Incomplete KLK7 Secretion and Upregulated LEKTI Expression Underlie Hyperkeratotic Stratum Corneum in Atopic Dermatis

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Atopic dermatitis (AD) is a common inflammatory skin disorder. Chronic AD lesions present hyperkeratosis, indicating a disturbed desquamation process. KLK7 is a serine protease involved in the proteolysis of extracellular corneodesmosome components, including desmocollin 1 and corneodesmosin, which leads to desquamation. KLK7 is secreted by lamellar granules and upregulated in AD lesional skin. However, despite increased KLK7 protein levels, immunostaining and electron microscopy indicated numerous corneodesmosomes remaining in the uppermost layer of the stratum corneum from AD lesions. We aimed to clarify the discrepancy between KLK7 overexpression and retention of corneodesmosomes on AD corneocytes. Western blot analysis indicated abnormal corneodesmosin degradation patterns in stratum corneum from AD lesions. The KLK activity of tape-striped corneocytes from AD lesions was not significantly elevated in situ zymography, which was our new attempt to detect the protease activity more precisely than conventional assays. This ineffective KLK activation was associated with impaired KLK7 secretion from lamellar granules and increased expression of LEKTI in AD. Such imbalances in protease-protease inhibitor interactions could lead to abnormal proteolysis of corneodesmosomes and compact hyperkeratosis. Upregulated expression of LEKTI might be a compensatory mechanism to prevent further barrier dysfunction in AD.


INTRODUCTION

Atopic dermatitis (AD) is a common chronic inflammatory skin disorder. Various factors are involved in AD pathogenesis. However, a T helper type 2-dominant environment and skin barrier dysfunction play key roles in AD. Although clinical manifestations vary with age, adult patients with AD often develop long-lasting scaling erythematous papules and lichenified plaques (Akdis et al., 2006). Histologically, the skin disorder varies with age, adult patients with AD skin barrier dysfunction play key roles in AD. Although various factors are involved in AD pathogenesis, various factors are involved in AD pathogenesis. However, a T helper type 2-dominant environment and skin barrier dysfunction play key roles in AD. Although clinical manifestations vary with age, adult patients with AD often develop long-lasting scaling erythematous papules and lichenified plaques (Akdis et al., 2006). Histologically, the stratum corneum (SC) of AD lesions shows compact hyperkeratosis, indicating a disturbed desquamation process (Ishida-Yamamoto et al., 2011). Proper degradation of modified SC desmosomes, corneodesmosomes (CDs), is essential for physiological desquamation. CDs present three extracellular components: desmoglein 1, desmocollin 1, and corneodesmosin (Cdsn). When these components are degraded by proteases, desquamation occurs (Haftek, 2015; Ishida-Yamamoto et al., 2011; Jonca et al., 2011; Rawlings and Voegeli, 2013). Various proteases, their inhibitors, the pH, and hydration of the SC regulate this process (Haftek, 2015; Ishida-Yamamoto et al., 2011; Miyai et al., 2014; Rawlings and Voegeli, 2013).

Kallikrein-related peptidase (KLK) 7, the sole chymotryptic enzyme in the epidermis, is a serine protease involved in CD degradation (Wang et al., 2004; Yousef and Diamandis, 2001). KLK7 is secreted by lamellar granules (LGs) into the intercellular spaces between the stratum granulosum and the SC (Ishida-Yamamoto et al., 2004), where it directly cleaves Cdsn and desmocollin 1 (Jonca et al., 2011). Increased KLK7 expression and activity were observed in the skin of patients with AD (Komatsu et al., 2007; Morizane et al., 2012; Voegeli et al., 2009). T helper type 2 cytokines increase KLK7 expression, but not the expression of other KLKs (Hatano et al., 2013; Morizane et al., 2012). We previously detected desmoglein 1, desmocollin 1, and Cdsn throughout the surface of AD corneocytes in the uppermost layer, indicating reduced CD degradation (Igawa et al., 2013). Reduced CD degradation was also detected in other scaling conditions such as xerosis (Rawlings and Voegeli, 2013; Simon et al., 2001) and psoriasis (Simon et al., 2008). A recent report showed reduced CD degradation in dandruff, accompanied by increased serum protease activities and overexpression of their inhibitors (Singh et al., 2014).

We aimed to clarify the mechanism underlying hyperkeratosis in AD and determined that KLK7 was overexpressed but insufficiently activated in AD. 

