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ORIGINAL ARTICLE

A New Reliable Method for Detecting Specific IgE Antibodies in the Patients with Immediate Type Wheat Allergy due to Hydrolyzed Wheat Protein: Correlation of Its Titer and Clinical Severity

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ABSTRACT

Background: Immediate-type wheat allergy caused by a specific hydrolyzed wheat protein (HWP-IWA), Glupearl 19S (GP19S), typically develops food-dependent exercise-induced anaphylaxis (FDEIA), but is different from conventional FDEIA, or simple wheat allergy in many aspects. The skin prick test (SPT) is considered to be the most effective method for diagnosis of HWP-IWA. As SPT is a relatively qualitative method, we developed quantitative and high-throughput test method for HWP-IWA.

Methods: An enzyme-linked immunosorbent assay (ELISA)-based GP19S-specific IgE assay was tested using sera from 14 HWP-IWA and five conventional wheat-dependent exercise-induced anaphylaxis (CO-WDEIA) patients, as well as five healthy subjects. Then a validation study at five different institutions was carried out using sera from 10 HWP-IWA and five CO-WDEIA patients, as well as five healthy subjects different from the previous studies.

Results: The mean unit values converted from measured absorbance of ELISA were 68.3, 1.3 and 1.1 respectively. Furthermore, the validation study revealed reproducible results across all five institutions, with the standard deviation (SD) being 0.3-0.4 for the healthy group, 0.2-0.6 for the CO-WDEIA group, and 3.8-9.6 for HWP-IWA group except for one case. One case of HWP-IWA was excluded from analysis due to the high SD of 53.3 units, indicating that samples with a unit value > 100.0 will affect inter-laboratory reproducibility.

Conclusions: Our findings suggest that the ELISA-based GP19S-specific IgE assay can be used to test HWP-IWA using venous blood samples, except for those with a unit value > 100.0.

KEY WORDS

enzyme-linked immunosorbent assay, Glupearl 19S, hydrolyzed wheat protein, immediate-type wheat allergy, test method

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INTRODUCTION

Many cases of Immediate-type allergy after wheat intake have been observed in Japanese consumers using cosmetics that contain hydrolyzed wheat protein (HWP), HWP-IWA, who had used "Cha no Shizuku" soap (sold by Yuuka, Fukuoka, Japan) that contained Glupearl 19S (GP19S), a substance manufactured by Katayama Chemical Industries, Osaka, Japan. HWP-IWA patients, but not conventional wheat allergy patients, react to GP19S. Therefore, GP19S hypersensitivity is essential for diagnosis of HWP-IWA.[1,2]

HWP is a cosmetic ingredient specified in the Japanese Standards of Quasi-drug Ingredients, and which is a collective term for water-soluble materials that are produced by hydrolyzing wheat glutens with acid, alkali, enzymes or other substances. Cases of HWP allergy have been reported in Western nations; notably, cases are fewer and less severe than those in Japan.[3-6] In order to address the problem of HWP allergy, the "Special Committee for the Safety of Protein Hydrolysates in Cosmetics" was organized by the Japanese Society of Allergology to study the epidemiology, pathogenesis, and establish diagnostic criteria, among other activities.

HWP-IWA is different from conventional wheat allergy. In contrast to the onset of conventional wheat allergy in children, HWP-related allergy arise in adults with a history of cosmetic use.[7] Although both conventional and HWP-related wheat allergy in adults can cause wheat-dependent exercise-induced anaphylaxis (WDEIA), unlike conventional WDEIA (CO-WDEIA), HWP-IWA is not mediated by ω-5 gliadin.[8]

Currently, the skin prick test (SPT) by GP19S is considered the most useful method in diagnosing HWP-IWA.[7] It would be advantageous to develop a quantitative, high-throughput method for the laboratory diagnosis of HWP-IWA that gives consistent results across different institutions. For this purpose, we evaluated the utility of GP19S-specific IgE antibody detection by enzyme-linked immunosorbent assay (ELISA) for the diagnosis of HWP-IWA using sera from healthy individuals and from patients with CO-WDEIA or HWP-IWA. Five institutions were involved in this study to assess the reliability of this method.

