博士論文

Incomplete KLK7 secretion and upregulated LEKTI expression underlie hyperkeratotic stratum corneum in atopic dermatitis

（アトピー性皮膚炎皮疹部の過角化を伴う鱗屑形成にはKLK7の不完全な分泌とLEKTIの発現亢進が関与している）

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Incomplete KLK7 secretion and upregulated LEKTI expression underlie hyperkeratotic stratum corneum in atopic dermatitis

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Short title: KLK-LEKTI imbalance in atopic dermatitis

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[1] **Abbreviations:** AD, atopic dermatitis; CD, corneodesmosome; Cdsn, corneodesmosin; Dsc1, desmocollin 1; Dsg1, desmoglein 1; KLK, kallikrein-related peptidase; LEKTI, lymphoepithelial Kazal-type-related inhibitor; LG, lamellar granule; NS, Netherton syndrome; PCA, 2-pyrrolidone-5-carboxylic acid; SC, stratum corneum; TEWL, transepidermal water loss; UA, urocanic acid

ABSTRACT

Atopic dermatitis (AD) is a common inflammatory skin disorder and its chronic lesions present compact hyperkeratosis, indicating a disturbed desquamation process. Timely proteolysis of extracellular corneodesmosome (CD) components, including desmocollin 1 (Dsc1) and corneodesmosin (Cdsn), is essential for normal desquamation. Kallikrein-related peptidase (KLK) 7 is a serine protease involved in the proteolysis of Dsc1 and Cdsn. KLK 7 is secreted by lamellar granules and upregulated in AD lesional skin. However, despite increased KLK7 protein levels, immunostaining and electron microscopy indicated numerous remaining CDs in the uppermost layer of the stratum corneum (SC) in AD lesions. Western blot analysis indicated abnormal Cdsn degradation patterns in SC from AD lesions. KLK activity of tape-stripped corneocytes from AD lesions was not significantly elevated in *in situ* zymography, which was our new attempt to detect the protease activity more precisely than by using conventional assay methods. This ineffective KLK activation was associated with impaired KLK7 secretion from lamellar granules and increased expression of lympho-epithelial Kazal-type-related inhibitor (LEKTI) in AD lesions. Such imbalance in protease–protease inhibitor interaction could lead to abnormal proteolysis of CDs 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 Th-2 dominant environment and skin barrier dysfunction play key roles in AD. Although clinical manifestations of AD vary with age, adult AD patients often suffer from long lasting scaling erythematous papules and lichenificated plaques (Akdis et al., 2006). Histologically, the stratum corneum (SC) of AD lesions shows compact hyperkeratosis, which indicates a disturbed desquamation process (Ishida-Yamamoto et al., 2011). Proper degradation of corneodesmosomes (CDs), which are modified desmosomes in the SC, is essential for physiological desquamation. CDs present three extracellular components, desmoglein 1 (Dsg1), desmocollin 1 (Dsc1), 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 and their inhibitors are involved in the regulation of CD degradation.(Miyai et al., 2014). SC pH and hydration also 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 one of the serine proteases 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 SC (Ishida-Yamamoto et al., 2004) and then directly cleaves Cdsn and Dsc1 (Jonca et al., 2011). Increased expression and activity of KLK7 have been shown in the skin of AD patients (Komatsu et al., 2007;Morizane et al., 2012;Voegeli et al., 2009). Th2 cytokines can increase KLK7 expression, but not other KLKs (Hatano et al., 2013;Morizane et al., 2012). We previously detected Dsg1, Dsc1, and Cdsn all over the surface of AD corneocytes in the uppermost layer, indicating reduced CD degradation (Igawa et al., 2013). Such 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). Interestingly, recent report showed that CD degradation is reduced in dandruff, accompanied with increased serine protease activities and overexpression of their inhibitors (Singh et al., 2014).

This study aimed to clarify the mechanism underlying hyperkeratosis in AD. We determined that KLK7 was overexpressed but insufficiently activated in AD. Increased expression of Lympho-epithelial Kazal-type-related inhibitor (LEKTI) and incomplete

secretion of KLK7 were associated with insufficient KLK activation in AD.

