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CONCISE COMMUNICATION

Japanese Cedar (*Cryptomeria japonica*) pollen allergen induces elevation of intracellular calcium in human keratinocytes and impairs epidermal barrier function of human skin ex vivo

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Abstract Cry j1 is the major peptide allergen of Japanese cedar (Sugi), *Cryptomeria japonica*. Since some allergens disrupt epidermal permeability barrier homeostasis, we hypothesized that Cry j1 might have a similar effect. Intracellular calcium level in cultured human keratinocytes was measured with a ratiometric fluorescent probe, Fura-2 AM. Application of Cry j1 significantly increased the intracellular calcium level of keratinocytes, and this increase was inhibited by trypsin inhibitor or a protease-activated receptor 2 (PAR-2) antagonist. We found that Cry j1 itself did not show protease activity, but application of Cry j1 to cultured keratinocytes induced a rapid (within 30 s) and transient increase of protease activity in the medium. This transient increase was blocked by trypsin inhibitor or PAR-2 antagonist. The effect of Cry j1 on transepidermal water loss (TEWL) of cultured human skin was measured in the presence and absence of a trypsin inhibitor and PAR-2 antagonist. Cry j1 significantly impaired the barrier function of human skin ex vivo, and this action was blocked by co-application of trypsin inhibitor or PAR-2 antagonist. Our results suggested that interaction of Cry j1 with epidermal keratinocytes leads to the activation of PAR-2, which induces elevation of intracellular calcium and disruption of barrier function. Blocking the interaction of Cry j1 with epidermal keratinocytes might ameliorate allergic reaction and prevent disruption of epidermal permeability barrier homeostasis.

Keywords Cry j1 · Protease-activated receptor 2 · PAR-2 · Trypsin inhibitor · FSLLRY-NH_2

Introduction

Allergic symptoms caused by IgE-mediated type 1 hypersensitivity reaction to pollen allergens occur worldwide [12]. For example, approximately 25 % of the population in Japan suffers from cedar pollinosis [1]. Further, hydration of the stratum corneum is impaired in patients with seasonal allergic rhinitis [16]. Mite and cockroach allergens activate protease-activated receptor 2 (PAR-2) in epidermal keratinocytes and delay epidermal permeability barrier recovery after disruption, and PAR-2 is known to be expressed in epidermal keratinocytes [3, 9]. The cedar pollen allergen CPA63 exhibits protease activity that might directly activate PAR-2 [8]. Moreover, the airway epithelial barrier is damaged by protein extracts from several pollens [17]. In addition, we previously demonstrated that elevation of intracellular calcium level induced by calcium ion influx into epidermal keratinocytes delayed barrier recovery [4]. Based on these findings, we hypothesized that the major cedar (*Cryptomeria japonica*) pollen allergen in Japan, Cry j1, might induce elevation of intracellular calcium in keratinocytes and impair barrier function via activation of PAR-2. Cry j1 is a basic glycoprotein with pectate lyase activity, consisting 353 amino acid residues [14, 15].

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To test the above idea, we first evaluated the effect of Cry j1 on intracellular calcium level in cultured human keratinocytes. Then, we examined the effect of the allergen in a human skin tissue culture system (skin ex vivo) after barrier disruption. We also evaluated the protease activity of Cry j1 and culture medium of Cry j1-stimulated keratinocytes, and the effects of a trypsin inhibitor and a PAR-2 antagonist on the actions of Cry j1.

Methods

Cells and cell culture

Normal human epithelial keratinocytes were purchased from Kurabo (Osaka, Japan) and cultured in EPILIFE-KG2 (Kurabo, Osaka, Japan). Keratinocytes were seeded onto collagen-coated glass coverslips (Matsunami, Osaka, Japan) and used within 4 days. Keratinocytes were first grown to 100 % confluency in low-Ca^{2+} (0.06 mM) medium for 48 h and then incubated with high-Ca^{2+} (1.8 mM) medium for 24–48 h.

