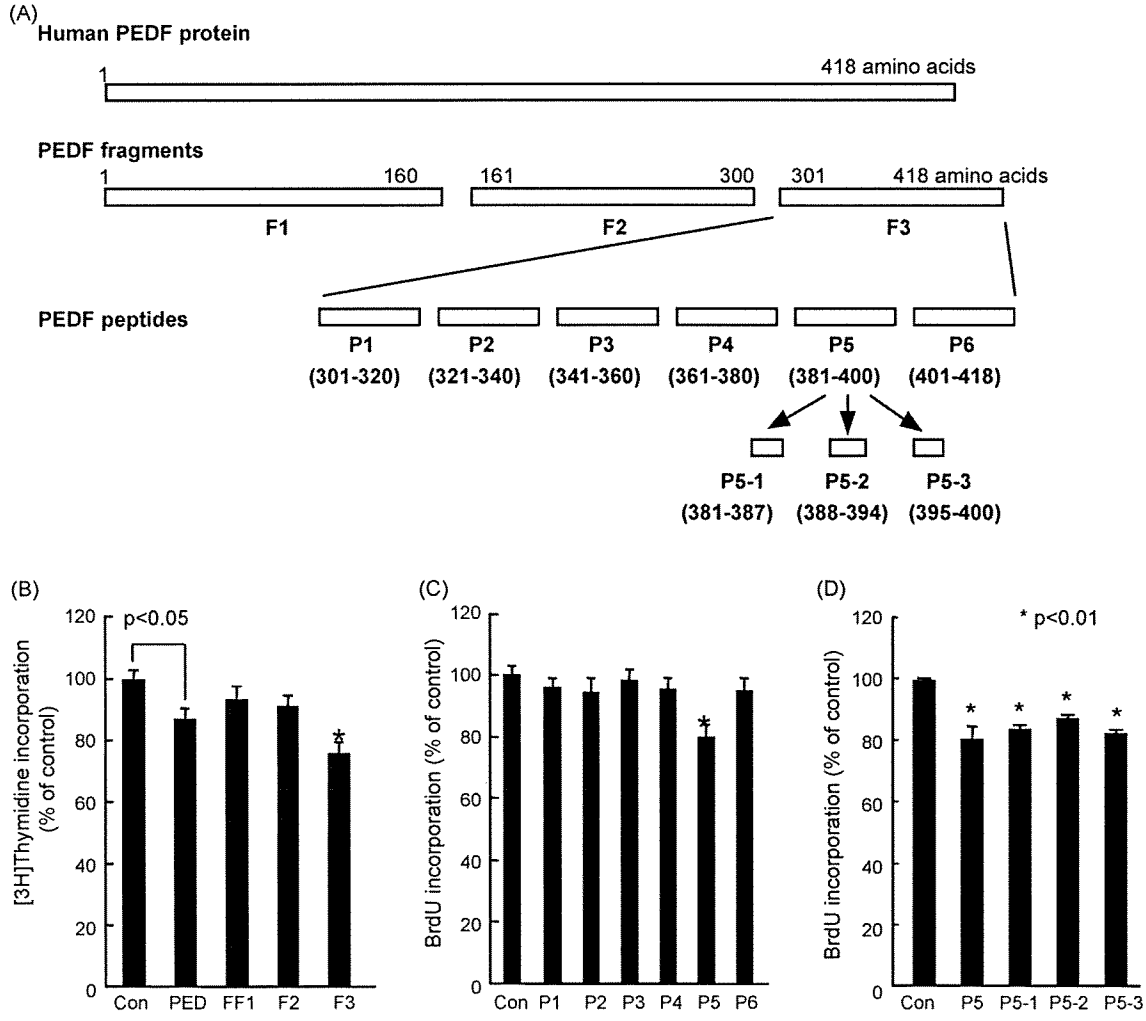

Fig. 5. Anti-angiogeneic activity of PEDF peptides. Diagram of the PEDF peptides studied for their effect on the growth of MG63 cells (B–D) or HUVEC (E). MG63 cells or HUVEC were treated with or without 100 nM PEDF, fragments or peptides and then [^{3}H]thymidine (B) and BrdU incorporation into the cells (C, D) were measured. The percentage of [^{3}H]thymidine or BrdU incorporation is indicated on the ordinate and related to the value of the control. *$p < 0.01$ compared to the value with 100 nM PEDF protein.