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 the AD skin into three conditions: non-treated chronic eczematous skin (lesion), moderate eczematous skin under treatment (under treatment), and non-eczematous skin (non-lesion). CD components were detected all over the surface of AD lesion corneocytes (Figure 1a), whereas they were localized mainly in the periphery and slightly in the central area in AD non-lesion areas (Figure 1c). These patterns are different from the normal pattern reported previously (Figure 1d) (Igawa et al., 2013). Topical steroid treatment gradually changed the diffuse staining pattern into a peripheral pattern along with clinical improvement (Figure 1b). However, in AD under treatment, the peripheral stained margins of corneocytes were significantly broader than those in AD non-lesion and normal skin (Supplementary Figure S1). Athough, CDs are detected only in the peripheral areas in the normal uppermost SC (Ishida-Yamamoto and Igawa, 2015;Naoe et al., 2010), our ultrastructural analysis revealed that CDs remained all over the surface of corneocytes

in the uppermost layer of the SC in AD lesion (Figure 1e and Supplementary Figure S2). 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 was different between AD lesions and normal tissues. Three bands around 52-kDa were detected in AD lesion SC, whereas only two bands were observed in normal SC (Figure 1f, black arrow). Additionally, around 38-kDa bands, the intermediate size of Cdsn, were markedly thicker (Figure 1g, slashed arrow), and 24-kDa bands of a putative degraded form of Cdsn were fainter in AD than in normal tissue (Figure 1g, white arrow).

To investigate the contributing factors to the retention of CDs, we used a linear regression model using the rate of non-peripheral Cdsn distribution as a dependent variable versus nine candidates of explanatory variables (Supplementary materials and methods). In AD, high TEWL, low SC water content, and high KLK activity were significantly associated with the increased rate of non-peripheral Cdsn distribution. Additionally, different effectors were identified in the normal skin (Supplementary Figure S3a and b). To evaluate individuals' barrier dysfunction, we measured filaggrin breakdown products such as total amino acids, 2-pyrrolidone-5-carboxylic acid (PCA) and urocanic acid (UA), in the SC of all normal control and AD lesion cases. In AD

lesion cases, the amounts of all three categories of filaggrin breakdown products were significantly lower than in normal cases (Supplementary table S1).

In situ **zymography using tape-stripped SC shows no significant difference in KLK protease activity between AD lesions and normal SC**

Despite disturbed CD degradation, previous assays using synthetic substrates showed increased serine protease activities in AD lesion SC (Voegeli et al., 2009). To detect the activity of extracellular protease only, we developed *in situ* zymography using tape-stripped corneocytes and a KLK inhibitor, which mainly inhibits chymotryptic enzymes (Supplementary Table S3). We observed very high KLK activity in Netherton Syndrome (NS) as a positive control (Supplementary Figure S4a and b). In AD lesions and normal control SC, a 10 μM concentration of the KLK inhibitor did not present a clear inhibitory activity (data not shown). We therefore used a 100 μM concentration for further analysis. In normal corneocytes, fluorescence images of *in situ* zymography revealed protease activity in the peripheral area (Figure 2a). The signal was significantly 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 KLK inhibitor (Figure 2d), which was consistent with the

results of *in situ* zymography using frozen skin section (Supplementary Figure S5a-d). To quantitate the fluorescence intensity, we measured the intensity per cell surface area in each group. 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 that corneocytes from AD lesions contained many intracellular vacuoles (Figure 3b-c), which were not observed in normal corneocytes. We speculated that KLK7 was trapped by these vacuoles derived from non-secreted LGs. To test that, post-embedding immunoelectron microscopy was employed to assess KLK7 localization. KLK7 was detected in secreting lamellar granules and intercellular areas in the normal skin (Figure 3d), whereas it was also detected in intracytoplasmic and intravesicular areas in AD lesions (Figure 3e). To quantify the KLK7 secretion rate, labeling density was calculated (Figure 3a). The KLK7 signals in AD SC were markedly higher than that in normal SC. They were detected in the intercellular, intracytoplasmic, and intravesicular areas with more than half of them being in the intercellular area in AD lesions (Figure 3f and g).

Imbalance between KLK7 and LEKTI underlies disturbed CD degradation in AD

Although intercellular KLK7 signals were higher in the SC of AD lesions than in normal SC, *in situ* KLK activity was not significantly elevated in the former. We speculated that LEKTI, a KLK inhibitor, might increase in the epidermis of AD lesions to suppress excessive protease activity. Indeed, immunofluorescence revealed expression of both LEKTI and KLK7 in the epidermis of AD lesions was markedly increased (Figure 4d-f) compared to those in the normal epidermis (Figure 4a-c). The order of expression, LEKTI being expressed earlier than KLK7, was similar in the epidermis from AD lesions and normal skin (Figure 4c and f). Western blot analysis confirmed that the expression of a mature form of KLK7 (24.3-kDa) increased in the SC and epidermis of AD lesions (Figure 4g-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 pro-form was detected in the SC of AD lesions (Figure 4g). LEKTI expression levels in AD patients were constantly higher than those in the normal skin (Figure 4h).

DISCUSSION

Our results indicate that impaired proteolysis of CDs, incomplete secretion of

KLK7, and increased expression of LEKTI in AD lesional skin.

