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Elevated Human Tissue Kallikrein Levels in the Stratum Corneum and Serum of Peeling Skin Syndrome-Type B Patients Suggests an Over-desquamation of Corneocytes

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TO THE EDITOR

Peeling skin syndrome type B (PSS-type B, MIN 270300) is a congenital skin disease associated with continual skin peeling and ichthyotic erythroderma, known to display various clinical similarities with Netherton syndrome (NS, caused by genetic defects of *serine protease inhibitor Kazal-type5* (*SPINK5*), MIN 256500) (Wile, 1924; Traupe, 1989; Magert *et al.*, 1999; Chavanas *et al.*, 2000). Human tissue kallikreins are a family of 15 trypsin- or chymotrypsin-like secreted serine proteases (hK1–hK15) found in a variety of tissues (Yousef and Diamandis, 2001). At least eight different hKs have been identified in the stratum corneum (SC) and sweat as desquamation-related proteases (Komatsu *et al.*, 2005b, 2006). Here, we aimed (1) to clarify the pathogenesis of PSS-type B, (2) to elucidate the relationship between PSS-type B and hK expression, and (3) to explain the reason for the clinical similarities between PSS-type B and NS.

Informed consent was obtained from all patients, their parents, and normal volunteers, and our studies were performed according to the Declaration of Helsinki Principles. The Medical Ethics Committee of the Graduate School of Medical Science, School of Medicine, Kanazawa University and Juntendo University, School of Medicine, approved all described studies. Two unrelated 8-year-old female Japanese patients (Patient M and Patient K) were studied (Figure S1 and Table S1). Patients M and K were born with erythroderma accompanied by scaling and their lesions have shown no improvement to date. Patient M suffered significant growth retardation (<-2 SD) since the age of 1 and occasional herpes simplex infections in her perioral region. Both patients experience severe pruritus, temperature instability, and low sweat secretion. Patient M exhibited overabsorption of topical agents from skin, which is also observed in NS (Smith *et al.*, 1995; Allen *et al.*, 2001). Asthma attacks were experienced in Patient M but no allergic diseases were apparent in Patient K. Both patients displayed eosinophilia and elevated serum IgE levels. As many clinical manifestations observed in the patients are common between NS and PSS-type B (Traupe, 1989; Judge *et al.*, 1994; Griffiths *et al.*, 1998), *SPINK5* gene mutation analysis by genomic polymerase chain reaction and sequencing was performed for both patients using specific primers (Komatsu *et al.*, 2002). However, no gene mutations were detected in both patients; therefore, NS was ruled out, leaving PSS-type B as the most likely diagnosis.

Pathologically, the patients showed an absence of the SC or a few layers of parakeratosis, which tended to be separated from the stratum granulosum, psoriasisforme acanthosis, and perivascular infiltration with mononuclear leukocytes (Figure S2).

Immunohistochemistry for hK6, hK8, hK13, and SPINK5 protein showed that they were mainly expressed in the stratum granulosum and SC in normal epidermis (Figure 1). In both patients, the hKs and SPINK5 protein expressions were similarly distributed, and their stainings were deeply expanded into the lower epidermis compared with those in normal skin. It is known that the skin of NS patients shows absent or only faint staining against the same anti-SPINK5 protein antibody (Raghunath *et al.*, 2004). Consequently, Patients M and K are unlikely to suffer from NS.

In the SC of both Patients M and K, all hK concentrations studied by ELISA were dramatically higher than those in the normal SC samples (Table 1a). The elevation of minor skin hKs (e.g., hK10, hK6, and hK13; <1.0 ng/mg dry weight for normal subjects) were prominent in the SC of the patients (Table 1a). In the serum, hK6, hK7, hK8, hK10, and hK13 concentrations were significantly elevated in the patients (Table 1b).

SC trypsin-like serine protease enzymatic activity examined using Boc-Pro-Phe-Arg-AMC (PFR-) as a substrate, and plasmin-like (for Boc-Val-Leu-Lys-AMC; VLK-) and furin-like (for Pyr-Arg-Thr-Lys-Arg-AMC; R-KR-) activities were significantly elevated, whereas trypsin-like (for Boc-Phe-Ser-Arg-AMC; FSR-) and chymotrypsin-like (for MeO-Suc-Arg-Pro-Tyr-pNA-HCl; RPY-) activities showed only mild elevations in both patients (Table 1c).

