



Increased mass levels of certain serine proteases in the stratum corneum in acute eczematous atopic skin

R. Voegeli*, S. Doppler*, P. Joller*, M. Breternitz†, J. W. Fluhr†,‡ and A. V. Rawlings§

*DSM Nutritional Products Ltd, Basel, Switzerland, †Skin Physiology Laboratory, Department of Dermatology, Friedrich Schiller University, Jena, Germany ‡Charité University Clinic, Department of Dermatology, Berlin, Germany and §AVR Consulting Ltd, Northwich, Cheshire, UK

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Synopsis

Acute eczematous atopic dermatitis (AD) is associated with increases in stratum corneum (SC) serine protease activity. The purpose of this study was to examine whether the increased SC protease activities in acute eczematous atopic dermatitis were associated with increased mass levels of SC proteases. Six subjects with healthy skin and six patients with AD each with non-lesional skin or lesional acute eczematous skin had the mass levels of their extractable SC kallikreins (KLK), plasmin and urokinase quantified using Luminex multiplex bead-based assays from SC tape strippings. The mass levels of KLK5 and KLK14 together with urokinase were not elevated in the SC in atopic skin. However, the mass levels of KLK7 and KLK11 together with plasmin were greatly elevated compared with the extracts from the non-lesional and the healthy skin and correlated with the corresponding enzymatic activities.

Résumé

La dermatite atopique eczémateuse aiguë (AD) est associée à une augmentation de l'activité protéasique dans la couche cornée (SC). Le but de cette étude était d'examiner si l'augmentation des activités protéasiques du SC dans la dermatite atopique eczémateuse aiguë est associée à des quantités élevées de protéases au sein du SC. Les kallikréines (KLK) extractibles du SC, la plasmine, et l'urokinase de six personnes avec une peau saine et six personnes avec une AD avec chacune soit la peau non lésionnelle, soit la peau lésionnelle ont été quantifiées en utilisant le multiplex Luminex sur des strippings de SC. Le niveau de KLK5 et de KLK14 ainsi que l'urokinase ne sont pas élevées dans le SC de la peau atopique. Toutefois, le niveau de KLK7 de KLK11 ainsi que la plasmine ont été considérablement élevés par rapport aux extraits de la peau non lésionnelle et de la peau saine. Nous mentionnons une augmentation du niveau des certaines serine protéases dans la dermatite eczémateuse atopique aiguë, incluant les KLK7 et KLK11 et plus précisément la plasmine.

Introduction

Atopic dermatitis (AD) is a chronic inflammatory disease associated with changes in stratum corneum (SC) structure and function. Focussing on the kallikreins, a serine protease family involved in

desquamation, Komatsu *et al.* [1] confirmed the presence of trypsin-like kallikreins (KLK5, KLK6, KLK8, KLK10, KLK11, KLK13) and chymotrypsin-like kallikreins (KLK7 and KLK14) in SC extracts immunologically. They found increased mass levels of all these enzymes, except KLK11, in atopic patients, but could not find increased KLK enzyme activity. Equally, they described increased SC plasmin and furin activities, but did not measure their mass levels. The patients studied were Japanese, only had mild lichenified AD lesions and were undergoing corticosteroid treatments. Conversely, we have previously examined SC extracts from Caucasian AD patients with acute eczematous lesions not undergoing any medical treatments and found increased enzymatic activities including SC chymotrypsin-like KLKs, SC trypsin-like KLKs, SC tryptase-like protease, plasmin, urokinase and leukocyte elastase activities which was associated with a thinner SC [2]. However, we did not measure immunochemically the mass levels of these enzymes.

The purpose of this study was to measure immunochemically the mass levels of KLK5, KLK7, KLK11, KLK14, plasmin and urokinase in the same SC extracts from the patients we had examined previously to ascertain whether the increase in SC enzyme activities was associated with increased epidermal production of these enzymes. Unfortunately, we did not succeed to establish antibody constructs for leukocyte elastase, tryptase and KLK8.

