REFERENCES

1 Tsubota A, Akiyama M, Sakai K, Goto M, Nomura Y, Ando S, et al. Keratin 1 gene mutation detected in epidermal nevus with epidermolytic hyperkeratosis. J Invest Dermatol 2007; 127: 1371-1374.

2 Lee DY, Ahn KS, Lee CH, Rho NK, Lee JH, Lee ES, et al. Two novel mutations in the keratin 1 gene in epidermolytic hyperkeratosis. J Invest Dermatol 2002; 119: 976-7.

3 Müller FB, Küster W, Wodecki K, Almeida H Jr, Bruckner-Tuderman L, Krieg T, et al. Novel and recurrent mutations in keratin KRT5 and KRT14 genes in epidermolysis bullosa simplex: implications for disease phenotype and keratin filament assembly.Hum Mutat 2006; 27: 719-20.

4 Lu Y, Guo C, Liu Q, Zhang X, Cheng L, Li J,et al. A novel mutation of keratin 9 in epidermolytic palmoplantar keratoderma combined with knuckle pads. Am J Med Genet A 2003; 120A: 345-9.

5 Coulombe PA, Fuch E. Elucidating the early stages of keratin filament assembly. J Cell Biol 1990; 111: 153-69.

6 Heald R, McKeon F. Mutations of phosphorylation sites in lamin A that prevent nuclear lamina disassembly in mitosis. Cell 1990; 61:579-89.

FIGURE LEGENDS

Fig. 1. Clinical, histopathological and ultrastructural features of the patient. Severe diffuse hyperkeratosis and scales are seen on the palms (a) and soles (b). Warty brown hyperkeratosis and scales are present on the margins and the dorsa of the foot (c). Generalized erythroderma and scaling on the trunk (d). Histopathological examination revealed acanthosis and hyperkeratosis, coarse keratohyaline granules, and severe granular degeneration in the entire spinous and granular layers of the epidermis (e). Ultrastructurally, clumping of the keratin filaments (arrows) is seen within an upper epidermal keratinocyte of the epidermis (f).

Fig 2. Summary of mutations in the helix initiation motif (HIM) and helix termination motif (HTM) of K1 from Human Intermediate Filament Database (http://www.interfil.org/). (a) Molecular structure of K1. (b) Heptad repeats in HIM and HTM of K1 and mutation sites. The majority of cases (22 out of 26) had mutations in the heptad repeat position ***a***, ***d***, ***e*** and ***g***. The present mutation is located at the ***a*** position leucine residue at codon no.486 (red characters) in the C-terminal-most heptad repeat. (c) Summary of the *KRT1* mutations in HIM and HTM, alterations of hydropathy index and levels of palmoplantar hyperkeratosis. Eight cases including the present one were reported as showing severe palmoplantar hyperkeratosis and 7 of those 9 patients harbored mutations in the important ***a, d, e*** and ***g*** position of heptad repeats. Mutations in this 486-leucine residue may seriously perturb the stability of keratin intermediate filaments. The substitution of arginine for leucine alters the character of amino acid from that of a hydrophobic, apolar amino acid (hydropathy index of leucine: +3.8) to that of the most hydrophilic, basic amino acid (hydropathy index of arginine: -4.5). (d) Heptad structure of

the rod domain: Schematic of a transverse cut through the last heptad (***abcdefg***) of the HTM of K1 and K10, showing hydrophobic interactions between positions ***a*** and ***d*** (dashed lines) and ionic hydrogen interactions between positions ***e*** and ***g*** (dotted lines). Position ***a*** is occupied by apolar, hydrophobic amino acids. The ***a*** residues are thought to interact with amino acids located in the ***d*** position of the partner molecule of the heterodimer through hydrophobic interactions which stabilize the two-chain coiled-coil molecules. When the two strands coil around each other, positions ***a*** and ***d*** are internalized, stabilizing the structure, while positions ***b,c,e,f,g*** are exposed on the surface of the protein. Residues at positions ***e*** and ***g*** stabilize dimer formation through ionic and hydrogen bonds.