Abbreviations: AMC, 7-amino-4-methyl-commarin; hK, kallikrein protein; NS, Netherton syndrome; pNA, para-nitroanilide; PSS, peeling skin syndrome; SC, stratum corneum; SPINK5, serine protease inhibitor Kazal-type 5

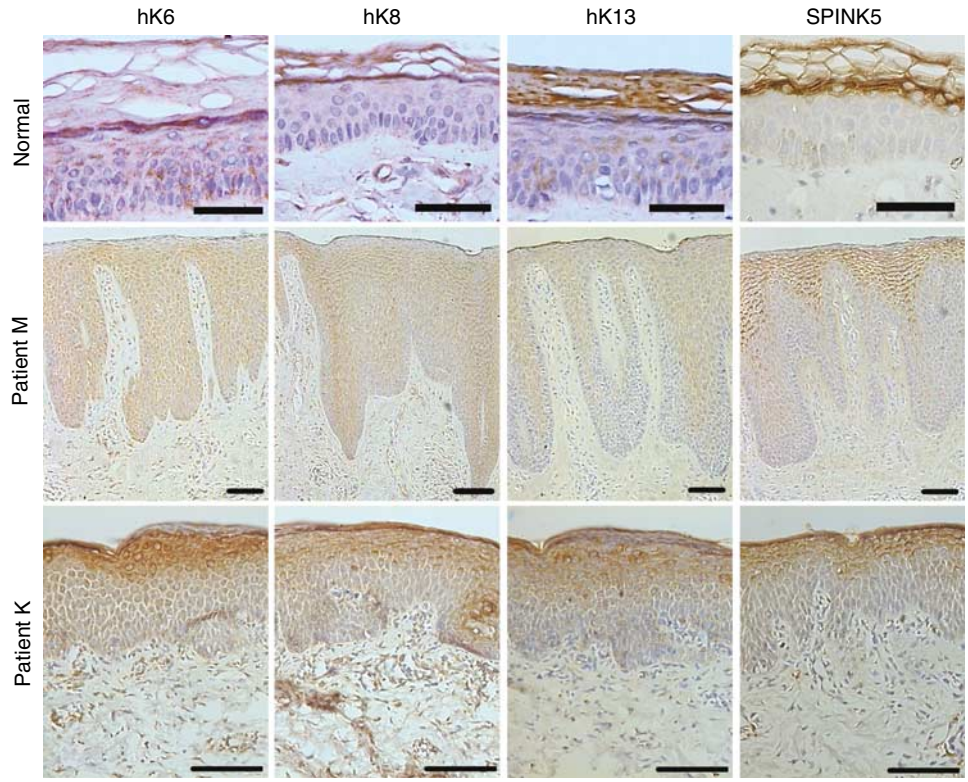

Figure 1. Immunohistochemical localization of hK6, hK8, hK13, and SPINK5 protein in the skin of PSS-type B patients. Bars indicate 50 μm for normal and 100 μm for the patients. It was previously demonstrated that NS patients show absent or only faint patchy cytoplasmic staining in the skin epidermis when the anti-SPINK5 protein antibody is applied (Raghunath *et al.*, 2004). The expression of the hKs in normal skin was referred from a previous study (Komatsu *et al.*, 2005a) and also displayed for comparison.

Table 1a. Kallikrein levels in the SC of PSS-type B patients by ELISA

hK (ng/mg dry weight)	Normal mean ± SD	Patient M	Patient K
Chymotrypsin-like hK			
hK7	10.9 ± 6.0	65.8*	130.7*
Trypsin-like hKs			
hK8	5.8 ± 1.8	62.9*	63.7*
hK11	8.7 ± 4.1	37.6*	56.9*
hK5	3.1 ± 1.4	8.3*	13.1*
hK10	0.67 ± 0.41	21.7*	29.5*
hK14	0.34 ± 0.13	2.3*	3.2*
hK6	0.28 ± 0.12	73.2*	30.0*
hK13	0.17 ± 0.14	24.1*	15.9*
Total of trypsin-like hKs	19.1 ± 5.4	230.1*	212.3*

