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ORIGINAL ARTICLE

Epicutaneous Administration of Papain Induces IgE and IgG Responses in a Cysteine Protease Activity-Dependent Manner

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ABSTRACT

Background: Epicutaneous sensitization to allergens is important in the pathogenesis of not only skin inflammation such as atopic dermatitis but also "atopic march" in allergic diseases such as asthma and food allergies. We here examined antibody production and skin barrier dysfunction in mice epicutaneously administered papain, a plant-derived occupational allergen belonging to the same family of cysteine proteases as mite major group 1 allergens.

Methods: Papain and *Staphylococcus aureus* V8 protease were patched on the backs of hairless mice. Transepidermal water loss was measured to evaluate the skin barrier dysfunction caused by the proteases. Papain or that treated with an irreversible inhibitor specific to cysteine proteases, E64, was painted onto the ear lobes of mice of an inbred strain C57BL/6. Serum total IgE levels and papain-specific IgE and IgG antibodies were measured by ELISA.

Results: Papain and V8 protease patched on the backs of hairless mice caused skin barrier dysfunction and increased serum total IgE levels, and papain induced the production of papain-specific IgG1, IgG2a, and IgG2b. Papain painted onto the ear lobes of C57BL/6 mice induced papain-specific IgE, IgG1, IgG2c, and IgG2b, whereas papain treated with E64 did not. IgG1 was the most significantly induced papain-specific IgG subclass among those measured.

Conclusions: We demonstrated that the epicutaneous administration of protease not only disrupted skin barrier function, but also induced IgE and IgG responses in a manner dependent on its protease activity. These results suggest that protease activity contained in environmental sources contributes to sensitization through an epicutaneous route.

KEY WORDS

epicutaneous sensitization, IgE, mouse model, papain, protease allergen

INTRODUCTION

The protease activity of allergens has been implicated as an important property that endows an allergen with its allergenicity.[1-7] The protease activity of the house dust mite major group 1 allergens Der f 1 and Der p 1, two of the most important allergens common worldwide, has been shown to cause barrier dysfunction in the mucosal epithelium and skin, induce the production of pro-inflammatory cytokines in epithelial cells and keratinocytes, cleave various molecules, modulate the functions of various cell types, and induce Th2 responses.[1,3,5-16] Previous studies reported that papain, a papaya fruit-derived occupational protease allergen, stimulated epithelial cells[17] and mouse basophils *in vitro*[16,18,19] and induces Th2 re-

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sponses[16,18-21] and the IL-33-dependent innate[22] and adaptive[16] responses of lung eosinophilia in mice. The repeated intranasal administration of papain and recombinant Der f 1 without using adjuvants such as Alum induced allergic airway inflammation and IgE/IgG1 production in a manner dependent of the protease activity of the protease allergens in mice.[16]

Skin barrier dysfunction has been reported as a major manifestation of atopic dermatitis, and its involvement in the pathogenesis has been reported.[1,2,23,24] Furthermore, epicutaneous sensitization through barrier-disrupted skin has been shown to cause the "atopic march" in allergic diseases such as asthma and food allergies.[1,23,25,26] We previously demonstrated that the recombinant mite major protease allergen Der f 1[9] and *Staphylococcus aureus* V8 protease[27] applied to the skin of mice caused skin barrier dysfunction. These results suggest that the proteases contained in allergen sources and those produced by skin-resident microbes contribute to a reduction in skin barrier function, which initiates and promotes allergen-sensitization through the skin.[1] However, whether exposure of the skin to proteases causes IgE production remains unknown. In the present study, we examined antibody production after the epicutaneous administration of proteases without using adjuvants.

METHODS

MICE

Six-to twelve-week-old female hairless mice (Skh: HR1 strain; Hoshino Laboratory Animals, Inc., Ibaraki, Japan) and C57BL/6N mice (Japan SLC, Inc., Hamamatsu, Japan) were maintained in a specific pathogen-free animal facility at Juntendo University and used in accordance with the guidelines of the institutional committee on animal experiments.

