Title:

Toll-like receptor signaling induces the expression of lympho-epithelial Kazal-type inhibitor in epidermal keratinocytes

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Abstract

Background: Lympho-epithelial Kazal-type inhibitor (LEKTI) tightly controls the activities of serine proteases such as kallikrein-related peptidase (KLK) 5 and KLK7 in the epidermis. LEKTI is known to be an essential molecule for the epidermal skin barrier, as demonstrated by SPINK5 nonsense mutation, which results in Netherton syndrome. Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns or damage-associated molecular patterns and produce inflammatory cytokines, chemokines, and antimicrobial peptides. However, the effect of TLR signaling on the expression of LEKTI is not clear.

Objective: To investigate whether TLR signaling can affect expression of LEKTI in epidermal keratinocytes.

Methods: We stimulated a panel of TLR ligands and investigated the expression of LEKTI in normal human epidermal keratinocytes (NHEKs). We further measured trypsin or chymotrypsin-like serine protease activity in NHEK cultured media under stimulation with TLR3 ligand, poly (I:C). Immunostaining for LEKTI was performed using skin samples from skin infectious diseases.

Results: TLR1/2, 3, 5, and 2/6 ligands induced the expression of LEKTI in NHEKs. The

trypsin or chymotrypsin-like serine protease activity in NHEKs was up-regulated with the stimulation of poly (I:C). The gene expressions of *KLK6*, *KLK10*, *KLK11*, and *KLK13* were also increased by poly (I:C). An immunohistochemical analysis demonstrated that the expression of LEKTI was up-regulated in the lesions of varicella, pyoderma, and rosacea.

Conclusions: TLR signaling induces the expression of LEKTI in epidermal keratinocytes, which might contribute to the control of aberrant serine protease activities in inflammatory skin diseases.

Keywords

serine protease inhibitor; Toll-like receptor; lympho-epithelial Kazal-type inhibitor; epidermal keratinocytes

1. Introduction

Human kallikrein-related peptidases (KLKs) are secreted serine proteases encoded by 15 genes located on chromosome 19 [1], and KLK5 and KLK7 are known as major serine proteases in the epidermis. KLK5, a trypsin-like serine protease, cleaves the carboxyl side of arginine or lysine, and KLK7, a chymotrypsin-like serine protease,

cleaves the carboxyl side of tyrosine or phenylalanine [2]. These proteases degrade corneodesmosome proteins (such as desmoglein 1, desmocollin 1, and corneodesmosin), leading to desquamation [3]. Aberrant serine protease activities are involved in skin diseases such as Netherton syndrome, atopic dermatitis, psoriasis, and rosacea [4]. The serine protease activity in the epidermis is tightly regulated by serine protease inhibitors such as lympho-epithelial Kazal-type inhibitor (LEKTI) encoded by *SPINK5*, secretory leukocyte protease inhibitor (SLPI), and elafin encoded by *peptidase inhibitor 3* (*PI3*) [4].

LEKTI is a 120-kDa major serine protease inhibitor in the skin [5]. LEKTI has 15 Kazal-type domains (D1–D15), and D2 through D15 (D1 is the exception) have been shown to inhibit several members of the serine protease family such as KLK5 and KLK7 [6]. In addition to each domain, the fragments of LEKTI processing (including D6D9, D7D9, D8D9, D10D15, and D10D13) are also biologically active [7]. The importance of LEKTI is highlighted by Netherton syndrome, a skin disease caused by loss-of-function mutations in *SPINK5* [8]. Individuals with Netherton syndrome present with hair abnormality, ichthyosis, and atopic manifestations. The absence of LEKTI in Netherton syndrome results in unopposed activities of KLK5, KLK7, and other KLK enzymes and aberrantly increases epidermal proteolysis [7, 9]. A single nucleotide

polymorphism (SNP) in *SPINK5*, p.K420E, has been reported to alter serine protease inhibitor function (resulting in protease deregulation) and to be associated with atopic dermatitis (AD) [10].

