

Title: TNF- α and IL-17A induce the expression of lympho-epithelial Kazal-type inhibitor in epidermal keratinocytes

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Funding Sources: This work was supported by a Grant-in-Aid for Scientific Research(C)(no.26461658) and a grant from the Japanese Dermatological Association (Shiseido Award).

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Conflicts of Interest: None declared.

Abstract: 245 words

Manuscript: 2,394 words

Tables: 0

Figures: 4

Abstracts

Background: Serine proteases have important roles in skin barrier function and desquamation, and the aberrant expression or the dysfunction of serine proteases is associated with the pathogenesis of skin diseases. Serine protease activities are tightly regulated by serine proteases such as kallikrein-related peptidases (KLKs) and serine protease inhibitors such as lympho-epithelial Kazal-type related inhibitor (LEKTI). For a better understating of diseases' pathogenesis, the regulation mechanism of serine proteases and the inhibitors' expression in epidermal keratinocytes must be clarified.

Objectives: To investigate the effects of the cytokines on the expression of LEKTI in epidermal keratinocytes.

Methods: Normal human epidermal keratinocytes (NHEKs) were stimulated with panels of inflammatory cytokines. The expression of serine protease inhibitors was analyzed using quantitative real-time PCR and ELISA. LEKTI expression in normal human skin and lesions from psoriasis or atopic dermatitis (AD) were analyzed by immunohistochemically and tape-stripping. Trypsin- and chymotrypsin-like serine protease activities in culture supernatants were measured by using specific substrates.

Results: TNF- α and IL-17A significantly induced the expression of LEKTI in NHEKs. The immunohistochemical and tape-stripping analysis revealed that psoriatic skin lesions had higher LEKTI expression compared to normal skin and AD lesions. Trypsin- and chymotrypsin-like protease activities in the culture media were upregulated 3-5 days later but attenuated 6-7 days later period by these cytokines.

Conclusions: In epidermal keratinocytes, the Th1&Th17 cytokines TNF- α and IL-17A induce the expression of serine protease inhibitor LEKTI, and it might occur to suppress

the increase in the serine protease activities under inflammation.

Keywords: lympho-epithelial Kazal-type inhibitor, serine protease inhibitor, TNF- α , IL-17A, epidermal keratinocyte

1. Introduction

Serine proteases have important roles in the skin barrier function and desquamation. Kallikrein-related peptidases (KLKs) are 15 trypsin- or chymotrypsin-like serine proteases, and in the skin, KLK5 and KLK7 in particular have a prominent role in desquamation. [1] These two KLKs are produced mainly by keratinocytes of the stratum granulosum, and they degrade the corneodesmosome proteins such as desmoglein 1, desmocollin 1, and corneodesmosin. [2] The aberrant expression or the dysfunction of serine proteases is associated with the pathogenesis of skin diseases such as atopic dermatitis (AD), psoriasis, Netherton syndrome, and rosacea. [1, 3-8] Hasson et al. reported that transgenic mice overexpressing human KLK7 developed chronic itchy dermatitis. [9] Furio et al. also showed that transgenic mice that express human KLK5 display severe skin inflammation and allergy with pruritus. [10]

The serine protease activities are tightly regulated by serine proteases such as KLKs and serine protease inhibitors including lympho-epithelial Kazal-type related inhibitor (LEKTI) encoded by *SPINK5*, secretory leukocyte peptidase inhibitor (SLPI), and elafin encoded by *PI3*. [1, 2] LEKTI is a major serine protease inhibitor in the skin; it is expressed in the differentiated epidermal layer. [1, 11] LEKTI has 15 Kazal-type domains (D1 to D15), and each of them functions as the protease inhibitor. [3, 12] Furthermore, the fragments of LEKTI processing, including D6D9, D7D9, D8D9, D10D15, and D10D13, are also bioactive. [3] The nonsense mutations of *SPINK5* lead to Netherton syndrome that is characterized by ichthyosis, hair abnormalities ('bamboo hair'), and atopic manifestations. [3, 6] *Spink5*-deficient mice replicate Netherton syndrome and demonstrate LEKTI as a key regulator of epidermal protease activity. [7]