Materials and methods

Eighteen Caucasian volunteers equally divided into three groups participated in this study: subjects with healthy skin (H) (age: 31.2 ± 7.3 years), non-lesional patients with AD (NL) (age: 31.2 ± 7.3 years) and AD patients with mild-to-moderate lesional eczema (L) (age: 20.3 ± 2.0 years) as described previously [2]. Before including a test person in the study, the modified Erlangen atopy score was defined, a questionnaire determining a possible atopic diathesis of the subject [3]. The total number of points thereby calculated varied from 0 to 20, an atopic skin diathesis being considered as certain from 10 points on. Only those persons with an atopy score of <10 points could join the group with healthy skin. Clinical evaluation was rated using adapted irritation score according to Frosch and Kligman [4–6]. The study was approved by the Ethical Commission of the Friedrich Schiller University of Jena, Germany. Written informed consent was obtained from each participant.

SC protein and SC serine protease mass measurements were performed from 20 subsequent tape strippings taken on the ventral forearm of the subjects.

Correspondence: Rainer Voegeli, DSM Nutritional Products Ltd, CH-4002 Basel, Switzerland. Tel.: +41 61 815 8298; fax: +41 61 815 80 50; e-mail: rainer.voegeli@dsm.com

Fifteen minutes before the tape stripping procedure, the skin was cleaned by gentle swabbing with a cotton pad soaked with distilled water of ambient temperature and allowed to dry. Subjects were acclimated for 15 min in an environmental room under standard conditions ($21 \pm 1^\circ\text{C}$ and $35 \pm 10\%$ relative humidity) prior to any measurements. The skin sites were marked with a surgical marker to ensure that the measurement probes and the tapes were consistently applied to the same area. Before conducting the sequential tape stripping on the ventral forearms, SC hydration, skin surface pH, TEWL and intensity of erythema, respectively, were measured as described and discussed in [2].

Standard D-Squame® discs with a diameter of 2.2 cm and an area of 3.8 cm^2 were placed on the skin under 225 g cm^{-2} of pressure with a pressure device (CuDerm Corporation, Dallas, TX, U.S.A.) for 5 s. To minimize variations, the procedure was conducted by the same technician. The interval between the strippings was $20 \pm 5\text{ s}$.

The SC protein content of the tape strippings was quantified by absorption measurements at 850 nm with the infrared densitometer SquameScan™ 850A (Heiland electronic, Wetzlar, Germany). For protein quantification, the following equation was used [7]:

$$C_{\text{protein}} (\mu\text{g cm}^{-2}) = 1.366 \times \text{absorption} (\%) - 1.557$$

Each tape strip was transferred into a 1.5 mL Eppendorf tube and extracted by shaking for 15 min at 25°C and 1000 rpm in $750\text{ }\mu\text{L}$ of a buffer composed of 0.1 M Tris/HCl and 0.5% Triton X-100 at pH 8.0, whereupon the mass levels of the proteases were determined on pooled tape strippings extracts (1–5, 6–10, 11–15). The average pooled sample of tapes 1–15 was also calculated. Correlation of mass vs. previous activity measurements was conducted [2].

Additional analysis on the deeper strippings (16–20) could not be performed because of a methodological problem: Only minimal amounts of protein could be removed from layers 16–20 especially in lesional skin of AD subjects.

The extracts of the tape strippings were analysed quantitatively and simultaneously for six serine proteases (KLK5, KLK7, KLK11,

KLK14, plasmin and urokinase) using Luminex¹⁰⁰ multiplex bead-based assays (Luminex Corp., Austin, TX, U.S.A.). The beads for measurement were prepared by first coupling BSA–biotin (A8549; Sigma-Aldrich, St. Louis, MO, U.S.A.) to the surface using carbodiimide chemistry. Method in short: wash 1.0×10^6 microspheres (beads) in a 96-well Millipore filter plate (MultiScreen HTS™, BV, Art. MSBVN1250; Millipore, Billerica, MA, U.S.A.) with PBS. Resuspend beads in $50\text{ }\mu\text{L}$ activation buffer (0.1 M phosphate, pH 6.0) and incubate for 5 min at room temperature (RT) on a platform shaker (Titramax, Heidolph Instruments, Schwabach, Germany), at 1000 rpm, aspirate buffer on a vacuum device. Repeat. Add $6\text{ }\mu\text{L}$ N-hydroxysulfosuccinimide solution, (ThermoScientific, Rockford, IL, U.S.A.), 50 mg mL^{-1} and $6\text{ }\mu\text{L}$ 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride solution, (Molecular Biosciences, Boulder, CO, U.S.A.), 50 mg mL^{-1} to the microsphere suspension and incubate for 20 min at RT on a Titramax at 1000 rpm. Wash with $500\text{ }\mu\text{L}$ wash buffer (PBS, pH 7.4). Add $200\text{ }\mu\text{L}$ of PBS coupling buffer, 0.01 M, pH 7.5, Titramax for 2 min, aspirate. Repeat. Add $200\text{ }\mu\text{L}$ coupling buffer containing $250\text{--}500\text{ }\mu\text{g mL}^{-1}$ BSA–biotin depending on the protease. Incubate 4 h at RT on Titramax and then overnight at 4°C . Add $200\text{ }\mu\text{L}$ of wash buffer to the coupled microspheres and Titramax for 2 min, aspirate. Repeat.

