

Letter to the Editor

T_H2 cytokines increase kallikrein 7 expression and function in patients with atopic dermatitis

To the Editor:

Atopic dermatitis (AD) is a chronic pruritic inflammatory skin disorder characterized by a T_H2 environment and epidermal barrier dysfunction.^{1,2} A primary defect in the development of AD because loss-of-function mutations in the filaggrin gene are associated with AD.² Excessive protease activity is another characteristic abnormality affecting the epidermal barrier in patients with AD. Human tissue kallikreins (KLKs) are secreted serine proteases encoded by 15 genes located on chromosome 19, and in the skin KLK5 and KLK7 are known as major serine

proteases. These proteases degrade corneodesmosome proteins, such as desmoglein 1, desmocollin 1, and corneodesmosin, leading to desquamation.³ KLK8 and KLK14 also have been shown to be involved in skin desquamation.³ Although KLK5, KLK8, and KLK14, trypsin-like serine proteases, cleave the carboxyl side of arginine or lysine, KLK7, a chymotrypsin-like serine protease, cleaves the carboxyl side of tyrosine or phenylalanine. An increase in KLK expression has been reported in the epidermis in patients with AD, and the increase in KLK7 expression is prominent among KLKs.⁴ An association between genetic variations of KLK7 and AD have been also reported.³ Patients with Netherton syndrome who have a defect in lymphoepithelial Kazal-type related inhibitor (LEKTI), a serine protease inhibitor that could control excessive KLK activity, have AD-like lesions.³