In addition, a single nucleotide polymorphism (SNP) pK420E has been reported to change the serine protease inhibitor activity and to be associated with AD. [13]

For a better understanding of the skin diseases' pathogenesis, the regulation mechanism of LEKTI expression in epidermal keratinocytes must be clarified. Previously our group reported that calcium but not 1,25-dihydroxyvitamin D3 or retinoic acid induces the expression of LEKTI in the cell, and that Toll-like receptor signaling induces the expression. [14, 15] However, the effect of inflammatory cytokines expressed in skin diseases' lesions on LEKTI expression has not been evaluated. Here we report that the Th1 cytokine TNF- α and the Th17 cytokine IL-17A induce the expression of LEKTI in epidermal keratinocytes.

2. Materials and methods

2.1. Human samples

This study was approved by the Ethics Committee of Okayama University (No. 1611-002). Skin samples were collected with written informed consent from patients with atopic dermatitis (AD) or psoriasis and from normal healthy volunteers at Okayama University Hospital. After the injection of local anesthesia, 3-mm punch biopsies were collected from the untreated lesional skin of the individuals with AD or psoriasis and from normal healthy volunteers. Formalin-fixed, paraffin-embedded skin samples were cut into 4- μ m sections, and the specimens were used for immunohistochemical studies. The scale samples were obtained from the forearm of normal healthy volunteers and acute skin lesions of trunk, limbs or neck of AD or psoriasis. We collect the scale samples using Nichiban tape (LP-24, Nichiban, Tokyo, Japan).

2.2. Tape-stripping protein extraction

The scale samples on the tapes were immediately stored at -20°C. The tapes were dipped into 10mL of toluene and shook strongly. After the insoluble tapes were removed, the samples were centrifuged at 1,700g for 15 min, 20°C. We aspirated the toluene leaving scale pellets and washed them with 5mL of toluene five times in order to remove any residual adhesive. After the last wash, the pellets were suspended in 1mL toluene and centrifuged at 1,700g for 15 min, 20°C. After decanting the toluene, and the pellets were dried up and then dissolved in RIPA buffer (Thermo scientific, Rockford,

IL, USA) with Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Indianapolis, IN, USA). The pellets were treated with Ultrasonic Homogenizer VP-050(TAITEC, Saitama, Japan) 20% power for 20 sec, 4°C. The samples were centrifuged at 1,700g for 15min at 4°C. Total protein concentration of the samples was measured with Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA, USA) and standardized by density. [16]

2.3. Cell culture and stimuli

Normal human epidermal keratinocytes (NHEKs) were obtained from Cascade Biologics/Invitrogen (Portland, OR, U.S.A.) and grown in serum-free EpiLife cell culture media (Cascade Biologics/Invitrogen) containing 0.06 mM Ca²⁺ and 1× EpiLife Defined Growth Supplement (EDGS, Cascade Biologics/Invitrogen) at 37 °C under standard tissue culture conditions. Cultures were maintained for up to seven passages in this media with the addition of 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. NHEKs were grown in 24-well flat bottom plates (Corning Incorporated, Corning, NY, U.S.A). Upon reaching 30-80% confluence, NHEKs were stimulated for up to 168h. Cells were stimulated with IL-4 (50 ng/ml; R&D Systems, Minneapolis, MN, U.S.A.), IL-13 (50 ng/ml; R&D systems), IL-17A (10-100 ng/ml; R & D systems), IL-22 (50 ng/ml; R&D Systems), IL-33 (50 ng/ml; R&D Systems), TNF- α (10-100 ng/ml; eBioscience, San Diego, CA, U.S.A.), IFN- γ (50 ng/ml, Sigma-Aldrich, St Louis, MO, U.S.A.), and phosphate-buffered saline (PBS) as vehicle in 24-well flat-bottom plates (Corning, Lowell, MA, U.S.A.) for up to 168 hr. After cell stimulation, the total cell media were stored at -20 °C until analysis. RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) after supernatant

collection. The RNA was stored at -80°C until use.

