congenital ichthyosiform erythroderma (NCIE) linked to chromosome 17p13.1. *Hum Mol Genet* 2002; **11**:107–13.

3 Eckl KM, de Juanes S, Kurtenbach J *et al.* Molecular analysis of 250 patients with autosomal recessive congenital ichthyosis: evidence for mutation hotspots in *ALOXE3* and allelic heterogeneity in *ALOX12B*. *J Invest Dermatol* 2009; **129**:1421–8.

4 Fischer J. Autosomal recessive congenital ichthyosis. *J Invest Dermatol* 2009; **129**:1319–21.

5 Brash AR, Yu Z, Boeglin WE, Schneider C. The hepoxilin connection in the epidermis. *FEBS J* 2007; **274**:3494–502.

6 Krieg P, Heidt M, Siebert M *et al.* Epidermis-type lipoxygenases. *Adv Exp Med Biol* 2002; **507**:165–70.

7 Anton R, Camacho M, Puig L, Vila L. Hepoxilin B3 and its enzymatically formed derivative trioxilin B3 are incorporated into phospholipids in psoriatic lesions. *J Invest Dermatol* 2002; **118**:139–46.

8 Lefèvre C, Bouadjar B, Ferrand V *et al.* Mutations in a new cytochrome P450 gene in lamellar ichthyosis type 3. *Hum Mol Genet* 2006; **15**:767–76.

9 Eckl KM, Krieg P, Küster W *et al.* Mutation spectrum and functional analysis of epidermis-type lipoxygenases in patients with autosomal recessive congenital ichthyosis. *Hum Mutat* 2005; **26**:351–61.

10 Yu Z, Schneider C, Boeglin WE, Brash AR. Mutations associated with a congenital form of ichthyosis (NCIE) inactivate the epidermal lipoxygenases 12R-LOX and eLOX3. *Biochim Biophys Acta* 2005; **3**:238–47.

11 Epp N, Fürstenberger G, Müller K *et al.* 12R-lipoxygenase deficiency disrupts epidermal barrier function. *J Cell Biol* 2007; **177**:173–82.

12 Moran J, Qiu H, Turbe-Doan A *et al.* A mouse mutation in the 12R-lipoxygenase, *Alox12b*, disrupts formation of the epidermal permeability barrier. *J Invest Dermatol* 2007; **127**:1893–7.

13 Harting M, Brunetti-Pierri N, Chan CS *et al.* Self-healing collodion membrane and mild nonbullous congenital ichthyosiform erythroderma due to 2 novel mutations in the *ALOX12B* gene. *Arch Dermatol* 2008; **144**:351–6.

14 de Juanes S, Epp N, Latzko S *et al.* Development of an ichthyosiform phenotype in *Alox12b*-deficient mouse skin transplants. *J Invest Dermatol* 2009; **129**:1429–36.

15 Hershkovitz D, Mandel H, Ishida-Yamamoto A *et al.* Defective lamellar granule secretion in arthrogryposis, renal dysfunction, and cholestasis syndrome caused by a mutation in *VPS33B*. *Arch Dermatol* 2008; **144**:334–40.

16 Elias PM. Stratum corneum defensive functions: an integrated view. *J Invest Dermatol* 2005; **125**:183–200.

17 Yanagi T, Akiyama M, Nishihara H *et al.* Harlequin ichthyosis model mouse reveals alveolar collapse and severe fetal skin barrier defects. *Hum Mol Genet* 2008; **17**:3075–83.

18 Sakai K, Akiyama M, Yanagi T *et al.* *ABCA12* is a major causative gene for non-bullous congenital ichthyosiform erythroderma. *J Invest Dermatol* 2009; **129**:2306–9.

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Short Communication

Transglutaminase1 Preferred Substrate Peptide K5 Is an Efficient Tool in Diagnosis of Lamellar Ichthyosis

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Lamellar ichthyosis (LI) is a genetically heterogeneous, severe genodermatosis showing widespread hyperkeratosis of the skin. Transglutaminase 1 (TGase1) deficiency by TGase1 gene (*TGM1*) mutations is the most prevalent cause of LI. Screening of TGase1 deficiency in skin is essential to facilitate the molecular diagnosis of LI. However, cadaverine, the most widely used substrate for TGase activity assay, is not isozyme specific. Recently, a human TGase1–specific highly preferred substrate peptide K5 (pepK5) was generated. To evaluate its potential as a diagnostic tool for LI, we performed pepK5 labeling of TGase1 activity in normal human and LI skin. Ca^{2+}-dependent labeling of FITC-pepK5 was clearly seen in the upper spinous and granular layers of normal human skin where it precisely overlapped with TGase1 immunostaining. Both specificity and sensitivity of FITC-pepK5 labeling for TGase1 activity were higher than those of FITC-cadaverine labeling. FITC-pepK5 labeling colocalized with involucrin and loricrin immunostaining at cornified cell envelope forming sites. FITC-pepK5 labeling was negative in LI patients carrying *TGM1* truncation mutations and partially abolished in the other LI patients harboring missense mutations. The present results clearly indicate that pepK5 is a powerful tool for screening LI patient TGase1 deficiency when we make molecular diagnosis of LI. ***(Am J Pathol 2010, 176:1592–1599; DOI: 10.2353/ajpath.2010.090597)***

One of the essential events during terminal differentiation of epidermal keratinocytes and skin barrier formation is the production of a 15-nm-thick layer of protein on the inner surface of the keratinocyte cell membrane, termed the cornified cell envelope (CCE). The CCE is assembled by the accumulation of several precursor proteins including involucrin and loricrin.[1] It is known that the precursor proteins are cross-linked together by the formation of N^{ε}-(γ-glutamyl) lysine isodipeptide bonds catalyzed by the action of transglutaminase isoforms. Transglutaminase 1 (TGase1) is a key enzyme in CCE formation in the epidermis.

Lamellar ichthyosis (LI) is a major subtype of autosomal recessive congenital ichthyosis and clinically characterized by large, thick, dark scales over the entire body without serious background erythroderma.[2] Since the identification of TGase1 gene (*TGM1*) mutations in a number of families with LI in 1995,[3,4] more than one hundred *TGM1* mutations have been reported in LI families. TGase1 deficiency attributable to *TGM1* mutations is a major underlying causative factor in LI patients,[5,6] although LI is thought to be a genetically heterogeneous disorder and several causative molecules including TGase1 have been identified.[3,4,7,8–11] Although genotype/phenotype correlations in autosomal recessive congenital ichthyosis including LI with *TGM1* mutations have been studied for years, the exact nature of the relationship has yet to be fully elucidated.[5,6,12–15] Thus, it is difficult to know whether a causative gene is *TGM1* or not in each LI patient from each patient's clinical features alone.

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To date, to facilitate molecular diagnosis in LI patients with *TGM1* mutations, *in situ* transglutaminase (TGase) activity assays have been performed using cadaverine as a substrate to detect TGase1 activity in the patients' skin,[16–20] despite the fact that cadaverine is not an isozyme-specific probe, and detects total TGase activity in the epidermis. Recently, a human TGase1 specific, highly preferred substrate peptide K5 (pepK5) was generated.[21] We hypothesized that, as previously shown in mouse skin, pepK5 would detect *in situ* TGase1 activity with high specificity and sensitivity in the human epidermis. If it is the case, pepK5 can be a useful tool to detect TGase1 deficiency in LI patients with *TGM1* mutations.

In the present study, we demonstrated that pepK5 can be used as an efficient probe to detect TGase1 activity in the human epidermis. In addition, we performed *in situ* TGase1 activity assay using pepK5 in skin specimens from LI patients with *TGM1* mutations and clearly revealed that this preferred substrate for TGase1, pepK5 is a powerful tool for evaluation of TGase1 activity in LI patients and for molecular diagnosis of LI.