We previously demonstrated that CD degradation was disturbed and extracellular CD components were detected all over the surface of AD corneocytes (Igawa et al., 2013). The present western blot analysis of Cdsn showed abnormal degradation patterns, further supporting the disturbed CD degradation in AD. It has been reported that both KLK7 expression and activity increase in AD lesion skin compared to AD nonlesion or normal skin (Komatsu et al., 2007;Morizane et al., 2012;Voegeli et al., 2009). In this study, we confirmed that KLK7 protein was increased in AD epidermis and SC. However, KLK activity in AD corneocytes was not significantly different from that in normal corneocytes (Figure 2). The assay method may be a reason for the difference between our and 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. Such severe procedures could easily induce the release of intracellular KLK7 into the solvent and increase apparent protease activity. Conversely, *in situ* zymography, used in this study, does not break corneocytes and can detect protease activity only on the extracellular surface. Therefore, we believe that KLK7 is overexpressed but not overactivated in the extracellular spaces. However, our method presents a drawback regarding 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 chymotrypsin-like protease inhibitor was needed to detect accurate KLK7 activity. The inhibitor used in this study inhibits KLK7 activity at a low concentration and trypsin-like protease activity at a much higher concentration (Supplementary Table S3). In *in situ* zymography, KLK activity in NS corneocytes was blocked by the KLK inhibitor in a concentration-dependent manner (Supplementary Figure S4). Therefore, the activities suppressed by a 100 μM concentration of the KLK inhibitor in AD corneocytes seemed to reflect KLK7 activity and partial trypsin-like activity. More specific protease inhibitors are required to detect proper KLK7 activity in the future.

Disturbed LG secretion in SC from AD lesions has been reported in ultrastructural studies (Elias and Wakefield, 2014;Fartasch et al., 1992). Consistent with those reports, vesicular structures were found inside AD corneocytes. Moreover, they encompassed KLK7 signals, suggesting that trapped KLK7 failed to degrade CD. Although NS corneocytes had intracytoplasmic vesicles, suggesting that LGs secretion was disturbed resembling AD (Supplementary Figure S6), KLK activity of NS corneocytes, evaluated by *in situ* zymography, was much greater than that in AD. Since LEKTI deficiency causes KLK hyperactivation resulting in early CD degradation in NS

(Igawa et al., 2013;Ishida-Yamamoto et al., 2005), we speculated that LEKTI could be another contributing factor to prevent KLK over-activation in AD. Indeed, our immunofluorescence and western blot analyses revealed increased LEKTI expression in AD. This might be a compensatory reaction against increased KLK7 expression to suppress KLK7 activity in AD. Further investigation is required to elucidate if other protease inhibitors can contribute to the development of compact hyperkeratosis in AD.

Various extrinsic factors are known to affect KLK activity (Haftek, 2015;Ishida-Yamamoto et al., 2011;Rawlings and Voegeli, 2013). Xerotic skin with low SC water content represents reduced KLK activity (Harding et al., 2000) and retained non-peripheral CDs in the upper SC (Simon et al., 2001). Our linear regression model showed that low SC water content was significantly associated with non-peripheral Cdsn distribution in AD (Supplementary Figure S3). Recently, Riethmuller et al (Riethmuller et al., 2015) reported that the correlation between low filaggrin breakdown products and disturbed Cdsn degradation was found in AD children. We measured total amino acids in SC as degraded products of filaggrin. Total amino acid content in SC was significantly correlated with non-peripheral Cdsn distribution in normal control SC, but not in AD lesion. The discrepancy might be caused by differences of methodology and age of patients group between our and previous study. However, at least, our model

suggests that SC water content influences KLK activity in AD lesion.

Genetic backgrounds also need 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 for barrier dysfunction (Palmer et al., 2006), and Th2 dominant condition also affects the filaggrin expression (Pellerin et al., 2013). SC from nonlesional area of our AD patients showed significantly lower amounts of filaggrin breakdown products compared to that of normal controls. This finding suggests that ordinal filaggrin gene mutation analysis might be effective to know patients' genetical background, but to know current skin barrier condition, analysis of filaggrin breakdown products is more reasonable.

In conclusion, impaired KLK7 secretion from lamellar granules and increased LEKTI expression could contribute for insufficient activation of KLK in AD. Such imbalance in protease–protease inhibitor interaction can induce delayed proteolysis of CD components, leading to compact hyperkeratosis. In contrast to NS, the upregulated expression of LEKTI might be one of the compensatory mechanisms to prevent excessive barrier dysfunction in AD.

MATERIALS AND METHODS

Human SC samples

All participants provided 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.

SC samples were obtained with sequential tape stripping from the forearm as previously described (Oyama et al., 2010). Patient characteristics are summarized in Supplementary Table S1. The first and second SC layers from all AD cases and normal controls were used for immunofluorescent staining. A sixth layer of AD lesion and normal control SC were used for *in situ* zymography, using SC samples of NS (Ishida-Yamamoto et al., 2005) as a positive control. For western blot analysis, a 24-mm wide adhesive tape (CELLOTAPE CT-24; Nichiban, Tokyo, Japan) was used to obtain SC samples from three AD lesions and three normal controls. The tape (a 24 × 150 mm piece) was pressed on the forearm for about a few seconds and stripped repeatedly until the stickiness of the tape was lost.