2.4. Quantitative real-time PCR

Complementary DNA (cDNA) was synthesized from RNA using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA, U.S.A.) as described by the manufacturer. TaqMan gene Expression Assays (Applied Biosystems ABI, Foster City, CA, U.S.A.) were used to analyze the expression of human *SPINK5* (assay ID: Hs00199260_m1), *SLPI* (assay ID: Hs00268204_m1), *PI3* (assay ID: Hs00160066_m1), *KLK5* (assay ID: Hs00202752_m1), *KLK6* (assay ID: Hs00160519_m1), *KLK7* (assay ID: Hs00192503_m1), *KLK8* (assay ID: Hs01012737_m1), *KLK10* (assay ID: Hs00173611_m1), *KLK11* (assay ID: Hs01100849_m1), *KLK13* (assay ID: Hs01087307_m1), and *KLK14* (assay ID: Hs00222788_m1) as described by the manufacturer (User Bulletin #2, Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control to validate the RNA for each cultured keratinocyte sample. *GAPDH* mRNA was detected using the VIC-CATCCATGACAACCTTTGGTA-MGB probe with the primers 5'-CTTAGCACCCCTGGCCAAG-3' and 5'-TGGTCATGAGTCCTTCCACG-3'. Each mRNA expression was calculated as the expression relative to *GAPDH* mRNA, and all data are presented as the fold-change in comparison with each control (the mean value of the non-stimulated cells).

2.5. ELISA

The LEKTI protein levels in the culture supernatants and tape-stripping standardized

samples were measured with a commercial sandwich ELISA, according to the manufacturer's protocol (Cloud-Clone, Houston, TX, U.S.A.) Absorbance at 450 nm was determined using a microplate reader (SH-1000Lab, Corona Electric, Hitachinaka, Japan).

2.6. Protease assay

Protease activity in cultured media was measured by protease assay with the 4-Methylcoumaryl-7-amide (MCA) as fluorescent substrates. When specific enzymes cleave the bond between the peptide moiety and MCA, fluorescent 7-amino-4-methylcoumarin (AMC) is released. We selected the specific substrates, Boc-Phe-Ser-Arg-MCA for trypsin-like protease, or Phe-MCA for chymotrypsin-like protease (3148-v or 3107-v, respectively, Peptide Institute, Osaka, Japan). One hundred μ l of the cultured media was mixed with 100 μ l of each substrate in 10 mM Tris-HCl buffer, pH 7.8, into a 96-well black plate (Corning) and incubated at 37 °C for 24 hr. Protease activity was monitored as increased fluorescence (excitation/emission = 380/460nm) with a FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, U.S.A.) at the Central Research Laboratory, Okayama University Medical School.

2.7. Immunohistochemistry

Formalin-fixed, paraffin-embedded skin samples were cut into 4- μ m sections and mounted on glass slides. The slides were deparaffinized and incubated with hydrogen peroxide for 5 min and blocked with 10% goat-serum in PBS for 10 min at room temperature. The sections were then incubated with the rabbit polyclonal anti-human

SPINK5 antibody (NBP1-90509, Novus Bio-Techne, Littleton, CO, U.S.A.) at 4 °C overnight. The slides were incubated with biotinylated anti-rabbit IgG polyclonal antibody and then streptavidin-HRP (K0675, Dako, Tokyo). Histochemical visualization was carried out with 3-Amino-9-ethylcarbazole (AEC) (Dako).

2.8. Statistical analysis

Results are expressed as the mean±standard error of the mean (SEM). Student's t-test was used to determine the significance of differences between the two groups. A one-way analysis of variance (ANOVA) with Tukey's test was used to determine the significance of differences among more than two groups. The analyses were performed using GraphPad Prism 4 (GraphPad Software, La Jolla, CA, U.S.A.). A p-value <0.05 was considered significant.

3. Results

Here we analyzed the effect of inflammatory cytokines on LEKTI expression in normal human epidermal keratinocytes (NHEKs). We performed an ELISA, which revealed that the Th1 cytokine TNF- α and the Th17 cytokine IL-17A significantly increased LEKTI protein in culture media of NHEKs (Fig. 1A). A real-time quantitative PCR analysis showed that the expression of the transcript, *SPINK5*, was also upregulated in parallel to protein in NHEKs (Fig. 1B). In addition, the induction of LEKTI by TNF- α and IL-17A was dose- and time-dependent (Fig. 1C-F). The mRNA expressions of other serine protease inhibitors, *SLPI* and *PI3*, were also significantly increased by TNF- α and IL-17A (Fig. 1G and H). On the other hand, the Th2 cytokines IL-4 and IL-13 significantly suppressed the expression of *SLPI* and *PI3* in NHEKs (Fig. 1G and H).

