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Establishment of an Epicutaneously Sensitized Murine Model of Shellfish Allergy and Evaluation of Skin Condition by Raman Microscopy

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Abstract: Background: Shellfish allergy is one of the most common food allergies. Recent studies have shown that sensitization to allergens via the skin is involved in the development of food allergies. In this study, a mouse model of shrimp allergy was generated by epicutaneous sensitization and used to identify skin conditions associated with susceptibility to sensitization. Methods: Four-week-old female BALB/c mice were sensitized by repeated application of 0.1 mg of tropomyosin to tape-stripped skin on days 0, 7, and 15, followed by a challenge on days 28 and 35. Results: Epicutaneously sensitized mice exhibited higher serum levels of tropomyosin-specific IgE on day 15 than control mice. After the oral challenge, model mice had higher anaphylaxis scores and lower rectal temperature. After three tape-strip treatments for sensitization, the skin was analyzed by Raman microscopy. The sensitized mice exhibited lower relative intensities of Raman bands at 399, 915, and 1073 cm^{-1} than control mice, which could be helpful noninvasive markers in screening for potential sensitization via the skin. Conclusions: An epicutaneous sensitization shellfish allergy model was generated. This model will be useful in studies to elucidate the pathogenesis of skin sensitization. Raman microscopy may also be valuable for capturing subtle skin changes leading to sensitization.

Keywords: food allergy; epicutaneous sensitization; shellfish; shrimp; tropomyosin; skin; Raman microscopy; tape-strip

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1. Introduction

Shellfish allergy is a common food allergy, especially in the Asia–Pacific region [1–3]. Allergic reactions to shellfish include gastrointestinal manifestations such as nausea, emesis, diarrhea, and abdominal cramping, as well as respiratory distress and life-threatening anaphylaxis [4,5]. Shellfish allergies tend to occur in childhood and persist throughout life [2],

and are therefore most common in adults [6]. Food allergies that develop in adulthood are more difficult to achieve clinical tolerance than those that develop in childhood; therefore, the only way to prevent allergic reactions in affected adults is to avoid consuming the allergen. Food allergy is associated with poorer quality of life for both the individual with the allergy, as well as their family.

One of the primary shellfish allergens is tropomyosin, a protein that forms thin myofibrils and constitutes 20% of the total protein in shrimp. Tropomyosin is present in many invertebrate species including other crustaceans (lobster, crab), mollusks (mussels, oysters, scallops, octopus, squids, snails, clams), as well as terrestrial arthropods (cockroaches and mites) [7]. As allergic cross-reactivity has been confirmed between tropomyosins from these sources, shellfish allergy patients must also exercise caution with these foods, including insect foods, which are being developed to address the food shortage crisis [6].

The mechanism leading to the onset of food allergies was thought to partly involve immature digestive function, as food allergies are more common in infancy [5]. However, recent research has demonstrated the importance of epicutaneous antigen exposure in the mechanism of sensitization [8]. Atopic dermatitis involving skin barrier damage is now recognized as a well-known risk factor for food allergies [9,10]. In addition, because of the cross-reactivity of tropomyosin between shrimp, cockroaches, and dust mites, skin sensitization to tropomyosin derived from cockroaches and mites may lead to oral intolerance to shrimp, leading to allergic reactions, which may explain the high prevalence of shellfish allergy in tropical and subtropical regions where mite sensitization is common [3]. We hypothesized that epicutaneous sensitization plays a significant role in adult-onset shellfish allergy, as the digestive tract has well-developed. To date, a mouse model of shrimp allergy by epicutaneous sensitization has not been reported [11]. An objective of the present study was to determine whether exposure to shrimp antigens via the skin can cause shellfish allergy.

