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Aggravation of Food Allergy by Skin Sensitization via Systemic Th2 Enhancement

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Keywords

Animal models · Epicutaneous immunotherapy · Food allergy · Immunoglobulin E · Skin sensitization

Abstract

Background: Recently, the relationship between antigen contact via skin (skin sensitization) and the development of food allergies has gained increasing attention. However, few studies have examined the effects of skin sensitization on healthy skin. ***Objective:*** To examine the effect of sensitization in healthy skin on IgE and cytokine production during food allergy development. ***Methods:*** The effect of skin sensitization on food allergy was evaluated using DO11.10 mice whose T cells express ovalbumin (OVA)-specific T-cell receptors. OVA was applied to the back skin of mice dehaired by various methods, and then food allergy was induced by providing them with an OVA-containing diet. OVA-specific IgE production in the sera and decreases in body temperature due to anaphylactic reaction were measured as indicators of food allergy. In addition, IL-4 production and proliferation of splenocytes were measured in mice with food allergy after skin sensitization. ***Results:*** Skin sensitization in healthy skin increased IgE production and exacerbated anaphylactic symptoms induced by ingesting the antigen. Moreover, skin sensitization enhanced IL-4 production from splenocytes during the onset of food allergy. In contrast, oral tolerance was induced even after establishing skin sensitization. ***Conclusion:*** Skin sensitization temporarily exacerbated food allergy by enhancing systemic Th2 responses. These findings will help identify the mechanisms involved in food allergy and help develop treatments.

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Introduction

Sites of allergen sensitization are gaining increasing attention to aid our understanding of the mechanisms involved in the onset and development of allergic dis-

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eases. The intestine is the most important site for the induction of food allergy because this is where immune cells initially encounter antigens. However, Lack advocated the "dual allergen exposure hypothesis" where sensitization of skin with food allergens, not the intestine [1], was proposed as the major cause for triggering food allergy. This hypothesis is supported by evidence from several studies. For example, patients with peanut and wheat allergies had used peanut oil and soaps containing hydrolyzed wheat proteins, respectively, before allergy onset [2, 3]. Moreover, some patients allergic to animal meats such as beef, pork, and lamb developed allergy after tick bites. It was revealed that a saccharide, galactose-α-1, 3-galactose (α-gal), present in ticks was also present in meats and that IgE antibodies induced by the tick allergen in skin cross-reacted with the meat allergen ingested orally [4, 5]. Other similar cases have been reported [6, 7]. In these patients, no allergic signs were observed during skin sensitization; however, antigen sensitization became obvious after the ingestion of relevant antigens.

Based on the abovementioned hypotheses and related evidence, several studies have reported the effects of skin sensitization on food allergy. In most of these studies, tape stripping of the skin was performed before antigen application [8, 9]. Presumably, slight dysfunctions in the skin barrier have a pivotal role in establishing skin sensitization. Of note, atopic dermatitis patients with a mutation in the filaggrin gene frequently develop food allergy [10–12]; however, the necessity of skin barrier dysfunction on skin sensitization has not been clarified because few studies have investigated the sensitization in healthy skin. Kubo et al. [13] reported that Langerhans cells took up antigens in the epidermis without disrupting the tight junctions, suggesting healthy skin can be sensitized.

The dual allergen exposure hypothesis suggests a major cause of food allergy although the specific mechanism involved remains unclear. The mechanism by which local antigen stimulation affects immune responses elicited at distant sites such as the intestine is important. Understanding the cell types and organs involved in skin sensitization will help us prevent skin sensitization and food allergy. For this purpose, appropriate animal models to investigate the influence of skin sensitization on food allergy are required.

Skin immunotherapy (also termed epicutaneous immunotherapy; EPIT) was recently applied to treat allergic diseases including food allergy [14–16]. The allergen is applied to the patients' skin using specialized materials. EPIT is thought to have comparable efficacy to oral immunotherapy, subcutaneous immunotherapy, and sublingual immunotherapy although the action of EPIT is similar to that of skin sensitization mentioned in the dual allergen exposure hypothesis. It is important to understand how EPIT inhibits allergic symptoms despite its resemblance to skin sensitization so it can be performed more effectively and safely. To understand the mechanism involved, it is important to develop an animal model to measure the effects of antigen application to skin on food allergy.