Antibodies

The following antibodies were used as primary antibodies: polyclonal rabbit antibody against the central part of Cdsn (Descargues et al., 2006) and polyclonal rabbit antibody

against the D12 domain of LEKTI (Miyai et al., 2014). The other commercially available primary and secondary antibodies are listed in Supplementary Table S2

Immunofluorescence microscopy

Formalin-fixed and paraffin-embedded tissue sections were obtained from normal control, NS, and AD lesional skin. After deparaffinization, sectioned 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 were detected using a laser scanning confocal microscope (Olympus FV1000-D, Tokyo, Japan).

Electron microscopy and immunoelectron microscopy (IEM)

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

Labeling density of KLK7

The cross-section surface of corneocytes in the first layer of SC (SC 1) was divided into three areas as shown in figure 3a. Immunogold particles in each area per unit length were counted manually and labeling densities were calculated. Ten units length in each sample were analyzed and data are described as an average.

Western blot analysis

For epidermis isolation, skin samples obtained from two AD lesion and normal control was treated with dispase (Godo Shusei, Tokyo, Japan) at 4°C overnight and the epidermis was mechanically separated from the dermis. The stripped corneocytes on the tape 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 then dried by using a vacuum concentrator (Thermo, SpeedVac, Waltham, MA, USA) for 4 h at 6,000 rpm. Based on the previous report (Descargues et al., 2005), the corneocytes or epidermis 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, USA). The protein concentration was determined by the BCA protein assay kit (Pierce, Rockford, IL, USA). Proteins (10–20 μg per lane) were

separated by SDS-PAGE and transferred onto Hybond-PVDF membranes (Amersham Bioscience, Piscataway, NJ, USA). Recombinant LEKTI protein (10 ng per lane) (Cloud-Clone Corp., Houston, TX, USA) was used as positive control. Immunoreactive bands were visualized using the ECL Advance Western Blotting Detection Kit (GE Healthcare UK Ltd., Buckinghamshire, UK) and detected using LAS-3000 Luminescent Image Analyzer (Fujifilm Corp., Tokyo, JAPAN).

In situ zymography

In situ zymography using tape stripped SC and frozen skin section were performed as previously described (Hachem et al., 2005;Kaneko et al., 2012). BODIPY-Fl casein (Molecular Probes, Eugene, OR, USA) (1 μg/mL) with or without 100 μM or 10 μM of a KLK inhibitor (Supplementary Table S3) was used. The signal was visualized under a confocal microscope (Olympus FV1000-D). The average of 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 ± S.E.M. 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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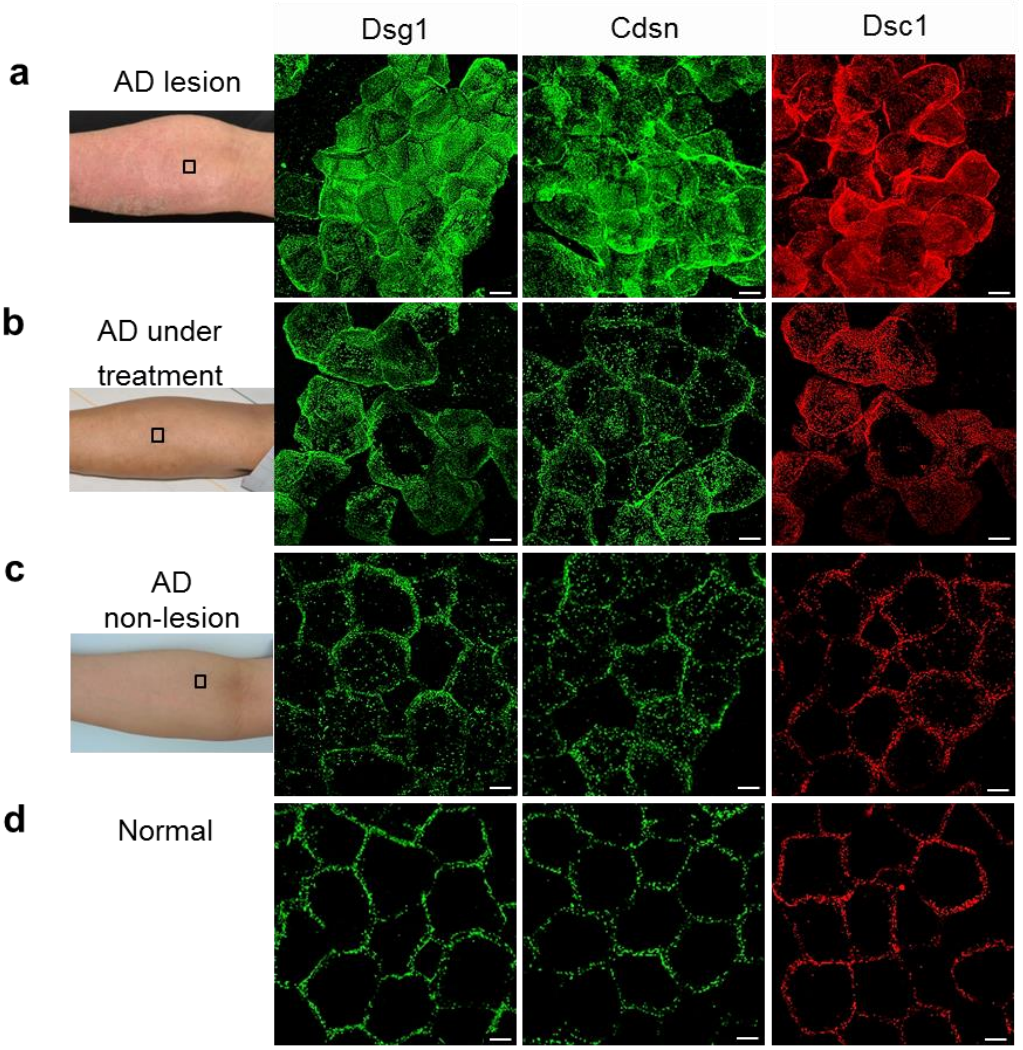
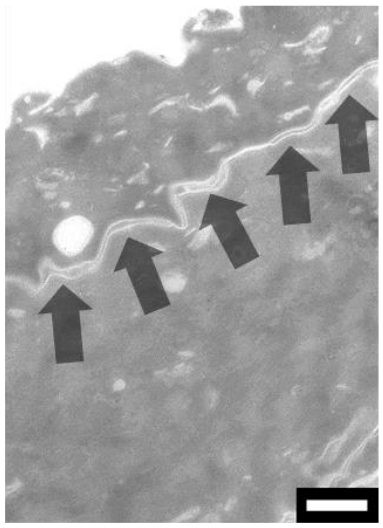
FIGURE AND FIGURE LEGENDS