SLPI and elafin are serine protease inhibitors functioning as antileukoproteinases inhibiting leukocyte elastase and neutrophil elastase [11]. These inhibitors are capable of targeting KLK7 [12]. The protease inhibitors SPINK9 and SPINK6 were also recently identified as KLK inhibitors and were reported to play a role in modulating the activity of KLKs in human skin [13, 14].

Toll-like receptors (TLRs) recognize both pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), and TLRs produce inflammatory cytokines, chemokines, and antimicrobial peptides [15]. Ten TLRs have been identified in humans, located at the cell surface or in the endosomal compartments [16]. Epidermal keratinocytes also express functional TLRs that contribute to the first line of defense against the body's external environment [16].TLRs also play a critical role in infectious and inflammatory diseases such as herpes zoster and rosacea [17, 18].

The regulation mechanism underlying the expression of LEKTI should be elucidated for a better understanding of skin homeostasis and the pathogenesis of skin diseases. We demonstrated that calcium induces the expression of LEKTI in epidermal keratinocytes

[19]. However, the effect of TLR signaling on LEKTI expression has not been reported, to our knowledge. In the present study, we observed that TLR signaling induces the expression of LEKTI in epidermal keratinocytes, and that the expression of LEKTI was up-regulated in lesions of varicella, pyoderma, and rosacea.

2. Materials and Methods

2.1 Cell culture and stimuli

Normal human epidermal keratinocytes (NHEKs) were obtained from Invitrogen/Cascade Biologics (Portland, OR, U.S.) and maintained in serum-free Epilife Medium containing 0.06 mM Ca^{2+}, 1× Epilife Defined Growth Supplement (Invitrogen/Cascade Biologics), 100 U/ml penicillin, and 50 μg/ml streptomycin. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C, and the medium was replaced every 2 days. Subconfluent NHEK monolayers were cultivated in 24-well plates. Cells were stimulated for 24–96 hr with poly (I:C) (0.1, 1, 5, or 10 μg/ml), Pam3CSK4 (10 μg/ml), lipopolysaccharides (LPS) (10 μg/ml), flagellin (100 ng/ml), MALP2 (100 ng/ml), or CpG (2 μM) (Invivogen, San Diego, CA).

2.2 Immunohistochemistry

This study was approved by the ethics committee of Okayama University (approval no. 1611-002). Human skin samples were collected from patients with varicella, pyoderma, and rosacea, and from normal healthy volunteers at Okayama University Hospital. Formalin-fixed, paraffin-embedded skin samples were cut into 4-μm sections. Deparaffinized sections were processed for rehydration, incubated with a peroxidase-blocking reagent for 5 min, and incubated with rabbit polyclonal anti-SPINK5 antibody (Novus Biotechne, Littleton, CO) or rabbit polyclonal anti-KLK5 antibody (LifeSpan BioSciences, Inc. Seattle, WA) at 4°C overnight. After being washed with phosphate-buffered saline, sections were incubated with secondary antibody for 30 min at 4°C, then with streptavidin-horseradish peroxidase for 10 min. Histochemical visualization was carried out with 3-amino-9-ethylcarbazole (AEC) (Dako, Carpinteria, CA).

2.3 Quantitative real-time polymerase chain reaction

Total RNA was extracted from NHEKs using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) and converted to complementary DNA using a ReverTra Ace qPCR RT Master Mix (Life Science Department, Toyobo, Osaka, Japan) according to the protocol described by the manufacturer. TaqMan® Gene Expression Assays

(Applied Biosystems, Foster City, CA) were used to analyze the expressions 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) according to the instructions from the manufacturer (the user bulletin provided by Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was detected using the probe VIC-CATCCATGACAACTTTGGTA-MGB and the primers 5'-CTTAGCACCCCTGGCCAAG-3' and 5'-TGGTCATGAGTCCTTCCACG-3', and was used as an internal control to validate RNA for each sample. The expression levels of each mRNA were calculated relative to that of GAPDH mRNA, and all data are presented as fold changes against the respective control (mean of non-stimulated cells).