EPICUTANEOUS ADMINISTRATION OF PAPAIN TO THE BACKS OF HAIRLESS MICE

A rectangular piece of filter paper 1 cm × 2 cm (3MM Chr, Whatmann, Middlesex, UK) was placed on the backs of hairless mice without tape stripping, and PBS (100 µl/2 cm^2/site), papain (Calbiochem, San Diego, CA, USA) (50 µg/100 µl-PBS/2 cm^2/site), or *S. aureus* V8 protease (Pierce, Rockford, IL, USA) (5 µg/100 µl-PBS/2 cm^2/site) was perfused to the paper. Paper containing PBS, papain, or V8 protease was wrapped and immobilized with a polyurethane film (Cathereep; Nichiban, Tokyo, Japan). The direct application of PBS without the patch and occlusion were performed in other mice as a control group. Applications were repeated on days 0-6 and days 9-15.

MEASUREMENT OF TRANSEPIDERMAL WATER LOSS IN HAIRLESS MICE

Transepidermal water loss (TEWL) was measured with a Mobile Tewameter (Courage + Khazaka Electronic GmbH, Köln, Germany) on days 0 and 9 before the application and on day 16.

ELISA FOR SERUM ANTIBODY PRODUCTION IN HAIRLESS MICE

Sera were collected on days 8 and 16 to measure antibody production by ELISA. Serum total IgE was measured by a sandwich ELISA as described previously.[28,29] Papain-specific antibodies were detected as described previously[16,30,31] with modifications as follows. Plates were coated with 30 µg/ml of papain, blocked with BlockAce (Snow Bland, Sapporo, Japan), and developed with HRP-conjugated antibodies specific to the murine IgG1 (rat monoclonal antibody, clone X56: BD Biosciences, San Jose, CA, USA), IgG2a (rat monoclonal antibody, clone R19-15; BD Biosciences), and IgG2b (Rabbit polyclonal antibody; Zymed, South San Francisco, CA, USA).[30,31] Sera and detection antibodies were diluted with Solutions 1 and 2 of CanGetSignal (TOYOBO, Osaka, Japan), respectively. Serum dilutions were 1/10000, 1/200, and 1/200 for detecting papain-specific IgG1, IgG2a, and IgG2b, respectively. Incubation with sera or detection antibodies to detect papain-specific IgG subclasses was for 1.5 h at room temperature. Dilutions of HRP-conjugated detection antibodies were 1/4000. The optical density (OD) at 450 nm, from which that at 570 nm was subtracted, was used as the signal for papain-specific IgGs.

TREATMENT OF PAPAIN WITH E64

Papain was treated with the irreversible inhibitor specific to cysteine proteases, E64 (Peptide Institute, Osaka, Japan), as described previously[14] with minor modifications.[16] After the treatment, free E64 was removed by dialysis. Papain, which was incubated similarly to E64-treated papain (but without the addition of E64) and dialyzed, was prepared and used for epicutaneous administration to C57BL/6 mice. Purity and the absence of both degradation and aggregation were confirmed by electrophoresis, and protease activity was analyzed using synthetic fluorogenic substrates.[14,32]

EPICUTANEOUS ADMINISTRATION OF PAPAIN TO THE EAR LOBES OF C57BL/6 MICE

This method was originally described for sensitization to mite extract by Gao *et al.*[33] and we modified it for sensitization to papain as follows. Tape stripping was not performed in the present study. 12.5 µl of 10 mg/ml of papain or E64-treated papain was applied onto both sides of both ear lobes (250 µg/25 µl/animal) on days 0, 3, 7, 10, 21, 24, and 28 for a total of 7 times.

