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Original Article

Human tissue kallikrein expression in the stratum corneum and serum of atopic dermatitis patients

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Abstract: Human tissue kallikreins are a family of 15 trypsin- or chymotrypsin-like secreted serine proteases (KLK1–KLK15). Many KLKs have been identified in normal stratum corneum (SC) and sweat, and are candidate desquamation-related proteases. We report quantification by enzyme-linked immunosorbent assay (ELISA) of KLK5, KLK6, KLK7, KLK8, KLK10, KLK11, KLK13 and KLK14 in the SC and serum of atopic dermatitis (AD) patients by ELISA, and examine their variation with clinical phenotype, correlation with blood levels of eosinophils, lactate dehydrogenase (LDH) and immunoglobulin E. The overall SC serine protease activities were also measured. In the SC of AD, all KLKs, except KLK11, were significantly elevated. The elevation of chymotrypsin-like KLK7 was predominant, compared with trypsin-like KLKs. The SC overall plasmin- and furin-like activities were significantly elevated, while trypsin- and chymotrypsin-like activities did not differ significantly. In the serum of AD patients, KLK8 was significantly elevated and KLK5 and KLK11 were significantly decreased. However, their serum levels were not modified by corticosteroid topical agents. The alterations of KLK levels in the SC of AD were more pronounced than those in the serum. KLK7 in the serum was significantly correlated with eosinophil counts in the blood of AD patients, while KLK5, KLK8 and KLK11 were significantly correlated with LDH in the serum. In conclusion, we report abnormal kallikrein levels in the SC and the serum of AD patients. KLKs might be involved in skin manifestation and/or focal/systemic inflammatory reactions in AD. Our data may contribute to a better understanding of the pathogenesis of AD.

Key words: atopic dermatitis – human kallikrein – serine protease – serum – stratum corneum – therapy

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Introduction

Atopic dermatitis (AD) is a common chronic inflammatory and allergic skin disease. 'Atopy', a personal and/or familial tendency to become sensitized and produce immunoglobulin E (IgE) antibodies in response to ordinary exposures to allergens (1), has been recognized as an important feature of diagnostic criteria for AD (2). AD patients (15–30%) do not show IgE-mediated sensitization; the involvement of IgE in the pathogenesis of AD is, therefore, controversial (3,4). However, AD patients with no elevation of serum IgE levels, are indistinguishable from other patients in terms of manifestations and therapeutic responses.

The pathogenesis of AD has been currently recognized as a complex interplay of numerous elements, including immune, genetic, metabolic, infectious and neuroendocrine factors, and their interaction with the environment (5). The skin barrier function is also known to be a key factor in AD, and many abnormalities in the skin barrier function have been linked to AD, e.g. transepidermal water loss, stratum corneum (SC) moisture content, permeability to hydrophilic substances, barrier to infectious agents and epicutaneous antigen absorption (5,6). An appropriate regulation of desquamation (i.e. renewal and removal of corneocytes), may be a crucial factor of the skin barrier function as well.

Abbreviations: Ab, antibody; AD, atopic dermatitis; AMC, 7-amino-4-methyl-coumarin; ELISA, enzyme-linked immunosorbent assay; IgE, immunoglobulin E; KLK, kallikrein protein; LDH, lactate dehydrogenase; LEKTI, lympho-epithelial Kazal-type-related inhibitor; PAR, protease-activated receptors; pNA, *para*-nitroanilide; SC, stratum corneum; SD, standard deviation.