Abbreviations: AD, atopic dermatitis; CD, corneodesmosome; Cdsn, corneodesmosin; KLK, kallikrein-related peptidase; LEKTI, lymphoepithelial Kazal-type-related inhibitor; LG, lamellar granule; NS, Netherton syndrome; SC, stratum corneum

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RESULTS

Abnormal CD degradation in AD
To assess CD distribution, we performed immunostaining for CD components in tape-stripped corneocytes (Figure 1a–d). We classified AD skin into three conditions: nontreated chronic eczematous skin (lesion), moderate eczematous skin under treatment (under treatment), and noneczematous skin (nonlesion). CD components were detected throughout the surface of AD lesion corneocytes (Figure 1a), whereas they were localized mainly in the periphery and slightly in the central region in AD nonlesional areas (Figure 1c). Topical steroid treatment gradually changed the staining pattern, accompanied by clinical improvement (Figure 1b). However, in AD under treatment, the desmoglein 1-peripheral-stained margins of corneocytes were significantly broader than those in AD nonlesional and normal skin (Supplementary Figure S1 online). Although CDs are detected only in peripheral areas in the normal uppermost SC (Ishida-Yamamoto and Igawa, 2015; Naoe et al., 2010), we ultrastructurally revealed that CDs remained throughout the surface of corneocytes in the uppermost AD lesional SC (Figure 1e, Supplementary Figure S2 online). Western blot analysis detected many Cdsn bands, which seemed to be progressively proteolyzed forms, in SC samples (Figure 1f and g). The Cdsn degradation pattern differed between AD lesions and normal tissues. Three bands of around 52 kDa were detected in AD lesional SC, whereas only two bands were observed in normal SC (Figure 1f, black arrow). In addition, the bands detected at around 38 kDa were markedly thicker (Figure 1g, slashed arrow), and the 24-kDa bands, indicating a putative degraded form of Cdsn, were fainter in AD than in normal tissue (Figure 1g, white arrow).

To investigate the factors contributing to CD retention, we used a linear regression model, with the rate of nonperipheral Cdsn distribution as a dependent variable versus nine candidate explanatory variables (Supplementary Materials and Methods online). In AD, high transepidermal water loss, low SC water content, and high KLK activity were significantly associated with an increased rate of nonperipheral Cdsn distribution. In addition, different effectors were identified in the normal control (Supplementary Figure S3a and b online). To evaluate factors affecting barrier function, we measured filaggrin breakdown products in the SC of normal controls and patients with AD. Although the SC was obtained from a clinically normal noneczematous area in patients with AD, the amounts of filaggrin breakdown products (2-pyrrolidone-5-carboxylic acid and urocanic acid) were significantly lower than in normal controls (Supplementary Table S1 online). In situ zymography by using tape-stripped SC showed no significant difference in KLK protease activity between AD lesions and normal SC

Despite disturbed CD degradation, previous assays with synthetic substrates showed increased serine protease activities in AD lesional SC (Voegeli et al., 2009). To only detect extracellular protease activity, we developed in situ zymography with tape-stripped corneocytes and a KLK inhibitor, which mainly inhibits chymotryptic enzymes (Supplementary Table S2 online). We observed very high KLK activity in Netherton syndrome (NS) used as a positive control (Supplementary Figure S4a and d online). In AD lesions and normal control SC, 10 μM KLK inhibitor did not present clear inhibitory activity (data not shown). We therefore used 100 μM for further analysis. In normal corneocytes, fluorescence images of in situ zymography revealed protease activity in the peripheral area (Figure 2a). The signal was attenuated by the KLK inhibitor (Figure 2b). On the other hand, AD corneocytes showed a disorganized fluorescence pattern (Figure 2c), and the reaction was only partially suppressed by the inhibitor (Figure 2d). The same tendency was detected with in situ zymography of frozen skin sections (Supplementary Figure S5a–d online). To quantitate the fluorescence intensity, we measured the intensity per cell surface area in each group. Although data tend to vary in individual cases (Supplementary Figure S6 online), there was no significant difference in KLK activity for AD and normal SC (Figure 2e).

Incomplete LG secretion and intracellularly trapped KLK7 in AD
To further address the functional state of KLK7 in AD, we analyzed the KLK7 secretion process. Ultrastructural analysis showed intracellular vacuoles in AD lesional corneocytes, which were not observed in normal corneocytes (Figure 3a and b). We speculated that KLK7 was trapped by these vacuoles derived from nonecreted LGs. To test this hypothesis, postembedding immunoelectron microscopy was employed. In normal SC, KLK7 was detected intercellularly (Figure 3c), whereas in AD SC, KLK7 was detected not only intercellularly (Figure 3d) but also in intracytoplasmic (Figure 3e) and intravesicular (Figure 3f) areas. In the intercellular areas, KLK7 was detected around degrading CD plaques, but not upon intact CD plaques (Figure 3c and d, dotted rectangles). To quantify the KLK7 secretion rate, the labeling density was calculated (Figure 3g). The KLK7 signals in AD SC were higher than those in normal SC (Figure 3h). The signals were detected in the intercellular, intracytoplasmic, and intravesicular areas, with more than 50% in the intercellular area in AD lesions (Figure 3i).