Materials and Methods

Synthesis of Transglutaminase Substrate Peptides

PepK5, peptide K5QN (pepK5QN), and peptide form T26 (pepT26) were synthesized as previously described.[21,22] Briefly, a phage-displayed random peptide library was used to screen primary amino acid sequences that are preferentially selected by human TGase1. The peptides selected as glutamine donor substrate exhibited a marked tendency in primary structure, conforming to the sequence: QxK/RψxxxWP (where x and ψ represent nonconserved and hydrophobic amino acids, respectively). Using glutathione S-transferase (GST) fusion proteins of the selected peptides, several sequences were identified as preferred substrates and confirmed that they were isozyme-specific. The 12-aa peptide pepK5 (YEQHKLPSSWPF) was synthesized. Even in peptide form, K5 appeared to have high and specific reactivity as substrate. In addition, a mutant peptide in which glutamine was substituted by asparagine was also synthesized as pepK5QN (YENHKLPSSWPF). pepT26 (HQSYVDPWMLDH) was synthesized as the transglutaminase 2 (TGase2) preferred substrate peptide for comparison.[22] Finally, these synthesized peptides were conjugated with FITC.[21]

In Situ *TGase1 Activity Assay*

Skin sections were prepared from skin biopsy patient specimens and normal control specimens using standard methods.[21,23] The frozen sections were dissected into 6-μm slices and stored frozen at −80°C until use.

Sections were dried and then blocked with 1% BSA in NaCl/Pi at room temperature. The sections were incubated for 90 minutes with a solution containing 100 mmol/L Tris/HCl pH 8.0, 5 mmol/L $CaCl_2$ or 1 mmol/L EDTA, and 1 mmol/L dithiothreitol, in the presence of 5 μmol/L (or other concentrations) of FITC-labeled substrate peptide or FITC-cadaverine (Sigma-Aldrich, St. Louis, MO). This *in situ* TGase1 activity assay works by measuring the fluorescence of fluorescein isothiocyanate (FITC)-labeled substrate peptide incorporated into cellular proteins by cross-linking catalyzed by TGase1. After washing with NaCl/Pi three times for 5 minutes, antifading solution was added to the sections, which were then sealed with a cover glass and mountant. In addition, we performed the above-mentioned pepK5 labeling using normal human skin specimens and LI patients' skin samples under various incubation conditions (pH 7.4, 8.0 and 8.4; temperature 25°C, 33°C and 37°C).

Double Labeling for in Situ *TGase1 Assay and Immunofluorescence Staining*

For double labeling (*in situ* TGase1 activity assay and immunofluorescence), at first, we performed *in situ* TGase1 activity assay as described above, then the sections were labeled with immunofluorescence methods below. Immunofluorescence labeling was performed as described previously.[23] Primary antibodies used in this study were as follows: mouse monoclonal anti-TGase 1 antibody (B.C1; Biomedical Technologies, Inc., Stoughton, MA), rabbit polyclonal anti-TGase1 antibody (Novus Biologicals, LLC, Littleton, CO), anti-loricrin antibody (Covance Lab., Richmond, CA), and anti-involucrin antibody (Biomedical Technologies, Inc., Stoughton, MA). We used FITC-conjugated or tetramethylrhodamine-isothiocyanate (TRITC)-conjugated rabbit anti-mouse immunoglobulin (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) or donkey anti-rabbit immunoglobulins (DAKO, Glostrup, Denmark), as secondary antibodies.

Ichthyosis Patients Involved in the Present Study

In total, four unrelated LI patients with *TGM1* mutations were included in this study. Patient 1 was a recently examined LI case and the other three patients were reported previously.[6,20,24] As controls, two *TGM1*-unrelated autosomal recessive congenital ichthyosis patients harboring ABCA12 mutations[25] were also included in the present study.

Fully informed consent was obtained from the participants or their legal guardians for this study. This study had been previously evaluated and approved by the ethics committee at Hokkaido University Graduate School of Medicine and was conducted according to the Declaration of Helsinki Principles.

Mutation Search

TGM1 mutation search was performed as previously reported.[19] Briefly, genomic DNA isolated from peripheral blood was subjected to polymerase chain reaction amplification, followed by direct automated sequencing and verification of the mutation by restriction enzyme digestions. Most oligonucleotide primers used for amplification of all 15 exons of *TGM1* have been reported elsewhere[12] and partially modified for the present study.[19] The entire coding regions of *TGM1* including the exon/intron boundaries were sequenced using genomic DNA samples from patients and their family members. One hundred normal alleles (50 unrelated, healthy Japanese individuals) were sequenced as normal controls.

Results

In Situ *Assay Using pepK5 Detected TGase1 Activity with High Specificity and Sensitivity in the Upper Epidermis of Normal Human Skin*

With the presence of $CaCl_2$ in the reaction mixture, we detected specific incorporation of FITC-labeled pepK5 (FITC-pepK5; 5 μmol/L) into substrate proteins in the epidermis, mainly at the cell periphery of the upper spinous and granular layers of normal human skin (Figure 1A). No signal was detected in the presence of EDTA (Figure 1B), or when we used FITC-conjugated pepK5QN mutant peptide (FITC-pepK5QN; Figure 1C), which indicated that the cross-linking reaction was catalyzed specifically by TGase1. Using FITC-conjugated pepT26 (FITC-pepT26), a preferable substrate for TGase2, only faint labeling was obtained around the granular layer cells and this labeling was abolished in the presence of EDTA (data not shown). Under various incubation conditions, pH 7.4, 8.0, and 8.4, temperature 25°C, 33°C, and 37°C, no significant difference in the pepK5 labeling intensity was observed in normal human epidermis (data not shown).

The FITC-pepK5 labeling pattern corresponded well with the localization of TGase1 by immunostaining with anti-TGase1 antibody. Double labeling for *in situ* TGase1 activity assay using FITC-pepK5 and immunostaining for TGase1 molecule showed completely overlapping colocalization of these moieties at the cell periphery of both the upper spinous and granular layer cells (Figure 1, D–F).

Double Labeling for TGase1 Activity with pepK5 and CCE Precursor Proteins Demonstrated that pepK5 Labeling Precisely Localized to Sites of CCE Formation

Immunofluorescence labeling for involucrin, a major CCE precursor protein, was seen in the upper half of the epidermis (Figure 1H). Double labeling for *in situ* TGase1 activity assay using pepK5, and involucrin immunolabeling showed that, in the upper spinous and granular cell layers, pepK5 labeling and involucrin co-localized at the cell periphery (Figure 1, G–I). In addition, double labeling for the *in situ* TGase1 activity assay using pepK5, and immunolabeling for loricrin, another major CCE precursor protein, revealed almost complete colocalization of

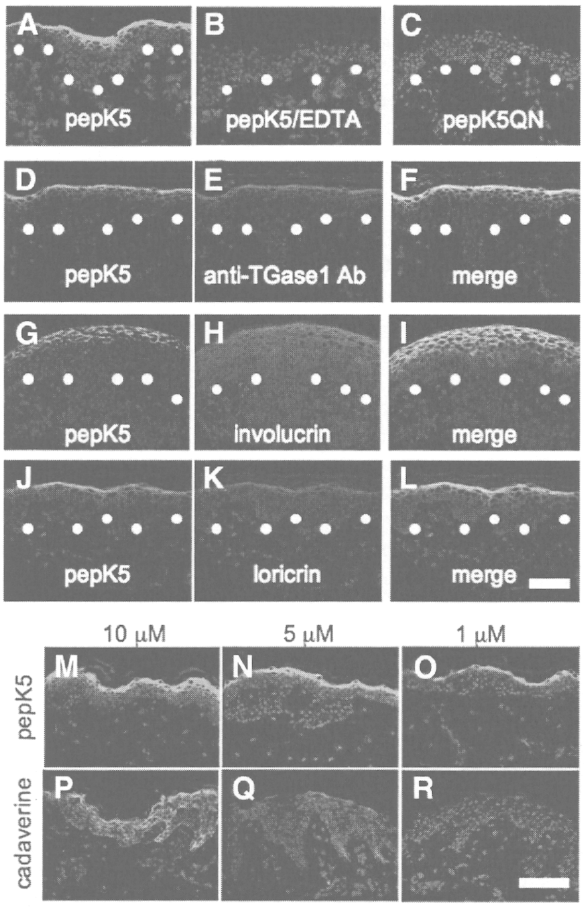
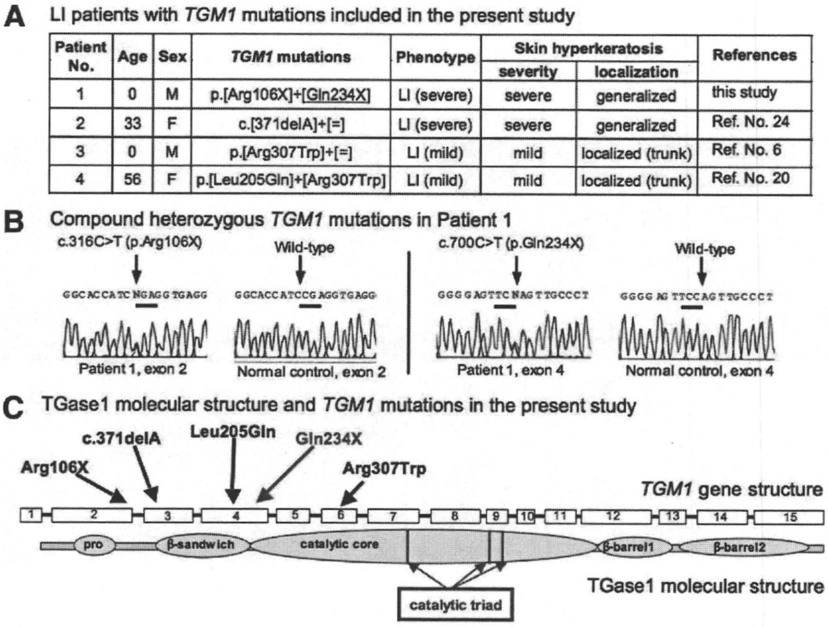