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

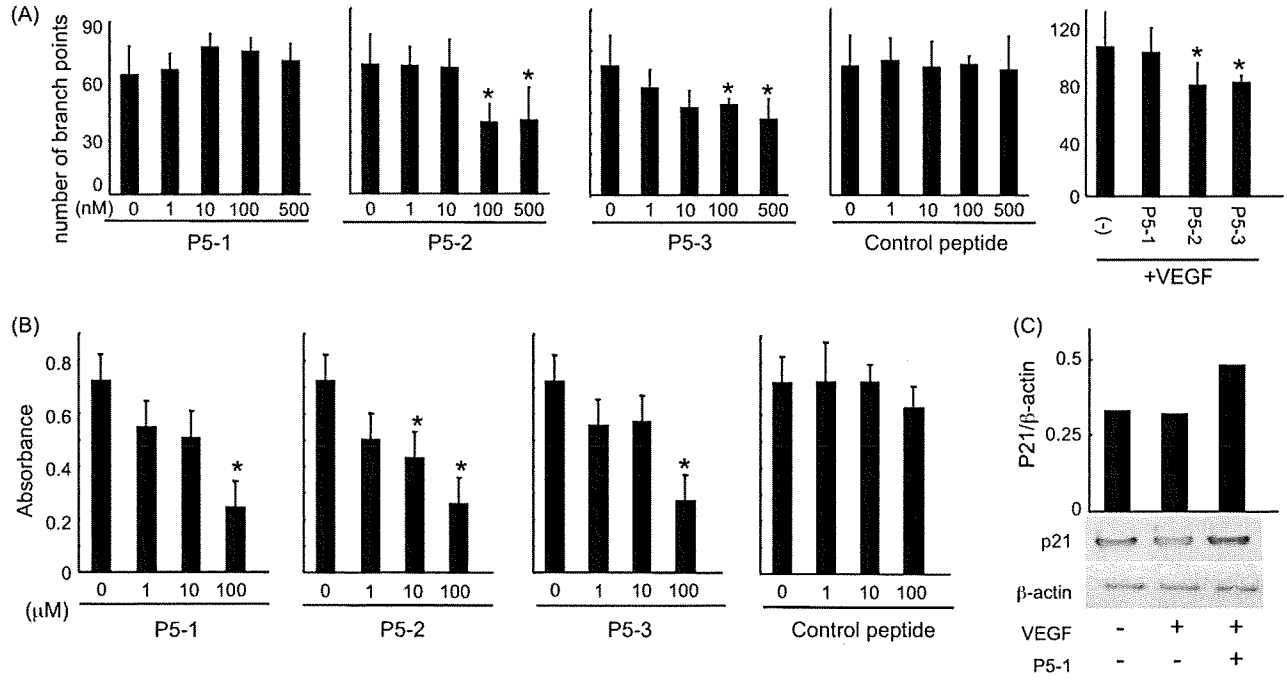

Fig. 6. (A) The endothelial tube formation assay. PEDF 1–3 showed a level of anti-angiogenic activity comparable to recombinant PEDF (*$p < 0.05$). (B) Diagram of the PEDF peptides studied for their effect on the growth of HUVEC. HUVEC were treated with or without 100 nM PEDF, fragments or peptides and 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.

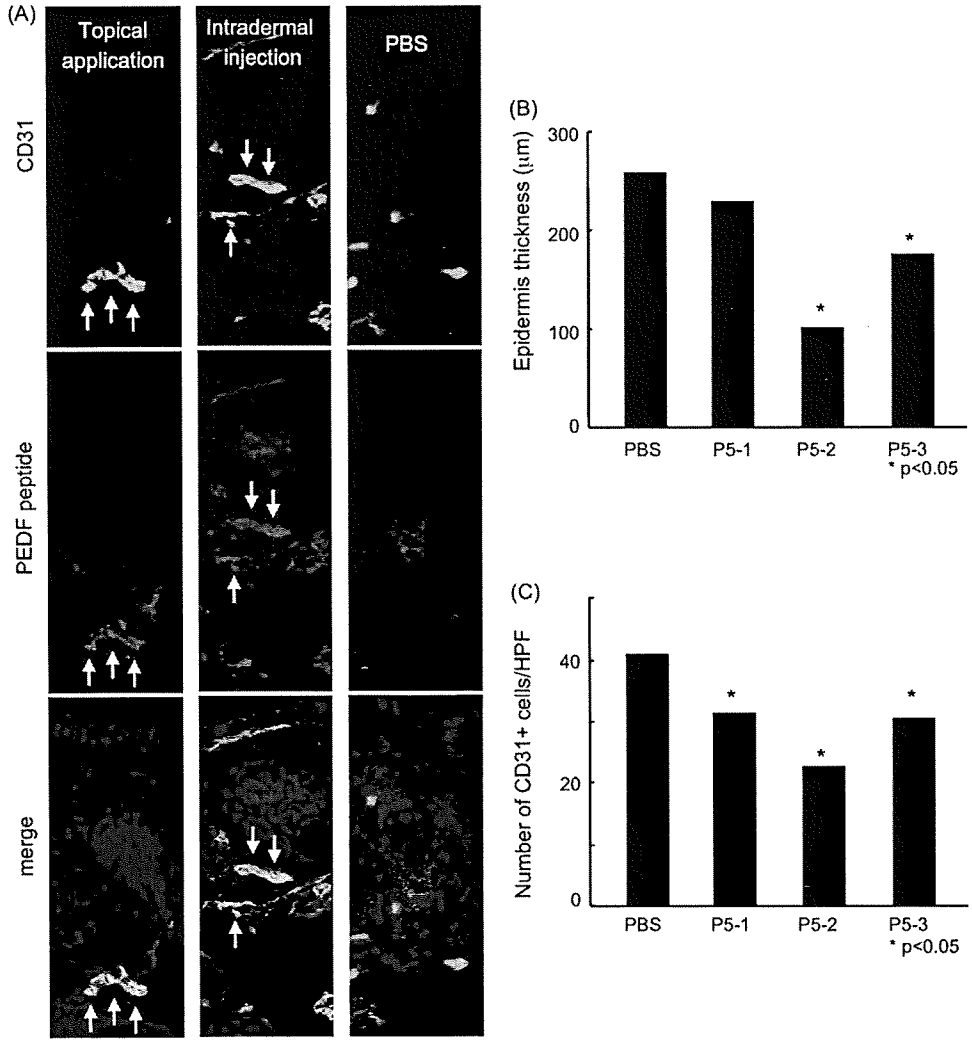

Fig. 7. Topically applied PEDF peptides penetrate into skin and reduce epidermal thickness and angiogenesis. (A) Biotin-labeled PEDF peptide (P5–1) was applied to the skin and its localization studied 2 h later using rhodamine–avidin staining as described in Section 2. Co-localization with PEDF peptide 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.

Acknowledgements

We thank Ms. Yuika Osaki for her excellent technical assistance and Prof. James R. McMillan for his manuscript proofreading.

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