

Increase in pruritogenic kallikrein 5 in the skin of NC mice with chronic dermatitis

Tsugunobu Andoh, Kenichiro Tsujii and Yasushi Kuraishi

Department of Applied Pharmacology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan

Correspondence: Tsugunobu Andoh, Department of Applied Pharmacology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan, Tel.: +81-76-434-7511; Fax: +81-76-434-5045, e-mail: andoh@pha.u-toyama.ac.jp

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Background

In the development of atopic dermatitis, epidermal barrier dysfunction is a key event (1) and scratching of the itchy skin worsens barrier function and pruritus. Although the control of itch (and scratching) is very important for the treatment of atopic dermatitis, a detailed understanding of the mechanisms and mediators of itching in atopic dermatitis is still unclear.

Kallikrein (KLK) 5, a member of the serine protease KLK family, is the most abundant in the skin (2) and plays a role in skin desquamation (3). The expression of KLK5 is increased in the epidermis of patients with atopic dermatitis (4), and its activity increases in atopic dermatitis-like lesions (5). In addition, KLK5 activates proteinase-activated receptor (PAR2; 6,7), which is involved in itching (8,9,s1–s4). However, it is unclear whether KLK5 is an endogenous pruritogen in a mouse model of atopic-like dermatitis.

Questions addressed

The overproduction of KLK5 in transgenic KLK5 mice has been shown to cause the development of dermatitis and intense, continuous scratching, an itch-related behaviour (s5). However, it is unclear whether KLK5 itself is an itch mediator. In this study, we investigated whether KLK5 would be pruritogenic in mice and whether it would be increased in the lesional skin of mice with atopic dermatitis-like pruritus.

Experimental design

Experimental procedures are detailed in Data S1. Briefly, male NC/jic mice with chronic dermatitis and scratching (s6) and healthy NC/jic mice were used. In a part of experiments, KLK5 was intra-dermally injected into the hair-clipped rostral back of healthy mice. The activity of KLK5 was measured using the KLK substrate *N*-benzoyl-Pro-Phe-Arg-*p*-nitroanilide. Proteins of KLK5 and lympho-epithelial Kazal-type inhibitor (LEKTI) were determined by western blotting. KLK5 mRNA was determined by real-time PCR.

Results

Compared with those treated with the vehicle control, an intra-dermal injection of KLK5 (300 ng/site) elicited significantly more hind-paw scratching of the injection site in healthy NC mice, with the effect peaking during the first 10-min period (Fig. 1a). The KLK5 effect was dose-dependent in the range of 10 to 300 ng/site of KLK5 (Fig. 1b). Intra-dermal pretreatment with the PAR2 antagonist FSLRY-NH₂ (375 nmol/site) significantly inhibited KLK5-induced scratching (Fig. 1c).

The enzyme activity of KLK5 was significantly increased in the skin of NC mice with chronic dermatitis (Fig. 2a). The serine protease inhibitor nafamostat mesilate (10 mg/kg, intravenous) significantly inhibited increased KLK5 activity in the lesional skin down to the level observed in healthy NC mice (Fig. 2a). An active form of KLK5 was significantly increased in the skin of NC mice with chronic dermatitis (Figs 2b, S1). Pro-KLK5 showed a tendency to decrease in the lesional skin (Figs 2c, S1). KLK5 mRNA was detectable in the skin of healthy NC mice and markedly increased in the lesional skin (Fig. 2d). The endogenous trypsin-type serine protease inhibitor LEKTI was significantly decreased in the skin of NC mice with chronic dermatitis (Figs 2e, S2).

Conclusions

The present study demonstrated that KLK5 elicited scratching mediated by the PAR2 receptors in healthy NC mice. This *in vivo* result is consistent with the prior *in vitro* findings that KLK5 itself activates the PAR2 receptor (7), suggesting that the pruritogenic action of KLK5 may be primarily mediated by PAR2 receptors, which are expressed in keratinocytes and primary afferents (s3,s7). NC mice with chronic dermatitis exhibit spontaneous scratching mediated by the serine protease and PAR2 receptor system (s3). Taken together, KLK5 may play an important role not only in skin barrier disruption (5) but also in atopic dermatitis-related itching.