Figure 1. PepK5 labeling detected *in situ* TGase1 activity with high specificity and sensitivity at CCE forming sites in normal human skin. **A–C:** *In situ* TGase1 activity detected by pepK5 in normal skin. Detection of *in situ* TGase 1 activity using FITC-labeled pepK5 (5 μmol/L) showed intense membrane-restricted staining within the upper spinous and granular layer keratinocytes of a normal human skin (**A**). In the presence of EDTA, the pepK5 labeling was completely abolished (**B**). No labeling was observed with FITC-labeled mutant K5 peptide (pepK5QN; **C**). Specific labeling, green (FITC); nuclear stain, red (propidium iodide). **White dots**, basement membrane zone. **D–F:** Double labeling with pepK5 and anti-TGase1 antibody in normal human skin. Both pepK5 labeling (**D**, green, FITC) and anti-TGase1 antibody (B.C1) labeling (**E**, red, TRITC) are seen in the upper epidermis, mainly in the granular layers. The merged image clearly demonstrates that both labeling patterns almost completely overlap (yellow) each other on the cell membrane of the upper epidermal keratinocytes (**F**). pepK5 labeling, green (FITC), anti-TGase1 antibody labeling, red (TRITC); nuclear stain, blue (TOPRO). White dots, basement membrane zone. **G–L:** Double labeling with anti-CCE precursor protein antibodies and pepK5 in normal human skin. Anti-involucrin antibody labeling (**H**, red, TRITC) is seen in the upper half of the epidermis, although pepK5 labeling (**G**, green, FITC) is observed mainly in the uppermost spinous and granular cell layers. Involucrin and pepK5 labeling overlap each other (yellow) on the cell membrane of the uppermost spinous and granular cell layer keratinocytes in the merged image (**I**). Both pepK5 labeling (**J**, green, FITC) and anti-loricrin antibody labeling (**K**, red, TRITC) are seen mostly within the uppermost spinous and granular layers. The merged image shows that loricrin and pepK5 labeling clearly overlap (yellow) each other on the cell membrane of the granular layer keratinocytes (**L**). FITC-pepK5 labeling, green; anti-involucrin and anti-loricirn antibodies, red (TRITC); nuclear stain, blue (TOPRO). **White dots**, basement membrane zone. **M–R:** Detection of TGase1 activity in normal human skin sections using graded concentrations of pepK5 or cadaverine. Intense labeling is seen in the upper epidermis with 10 μmol/L (**M**) and 5 μmol/L (**N**) of FITC-pepK5. Only the granular layer keratinocytes are labeled with 1 μmol/L (**O**) of FITC-pepK5. Using 10 μmol/L (**P**) of FITC-cadaverine, all epidermal keratinocytes are labeled. With 5 μmol/L (**Q**) of FITC-cadaverine, entire epidermis is faintly labeled. No labeling is observed with 1 μmol/L (**R**) of FITC-cadaverine. **M–O:** FITC-pepK5 labeling, green; **P–R:** FITC-cadaverine labeling, green; nuclear stain, red (propidium iodide). Substrate concentrations, 10 μmol/L (**M**, **P**), 5 μmol/L (**N**, **Q**), 1 μmol/L (**O**, **R**). Scale bars = 50 μm.



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

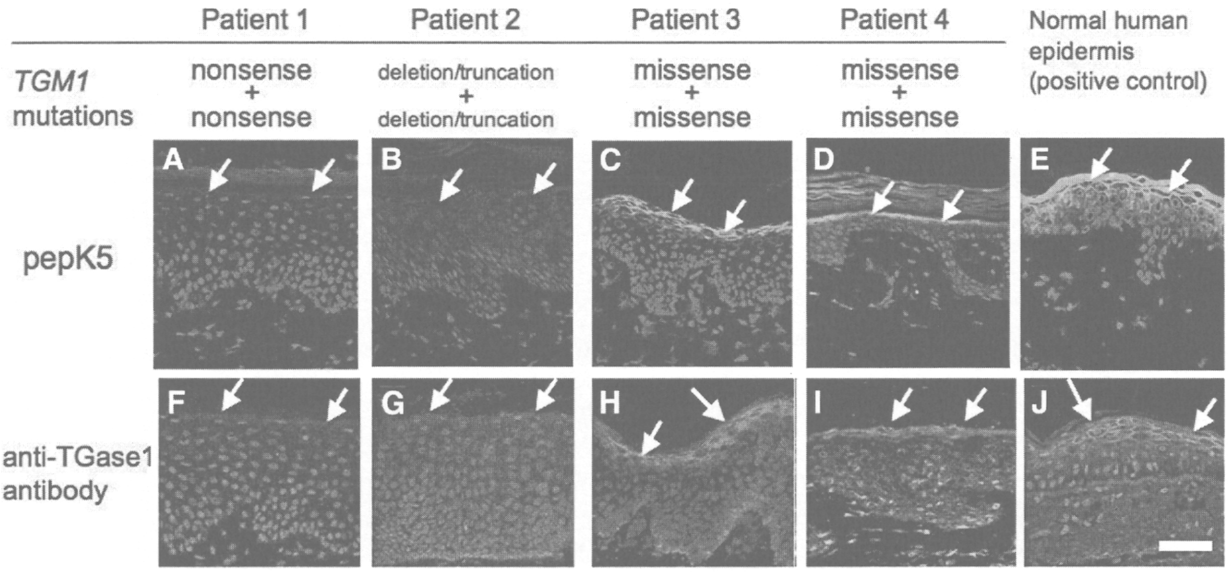


Figure 3. TGase1 deficiency detected by pepK5 labeling in the LI patients. **A and F:** Patient 1, a compound heterozygote for two *TGM1* nonsense mutations: FITC-pepK5 labeling (green) shows complete absence of TGase1 activity in the upper epidermis (**arrows**; **A**), and TGase1 immunostaining (green) is also negative in the upper epidermis (**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 (**arrows**; **B**) and no TGase1 immunolabeling (in green) is seen in the upper epidermis (**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 (**arrows**) by FITC-pepK5 labeling (green; **C**). TGase1 immunostaining (green) in the upper epidermis (**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 (**arrows**; **D**). Immunofluorescence labeling for TGase1 (green) reveals a positive staining in the granular layer (**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 (**arrows**) using FITC-pepK5 labeling (green; **E**). TGase1 immunolabeling (green) is also positive in the upper epidermis (**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.