Materials

We purchased Cry j1 from Hayashibara Co., Ltd (Okayama, Japan), soybean trypsin inhibitor (SBTI) from Sigma-Aldrich (St. Louis, MO, USA), and PAR2 antagonist FSLLRY-NH_2 (FSY-NH_2) [13] from Tocris (Bristol, UK).

Ratiometric fluorescence measurement of intracellular calcium

Changes of intracellular calcium concentration in single cells were measured with Fura-2 AM according to the manufacturer's instructions (Molecular Probes Inc., OR, USA). Briefly, cells were loaded with 5 μM Fura-2 AM at 37 °C for 45 min. After loading, the cells were rinsed with balanced salt solution containing (in mM): NaCl 150, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1.2, HEPES 25, and D-glucose 10 (pH 7.4), abbreviated as BSS (+), and incubated for a further 10 min at room temperature to allow de-esterification of the loaded dye.

The coverslip was mounted on an inverted epifluorescence microscope (ECLIPSE Ti, Nikon, Tokyo, Japan), equipped with a 75 W xenon lamp and band-pass filters of 340 and 380 nm. Imaging was done with a high-sensitivity CCD camera (ORCA-R2, Hamamatsu Photonics, Hamamatsu, Japan) under the control of a Ca^{2+} analyzing system (AQUACOSMOS/RATIO, Hamamatsu Photonics).

Protease assay

For the assay of protease activity, we used two assay kits: Amplite™ Universal Fluorimetric Protease Activity Assay Kit *Green Fluorescence* (AAT Bioquest, Sunnyvale, CA, USA) and Protease Activity Fluorometric Assay Kit (BioVision, Milipitas, CA, USA). Cry j1 itself was evaluated at concentrations of 10, 100 ng/ml and 1 μg/ml in 500 μl of assay buffer. For evaluation of protease activity in culture medium, human keratinocytes were incubated with 500 μl of 100 ng/ml, 1 and 100 μg/ml Cry j1 in 10 cm dishes with gentle shaking for 1 min, or with Cry j1 in 5 ml EPILIFE-KG2 for 24 h, and protein in the medium was concentrated with Amicon Ultra-0.5 ml Centrifugal Filters for Protein Purification and Concentration (Merck Millipore, Darmstadt, Germany), which gave a concentration factor of 13.1–18.1 times. Further, to examine the time course of protease activity in the medium with or without SBTI (final concentration 1 μM) or FSY-NH_2 (final concentration 100 μM), we added 500 μl of the Amplite™ kit substrate (fluorescent casein conjugate, diluted 1:100) to the culture of differentiated human keratinocytes, then added 10 μl of BSS (+) solution with/without Cry j1 (100 ng/ml), and imaged the fluorescence (excitation filter of 455–485 nm and emission filter of 500–545 nm) with a high-sensitivity CCD camera (ORCA-R2, Hamamatsu Photonics, Hamamatsu, Japan) under the control of a Ca^{2+} analyzing system (AQUACOSMOS/RATIO, Hamamatsu Photonics).

Human skin tissue culture

Human tissues were purchased from Biopredic International (Rennes, France) via KAC Co., Ltd. (Kyoto, Japan). The samples had been obtained following plastic surgery, with informed consent. The excised skin was dermatomed to 340–440 μm thickness (containing epidermis and dermis), and then discs (10 mm in diameter, thickness about 2 mm) were punched out and transferred to our laboratory. Four samples of skin tissues from abdomen of healthy, independent subjects were used for the study (32, 36, 37, 39 years old, Caucasian females). This study was approved by the ethics committee of Shiseido, in accordance with the guideline of the National Institute of Health. Tissues were cultured in long-term skin culture medium (LTSC medium), provided by Biopredic International.