In the present study, we examined the effects of healthy skin sensitization on food allergy using DO11.10 mice, which develop food allergy by ingesting OVA (ovalbumin) without any adjuvant [17]. This study used DO11.10 mice to investigate the influence of skin sensitization at the onset of food allergy and the cellular mechanism involved.

Methods

Mice

OVA-specific T-cell receptor-transgenic DO11.10 mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The T cells of these mice recognize OVA323-339 restricted to I-A^d. All mice used in this study were heterozygous and were produced by breeding male homozygous and female BALB/c mice. BALB/c mice were purchased from Clea Japan Inc. (Tokyo, Japan). We used female mice at 6–12 weeks of age in this study. The mice were bred at the animal facility of our university and were maintained on irradiated food and autoclaved distilled water.

Skin Sensitization and Induction of Food Allergy

For skin sensitization, a pet clipper (Panasonic, Osaka, Japan) was used to remove hair on the back skin of the mice. To analyze the effects of different hair removal techniques on skin sensitization, hair removal was also performed with scissors. In general, 100 µg of OVA (Wako Junyaku, Osaka, Japan) dissolved in 50-µL water was applied to the shaved back skin 7 times every other day from days 0 to 12. As a control, the same volume of water was applied at the same time points to the study mice.

Two days after skin sensitization, mice were fed a diet containing 20% OVA for 2–21 days to induce food allergy depending on each experimental procedure. Specifically, we provided the OVA-containing diet for 10 days and bled mice at days 7 and 9 and then performed an anaphylaxis assay at day 10 in the 1st experiment. We provided the same diet for 21 days and bled mice at days 7, 14, and 21 in the 2nd experiment. For the T-cell assay, we provided the OVA-containing diet for 2 or 7 days.

Serum Collection

Blood was collected from the tail artery of mice before and after the onset of skin sensitization and after inducing food allergy. The obtained blood was centrifuged (1,500 *g*, 4°C, 10 min) using a centrifuge (05PR-22; Hitachi, Tokyo, Japan), followed

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by centrifugation of the supernatant. Then, the remaining supernatant was collected to obtain serum, which was frozen and stored as a sample for the analysis of OVA-specific IgE antibody titers.

Induction and Measurement of Systemic Anaphylaxis

After inducing food allergy for 10 days, mice were injected intraperitoneally (i.p.) with 25 mg/mL of OVA solution (500 μL/mice) to elicit a systemic anaphylactic reaction. Changes in body temperature were observed by measuring the body surface temperature of the abdomen for 120 min using a noncontact thermometer UT-701 (A&D, Tokyo, Japan).

Cell Culture

For measuring the IL-4 production and cell proliferation, splenocytes were isolated from the mice on days 14, 16, and 21. Splenocytes isolated from the mice were cultured in 96-well plates (Nunc, Thermo Fisher Scientific, MA, USA) at 1×10^6 cells/well in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal calf serum (Sigma, St Louis, MO, USA), 100 U/mL of penicillin, 100 μg/mL of streptomycin (Gibco, Thermo Fisher Scientific), and 5×10^{-5} mol/L of 2-mercaptoethanol (Wako Junyaku) for 3 days in the presence of OVA (500 μg/mL). The supernatant was collected from each well for the cytokine production assay, whereas the remaining cells were used for the cell proliferation assay.

ELISA

To measure OVA-specific IgE antibody in sera, Maxisorp immunoplates (Nunc, Roskilde, Denmark) were coated with 0.01% OVA/PBS. Samples and standards were added after washing and blocking of the plates. Standards were prepared by diluting an OVA-specific IgE standard reagent in a mouse OVA-specific IgE measurement reagent (DS Pharma Biomedical, Osaka, Japan) with 0.05% PBS-Tween. Bound IgE antibody was detected using biotinylated anti-mouse IgE antibody (BD Pharmingen, San Diego, CA, USA) before incubating it with alkaline phosphatase-streptavidin (Invitrogen, Carlsbad, CA, USA). The substrate (*p*-nitrophenol phosphate) was added, and the absorbance was determined at 405 nm. The antibody concentration in the sample was presented as the absolute concentration based on the standard reagent.