SC, stratum corneum; SD, standard deviation; PSS, peeling skin syndrome;
The values indicate the mean ± SD (ng/mg dry weight). The normal subjects (< 11 years) were referred from a previous study (Komatsu *et al.*, 2005b). Kallikreins are subdivided into chymotrypsin-like hK (hK7) and trypsin-like hKs (the rest of hKs) (Yousef and Diamandis, 2001). (*) Smirnov test showed significant differences between normal SC samples and individual patients ($P<0.05$).

The overall SC trypsin-like (FSR-) and chymotrypsin-like (RPY-) activities are known as desquamation-related SC protease activities (Suzuki *et al.*, 1996). These activities are regulated in a consistent manner across different age groups, indicating that maintenance of a stable SC serine protease activity may be essential for retaining a constant number of SC layers which is known to be unaffected by aging (Ya-Xian *et al.*, 1999; Komatsu *et al.*, 2005b).

hKs may contribute to the overall SC protease activities and the degradation of intercellular adhesion molecules resulting in desquamation of corneocytes (Simon *et al.*, 2001; Komatsu *et al.*, 2002; Caubet *et al.*, 2004) (Figure 2a). SPINK5 inhibitory domains are believed to be negative regulators of desquamation-related proteases including hKs (Mitsudo *et al.*, 2003; Descargues *et al.*, 2005; Egelrud *et al.*, 2005; Schechter *et al.*, 2005) (Figure 2a). SPINK5 proprotein can be proteolytically processed at (R-KR↓) by furin-like activity to 15 individual bioactive domains (Seidah and Chretien, 1999; Komatsu *et al.*, 2002; Mitsudo *et al.*, 2003) (Figure 2a).

SPINK5 knockout mice displayed high trypsin-like and chymotrypsin-like activities in their SC (Descargues *et al.*, 2005). Similarly, NS patients, who lack the downstream of *SPINK5* products, display significantly high trypsin-like (FSR-) (Komatsu *et al.*, 2002) and

Table 1b. Kallikrein levels in the serum of PSS-type B patients by ELISA

hK (ng/mg)	Normal mean±SD	Patient M	Patient K
Chymotrypsin-like hK			
hK7	5.1±2.1	17.0*	35.5*
Trypsin-like hKs			
hK6	4.4±1.5	15.7*	10.4*
hK8	1.9±0.77	15.2*	7.1*
hK10	1.2±0.56	5.6*	5.4*
hK5	0.68±0 15	2.0*	0.47
hK11	0.54±0.16	0.38	0.67
hK14	0.22±0.091	0.43	2.6*
hK13	<0.01	0.26*	0.14*
Total of trypsin-like hKs	4.5±5.4	23.9*	16.4*

PSS, peeling skin syndrome; SD, standard deviation.
The values indicate the mean±SD (ng/ml). (*) Smirnov test showed significant differences between the normal serum samples and individual patients ($P<0.05$). The amount of hK13 in normal samples usually is very low or undetectable; hK13 in normal serum was described as <0.01 ng/ml without SD. The statistics for hK13 was performed considering hK13 values for normal subjects as 0.01 ng/ml.

Table 1c. SC enzymatic activities in normal subjects and PSS-type B patients

Substrate		Normal	PSS-type B	
Released AMC or pNA		n=9 or 16[1]	Patient M	Patient K
(nmol/mg dry weight)	Time (hours)	mean±SD	Mean	Mean
Trypsin-like activity				
Phe-Ser-Arg-AMC	2	15.5±1.5	21.0	15.5
Pro-Phe-Arg-AMC	2	5.7±3.1[1]	24.0*	18.6*
Chymotrypsin-like activity				
Arg-Pro-Tyr-pNA	4	13.9±5.5	11.9	13.7
Plasmin-like activity				
Val-Leu-Lys-AMC	2	1.7±1.0[1]	8.9*	19.6*
Furin-like activity				
Pyr-Arg-Thr-Lys-Arg-AMC	2	3.0±1.3[1]	43.0*	28.5*