Transepidermal water loss

Gravimetric transepidermal water loss (TEWL) was measured as described by Hanley et al. [7]. Skin sections were placed dermis-side down onto glass-based dishes and the lateral edges and dermal surface were sealed with petrolatum, so that water loss occurred only through the

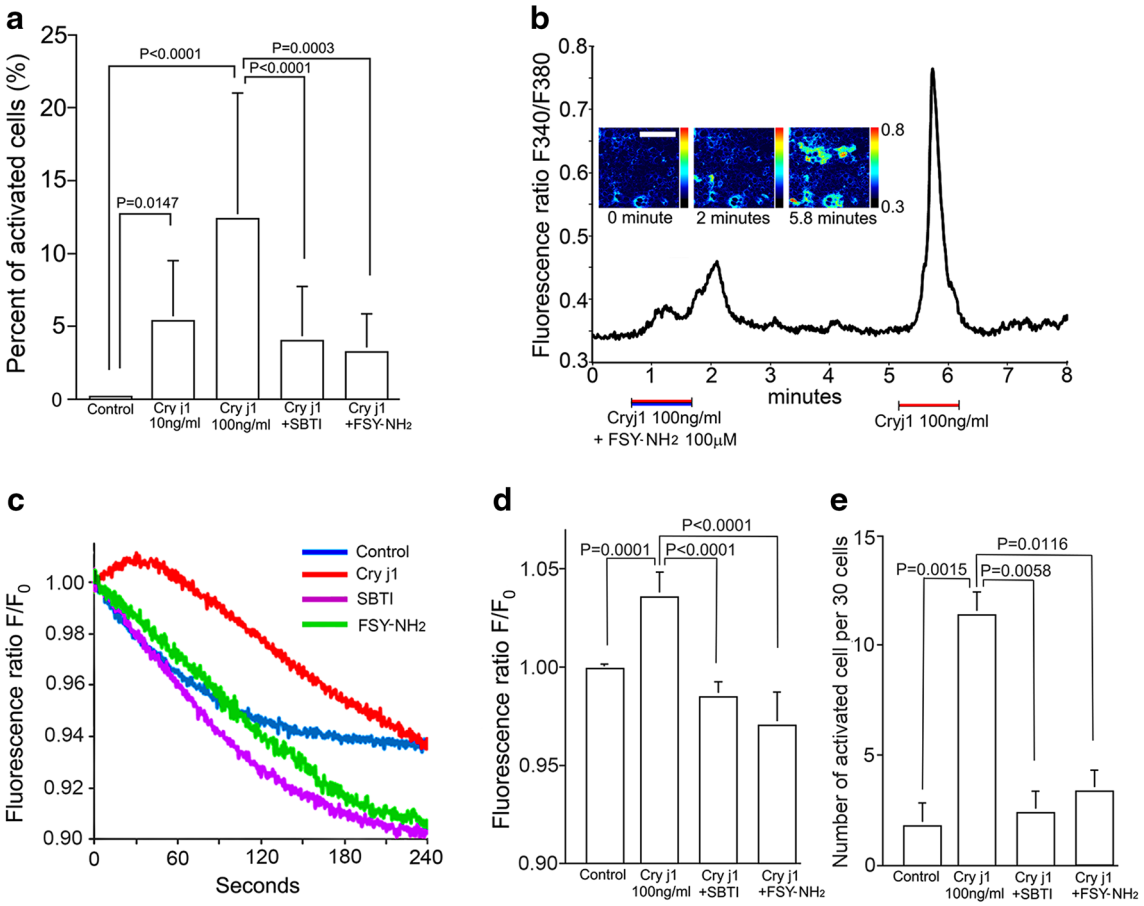

Fig. 1 Effects of Cry j1 on cultured human keratinocytes. **a** Quantitative determination of percentage of activated cells after application of Cry j1 with or without SBTI and FSY-NH_2. The intracellular calcium elevation induced by 100 ng/ml Cry J1 was significantly reduced by pre-application of 1 μM SBTI or 100 μM FSY-NH_2. *Bars* and *lines* represent mean + SD. **b** Changes of intracellular calcium (expressed as the ratio of fluorescence intensities at 340 and 380 nm). Application of 100 ng/ml Cry j1 with 100 μM FSY-NH_2 increased the level of intracellular calcium by approximately 0.1 unit, while the same amount of Cry j1 without FSY-NH_2 increased the level of intracellular calcium increased by approximately 0.4 units. Images obtained at 0 (control), 2 min (peak for Cry j1 plus FSY-NH_2), and 5.8 min (peak for Cry j1 alone) after the start of observation are also shown. *Bar* 200 μm. **c** Time course of protease activity (expressed as fluorescence ratio of Amplite^{TM} kit substrate). Application of Cry j1 to cultured human keratinocytes at time 0 s induced a rapid, transient increase of protease activity (*red line*). After application of BSS (+) only (control), the fluorescence level gradually decreased (*blue line*). The transient increase of protease activity was blocked by SBTI (*purple line*) or FSY-NH_2 (*green line*). Vertical scale is normalized by the fluorescence at time 0. Note vertical scale is different from (**b**). **d** Quantitation of fluorescence change at 30 s after application ($n = 4$). A significant difference was observed between the control and Cry j1 application groups. SBTI and FSY-NH_2 each significantly blocked the increase. **e** *Numbers* of activated cells per 30 cells showed similar differences to (**d**) ($n = 3$–5). *Bars* and *lines* represent mean + SD