For the measurement of IL-4 in the culture supernatants, Maxisorp immunoplates were coated with purified 11B11 anti-mouse IL-4 mAb (BD Pharmingen). Samples and standards were added after washing and blocking of the plates. Recombinant mouse IL-4 (BD Pharmingen) (5 μg/mL) diluted 1,000-fold with 0.05% PBS-Tween was prepared as the standard. Bound IL-4 was detected by using BVD6-24G2 biotinylated anti-mouse IL-4 (BD Pharmingen) before incubation with alkaline phosphatase-streptavidin. The substrate (*p*-nitrophenol phosphate) was added, and the absorbance was determined at 405 nm. The IL-4 concentration in the sample was determined as the absolute concentration based on the standard reagent.

Cell Proliferation Assay

Cell proliferation was evaluated using the BrdU ELISA kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions.

Purification and Labeling of Anti-OVA Antibody

IgG antibodies enriched in OVA-specific antibodies were purified from the sera of numerous DO11.10 mice that were fed a diet containing 20% OVA. An IgG purification kit A/G (Dojindo Laboratories, Kumamoto, Japan) was used for purification. After purification, the solvent was replaced with PBS and labeled with FITC using Pierce FITC Antibody Labeling Kit (Thermo Fisher Scientific).

Preparation and Observation of Skin Section

Approximately 100 μg of OVA dissolved in 50-μL water was applied to the shaved back skin of DO11.10 mice once or 5 times. Then, the mice were euthanized, and the skin was removed from the application area. The obtained skin was cut to an appropriate size and fixed using the AMeX method. After fixing, it was embedded in paraffin. Paraffin blocks were cut into sections with a thickness of 4 μm, attached to glass slides coated with poly-L-lysine as an adhesive, and then dried. Thereafter, the paraffin was melted in an oven at 65°C overnight, and the section samples were fixed on the slide glass. Next, they were immersed in xylene 3 times for 1 h or more followed by immersion in ethanol 3 times for 10 min or more. Eventually, they were washed twice with MQ water for 5 min each. After blocking with 0.5% casein/0.15 M NaCl (saline)/10 mM Tris-HCl buffer for 1 h, immunostaining was performed. For immunostaining, FITC-labeled OVA-specific antibody prepared as described above was used. After staining for 20 h or more, the samples were washed, and the nuclei were stained with DAPI. Eventually, glycine containing a fluorescence decay inhibitor was dropped on the washed samples, and the slide was coverslipped with glass covers.

The prepared sections were observed with a confocal laser microscope (LSM710NLO; Carl Zeiss, Jena, Germany). DAPI and FITC were excited with a blue diode laser (405 nm) and an argon laser (488 nm), respectively. FITC fluorescence was detected with a gallium arsenide phosphide (GaAsP) detector, and DAPI fluorescence was detected between 410 and 528 nm. Fluorescence images were processed using ZEN 2009 software (Carl Zeiss).

Statistical Analysis

Statistical significance was determined by the Tukey-Kramer method or Student's *t* test. Results were considered statistically significant when p values were <0.05. The number of experiments performed is indicated at the end of each figure legend.

Results

Skin Sensitization Enhanced IgE Production Induced by Orally Administered Antigen

We examined the influence of skin sensitization on IgE production in mice with food allergy. Antigen application to the back skin did not induce IgE production (Fig. 1). In contrast, IgE production was significantly enhanced in mice on the OVA diet subjected to antigen application compared with mice on the OVA diet without antigen application (Fig. 1).