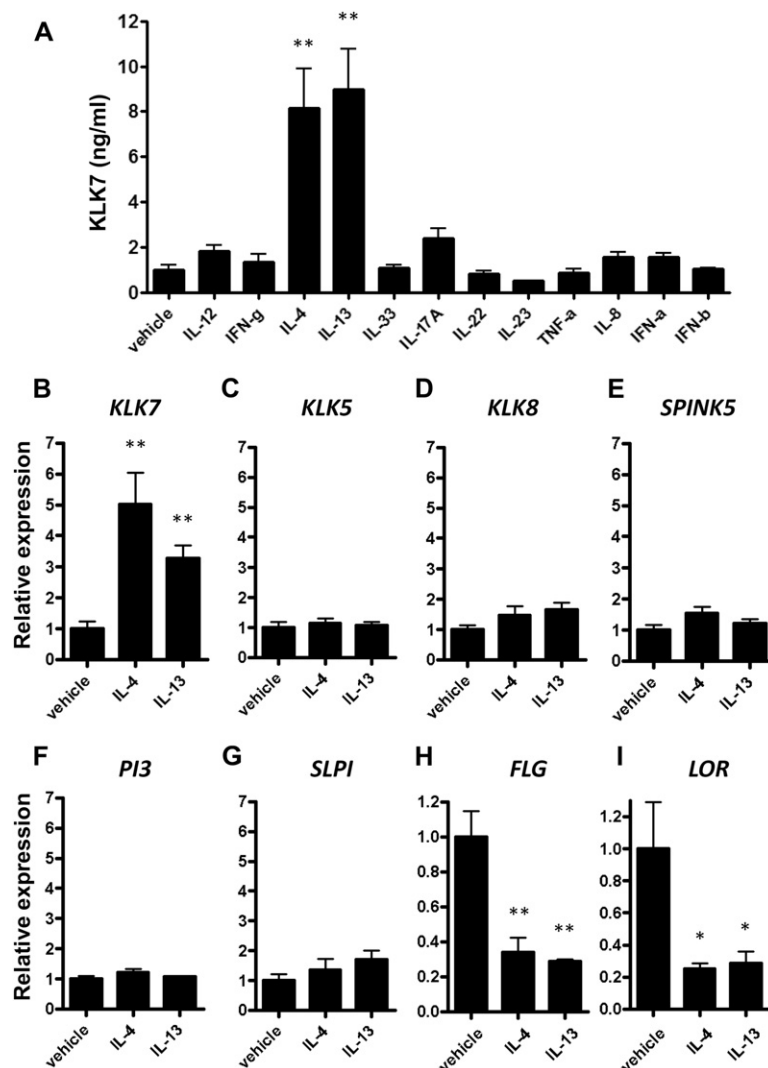


FIG 1. A, NHEKs were treated with inflammatory cytokines, and levels of KLK7 protein in the cultured media were measured by using ELISAs. B-I, NHEKs were stimulated with IL-4 (50 ng/mL) or IL-13 (50 ng/mL), and the relative mRNA abundance of KLK7 (*KLK7*), KLK5 (*KLK5*), KLK8 (*KLK8*), LEKTI (*SPINK5*), elafin (*PI3*), secretory leukocyte protease inhibitor (*SLPI*), filaggrin (*FLG*), and loricrin (*LOR*) was analyzed by using real-time PCR. * $P < .05$ and ** $P < .01$. Data are means $\pm$ SEMs of triplicate samples and representative of 3 independent experiments.