2.4 Enzyme-linked immunosorbent assay

LEKTI protein in NHEK culture media was measured by a commercial sandwich enzyme-linked immunosorbent assay (ELISA) (Cloud-Clone, Houston, TX) following

the manufacturer's instructions.

SLPI and elafin proteins in the NHEK culture media were measured as follows: Ninety-six-well EIA plates (Corning, Corning, NY) were coated with mouse monoclonal anti-SLPI or elafin antibody (R&D Systems, Minneapolis, MN). Following incubation with the samples or recombinant SLPI or elafin (R&D Systems) as standards for 24 hr, biotinylated goat anti-mouse IgG antibody (R&D Systems) was used as a detection antibody. Streptavidin-conjugated horseradish peroxidase and 3,3',5,5'-tetramethylbenzidine substrate were used for colorimetric quantification, and reactions were stopped with sulfuric acid. Absorbance at 450 nm was determined using an SH-1000Lab microplate reader (Corona Electric, Hitachinaka, Japan).

2.5 Protease assay

Serine protease activities in NHEK culture media were measured using Boc-Phe-Ser-Arg-MCA as a specific substrate for trypsin-like serine protease activity [20] and MeO-Suc-Arg-Pro-Tyr-MCA for chymotrypsin-like serine protease activity [12] (Peptide Institute, Osaka, Japan). First, 100 μL of culture supernatant was mixed with 100 μL substrate diluted with Tris-HCl buffer (pH 7.8). The concentrations of substrate Boc-Phe-Ser-Arg-MCA and MeO-Suc-Arg-Pro-Tyr-MCA were 530 μg mL^{-1}

and 800 μg mL^{-1}, respectively.

After incubation for 24 hr, fluorescence was measured by a FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA) (excitation 380 nm, emission 460 nm) at the Central Research Laboratory, Okayama University Medical School.

2.6 Statistical analysis

All statistical analyses were conducted using Graphpad Prism ver. 4.03 software (GraphPad, La Jolla, CA). We performed a one-way analysis of variance (ANOVA) with Tukey's test to determine significance among more than two groups. Values of $p<0.05$ were considered significant.

3. Result

3.1 TLR ligands induce the expression of LEKTI in epidermal keratinocytes

To investigate whether TLR signaling affects the expression of LEKTI in epidermal keratinocytes, we stimulated NHEKs with a panel of TLR ligands. Of the panel of TLR ligands, Pam3CSK4 (TLR1/2), poly (I:C) (TLR3), flagellin (TLR5) and MALP2 (TLR2/6) significantly induced the mRNA expression of *SPINK5* (Fig. 1a). A corresponding increase of LEKTI protein in cultured media was confirmed by the

stimuli of the same four TLR ligands, i.e., Pam3CSK4, poly (I:C), flagellin and MALP2 (Fig. 1b). In addition, these TLR ligands similarly induced the expression of SLPI and elafin at the transcriptional and protein synthesis levels (Fig. 1c–f).

3.2 Poly (I:C) induces the expression of LEKTI in a dose-dependent manner in epidermal keratinocytes

Since poly (I:C), a TLR3 ligand, was the strongest inducer of serine protease inhibitors among the TLR ligands, we focused on the effects of poly (I:C) on these inhibitors in NHEKs. We observed that the LEKTI protein expression was up-regulated by poly (I:C) in a dose-dependent manner in NHEKs (Fig. 2a). SLPI and elafin were also induced by poly (I:C) in the same manner in the NHEKs (Fig. 2b,c).

3.3 Poly (I:C) up-regulates serine protease activities in epidermal keratinocytes

We next examined trypsin-or chymotrypsin-like serine protease activity in the NHEK cultured media. Unexpectedly, the trypsin- or chymotrypsin-like serine protease activity was significantly up-regulated with the stimulation of poly (I:C), in a dose-dependent manner (Fig. 3a,b).