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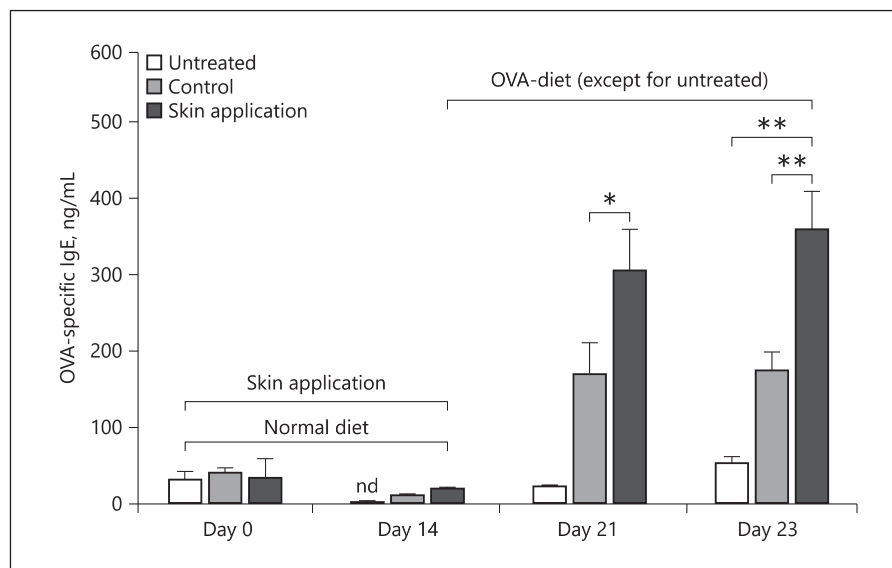

Fig. 1. Effect of skin sensitization on IgE production in food allergy mice. DO11.10 mice were subjected to skin sensitization as described in the section Methods from days 0 to 12 and were then fed a diet containing 20% OVA to induce food allergy. The amount of IgE antibody production in the sera of the mice was measured on days 0, 14, 21, and 23. Data are representative of 2 independent experiments with 5 mice per group. Error bars indicate means ± SD. Tukey-Kramer test was used for statistical analysis. *$p < 0.05$; **$p < 0.01$. OVA, ovalbumin.

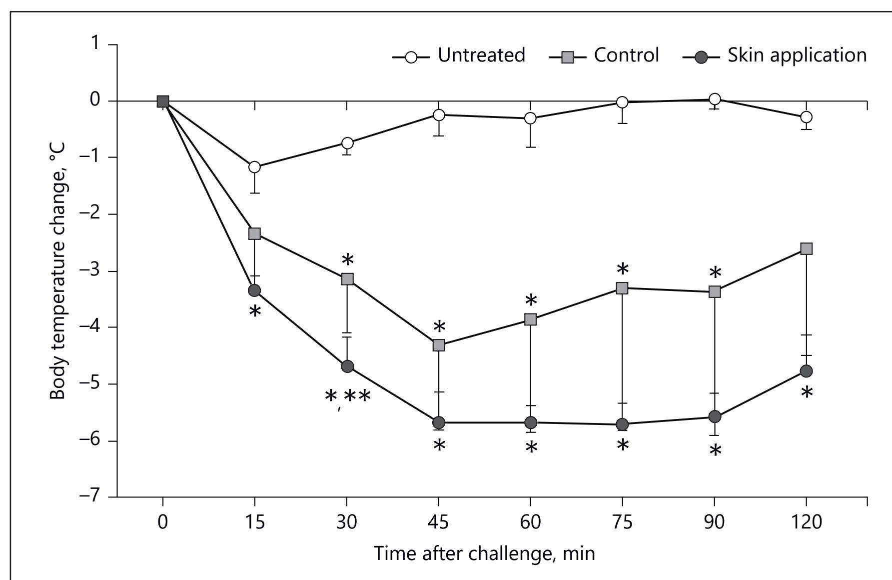

Fig. 2. Effect of skin sensitization on body temperature reduction caused by anaphylactic reaction in food allergy mice. DO11.10 mice were subjected to skin sensitization as described in the section Methods from days 0 to 12 and were then fed a diet containing 20% OVA to induce food allergy. The mice were injected i.p. with 25 mg/mL of OVA solution (500 µL/mice) to elicit a systemic anaphylactic reaction 10 days after inducing food allergy. Data are representative of 2 independent experiments with 5 mice per group. Error bars indicate means ± SD. Significant differences were determined with the Tukey-Kramer test and were indicated as follows: *$p < 0.05$ versus untreated; **$p < 0.05$ versus control. OVA, ovalbumin; i.p., intraperitoneally.

Skin Sensitization Exacerbated Anaphylactic Reaction in Food Allergy Mice

We confirmed the allergic symptoms in the food allergy mice that received the antigen on their back skin. The i.p. administration of OVA induced a temporary decrease in body temperature in the food allergy mice, which was significantly exacerbated in mice exposed to the antigen before the induction of food allergy (Fig. 2).