Figure 1. The corneodesmosome (CD) components degradation in atopic dermatitis (AD) is different from that in the normal skin. (a-d) CD components (Dsg1, Cdsn, and Dsc1) immunostainings using tape-stripped stratum corneum (SC) from AD lesions, AD under treatment, AD non-lesions, and normal skin. Scale bars: 10 μm. (e) Transmission electron microscopy of the most superficial SC of AD lesion skin (also see supplementary Figure S2.) Black arrows indicate CDs. Scale bar: 500 nm. (f-g) Cdsn western blot analysis in normal epidermis, normal and AD lesion SC. Equal loading was verified by Coomassie Brilliant Blue staining. Figure f is a longer exposure image of the rectangular area in Figure g. Black, slashed and white arrows indicate the 52 k-Da range, 38 k-Da range and 24 k-Da bands, respectively.


Dsg1
Cdsn
Dsc1
a
AD lesion
b
AD under treatment
c
AD non-lesion
d
Normal


e

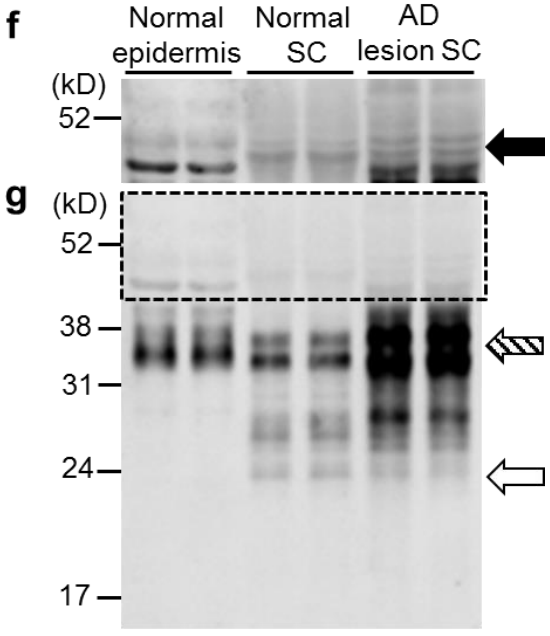

f
Normal epidermis
Normal SC
AD lesion SC
(kD)
52
g
(kD)
52
38
31
24
17

Figure 2. Kallikrein (KLK) protease activities of AD and normal corneocytes using *in situ* zymography of tape-stripped SC. (a-d) Fluorescence image of *in situ* zymography of tape-stripped normal SC and SC from AD lesions with or without 100 μM KLK inhibitor. Scale bars: 10 μm. (e) Fluorescence intensity per cell surface area was measured in each corneocyte with or without the KLK inhibitor. KLK activity (slashed bar) was determined by subtracting the intensity with the KLK inhibitor (white bar) from that without the KLK inhibitor (black bar).