Numerous studies have described molecules associated with the dysfunction of the skin barrier. For example, mutations in the gene encoding filaggrin protein are associated with the development of atopic dermatitis [12]. Reduced ceramide has also been associated with food allergies in patients with atopic dermatitis [10]. However, lipid reduction and other slight molecular changes cannot be detected morphologically using histopathological techniques. Confocal Raman microscopy is a non-invasive technique that uses non-visible light to provide information on the molecular structure of the skin, and its use has recently become common in dermatological research [13]. Confocal Raman microscopy enables the estimation of substances by measuring laser excitation–induced vibrational modes of chemical molecules, which have unique values for each functional group [14]. The use of confocal Raman microscopy could provide for early detection of changes in the skin that lead to sensitization, thereby helping prevent food allergies associated with epicutaneous sensitization. The aim of this study was to generate a mouse model of shrimp allergy via epicutaneous sensitization and elucidate the changes in the skin that cause sensitization using Raman microscopy.

2. Materials and Methods

2.1. Sensitization and Challenge of Mice

Three-week-old female BALB/c mice were purchased from CREA Japan (Shizuoka, Japan) and maintained on AIN-93G, a shrimp-free diet (CLEA Japan), under specific pathogen-free conditions. After 7 days of acclimation, the dorsal fur was removed from the mice using depilatory cream (Veet; RB Japan, Tokyo, Japan), and the skin was 3-times tape-stripped using mending tape (Scotch; 3M Japan, Tokyo, Japan) [15]. Next, the mice (n = 6) were sensitized with 0.1 mg of shrimp tropomyosin. Briefly, tropomyosin solution was soaked into 8 mm diameter filter paper, attached to the tape-stripped dorsal skin, and covered with a Finn Chamber (Finn Chamber disk; Smart Practice, Phoenix, AZ, USA) for 24 h on days 0, 7, and 15. Control mice (n = 5) were treated with phosphate-buffered saline (PBS). All mice were challenged with intragastric administration of 0.5 mg tropomyosin on

days 28 and 35. At 24 h after the final challenge, all mice were sacrificed under anesthesia with isoflurane.

All animal experimentation procedures were approved by the Ethics Committee of Tokushima University (approval number T30-64), and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals of Tokushima University.

2.2. Preparation of Recombinant Shrimp Tropomyosin

Recombinant shrimp tropomyosin was prepared as described in a previous study [16]. In brief, a cDNA encoding shrimp tropomyosin (Met e 1) was cloned into the 6×His-tag expression vector pET30 (a)$^+$ (Novagen, Madison, WI, USA) and expressed in Escherichia coli BL21 (Invitrogen, Carlsbad, CA, USA) by overnight culture in MagicMedia (Invitrogen). Recombinant His-tag Met e 1 fusion protein (rMet e 1) was purified using a Hispur Cobalt spin column (Thermo Scientific, Rockford, IL, USA) in accordance with the manufacturer's instructions. The purity and concentration of rMet e 1 were determined by SDS polyacrylamide gel electrophoresis and bicinchoninic acid protein assay (BCA Protein Assay, Thermo Scientific, Waltham, MA USA), respectively. rMet e 1 was diluted with PBS and used as an allergen solution.

2.3. Assessment of Systemic Anaphylaxis

Anaphylactic symptoms were evaluated within 15 min after the oral challenge in a blinded manner as follows: score 0, no symptoms; score 1, scratching and puffiness; score 2, puffiness around the eyes or reduced activity; score 3, labored respiration; score 4, no activity after prodding, or tremors and convulsions; score 5, death [4,17]. The rectal temperature was measured using an animal thermometer (A&D, Tokyo, Japan) at an interval of 15 min for 1 h post-challenge.