Imbalance between KLK7 and lymphoepithelial Kazal-type-related inhibitor underlies disturbed CD degradation in AD
Although intercellular KLK7 signals were higher in the AD SC than in normal SC, in situ KLK activity was not significantly elevated in AD SC. We speculated that increased expression of lymphoepithelial Kazal-type-related inhibitor (LEKT1), a KLK inhibitor, suppresses excessive protease activity in AD SC. Indeed, immunofluorescence revealed increased expression of both LEKT1 and KLK7 in the AD lesional epidermis (Figure 4d–f) compared with those in the normal epidermis (Figure 4a–c). The order of expression, with LEKT1 being expressed earlier than KLK7, was similar between AD and normal epidermis (Figure 4c and f). Western blot analysis confirmed increased expression of a mature form of KLK7 (24.3 kDa) in the SC and epidermis of AD lesions (Figure 4g and h). Interestingly, not only a 25.8-kDa band corresponding to the full-length form but also a 27.4-kDa band corresponding to a possible proform was
detected in the SC of AD lesions (Figure 4g). The LEKTI expression levels in patients with AD were consistently higher than those in normal skin (Figure 4h), but immuno-electron microscopy showed trapped LEKTI in the intracellular area of AD SC (Supplementary Figure S7a–d online).
DISCUSSION

Our results indicate that AD lesional skin shows impaired CD proteolysis and increased expression and incomplete secretion of KLK7 and LEKTI.

We previously demonstrated that extracellular CD components were detected throughout the surface of AD corneocytes (Igawa et al., 2013). The present Cdsn western blot analysis showed abnormal degradation patterns, providing further evidence of disturbed CD degradation in AD. It was reported that both KLK7 expression and activity are increased in AD lesional skin (Komatsu et al., 2007; Morizane et al., 2012; Voegeli et al., 2009). In this study, we confirmed that KLK7 protein was increased in the epidermis and SC in AD. However, KLK activity in AD corneocytes was not significantly different from that in normal corneocytes (Figure 2). Differences in assay methods may explain the discrepancy between our results and those of previous studies (Komatsu et al., 2007; Voegeli et al., 2009). In previous studies, tape-stripped corneocytes were processed in organic solvent, centrifuged at high speeds, frozen, and homogenized. These procedures could easily induce the release of intracellular KLK7 into the solvent and increase apparent protease activity. Conversely, our in situ zymography does not break corneocytes and can detect protease activity only on the extracellular surface. Although results tended to vary in individual cases, we believe that KLK7 is overexpressed but not overactivated in the extracellular spaces. However, our method presents a drawback concerning the specificity of the protease inhibitor used in the assay. Casein, used as a substrate, can be proteolyzed by a wide range of proteases. Thus, a specific KLK7 inhibitor was needed to accurately detect the KLK7 activity. Our inhibitor inhibits KLK7 activity at a low concentration, and trypsin-like protease activity at a much higher concentration (Supplementary Table S2). In in situ zymography, KLK activity in NS corneocytes was blocked by the inhibitor in a concentration-dependent manner (Supplementary Figure S4). Therefore, the activities suppressed by a 100 μM concentration of the inhibitor in AD corneocytes seemed to reflect KLK7 activity and partial trypsin-like activity. More specific protease inhibitors are required to detect KLK7 activity with greater specificity in the future.

Previous ultrastructural studies reported disturbed LG secretion in AD SC (Elias and Wakefield, 2014; Fartasch et al., 1992). Consistent with those reports, vesicular structures were found inside AD corneocytes. Moreover, they encompassed KLK7 and LEKTI signals, suggesting that both were trapped in LGs in the corneocytes. Although NS corneocytes also had intracytoplasmic vesicles, suggesting that LG secretion was incomplete (Supplementary Figure S8 online), the KLK activity of NS corneocytes, evaluated with in situ zymography, was much higher than that of AD corneocytes. We speculated that LEKTI could be another contributing factor preventing KLK overactivation in AD because LEKTI deficiency causes KLK hyperactivation resulting in early CD degradation in NS (Igawa et al., 2013; Ishida-Yamamoto et al., 2005). Indeed, our immunofluorescence and western blot analyses revealed increased LEKTI expression in AD. Although we need further investigation to elucidate the effects of intracellular LEKTI or other protease inhibitors on the KLK inhibition, this might be a compensatory reaction against increased KLK7 expression to suppress KLK7 activity in AD. A similar mechanism might be occurring in other conditions with hyperkeratosis and defective permeability barrier. Although further investigation is required, in some of such conditions, both KLK7 and LEKTI expression were upregulated, but the expression order in LEKTI and KLK7 was different from AD (Supplementary Figure S9b–f online). Only in ichthyosis vulgaris, LEKTI is expressed earlier than KLK7, but their expression was not upregulated (Supplementary Figure S9a).