Human tissue kallikreins are a family of 15 trypsin- or chymotrypsin-like secreted serine proteases (KLK1–KLK15) found in a variety of tissues (7), where they have been suggested to function as an enzymatic cascade pathway (8). At least eight different KLKs are found in the SC and sweat (9,10), and KLKs may contribute to the overall SC protease activities and to degradation of intercellular adhesion molecules, resulting in the desquamation of corneocytes (9,11–13). Lympho-epithelial Kazal-type-related inhibitor (LEKTI) and its processed bioactive domains, co-localize with KLKs in skin, and are believed to be negative regulators of desquamation-related proteases, including KLKs (14–17). The localization of KLKs in normal skin is in the stratum granulosum, SC and skin appendages (18,19), but KLK expressions are expanded into the lower epidermis in the skin lesion of AD (19). Transgenic mice, expressing KLK7 in suprabasal epidermal keratinocytes, develop pathologic skin changes with increased epidermal thickness, hyperkeratosis, dermal inflammation and severe pruritus (20). These findings suggest an involvement of KLKs in the pathogenesis of AD, including inflammatory reactions and skin manifestations. Therefore, this study aimed to quantitatively measure KLK5, KLK6, KLK7, KLK8, KLK10, KLK11, KLK13 and KLK14 both in the SC and the serum, as well as assess the overall serine protease activities in the SC of AD patients. These data were further associated with the patient's age, the therapy, the severity and the blood levels of IgE, eosinophils and LDH, to reveal relationships among multiple kallikreins and AD manifestations.

Methods

Sample preparation for the stratum corneum

The SC samples of normal subjects were the same as in our previous study (9), and were obtained from the forearms of 60 normal volunteers [30 (15 females and 15 males) in each of two age groups: 20–29 and 30–39 years]. The SC samples for AD were obtained from the forearms of 14 patients [nine females and five males, 27.4 ± 4.2 years old (mean ± standard deviation (SD))] who had been diagnosed with AD and were under medication with corticosteroid topical agents. All patients who provided the SC samples were well-controlled by the medication. The SC samples were collected only from dry skin with or without very mild lichenification, and joint areas were excluded. For consistency, the patients who showed moderate to strong lichenification, excoriation, crust, apparent scratch mark and secondary skin infection were excluded.

SC was obtained by stripping using the Nichiban^{TM} tape (organic solvent-stable tape with organic solvent-soluble adhesive; Nichiban, Tokyo, Japan). For optimization purposes, samples collected from several subjects using various tape brands were weighed and tested with a KLK6 enzyme-linked immunosorbent assay (ELISA). The Nichiban tape yielded the highest levels of KLK6 among all the tapes tested, which indicated the lowest contamination of adhesive material after purification. The tape stripping was performed as follows: subjects took a shower the night before and used no topical agents afterwards. The following afternoon, the tape was applied to the skin surface of the subjects' forearm and upper arm. As the number of SC layers in the forearm is known to be consistent regardless of age (21), we performed the stripping at different places until around 2 mg of SC had been visibly obtained. With this procedure, we assumed that a similar number of SC layers had been removed in each case. The histology of these skin samples was not examined. The SC samples on the tape were immediately stored at −20°C until the toluene treatment was performed. When the tape was dipped in 10 ml of toluene, all adhesives were dissolved and any attached SC was suspended. After the insoluble tape backing was removed, the sample was centrifuged at 1700 *g* for 15 min. The precipitate was washed with 5 ml of toluene six times in order to remove any residual adhesive. The amount of SC obtained was typically around 2 mg regardless of the subjects' age and gender. After the toluene treatment, the purified samples were air dried, weighed and kept at −20°C until immunofluorometric assays and enzymatic activity measurements could be performed.

For the immunofluorometric assays, 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 37°C for 1 h on a shaker. After incubation, samples were then centrifuged at 1700 *g* for 10 min and the supernatants were retrieved. Aliquots (10–50 μl) were used in each immunofluorometric assay as described (9). The SC extract for ELISA was prepared, aliquoted for each KLK, and then frozen immediately. The aliquoted and frozen extract was thawed only once for ELISA and was never be re-used, re-frozen or re-thawed. Therefore, the quality of the extract for each subject is consistent.