METHODS

SUBJECTS

The HWP-IWA group consisted of 24 patients diagnosed with immediate-type wheat allergy induced by GP19S according to the diagnostic criteria by Special Committee for the Safety of Protein Hydrolysates in Cosmetics; CO-WDEIA group consisted of 10 patients with conventional WDEIA; and the healthy control group consisted of 10 individuals without wheat allergy (Table 1, 2). HWP-IWA patient 1 to 14, CO-WDEIA patient and healthy control 1 to 5 were used for utility evaluation of ELISA-based GP19S-specific IgE assay. HWP-IWA patient 15 to 24, CO-WDEIA patient and healthy control 6 to 10 were used for validation this method. Patients 1 to 14 in the HWP-IWA group were classified into the following four grades of severity based on symptoms after wheat ingestion: grade 1, eyelid swelling, symptoms limited to the face and nasal mucosa; grade 2, generalized urticaria in addition to grade 1 symptoms; grade 3, systemic symptoms (e.g. dyspnea, diarrhea) in addition to dermal swelling; and grade 4, anaphylactic shock. The presence of specific serum IgE was determined using ImmunoCAP (Thermo Fisher Scientific, Phadia AB, Uppsala, Sweden). Sensitivity to GP19S was evaluated using SPT.

ELISA-BASED GP19S-SPECIFIC IgE ASSAY

GP19S (Katayama Chemical Industries) at 1 mg/ml dissolved in phosphate-buffered saline (PBS) was centrifuged and the supernatant was recovered (GP19S solution). Next, 100 µl of GP19S solution was added to each well of a Nunc MaxiSorp flat bottom 96-well plate (Thermo Fisher Scientific, Waltham, MA, USA), and the plate was sealed and left overnight at 4°C. The plate was blocked with 1% skim milk/PBS with 0.1% Tween 20 (PBS-T) for 1 hour at room temperature, after which 100 µl patients' sera diluted to 20% in 1% skim milk/PBS-T were added to the wells, followed by a further incubation for 1 hour at room temperature. The plate was then washed with 1% skim milk/PBS-T. A total of 100 µl of 0.1 µg/ml anti-human IgE-HRP conjugate (KPL, Gaithersburg, MD, USA) in 1% skim milk/PBS-T was added to the wells, and the plate was incubated for 1 hour at room temperature. The plate was washed, and the colorimetric reaction was developed by adding 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific) and incubated for 15 min at room temperature. The reaction was stopped by adding 2 M H_2SO_4. Absorbance was measured by multi-plate optic densitometories, VersaMax (Molecular Devices, Sunnyvale, CA, USA), with a wavelength of 450 nm.

CONVERSION OF ABSORBANCE INTO "UNIT" VALUES

Serum taken from HWP-IWA patient 5 was chosen as the standard. Serial dilution was performed using 1% skim milk/PBS-T, starting at 40 times dilution, with subsequent doubling of the dilution factor up to 5120 times dilution. The GP19S-specific IgE ELISA was performed as described above. To create a curve for the conversion of absorbance values to "unit" values, the absorbance of the 40 times-diluted serum was defined as that corresponding to 100.0 units, with the absorbance of the 80 times-diluted serum as 50.0 units, and that of the 640 times-diluted serum as 6.3 units, and so forth, such that the absorbance at each dilution factor corresponds to a "unit" value. For each