epidermal surface. Before application of Cry j1 with/without other reagents, the stratum corneum was stripped 10 times with adhesive tape. Skin sections were kept at ambient temperature (37 °C) and humidity (30–35 %), and weighed every 2 h. TEWL levels are reported as milligrams of water lost per square millimeter per hour. Skin sections from four different subjects were used.

Electron-microscopic observation

Skin samples for electron microscopy minced (<0.5 mm^3 pieces) and fixed overnight in modified Karnovsky's fixative. They were then post-fixed in 2 % aqueous osmium tetroxide or 0.2 % ruthenium tetroxide as described previously [6]. After fixation, all samples were dehydrated in graded ethanol solutions, and embedded in an Epon-epoxy mixture. The area ratio of stratum corneum/stratum granulosum (SC/SG) lipid domains was quantified using osmium post-fixed material. Measurements were made without knowledge of the prior experimental treatment. These parameters were evaluated from photographs of randomly selected sections at a constant magnification, using computer software (NIH Image).

Statistics

Statistical significance of differences among three or more groups was determined by ANOVA with Fisher's protected least significant difference. $P < 0.05$ was considered significant. Student's *t* test was used to determine the significance of differences between the two groups.

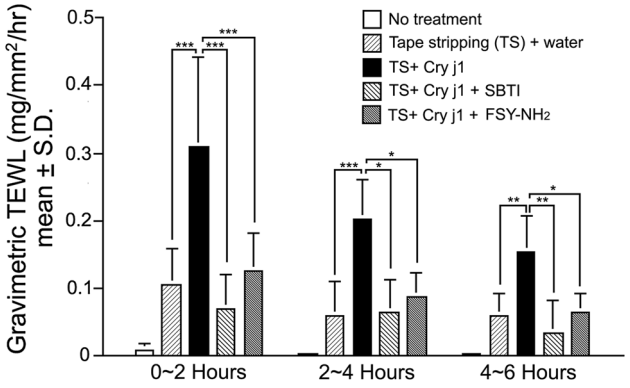

Fig. 2 Ex vivo evaluation of water-impermeable barrier function after application of Cry j1 with or without SBTI and FSY-NH_2. The *vertical axis* shows the amount of water loss during 0–2, 2–4 or 4–6 h after TS. Application of Cry j1 dramatically increased the water loss. The Cry j1-induced increase was significantly reduced by co-application of SBTI or FSY-NH_2

Results

In vitro study

Quantitative data for intracellular calcium concentration are shown in Fig. 1. The intracellular calcium concentration was significantly increased by 10 and 100 ng/ml Cry j1. The intracellular calcium elevation induced by 100 ng/ml Cry j1 was significantly reduced by pre-application of 1 μM SBTI or 100 μM of FSY-NH_2. We observed 4 pools for each treatment. Each microscope field covered approximately 200 cells (Fig. 1a).