2.4. Measurement of Serum Tropomyosin-Specific IgE Levels

Serum levels of shrimp tropomyosin-specific IgE were measured by ELISA, as described in a previous study [16]. In brief, 96-well ELISA plates were coated overnight at 4 °C with rMet e 1 (10 μg/mL) in carbonate buffer (0.05 M Na_2CO_3, 0.13 M $NaHCO_3$, 0.003 M NaN_3 (pH 9.6)), blocked with 5% FBS/PBS for 2 h, and then incubated with serum samples from all mice (1:10) overnight at 4 °C. After washing with PBS/0.05% Tween-20, the plates were incubated with biotin-conjugated anti-mouse IgE (1:1000, Pharmingen, San Diego, CA, USA) and Avidin D peroxidase (1:1000, Vector Laboratories, Burlingame, CA, USA) at room temperature for 1 h and 30 min, respectively. The plates were then washed and developed for 15 min using the TMB substrate reagent set (BD Biosciences, Franklin Lakes, NJ, USA). The reaction was terminated by addition of 2 N H_2SO_4, and the optical density at 450 nm was measured using a microplate reader. Serum was prepared from blood samples collected from the mice 24 h after sensitization (days 1, 8, and 16) and the challenge (days 29 and 36).

2.5. Raman Microscopy

To capture the condition of the skin into which the allergen enters, four-week-old female BALB/c mice were treated with tape-stripping or the application of 4% SDS to the dorsal skin to establish a sensitized skin condition on days 0, 7, and 15. The application of SDS solution is a commonly used technique for epicutaneous sensitization as well as tape-stripping [18]. After the third treatment with tape-stripping or 4% SDS, the mice were immediately sacrificed, and skin from the back was collected. Raman spectra were acquired with a home-built laser-scanning confocal Raman microscope. A single-mode frequency-doubled Nd:YAG laser (MSL-FN-532-S-100mW; CNI Laser, Changchun, China) operating at the wavelength of 532 nm was used as an excitation laser light. The excitation laser light was focused on a sample through a 60× objective lens (CFI Plan Apo Lambda 60XC, 60×, NA = 1.2; Nikon, Tokyo, Japan). The back-scattered Raman signal was collected

with the same objective lens and detected by a spectrometer (IsoPlane 320, Princeton Instruments, Trenton, NJ, USA) with a cooled CCD image sensor (Pixis 400BR, −70 °C, 1340 × 400 pixels; Princeton Instruments, Trenton, NJ, USA). The Raman microscope was controlled with imaging software (MwMapper, version 1.4.5; ScienceEdge Inc., Shizuoka, Japan, https://scienceedge.com, accessed on 30 December 2021). The Raman spectrum from −30 to 3588 cm^{-1} was simultaneously obtained with a single exposure. The excitation laser power and the exposure time were 25 mW on the sample plane and 5 s, respectively. Three independent sites were analyzed at a depth of 5, 10, and 15 μm from the skin surface.

2.6. Histopathological Examinations

Skin tissues were fixed in 10% phosphate-buffered formalin after Raman microscopy analysis. The tissues were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

2.7. Statistical Analysis

Data are expressed as mean ± standard error (SE). Differences between groups were tested for statistical significance using a Student's *t*-test or one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test. All analyses were performed using SPSS 24.0 software (SPSS, Inc., Chicago, IL, USA) for Windows. $p < 0.05$ was considered indicative of significance.

3. Results

3.1. Elevated Levels of Serum Tropomyosin-Specific IgE after Epicutaneous Sensitization and Intragastric Challenge

BALB/c mice were subjected to three rounds of epicutaneous sensitization with tropomyosin (n = 6) or PBS (n = 5) as a control and then orally challenged with tropomyosin twice (Figure 1A). Levels of shrimp tropomyosin-specific IgE increased from day 8 (control group, 0.01 ± 0.00; sensitized group, 0.36 ± 0.09; $p = 0.002$) through day 16 (control group, 0.03 ± 0.01; sensitized group, 0.46 ± 0.14; $p = 0.002$) in mice sensitized epicutaneously with tropomyosin (Figure 1B). Significantly higher levels of shrimp tropomyosin-specific IgE were also observed in the sensitized group after the intragastric challenge, suggesting that sensitization was successful (first challenge: control group, 0.04 ± 0.01; sensitized group, 0.54 ± 0.16; $p = 0.037$; second challenge: control group, 0.02 ± 0.01; sensitized group, 0.54 ± 0.28; $p = 0.030$). In contrast, levels of serum tropomyosin-specific IgE were lower after the fourth epicutaneous sensitization than after the third sensitization and did not increase after oral administration (Supplemental Figure S1).