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References

1. Steinert PM, Marekov LN: The proteins elafin, filaggrin, keratin intermediate filaments, loricrin, and small proline-rich proteins 1 and 2 are isodipeptide cross-linked components of the human epidermal cornified cell envelope. J Biol Chem 1995, 70:17702–17711
2. Akiyama M: Harlequin ichthyosis and other autosomal recessive congenital ichthyoses: the underlying genetic defects and pathomechanisms. J Dermatol Sci 2006, 42:83–89
3. Huber M, Rettler I, Bernasconi K, Frenk E, Lavrijsen SP, Ponec M, Bon A, Lautenschlager S, Schorderet DF, Hohl D: Mutations of keratinocyte transglutaminase in lamellar ichthyosis. Science 1995, 267:525–528
4. Russell LJ, DiGiovanna JJ, Rogers GR, Steinert PM, Hashem N, Compton JG, Bale SJ: Mutations in the gene for transglutaminase 1 in autosomal recessive lamellar ichthyosis. Nat Genet 1995, 9:279–283
5. Herman ML, Farasat S, Steinbach PJ, Wei MH, Toure O, Fleckman P, Blake P, Bale SJ, Toro JR: Transglutaminase-1 gene mutations in autosomal recessive congenital ichthyosis: summary of mutations (including 23 novel) and modeling of TGase-1. Hum Mutat 2009, 30:537–547
6. Sakai K, Akiyama M, Yanagi T, McMillan JR, Suzuki T, Tsukamoto K, Sugiyama H, Hatano Y, Hayashitani M, Takamori K, Nakashima Keiko, Shimizu H: ABCA12 is a major causative gene for non-bullous congenital ichthyosiform erythroderma. J Invest Dermatol 2009, 129:2306–2309
7. Akiyama M, Shimizu H: An update on molecular aspects of the non-syndromic ichthyoses. Exp Dermatol 2008, 17:373–382
8. Jobard F, Lefévre C, Karaduman A, Blanchet-Bardon C, Emre S, Weissenbach J, Ozgüc M, Lathrop M, Prud'homme JF, Fischer J: Lipoxygenase-3 (ALOXE3) and 12(R)-lipoxygenase (ALOX12B) are mutated in non-bullous congenital ichthyosiform erythroderma (NCIE) linked to chromosome 17p13.1. Hum Mol Genet 2002, 1:107–113
9. Lefévre C, Audebert S, Jobard F, Bouadjar B, Lakhdar H, Boughdene-Stambouli O, Blanchet-Bardon C, Heilig R, Foglio M, Weissenbach J, Lathrop M, Prud'homme JF, Fischer J: Mutations in the transporter ABCA12 are associated with lamellar ichthyosis type 2. Hum Mol Genet 2003, 12:2369–2378
10. Lefévre C, Bouadjar B, Karaduman A, Jobard F, Saker S, Ozgüc M, Lathrop M, Prud'homme JF, Fischer J: Mutations in ichthyin a new gene on chromosome 5q33 in a new form of autosomal recessive congenital ichthyosis. Hum Mol Genet 2004, 13:2473–2482
11. Lefévre C, Bouadjar B, Ferrand V, Tadini G, Mégarbané A, Lathrop M, Prud'homme JF, Fischer J: Mutations in a new cytochrome P450 gene in lamellar ichthyosis type 3. Hum Mol Genet 2006, 15:767–776
12. Laiho E, Ignatius J, Mikkola H, Yee VC, Teller DC, Niemi KM, Saarialho-Kere U, Kere J, Palotie A: Transglutaminase 1 mutations in autosomal recessive congenital ichthyosis: private and recurrent mutations in an isolated population. Am J Hum Genet 1997, 61:529–538
13. Hennies HC, Küster W, Wiebe V, Krebsová A, Reis A: Genotype/phenotype correlation in autosomal recessive lamellar ichthyosis. Am J Hum Genet 1998, 62:1052–1061
14. Laiho E, Niemi K-M, Ignatius J, Kere J, Palotie A, Saarialho-Kere U: Clinical and morphological correlations for transglutaminase 1 gene mutations in autosomal recessive congenital ichthyosis. Eur J Hum Genet 1999, 7:625–632
15. Shevchenko YO, Compton JG, Toro JR, DiGiovanna JJ, Bale SJ: Splice-site mutation in TGM1 in congenital recessive ichthyosis in American families: molecular, genetic, genealogic, and clinical studies. Hum Genet 2000, 106:492–499
16. Aeschlimann D, Wetterwald A, Fleisch H, Paulsson M: Expression of tissue transglutaminase in skeletal tissues correlates with events of terminal differentiation of chondrocytes. J Cell Biol 1993, 120:1461–1470
17. Raghunath M, Hennies HC, Velten F, Wiebe V, Steinert PM, Reis A, Traupe H: A novel in situ method for the detection of deficient transglutaminase activity in the skin. Arch Dermatol Res 1998, 290:621–627
18. Hohl D, Aeschlimann D, Huber M: In vitro and rapid in situ transglutaminase assays for congenital ichthyoses—a comparative study. J Invest Dermatol 1998, 110:268–271
19. Akiyama M, Takizawa Y, Kokaji T, Shimizu H: Novel mutations of TGM1 in a child with congenital ichthyosiform erythroderma. Br J Dermatol 2001, 144:401–407
20. Akiyama M, Takizawa Y, Suzuki Y, Ishiko A, Matsuo I, Shimizu H: Compound heterozygous TGM1 mutations including a novel missense mutation L204Q in a mild form of lamellar ichthyosis. J Invest Dermatol 2001, 116:992–995
21. Sugimura Y, Hosono M, Kitamura M, Tsuda T, Yamanishi K, Maki M, Hitomi K: Identification of preferred substrate sequences for transglutaminase 1 – development of a novel peptide that can efficiently detect cross-linking enzyme activity in the skin. FEBS J 2008, 275:5667–5677
22. Sugimura Y, Hosono M, Wada F, Yoshimura T, Maki M, Hitomi K: Screening for the preferred substrate sequence of transglutaminase using a phage-displayed peptide library. Identification of peptide substrates for TGase2 and factor XIIIa J Biol Chem 2006, 281:17699–17706
23. Akiyama M, Smith LT, Shimizu H: Expression of transglutaminase activity in developing human epidermis. Br J Dermatol 2000, 142:223–225
24. Akiyama M, Takizawa Y, Suzuki Y, Shimizu H: A novel homozygous mutation 371delA in TGM1 leads to a classic lamellar ichthyosis phenotype. Br J Dermatol 2003, 148:149–153
25. Natsuga K, Akiyama M, Kato N, Sakai K, Sugiyama-Nakagiri Y, Nishimura M, Hata H, Abe M, Arita K, Tsuji-Abe Y, Onozuka T, Aoyagi S, Kodama K, Ujiie H, Tomita Y, Shimizu H: Novel ABCA12 mutations identified in two cases of non-bullous congenital ichthyosiform erythroderma associated with multiple skin malignant neoplasia. J Invest Dermatol 2007, 127:2669–2673
26. Oji V, Hautier JM, Ahvazi B, Hausser I, Aufenvenne K, Walker T, Seller N, Steijlen PM, Küster W, Hovnanian A, Hennies HC, Traupe H: Bathing suit ichthyosis is caused by transglutaminase-1 deficiency: evidence for a temperature-sensitive phenotype. Hum Mol Genet 2006, 15:3082–3097
27. Esposito G, Tadini G, Paparo F, Viola A, Ieno L, Pennacchia W, Messina F, Giordano L, Piccirillo A, Auricchio L: Transglutaminase 1 deficiency and corneocyte collapse: an indication for targeted molecular screening in autosomal recessive congenital ichthyosis. Br J Dermatol 2007, 157:808–810
28. Furutani Y, Kato A, Notoya M, Ghoneim MA, Hirose S: A simple assay and histochemical localization of transglutaminase activity using a derivative of green fluorescent protein as substrate. J Histochem Cytochem 2001, 49:247–258
29. Oji V, Oji ME, Adamini N, Walker T, Aufenvenne K, Raghunath M, Traupe H: Plasminogen activator inhibitor-2 is expressed in different types of congenital ichthyosis: in vivo evidence for its cross-linking into the cornified cell envelope by transglutaminase-1. Br J Dermatol 2006, 154:860–867
30. Hiiragi T, Sasaki H, Nagafuchi A, Sabe H, Shen SC, Matsuki M, Yamanishi K, Tsukita S: Transglutaminase type 1 and its cross-linking activity are concentrated at adherens junctions in simple epithelial cells. J Biol Chem 1999, 274:34148–34154
31. Iizuka R, Chiba K, Ohmi-Imajoh S: A novel approach for the detection of proteolytically activated transglutaminase 1 in epidermis using cleavage site-directed antibodies. J Invest Dermatol 2003, 121:457–464
32. Yee VC, Pedersen LC, Le Trong I, Bishop PD, Stenkamp RE, Teller DC: Three-dimensional structure of a transglutaminase: human blood coagulation factor XIII. Proc Natl Acad Sci USA 1994, 91:7296–7300
33. Maquat LE: Nonsense-mediated mRNA decay: splicing, translation and mRNP dynamics. Nat Rev Mol Cell Biol 2004, 5:89–99