The Back Hair Removal Technique Did Not Influence the Skin Sensitization Effect

We checked whether epilation with hair clippers caused any damage to the skin and affected skin sensitization. The antigen application enhanced IgE production induced by the orally administered antigen regardless of the epilation method used (Fig. 3). No difference was observed between mice subjected to hair removal with clippers or scissors. Moreover, similar results were obtained while using a depilatory to remove hair (data not shown).

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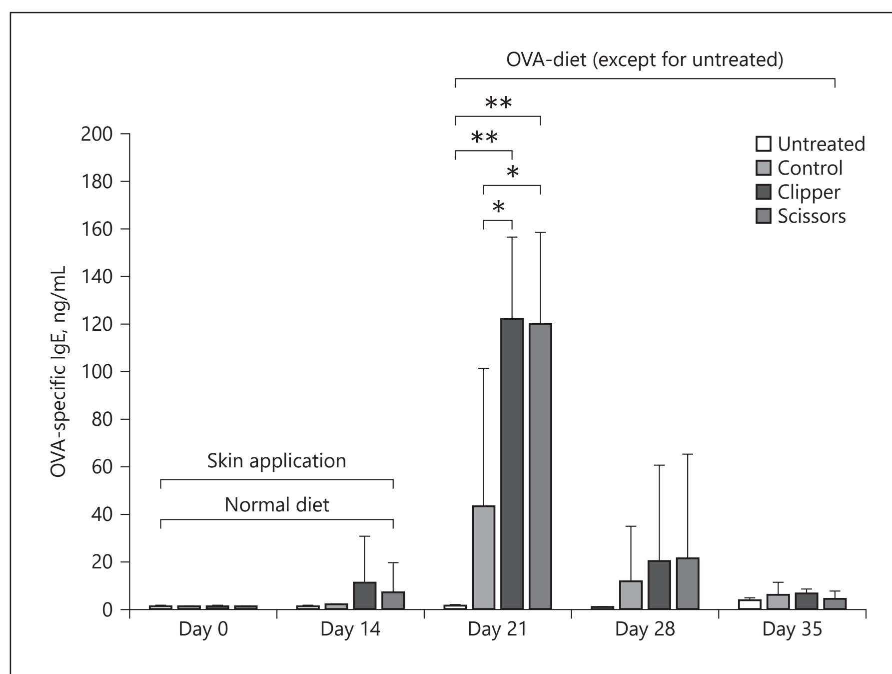

Fig. 3. Effect of different epilation methods on skin sensitization in food allergy mice. DO11.10 mice were subjected to skin sensitization as described in the section Methods from days 0 to 12 and were then fed a diet containing 20% OVA to induce food allergy. The amount of IgE antibody production in the serum of the mice was measured on days 0, 14, 21, 28, and 35. Data are representative of 2 independent experiments with 5 mice per group. Error bars indicate means ± SD. Tukey-Kramer test was used for statistical analysis. $^{*}p < 0.05$; $^{**}p < 0.01$. OVA, ovalbumin.

Skin Sensitization Did Not Inhibit the Induction of Oral Tolerance

Our results clearly demonstrated that skin sensitization aggravated food allergy even in healthy skin. In contrast, IgE values in the sera gradually decreased 2 weeks after starting the OVA diet with or without antigen application (Fig. 3). This result indicated that enhanced IgE production by skin sensitization was not caused by the abrogation of oral tolerance.

Skin Sensitization Upregulated IL-4 Production from Splenocytes in Food Allergy Mice

Next, we addressed the mechanism by which the local skin sensitization enhanced systemic IgE production induced by orally ingested antigens. Splenocytes from mice subjected to antigen application in advance produced higher amounts of IL-4 than mice without skin sensitization at 2 and 7 days after feeding the diet containing OVA in response to antigen stimulation (Fig. 4a). A minor proliferative response was also enhanced by skin sensitization (Fig. 4b).

Antigen Applied to Skin Accumulated in the Epidermis

We investigated the antigen distribution after antigen application to the skin using FITC-labeled antibody specific for the antigen. Confocal microscopy revealed that the antigen accumulated mostly in the epidermis of mice after multiple application of the antigen to the skin (Fig. 5).