ELISA FOR SERUM ANTIBODY PRODUCTION IN C57BL/6 MICE

Sera were collected on days 17 and 35 to measure an-

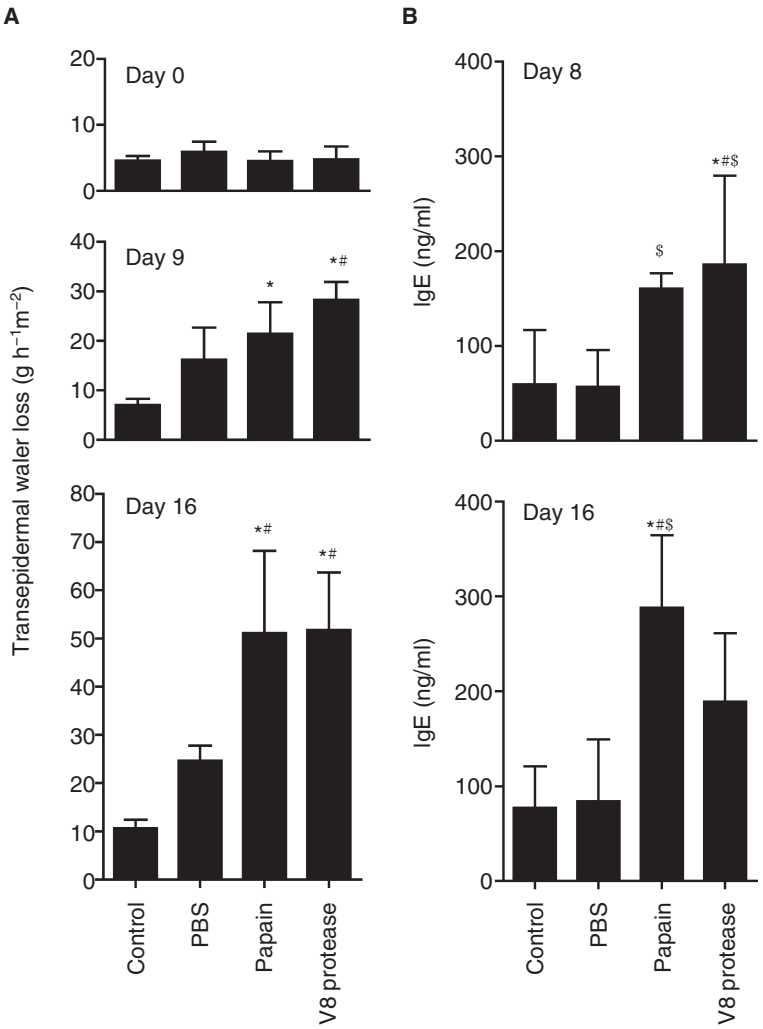

Fig. 1 Papain and *S. aureus* V8 protease patched on the backs of hairless mice caused skin barrier dysfunction and increased serum total IgE levels. A patch containing PBS, papain, or V8 protease with occlusion was applied to the backs of hairless mice and immobilized with a polyurethane film every day on days 0-6 and days 10-15. In the control group, PBS was directly applied without the patch and occlusion (*Control*). TEWL was measured on days 0 and 9 before the application and day 16 (**A**). Sera were collected from mice in on days 8 and 16. IgE concentrations were measured by ELISA (**B**). $^{*}p < 0.05$ significantly different from *Control* and $^{\#}p < 0.05$ significantly different from *PBS* by a one-way ANOVA and Tukey *post hoc* test, and $^{\$}p < 0.05$ significantly different from *PBS* by a *t*-test (two-tailed). Data shown are the means ± SDs for four or three mice/group and are representative of two or three independent experiments.

tibody production by ELISA. Serum total IgE was measured by a sandwich ELISA as described previously.[28,29] Papain-specific IgG antibodies were detected as described previously[16] with modifications as follows. Plates were coated with 10 μg/ml of papain and blocked with ImmunoBlock (DS Pharma Biomedical, Osaka, Japan). Sera, biotinylated anti-mouse IgE, and horseradish peroxidase (HRP)-conjugated streptavidine were diluted with PBS containing 0.05% Tween 20 and 1/20 volume of ImmunoBlock (1/50, 1/250, and 1/2500 dilutions, respectively).[16] To detect papain-specific IgE, incubations with sera, biotinylated anti-mouse IgE, and HRP-conjugated streptavidine were conducted overnight at 4°C, for 60 min at