Sample preparation for serum and blood cells

Serum samples were obtained from 90 normal volunteers (a mixture of males and females, 21–50 years old). Serum and blood samples from AD patients were obtained from 104 subjects who were diagnosed with AD: <15 years, $n = 19$ [10 females and 9 males, 9.6 ± 3.9 years old (mean ± SD)]; 15–40 years, $n = 80$ [38 females and 42 males, 23.7 ± 6.1 years old]; >40 years, $n = 5$ [3 females and 2 males, 46.8 ± 4.4 years old].

The severity of the AD skin lesion was evaluated using the severity score (22) for each patient. The patients for the age group of 15–40 years were also subdivided by types of

therapy, i.e. with or without therapy of corticosteroid topical agents.

Immunofluorometric ELISA assays for human tissue kallikreins

These were described elsewhere (9).

Assay of serine protease enzymatic activities in the stratum corneum

The synthetic peptide substrates Boc-Phe-Ser-Arg-AMC (FSR-), Boc-Pro-Phe-Arg-AMC (PFR-), Pyr-Arg-Thr-Lys-Arg-AMC (R-KR-) and Boc-Val-Leu-Lys-AMC (VLK-) (BACHEM, Torrance, CA, USA) were used at 0.1 mM final concentration. MeO-Suc-Arg-Pro-Tyr-pNA-HCl (RPY-) (Chromogenix, Milan, Italy) was used at 0.4 mM final concentration. Further details are described elsewhere (9,12). For protease activity assay we performed: (i) blank analysis, (ii) the premix with substrate but without SC sample, and (iii) the premix without both substrate and SC sample. These assays were performed every time as negative controls to evaluate assay quality. In addition, a few samples, for which we had sufficient quantity were measured twice or more in different days/times to check the assay consistency and reproducibility.

Statistical analysis

Bartlett's test was performed to determine the equality of variances among the specified groups. If significant differences were found with Bartlett's test, then the Kruskal–Wallis test was performed. Reported *P*-values have been adjusted by Dunn's method to reflect multiple comparisons. When a significant difference was not found with Bartlett's test, both the Kruskal–Wallis test and one-way ANOVA were performed. However, the statistical results were consistently presented with the Kruskal–Wallis test. When comparing two groups, the Mann–Whitney test was used. All statistical tests were performed using GraphPad Prism 4 version 4.02 software (GraphPad Software, Inc., San Diego, CA, USA).

Results

Kallikrein levels in the stratum corneum of normal and atopic dermatitis lesions

Informed consent was obtained from all patients and normal volunteers, and our studies were performed according to the Declaration of Helsinki. The Medical Ethics Committee of the Graduate School of Medical Science, School of Medicine, Kanazawa University, approved all described studies.

The concentrations of KLK5, KLK6, KLK8, KLK10, KLK11, KLK13 and KLK14 (trypsin-like KLKs), and KLK7 (chymotrypsin-like KLK) (7) in the SC from normal and AD subjects were determined (ng/mg of dry weight) by immunofluorometric ELISAs (Fig. 1a–h). The total concentration (sum) of trypsin-like KLKs is indicated in Fig. 1i. In AD, all KLK levels, with an exception of KLK11, were significantly higher than those in the normal subjects ($P < 0.05$ for each comparison) (Fig. 1a–h). Both the sum of trypsin-like KLKs and chymotrypsin-like KLK7 in AD ranged approximately from 12 to 90 ng/mg of dry weight and displayed significant differences in comparison with the normal subjects (Fig. 1a and i). For KLK5, KLK10 and KLK13, the median of AD was outside the normal range (Fig. 1d,e,h).