Discussion

The present study aimed to confirm the effects of skin sensitization on the onset and development of food allergy using OVA-specific T-cell receptor-transgenic DO11.10 mice as a murine model for food allergy. The mice presented with high levels of OVA-specific serum IgE upon feeding of OVA in the absence of adjuvants [17]. We observed the effects of skin sensitization in food allergy mice, which might be applicable to the mechanisms expected in patients with food allergy.

In this study, we clearly demonstrated that skin sensitization with small amounts of the antigen enhanced IgE production and triggered severe allergic symptoms induced by the orally administered antigen. The “dual allergen exposure hypothesis” advocated by Lack [1] indicated the risk of skin sensitization for developing food allergy; however, the molecular mechanism involved remains unclear. Understanding the mechanism by which antigens applied to the skin affect the immune responses elicited by orally administered antigens is crucial to preventing skin sensitization. These mechanisms were inves-

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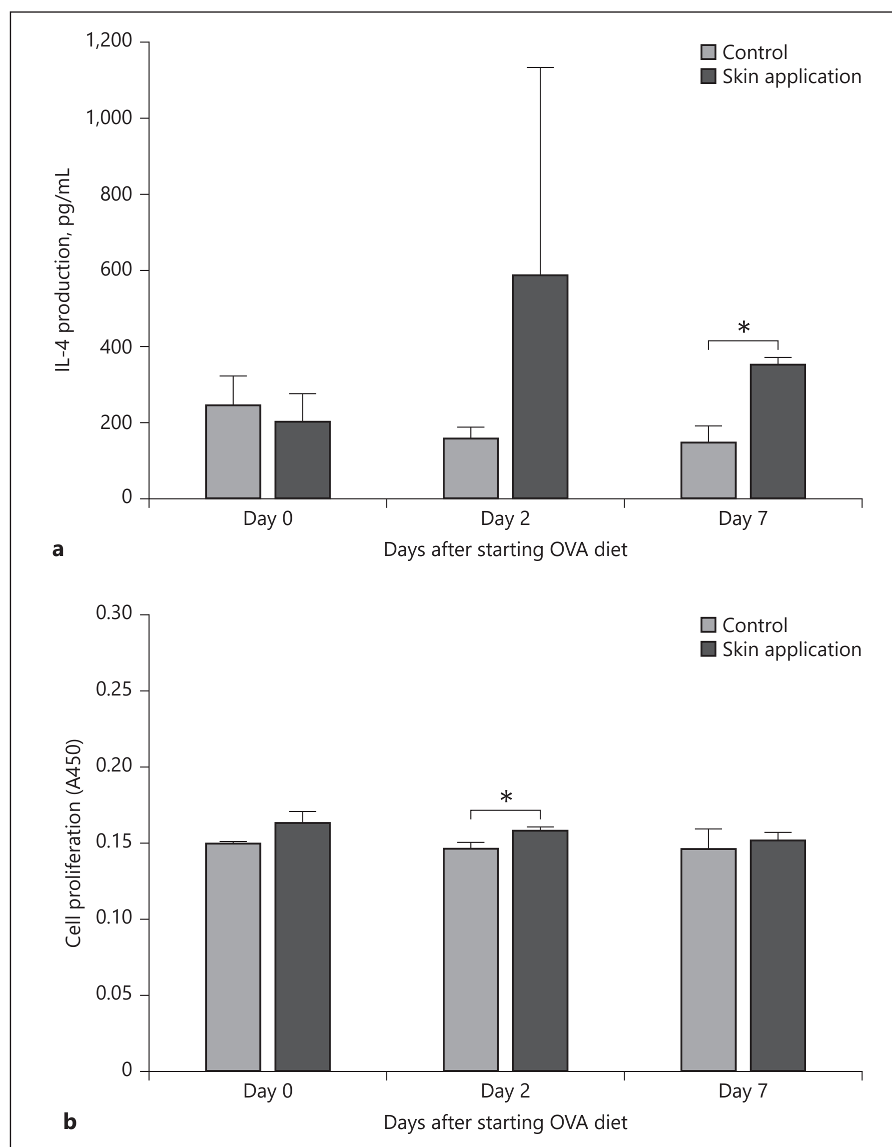