34. Lejeune F, Maquat LE: Mechanistic links between nonsense-mediated mRNA decay and pre-mRNA splicing in mammalian cells. Curr Opin Cell Biol 2005, 17:309–315

35. Raghunath M, Hennies HC, Ahvazi B, Vogel M, Reis A, Steinert PM, Traupe H: Self-healing collodion baby: a dynamic phenotype explained by a particular transglutaminase-1 mutation. J Invest Dermatol 2003, 120:224–228

36. Arita K, Jacyk WK, Wessagowit V, van Rensburg EJ, Chaplin T, Mein CA, Akiyama M, Shimizu H, Happle R, McGrath JA: The South African "bathing suit ichthyosis" is a form of lamellar ichthyosis caused by a homozygous missense mutation, p.R315L, in transglutaminase 1. J Invest Dermatol 2007, 127:490–493

37. Aufenvenne K, Oji V, Walker T, Becker-Pauly C, Hennies HC, Stöcker W, Traupe H: Transglutaminase-1 and bathing suit ichthyosis: molecular analysis of gene/environment interactions. J Invest Dermatol 2009, 129:2068–2071

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

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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

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

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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

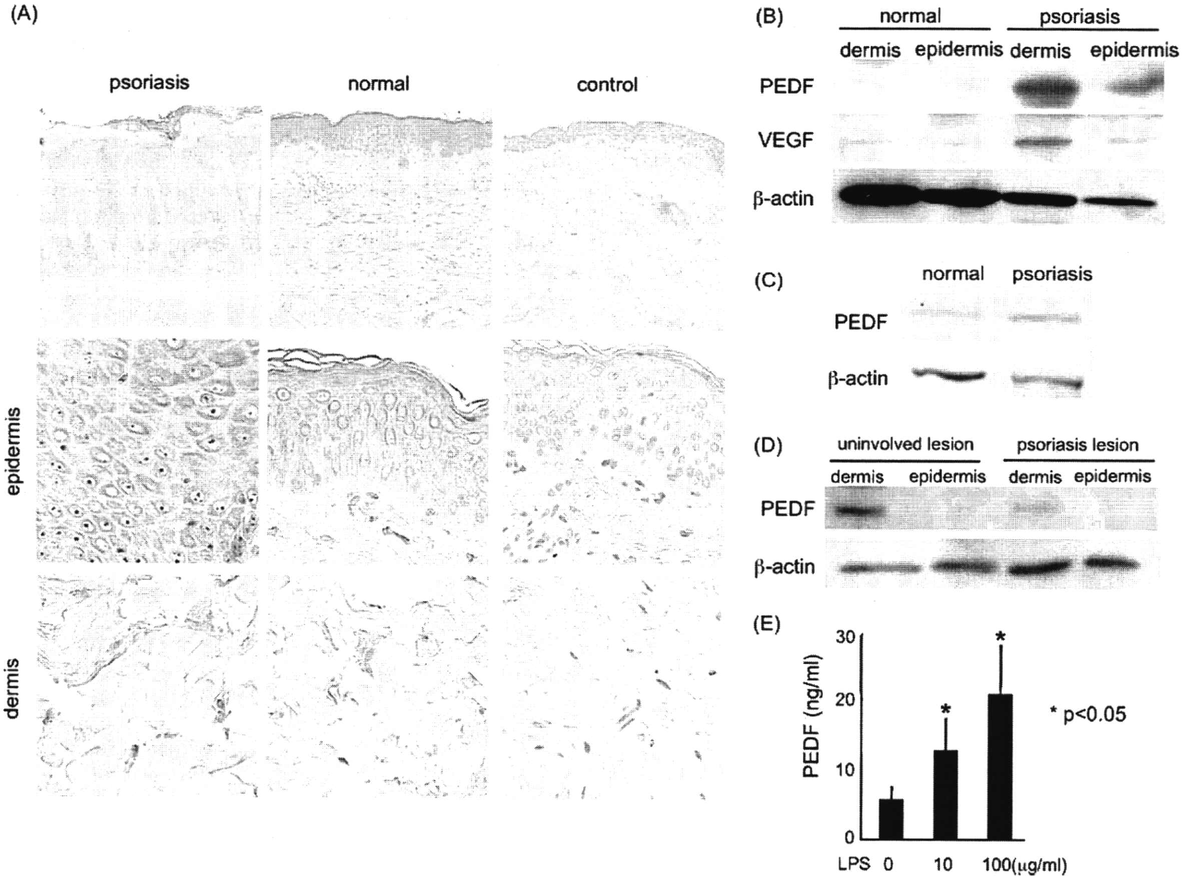

Fig. 1. PEDF is expressed in both the epidermis and dermis. (A) Immunohistochemistry of normal and psoriatic skin lesions. In normal skin, PEDF was detected in both the epidermis and the dermis. PEDF was significantly up-regulated in psoriatic epidermis in comparison with normal epidermis. (B) The expression 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 ($^*p < 0.05$).

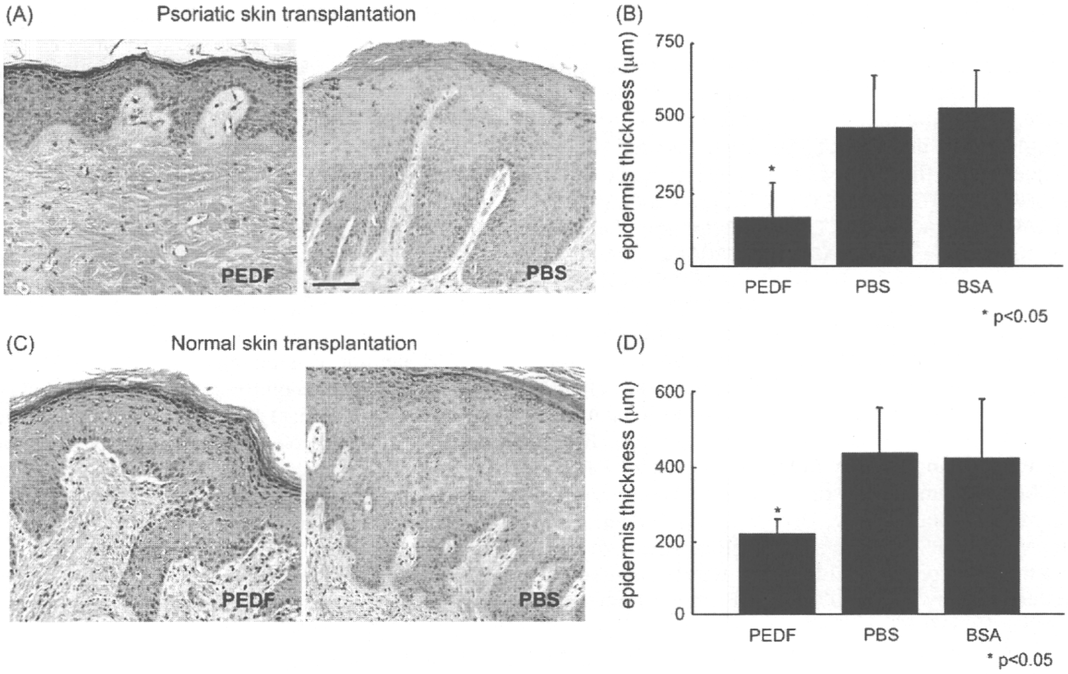

Fig. 2. Intradermal PEDF administration reduced the thickness of grafted epidermis in xenotransplanted SCID mice. Acanthosis was significantly reduced both in psoriatic (A, B) and normal (C, D) skin when compared to PBS or BSA injected groups. Scale bar, 50 μm. Values shown are means and SDs based on 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.