As well as spontaneous scratching (s3), serine protease inhibitor nafamostat inhibited the increased in the activity of cutaneous KLK5 in NC mice with chronic dermatitis. This suggests that increased activity of KLK5 is implicated in itching of chronic dermatitis. An active form of KLK5 and KLK5 mRNA were markedly increased in the skin of NC mice with chronic dermatitis. The increase in KLK5 may primarily contribute to the increased KLK5 activity in the lesional skin. In contrast to KLK5, pro-KLK5 showed a tendency to decrease, suggesting increased processing of pro-KLK5 to mature KLK5. Thus, it is considered that both KLK5 gene expression and processing of KLK5 are enhanced in the lesional skin of NC mice.

LEKTI, an endogenous trypsin-type serine protease inhibitor, was decreased in the skin of mice with chronic dermatitis. The inhibition of KLK5 by LEKTI is stronger than that of the other KLKs (s8). In addition to the increased production of KLK5, the decrease in LEKTI may also contribute to the increase in KLK activity in the lesional skin of NC mice. In fact, lack of LEKTI in a mouse model of Netherton syndrome induces atopic dermatitis-like lesions via the KLK5-PAR2 system (5).

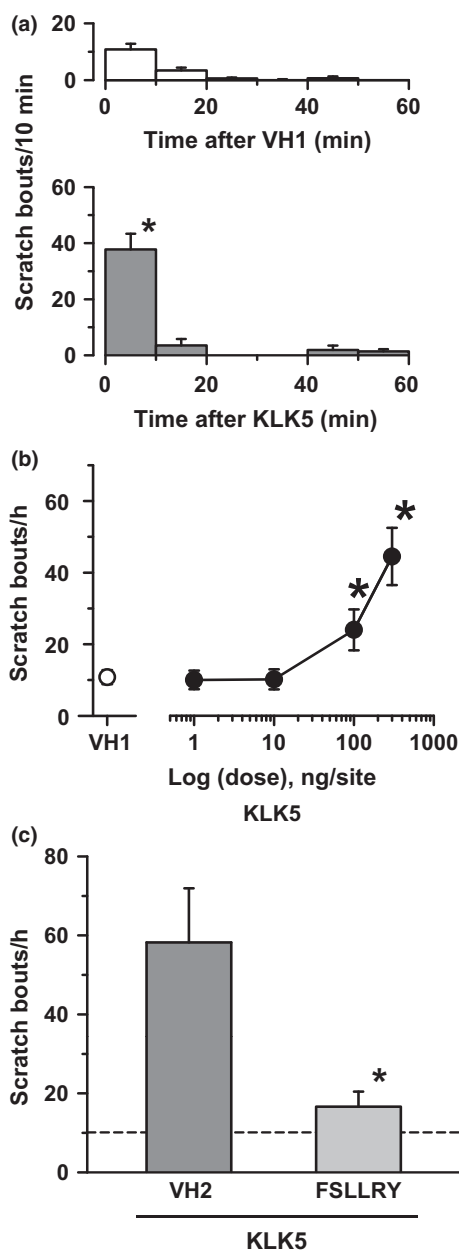


Figure 1. Intra-dermal KLK5-induced scratching and inhibition by PAR2 antagonist in healthy NC mice. KLK5 or vehicle (VH1) was injected intra-dermally, and hind-paw scratching of the injection site was counted for 1 h. (a) Time course of scratching after VH1 (upper panel, $n = 12$) and KLK5 (lower panel, 300 ng/site, $n = 8$) injections. $*P < 0.05$, compared with VH1 (Holm–Šidák test). (b) Dose–response curve for the scratch-inducing effects of KLK5 ($n = 12$ per group except 300 ng/site, $n = 8$). $*P < 0.05$, as compared with VH1 (Dunnett’s test). (c) Effect of the PAR2 antagonist FSLRY-NH₂ (FSLRY) on the KLK5-induced scratching. FSLRY (375 nmol/site, $n = 8$) or vehicle (VH2) was injected intra-dermally, and 30 min later, KLK5 (300 ng/site, $n = 8$) was injected into the same site. The dashed line represents the average of the VH1-injected group ($n = 12$). $*P < 0.05$ (Student’s *t*-test). Values represent means $\pm$ SEM.

KLK5 plays a role in not only itch (present study) but also skin desquamation (3). Serine protease inhibitors inducers may be useful for the treatment of itching and barrier dysfunction in atopic dermatitis.