To investigate the clinical relevance of these *in vitro* findings, we analyzed the LEKTI expression in normal human skin and lesions from psoriasis or AD. We evaluated immunostaining results by scoring the degrees of stain color and pattern. Since the granular layer is always strongly positive for LEKTI staining, we scored the epidermis excluding this layer. The immunohistochemical analysis and LEKTI ELISA using the scale samples revealed that keratinocytes from psoriasis skin lesions had significantly higher LEKTI expression compared to normal skin and AD lesions (Fig. 2A and B). There was no significant difference in LEKTI expression between the normal skin and AD lesions.

Furthermore, we examined the functional relevance of our results, i.e., the trypsin- and chymotrypsin-like serine protease activities, in NHEKs stimulated with TNF- α and

IL-17A. Both the trypsin- and chymotrypsin-like serine protease activities in the cells were increased at 72, 96, and 120 hr after the TNF- α and IL-17A stimulation (Fig. 3). However, the increases were attenuated at 144 and 168 hr after the stimulation. Since the serine protease activities were upregulated by TNF- α and IL-17A, we also examined the expressions of KLK5, 6, 7, 8, 10, 11, 13, and 14 in NHEKs. [16] TNF- α and IL-17A significantly increased the expression of KLK6, 10, 11, and 13 in NHEKs (Fig. 4). KLK14 expression was not consistently detectable in our keratinocyte culture system, as previously reported. [17]

4. Discussion

KLK5 and KLK7 are known as the major serine proteases in the skin. Previously we reported that Th2 but not Th1 or Th17 cytokines induce the expression of KLK7 in epidermal keratinocytes. [17, 18] Here we demonstrated that the Th1 cytokine TNF- α and the Th17 cytokine IL-17A induce the expression of LEKTI, the major inhibitor of KLK5 and KLK7, in epidermal keratinocytes. *SPINK5* mRNA expression was only induced 1.25 or 1.5-fold by these cytokines. TNF- α and IL-17A might increase mainly LEKTI release dependent on calcium flux rather than the transcription. [19]

The Th1 cytokine IFN- γ and the Th17 cytokine IL-22 did not induce LEKTI (*SPINK5*), SLPI, and elafin (*PI3*) expression (Fig. 1A, B, G and H). Both TNF- α and IL-17A signaling leads to NF- κ B and AP-1 activation but IFN- γ and IL-22 do not. We hypothesized that the induction of LEKTI is dependent on these transcription factors, and performed siRNA experiments by using NF- κ Bp65 siRNA and c-Jun siRNA. The knockdown of NF- κ Bp65 or c-Jun did not significantly suppress the induction of LEKTI, SLPI, or elafin by TNF- α and IL-17A in NHEKs (data not shown). These results suggest that some NF- κ B/AP-1-independent mechanisms are involved in the induction of the serine protease inhibitors by TNF- α and IL-17A in epidermal keratinocytes. This is the future direction to be investigated.

Th1 and Th17 cytokines are thought to play critical roles in the pathogenesis of psoriasis, whereas AD is a Th2 disease characterized by the predominance of the Th2 cytokines IL-4 and IL-13. [11, 20] In previous studies, LEKTI expression was reported to be increased in upper epidermis of psoriatic lesions. [21, 22] The expression in AD lesions were controversial. [21-24] In addition, we recently reported that the expression

of LEKTI was up-regulated in lesions of varicella, pyoderma, and rosacea. [14] Our present analyses showed that keratinocytes from psoriasis but not AD skin lesions had significantly higher LEKTI expression compared to normal skin. Granular layers always highly express LEKTI. However, psoriatic lesions do not have granular layers, but the psoriatic basal and spinous layers visibly express LEKTI in the lesions but not in normal skins. We needed to evaluate the increase in LEKTI expression in skin diseases fairly. Therefore, we excluded granular layers in order to correctly evaluate spinous and basal layers in this study. Our *in vitro* results might explain why LEKTI expression in AD patients is not increased like that in psoriasis patients.