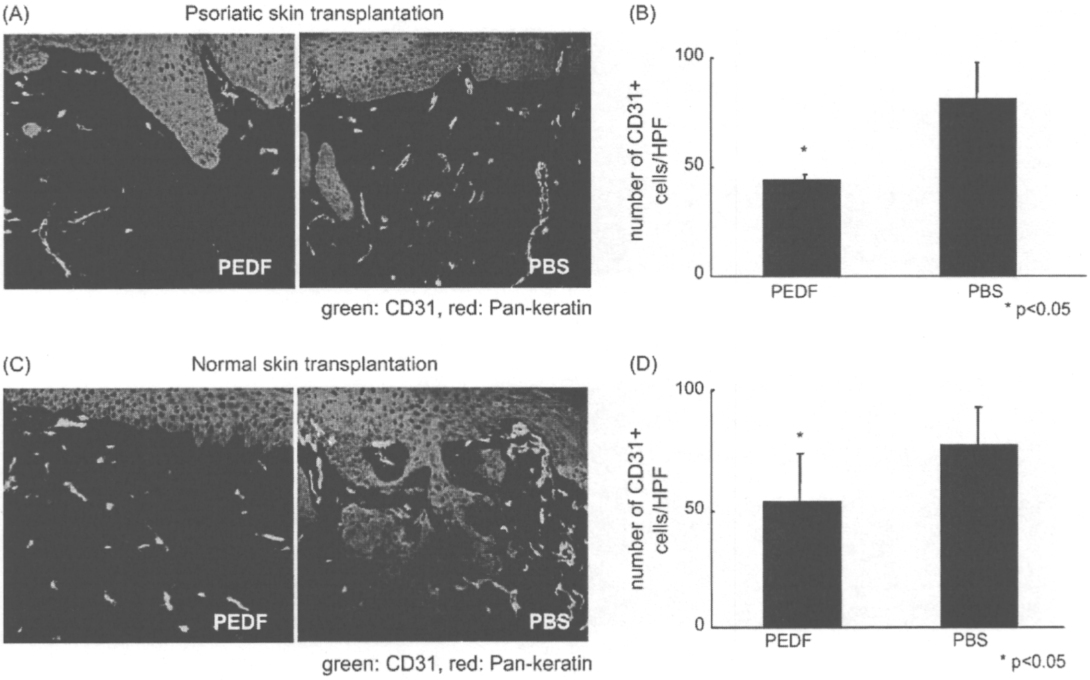

Fig. 3. Intradermal PEDF administration reduced angiogenesis of grafted epidermis. CD31-positive cells (capillary endothelial cells) were enumerated by immunofluorescence after the treatment of psoriatic (A, B) and normal skin (C, D) with PEDF or PBS. Quantification of CD31-positive blood vessels per 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).

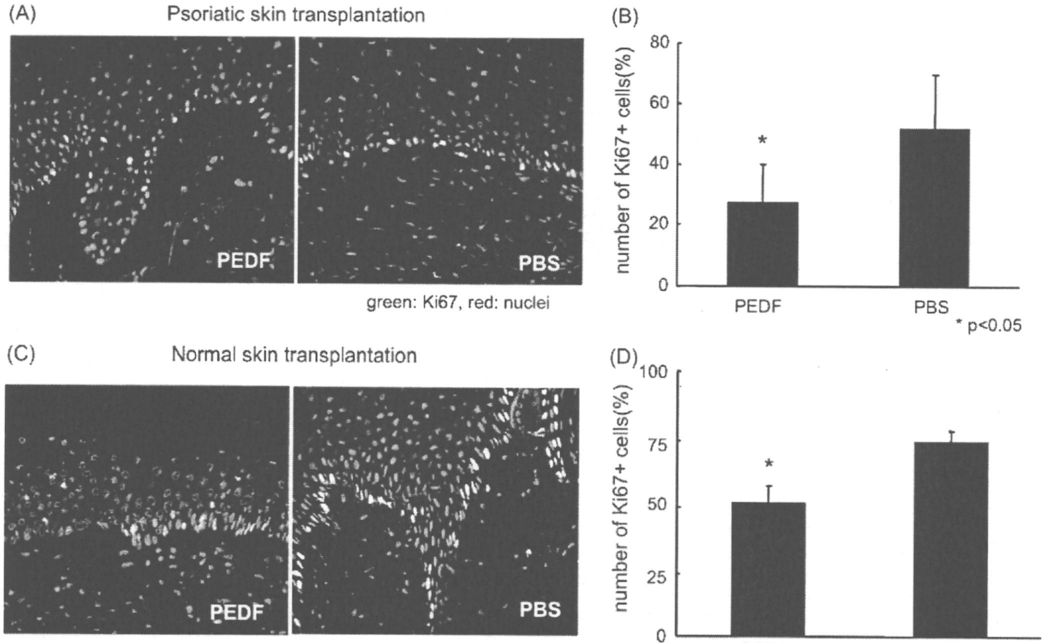

Fig. 4. Epidermal proliferation in basal keratinocytes is inhibited by PEDF. Ki-67-positive (proliferating) cells were stained and enumerated by immunofluorescence after the treatment of psoriatic (A, B) and normal skin (C, D) with PEDF or PBS. The Ki-67-positive keratinocytes in the basal layer were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

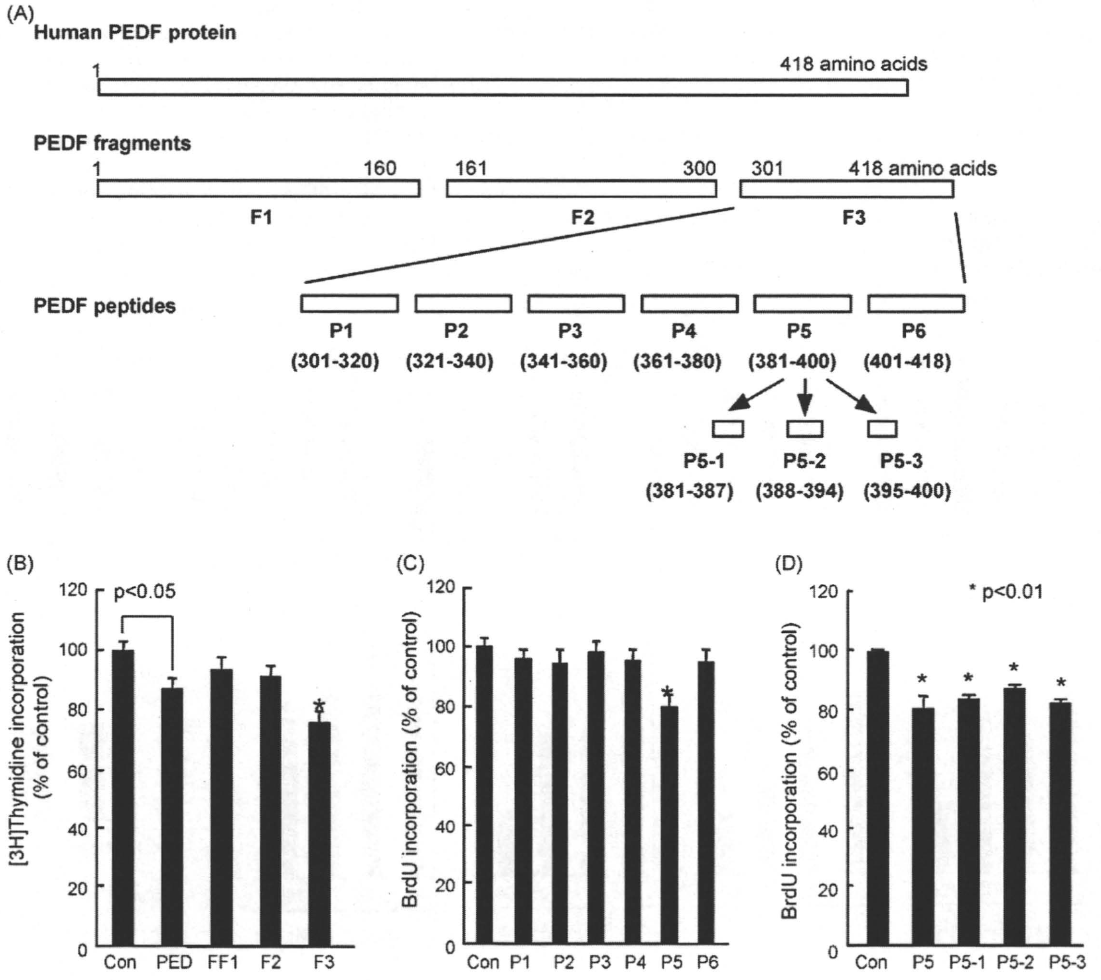

Fig. 5. Anti-angiogeneic activity of PEDF peptides. Diagram of the PEDF peptides studied for their effect on the growth of MG63 cells (B–D) or HUVEC (E). MG63 cells or HUVEC were treated with or without 100 nM PEDF, fragments or peptides and then [^{3}H]thymidine (B) and BrdU incorporation into the cells (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