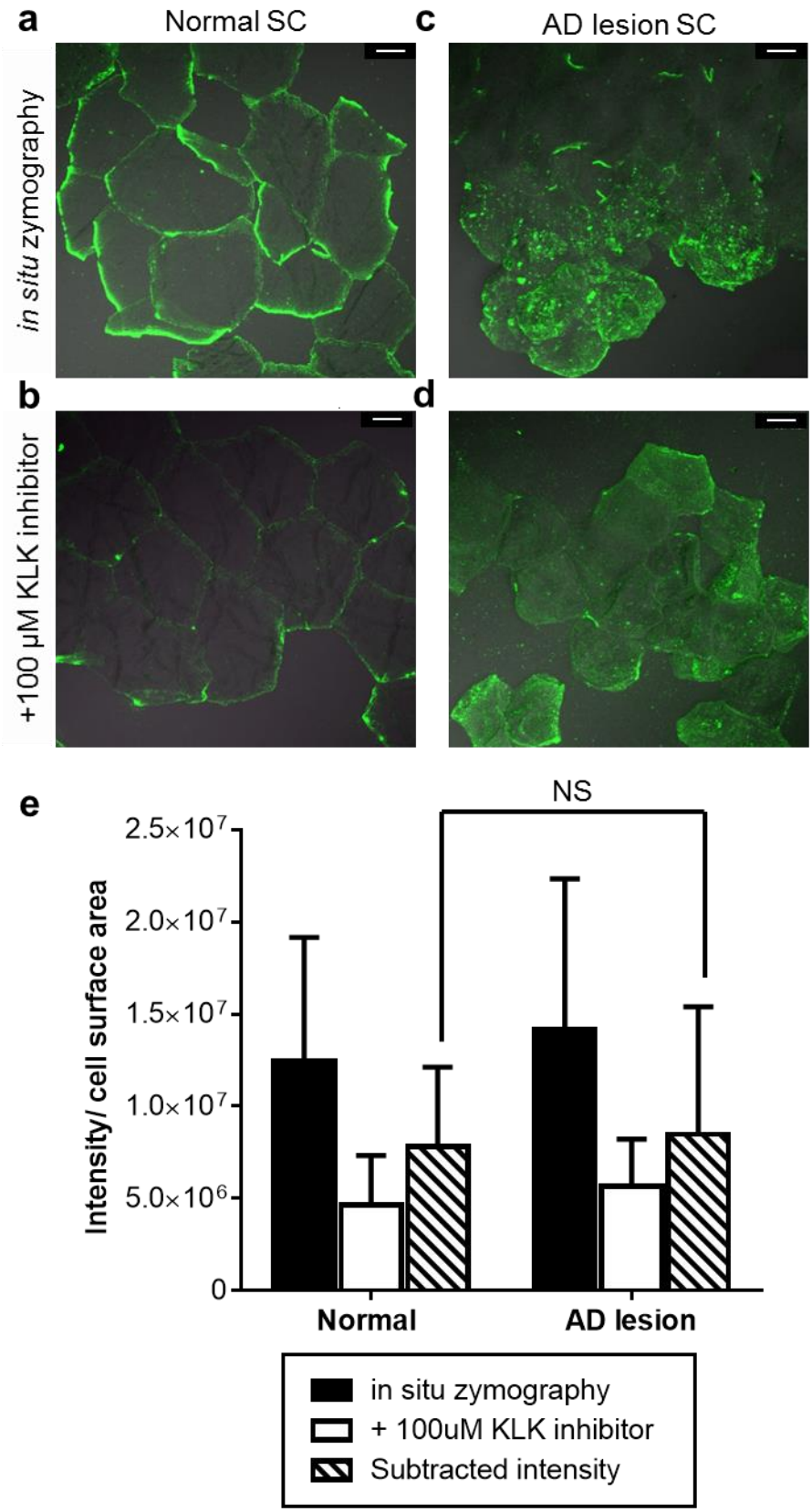

a
Normal SC
c
AD lesion SC
in situ zymography
b
d
+100 μM KLK inhibitor
e
NS
Intensity/ cell surface area
2.5×10^7
2.0×10^7
1.5×10^7
1.0×10^7
5.0×10^6
0
Normal
AD lesion
in situ zymography
+ 100uM KLK inhibitor
Subtracted intensity

Figure 3. Aberrant lamellar granule secretion induces KLK7 hyposecretion in AD.