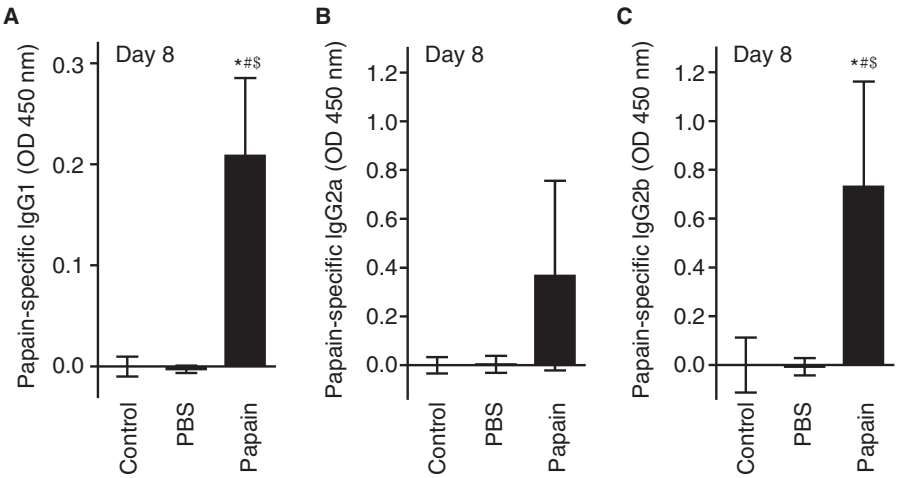

Fig. 2 Papain patched on the backs of hairless mice induced papain-specific IgG antibodies. Sera were collected from hairless mice shown in Figures 1 on day 8. Papain-specific IgG1 (**A**), IgG2a (**B**), and IgG2b (**C**) were measured by ELISA (serum dilutions: 1/10000, 1/200, and 1/200, respectively). The average of the values for the *Control* group was set to zero in each of the panels. $^{*}p < 0.05$ significantly different from *Control* and $^{\#}p < 0.05$ significantly different from *PBS* by a one-way ANOVA and Tukey *post hoc* test, and $^{\$}p < 0.05$ significantly different from *PBS* by a *t*-test (two-tailed). Data shown are the means ± SDs for four or three mice/group and are representative of two or three independent experiments.

room temperature, and for 30 min at room temperature, respectively. The HRP-conjugated anti-IgG2a monoclonal antibody (clone R19-15; BD Biosciences) cross-reactive to IgG2c was used for detection of IgG2c. To detect papain-specific IgG1, IgG2c, and IgG2b, incubations with sera (1/5000, 1/200, and 1/200 dilutions, respectively) and HRP-conjugated anti-mouse IgG subclasses were conducted for 80 min at 37°C, respectively. The OD at 450 nm, from which that at 570 nm was subtracted, was used as the signal for papain specific antibodies.

STATISTICAL ANALYSIS

A one-way analysis of variance (ANOVA) with Tukey's multiple comparison test and unpaired t-test (two-tailed) were used. Values of $P < 0.05$ were regarded as significant.