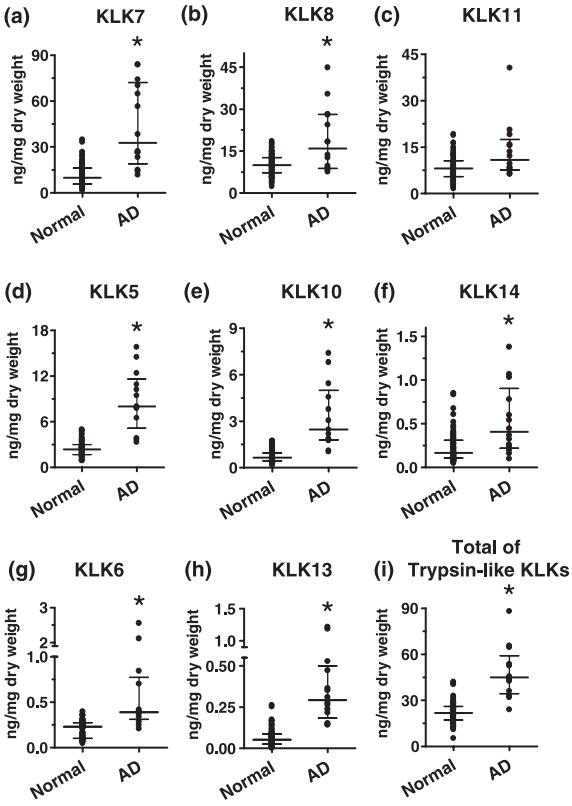

Figure 1. Differences of kallikrein concentrations in the stratum corneum of normal subjects and atopic dermatitis patients. Kallikrein levels in the SC were measured by ELISA (ng/mg of dry weight). Bars indicate median with interquartile range. *Significant differences between normal and AD subjects ($P < 0.05$, Mann–Whitney test).

SC serine protease enzymatic activities in normal and atopic dermatitis subjects

The overall SC enzymatic activities were measured in the normal and AD samples (Table 1). In this study, 'trypsin-like activities' refer to the activities of enzymes towards Boc-Phe-Ser-Arg-AMC (FSR-) and Boc-Pro-Phe-Arg-AMC (PFR-) substrates. 'Chymotrypsin-like activity', 'plasmin-like activity' and 'furin-like activity' refer to the activities of enzymes towards MeO-Suc-Arg-Pro-Tyr-pNA-HCl (RPY-), Boc-Val-Leu-Lys-AMC (VLK-) and Pyr-Arg-Thr-Lys-Arg-

Table 1. Stratum corneum serine protease enzymatic activities in normal subjects and atopic dermatitis patients[1]

Substrate	Normal ($n = 12$)	Atopic dermatitis ($n = 8$)
Trypsin-like activity		
Phe-Ser-Arg-AMC	13.6 ± 4.5	13.3 ± 4.8
Pro-Phe-Arg-AMC	5.7 ± 3.0	5. 5 ± 3.4
Chymotrypsin-like activity		
Arg-Pro-Tyr-pNA	10.7 ± 4.1	14.3 ± 5.2
Plasmin-like activity		
Val-Leu-Lys-AMC	1.7 ± 1.0	3.1 ± 1.8*
Furin-like activity		
Pyr-Arg-Thr-Lys-Arg-AMC	3.0 ± 1.3	11.0 ± 3.2*

[1]The overall SC serine protease enzymatic activities (mean ± SD) represent released 7-amino-4-methyl-coumarin (AMC) or *para*-nitroanilide (pNA) from the synthetic substrates (nmol/mg dry weight). The amount of released AMC or pNA was measured at 2 or 4 h, respectively. Mann-Whitney test presented significant differences for each substrate comparison, $*P < 0.05$.

AMC (R-KR-) substrates, respectively. Because of the limited amount of SC sample from all individuals, the subjects ($n = 12$ for normal or $n = 8$ for AD) for each substrate were randomly chosen from the normal ($n = 60$) or AD ($n = 14$) groups (also see Methods). In other words, the number of subjects is consistent, while the individuals are not always the same for each enzymatic activity determination.