(a) We defined three SC areas. Immuno-gold particles were counted in each area per unit length. (b-e) TEM (b-c) and IEM (d-e) in normal (b, d) and AD lesional (c, e) stratum granulosum and SC. Dots are KLK7 labeling, indicated with black and white arrows for those in secreting lamellar granules and in intercellular areas, and with black and white circles for intravesicular and intracytoplasmic areas, respectively. Rectangles of dotted lines indicates CDs (d-e). Scale bars: 200 nm. (f) The number of KLK7 labeling in the three areas. Black and slashed bars show normal and AD lesion SC, respectively. (g) The distribution rate of KLK7 labeling in normal (upper bar) and AD lesion (lower bar) SC.

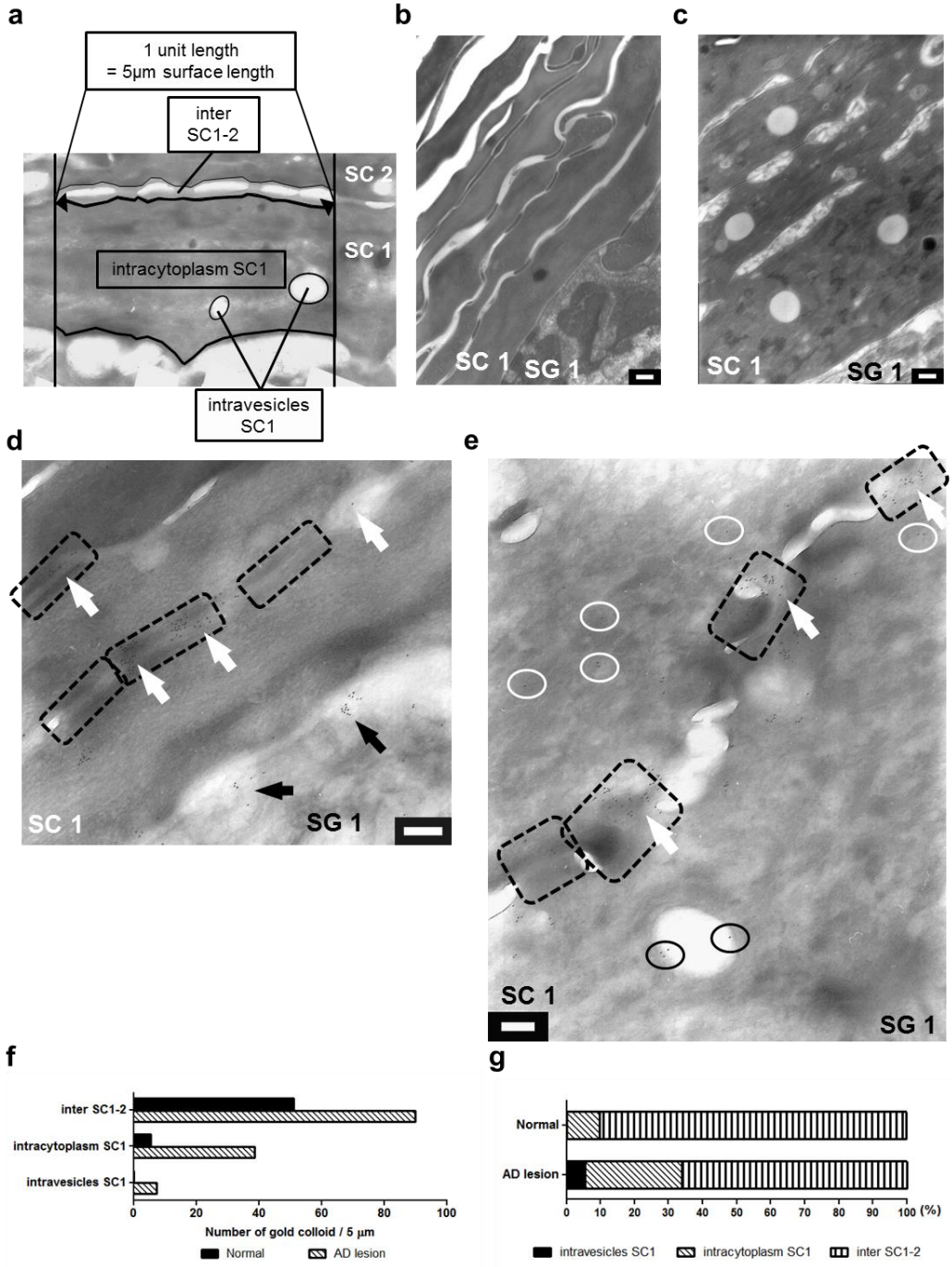
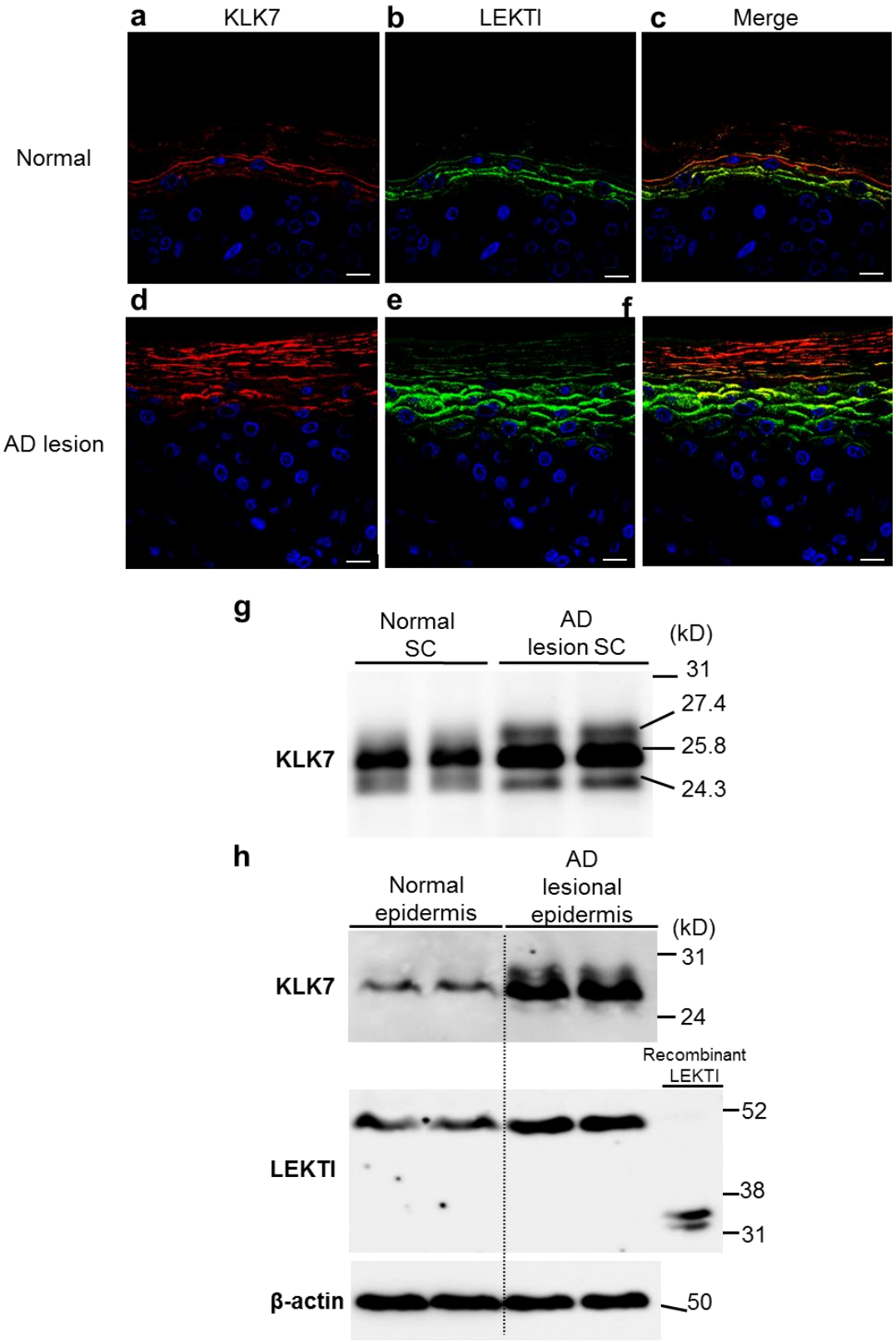