RESULTS

EPICUTANEOUS EXPOSURE OF THE BACKS OF HAIRLESS MICE TO PAPAIN INDUCED SKIN BARRIER DYSFUNCTION

Papain was applied in a patch to the backs of hairless mice on days 0-6 and days 9-15. TEWL was measured on days 9 and 16. The hairless phenotype of this strain simplified the experimental protocol. V8 protease,[27] a *Staphylococcus aureus* extracellular serine protease showing a specificity of glutamate-specific cleavage similar to *S. aureus* exfoliative toxins, was used as a positive control in the measurement of TEWL. In our previous report, TEWL was increased in V8 protease-patched mice on day 7 after the application on days 0-6.[27] On day 9 after the two-day interval from the application on days 0-6 (Fig. 1A, middle panel), TEWL was higher in V8 protease-patched mice than PBS-patched mice but was not in papain-patched mice. TEWL was also higher in papain-patched and V8 protease-patched mice than in mice directly administered PBS onto their backs without the patch with occlusion (Fig. 1A, *Control*). TEWL was higher in papain-patched mice and V8 protease-patched mice than in PBS-patched mice on day 16 (Fig. 1A, lower panel), the day after the application on days 9-15. Thus, epicutaneous exposure to papain on the backs of hairless mice induced skin barrier dysfunction, although the amount of papain (50 μg/100 μl/2 cm^2/site) used to reduce barrier function was more than that of V8 protease (5 μg/100 μl/2 cm^2/site).

EPICUTANEOUS EXPOSURE OF THE BACKS OF HAIRLESS MICE TO PAPAIN UPREGULATED SERUM TOTAL IgE LEVELS AND INDUCED PAPAIN-SPECIFIC IgG PRODUCTION

Serum total IgE levels were higher in papain-patched hairless mice than in PBS-patched mice on day 8, and were also higher in V8 protease-patched mice on day 8 (Fig. 1B). On day 8 (Fig. 2), papain-specific IgG1 and IgG2b, but not IgG2a (Fig. 2A, C, B, respectively), were induced in papain-patched hairless mice.

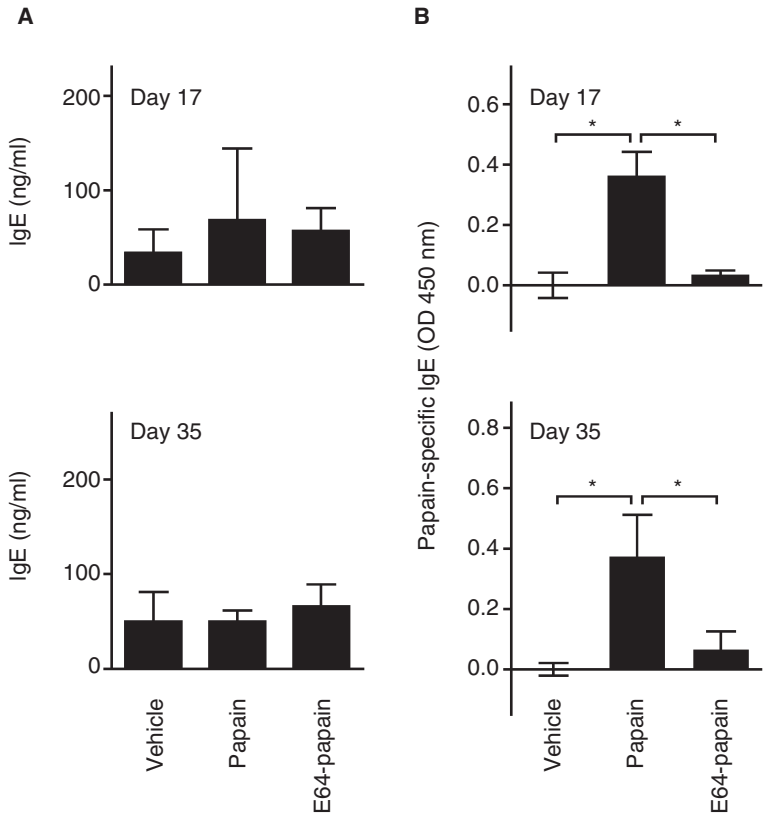

Fig. 3 Papain painted on the ear lobes of C57BL/6 mice induced papain-specific IgE in a manner dependent on the protease activity of papain. Papain or that treated with E-64 was painted on the ear lobes of C57BL/6 mice on days 0, 3, 7, 10, 21, 24, and 28. Sera were collected on days 17 and 35. Total IgE (**A**) and papain-specific IgE (serum dilution: 1/50) (**B**) were measured by ELISA. The average of the values for the *Vehicle* group was set to zero in each of the panels in **B**. *$p < 0.05$ by a one-way ANOVA and Tukey *post hoc* test. Data shown are the means ± SDs for four or three mice/group and are representative of three independent experiments.