Various extrinsic factors affect KLK activity (Haftek, 2015; Ishida-Yamamoto et al., 2011; Rawlings and Voegeli, 2013). Xerotic skin with low SC water content showed...
Figure 3. Aberrant lamellar granule (LG) secretion underlies kallikrein-related peptidase (KLK) 7 hyposecretion in atopic dermatitis (AD). (a–f) Transmission electron microscopy (a, b) and immunoelectron microscopy (c–f) in normal (a, c) and AD (b, d–f) stratum granulosum (SG) and stratum corneum (SC). Dots show KLK7 labeling, with black and white arrows in secreting LGs and intercellular areas, and black and white circles in intravesicular and intracytoplasmic areas, respectively. Dotted rectangles indicate corneodesmosomes (c, d). Scale bars = (a–c, g–f) 200 nm and (d) 500 nm. (g) Three areas are defined: inter SC1–2, intracytoplasm SC1, and intravesicles SC1. Immunogold particles were counted in each area per μm². (h) KLK7 labeling count. Black and slashed bars show normal and AD SC, respectively. (i) Distribution of KLK7 labeling per compartment in normal SC (upper) and AD lesions (lower).

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reduced KLK activity (Harding et al., 2000) and retained nonperipheral CDs in the upper SC (Simon et al., 2001). Our linear regression model showed that low SC water content was significantly associated with nonperipheral Cdsn distribution in AD (Supplementary Figure S3). Recently, Riethmuller et al. (2015) reported a correlation between low levels of filagrin breakdown products and disturbed Cdsn degradation in children with AD.

Figure 4. Lymphoepithelial Kazal-type-related inhibitor (LEKTI) and kallikrein-related peptidase (KLK)7 expression levels are upregulated in atopic dermatitis (AD) lesions. (a–f) Immunostaining of KLK7 (a, d), LEKTI (b, e), and their merged images (c, f) in normal and AD lesional skin. Scale bars = 10 μm. (g, h) Western blot analysis of KLK7 (g, h) and LEKTI (h) in normal and AD lesional stratum corneum (SC) (g), and in the whole epidermis (h). Recombinant LEKTI and β-actin were used as positive and loading controls, respectively.
Genetic background also needs to be considered when discussing AD pathogenesis (Fortugno et al., 2012; Margolis et al., 2014; Saunders et al., 2013). Filaggrin gene mutation is a major factor in barrier dysfunction (Palmer et al., 2006), and T helper type 2-dominant conditions also affect filagrin expression (Pellerin et al., 2013). The SC from the non-eczematous area of all patients with AD in this study showed significantly lower amounts of filaggrin breakdown products than that of normal controls. Although filaggrin gene mutation analysis might elucidate the genetic background underlying barrier deficiency, analysis of filaggrin breakdown products is more informative in defining current skin barrier conditions. The patients with AD in this study could be defined as a group with barrier deficiency, eliminating the so-called intrinsic-type AD, in which skin barrier function is normal (Tokura, 2010).

In conclusion, impaired KLK7 secretion from LGs and increased LEKTI expression could underlie the insufficient activation of KLK in AD. Recently, it was reported that human skin equivalent models, which commonly show thick SC, showed similar results (McGovern et al., 2016). Such imbalances in protease-protease inhibitor interactions underlie delayed proteolysis of CD components, leading to compact hyperkeratosis.

**MATERIALS AND METHODS**

**Human SC samples**

All participants provided written informed consent, and the protocol was approved by the medical ethics committee of the Asahikawa Medical University. The study was conducted according to the principles of the Helsinki Declaration.