Journal Name	Manuscript No.	B	Dispatch: 17.8.09	Journal: IJD	CE: Vijay
IJD	4324		Author Received:	No. of pages: 2	PE: Prasanna

doi: 10.1111/j.1365-4632.2009.04324.x

Correspondence

Type XVII collagen ELISA indices significantly decreased after bullous pemphigoid remission

The major pathogenic epitope of bullous pemphigoid (BP) is known to be the noncollagenous extracellular domain (NC16A) of type XVII collagen (COL17).[1] Here we investigated indirect immunofluorescence (IIF) and COL17 NC16A domain enzyme-linked immunosorbent assay (ELISA)[2–5] data before treatment and after remission to evaluate the usefulness of ELISA analyses as indicators for BP disease activity.

We included ten consecutive BP patients [eight women and two men: between 33 and 80-year-old (mean; 59-years-old)] who showed typical clinical features before treatment and were successfully treated, resulting in complete or partial remission at our institute. The first day of each patient visit was within the last 3 years. In all patients, the diagnosis was confirmed by histopathological observation and immunofluorescence study, i.e. histopathological subepidermal blister formation was observed and direct and IIF studies revealed the presence of autoantibodies along the dermal–epidermal junction. All patients were successfully treated with oral prednisolone therapy of 30–50 mg/d with or without azathioprine or a combination therapy using tetracycline and nicotinamide. Treatment periods from initial diagnosis to remission ranged from 4 months to 35 months (mean; 14.6 ± 10.8 months). Serum samples were obtained for ELISA and IIF at least twice during the disease course for each patient.

Concentration of autoantibodies in the patients' sera directed against the NC16A domain of COL17 was measured using the COL17 NC16A ELISA kit following the kit's instructions.[6] IIF staining and evaluation were performed as previously described using normal human skin as a substrate.[7]

In all the cases, the ELISA indices showed a decrease during the successful treatment course. ELISA indices after remission were significantly reduced compared with those before treatment ($P < 0.0001$) (Fig. 1a). IIF titers

POOR QUALITY COLOUR FIG

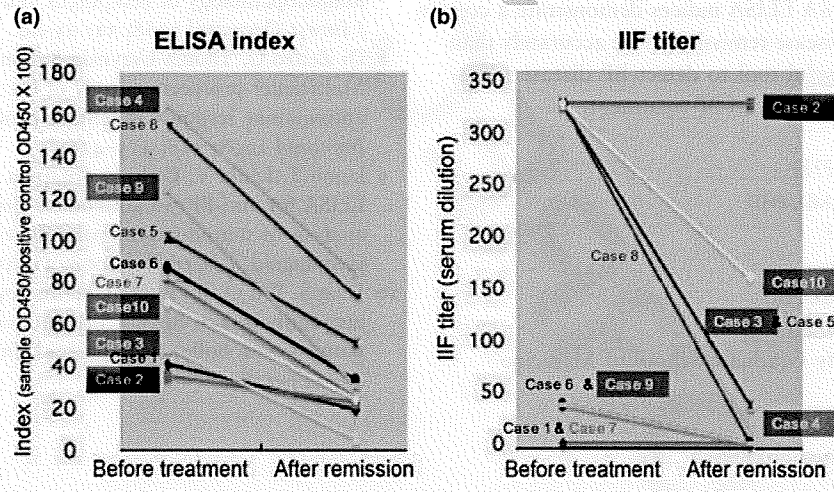

Figure 1 ELISA indices and indirect immunofluorescence (IIF) titers before treatment and after remission. (a) ELISA indices of successfully treated BP patients. Disease remission was defined as when erythema, bullae and erosions had completely healed (complete remission) or no more than three bullae or erythema were seen in a week (partial remission) and only a low dose of oral prednisolone (<5 mg/d) or no treatment was needed to maintain this condition. As ELISA indices after remission, we adopted ELISA indices at the time when each patient's disease activity was evaluated as being in "complete remission" or "partial remission" (as defined above) for the first time after treatment. Mean ELISA index of the 10 patients before treatment was 91.3 ± 45.7 (range: 35.6–165.6) and the mean index after remission was 37.4 ± 25.3 (range: 6.0–86.4). After complete or partial remission, the ELISA indices were significantly reduced ($P < 0.0001$). (b) IIF titers of the same patients. Apparent decreases in IIF titers after remission were seen only in six patients. Mean IIF titer of the 10 patients before treatment was 201 ± 154 (range: 5–320) and the mean titer after remission was 60.5 ± 102.8 (range: 5–320). A statistically, significant reduction was observed in combined IIF titers after remission compared with those before treatment ($P < 0.05$). Colors of the lines are specific for each patient in both figures (a) and (b)