3.2. Systemic Anaphylactic Response after Intragastric Challenge with Tropomyosin

Following the intragastric challenge, all mice sensitized with tropomyosin exhibited puffiness around the eyes and mouth (score 2) after the first challenge on day 28 (Figure 2A,B). One mouse exhibited no activity after prodding (score 4) after the second challenge on day 35. The rectal temperature of mice sensitized with tropomyosin declined after the challenge (at 45 and 60 min in the first challenge: −2.0 ± 0.3, −1.6 ± 0.4 °C, respectively) and was significantly lower than that of the controls (at 45 and 60 min in 1st challenge: −0.3 ± 0.1, −0.3 ± 0.2 °C; $p = 0.049$, 0.050, respectively, Figure 2C).

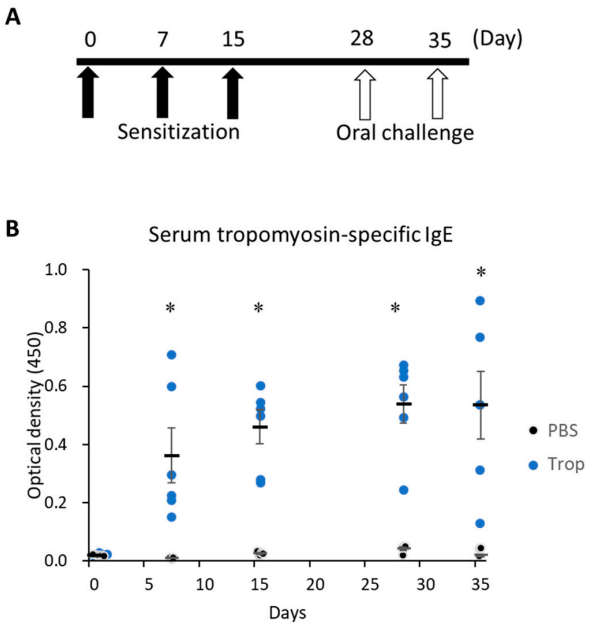

Figure 1. Epicutaneous sensitization with tropomyosin. (**A**) Epicutaneous sensitization and oral challenge protocol. (**B**) Serum levels of tropomyosin-specific IgE. Serum samples were collected from the mice 24 h after sensitization (days 1, 8, and 16) and challenge (days 29 and 36). Data are mean ± SE. * $p < 0.01$, by Student's *t*-test. The number of animals was as follows: 5 (PBS), 6 (Trop).

