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

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

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ABSTRACT

Atopic dermatitis (AD) is a common inflammatory skin disorder. Chronic AD lesions present hyperkeratosis, indicating a disturbed desquamation process. Kallikrein-related peptidase (KLK) 7 is a serine protease involved in the proteolysis of extracellular corneodesmosome (CD) components, including desmocollin1 and corneodesmosin (Cdsn), 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 CDs remaining in the uppermost layer of the stratum corneum (SC) from AD lesions. We aimed to clarify the discrepancy between KLK7 overexpression and retention of CDs on AD corneocytes. 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 conventional assays. 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. Such imbalances in protease–protease inhibitor interactions 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 Th2-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 (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, 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 (SG) and the SC (Ishida-Yamamoto et al., 2004), where it directly cleaves Cdsn and Dsc1 (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). Th2 cytokines increase KLK7 expression, but not the expression of other KLKs (Hatano et al., 2013; Morizane et al., 2012). We previously detected Dsg1, Dsc1, and Cdsn throughout 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). A recent report showed reduced CD degradation in dandruff, accompanied with increased serine 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.

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 Dsg1 peripheral stained margins of corneocytes were significantly broader than those in AD nonlesional and normal skin (Supplementary Figure S1). Although CDs are detected only in peripheral areas in 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 and Supplementary Figure S2). Western blot analysis detected many Cdsn bands, which seemed to be progressively proteolyzed forms, in SC samples (Figure 1f,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). Additionally, 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). 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. Additionally, different effectors were identified in the normal control (Supplementary Figure S3a,b). 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).

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 S3). We observed very high KLK activity in Netherton syndrome (NS) used as a positive control (Supplementary Figure S4a,d). 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). 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), 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,b). We speculated that KLK7 was trapped by these vacuoles derived from nonsecreted LGs. To test this hypothesis, postembedding immunoelectron microscopy (IEM) 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,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 lympho-epithelial Kazal-type-related inhibitor (LEKTI) 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 LEKTI, a KLK inhibitor, suppresses excessive protease activity in AD SC. Indeed, immunofluorescence revealed increased expression of both LEKTI 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 LEKTI being expressed earlier than KLK7, was similar between AD and normal epidermis (Figure 4c,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,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). The LEKTI expression levels in patients with AD were consistently higher than those in normal skin (Figure 4h), but IEM showed trapped LEKTI in intracellular area of AD SC (Supplementary Figure S7a–d).

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 S3). 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), 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 was upregulated, but the expression order in LEKTI and KLK7 was different from AD (Supplementary Figure S9 b-f). Only in ichthyosis vulgaris, LEKTI is expressed earlier than KLK7, but their expression was not upregulated (Supplementary Figure S9 a).

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 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 filaggrin breakdown products and disturbed Cdsn degradation in children with AD.

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 Th2-dominant conditions also affect filaggrin expression (Pellerin et al., 2013). The SC from the noneczematous 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 to normal controls (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 were 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 (CELLOTAPE CT-24; Nichiban, Tokyo, Japan) from three AD lesions and normal controls. The tape (a 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 S2.

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 in order to clarify the location of the staining (FV1000-D; Olympus, Tokyo, Japan).

Electron microscopy and IEM

Conventional transmission electron microscopy and IEM 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 μm^2 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, USA). 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, USA). The protein concentration was determined with the BCA protein assay kit (Pierce, Rockford, IL, USA). Proteins (10–20 μg/lane) were separated using SDS-PAGE and transferred onto Hybond-PVDF membranes (Amersham Bioscience, Piscataway, NJ, USA). Recombinant LEKTI protein (10 ng/lane) (Cloud-Clone Corp., Houston, TX, USA) was used as a positive control. Immunoreactive bands were visualized by using the ECL Advance Western Blotting Detection Kit (GE Healthcare UK Ltd., Buckinghamshire, UK) and detected with a LAS-3000 Luminescent Image Analyzer (Fujifilm Corp., 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-Fl casein (Molecular Probes, Eugene, OR, USA) (1 μg/mL) with or without 100 μM or 10 μM KLK inhibitor (Supplementary Table S3) 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 ± SEM. Welch's *t*-test was applied to analyze the differences between two groups. $P < 0.05$ was considered significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