also decreased after remission in six cases, but the titers were not apparently reduced in the other four cases, although a statistically significant reduction in combined IIF titer was observed after remission compared with those before treatment ($P < 0.05$) (Fig. 1b).

Positive correlation between ELISA indices and BP disease activity has been reported previously in the literature. Di Zenzo *et al.*[8] demonstrated that disease severity before treatment was well correlated with ELISA indices in BP patients. Izumi *et al.*[9] described ELISA indices and alteration of disease activity of five BP patients during various treatments. In this study, we compared the ELISA indices before treatment and after remission in our BP patient cohort and clearly demonstrated that ELISA indices significantly decreased after remission. Feng *et al.*[10] reported similar results on correlation of ELISA indices with disease course in BP patients, although the time points for ELISA after treatment were just before the decrease in corticosteroid and when the dosage of corticosteroid was successfully decreased to half the initial dose in the report. In this study, we employed ELISA indices at the time when each patient's disease activity was evaluated as "complete remission" or "partial remission" for the first time after treatment. Thus, this study is unique in the point that we evaluated exact correlation between ELISA indices and disease remission.

In conclusion, the present results further support the idea that the COL17 NC16A ELISA indices demonstrate a correlation with the BP disease remission more accurately than IIF titers and are a useful tool to detect BP disease remission and to assess the efficacy of BP treatment.

Erika Kusajima, ??????
Masashi Akiyama, MD, PHD
Megumi Sato, ?????
Ken Natsuga, MD
Hiroshi Shimizu, MD, PHD
1 Department of Dermatology
Hokkaido University Graduate School of Medicine
Sapporo
Japan
E-mail: akiyama@med.hokudai.ac.jp

Conflict of interest: the authors state no conflict of interest.

References

1 Giudice GJ, Emery DJ, Zelickson BD, *et al.* Bullous pemphigoid and herpes gestationis autoantibodies recognize a common non-collagenous site on the BP180 ectodomain. *J Immunol* 1993; 151: 5742–5750.
2 Zillikens D, Mascaro JM, Rose PA, *et al.* A highly sensitive enzyme-linked immunosorbent assay for the detection of circulating anti-BP180 autoantibodies in patients with bullous pemphigoid. *J Invest Dermatol* 1997; 109: 679–683.
3 Hata Y, Fujii Y, Tsunoda K, Amagai M. Production of the entire extracellular domain of BP180 (type XVII collagen) by baculovirus expression. *J Dermatol Sci* 2000; 23: 183–190.
4 Schmidt E, Obe K, Brocher EB, Zillikens D. Serum levels of autoantibodies to BP180 correlate with disease activity in patients with bullous pemphigoid. *Arch Dermatol* 2000; 136: 174–178.
5 Kobayashi M, Amagai M, Kuroda-Kinoshita K, *et al.* BP180 ELISA using bacterial recombinant NC16a protein as a diagnostic and monitoring tool for bullous pemphigoid. *J Deramtol Sci* 2002; 30: 224–232.
6 Tsuji-Abe Y, Akiyama M, Yamanaka Y, *et al.* Correlation of clinical severity and ELISA indices for the NC16A domain of BP180 measured using BP180 ELISA kit in bullous pemphigoid. *J Dermatol Sci* 2005; 37: 145–149.
7 Beutner EH, Jordon RE, Chorzelski TP. The immunopathology of pemphigus and bullous pemphigoid. *J Invest Dermatol* 1968; 51: 63–80.
8 Di Zenzo G, Thoma-Uszynski S, Fontao L, *et al.* Multicenter prospective study of the humoral autoimmune response in bullous pemphigoid. *Clin Immunol* 2008; 128: 415–426.
9 Izumi T, Ichiki Y, Esaki C, Kitajima Y. Monitoring of ELISA for anti-BP180 antibodies: clinical and therapeutic analysis of steroid-treated patients with bullous pemphigoid. *J Dermatol* 2004; 31: 383–391.
10 Feng S, Wu Q, Jin P, *et al.* Serum levels of autoantibodies to BP180 correlate with disease activity in patients with bullous pemphigoid. *Int J Dermatol* 2008; 47: 225–228.