The beads are now ready for streptavidin binding. Two hundred microlitre streptavidin (S-0677; Sigma-Aldrich), at a concentration of $100\text{--}200\text{ }\mu\text{g mL}^{-1}$ depending on the protease, is added per well with the BSA–biotin-coupled beads. Incubate the mixture for 1 h in the dark at RT on the Titramax. Aspirate. Wash with $3 \times 70\text{ }\mu\text{L}$ wash buffer. These steps are identical for all enzymes to be analysed.

After a further washing step on the filter plate, the biotinylated polyclonal antibody for the desired specificity (see Table I) was then attached to the streptavidin on the respective beads. After a further washing step, the beads were ready for combination with the other five bead specificities (multiplexing) in a new filter plate and incubation with $50\text{ }\mu\text{L}$ of the tape stripping extracts for 1 h at RT on Titramax and 16 h at 4°C . After washing the beads with the bound enzymes, the six required anti-enzyme monoclonal antibod-

Table I Antibodies and protease standards used in the Luminex multiplex assays

	Polyclonal antibody	Monoclonal antibody	Antigen (standards)
KLK5	Human Kallikrein 5 Biotinylated Affinity Purified PAb, Goat IgG R&D Systems, BAF 1108	Human Kallikrein 5 MAb (Clone 193316) Mouse IgG2B R&D Systems, MAB 11081	Recombinant Human Kallikrein 5, CF R&D Systems, 11081-SE
KLK7	Human Kallikrein 7 Biotinylated Affinity Purified PAb, Goat IgG R&D Systems, BAF 2624	Human Kallikrein 7 MAb (Clone 333931) Mouse IgG2A R&D Systems, MAB 2624	Recombinant Human Kallikrein 7, CF R&D Systems, 2624-SE
KLK11	Human Kallikrein 11 Biotinylated Affinity Purified PAb, Goat IgG R&D Systems, BAF 1595	Human Kallikrein 11 MAb (Clone FB6MA11) Mouse IgG1 AbD Serotec, MCA2156	Recombinant Human Kallikrein 11, CF R&D Systems, 1595-SE
KLK14	Human Kallikrein 14 Biotin Affinity Purified PAb, Goat IgG R&D Systems, BAF 2626	Human Kallikrein 14 MAb (Clone 337903) Mouse IgG1 R&D Systems, MAB 2626	Recombinant Human Kallikrein 14, CF R&D Systems, 2626-SE
Plasmin	Human Plasminogen Biotin Purified Pab, Goat IgG US Biological, P4256-50	Human Plasminogen MAb (Clone 0.B.32) Mouse IgG1 US Biological, P4256-26	Plasminogen, Human, 98% (Profibrinolysin) US Biological, P4256-25
Urokinase	Human u-Plasminogen Activator Biotinylated Affinity Pur PAb, Goat IgG R&D Systems, BAF 1310	Human uPA MAb (Clone 204212) Mouse IgG2A R&D Systems, MAB 1310	Urokinase, Human Urine Calbiochem 672112

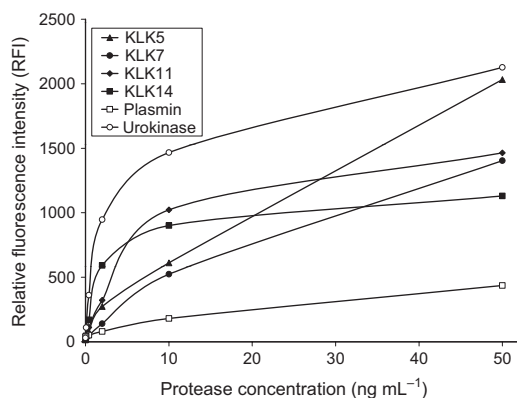


Figure 1 Standard curves of proteases for the determination of extracted stratum corneum protease mass.

ies (50 μ L) were added (see Table I). Incubation at RT was for 30 min.