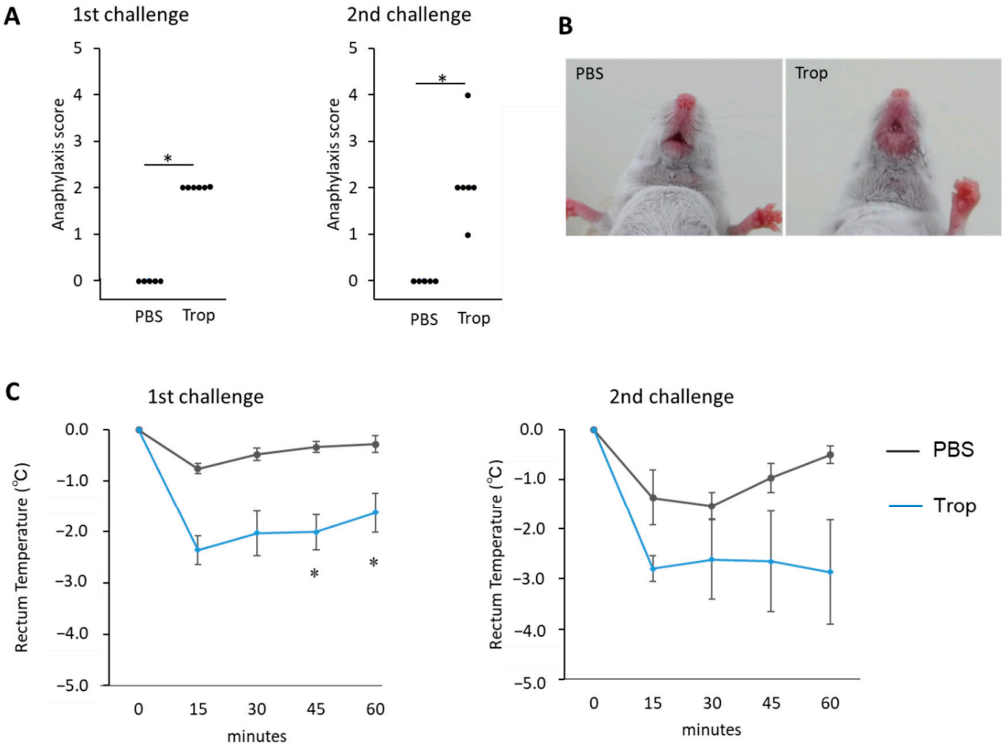

Figure 2. Anaphylaxis following oral tropomyosin challenge. (**A**) Systemic anaphylaxis score after the first and second oral challenges. Anaphylactic symptoms were evaluated within 15 min after the oral challenge. (**B**) Representative images showing puffiness around the mouth after oral challenge (score 2). (**C**) Change in rectal temperature after the first and second oral challenge. The data show the changes in rectal temperature from the levels at 0 min. Data are mean ± SE. * $p < 0.05$ by Student's *t*-test. The number of animals was as follows: 5 (PBS), 6 (Trop).

3.3. Use of Raman Microscopy to Detect Changes in Skin Exhibiting Impaired Barrier Function

Skin susceptible to sensitization due to the disruption of the barrier function by tape stripping and SDS application following the removal of fur using depilatory cream was analyzed using Raman microscopy. Major Raman bands were observed at 484, 915, 1442, 2855, and 2935 cm^{-1}, as shown in Figure 3A. After the integration of data for analyses at all skin depths, the intensity of the Raman bands at 399, 915, and 1073 cm^{-1} was significantly lower in treated mice than in controls (Figure 3B). A significant difference in band intensity at 399, 915, and 1073 cm^{-1} was also observed at a depth of 5 µm, while no significant differences were observed at depths of 10 and 15 µm, although a similar trend was observed. These results suggest that the damage occurring on the extreme surface of the skin is related to sensitization. Histological analyses did not reveal any significant changes in the stratum corneum (Figure 3C).