Figure 1b shows a representative profile of the changes of intracellular calcium concentration after application of 100 ng/ml Cryj1 with or without FSY-NH_2. The parameter of the vertical scale is the ratio of the emission intensity at 340 nm to that at 380 nm. When we applied Cry j1 with FSY-NH_2, the fluorescence ratio showed a small, rather broad increase of 0.1 unit. When we applied the same

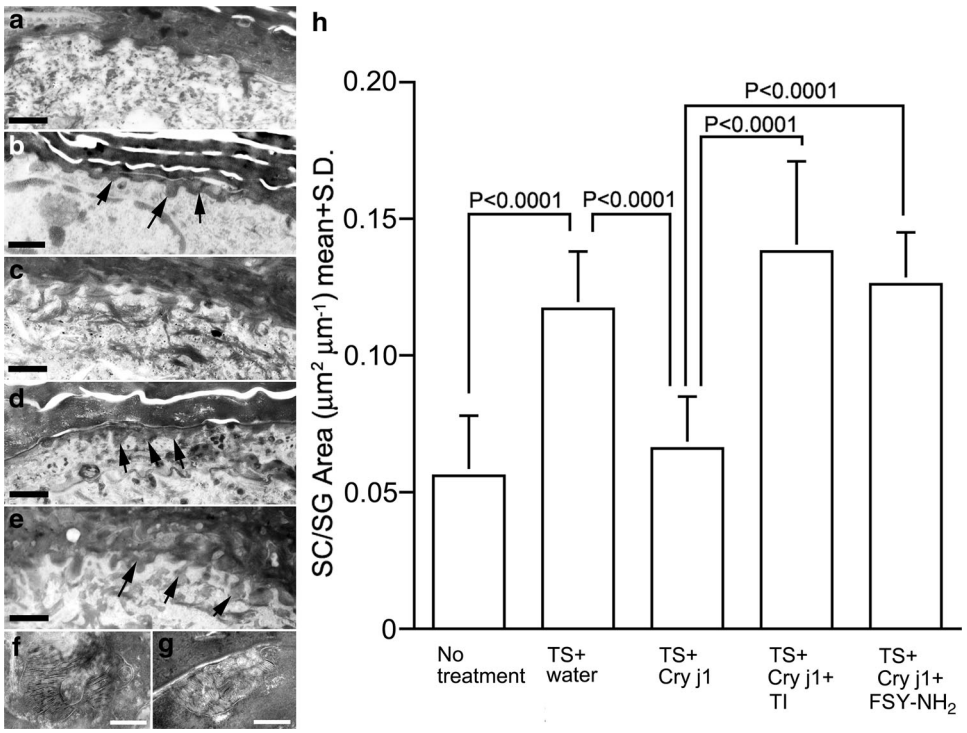

Fig. 3 Electron-microscopic observation of ex vivo skin samples. **a** No treatment. **b** Tape stripping (TS) 10 times and application of water. **c** TS and application of 100 ng/ml Cry j1. **d** TS and application of 100 ng/ml Cry j1 and 1 μM SBTI. **e** TS and application of 100 ng/ml Cry j1 and 100 μM FSY-NH_2. *Arrows* indicate secreted lipids between stratum corneum (SC) and stratum granulosum (SG). *Bars* 1 μM. **f** Magnified image of secreted lipids after TS and application of water. **g** Magnified image of secreted lipids after TS and application of Cry j1 and FSY-NH_2. *Bars* 100 nm. **h** Quantitative evaluation of the area ratio of stratum corneum/stratum granulosum (SC/SG) lipid domains. A significant increase of the intercellular lipid domain was observed after tape stripping and it was reduced by application of Cry j1. The effect of Cry j1 was blocked by co-application with SBTI or FSY-NH_2

amount of Cry j1 without FSY-NH_2, a much larger, sharp increase of 0.4 unit was observed. Images obtained at 0 min (control), 2 min (peak for Cry j1 plus FSY-NH_2), and 5.8 min (peak for Cry j1 alone) after the start of observation are also shown.