Table 1 Clinical characteristics of the patients with HWP-IWA

ID	Age	Sex	Past allergic history	Severity	CAP-FEIA						GP19S Skin prick test
					wheat-sIgE		gluten-sIgE		ω-5 gliadin-sIgE		Threshold for positive prick reaction (%)
					(UA/mL)	(Class)	(UA/mL)	(Class)	(UA/mL)	(Class)	
HWP-IWA 1	47	F	Pollinosis	4	4.52	3	7.16	3	0.34>	0	0.001
HWP-IWA 2	38	F	Pollinosis, Graves disease	4	4.1	3	4.71	3	0.34>	0	0.0001
HWP-IWA 3	43	F	Non	4	2.21	2	1.94	2	0.34>	0	0.01
HWP-IWA 4	18	F	Atopic dermatitis, Asthma, Pollinosis	4	2.28	2	5.37	3	0.71	2	0.01
HWP-IWA 5	45	F	Atopic dermatitis (Childhood), Rhinitis, Pollinosis	4	25.1	4	57.3	5	0.68	1	0.001
HWP-IWA 6	61	F	Pollinosis	3	0.72	2	0.98	2	0.34>	0	0.001
HWP-IWA 7	62	F	Non	4	4.44	3	6.41	3	0.34>	0	0.001
HWP-IWA 8	33	F	Pollinosis	3	<0.35	0	<0.35	0	0.34>	0	0.01
HWP-IWA 9	44	F	Non	3	0.35	1	0.73	2	0.34>	0	0.001
HWP-IWA 10	49	F	Rhinitis	2	1.08	2	1.53	2	0.34>	0	0.001
HWP-IWA 11	43	F	Non	3	3.6	3	7.89	3	1.29	2	0.001
HWP-IWA 12	37	F	Non	2	0.67	1	1.41	2	0.34>	0	0.001
HWP-IWA 13	63	F	Contact dermatitis	1	<0.35	0	0.56	1	0.34>	0	0.01
HWP-IWA 14	30	F	Rhinitis, Pollinosis, Metal allergy	1	0.45	1	0.75	2	0.34>	0	0.10

sIgE, specific IgE.

Severity: 1: eyelid swelling, symptoms limited to face and nasal mucosa; 2: generalized urticaria besides Stage 1 symptoms; 3: general symptoms in addition to dermal disorders (diarrhea, dyspnea, etc.); 4: anaphylactic shock.

GP19S Skin prick test : GP19S was diluted to 100 μg/ml in sterile physiologic saline (PS) and then made into solutions at concentration from 0.00001% to 0.1%. Reactions were read at 15 min, a wheal at least half the size of that caused by histamine dihydrochloride (10 mg/ml) or 3 mm was considered a positive reaction.

serum, a "unit" value was obtained from the measured absorbance with 5 times-diluted serum samples according to this curve.

CORRELATION BETWEEN LABORATORY VALUES AND CLINICAL SEVERITY

Pearson's correlation coefficient (Pearson's product-moment correlation coefficient, represented by the letter *r*), between grades of severity and the following test values were calculated: wheat-specific IgE (UA/mL), gluten-specific IgE (UA/mL), ω-5 gliadin-specific IgE (UA/mL), GP19S SPT positive concentration (%), and GP19S-specific IgE (unit).

VALIDATION STUDY OF THE ELISA-BASED GP19S-SPECIFIC IgE ASSAY AT FIVE INSTITUTIONS

In order to validate and determine the inter-laboratory reproducibility of the ELISA-based GP19S-specific IgE Assay, the method was performed at five institutions affiliated with members of the Special Committee for the Safety of Protein Hydrolysates in Cosmetics. A manual was complied and distributed prior to the study to ensure common understanding of the technique and to allow discussion of uncertainties. All participating institutions used the same reagents and consumables that were prepared by members of Fujita Health University School of Medicine. The microplate reader for absorbance detection and other laboratory equipment were prepared by each institution. ELISA was performed using sera from 10 HWP-IWA and five CO-WDEIA patients, as well as five healthy subjects. Each sample was tested in duplicates to obtain absorbance and unit values. The absorbance and unit values obtained by all five institutions were examined to determine the validity of the test conditions. The standard deviation (SD) of absorbance and unit values was calculated to assess inter-laboratory reproducibility.

ETHICAL CONSIDERATION

This study was approved by the Ethics Committee of Fujita Health University (No. 11-210). Venous blood samples were collected with patients' informed consent.