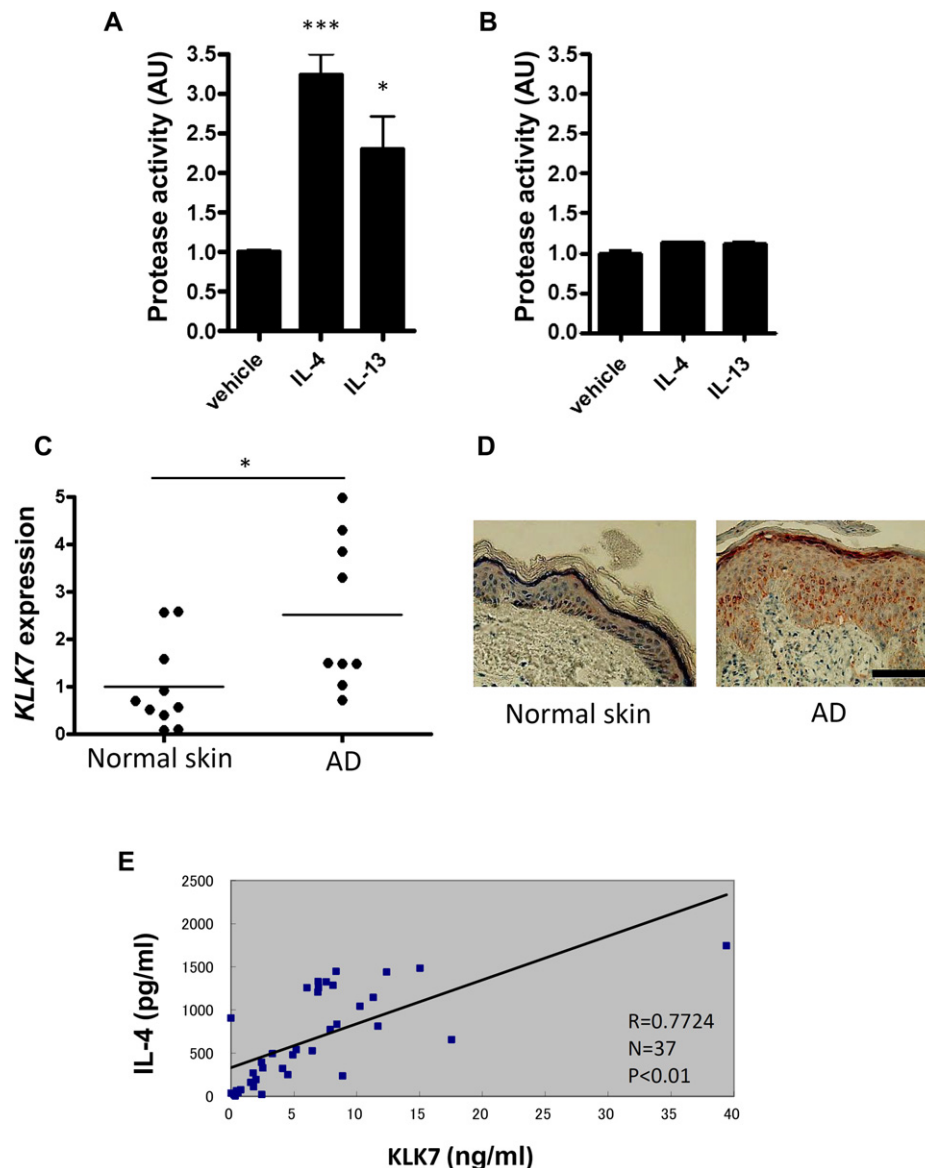


FIG 2. **A** and **B**, NHEKs were stimulated with IL-4 (50 ng/mL) or IL-13 (50 ng/mL), and chymotrypsin-like (Fig 2, **A**) or trypsin-like (Fig 2, **B**) serine protease activity was measured by using the specific substrates. * $P < .05$ and *** $P < .001$. Data are means $\pm$ SEMs of triplicate samples and representative of 3 independent experiments. **C**, KLK7 mRNA expression was analyzed by using real-time PCR and normalized with the mean of normal skin. * $P < .05$. **D**, KLK7 protein expression was examined by using immunohistochemistry. Bar = 100 μ m. **E**, KLK7 and IL-4 levels in the sera of patients with AD were measured by using ELISAs and the correlation was analyzed with the Spearman rank correlation coefficient.

Correspondingly, the overexpression of human KLK7 in murine epidermis results in chronic itchy dermatitis, which is similar to that seen in patients with chronic AD.³ These findings suggest that an increase in KLK7 expression is involved in AD pathogenesis, although the regulation of KLK expression in patients with AD has not been fully studied.

We sought molecular mechanisms that might explain a KLK7 increase in patients with AD and hypothesized that some inflammatory cytokines expressed in AD lesions induce KLK7 expression in keratinocytes. We stimulated normal human epidermal keratinocytes (NHEKs) with panels of inflammatory cytokines and revealed, using ELISA analysis, that the T_H2 cytokines IL-4 (50 ng/mL) and IL-13 (50 ng/mL) significantly induced KLK7

expression in the cultured media, but the T_H1 cytokines IFN- γ (100 U/mL) and IL-12 (50 ng/mL) and the T_H17 cytokines IL-17A (50 ng/mL), IL-22 (50 ng/mL), and IL-23 (50 ng/mL) did not (Fig 1, **A**, and see the Methods section in this article's Online Repository at www.jacionline.org). IL-33 (50 ng/mL), a pro- T_H2 cytokine that has been recently highlighted as a strong inducer of T_H2 cytokines in T_H2 lymphocytes,¹ did not affect KLK7 expression in NHEKs. We also confirmed that IL-4 (50 ng/mL) and IL-13 (50 ng/mL) significantly induced KLK7 mRNA expression in NHEKs (Fig 1, **B**, and see the Methods section in this article's Online Repository). On the other hand, KLK5 and KLK8 expression was not altered by these T_H2 cytokines (Fig 1, **C** and **D**). KLK14 expression was not consistently detectable in cultured

keratinocytes (data not shown). We also examined the effect on LEKTI, elafin, and secretory leukocyte protease inhibitor, which are serine protease inhibitors expressed by keratinocytes, but their expression was not affected (Fig 1, E-G). Filaggrin and loricrin expression was significantly decreased by T_H2 cytokines, as previously reported (Fig 1, H and I).⁵ Protease assays using specific substrates were performed to examine whether levels of serine protease activities increase in parallel to protein and mRNA levels (see the Methods section in this article's Online Repository). As expected, KLK7 activity, namely chymotrypsin-like serine protease activity, was significantly enhanced by IL-4 (50 ng/mL) and IL-13 (50 ng/mL; Fig 2, A). Activities of trypsin-like serine proteases, such as KLK5, KLK8, and KLK14, were not changed (Fig 2, B). In addition to *in vitro* data, analyses using real-time PCR and immunohistochemistry showed an increase in KLK7 expression in AD lesions compared with that seen in normal skin (Fig 2, C and D, see the Methods section in this article's Online Repository). Furthermore, the KLK7 protein level in the sera of patients with AD significantly correlated with IL-4 levels in sera (Fig 2, E, and see the Methods section in this article's Online Repository).