AMC, 7-amino-4-methyl-commarin; pNA, para-nitroanilide; PSS, peeling skin syndrome; SC, stratum corneum; SD, standard deviation.
The overall SC serine protease enzymatic activities represent released AMC or pNA from the synthetic substrates for the normal subjects (mean±SD; nmol/mg dry weight). The amount of released AMC or pNA was measured at 2 or 4 h, respectively. Each assay was performed in triplicate for the patients and the mean values are indicated. (*) Significant differences ($P<0.05$) between the mean of normal samples and each patient at the specified time (Smirnov test for extreme values).
[1]Refer to the supplementary text for the details. According to their kinetic properties, hK5, hK6, hK8, hK13, and hK14 strongly display trypsin-like (FSR-) activity (Oka *et al.*, 2002; Magklara *et al.*, 2003; Kapadia *et al.*, 2004; Felber *et al.*, 2005; Michael *et al.*, 2005, respectively). hK7 may be largely responsible for the chymotrypsin-like (RPY-) activity (Franzke *et al.*, 1996; Komatsu *et al.*, 2005b). The identity of enzymes contributing to PFR- and VLK-activities in the skin is unknown. Arg-X-Lys-Arg- (R-X-KR) sequences are repeatedly found between an inhibitory domain and other domains of SPINK5 proprotein, and the sequence is the specific proteolytic target (R-KR-↓) of furin (Seidah and Chretien, 1999; Komatsu *et al.*, 2002; Mitsudo *et al.*, 2003). The co-localization of SPINK5 protein and furin in normal skin (Bergeron *et al.*, 2000; Bitoun *et al.*, 2003) allowed us to predict the presence of a furin-like activity in normal SC.

chymotrypsin-like (RPY-) activities (unpublished data) (Figure 2b). This indicates that SPINK5 protein possesses strong inhibitory functions towards FSR- and RPY-activities. The increased epidermal cell layers expressing SPINK5 protein in the patients' skins could lead to an enhanced expression of SPINK5 proprotein, and then the elevation of furin-like (R-KR-) activity may yield an excess amount of SPINK5 inhibitory domains by proteolysis of SPINK5 proprotein (Figure 2c). Ultimately, the mild elevation of FSR-activity and the normal RPY-activity in the two PSS-type B patients, both of whom with ''intact'' (and may be increased) SPINK5 protein/domain expression, could be explained by an efficient inhibitory function by SPINK5 proteins.

As SPINK5 protein possesses anti-''plasmin'' function (Mitsudo *et al.*, 2003), we measured overall SC ''plasmin-like (VLK-) activity'' in the SC. However, the elevated VLK-activity in the patients suggested that SPINK5 protein may not be an adequate inhibitor for the overall SC ''plasmin-like activity''. The trypsin-like (FSR-) and (PFR-) activities in the patients showed different preferences; therefore, the composition of proteases that play a role in FSR- and PFR-activities may be different.

The elevated SC protease activities in the PSS-type B patients with ''intact'' *SPINK5* implied an abnormal regulation of SC protease expressions/activities. Hence, we focused on the SC proteases, especially hKs, assuming an aberrant hK expression in these patients. As predicted, the amount of all hKs in the SC and serum of the PSS-type B patients was substantially higher than those in normal subjects.

hKs may function as an enzymatic cascade pathway in many tissues (Yousef and Diamandis, 2002), for example, hK5 can activate the proforms of hK7 and hK14 (Caubet *et al.*, 2004), and activation of pro-hK5 is either autocatalytic or is mediated by hK14 (Brattsand *et al.*, 2005). As hK7 exists in the SC as a mixture of the proform and the active form (Ekholm and Egelrud, 1999), other hKs in the SC may also exist in a similar manner as mixtures. It is conceivable that the abnormally increased concentration of multiple hKs in PSS-type B patients would result in an aberrant activation of other hKs (an altered ratio of pro- and active-form in hKs) and/or activation (and possibly amplification) of an hK enzymatic cascade. This could lead to a