To measure the amount of bound monoclonal antibodies, which is equivalent to the bound enzymes from the tape stripping extracts, 100 μ L anti-mouse phycoerythrin-labelled antibodies (ImmunoPure 31861; ThermoScientific) was introduced, incubated for 30 min and washed. Measurements in the Luminex apparatus were performed directly from the filter plate. To achieve a good statistical basis, at least 200 beads of each antigenic specificity were acquired. The tape stripping extracts were analysed in triplicates. A six-point standard curve with the six purified proteases was run at the same time. The Luminex results [relative fluorescence intensity (RFI)] of bound phycoerythrin of the dilutions were plotted against the ng enzymes per mL, and the quantities of the unknowns were derived from these standard curves.

All data were collected in Microsoft Excel 2003 and checked for normality using the D'Agostino and Pearson omnibus normality test; then, ANOVA tests (SPSS 17.0.2 ML R1, SPSS Inc., Chicago, IL, U.S.A.) with subsequent *post-hoc* comparisons were made using the Bonferroni test to adjust for multiple comparisons. Statistical significance was set at $\alpha = 0.05$.

Results

The standard curves for the protease mass measurements using the Luminex multiplex bead-based assays are shown in Fig. 1.

Although mass levels of serine proteases tended to be higher in the deeper SC layers for the lesional skin, there was no change in the mass levels of any enzymes measured with increasing depth in the SC extracts from either the healthy subjects or the extracts from the non-lesional (not shown).

The mass levels of the proteases in H, NL and L pooled tape strippings (tapes 1–15) are shown in Fig. 2. As can be seen, there were no differences in the mass levels of KLK5, KLK14 and urokinase between H, NL and L tape strippings. There was a trend of increasing KLK7 levels ($P = 0.1$). However, the mass levels of KLK11 and plasmin were very significantly elevated in the lesional group (KLK11: L vs. NL and vs. H $P = 0.006$, plasmin: L vs. NL and vs. H $P = 0.00001$, Fig. 2), but the variances were also substantially larger than in the healthy and non-lesional group. To satisfy the assumption of equal variances, the ANOVA and the subsequent *post-hoc* Bonferroni test were performed on log-transformed

values. The results were again highly significant and consistent with the ANOVA on untransformed values. The *post-hoc* P -values on log-transformed values were as follows: KLK11 L vs. NL $P = 0.0003$, L vs. H $P = 0.0004$, plasmin L vs. NL $P = 0.00004$, L vs. H $P = 0.00005$. Levene's test for homogeneity of variances showed no significant differences of variances after the log transformation. As a further validation of the results, a Kruskal–Wallis test was performed on each marker/parameter. This is a non-parametric version of the ANOVA, which is more conservative and robust towards outliers and non-normal distributions. This also gave a highly significant result, with $P = 0.003$ for KLK11 and plasmin.

Correlations of immunofluorometrically determined protease mass vs. previously reported enzyme activity measurements [2] were also conducted (Table II). Plasmin and KLK11 mass showed a good (Fig. 3), and KLK7 showed a moderate correlation with activity ($R^2 = 0.8$, $R^2 = 0.9$ and $R^2 = 0.5$, respectively).

Discussion

Changes in the expression and activity of serine proteases in the SC have been observed in samples taken from subjects with AD [8–10]. Komatsu *et al.* [10] examined the SC mass levels of kallikreins (KLK5, KLK6, KLK7, KLK8, KLK10, KLK11, KLK13 and KLK14) from subjects with AD together with enzyme activities for the trypsin-like kallikreins (KLK5, KLK6, KLK8, KLK10, KLK11, KLK13 and KLK14), chymotrypsin-like kallikreins (KLK7), plasmin-like and furin-like proteases.