The trypsin-like (FSR- and PFR-) and chymotrypsin-like (RPY-) activities in the AD patients did not differ significantly from those of the normal subjects. The plasmin-like (VLK-) and furin-like (R-KR-) activities were significantly elevated in the AD group ($P < 0.05$ for each) compared with those of the normal group (Table 1).

Kallikrein levels in the serum of normal and atopic dermatitis patients

The concentrations of KLKs in serum were quantitatively determined for the normal and AD subjects (ng/ml) (Table 2). As KLK13 concentrations in a serum were undetectable in the majority of normal and AD subjects (data not shown), the serum KLK13 data were excluded.

The AD subjects were further subdivided as follows: under 15 years without therapy ['<15 years therapy (−)', $n = 19$; the average of severity scores (22) were 7.1 ± 1.4 (mean ± SD)]; '15–40 years therapy (−)' [$n = 65$, 7.6 ± 1.4]; and 15–40 years with therapy ['15–40 years therapy (+)', $n = 10$, 5.4 ± 1.3]. Because of sample collection unavailability, the group of '<15 years therapy (+)' was not included.

Table 2. Kallikrein levels in the serum of atopic dermatitis patients among age or therapy groups[1]

KLKs (ng/ml)	Normal $n = 85$ (20–40 years)	Atopic dermatitis: Therapy (−) $n = 19$ (<15 years)	Atopic dermatitis: Therapy (−) $n = 65$ (15–40 years)	Atopic dermatitis: Therapy (+) $n = 10$ (15–40 years)
Chymotrypsin-like KLK				
KLK7				
75th Percentile	6.1	8.2	6.3	6.7
Median	4.7	5.9	4.1	4.5
25th Percentile	3.8	3.8	3.1	3.3
Trypsin-like KLKs				
KLK6				
75th Percentile	5.5	4.9	5.4	6.1
Median	4.4	4.1	4.1	4.2
25th Percentile	3.1	3.3	3.1	3.7
KLK8				
75th Percentile	2.2	3.1	2.9	3.2
Median	1.8	2.7*	2.2*	2.4
25th Percentile	1.5	2.0	1.8	1.9
KLK10				
75th Percentile	1.5	1.5	1.4	1.8
Median	1.2	1.2	1.1	1.3
25th Percentile	0.77	0.88	0.79	0.76
KLK5				
75th Percentile	0.76	0.70	0.59	0.73
Median	0.67	0.52*	0.48*	0.54
25th Percentile	0.58	0.37	0.30	0.35
KLK 11				
75th Percentile	0.67	0.60	0.65	0.44
Median	0.54	0.44	0.41*	0.35*
25th Percentile	0.43	0.24	0.29	0.22
KLK 14				
75th Percentile	0.29	0.27	0.26	0.27
Median	0.22	0.15	0.15	0.15
25th Percentile	0.16	0.11	0.11	0.075

[1]Kallikrein levels in serum were examined by ELISA (ng/ml). After the Kruskal–Wallis test, significant differences ($*P < 0.05$ for each) between normal and the specified groups were confirmed by the post hoc test (Dunn's multiple comparison test).

The levels of chymotrypsin-like KLK7 displayed no significant difference among the groups. For trypsin-like KLKs, all AD groups tended to display higher KLK8 levels, and the <15 and 15–40 years therapy (−) groups exhibited significant differences, in comparison with normal subjects ($P < 0.05$ for each comparison). In contrast, the KLK5 and KLK11 levels in the AD were slightly lower than those of normal subjects. KLK5 in the groups of <15 and 15–40 years therapy (−) differed significantly from those of normal subjects ($P < 0.05$ for each). The AD groups of 15–40 years, for both therapy (−) and (+), displayed significantly lower KLK11 levels than the normal group ($P < 0.05$