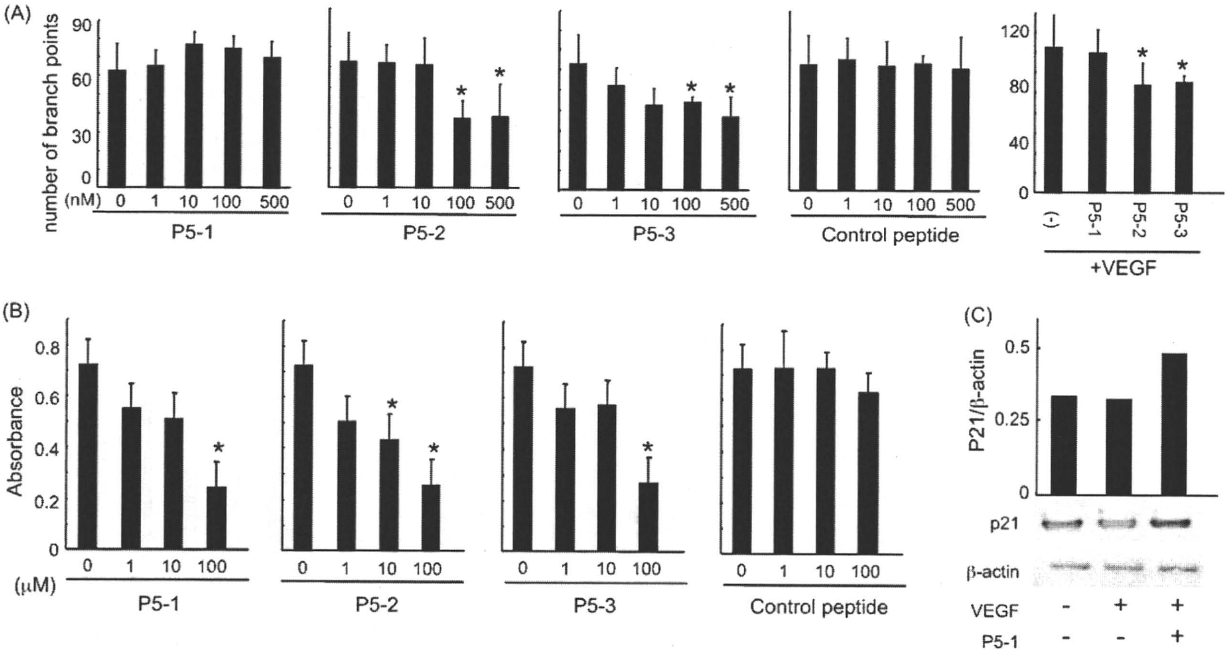

Fig. 6. (A) The endothelial tube formation assay. PEDF 1–3 showed a level of anti-angiogenic activity comparable to recombinant PEDF ($^*p < 0.05$). (B) Diagram of the PEDF peptides studied for their effect on the growth of HUVEC. HUVEC were treated with or without 100 nM PEDF, fragments or peptides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

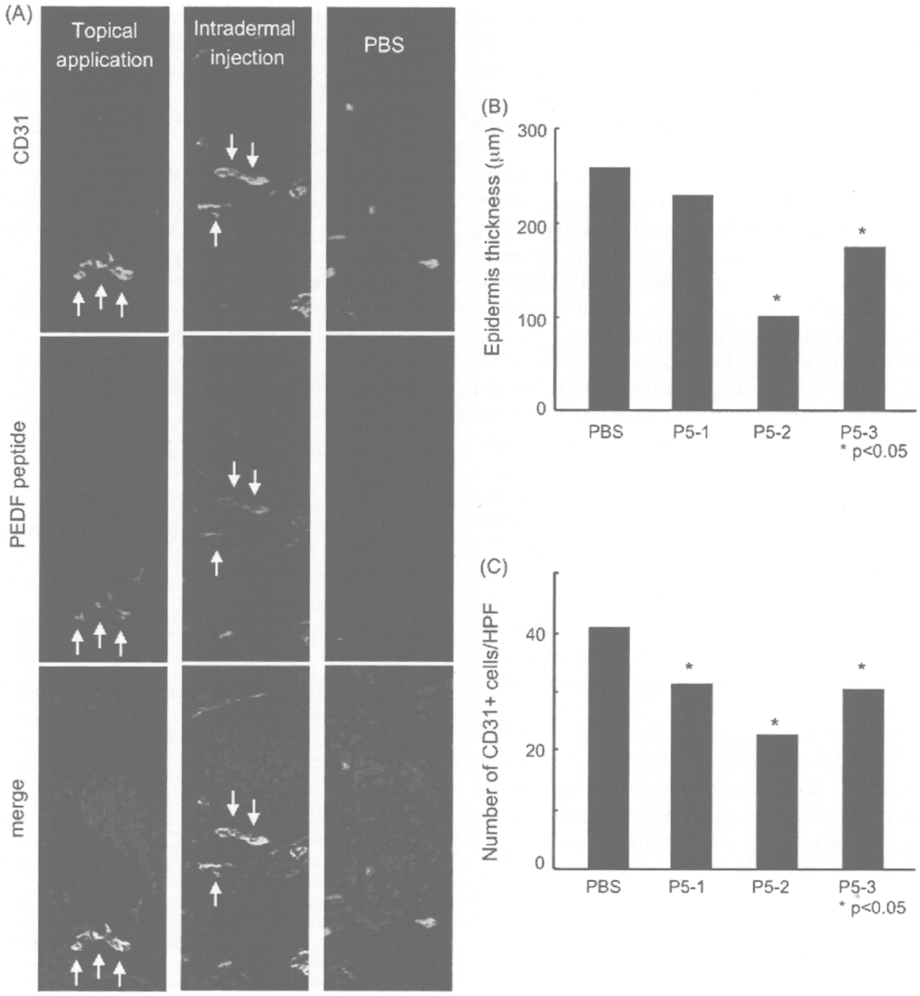

Fig. 7. Topically applied PEDF peptides penetrate into skin and reduce epidermal thickness and angiogenesis. (A) Biotin-labeled PEDF peptide (P5–1) was applied to the skin and its localization studied 2 h later using rhodamine–avidin staining as described in Section 2. Co-localization with PEDF peptide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

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References

[1] Schon MP, Boehncke WH. N Engl J Med 2005;352:1899–912.
[2] Bowcock AM, Krueger JG. Nat Rev Immunol 2005;5:699–711.
[3] Creamer JD, Barker JN. Clin Exp Dermatol 1995;20:6–9.
[4] Detmar M, Brown LF, Claffey KP, Yeo KT, Kocher O, Jackman RW, et al. J Exp Med 1994;180:1141–6.
[5] Ballaun C, Weninger W, Uthman A, Weich H, Tschachler E. J Invest Dermatol 1995;104:7–10.
[6] Bhushan M, McLaughlin B, Weiss JB, Griffiths CE. Br J Dermatol 1999;141:1054–60.
[7] Lowes MA, Bowcock AM, Krueger JG. Nature 2007;445:866–73.
[8] Tombran-Tink J, Chader GG, Johnson LV. Exp Eye Res 1991;53:411–4.
[9] Dawson DW, Volpert OV, Gillis P, Crawford SE, Xu HJ, Benedict W, et al. Science 1999;285:245–8.
[10] Abe R, Shimizu T, Yamagishi S-i, Shibaki A, Amano S, Inagaki Y, et al. Am J Pathol 2004;164:1225–32.
[11] Amano S, Yamagishi S-i, Inagaki Y, Nakamura K, Takeuchi M, Inoue H, et al. Microvasc Res 2005;69:45–55.
[12] Yamagishi S, Inagaki Y, Amano S, Okamoto T, Takeuchi M, Makita Z. Biochem Biophys Res Commun 2002;296:877–82.
[13] Walker PA, Leong LE, Ng PW, Tan SH, Waller S, Murphy D, et al. Biotechnology (N Y) 1994;12:601–5.
[14] Takenaka K, Yamagishi S-i, Jinnouchi Y, Nakamura K, Matsui T, Imaizumi T. Life Sci 2005;77:3231–41.
[15] Fukami K, Ueda S, Yamagishi S-i, Kato S, Inagaki Y, Takeuchi M, et al. Kidney Int 2004;66:2137–47.
[16] Brizzi MF, Dentelli P, Pavan M, Rosso A, Gambino R, Grazia De Cesaris M, et al. J Clin Invest 2002;109:111–9.
[17] Francis MK, Appel S, Meyer C, Balin SJ, Balin AK, Cristofalo VJ. J Invest Dermatol 2004;122:1096–105.
[18] Bhagavathula N, Nerusu KC, Fisher GJ, Liu G, Thakur AB, Gemmell L, et al. Am J Pathol 2005;166:1009–16.
[19] Zeigler M, Chi Y, Tumas DB, Bodary S, Tang H, Varani J. Lab Invest 2001;81:1253–61.
[20] Nickoloff BJ, Nestle FO. J Clin Invest 2004;113:1664–75.
[21] Segre JA. J Clin Invest 2006;116:1150–8.
[22] Bos JD, Meinardi MMHM. Exp Dermatol 2000;9:165–9.
[23] Doll JA, Stellmach VM, Bouck NP, Bergh ARJ, Lee C, Abramson LP, et al. Nat Med 2003;9:774–80.
[24] Notari L, Baladron V, Aroca-Aguilar JD, Balko N, Heredia R, Meyer C, et al. J Biol Chem 2006;281:38022–37.
[25] Distler JH, Hirth A, Kurowska-Stolarska M, Gay RE, Gay S, Distler O. Q J Nucl Med 2003;47:149–61.
[26] Filleur S, Volz K, Nelius T, Mirochnik Y, Huang H, Zaichuk TA, et al. Cancer Res 2005;65:5144–52.