On day 16, higher titers of all three subclasses of papain-specific IgG examined were observed (data not shown). Thus, the epicutaneous exposure of the backs of hairless mice to papain upregulated serum total IgE levels (Fig. 1B) and induced papain-specific IgG production (Fig. 2). V8 protease also upregulated serum total IgE levels (Fig. 1B).

EPICUTANEOUS EXPOSURE OF THE EAR LOBES OF C57BL/6 MICE TO PAPAIN INDUCED PAPAIN-SPECIFIC IgE AND IgG PRODUCTION IN A MANNER DEPENDENT ON THE CYSTEINE PROTEASE ACTIVITY OF PAPAIN

Recently we reported that intranasal administration of papain and recombinant Der f 1 without using adjuvants such as Alum induced allergic airway inflammation and IgE/IgG1 production in a manner dependent of the protease activity of the protease allergens in an inbred murine strain, C57BL/6.[16] We examined the epicutaneous administration of papain in the same strain. We painted the papain solution onto the ear lobes of C57BL/6 without tape stripping and without the patch and occlusion on days 0, 3, 7, 10, 21, 24, and 28. Although no significant difference was observed in serum total IgE on days 17 and 35 between the papain-treated and the control vehicle-treated mice of this strain (Fig. 3A), the production of IgE and IgG subclasses specific to papain was observed (Fig. 3B, Fig. 4, Papain). In contrast, papain treated with E64, an irreversible inhibitor specific to cysteine proteases, did not induce significant IgE and IgG production specific to papain (Fig. 3B, 4, E64-papain). Thus, epicutaneous exposure of the ear lobes of C57BL/6 mice to papain induced the production of papain-specific IgE and IgG antibodies and this was dependent on the protease activity of papain. As the C57BL/6 strain lacks the gene for IgG2a but instead express IgG2c,[34] the HRP-conjugated anti-IgG2a monoclonal