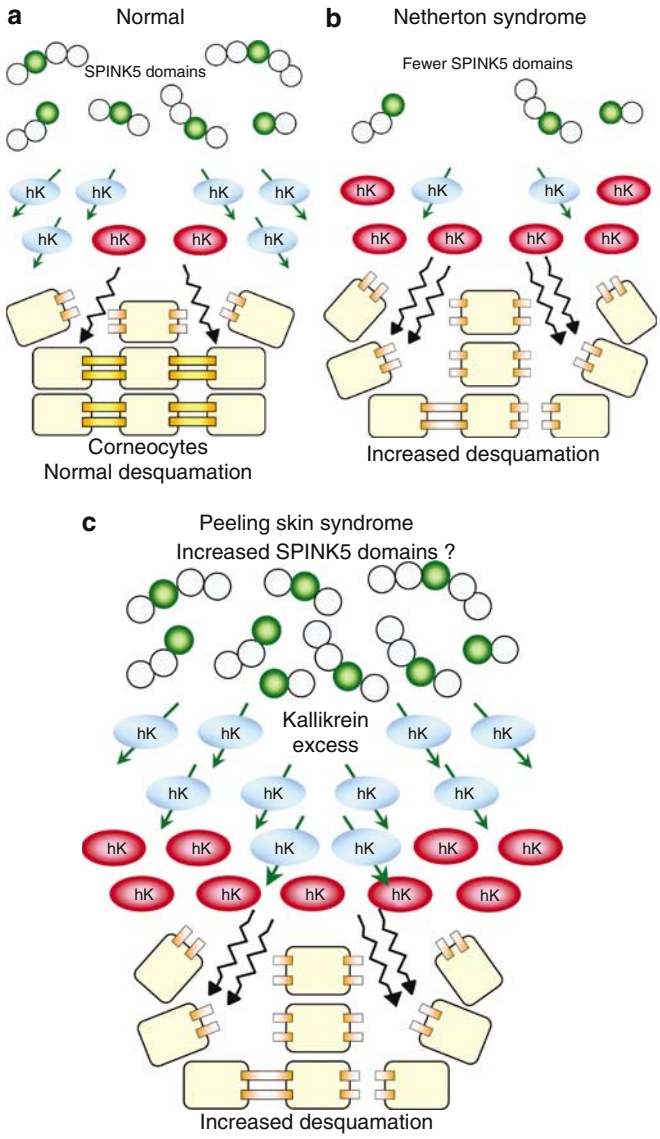

Figure 2. A model for desquamation regulation in normal individuals, and NS (modified from Komatsu *et al.*, 2002) and PSS-type B patients. (**a**) In normal skin, serine proteases in the SC, such as the kallikreins, may degrade the intercellular adhesion molecules, for example, desmoglein1, desmocollin 1, and corneodesmosin (Simon *et al.*, 2001; Caubet *et al.*, 2004; Descargues *et al.*, 2005), leading to desquamation of corneocytes. Fifteen SPINK5 domains may be inhibitory regulators of desquamation. (**b**) In NS patients, *SPINK5* genetic defects lead to the production of truncated proprotein containing fewer functional SPINK5 domains. This is followed by relatively elevated SC protease activities, excessive degradation of the adhesion molecules, and over-desquamation of corneocytes. (**c**) In PSS-type B patients, an unknown mechanism may lead to over-expression of multiple kallikreins. The production of SPINK5 proteins/domains could be normal or elevated, to inhibit kallikrein activity. However, overall protease activities may override SPINK5 domains' inhibitory function. The over-expression of kallikreins results in elevated SC protease activity, which is followed by over-degradation of the adhesion molecules, and ultimately, over-desquamation of corneocytes. Although the skin lesions in NS and PSS-type B are caused by different pathways, the phenotype might be the same for both diseases, that is, an over-desquamation of corneocytes. Active hKs are shown as red circles. SPINK-inactivated hKs are shown as blue circles with arrows.

high SC enzymatic protease activity, an overdegradation of intercellular adhesion molecules, and finally, an abnormal desquamation (Figure 2c).

NS and PSS-type B may have totally different pathogenesis; however, the final stages for each disease, that is, an elevation of SC protease activities and detachment of the SC (probably owing to over-desquamation) seem to be similar (Figure 2b and c), which may explain the clinical similarities between them.