In the work of Komatsu *et al.* [10], the chymotrypsin-like and trypsin-like kallikrein enzyme activities were not significantly increased in marked contrast to the plasmin-like and furin-like activities. Conversely, we have reported increased chymotrypsin-like kallikrein and especially trypsin-like kallikrein enzyme activities in acute eczematous AD [2]. Like Komatsu *et al.* [10], we also found elevated SC plasmin-like activities, although the levels were reported were dramatically higher [2]. We also determined increased activities of SC tryptase-like enzyme, urokinase and leukocyte elastase.

According to Komatsu *et al.* [10] in the SC of atopic patients, the mass levels of all trypsin-like kallikreins, except KLK11, were significantly elevated. The elevation of the major chymotrypsin-like kallikrein in the SC, KLK7, was predominant, compared with trypsin-like KLKs [10]. In the present study, we only observed increased mass levels of KLK11 but not of KLK5 or KLK14 and a tendency of KLK7 levels to be increased in L. We also found differences in some of the mass levels of the kallikreins in healthy skin. We observed greater quantities of KLK5 but similar quantities of KLK7, KLK11 and KLK14 compared with the study described by Komatsu *et al.* [10].

The differences in protease mass and activity levels to the Komatsu *et al.* study [10] may be related to the differences in the skin condition of the AD patients as well as to different sampling procedures and assays having been used. We examined subjects with active lesions having an exacerbated inflammatory condition, whereas the subjects of the Komatsu *et al.* study showed very mild lichenification only, a condition with retention hyperkeratosis and not premature desquamation. Komatsu *et al.* collected uppermost corneocytes only, whereas we did 15 subsequent tape strippings, allowing depth profiling.

Perhaps one of the major reasons for the differing results between the two studies is the corticosteroid medication of the AD patients in the Komatsu *et al.* study. It has been shown that the