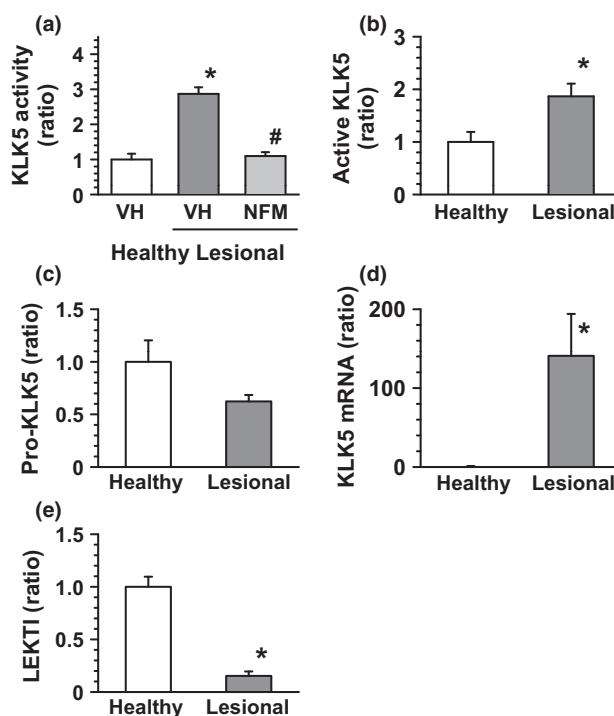


Figure 2. Increased activity and RNA expression levels of KLK5, and decreased protein expression levels of lympho-epithelial Kazal-type inhibitor (LEKTI) in the skin of NC mice with chronic dermatitis. Assays were performed in the skin samples from the rostral backs of mice. Activity and expression levels were normalized to the average level of the healthy controls. (a) KLK5 activity. Nafamostat mesilate (NFM, 10 mg/kg) and vehicle (VH) were administered intravenously 5 min before sample isolation ($n = 10$ per group). $*P < 0.001$, as compared with healthy mice treated with VH; $*P < 0.001$, as compared with the lesional group treated with VH (Holm–Šidák test). (b) Expression level of an active form of KLK5 (approx. 25 kDa) ($n = 4$ per group). $*P < 0.01$ (Student’s *t*-test). (c) Expression level of pro-KLK5 (approx. 32 kDa) ($n = 4$ per group). (d) Expression level of KLK5 mRNA ($n = 6$ per group). $*P < 0.01$ (Mann–Whitney rank sum test). (e) Expression level of LEKTI ($n = 3$ per group). $*P < 0.001$ (Student’s *t*-test). Values represent means $\pm$ SEM. The data in (b) and (c) were obtained from the same mice.

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Author contributions

TA and YK designed experiments, and TA and KT performed experiments. TA and YK wrote the manuscript.

Conflict of interests

The authors have declared no conflicting interests.

Supporting Information

Additional supporting data may be found in the supplementary information of this article.

Figure S1. Increases in an active form of KLK5 in the skin of NC mice with chronic dermatitis.

Figure S2. Decrease in lympho-epithelial Kazal-type inhibitor (LEKTI) in the skin of NC mice with chronic dermatitis.

Data S1. Experimental design.

Data S2. Supplementary references.

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Figure S1

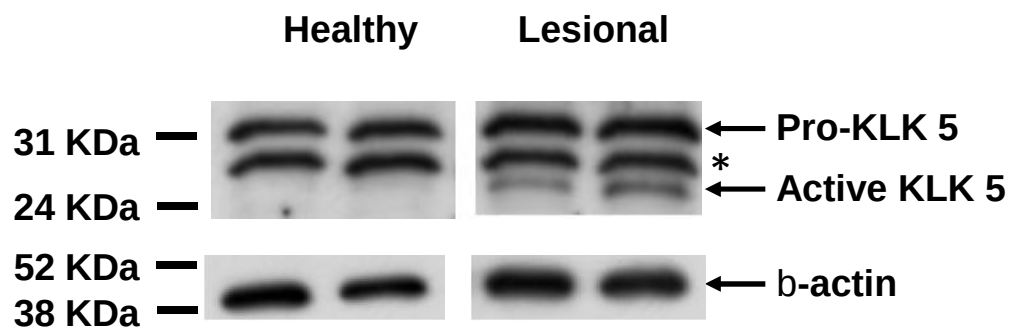


Figure S1. Increases in an active form of KLK5 in the skin of NC mice with chronic dermatitis. The photographs show typical examples of western blot bands (Pro-KLK5, aprox. 32 kDa; Active KLK5, aprox. 25 kDa). An asterisk indicates unknown bands.