Letters to the Editor

Title:

Two cases of cutaneous sporotrichosis in continental/ microthermal climate zone: global warming alert?

Authors:

Daisuke Inokuma, Akihiko Shibaki, Hiroshi Shimizu

Affiliation:

Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Correspondence to

Daisuke Inokuma, M.D., Ph.D.
Department of Dermatology: Hokkaido University Graduate School of Medicine
N15 W7, Sapporo 060-8638, Japan.
Tel: +81-11-716-1161 ex.5962 Fax: +81-11-706-7820
e-mail:inokuma@med.hokudai.ac.jp

Key words:

Sporotrichosis, *Sporothrix schenckii*, continental/ microthermal climate zone, global warming

Word count: 498 words

Conflicts of interest: None to declare

Case report

Sporotrichosis is commonly encountered in tropical and subtropical areas, but rarely in continental/ microthermal climate zone of the Köppen-Geiger Climate Classification[1]. Here, we report two cases of cutaneous sporotrichosis in Hokkaido, an island in the continental/ microthermal climate zone in Japan.

A 55-year-old Japanese female presented with a 6-month history of two dark-red-crusted infiltrated skin lesions measuring about 10 mm and 2 mm on the left upper eyelid on 2009 (Fig. 1a). She was working as a farmer in Hokkaido and had never lived in any other part of Japan. Histopathological examination of hematoxylin and eosin stained specimens revealed a prominent epidermal hyperplasia and abundant inflammatory infiltration in the dermis (Fig. 1b, c). Periodic acid Schiff stain (Fig. 1d) and Grocott's methenamine silver stain (Fig. 1e) showed a few round and budding yeast-like cells scattered in the dermis, occasionally within a giant cell. Cultures of the tissue sections of lesion on Sabouraud's dextrose agar and potato dextrose agar grew dark brown velvety colonies (Fig. 1f). Slide cultures from the colonies showed septate branching hyphae with slender, tapering conidiophores arising at right angles (Fig. 1g). A sporotrichin skin test showed positive reaction.

A 55-year-old male patient presented with a chronic erosive nodule measured 20 x 10 mm on the left mandible, which was present for over 1 year on 2002 (Fig. 2a). He was working as a carpenter in Hokkaido, and he had never lived in any other part out of Hokkaido. Histopathological analysis showed prominent epidermal hyperplasia and chronic granulomatous inflammatory cells infiltrate (Fig. 2b, c). The periodic acid Schiff stain and Grocott's staining sections revealed the presence of round yeast-like cells scattered in the dermis (Fig. 2d). Cultures of the skin biopsied tissue samples on Sabouraud's dextrose agar produced dark brown velvety colonies (Fig. 2e). Slide cultures from the colonies showed septate, branching hyphae (Fig. 2f). Sporotrichin skin test showed positive reaction.

Cutaneous sporotrichosis is a fungal infection commonly encountered in tropical and subtropical areas[1]. In Japan, more than 3,500 cases of cutaneous sporotrichosis had been reported as of 2001 on Honshu Island, which falls in temperate/ mesothermal climates zone[2]. In contrast, no case reports were available in Hokkaido in English journals as of 2004. Similarly, few cases had been reported in continental/ microthermal climate zone around the world[3], suggesting that cutaneous sporotrichosis is extremely rare in that zone. This

geographic difference in reported cases may be due to the fact that *Sporothrix schenckii,* the pathogenic fungus that causes sporotrichosis, prefers moderate temperatures (around 22 degrees Celsius) [1]. The yearly temperature in Hokkaido averaged for the years 2000 to 2008 was 9.1 degrees Celsius, so these results further suggest that *Sporothrix schenckii* rarely develops in Hokkaido. Interestingly, three cases of cutaneous sporotrichosis, including the two abovementioned cases, have been reported from Hokkaido in the Japanese literature since 2000, whereas only one case was recorded before 2000. It is suggested that the prevalence of cutaneous sporotrichosis in Hokkaido may be increasing as a result of recent global warming.