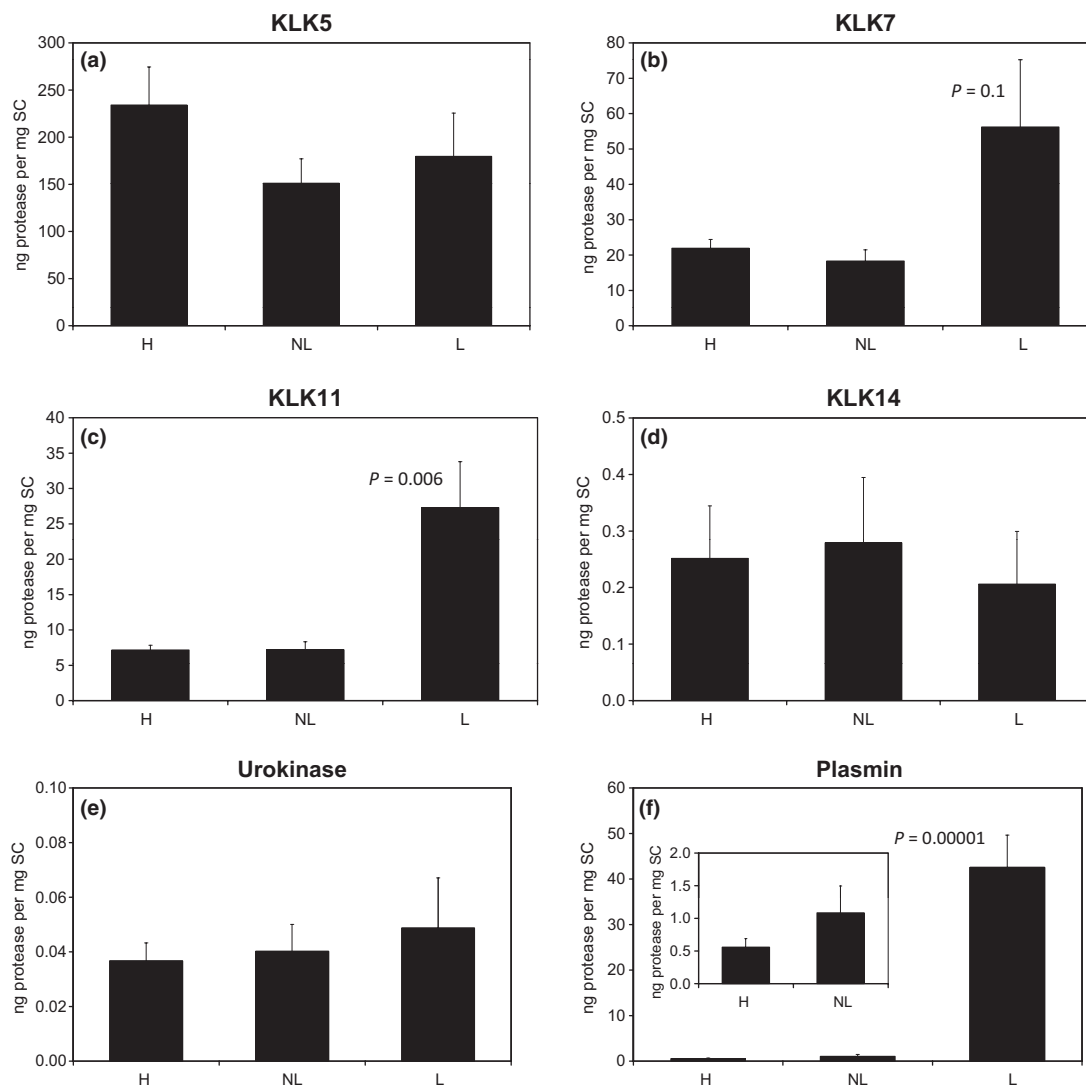


Figure 2 Mass levels of stratum corneum (SC) serine proteases for healthy subjects (H) together with non-lesional (NL) and lesional (L) skin of atopic patients comparing pooled subsequent tape strippings (tapes 1–15), respectively. All bars represent ng protease per μ g SC protein; mean $\pm$ SEM, values $n = 6$ per group. P -values = L vs. H and NL. (a) KLK5, (b) KLK7, (c) KLK11, (d) KLK14, (e) urokinase and (f) plasmin.

Table II Correlation of protease mass and protease activities

Protease mass vs. activity	Correlation coefficient
KLK5 mass vs. trypsin-like kallikrein activity	$R^2 = 0.08$
KLK7 mass vs. chymotrypsin-like kallikrein activity	$R^2 = 0.5$
KLK11 mass vs. trypsin-like kallikrein activity	$R^2 = 0.9$
KLK14 mass vs. chymotrypsin-like kallikrein activity	$R^2 = 0.006$
Urokinase mass vs. urokinase-like activity	$R^2 = 0.002$
Plasmin mass vs. plasmin-like activity	$R^2 = 0.8$

application of clobetasol propionate to normal human skin induces the expression of the mRNA for KLK7 [11]. Obviously the results of the two.

In the present study, the mass levels of KLK14 in lesional skin were very low and not elevated compared with healthy or non-lesional skin. These results would suggest that the increases in SC chymotrypsin-like kallikrein activity we observed in our previous study [2] is probably due to the increased mass levels of KLK7, although KLK14 exerts a high catalytic efficiency. Indeed, the mass levels of KLK7 were elevated approximately three-fold in lesional skin. Also, the enzymatic activity measurements of KLK7 made previously were 1.9 $\times$ and 3.9 $\times$ higher in lesional skin compared with healthy and non-lesional skin. As we did not measure the mass levels of all SC trypsin-like kallikrein activities but did not observe increased KLK5 mass levels in lesional skin, other trypsin-like kallikrein activities are probably contributing to the increased SC trypsin-like enzyme activity we previously reported [2]. KLK11 would appear to be a candidate but not KLK14 from the present studies.