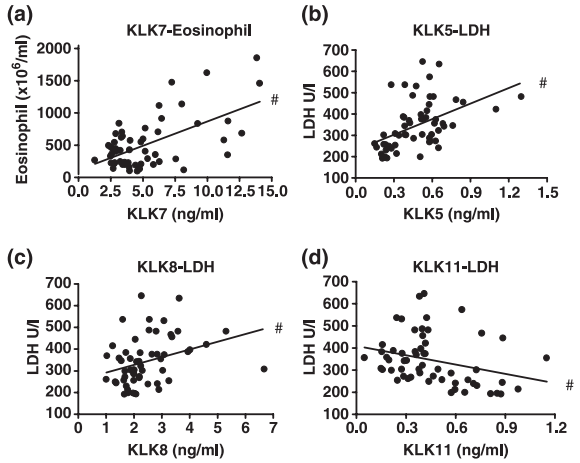

Figure 2. Regression analyses between serum kallikrein levels and blood eosinophil counts or serum LDH concentration in atopic dermatitis patients. Regression analyses were performed with AD subjects [15–40 years with no therapy ($n = 65$)]. Slopes significantly different from zero ($^{\#}P < 0.05$ for each) are shown. The equations of the regression lines are as follows: (a) $y = 58.5x + 210$; (b) $y = 244x + 230$; (c) $y = 35.5x + 256$; and (d) $y = -141x + 410$.

for each). The levels of KLK6, KLK10 and KLK14 did not differ significantly among the normal and AD groups.

Regression analysis was performed between serum KLKs and eosinophil counts and LDH concentrations of AD patients in the group [15–40 years with no therapy ($n = 65$)] (Fig. 2). There was a significant correlation between eosinophil counts and KLK7 levels in the AD subjects ($r = 0.48$, $P < 0.05$, Fig. 2a). The KLK5 and KLK8 levels were significantly correlated with LDH [$r = 0.47$ (Fig. 2b) and $r = 0.33$ (Fig. 2c), $P < 0.05$ for each, respectively]. The KLK11 levels were weakly but inversely correlated with LDH ($r = -0.30$, $P < 0.05$) (Fig. 2d). None of the KLKs show any correlation with the eosinophil counts or LDH (data not shown). In addition, the KLK levels were correlated with either the AD skin severity score (22) or IgE in the AD subjects (data not shown).

Discussion

In the SC of AD, the KLK levels are elevated (both the chymotrypsin-like KLK7 and trypsin-like KLKs). The ratio of total concentration of KLK7 versus trypsin-like KLKs in AD was approximately 1:1, whereas it was 1:2 in the normal SC (9). Thus, the elevation of KLK7 was prominent in the SC of AD, suggesting that the increase in chymotrypsin-like KLK7 may be crucial in AD pathogenesis or manifestations.

In the case of trypsin-like KLKs, KLK8, KLK5 and KLK10 were predominantly elevated in the SC of AD, and these KLKs may play a role in AD.

In general, changes in the KLK levels in the SC of AD were much more evident compared with those in the serum, suggesting that KLKs could be involved in a focal inflammatory reaction.

KLK7 and other KLKs (at least KLK5, KLK6, KLK8, KLK13 and KLK14) are believed to participate in RPY- and FSR- activities, respectively (12). However, the overall SC trypsin-like (FSR-) and chymotrypsin-like (RPY-) activities remained within normal ranges. Thus, it seems that the elevation of these KLKs was not reflected in the enzymatic activity in AD patients.

Furin-like activity may contribute to process pro-LEKTI into 15 individual bioactive inhibitory domains (14,23,24). LEKTI possesses an efficient inhibitory function towards the overall FSR- and RPY- activities in the SC (15,24) (our unpublished data). The elevation of furin-like (R-KR-) activity, observed in the SC of AD, could efficiently process pro-LEKTI, which is expressed in the skin lesion of AD (25) to release increased amounts of LEKTI-active inhibitory domains. Accordingly, the discrepancy between the KLK levels and FSR-/RPY- activities in the SC of AD could be explained by an efficient inhibitory function by LEKTI domains.