3.4 Poly (I:C) increases the expression of KLK6, KLK10, KLK11, and KLK13 in epidermal keratinocytes

KLK family proteins consists of 15 members known as KLK1 to KLK15 [21]. Among all of the KLKs, KLK5, KLK6, KLK7, KLK8, KLK10, KLK11, KLK13, and KLK14 are reported to be detectable in normal human skin [21]. Since poly (I:C) significantly induced the serine protease activities in NHEKs, we next analyzed the effect of poly (I:C) on the KLKs' expression in the cells. The gene expressions of *KLK6*, *KLK10*, *KLK11*, and *KLK13* were significantly increased by poly (I:C) (Fig. 4a–d), whereas the expressions of *KLK5*, *KLK7*, and *KLK8* were not changed by poly (I:C) (Fig. 4e–g). *KLK14* expression was not consistently detectable in our keratinocyte culture system, as described [22].

3.5 LEKTI expression is up-regulated in the lesions of various skin diseases

Since the results of our in vitro experiment suggested that LEKTI might be up-regulated by TLR signaling, we next investigated the expression of LEKTI in skin infections and inflammations. LEKTI is a secretory protein that is localized mainly in the granular layer of the epidermis [23]. As expected, our immunohistochemical analysis revealed that LEKTI-positive keratinocytes were increased in the whole epidermis in the lesion

of an acute viral infectious disease, i.e., varicella, a chronic skin inflammatory disease, i.e., pyoderma, and a TLR2-activated disease, rosacea (Fig. 5a–d). Furthermore KLK5 expression were up-regulated in varicella compared to the normal skin (Fig. 5e,f).

4. Discussion

The proteolytic pathways are deregulated in multiple skin disorders that are characterized by a defective skin barrier, and the associated epidermal inflammation is an emerging concept. It is well known that aberrant serine protease activities are observed in the lesions of Netherton syndrome and AD. KLK activity also might have a core role in the pathology of multiple peeling skin diseases. For example, KLK activity is enhanced in patients with peeling skin syndrome-type B and acral peeling skin syndrome [24, 25], whereas KLK activity is reduced in patients with Harlequin ichthyosis [26]. LEKTI is the major inhibitor of KLK5 and KLK7 in the skin. To the best of our knowledge, our present findings show for the first time that TLR signaling induces the expression of LEKTI in epidermal keratinocytes.

Since we observed that the TLR3 ligand poly (I:C), a synthetic double-stranded RNA, was the strongest inducer of serine protease inhibitors among the TLR ligands studied, we focused on the effects of poly (I:C) on NHEKs. Our group already demonstrated that

a sensor of viral dsRNA (i.e., TLR3) and the catheliciden antimicrobial peptide LL-37 are increased in keratinocytes surrounding herpetic vesicles [27]. In addition, poly (I:C)-induced TLR3 signaling was reported to enhance antiviral activity in keratinocytes [28].

Poly (I:C)-mediated TLR3 activation of keratinocytes also leads to changes in the expression of some genes in keratinocytes that are associated with the epidermal structure [29]. In the present study, we observed that poly (I:C) enhanced the LEKTI expression in a dose-dependent manner in NHEKs. However, poly (I:C) also increased both trypsin- and chymotrypsin-like serine protease activities in a dose-dependent manner in the cells. Our further analysis revealed that the expressions of KLK6, KLK10, KLK11, and KLK13 but not those of KLK5, KLK7, or KLK8 were induced by poly (I:C) in the cells, which might explain the increase in the trypsin-like serine protease activities. On the other hand, TLR signaling also might activate some chymotrypsin-like serine proteases rather than KLK7 in NHEKs.