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Identification of a preferred substrate peptide for transglutaminase 3 and detection of *in situ* activity in skin and hair follicles

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Keywords
epidermis; hair follicle; phage-display; skin; transglutaminase

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Transglutaminases (TGases) are a family of enzymes that catalyze cross-linking reactions between proteins. During epidermal differentiation, these enzymatic reactions are essential for formation of the cornified envelope, which consists of cross-linked structural proteins. Two main transglutaminases isoforms, epidermal-type (TGase 3) and keratinocyte-type (TGase 1), are cooperatively involved in this process of differentiating keratinocytes. Information regarding their substrate preference is of great importance to determine the functional role of these isozymes and clarify their possible co-operative action. Thus far, we have identified highly reactive peptide sequences specifically recognized by TGases isozymes such as TGase 1, TGase 2 (tissue-type isozyme) and the blood coagulation isozyme, Factor XIII. In this study, several substrate peptide sequences for human TGase 3 were screened from a phage-displayed peptide library. The preferred substrate sequences for TGase 3 were selected and evaluated as fusion proteins with mutated glutathione *S*-transferase. From these studies, a highly reactive and isozyme-specific sequence (E51) was identified. Furthermore, this sequence was found to be a prominent substrate in the peptide form and was suitable for detection of *in situ* TGase 3 activity in the mouse epidermis. TGase 3 enzymatic activity was detected in the layers of differentiating keratinocytes and hair follicles with patterns distinct from those of TGase 1. Our findings provide new information on the specific distribution of TGase 3 and constitute a useful tool to clarify its functional role in the epidermis.

Introduction

Transglutaminases (TGases: EC 2.3.2.13) are a family of enzymes that catalyze the calcium-dependent formation of isopeptide cross-links between glutamine and lysine residues in various proteins [1,2]. Furthermore, these enzymatic reactions include the attachment of primary amines to peptide-bound glutamine residues, and the conversion of glutamine to glutamic acid. To date, eight TGase isozymes (Factor XIII, TGases 1–7),

Abbreviations
bio-Cd, 5-(biotinamido)pentylamine; CE, cornified envelope; Dansyl-Cd, monodansylpentylamine; FITC, fluorescein isothiocyanate; GST, glutathione *S*-transferase; SPR, small proline-rich protein; TBS, Tris/buffered saline; TGase, transglutaminase.

comprising a protein family with unique substrate specificities and different tissue distributions, have been identified in mammals. Factor XIII and TGase 2 are involved in the stabilization of fibrin clots and various roles including apoptosis, extracellular matrix formation and wound healing, respectively [3–6]. TGase 1 and TGase 3 have been reported to contribute to the formation of the epidermis by cross-linking structural proteins in keratinocytes [7–9]. TGase 4 is expressed in the prostate and is reported to be involved in plug formation in rodents [10]. The biochemical characterization and physiological roles of TGase 5 (expressed in keratinocytes), TGase 6 and TGase 7 remain unknown [11,12].

In a TGase-catalyzed reaction, a glutamine residue in the substrate binds to the cysteine residue at the active site of the enzyme, resulting in the formation of an intermediate. This is a rate-limiting step because not all glutamine residues participate in the reaction. By contrast, the reaction with the second substrate, a lysine residue or a primary amine, is less selective. Moreover, distinct isozymes recognize distinct glutamine residues in the same protein. Therefore, primary and secondary structures surrounding the reactive glutamine residues are critical in the formation of an intermediate enzyme–substrate complex. Each isozyme in the TGase family, mainly characterized as TGase 1, TGase 2 and Factor XIII, demonstrates different substrate recognition patterns because the glutamine residues in the substrate involved in binding to the enzyme are isozyme specific [13,14].

We have established a screening system that employs a phage-displayed random peptide library to characterize the preferred substrate sequences for TGase [15–18]. In a series of studies, 12-mer sequences acting as isozyme-specific substrates for Factor XIII, TGase 1 and TGase 2 were obtained. From these studies, we selected the most reactive and isozyme-specific substrate sequences that were functional not only as phage-display proteins, but also as peptide forms. Furthermore, in our recent reports, the most reactive peptide sequence (K5), selected as a TGase 1-preferred substrate, was successfully used as a probe to detect *in situ* enzymatic activity in both human and mouse skin [16,19]. These studies have provided new insight into the substrate specificity of Tgases and have expanded the range of application of the enzyme reaction [20].

TGase 3, initially designated as an epidermal-type enzyme, is responsible for formation of the epidermis [21,22]. In the current model of TGase function, during keratinocyte differentiation, TGase 1 and TGase 3 are believed to act cooperatively in the cross-linking of proteins, including involucrin, loricrin and small proline-rich proteins (SPRs). Such concerted reactions result in formation of the cornified envelope (CE), a specialized component consisting of covalent cross-links of proteins beneath the plasma membrane of terminally differentiated keratinocytes [23,24]. Furthermore, TGase 3 in hair follicles is involved in cross-linking structural proteins such as trichohyalin and keratin intermediate to hardening the inner root sheath. In this case, TGase 1 co-operates with TGase 3 through a cross-linking reaction to produce stable hair fibers.

During differentiation in these processes, a zymogen form of TGase 3 (77 kDa) is activated by limited proteolysis with cathepsins S and/or L [25,26]. Although several studies have focused on the localization, structural analysis and activation mechanism of TGase 3 zymogen, not much information is available about the substrate specificity and physiological function of the active form [27–31]. In particularly, the precise substrate specificity and local activation areas of TGase 1 and TGase 3 in the epidermis have not been fully identified.

In this study, we applied a screening system to obtain the preferred substrate peptides for human TGase 3. The selected phages displayed a unique tendency toward the primary sequences, and the most reactive and isozyme-specific sequence among the peptide sequences was determined. Furthermore, this sequence proved to be a prominent substrate in the peptide form. Specific localization of activated TGase 3, which was found to display a pattern distinct from that of TGase 1, was observed by the detection of *in situ* activities using this peptide.

Results

Screening of candidate substrate sequences from a random peptide library

Phage clones in a random peptide library were incubated with biotinylated cadaverine (bio-Cd), a glutamine-acceptor substrate, in the presence of the activated form of human TGase 3 (Fig. S1). By the enzymatic reaction, phage particles displaying the reactive glutamine residues preferably incorporate bio-Cd. Avidin affinity purification resulted in the selection of phage particles that covalently bound bio-Cd. The phage particles were amplified and subjected to four additional enzymatic reactions and panning. Sequence analysis of the finally selected individual phage clones (125 clones) revealed that ~ 93.6% (117/125) of the clones displayed peptide sequences containing glutamine residue. In this process, false-positive clones containing no glutamine residue might be co-purified if the sequence has an affinity to avidin.