We next examined protease activity. Cry j1 itself showed no protease activity with the two different assay kits (data not shown). Culture medium from human keratinocytes incubated with Cry j1 for 1 min or for 24 h also showed no detectable activity (data not shown). However, when we examined the time course of protease activity in culture medium, we observed a rapid and transient increase of fluorescence of the Amplite^{TM} kit substrate in the presence of Cry j1, whereas the control (without Cry j1) showed a continuous gradual decrease of fluorescence due to quenching. The transient increase of the protease activity was blocked by SBTI or FSY-NH_2 (Fig. 1c, see also supplement file, Online Resource ESM_1. pdf). Quantitative comparison ($n = 4$) showed that the fluorescence ratio at 30 s after application of Cry j1 was significantly greater than that of the control. Inhibition of the transient increase of the activity by SBTI and/or FSY-NH_2 was also statistically significant in terms of both fluorescence ratio and number of activated cells (Fig. 1d, e).

Ex vivo study

The changes of TEWL in response to application of Cry j1 alone or with SBTI and FSY-NH_2 are shown in Fig. 2. Compared with the control (tape stripping + water), application of Cry j1 dramatically impaired barrier function. However, when we applied SBTI or FSY-NH_2 with Cry j1, the decrease of the barrier function by Cry j1 was almost completely blocked.

Electron-microscopic observations supported the results of the barrier study. Application of Cry j1 prevented lamellar body secretion, as compared with the tape-stripped control, while co-application of trypsin inhibitor and PAR-2 antagonist with Cry j1 normalized the lamellar body secretion (Fig. 3).

Discussion

The changes of intracellular calcium concentration measured in vitro suggested that the PAR-2 receptor was activated by Cry j1, leading to elevation of intracellular calcium. Activation of PAR-2 requires protease activity, and although Cry j1 itself showed no detectable activity, we observed a rapid (within 30 s) and transient increase of protease activity in the medium after addition of Cry j1 to the cultured keratinocytes. Assays of protease activity in the medium with two commercial kits had failed to detect this increase, because of insufficient time resolution (it took at least 4 min to measure the activity). We previously demonstrated that trypsin-type protease activity increases in the epidermis immediately after barrier disruption of human skin [5]. Thus, we speculate that a trypsin-type protease might have been induced immediately upon contact of Cry j1 with keratinocytes and then rapidly autolyzed or inactivated. This seems plausible, because if the protease remained active, barrier recovery might be extremely slow, and serious inflammation might be induced. Many endogenous serine proteases are expressed in skin [10]. We speculate that epidermal keratinocytes contain an endogenous factor(s) that regulates protease activity.

The ex vivo studies supported the idea that Cry j1 impaired epidermal barrier homeostasis by inducing activation of PAR-2. We previously demonstrated that influx of calcium into keratinocytes at the uppermost layer of the epidermis after barrier disruption prevented the lamellar body secretion, resulting in delayed barrier recovery [4]. In the present study, electron-microscopic observation confirmed that Cry j1 blocked lamellar body secretion.

An interesting possibility is that the impairment of epidermal barrier function by Cry j1 might promote entry of Cry j1 and/or other allergens into the skin, exacerbating allergic reaction. Moreover, PAR-2 activation sensitizes TRPV1 and TRPA1, which are well-known pain and itching receptors [2, 11, 18]. Thus, Cry j1 might directly induce itching or pain, which is a common feature of skin allergic reaction. Our results suggest that topical application of trypsin-type protease inhibitor or PAR-2 inhibitor might clinically effective for the treatment of Cry j1-induced allergy.

Compliance with ethical standards

Conflict of interest The authors report no conflict of interest.

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