RESULTS

ELISA-BASED GP19S-SPECIFIC IgE ASSAY

The range of measured absorbance was 0.01-0.09 optic density (OD) (mean, 0.04 OD) for the healthy control group (n = 5), 0.00-0.11 OD (mean, 0.05 OD) for the CO-WDEIA group (n = 5), and 0.21-3.92 OD

Table 2 Laboratory findings of the sera from patients and controls in the validation study

ID	Total IgE	CAP-FEIA						GP19S Skin prick test
		wheat-sIgE		gluten-sIgE		ω-5 gliadin-sIgE		
	(U/ml)	(UA/mL)	(Class)	(UA/mL)	(Class)	(UA/mL)	(Class)	
Healthy 6	8.16	0.34>	0	0.34>	0	0.34>	0	Nagative
Healthy 7	138	0.34>	0	0.34>	0	0.34>	0	Nagative
Healthy 8	NT	NT	NT	NT	NT	NT	NT	NT
Healthy 9	NT	NT	NT	NT	NT	NT	NT	NT
Healthy 10	NT	NT	NT	NT	NT	NT	NT	NT
CO-WDEIA 6	NT	0.57	1	2.65	2	0.34>	0	Nagative
CO-WDEIA 7	NT	2.37	2	1.19	2	9.73	3	Nagative
CO-WDEIA 8	148	0.46	1	2.51	2	13.3	3	Nagative
CO-WDEIA 9	NT	3.36	3	1.48	2	NT	NT	Nagative
CO-WDEIA 10	NT	0.55	1	3.85	3	9.39	3	Nagative
HWP-IWA 15	3650	8.91	3	NT	NT	NT	NT	Positive
HWP-IWA 16	36	0.34>	0	0.39	1	0.34>	0	Positive
HWP-IWA 17	101	0.85	2	2.91	2	0.34>	0	NT
HWP-IWA 18	285	0.77	2	1.84	2	0.34>	0	Positive
HWP-IWA 19	82	4.25	3	7.18	3	0.34>	0	Positive
HWP-IWA 20	738	13.1	3	24.3	4	0.34>	0	Positive
HWP-IWA 21	148	4.44	3	6.41	3	0.34>	0	Positive
HWP-IWA 22	442	0.54	1	1.23	2	0.34>	0	Positive
HWP-IWA 23	2343	3.55	3	4.27	3	0.34>	0	Positive
HWP-IWA 24	67	0.4	1	0.6	1	0.34>	0	Positive

NT, Not tested; sIgE, specific IgE.

Skin prick test: Reactions were read at 15 min, a wheal at least half the size of that caused by histamine dihydrochloride (10 mg/ml) or 3 mm was considered a positive reaction.

(mean, 2.10 OD) for the HWP-IWA group (n = 14). The absorbance values of the healthy and CO-WDEIA groups were relatively low and no marked difference was observed between the two groups. On the other hand, a wide range of absorbance values were observed in the HWP-IWA group, and were markedly different from those in the healthy and CO-WDEIA groups (Table 3).

CONVERSION OF ABSORBANCE INTO "UNIT" VALUES

Serial dilution of the serum sample resulted in a decrease in the measured absorbance, producing a standard curve. After assigning arbitrary "unit" values to the measured absorbance at each dilution factor, the following unit values were obtained from the measured absorbance: a range of 0.2-2.1 (mean, 1.1) for the healthy group, 0.0-2.5 (mean, 1.3) for the CO-WDEIA group, and 5.2-115.5 (mean, 59.5) for the HWP-IWA group (Table 3).

CORRELATION BETWEEN LABORATORY VALUES AND CLINICAL SEVERITY

The correlation coefficients of clinical severity and wheat-, gluten- and ω-5 gliadin-specific IgE antibodies were 0.43, 0.36 and 0.24, respectively. The correlation coefficient of clinical severity and GP19S SPT positive concentration was -0.53, which is high enough for quantitative diagnosis of HWP-IWA, but not high enough to indicate a correlation with severity. The correlation coefficient of severity and GP19S-specific IgE was 0.76, which was higher than all other parameters (Table 4).