Fig. 4. Effect of skin sensitization on systemic immune responses in food allergy mice. DO11.10 mice were subjected to skin sensitization as described in the section Methods from days 0 to 12 and then were fed a diet containing 20% OVA to induce food allergy. Splenocytes were prepared from mice on days 14, 16, and 21 (on days 0, 2, and 7 after starting the OVA diet) and cultured with OVA (500 µg/mL). **a** IL-4 production in the supernatants was measured at day 3 of culture. **b** Cell proliferation assay was performed using the remaining cells. Data are representative of 2 independent experiments with 3 wells for each group. The cells were used by pooling the splenocytes from 2 mice per group. Error bars indicate means ± SD. Student's *t* test was used for statistical analysis. *$p < 0.05$. OVA, ovalbumin.

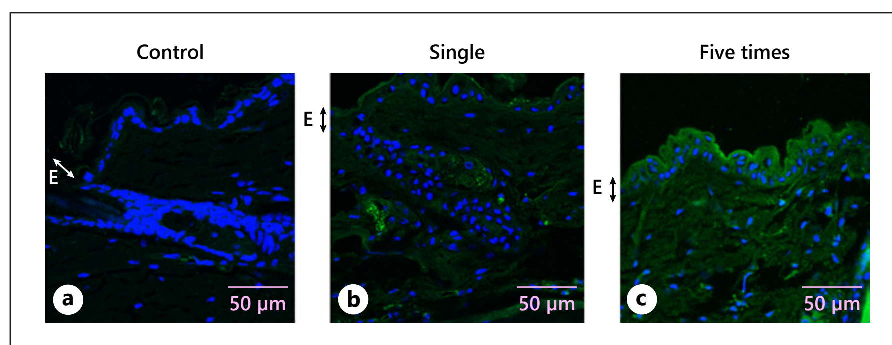

Fig. 5. Location of the antigen applied to healthy skin. About 100 µg of OVA dissolved in 50-µL water was applied to the back skin of DO11.10 mice after shaving the skin with a clipper. E indicates the epidermis. Green: FITC anti-OVA (OVA); blue: DAPI (nucleus). **a** Control group. **b** Single application group. **c** Five times application group. OVA, ovalbumin.

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tigated using the OVA-specific T-cell receptor-transgenic DO11.10 mice.

In previous studies, skin sensitization was commonly induced in the skin upon tape stripping or by treating skin with distinct detergents to slightly damage the skin's barrier function [8, 9]. Therefore, it is thought that skin sensitization requires a certain amount of skin damage; however, the results of the present study demonstrated that sensitization in healthy skin also aggravated food allergy. We tested several methods to remove the back hair and revealed that the skin sensitization was similarly established regardless of the method used. In particular, the fact that the aggravation of food allergy was observed in mice that had hair removed using scissors strongly suggests the potential risk of contact between skin and food in our daily life because the hair removal procedure did not damage or stimulate the skin.

During each application, only 100 μg of antigen was applied to the skin. In contrast, each mouse was fed about 600 mg of the antigen every day. Interestingly, even slight skin sensitization had a huge impact on food allergy. Although we did not determine the cellular mechanism of skin sensitization, Langerhans cells and/or other skin-resident antigen-presenting cells (APCs) might play a pivotal role in sensitization. Langerhans cells are present in the epidermis and are potent APCs that collect antigens in the epidermis [18–21]. However, it was also reported that Langerhans cells induced suppressive immune responses rather than active responses according to circumstances [22, 23]. Our results suggest that Langerhans cells or other APCs ingested the antigen applied to the skin and induced certain changes systemically. Of note, we observed the accumulation of the applied antigen in the epidermis. Identifying the cells presenting the antigen and the site where the antigen presentation occurs is crucial and needs to be elucidated in a future study.

Skin sensitization did not induce any detectable change by itself; for example, IgE production was very low in the sera and IL-4 production from splenocytes induced by in vitro antigen stimulation remained unaltered before the oral administration of the antigen. In contrast, IgE production was rapidly enhanced after the intake of the OVA diet, and IL-4 production from splenocytes was also simultaneously upregulated. These findings suggest that memory T and B cells were induced systemically via application of the antigen to the skin. APCs collecting the antigen in the skin may transfer to specific sites to elicit systemic responses, and induced memory cells might gradually accumulate in the spleen or elsewhere. Nevertheless, the antigen applied to the skin did not reach these accumulation sites because we used small amounts. In contrast, memory cells respond quickly after major antigen intake from the intestine. In the case of food allergy induced by skin sensitization with peanut oil or soap including hydrolyzed wheat proteins, it was reported that patients did not suffer from any allergic symptoms before eating peanuts or wheat [2, 3]. Our findings are in-line with these previous reports.