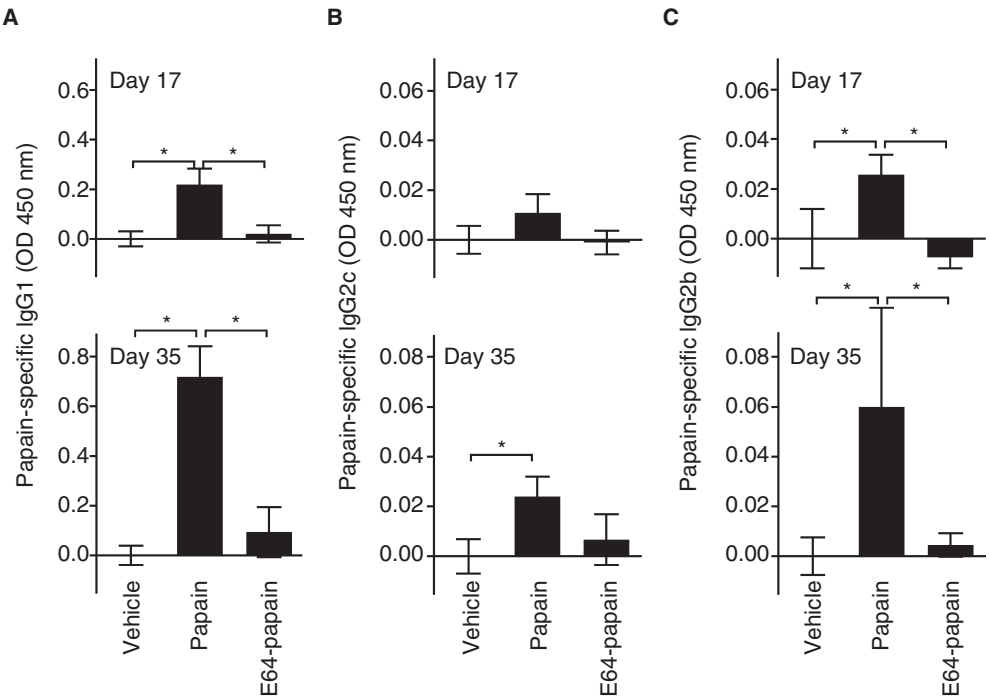

Fig. 4 Papain painted on the ear lobes of C57BL/6 mice induced papain-specific IgG antibodies in a manner dependent on the protease activity of papain. Sera used were identical to those analyzed in Figure 3. Papain-specific IgG1 (**A**), IgG2c (**B**), and IgG2b (**C**) were measured by ELISA (serum dilutions: 1/5000, 1/200, and 1/200, respectively). As the C57BL/6 mice lack the gene for IgG2a but instead express IgG2c, the HRP-conjugated anti-IgG2a monoclonal antibody cross-reactive to IgG2c was used for detection of IgG2c. The average of the values for the *Vehicle* group was set to zero in each of the panels. *$p < 0.05$ by a one-way ANOVA and Tukey *post hoc* test. Data shown are the means ± SDs for four or three mice/group and are representative of three independent experiments.

antibody cross-reactive to IgG2c was used for detection of IgG2c (Fig. 4B).

DISCUSSION

The mite-derived indoor allergens, Der f 1 and Der p 1, and plant-derived occupational allergen, papain, belong to the same family of cysteine proteases, family C1 of clan CA.[3,35,36] In the present study, we used papain as a model protease allergen that mimics the cysteine proteases contained in natural allergen sources such as mites and pollen.[1,37,38] We demonstrated that the epicutaneous administration of papain did not only disrupt skin barrier function (Fig. 1A), but also induced IgE and IgG responses (Fig. 1B, 2-4). Additionally, epicutaneously administered *Staphylococcus aureus* V8 protease also exhibited the capacity to reduce skin barrier function (Fig. 1) and upregulate serum total IgE levels (Fig. 2). Upregulation of total IgE levels was not observed in C57BL/6 mice and this could be due to differences of the strains and methods of administration between the two experimental models.

The protease activity of papain markedly contributed to serum antibody production of IgE and IgG subclasses (Fig. 3B, 4). We used the cysteine protease inhibitor, E64, which irreversibly binds to the catalytic site of papain without altering its tertiary structure.[14,39] The inactivation of protease activity by heat denaturation has been reported to disrupt the tertiary structure and/or cause oligomerization, which are likely to affect the immunochemical properties of allergens such as antibody binding[40,41] and the T-cell response.[14] Our results using E64-treated papain indicate that elevated serum antibody production is dependent on the cysteine protease activity of papain, but not to conformational changes or aggregation, which may cause different immune responses. Considering the serum dilution factors and ODs in ELISA for the papain-specific IgG subclasses, the epicutaneous administration of papain induced the most significant production of IgG1, the class-switch to which and IgE Th2 cytokines contribute to, among the IgG subclasses measured (Fig. 2, Fig. 4).

To the best of our knowledge, this is the first study

to report epicutaneous sensitization dependent on protease activity of the antigen, which suggests that epicutaneous exposure to environmental proteases promotes allergic sensitization. Allergen source-derived or microbe-derived proteases, some of which are allergens (protease allergens), have been shown to cause barrier breakdown and induce innate immune responses in various cell types.[1,3-7] The mouse models described here should be prototype models, which will be useful for analyzing the natural history of allergic diseases initiated with epicutaneous sensitization to proteases. We are now conducting further analyses using these models to explore the mechanisms of the protease-dependent epicutaneous sensitization and its roles in allergic diseases.

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