ACKNOWLEDGMENTS

We thank Hideomi Shibaki M.D. for his technical assistance with the cultures.

FIGURE LEGENDS

Figure 1

(a)

Clinical appearance of the nodule and papule on the left upper eyelid.

(b-e)

Skin biopsy findings: Hematoxylin and eosin staining reveals abundant infiltrate of lymphocytes, plasma cells, histiocytes, and giant cells. Low-power magnification (b), and high-power magnification (c). The periodic acid-Schiff stain (d) and Grocott methenamine silver stain (e) show the presence of round and budding yeast-like cells in the dermis.

(f, g)

Cultures of the biopsy specimen on Sabouraud's dextrose agar and potato dextrose agar: dark brown velvety colonies form (f). From slide cultures, branching hyphae with slender, tapering conidiophores rising at right angles are seen. The apex of the conidiophore bears pear-shaped or almost round conidia, giving it a flower-like appearance. Dark brown conidia are produced singly along the hyphae and conidiophores (g).

Figure 2

(a)

Clinical appearance of the nodule on the left mandible.

(b-d)

Skin biopsy findings: Hematoxylin and eosin staining reveals chronic inflammatory granuloma with infiltration of neutrophils, histiocytes, and giant cells. Low-power magnification (b), and high-power magnification (c). The Grocott methenamine silver stain (d) shows a few round yeast-like cells in the dermis.

(e, f)

Cultures of the biopsy specimen using Sabouraud's dextrose agar: a dark brown velvety colonies yield grows (e). Slide cultures from colonies show fine branching, septate hyphae with spherical conidia at the tips of the conidiophores, and thick-walled dematiaceous conidia along the conidiophores and hyphae (f).

REFERENCES

1. Bustamante B, Campos P. Endemic sporotrichosis. *Curr Opin Infect Dis* 2001; 14: 145-9

2. Kikuchi I, Morimoto K, Kawana S et al. Usefulness of itraconazole for sporotrichosis in Japan: study of three cases and literature comparison of therapeutic effects before and after release on the market. *Eur J of Dermatol* 2006; 16: 42-7

3. Bargman H. Successful treatment of cutaneous sporotrichosis with liquid nitrogen: report of three cases. *Mycoses* 1995; 38: 285-7

REVISION OF MANUSCRIPT #08-01362

Case Letter for *The Journal of the American Academy of Dermatology*

Aleukemic leukemia cutis with extensive bone involvement

Maria Maroto Iitani, Riichiro Abe, Teruki Yanagi, Asuka Hamasaka, Yasuki Tateishi, Yukiko Abe, Miki Ito*,Takeshi Kondo**, Kanako Kubota***, Hiroshi Shimizu

Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

* Department of Dermatology, National Hospital Organization Hokkaido Cancer Center, Sapporo, Japan

** Department of Gastroenterology and Hematology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

***Department of Surgical Pathology, Hokkaido University Hospital, Sapporo, Japan

Running head: Bone metastases in aleukemic leukemia cutis

Key words: aleukemic leukemia cutis, acute myelocytic leukemia, multiple bone involvement

Word counts: 507

Number of Figures: 3

Number of References: 8

Statement of all funding sources for this work: None

The authors have no conflicts of interest to disclose.

Correspondence and reprint requests to:

Maria Maroto Iitani, M.D.

Department of Dermatology,

Hokkaido University Graduate School of Medicine.

North 15, West 7, Kita-ku, Sapporo 060-8638, Japan,

Phone: +81-11-716-1161 ext. 5962

Fax: +81-11-706-7820

e-mail: miitani@med.hokudai.ac.jp

To the editor: Aleukemic leukemia cutis (ALC) is a rare condition that is characterized by the invasion of leukemic cells into the skin prior to such cells being observed in the peripheral blood.[1] Here, we present a case of ALC with multiple bone metastases.