NS patients exhibit severe over-absorption from their skin owing to the over-desquamation of corneocytes (Komatsu *et al.*, 2002), and skin permeability barrier dysfunction, causing iatrogenic Cushing's syndrome (Smith *et al.*, 1995) and elevated tacrolimus serum levels with a kidney disorder (Allen *et al.*, 2001). Patient M showed over-absorption through her skin, highly suggesting that the PSS-type B patients, like NS patients, suffer from over-desquamation.

In conclusion, we suggest that PSS-type B may not be an ichthyosis characterized by the retention of thick adherent scales (Frost and Van Scott, 1966) but an over-desquamation disease owing to an over-expression of hKs and an elevation of the SC protease activities. The over-desquamation of corneocytes may explain the clinical similarities between PSS-type B and NS. The elevated SC enzymatic activities may be good therapeutic targets for PSS-type B patients.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Materials and Methods, References.

Figure S1. Clinical features of PSS type B patients from two unrelated Japanese families.

Figure S2. Histopathological features of the skin in peeling skin syndrome type B patients.

Table S1. Comparison of clinical and laboratory findings between PSS type B and Netherton syndrome, including our two patients.

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Supplementary Text and Figures

Supplementary Materials and Methods

Mutation analysis

Genomic DNA was isolated from peripheral blood samples using standard methods. Further details for the polymerase chain reaction (PCR) conditions and the sequences of primers were described elsewhere (Komatsu et al, 2002).

Immunohistochemistry

Skin samples were obtained from the forearm of both patients at the age of one month. The formalin-fixed, paraffin-embedded specimens were cut into 4 μm sections and mounted on silane-coated glass slides for hematoxylin-eosin staining and immunohistochemistry. Anti-hK6 rabbit polyclonal antibody (Ab), anti-hK8 rabbit polyclonal Ab, and anti-hK13 mouse monoclonal Ab (clone 13C11) were developed in our laboratory (Komatsu et al, 2005a). Anti-SPINK5 protein mouse monoclonal Ab was also prepared (Raghunath et al, 2004). Negligible cross-reactivity for each Ab against other kallikreins has been verified, and the immunostaining procedures are described in detail elsewhere (Komatsu et al, 2005a).

Sample preparation of the SC and serum

The SC samples were obtained from the forearm of patients and 25 normal volunteers, who were less than 11yrs (4 females and 5 males) or 20-29 yrs of age (8 females and 8 males) (Komatsu et al, 2005b), by stripping using Nichiban^{TM} tape (organic-solvent-stable tape with organic solvent-soluble adhesive, Nichiban, Tokyo, Japan). The SC samples were washed and purified using toluene. After toluene treatment, the purified samples were air dried and weighed. The detailed

procedure for purification is described elsewhere (Komatsu et al, 2005b).

Serum samples were obtained from the two patients (at age 8 years) and 90 normal Japanese volunteers (a mix of males and females, 21-35 yrs old).

Immunofluorometric assays for human tissue kallikreins

For the immunofluorometric assays for the SC samples, 0.5 mg dry weight of the SC samples were mixed with 20 μl of N, N-dimethylformamide, 480 μl of 0.1 % Triton X-100, 350 μl of 0.2 M Tris-HCl buffer (pH8.0), and 100 μl of H_2O. The mixtures were incubated at 37ºC for 1hr on a shaker. After incubation, samples were centrifuged at 1,700 g for 10 min and the supernatants retrieved. Further details of sample preparation for the SC samples are described elsewhere (Komatsu et al, 2005b).

For the immunofluorometric assays, the serum samples were diluted 5 times with a solution containing 60 g/L bovine serum albumin; 50 mM Tris, pH7.8; 0.5 g/L NaN_3, for hK6, hK7, hK8 and hK10. No dilution was necessary for hK5, hK11, hK13 and hK14 assays.

We used in-house developed immunofluorometric assays (ELISA) to quantitatively determine the amounts of hKs. With the exception of Fuso-FB6MA53 anti-hK11 Ab which was purchased (Fuso, Osaka, Japan), all other monoclonal and polyclonal anti-kallikrein Abs were developed in our laboratory (Komatsu et al, 2005a). Each of the Abs displayed negligible cross-reactivity with other kallikreins (data not shown). More details are described elsewhere (Komatsu et al, 2005b).