On the other hand, TNF- α and IL-17A significantly enhanced trypsin- and chymotrypsin-like protease activities in the culture supernatants of epidermal keratinocytes 72–120 hr post-stimulation, but the enhancement gradually disappeared by 144–168 hr. Serine protease activities are controlled by the balance between serine proteases and the inhibitors. These cytokines induced the expression of KLK6, KLK10, KLK11, and KLK13 as well as the inhibitors in NHEKs. The expression of KLK5, KLK7, and KLK8 were not changed by the stimulation in the cells (data not shown). The increases in trypsin-like serine protease activity after the stimulation might be due to the enhancement of transcription of KLKs. On the other hand, TNF- α and IL-17A also might activate some chymotrypsin-like serine proteases rather than KLK7 in NHEKs. The reduction at 144 and 168 hr after the stimulation might be due to the induction of LEKTI, SLPI, and elafin expression.

The lesional skin of AD and psoriasis has various degrees of scales. The epidermis might upregulate the serine protease activities under inflammation based on the

necessity of removing scales including pathogens and allergens, and it might also stop the activities through the increase of serine protease inhibitors after inflammation. Also, biologics including anti-TNF- α and anti-IL-17 antibodies improves psoriasis lesions quite effectively by suppressing inflammation that is induced by those cytokines. These agents might improve the dyskeratosis by inhibiting the LEKTI secretion. Further investigations are required to clarify which KLKs are responsible for the increase in the activities of the cells, and how the inhibitors induced by TNF- α and IL-17A are involved in dyskeratosis in psoriasis.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research(C)(no.26461658) and a grant from the Japanese Dermatological Association (Shiseido Award).

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Figure legends

Fig. 1 TNF- α and IL-17A induce the expression of LEKTI in NHEKs. NHEKs were stimulated with TNF- α , IL-4, -13, -17A, and -22, IFN- γ , IL-33 (50 ng/ml) and non-stimulated cells(vehicle) for 24hr (**A, B, G and H**). NHEKs were stimulated with TNF- α and IL-17A as 10, 50, 100 ng/ml and non-stimulated cells(vehicle) for 24hr (**C, D**). NHEKs were stimulated with TNF- α and IL-17A (50 ng/ml) for 0-72hr (**E, F**). LEKTI protein in the cultured media were measured by using ELISA (**A and C-F**). The relative *SPINK5*, *SLPI* and *PI3*(elafin) mRNA abundance against *GAPDH* were analyzed by qPCR (**B, G and H**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are given as mean \pm SEM of triplicate samples and are representative of three independent experiments.

Fig. 2 The expression of LEKTI in psoriatic lesions is upregulated compared to normal skin or AD lesions. Skin samples were stained with rabbit polyclonal antibodies to LEKTI. NS: normal skin (n=9) AD: atopic dermatitis (n=25) PS: Psoriasis (n=21) We excluded granular layers and scored as follows. 0: not stained. 1: stained weakly in the whole epidermis. 2: stained strongly in the upper epidermis. 3: stained strongly in the whole epidermis (**A**). Tape-stripping scale samples were collected from normal healthy volunteers, AD and PS patients. NS: (n=5) AD: (n=5) PS: (n=5) LEKTI protein in the scale samples were measured by using ELISA (**B**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are given as mean \pm SEM.

Fig. 3 TNF- α and IL-17A change the activities of serine protease inhibitors in

NHEKs. NHEKs were stimulated with TNF- α (50 ng/ml) and IL-17A (50 ng/ml) for 24–168hr (**A-D**). Relative trypsin- (**A, B**) or chymotrypsin- (**C, D**) like serine protease activity to that of non-stimulated cells (vehicle) in the culture media were analyzed.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are given as mean \pm SEM of triplicate samples and are representative of three independent experiments.

Fig. 4 TNF- α and IL-17A induce the expression of KLK6, 10, 11, and 13 in NHEKs.

NHEKs were stimulated with TNF- α (50 ng/ml) and IL-17A (50 ng/ml) for 24hr (**A-D**). The relative *KLK 6, 10, 11, and 13* mRNA abundance against *GAPDH* were analyzed

by qPCR. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are given as mean \pm SEM of triplicate samples and are representative of three independent experiments.