An 81-year-old male presented with a two-month history of an asymptomatic nodule on his left thigh. A careful physical examination revealed five subcutaneous nodules measuring up to 10mm in size on his trunk and legs together with a firm, slightly violaceous nodule measuring 20 mm in diameter on his left thigh (Fig. 1). A complete blood cell count and chemical analysis showed no pathologic changes. Histopathologic examination revealed dense, nodular, diffuse infiltrate of monotonous uniform cells with round nuclei, prominent single or multiple nucleoli, and abundant pale, slightly eosinophilic cytoplasmic cells throughout the dermis and subcutaneous fat (Fig. 2a). Atypical mitotic figures were scant (Fig. 2b). Histological diagnosis of myeloid leukemia cutis with possible monocytic lineage was made. However, bone marrow aspiration showed neither an increase in blasts nor abnormal cell infiltration, and repeated peripheral blood counts were normal, with no atypical cells. A diagnosis of ALC was established. Positron emission tomography revealed extensive high-density areas on the bone and the subcutaneous tissue, suggesting multiple metastases (Fig. 3). At seven weeks after the first visit, peripheral blood examination disclosed 8% of atypical monocytic cells indicating a diagnosis of acute myeloid leukemia (AML). The patient died about one week later.

ALC is a rare form of leukemia with poor prognosis. It was first reported by Yoder in 1976.[2] The term aleukemic has been used to designate a form of leukemia in which there are no leukemic cells in the blood.[2] ALC precedes to the peripheral blood or bone marrow abnormalities at least one month before the systemic findings. Once leukemic cells appear in the peripheral blood or bone marrow, the mean survival time ranges from 3 to 30 months.[3,4] The clinical features of ALC include multiple papules, nodules or infiltrated plaques with a red-brown or plum coloured surface. Histological findings show infiltration of leukemic cells in the dermal or subcutaneous tissues. The cytologic features of the tumour cells include the large size, vesicular nuclei, and multiple prominent nucleoli.[3] There is no consensus on the treatment of choice for ALC, due to rarity of the disease; radiotherapy, chemotherapy and total body electron therapy have been reported to achieve varying results.[1,3,4,5,6,7,8] A study of the largest group of ALC patients, by Chang et al., showed the commonest extramedullary site in ALC after the skin to be the lymph node (8 patients out of 31), followed by the spleen (2 patients out of 31).[4] Although no reports of clinical presentation of ALC with multiple bone infiltration were found in a thorough search of the English-language literature, extramedullary leukemia is known to occur in the bone.[5] It is to be emphasized that routine assessment of a patient with ALC should include systemic investigations such as PET scan in our case, taking into consideration the possibility of bone involvement..

References

1 Ohno S, Yokoo T, Ohta M, et al. Aleukemic leukemia cutis J Am Acad Dermatol 1990;22:374-7

2 Yoder FW, Shuen RL. Aleukemic leukemia cutis. Arch Dermatol 1976;112:367-9

3 Okun MM, Fitzgibbon J, Nahass GT, et al. Aleukemic leukemia cutis, myeloid subtype. Eur J Dermatol 1995;5:290-3

4 Chang H, Shin LY, Kuo TT. Primary aleukemic myeloid leukemia cutis treated successfully with combination chemotherapy: report of a case and review of the literature. Ann Hematol 2003;82:435-9

5 Lee B, Fatterpekar GM, Kim W, et al. Granulocytic sarcoma of the temporal bone, Am J Neuroradiaol 2002;23:1497-9

6 Torok L, Lueff S, Garay G, et al. Monocytic aleukemic leukemia cutis. J Eur Acad Dermatol Venerol 1999;13:54-8

7 Tomasini C, Quaglino P, Novelli M, et al. "Aleukemic" granulomatous leukemia cutis. Am J Dermatopathol 1998;20:417-21

8 Imanaka K, Fujiwara K, Satoh K, et al. A case of aleukemic monocytic leukemia cutis treated with total body electron therapy. Radait Med 1988;6:229-31

Fig. 1. A slightly violaceous nodule on the left thigh.