Proteases that are responsible for the overall SC plasmin-like activity are unknown. Pro-LEKTI, particularly LEKTI domain 15 (14,16), shows an anti-plasmin function. However, the SC of AD showed a significant elevation of the 'overall SC plasmin-like activity'. Thus, LEKTI domains may not be efficient inhibitors of plasmin-like activity.

The alteration of the KLK levels in the SC of AD patients was evident (Fig. 1), but changes in serum were modest (Table 2). SC KLK levels could be crucial and might be linked to focal inflammation or to skin manifestations in AD. No significant differences were observed in the serum levels of KLKs between the various age or therapy groups (Table 2). Thus, the KLK levels in the serum are unlikely to be useful serological markers of AD.

Serum KLK5, KLK8 and KLK11 levels differed slightly between normal and AD subjects. However, such changes were also observed in other inflammatory skin diseases (our unpublished data). KLK5, KLK8 and KLK11 were also significantly correlated with serum LDH (Fig. 2), a finding that may represent a non-specific inflammatory response rather than a specific reaction to AD.

Because of limitation in the amount of samples, the data of KLK levels and protease activities were not always obtained from the same individuals. Thus, the data may not be fully representative of what is occurring in each individual. Further studies are necessary to confirm these data.

A polymorphism in the 3′-untranslated region of KLK7, which could cause KLK7 dysregulation, was frequently detected in AD patients who show no IgE elevation (26).

In the present study, KLKs in the serum of AD patients were not significantly correlated with IgE levels (data not shown). Hence, KLK expression could be an independent factor from 'atopy' (1) or IgE-mediated sensitization.

On the other hand, a bioactive chymotrypsin-like protease (MW 28 kDa; neither chymase nor cathepsin G) is contained in eosinophil granules, which might be related to lymphocyte proliferation and release of eosinophil peroxidase (27,28). Given its molecular weight (29), KLK7 could be a candidate. As the KLK7 and eosinophil levels in the blood of AD were significantly correlated (Fig. 2), it is possible that KLK7 might be involved in a pathway of eosinophilia and/or inflammation mediated by eosinophils. Further studies are necessary to elucidate this point.

Protease-activated receptors (PARs) are members of the transmembrane G-protein-coupled receptors which are cleaved and activated by serine proteases (e.g. trypsin and thrombin). The signals are transmitted to the nucleus to mediate cell proliferation, differentiation, pain transmission and inflammatory responses (30). KLK5, KLK6 and KLK14 cleave PAR-2 at its activation site in-vitro (31,32). Hence, the presence of a KLK-PAR signalling pathway is conceivable. KLKs and PAR-2 are co-localized not only in normal skin, but also in skin lesions of AD (19,33,34). It is thus likely that KLKs might be contributing to a focal inflammatory reaction in AD skin lesions, by way of the PAR trans-signalling pathway.

It is not known whether the elevation of the KLK levels in the SC of AD is due to accelerated production of KLKs in the stratum granulosum, and/or to the increased layers with KLK expression in the skin lesion of AD (19). The mechanism of regulation of KLK expression in the skin is not clear. For example, the amount of active KLK7 is not affected by glycolic acid in the human skin (35). Further studies are necessary to elucidate the mechanism of KLK regulation in the skin of normal individuals and AD patients.

In summary, we report for the first time the quantification of multiple kallikreins in the SC and serum of AD patients. Our data suggest that kallikreins, along with other proteolytic enzymes, might be involved in the skin manifestations of AD. Chymotrypsin-like KLK7 could be a key enzyme in skin lesions and in concert with eosinophil and trypsin-like KLKs (e.g. KLK5, KLK8 and KLK11), it might be involved in a systemic inflammatory response in AD. The kallikrein proteolytic system might help in the understanding of the mechanisms of focal and systemic inflammatory reactions in AD.

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