Assay of serine protease enzymatic activities in the stratum corneum

The synthetic peptide substrates Boc-Phe-Ser-Arg-AMC, Boc-Pro-Phe-Arg-AMC, Pyr-Arg-Thr-

Lys-Arg-AMC and Boc-Val-Leu-Lys-AMC (BACHEM, Torrance, CA) were used at 0.1 mM final concentration. MeO-Suc-Arg-Pro-Tyr-pNA-HCl (Chromogenix, Milano, Italy) was used at 0.4 mM final concentration.

The reaction mixtures for the SC samples consisted of 0.5 mg dry weight of the SC, 10 µl of N, N-dimethylformamide, 240 µl of 0.1% Triton X-100, 175 µl of 0.2 M Tris-HCl buffer (pH 8.0), and 50 µl of either 1 mM FSR-, PFR- or VLK-substrates or 4 mM of RPY-substrate (Komatsu et al, 2005b). They were incubated at 37°C with shaking for 1 to 4 hrs.

Released AMC was measured using a fluorescence spectrophotometer (Wallac Victor^2 1420 Multilabel counter, Perkin Elmer, Boston, MA). Porcine trypsin type II (Trypsin tablets, Sigma, St. Louis, MO; molecular weight 23.8kDa) and plasmin (Sigma) were used as positive controls. Released pNA by the chymotrypsin-like activity was measured spectrophotometrically at 405 nm (Wallac Victor^2 1420 Multilabel counter). Each assay was performed in triplicate for the patients and the mean values are indicated in Table Ic (the difference in triplicate measurements was within 10%). For normal subjects, due to the limited amount of samples, each assay was performed only once, but all measurements were taken three times.

The normal subjects for PFR-, R-KR- and VLK-activities were 20-29 years old (n=16, 8 females and 8 males) due to the difficulty of obtaining sufficient SC samples (consuming 0.5mg of SC sample per assay) from younger (<11years) subjects.

Supplementary Figure Legends

Supplementary Figure 1. Clinical features of peeling skin syndrome type B patients from two unrelated Japanese families

a and b, Patient M at 8 years old. c, d and e, Patient K at 8 years old.

Erythematous scaling and migratory patches appear everywhere on the body, including palms and soles (a, c and e). No blister has been observed. The scales can easily be peeled back as a sheet (b and d). The denuded skin exhibits redness and pain but no bleeding. Both patients reported that denuded areas of skin would be re-covered with scales by the following day, and that these new scales were already peelable. Both patients estimated the scaling turnover as 1-3 days. The redness appears when the skin is peeled or is covered with scales subsided. Their palms and soles have shown mild scaling with chapping but no keratoderma (e).

Supplementary Figure 2. Histopathological features of the skin in peeling skin syndrome type B patients