Fig. 2. Skin biopsy findings: (a) Dense, nodular, diffuse infiltrate of monotonous uniform cells involving the dermis and subcutaneous fat. (b) A nodule of cells with round nuclei, prominent single or multiple nucleoli and abundant pale, slightly eosinophilic cytoplasm and scant atypical mitotic figures.

Fig. 3. PET reveals extensive high-density areas on the bone and in the subcutaneous tissue on the trunk and the extremities, suggesting multiple metastases.

The American Journal of Pathology, Vol. 176, No. 4, April 2010

DOI: 10.2353/ajpath.2010.090597

Short Communication

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

Masashi Akiyama,* Kaori Sakai,* Teruki Yanagi,* Satoshi Fukushima,† Hironobu Ihn,† Kiyotaka Hitomi,‡ and Hiroshi Shimizu*

From the Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan; the Department of Dermatology and Plastic Surgery,† Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan; and the Department of Applied Molecular Biosciences,‡ Graduate School of Bioagricultural Sciences, Nagoya University, Japan*

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, 176:000–000; 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.

Supported in part by a grant from the Ministry of Education, Science, and Culture of Japan to M.A. (Kiban B 20390304) and by a grant from Ministry of Health, Labor, and Welfare of Japan (Health and Labor Sciences Research Grants; Research on intractable diseases; H21-047) to M.A.

Accepted for publication November 23, 2009.

Address reprint requests to Masashi Akiyama, M.D., Ph.D., Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15 West 7, Kita-ku, Sapporo 060-8638, Japan. E-mail: akiyama@med.hokudai.ac.jp.

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 non-conserved 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 diges-

tions. 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

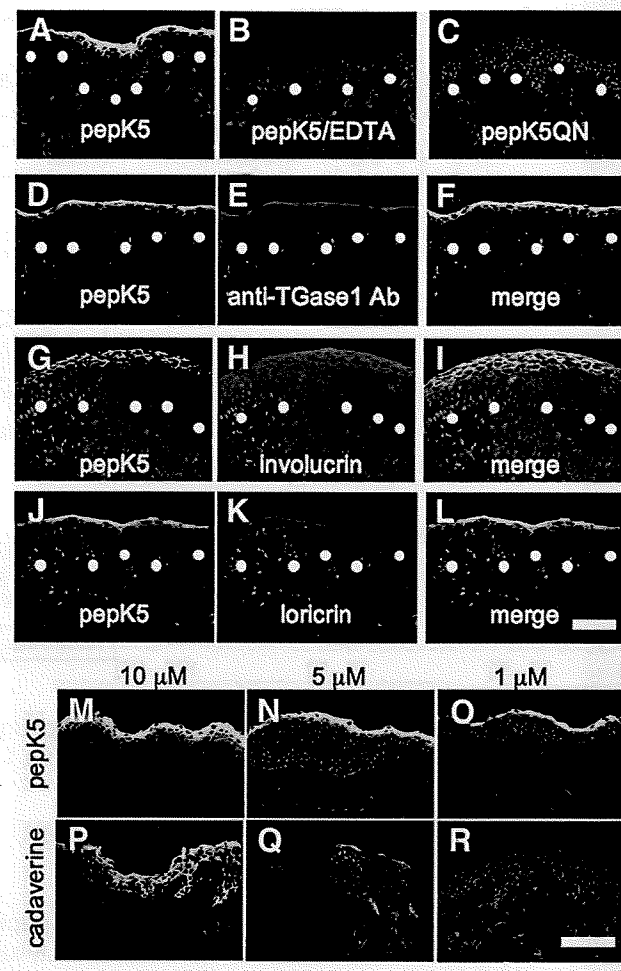

Figure 1. PepK5 labeling detected *in situ* TGase1 activity with high specificity and sensitivity at CCE forming sites in normal human skin. **A–C:** *In situ* TGase1 activity detected by pepK5 in normal skin. Detection of *in situ* TGase 1 activity using FITC-labeled pepK5 (5 μmol/L) showed intense 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.