Figure S2

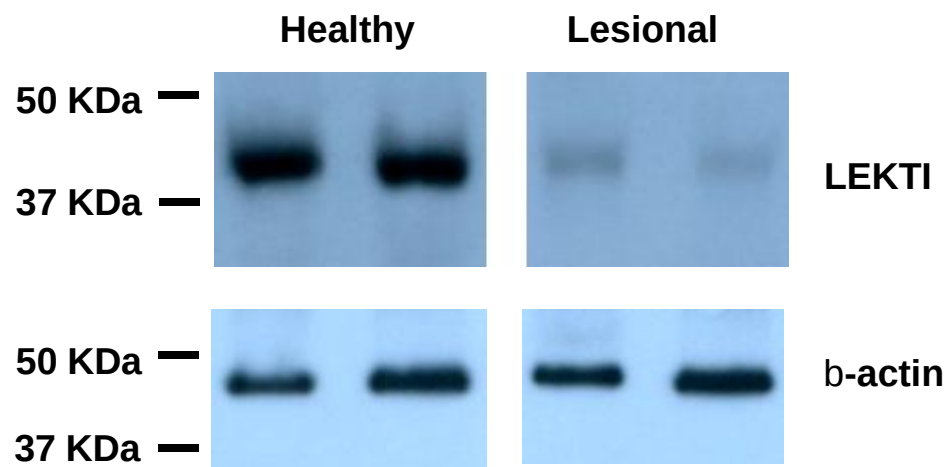


Figure S2. Decrease in lympho-epithelial Kazal-type inhibitor (LEKTI) in the skin of NC mice with chronic dermatitis. Typical example of western blot bands.

Data S1. Experimental design

Animals

Procedures in the animal experiments were approved by the Committee for Animal Experiments at the University of Toyama and were conducted in accordance with the guidelines for proper conduct of animal experiments (Science Council of Japan, 2006).

Breeding, rearing, and behavioural experiments were conducted under controlled temperature (22–24°C), humidity (55–65%), and light (lights on 07:00–19:00 h).

Animals were housed six per cage and allowed free access to food and water.

Nishiki-nezumi Cinnamon (NC)/Jic mice (1) were bred in a specific pathogen-free environment at the Division of Animal Resources and Development, Life Science Research Centre, University of Toyama. All mice, except for healthy controls were transferred to a conventional environment at 4–5 weeks of age; they were kept together with mite-infected mice with chronic dermatitis for 2 weeks to increase the incidence of chronic dermatitis (2). In the present results, we used male mice that showed chronic dermatitis and frequent spontaneous scratching at 13–25 weeks of age ($n = 34$) and as well as age-matched healthy littermates (healthy control mice, $n = 84$).

Materials

FSLLRY-NH₂ was synthesized and identified using the peptide synthesizer PSSM-8 (Shimazu Co., Kyoto, Japan) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometer Autoflex T1 (Bruker Daltonics, Billerica, MA, USA), respectively (3). Recombinant human KLK5 (R&D Systems, Minneapolis, MN, USA) and FSLLRY-NH₂, dissolved in 50 mM Tris-HCl (pH 8.0) and physiological saline, were injected intradermally into the rostral back in a volume of 50 µl. FSLLRY-NH₂ was injected 30 min before KLK5 injection. Nafamostat mesilate (Torii Pharmaceutical Co., Ltd., Tokyo, Japan) was dissolved in 5% glucose solution and was injected intravenously 5 min before excision of the skin.

Behavioural experiments

Mice were placed individually in an acrylic cage composed of four equal-sized cells (13 × 9 × 40 cm) for at least 1 h to allow them to acclimatise. To observe spontaneous scratching, they were kept in the same cells and their behaviour was videotaped for 1 h. Additionally, to observe evoked scratching, mice were returned to the same cells immediately after intradermal injection and their behaviour was videotaped for 1 h. Behavioural recording was performed with personnel kept out of the observation room.

Scratching by the hind paws were observed during video playback; hind-paw scratching of the injection site was considered to represent evoked scratching (2). Mice stretched either hind paw, leaned the head toward it, rapidly scratched themselves, and brought their toenails to their mouth; a series of these movements was counted as one bout of scratching (4).