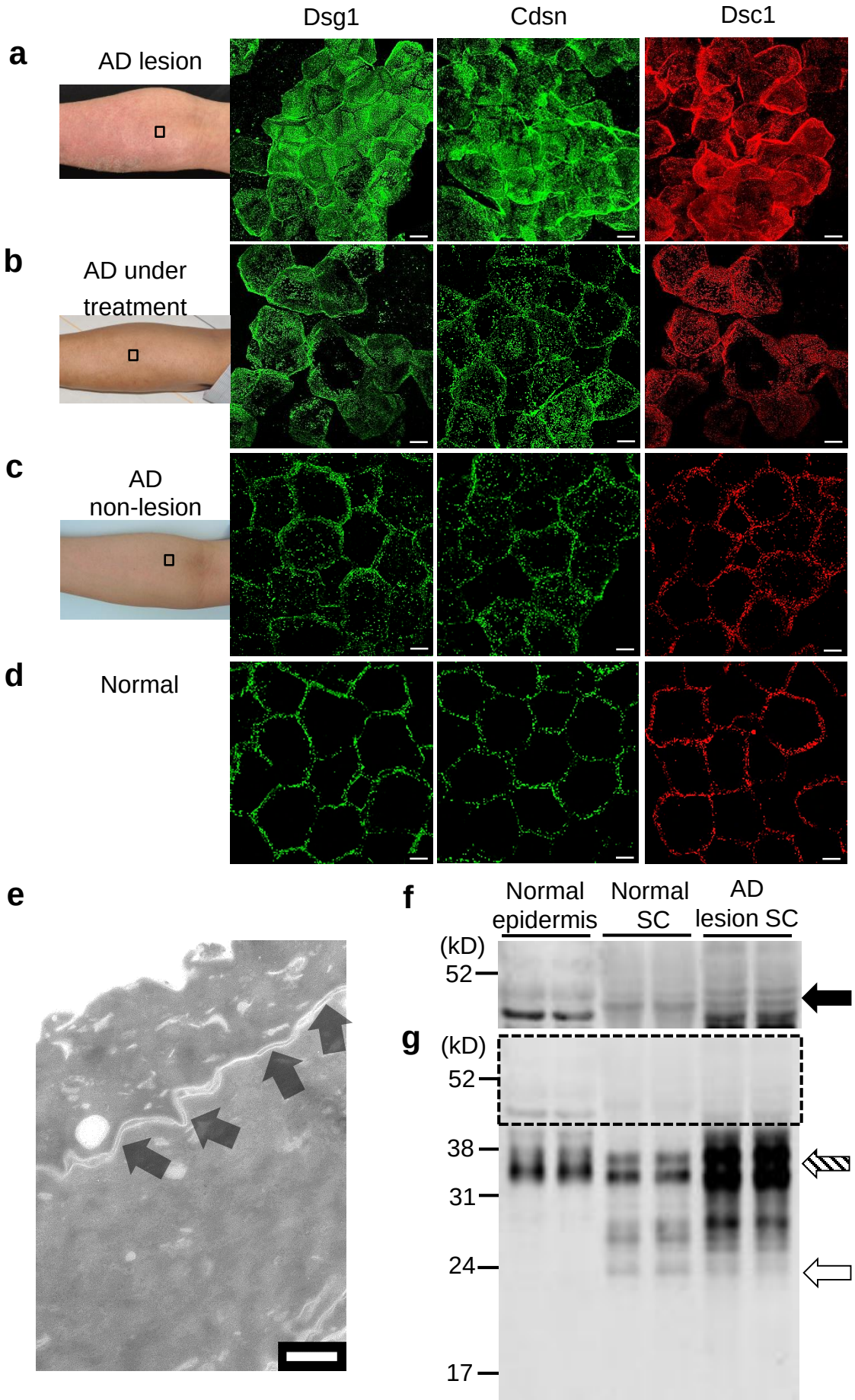
Figure 1. The degradation of corneodesmosome (CD) components in atopic dermatitis (AD) differs from that in normal skin. (a–d) Immunostaining of CD components (Dsg1, Cdsn, and Dsc1) on tape-stripped stratum corneum (SC) from AD lesions, AD under treatment. AD nonlesional, and normal skin. Scale bars: 10 μm. (e) Transmission electron microscopy of the most superficial SC of AD lesional skin (also see Supplementary Figure S2.) Black arrows indicate CDs. Scale bar: 500 nm. (f–g) Cdsn western blot analysis in normal epidermis, normal SC, and AD lesional 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 bands in the 52-, 38-, and 24-kDa ranges, respectively.