T_H2 cytokines are generally expressed by T_H2 lymphocytes, basophils, eosinophils, and mast cells and play roles in T_H2 cell differentiation, IgE production, eosinophil recruitment, and so forth.¹ These cytokines also affect epidermal barrier functions through signal transducer and activator of transcription 6 because IL-4 and IL-13 decrease the expression of filaggrin, loricrin, and involucrin in keratinocytes.⁵ Hatano et al⁶ reported that IL-4 suppresses the expression of ceramide and cutaneous permeability barrier functions induced by TNF- α and IFN- γ and the recovery of cutaneous permeability barrier dysfunction *in vivo*.⁵ Desmoglein 3 expression is also inhibited by IL-4.⁵ IL-4 transgenic mice spontaneously have AD-like dermatitis, which supports the importance of T_H2 cytokines in AD pathogenesis.¹ We here report how T_H2 cytokines could further alter the skin barrier by showing that IL-4 and IL-13 increase KLK7 expression and function in keratinocytes. Excessive protease activity is known to induce epidermal barrier dysfunction, and thus the increase in KLK7 by IL-4 and IL-13 would count on the skin barrier disruption in patients with AD.

Interestingly, among skin KLKs, only KLK7 is a chymotrypsin-like serine protease, and others are trypsin-like serine proteases. Chymotrypsin-like serine protease KLK7 degrades the human cathelicidin antimicrobial peptide LL-37,⁷ and a decrease in LL-37 levels is documented in AD skin.⁸ Along with cathelicidin mRNA suppression by IL-4 and IL-13,⁹ the increase in KLK7 expression by IL-4 and IL-13 would further decrease the

antimicrobial skin barrier in patients with AD. Taken together with previous reports suggesting an association between KLK7 and AD, the enhancement of protease activity through increased KLK7 expression by the T_H2 cytokines IL-4 and IL-13 might be an important factor for mechanical and chemical epidermal barrier dysfunction in patients with AD.

Shin Morizane, MD, PhD^a
Kenshi Yamasaki, MD, PhD^b
Ai Kajita, MD^a
Kazuko Ikeda, MD^a
Maosheng Zhan, MD^a
Yumi Aoyama, MD, PhD^a
Richard L. Gallo, MD, PhD^c
Keiji Iwatsuki, MD, PhD^a

From ^athe Department of Dermatology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan; ^bthe Department of Dermatology, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan; and ^cthe Division of Dermatology, Department of Medicine, University of California, San Diego, and VA San Diego Healthcare System, San Diego, Calif. E-mail: zanemori@cc.okayama-u.ac.jp.

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METHODS

Human samples

This study was approved by the Ethics Committee of Okayama University. Human skin samples and sera were collected from patients with AD and from healthy volunteers at Okayama University Hospital. Informed consent was obtained for all procedures. After the injection of a local anesthetic, 3-mm punch biopsy specimens were taken from the untreated lesional skin of patients with AD and from healthy volunteers. Formalin-fixed, paraffin-embedded skin samples were cut into 4- μ m sections, and the specimens were used for immunohistochemical studies or RNA extraction by using the FFPE RNA Purification Kit (Norgen Biotek Corp, Thorold, Ontario, Canada). RNA and sera were stored at -80°C until use.

Cell culture and stimuli

NHEKs (catalog no. C-001-5C) were obtained from Cascade Biologics/Invitrogen (Portland, Ore), and grown in serum-free EpiLife cell culture media (Cascade Biologics/Invitrogen) containing 0.06 mmol/L Ca^{2+} and $1\times$ EpiLife Defined Growth Supplement (EDGS, Cascade Biologics/Invitrogen) at 37°C under standard tissue culture conditions. Cultures were maintained for up to 8 passages in this media with the addition of 100 U/mL penicillin and 50 $\mu\text{g}/\text{mL}$ streptomycin. Cells were stimulated with IL-4 (50 ng/mL; R&D Systems, Minneapolis, Minn), IL-8 (20 ng/mL, R&D Systems), IL-12 (50 ng/mL, R&D Systems), IL-13 (50 ng/mL, R&D Systems), IL-17A (50 ng/mL, R&D Systems), IL-22 (50 ng/mL, R&D Systems), IL-23 (50 ng/mL, R&D Systems), IL-33 (50 ng/mL, R&D systems), TNF- α (5 ng/mL; Chemicon, Temecula, Calif), IFN- α (200 U/mL, Chemicon), IFN- β (200 U/mL; Roche, Indianapolis, Ind), and IFN- γ (100 U/mL; Sigma-Aldrich, St Louis, Mo) in 24-well flat-bottom plates (Corning Incorporated Life Sciences, Lowell, Mass) for up to 96 hours. After cell stimulation, total cell media was stored at -20°C until analysis. RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, Calif) after supernatant collection. RNA was stored at -80°C until use.