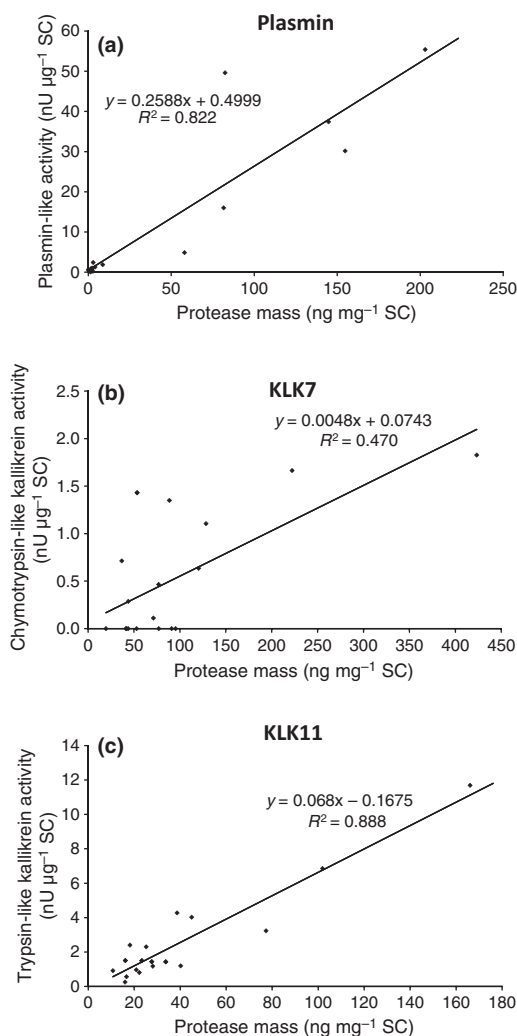


Figure 3 Correlations of immunofluorometrically determined protease mass vs. enzyme activity measurements: (a) plasmin, (b) KLK7, and (c) KLK11.

Urokinase activity levels were reported to be increased 8× and 7× for lesional skin compared with healthy and non-lesional skin samples but in this study the mass levels had not increased. Like KLK14, the mass levels of urokinase were close to the baseline. However, we do report for the first time increased mass levels of plasmin in SC extracts from atopic patients with acute eczema, which were increased 100× and 53×, respectively, in healthy vs. non-lesional skin. This compares with 69× and 30× increase in enzyme activity levels [2]. Urokinase, the major plasminogen activator, is present at very low concentration within the studied SC layers. This fact raises the question: where is plasminogen activated and are there other plasminogen activators than urokinase within the SC?

Differences in the mass levels of SC protease inhibitors, especially the lymphoepithelial Kazal type-related inhibitor (LEKTI), may be contributing to the differences in enzyme activities and the mass levels we record here (for a review see Roelandt *et al.* [12]). For

Table III Comparison of study parameters of Komatsu *et al.* [10] and the present study

	Komatsu <i>et al.</i>	Present study
Human race	Japanese	Caucasian
Medication	Topical corticosteroids	None
SC collection	Tape stripping on non-lesional dry skin, with or without very mild lichenification	Tape stripping on acute eczema lesions
Tapes	Nichiban	D-Squame
Tape extraction	Toluene	Aqueous TRIS buffer incl. non-ionic surfactant
SC collection area	Forearm and upper arm	Ventral forearm
SC collection procedure	Uppermost corneocytes until 2 mg of SC had been visibly obtained	20 subsequent tape strippings at the same test site

SC, stratum corneum.

instance, Roedl *et al.* [13] have reported reduced LEKTI expression in atopic keratinocytes.

The reason for the increased kallikrein protease mass levels in AD is not known. Nevertheless, increased expression of kallikreins may occur because of *KLK* gene polymorphisms such as those reported for *KLK7* by Vasilopoulos *et al.* [14]. However, perturbed barrier function is also known to increase the activities of SC proteases [15–17].

Also, not much is known about the interaction of desquamatory kallikreins and proteases of the thrombostasis axis. Yoon *et al.* [18] demonstrated the potential for important regulatory interactions between these two major protease families. It might be hypothesized that the activation of proKLK7 and/or proKLK11 by plasmin in AD and *vice versa* may occur and that these KLKs may play a role in the inflammatory cascade in AD. Certainly, we find good correlations between mass levels of KLK7, KLK11 and plasmin together with the activity measurements of plasmin and the SC chymotrypsin-like and trypsin-like kallikrein activities we previously reported [2].

In conclusion, mass levels of KLK7 and KLK11 together with plasmin are increased in acute atopic eczematous lesions compared with non-lesional and healthy skin, and these results add to the growing data to support the use of serine protease inhibitors as a potential treatment for barrier-compromised conditions such as acute atopic eczema [19]. This would not discount the use of other occlusives and emollients that improve barrier function [20] and reduce drying stresses on the SC [21, 22] and/or other agents to improve barrier recovery [23].

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