Oral tolerance is an immunological hyporesponsiveness induced by orally administered antigens. The abrogation of oral tolerance to a specific antigen is considered to greatly contribute to food allergy. In contrast, our results revealed that IgE levels in the sera gradually decreased 2 weeks after providing an OVA diet regardless of antigen skin application, indicating that oral tolerance was successfully established even in mice with skin sensitization. This finding suggests that enhanced food allergy by skin sensitization was related to the temporary accumulation of memory cells and not due to the interference of oral tolerance induction. This suggests that oral immunotherapy might be effective even for patients with food allergy accompanied with skin sensitization.

The results illustrated in Figure 4 revealed that skin sensitization affected IL-4 production more than the proliferation of splenocytes. It was reported that Langerhans cells preferentially induced Th2-type responses [19–21]. Taken together, this suggests that skin sensitization might enhance systemic Th2-type immune responses resulting in allergic diseases.

Our results demonstrate that the application of food antigens to the skin might increase the risk of inducing or aggravating food allergy; however, EPIT is practically applied to desensitization in allergic patients [14–16]. In addition, Li et al. [24] reported that the application of allergens to healthy skin prevented the onset of food allergy. The mechanism by which EPIT prevents food allergy symptoms remains unclear, but it may be related to the condition of skin, the application site of the antigen, the amount or frequency of the antigen applied, or the use of materials to adhere the antigen to the skin. Skin condition is thought to be the most critical factor that determines whether the skin application of an antigen induces the aggravation or inhibition of food allergy although our present study clearly denied the hypothesis. The molecular and cellular mechanisms of EPIT for inhibiting allergy need to be elucidated immediately so it can be performed more effectively and safely. We have examined the influence of application sites and found no differences between the sites we studied (unpublished data). Interestingly, Li et al. [24] reported that the responses of T cells

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were enhanced by the skin application of an allergen even though IgE production and symptoms induced by subsequent feeding of the allergen were inhibited. This indicated that the skin application of antigens might generally activate T cells systemically as shown in this present study. It is unclear why the skin application of the allergen aggravated food allergy in this study but suppressed it in the study by Li et al. [24] even though T cells were activated by allergen application in both studies; however, it might be related to the different experimental procedures used to induce food allergy. The study by Li et al. [24] used an adjuvant to induce food allergy while we did not, and they fed a small amount of antigen 5 times a week while we provided a diet containing 20% of the antigen ad libitum. Furthermore, the antigen application might have enhanced food allergy at the early period of antigen ingestion in Li's study. Li et al. [24] did not observe IgE production earlier than week 3 after starting antigen feeding, indicating antigen application might have enhanced allergic responses at an earlier period in their study. Indeed, such activation followed by inhibition was observed for T cells in the study as discussed above. We are now studying the difference in skin sensitization and EPIT using our murine model.

In conclusion, in accordance with observations in certain food allergy patients, we successfully demonstrated that skin sensitization with a food allergen aggravated food allergy using a murine model. We also revealed that IL-4 production from the splenocytes of mice with food allergy was enhanced by skin sensitization. We consider that memory T and/or B cells accumulate systemically after skin sensitization. The murine model for skin sensitization used in this study might contribute to future investigations of the specific mechanism involved in the "dual allergen exposure hypothesis" and EPIT.

Statement of Ethics

All mice used in this study were maintained and used in accordance with the guidelines for the care and use of experimental animals of the Tokyo University of Agriculture and Technology (27-2, April 17, 2015; 28-2, April 19, 2016; 30-5, April 6, 2018).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

T.Y.: conceptualization, data curation, and writing the manuscript; R.K., T.O., A.F., K.A., N.S., G.K., and M.H.: data curation and writing the manuscript; H.M.: conceptualization, writing the manuscript, and funding acquisition.

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