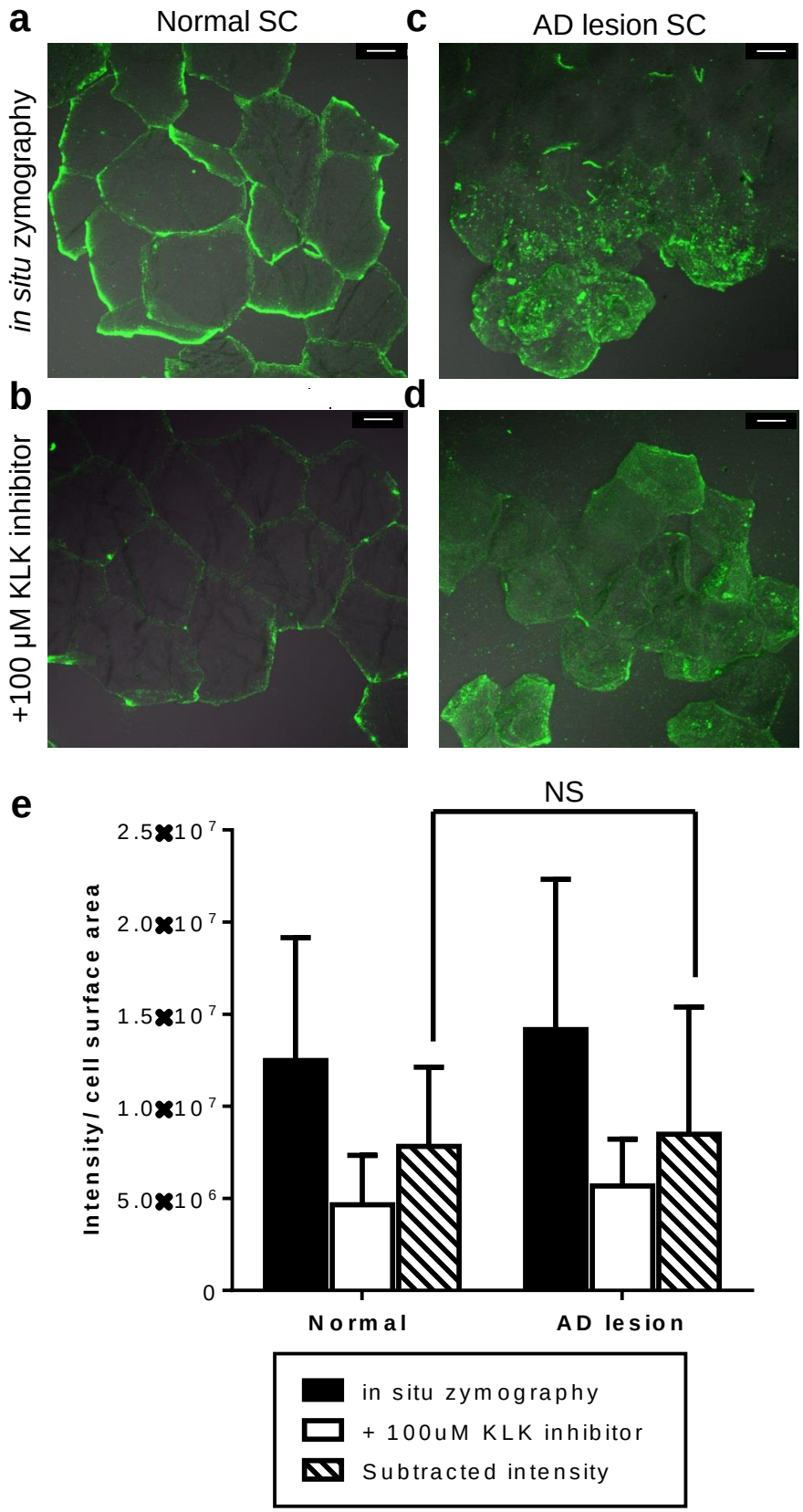
Figure 2. Kallikrein (KLK) protease activities of atopic dermatitis (AD) and normal corneocytes on *in situ* zymography of tape-stripped stratum corneum (SC). (a–d) Fluorescence image of *in situ* zymography of tape-stripped normal SC and SC from AD lesions with or without 100 μM KLK inhibitor. Scale bars: 10 μm. (e) The 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. KLK activities in AD and