VALIDATION STUDY OF ELISA-BASED GP19S-SPECIFIC IgE ASSAY AT FIVE INSTITUTIONS

The results for GP19S-specific IgE were obtained by each institution. Notably, the absorbance and unit values were low in the healthy and CO-WDEIA groups but high in the HWP-IWA group across all institutions. All samples were tested in duplicates and similar absorbance and unit values were obtained. The respective SD for absorbance and unit values were 0.02-0.05 OD and 0.3-0.4 in the healthy group, and 0.03-0.04 OD and 0.2-0.6 in the CO-WDEIA group. In the HWP-IWA group, the SD ranged from 0.19-0.31 OD and 3.8-9.6 for HWP-IWA 16-24, and it was as high as 0.93 OD and 53.3 for HWP-IWA 15. It was observed that the SD became higher as the GP19S-specific IgE level increased. We consider that a high inter-laboratory reproducibility is achieved only when the "unit" value is below 100.0 (Table 5).

Table 3 Results of the ELISA-based GP19S-specific IgE assay

ID	Absorbance	Unit
Healthy 1	0.01	0.2
Healthy 2	0.01	0.2
Healthy 3	0.05	1.2
Healthy 4	0.07	1.6
Healthy 5	0.09	2.1
CO-WDEIA 1	0.11	2.5
CO-WDEIA 2	0.06	1.4
CO-WDEIA 3	0.00	0.0
CO-WDEIA 4	0.03	0.7
CO-WDEIA 5	0.07	1.7
HWP-IWA 1	3.92	115.5
HWP-IWA 2	3.89	114.5
HWP-IWA 3	2.60	71.3
HWP-IWA 4	3.89	114.5
HWP-IWA 5	3.89	114.5
HWP-IWA 6	3.60	104.0
HWP-IWA 7	2.54	69.7
HWP-IWA 8	0.28	6.9
HWP-IWA 9	0.43	10.8
HWP-IWA 10	1.49	39.0
HWP-IWA 11	1.20	30.9
HWP-IWA 12	1.10	28.3
HWP-IWA 13	0.35	8.7
HWP-IWA 14	0.21	5.2

Absorbance, absorbance at 450 nm.

Table 4 Correlation between clinical severity and laboratory findings

	r	*p*
wheat-specific IgE (UA/mL)	0.43	0.12
gluten-specific IgE (UA/mL)	0.36	0.20
ω-5 gliadin-specific IgE (UA/mL)	0.24	0.41
GP19S SPT positive concentration (%)	-0.53	0.052
GP19S-specific IgE (unit)	0.76	0.0015

Positive concentration, threshold for positive prick reaction.

Correlation coefficient *r* were calculated by Pearson's product-moment correlation coefficient.

Table 5 Combined ELISA results from the five institutions

ID	Absorbance		Unit	
	Mean	SD	Mean	SD
Healthy 6	0.08	0.05	0.8	0.4
Healthy 7	0.07	0.04	0.7	0.3
Healthy 8	0.07	0.03	0.6	0.3
Healthy 9	0.08	0.02	0.8	0.3
Healthy 10	0.08	0.05	0.8	0.3
CO-WDEIA 6	0.11	0.03	1.5	0.5
CO-WDEIA 7	0.10	0.03	1.3	0.5
CO-WDEIA 8	0.08	0.03	0.8	0.2
CO-WDEIA 9	0.08	0.03	0.9	0.2
CO-WDEIA 10	0.10	0.04	1.2	0.6
HWP-IWA 15	4.36	0.93	154.1	53.3
HWP-IWA 16	3.22	0.31	97.0	9.2
HWP-IWA 17	3.06	0.23	90.1	9.6
HWP-IWA 18	3.10	0.19	92.0	8.8
HWP-IWA 19	2.60	0.28	72.0	5.9
HWP-IWA 20	2.59	0.30	71.8	5.7
HWP-IWA 21	2.38	0.24	63.6	6.1
HWP-IWA 22	1.55	0.25	36.7	3.8
HWP-IWA 23	1.73	0.24	42.3	3.9
HWP-IWA 24	1.32	0.24	30.1	4.5

Absorbance, absorbance at 450 nm.