TLRs were first discovered as microbe sensors. Some evidence has shown that microbials can influence skin protease functions. Williams et al. reported that *Staphylococcus aureus* induces increased KLK expression and serine protease activity in keratinocytes [30]. Yamasaki et al. reported that *Propionibacterium acnes* affects the

serine protease expression and activity in NHEKs [18]. We showed here that LEKTI expression is increased in skin infectious or inflammatory diseases. Varicella is a primary infectious herpetic disease, and Varicella Zoster Virus is reported to up-regulate the expression of the majority of kallikrein genes [31]. We already reported TLR3 expression is up-regulated in herpes simplex lesions [27]. It is reasonable that TLR3 response against virus are activated and the expression is up-regulated in viral infection such as herpes zoster and varicella. Rosacea is an inflammatory disease of the skin, the pathogenesis of which is known to involve TLR2 and KLK5 [18], and TLR2 signaling might increase LEKTI expression in the disease [31]. Pyoderma is a relapsing infectious disease involving long-term exposure to pathogenic microbials, and TLR2 and TLR4 might be involved in the pathogenesis. Further investigation is required to clarify the upregulation mechanism of LEKTI in pyoderma.

Herpes viral infection might represent serious cases in Netherton syndrome because of the skin barrier disorder and the immunological abnormality. There is a case report of recurrent herpes viral infection in Netherton syndrome patient [32]. In addition, the patients also have the susceptibility to bacterial infections. It looks like that the aberrant increase of serine proteases leads to the susceptibility. On the other hand, in some inflammatory diseases such as atopic dermatitis, psoriasis, and rosacea, it has been

already reported that serine protease activities are increased in these diseases. The increases in serine protease inhibitors might also be important to attenuate protease activities after the inflammation. Further investigations are required to understand the TLR signaling control mechanisms for serine protease activities regulated by serine proteases and their inhibitors in keratinocytes.

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Legends

Fig 1. TLR ligands induce the expression of LEKTI in NHEKs.

NHEKs were stimulated with Pam3CSK4 (10 μg/ml), poly (I:C) (10 μg/ml), flagellin (100 ng/ml), MALP2 (100 ng/ml), LPS (10 μg/ml), or CpG (2 μM) for 24 hr. (**a-c):** The mRNA expressions of *SPINK5, SLPI,* and *PI3* were analyzed by quantitative real-time polymerase chain reaction (qPCR). (**d-f):** LEKTI, SLPI and elafin protein in the media were analyzed by ELISA. Data are the mean ±SEM of triplicate samples and are representative of three independent experiments. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

Fig 2. Poly (I:C) induces the expression of LEKTI in NHEKs.

NHEKs were stimulated with poly (I:C) (0.1, 1, 5, 10 μg/ml) for 24 hr. LEKTI, SLPI, and elafin protein in the media were analyzed by ELISA. Data are the mean ±SEM of triplicate samples and are representative of three independent experiments. ***$p<0.001$, **$p<0.01$, *$p<0.05$.

Fig 3. The effect of poly (I:C) on protease activities in NHEKs.

NHEKs were stimulated with poly (I:C) (0.1, 1, or 10 μg/ml) for 24 hr. Trypsin- and chymotrypsin-like serine protease activities in the media were measured with protease-specific substrates. Data are the mean ±SEM of triplicate samples and are representative of three independent experiments. ***p<0.001, **p<0.01, *p<0.05.

Fig 4. The effect of poly (I:C) on KLK gene expressions in NHEKs.

NHEKs were stimulated with poly (I:C) (0.1, 1, or 10 μg/ml) for 24 hr. The mRNA expressions of *KLK5, KLK6, KLK7, KLK8, KLK10, KLK11* and *KLK13* were analyzed by qPCR. Data are the mean ±SEM of triplicate samples and are representative of three independent experiments. ***p<0.001, **p<0.01, *p<0.05.

Fig 5. LEKTI and KLK5 expression in lesions of various skin diseases.

The expression of LEKTI or KLK5 was examined by immunohistochemistry using biopsy specimens from normal skin (**a,e**) and lesional skin from varicella (**b,f**), pyoderma (**c**), and rosacea (**d**). Scale bars=200 μm.