Determination of KLK5-like serine protease activity

Animals were euthanized by transcardial perfusion with phosphate-buffered saline (PBS) under ethyl carbamate (1.5 g/kg) anaesthesia, and the skin, each 1-cm² squares of skin were excised. The skin sample was homogenized in 50 mM Tris-HCl buffer (pH 8.0) with a Polytron homogenizer (Kinematica AG, Lucerne, Switzerland). The homogenate was centrifuged at 600 ×*g* at 4°C for 5 min. The supernatant was collected and diluted to 1 µg/µl of protein with the Tris-HCl buffer. The sample solution (100 µl) was mixed with 100 µl of a solution of the KLK substrate *N*-benzoyl-Pro-Phe-Arg-*p*-nitroanilide (Bachem, Bubendorf, Switzerland), which had been dissolved in dimethylsulfoxide and diluted to 500 µg/ml with the Tris-HCl buffer. This was incubated at 37°C for 75 min (5). The amount of *p*-nitroanilide released was colorimetrically determined at 420 nm. KLK5 activity was calculated from this data.

Western blotting

Animals were euthanized and the skin was excised, as mentioned above. The skin sample was stored at -80°C until the assay. The Protein extraction, electrophoresis and membrane transfer were performed as described previously (2). After blocking a polyvinylidene difluoride membrane with 5% skim milk solution for 1 h, the membrane was reacted with rabbit anti-KLK5 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rabbit anti-LEKTI antibody (Santa Cruz Biotechnology Inc.), or mouse anti- β -actin monoclonal antibody (Sigma-Aldrich) at 4°C overnight. Subsequently, it was incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (GE Healthcare Bio-Sciences Co., Piscataway, NJ, USA) or horseradish peroxidase-conjugated anti-mouse IgG antibody (GE Healthcare Bio-Sciences Co.) for 1 h at room temperature and then treated with chemiluminescence reagents (GE Healthcare Bio-Sciences Co.). Chemiluminescent signals were detected using X-ray film and analysed with Image J (upper band: pro-KLK5 (aprox. 32 kDa), lower band: KLK5 (aprox. 25 kDa)) (6). Signal intensity was normalized to that of β -actin.

Real-time polymerase chain reaction (PCR)

Animals were euthanized, and the skin was isolated and stored, as mentioned before. Total RNA extraction, reverse transcription, and real-time polymerase chain reaction (PCR) were performed as described previously (7). The sequences of the primers used were as follows: glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*), 5'-tcagaaggactcctatgtgg-3' (sense) and 5'-tctctttgatgtcacgcacg-3' (antisense); *KLK5*, 5'-gggactaggtgcatggtgtc-3' (sense) and 5'-cgaactcacacaggttggtg-3' (antisense). The mRNA expression of *GAPDH* and *KLK5* was analysed using Mx Pro QPCR software (Agilent Technologies, Inc., Santa Clara, CA). PCR involved 40 cycles of denaturation (for 30 s at 95°C), primer annealing (for 30 s at 60°C), and elongation (for 30 s at 72°C). The relative amount of *KLK5* mRNA was first normalized to the level of *GAPDH* mRNA in each sample and then to average expression levels in vehicle controls.

Data processing

Data are presented as means $\pm$ standard error of the mean. In Fig. 1A, the data were analysed with a two-way repeated measures analysis of variance for comparing the time courses and the different treatments, followed by a post-hoc Holm–Šidák test for all pairwise comparisons. Since two sets of quantitative data were compared in Figs. 1C, 2B, and 2E, we analysed the data with the Student's *t*-test. In Fig. 2D, since the data

regarding healthy mice were minimal, we used the Mann–Whitney rank sum test. In Fig. 1B, the data were analysed with one-way analysis of variance followed by a post-hoc Dunnett’s test for comparisons between the control and each treatment group. In Fig. 2A, the data were assayed using one-way analysis of variance followed by a post-hoc Holm–Šidák test for all pairwise comparisons. *P* values less than 0.05 were considered to be statistically significant. Statistical analysis was performed using Sigmaplot graphing and statistical analysis software (version 11; Systat Software, Inc., Chicago, IL, USA). In addition, experiments were repeated at least twice more, on different days.

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