a
1 unit length
= 5μm surface length
inter
SC1-2
SC 2
SC 1
intracytoplasm SC1
intravesicles
SC1
b
SC 1
SG 1
c
SC 1
SG 1
d
SC 1
SG 1
e
SC 1
SG 1
f
inter SC1-2
intracytoplasm SC1
intravesicles SC1
0
20
40
60
80
100
Number of gold colloid / 5 μm
Normal
AD lesion
g
Normal
AD lesion
0
10
20
30
40
50
60
70
80
90
100 (%)
intravesicles SC1
intracytoplasm SC1
inter SC1-2

Figure 4. Both LEKTI and KLK7 expression are upregulated in AD lesions. (a–f) Immunostaining of KLK7 (a and d), LEKTI (b and e), and their merged images (c and f) in normal and AD lesion skin. (g and h) Western blot analysis of KLK7 (g and h) and LEKTI (h) in normal and AD lesion SC (g), and whole epidermis (h). Recombinant LEKTI and β-actin were used as positive and loading controls, respectively.


a
KLK7
b
LEKTI
c
Merge
Normal
d
e
f
AD lesion
g
Normal SC
AD lesion SC
(kD)
31
27.4
25.8
24.3
KLK7
h
Normal epidermis
AD lesional epidermis
(kD)
31
24
KLK7
Recombinant LEKTI
52
38
31
LEKTI
β-actin
50