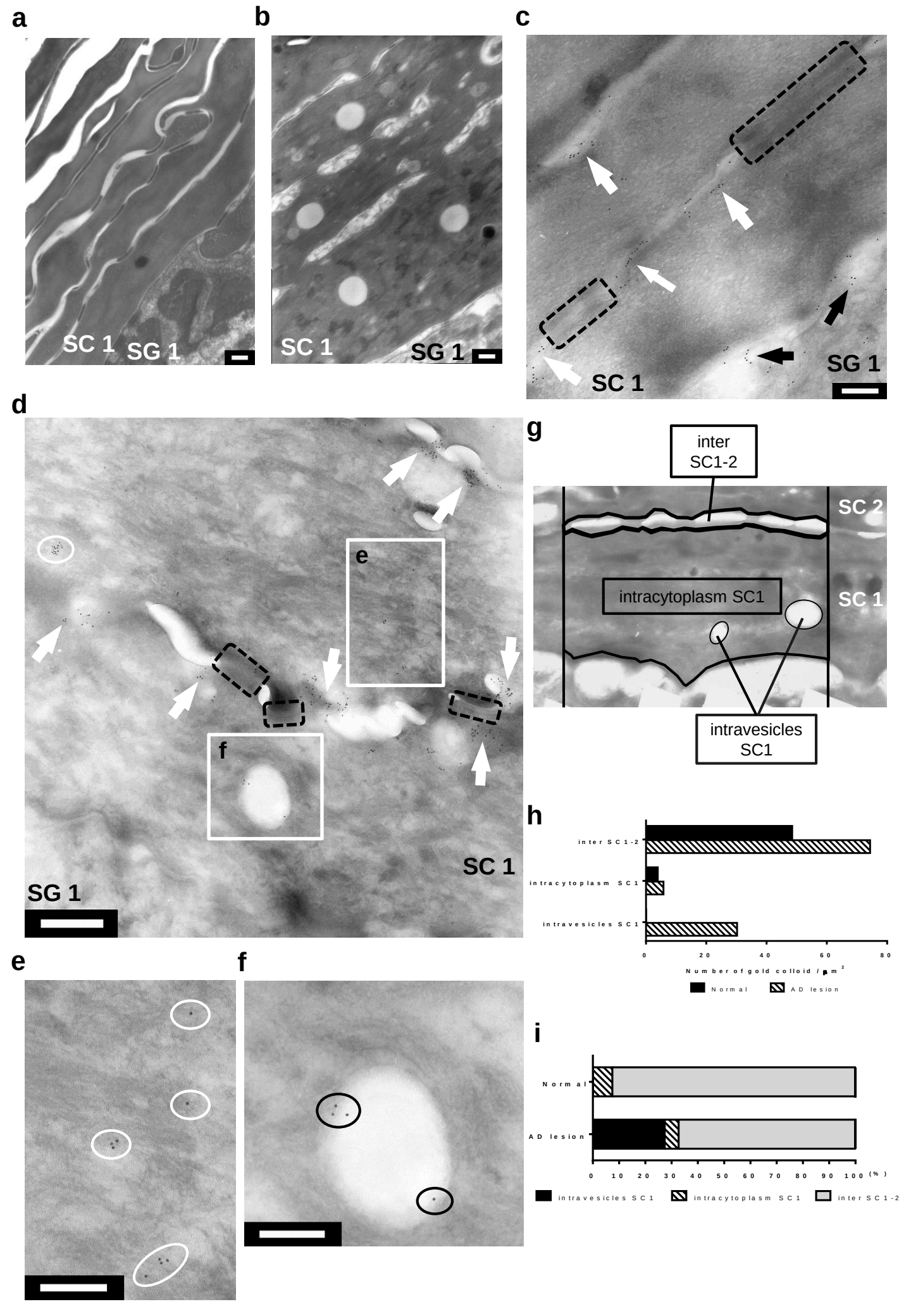
normal corneocytes showed no significant difference.