The patient characteristics are summarized in Supplementary Table S1. The patients with AD included in this study had barrier deficiency, as shown by the significantly reduced amount of filaggrin breakdown products in noneczematous skin compared with normal controls (Supplementary Table S1). SC samples were obtained with sequential tape stripping from the forearm, as previously described (Oyama et al., 2010). The first and second layers from all AD cases and normal controls were used for immunofluorescence staining. A sixth layer of AD lesion and normal control SC was used for in situ zymography, with SC samples of NS (Ishida-Yamamoto et al., 2005) as positive controls. For western blot analysis, we obtained SC samples with another adhesive tape (CELLTOAPE CT-24; Nichiban, Tokyo, Japan) from three AD lesions and normal controls. The tape (24 × 150 mm piece) was pressed on the forearm and stripped repeatedly until the tape was no longer sticky.

**Antibodies**

The followings were used as primary antibodies: polyclonal rabbit Cdsn antibody (Descargues et al., 2006) and polyclonal rabbit antibody against the D12 domain of LEKTI (Miyai et al., 2014). The other primary and secondary antibodies are listed in Supplementary Table S3 online.

**Immunofluorescence microscopy**

Formalin-fixed and paraffin-embedded tissue sections were obtained from normal control, NS, ichthyosis vulgaris without AD, lichen planus, prurigo nodularis, psoriasis vulgaris, and AD lesional skin. After deparaffinization, samples were steamed with Tris-EDTA buffer (pH 9.2) for antigen retrieval. Immunofluorescence analysis of these skin samples and tape-stripped corneocytes was performed as described previously (Igawa et al., 2013). Fluorescence images taken by using a laser scanning confocal microscope were compared with differential interference contrast microscopy images to clarify the location of the staining (FV1000-D, Olympus, Tokyo).

**Electron microscopy and immunoelectron microscopy**

Conventional transmission electron microscopy and immunoelectron microscopy for KLK7 and LEKTI by using Lowicryl K11M resin-embedded skin samples were performed as described previously (Ishida-Yamamoto et al., 2005). Incubation with secondary antibodies only served as a negative control.

**Labeling density of KLK7**

The cross-sectional surface of corneocytes in the first layer of SC (SC 1) was divided into three areas (Figure 3g). The immunogold particles and areas in each sample were measured manually, and labeling densities per μm² were calculated. Ten view fields were analyzed in each sample, and the data are presented as an average.

**Western blot analysis**

For epidermis isolation, skin samples obtained from two AD lesions and normal controls were treated with dispase (Godo Shusei, Tokyo, Japan) at 4 °C overnight. The tape-stripped corneocytes were dipped in toluene for 2 days at 4 °C to remove the tape. The precipitates were washed with toluene to remove any residual adhesive. The samples were dried with a vacuum concentrator (Thermo SpeedVac; Thermo Scientific, Waltham, MA). According to a previous report (Descargues et al., 2005), the corneocytes or epidermal samples were lysed in protein extraction buffer (150 mM NaCl, 50 mM Tris HCl [pH 8], 5 mM EDTA [pH 8], 1% Nonidet-P40, 9 M urea, 50 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The protein concentration in to remove the tape. The precipitates were washed with toluene to remove any residual adhesive. The samples were dried with a vacuum concentrator (Thermo SpeedVac; Thermo Scientific, Waltham, MA). According to a previous report (Descargues et al., 2005), the corneocytes or epidermal samples were lysed in protein extraction buffer (150 mM NaCl, 50 mM Tris HCl [pH 8], 5 mM EDTA [pH 8], 1% Nonidet-P40, 9 M urea, 50 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The protein concentration was determined with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Proteins (10–20 μg/lane) were separated using SDS-PAGE and transferred onto Hybrid-poly vinylidene difluoride membranes (Amersham Bioscience, Piscataway, NJ). Recombinant LEKTI protein (10 ng/lane) (Cloud-Clone, Houston, TX) was used as a positive control. Immunoreactive bands were visualized by using the ECL Advance Western Blotting Detection Kit (GE Healthcare UK, Buckinghamshire, UK) and detected with a LAS-3000 Luminescent Image Analyzer (Fujiﬁlm, Tokyo, Japan).

**In situ zymography**

In situ zymography with tape-stripped SC and frozen skin sections was performed as previously described (Hachem et al., 2005; Kaneko et al., 2012). BODIPY-F1 casein (Molecular Probes, Eugene, OR) (1 μg/ml) with or without 100 or 10 μM KLK inhibitor (Supplementary Table S2) was used. The signal was visualized under a confocal microscope (Olympus FV1000-D). The average intensity per cell surface area was calculated. KLK activity was evaluated by subtracting the intensity obtained with the KLK inhibitor from that without the inhibitor.

**Statistical analysis**

Values are expressed as means ± standard error of the mean. Welch's t-test was applied to analyze the differences between two groups. P < 0.05 was considered significant.

**CONFLICT OF INTEREST**

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.10.015.

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