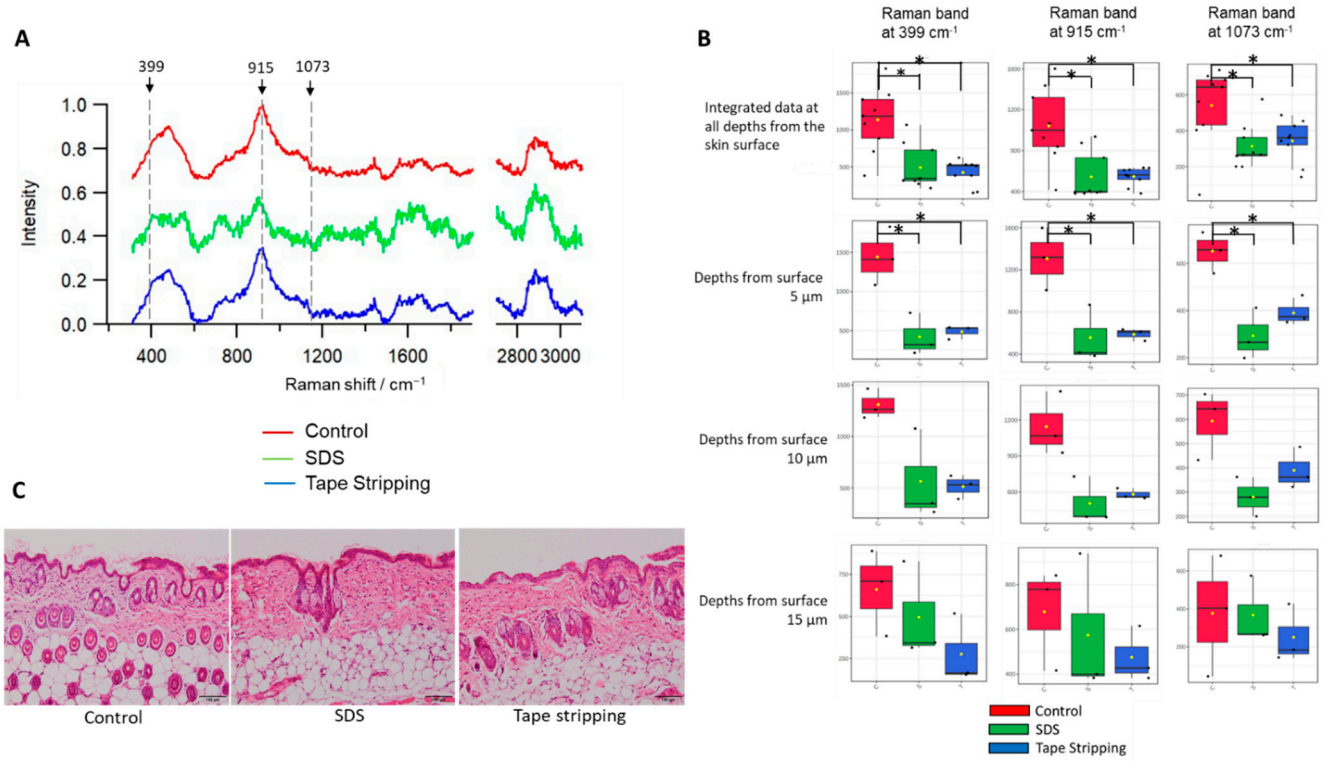

Figure 3. Use of Raman microscopy to detect changes in the skin with impaired barrier function. (**A**) Typical Raman spectra of the skin of a mouse subjected to tape-stripping and SDS application. (**B**) Box plots for the intensity of Raman bands at 399, 915, and 1073 cm^{-1}. Raman bands were obtained from 3 independent sites at a depth of 5, 10, and 15 µm from the skin surface following tape-stripping and SDS application. The differences in intensity between treatments indicate that there is a change in the molecular species and structure that constitute the skin. * $p < 0.05$, by ANOVA followed by Bonferroni test. (**C**) Hematoxylin and eosin staining of dorsal skin.

4. Discussion

A mouse model of shrimp allergy was established in the present study by repeated application of tropomyosin to the skin. It was also shown that Raman microscopy can capture the changes in skin causing sensitization. These changes were characterized as relative reductions in the intensity of Raman bands at 399, 915, and 1073 cm^{-1} on the outermost surface of the skin. The method of skin treatment might be important for making animal models of food allergy through epicutaneous sensitization. Both the frequency and method of skin sensitization have varied in previous studies. For example, Bartnikas et al. employed three 1-week ovalbumin exposures to tape-stripped skin [19], whereas Muto et al. treated mice with 4% SDS for three exposures per week over a 2-week period [18]. As shown

by our data, four rounds of epicutaneous sensitization with shrimp tropomyosin appeared to lead to desensitization, as reflected by the drop of specific IgE levels upon the fourth epicutaneous sensitization on day 21, as well as during the two intragastric challenges on days 28 and 35, suggesting that the allergen-loading dose and frequency of administration must be optimized to generate an anaphylactic reaction. As our model induced anaphylaxis with only three epicutaneous sensitizations, it could be useful in studies to elucidate allergic pathogenesis or to establish treatment methods. It would be preferable to establish a unified model of epicutaneous sensitization to enable the comparison of results from different studies.