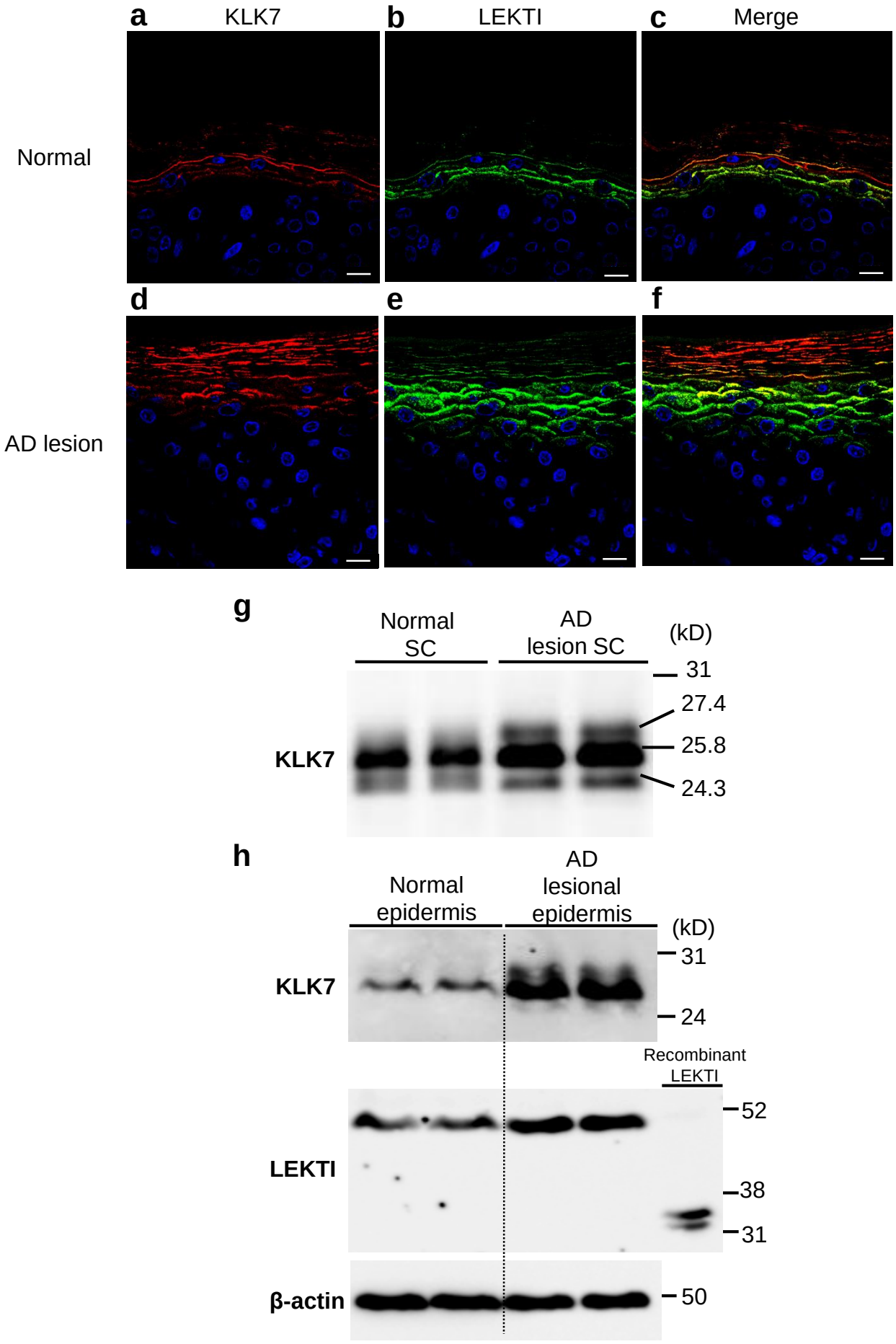
Figure 3. Aberrant lamellar granule (LG) secretion underlies KLK7 hyposecretion in AD. (a–f) Transmission electron microscopy (a, b) and immunoelectron microscopy (c-f) in normal (a, c) and AD (b, d-f) stratum granulosum and SC. Dots show KLK7 labeling, with black and white arrows in secreting LGs and intercellular areas, and black and white circles in intravesicular and intracytoplasmic areas, respectively. Dotted rectangles indicate CDs (c, d). Scale bars: (a-c, g-f) 200 nm, (d) 500nm. (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).

Figure 4. LEKTI and KLK7 expression levels are upregulated in 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 SC (g), and in the whole epidermis (h). Recombinant LEKTI and β-actin were used as positive and loading controls, 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


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


a
SC 1
SG 1
b
SC 1
SG 1
c
SC 1
SG 1
d
e
f
SC 1
SG 1
g
inter
SC1-2
SC 2
intracytoplasm SC1
SC 1
intravesicles
SC1
h
inter SC1-2
intracytoplasm SC1
intravesicles SC1
0
20
40
60
80
Number of gold colloid / µm²
Normal
AD lesion
e
f
i
Normal
AD lesion
0
10
20
30
40
50
60
70
80
90
100
(%)
intravesicles SC1
intracytoplasm SC1
inter SC1-2


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