Quantitative real-time PCR

cDNA was synthesized from RNA by using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, Calif), as described by the manufacturer's protocol. TaqMan Gene Expression Assays (Applied Biosystems, Foster City, Calif) were used to analyze the expression of human *KLK5* (assay ID: Hs00202752_m1), *KLK7* (assay ID: Hs00192503_m1), *KLK8* (assay ID: Hs01012737_m1), *KLK14* (assay ID: Hs00222788_m1), *LEKTI* (*SPINK5*; assay ID: Hs00199260_m1), *elafin* (*PI3*; assay ID: Hs00160066_m1), *secretory leukocyte peptidase inhibitor* (*SLPI*; assay ID: Hs00268204_m1), *filaggrin* (*FLG*; assay ID: Hs00863478_g1), and *loricrin* (*LOR*; assay ID: Hs01894962_s1), as described by the manufacturer's instruction (User Bulletin #2, Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) or *18S* ribosomal RNA was used as an internal control to validate RNA for each cultured keratinocyte sample or skin sample, respectively. *GAPDH* mRNA was detected by using the probe VIC-CATCCATGACAAC

TTTGGA-MGB and the primers 5'-CTTAGCACCCCTGGCCAAG-3' and 5'-TGGTCATGAGTCCTTCCACG-3'. *18S* ribosomal RNA was detected by using the primers 5'-CGGAGGTTCTGAAGACGATCA-3' and 5'-CATCGTTTATGGTCGGAACCTACG-3' with SYBER Green. Each mRNA expression was calculated as the relative expression to *GAPDH* mRNA (cultured cell samples) or *18S* ribosomal RNA (skin samples), and all data are presented as the fold change against each control (mean of nonstimulated cells or mean of normal skin).

ELISA

KLK7 protein in NHEK-cultured media was measured by using ELISA. Ninety-six-well EIA plates (Corning) were coated with murine anti-KLK7 mAb (1 $\mu\text{g}/\text{mL}$ in PBS, catalog no. MAB2624, R&D Systems) at 20°C to 25°C overnight. After washing with PBS containing 0.05% Tween 20 (washing buffer), the wells were blocked with PBS containing 1% BSA for 1 hour at 20°C to 25°C . After incubation with the cultured media or recombinant KLK7 (R&D Systems) as standards overnight, biotinylated goat anti-KLK7 antibody (0.1 $\mu\text{g}/\text{mL}$, catalog no. BAF2624, R&D Systems) was used as detection antibody. Streptavidin-conjugated horseradish peroxidase (R&D Systems) and 3,3',5,5'-tetramethylbenzidine substrate (BD Biosciences, San Jose, Calif) were used for colorimetric quantification, and reactions were stopped with 0.2 mol/L sulfuric acid (Sigma-Aldrich). The absorbance at 450 nm was monitored with a SpectraMax Plus384 (Molecular Devices, Sunnyvale, Calif), and concentrations in the samples were calibrated from the standard curve of recombinant KLK7 by using SoftMax Pro4.6 (Molecular Devices).

Protease assay

One hundred milliliters of the cultured media was mixed with 100 mL of a specific substrate for chymotrypsin-like serine protease (Suc-Ala-Ala-Phe-AMC; Bachem, Bubendorf, Switzerland) or trypsin-like serine protease (Boc-Val-Pro-Arg-AMC, Bachem) in 10 mmol/L Tris-HCl, pH 7.8, and incubated at 37°C for 24 hours. Protease activity was monitored as increased fluorescence with the FlexStation 3 (Molecular Devices).

Immunohistochemistry

Formalin-fixed, paraffin-embedded skin samples were cut into 4-mm sections and examined with mAbs to KLK7. Histochemical visualization was carried out with an LSAB 2 kit (Dako, Carpinteria, Calif), according to the manufacturer's instructions.

Statistical analysis

The Student *t* test was used for statistical analysis, and a *P* value of less than .05 was considered significant. A correlation between KLK7 and IL-4 levels in the sera of patients with AD was analyzed with the Spearman rank correlation coefficient.