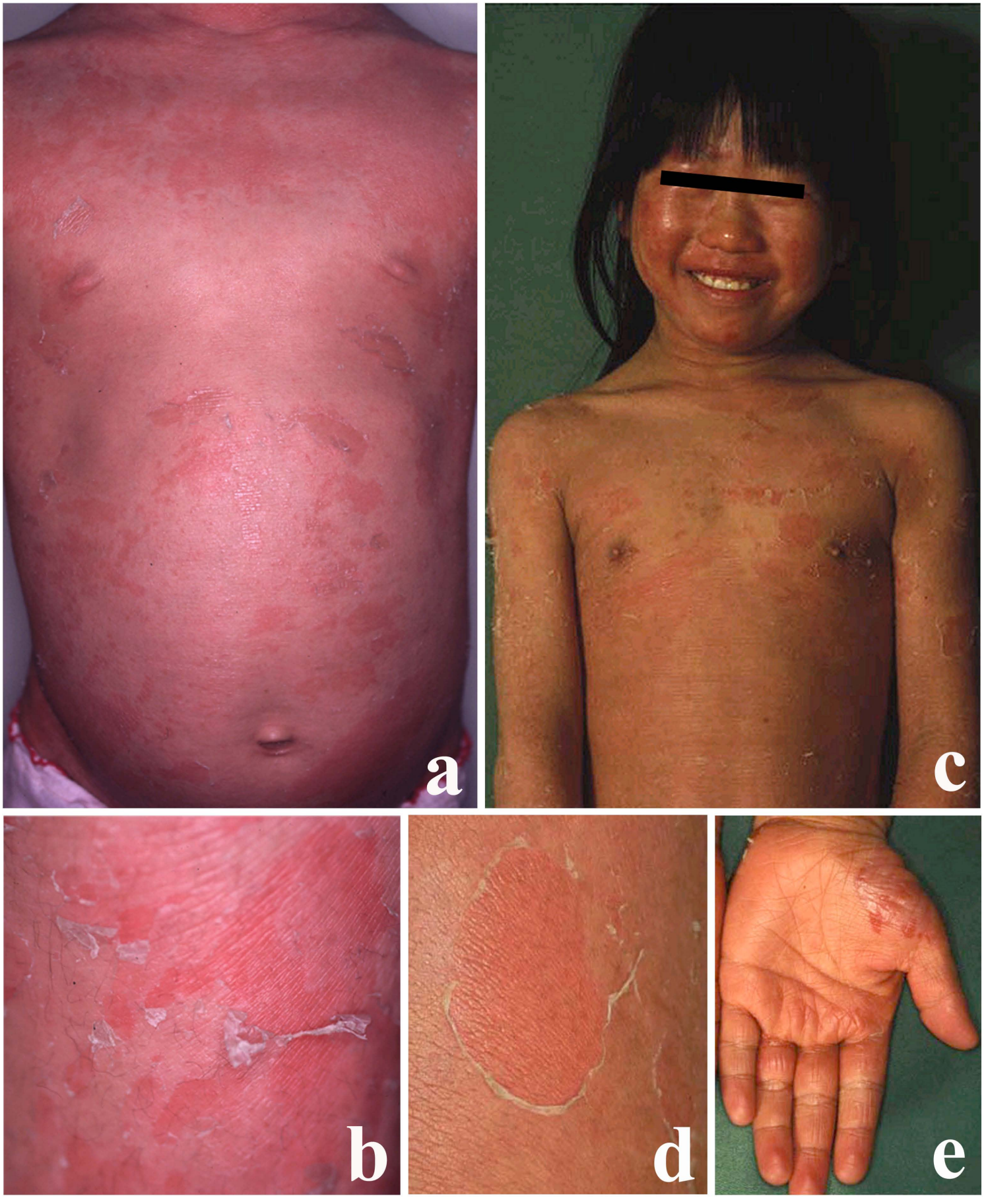
Hematoxylin-eosin staining. Scale bars indicate 100μm (a, c and d) and 50 μm (b). Arrowheads indicate eosinophils. Skin sections were obtained from an erythematous and scaling lesion one month after birth for both patients and stained with hematoxylin-eosin.

In Patient M, the skin section mostly lacked the SC, and the split of the SC appeared to have occurred directly above the STRATUM GRANULOSUM (a and b). The STRATUM GRANULOSUM consisted of a few layers. The stratum malpighii showed a psoriasisforme acanthosis. The upper dermis showed a considerable perivascular infiltration with mononuclear leukocytes and very few eosinophils and neutrophils (a).

In Patient K, compact parakeratosis with 1-6 layers was observed (c and d), which tended to be separated from the STRATUM GRANULOSUM. Normal SC was not confirmed in either the skin section or the peeled scales (data not shown). When layers of parakeratosis were thicker, the STRATUM GRANULOSUM tended to be thinner, and vice versa (c). The stratum malpighii showed a moderate acanthosis, displaying mildly dilated rete ridges of the epidermis. The upper dermis showed a mild perivascular infiltration of mononuclear leukocytes, some eosinophils, and very few neutrophils (d).

Supplementary references

Mevorah B, Frenk E, Saurat JH, Siegenthaler G: Peeling skin syndrome: a clinical, ultrastructural and biochemical study. *Br J Dermatol* **116**: 117-125, 1987


a
c
b
d
e

Patient K

Patient M