The skin consists of epidermis, dermis, and subcutaneous tissue layers, and the epidermis is composed of the stratum corneum and stratified epithelium. The stratum corneum constitutes the outermost epidermal barrier and consists of corneocytes, with intercellular lipids filling the spaces between cells. The inner layer of the stratum corneum, the stratified epithelium, consists of multiple layers of flattened keratinocytes with tight junctions and other barrier structures that restrict the movement of materials through the cell layer [20]. The stratum corneum, stratified epithelium, and dermis of mouse skin were evaluated in the present study at depths of 5, 10, and 15 μm from the surface using confocal Raman microscopy [21]. These analyses suggested that changes in the stratum corneum are associated with susceptibility to sensitization. It is known that dendritic cells, which are normally located inside the tight junctions of stratified epithelium, extend their dendrites to below the stratum corneum to capture antigens when the stratum corneum is damaged [22], which connects to our result that only changes in the outermost layer of the skin were significant. The lack of findings of changes in the skin under optical microscopy suggests that some substances indicated by the Raman bands had little effect on the morphology of the cell cytoskeleton or degenerated deposits. The structure of the outermost surface of the skin is easily disrupted by surfactants and abrasives contained in cosmetics and detergents [23]. As there are few established treatments for adult food allergies, determining how to protect the skin from allergens is considered important.

The disruption of the stratum corneum barrier caused by endogenous factors such as predisposition to atopic dermatitis or changes in moisturizing factors such as filaggrin is thought to play an important role in epicutaneous sensitization. Many epidemiologic studies have reported that atopic dermatitis and mutations in the filaggrin gene are significant risk factors for food allergies [24]. However, the results of the present study, in which a food allergy was induced in normal mice without manipulation of the filaggrin gene, suggest that anyone can develop food allergies, even in the absence of an endogenous predisposition. It is therefore important to recognize the condition of one's own skin to prevent sensitization to allergens via the skin. Raman microscopy is an ideal tool for skin evaluation and diagnosis because it is non-invasive, in contrast to skin biopsy. Accumulating evidence indicates that data regarding intradermal composition based on Raman microscopy analysis are applicable to the classification of skin cancers as well as allergic dermatitis [25,26]. Several Raman bands observed in this study could be useful markers in screening for potential sensitization via the skin, although what substance(s) these bands represent remains unclear.

A limitation of the present study is that the skin barrier disruption, leading to sensitization, was induced by relatively strong physical and chemical reactions in mice, which may differ from the physiological condition of humans. In addition, the association between the depth of skin damage and molecular information obtained by confocal Raman microscopy still needs to be examined in humans since the thickness of the skin layers differs among species [21]. However, the ability of Raman microscopy to histologically capture undetectable changes was a noteworthy result in the present study. Further accumulation of evidence showing the usefulness of Raman microscopy in the diagnosis of skin disorders would lead to its introduction as a diagnostic modality in dermatology clinics and thus to the early detection of individuals with a high risk of epicutaneous sensitization.

In summary, a mouse model of shellfish allergy induced by epicutaneous sensitization was generated in the present study. Further elucidation of the mechanism of skin sensitization using Raman microscopy may facilitate the development of diagnostic techniques to prevent food allergies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12073566/s1, Figure S1: Serum levels of tropomyosin-specific IgE in the mice treated with 4 time-sensitization before oral challenge.

Author Contributions: M.I.-S., C.W. and K.T. designed the research; M.I.-S., S.I., T.T., A.E., T.K. (Takumi Kojima), T.M. and T.K. (Takumi Kakimoto) performed the experiments; M.I.-S., S.I., M.M., T.K. (Tomoko Kobayashi), S.S., T.K. (Takumi Kakimoto), Y.M., H.O., T.O. and K.T. performed data validation and analysis; M.I.-S., S.I., C.W., T.M. and K.T. prepared the original draft of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All animal experimentation procedures were approved by the Ethics Committee of Tokushima University (approval number T30-64).

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