DISCUSSION

In Japan, approximately 4.7 million people bought 46.7 million cakes of "Cha no Shizuku" soap that contained GP19S. According to an epidemiological study released online by the Japanese Society of Allergology on 20 November 2013, there were 2026 cases of HWP-IWA, of which 95.9% were females mainly in their 40s. About half of these cases experienced anaphylactic symptoms, of which half experienced anaphylactic shock. Many of these patients developed WDEIA suddenly, manifesting as eyelid edema after eating wheat-containing food, even though no symptoms appeared while using the soap.[7] This phenomenon was also noted in patients in our study (Table 1). Unsuspectingly, patients with oral wheat allergy continued to use the offending soap, highlighting the possibility of a large number of patients who are unaware of their condition.

As a result of sensitization to GP19S contained in the soap, the produced IgE cross-reacts with orally ingested wheat protein.[8] SPT using GP19S is considered a fast and sensitive method for the diagnosis of HWP-IWA; the condition is ruled out if the SPT using 0.1% GP19S solution is negative. However, some patients decline SPT, which causes discomfort and can induce a severe allergic reaction. In addition to SPT, the Special Committee for the Safety of Protein Hydrolysates in Cosmetics recommends other immunological methods such as dot blotting, ELISA, Western blotting (patient is considered HWP-IWA-positive if GP19S-specific IgE is detected in the blood), or basophil activation test that uses GP19S as the antigen (a positive result suggests HWP-IWA).

Reports exist regarding the diagnosis of wheat allergy using various immunological methods. Western blotting for GP19S using serum IgE antibody has been employed at many institutions.[1,2,8] Using patients' basophils, Hiragun *et al.* conducted the histamine release test[9] and Chinuki *et al.* performed the

CD203c expression-based basophil activation test.[10] Nakamura *et al.* described the EXiLE (IgE Cross linking-induced Luciferase Expression) method, which uses a rat mast cell line expressing human IgE antibody receptors.[11] Though useful, the abovementioned methods have drawbacks. Quantitative evaluation is difficult in Western blotting. Basophil-based tests and the EXiLE method are not widely available, and samples cannot be preserved in the former.

Continuing investigation into HWP-IWA will be required to assess the incidence, natural clinical course, allergenicity, treatment, and appropriate patient education. We believe that the most important issue was the development of a quantitative, high-throughput, GP19S-specific IgE diagnostic test that can provide consistent results at any institution. For these reasons, we tested an ELISA-based assay that would satisfy these conditions.

First, we compared the results of GP19S-specific IgE measurement by ELISA between healthy controls (five subjects), CO-WDEIA (five patients) and HWP-IWA patients (14 patients). Under the described test conditions, the measured absorbance was high in the HWP-IWA group but low in the healthy and CO-WDEIA groups, suggesting that ELISA is effective for the diagnosis of HWP-IWA (Table 3). Using the absorbance results from one patient's serum sample (HWP-IWA 5), "unit" values were assigned to absorbance values in order to improve inter-test and inter-laboratory reproducibility. Using one patient's serum as a standard is disadvantageous, as the created standard cannot be reproduced at other institutions. Therefore, dilution series using sera from other HWP-IWA patients were performed to determine whether the resulting curves are comparable to that of HWP-IWA 5; similar curves were obtained in each case (data not shown). This suggests that it is unnecessary to use the sample of HWP-IWA 5 as the standard if the relative concentration of antibodies against GP19S to HWP-IWA 5 can be determined.

The ELISA results differed widely among HWP-IWA patients (1 to 14); therefore, we examined the correlation between clinical severity and laboratory values to analyze the nature of these differences. GP19S-specific IgE had a higher degree of correlation with severity than gluten and wheat (Table 4), suggesting that it may be predictive of symptoms experienced by HWP-IWA patients. We believe that such data will be useful in gauging the effect of treatment and for patient education. The ELISA-based GP 19S-specific IgE assay provides quantitative results that are meaningful in predicting disease severity.

In the validation study conducted at five different institutions, repeat tests demonstrated reproducible results, with the HWP-IWA group consistently showing higher values. This suggests that the test conditions were appropriate. However, it was noted that one sample (HWP-IWA 15) had a high SD of 53.3, which was different from the SD of the HWP-IWA (<9.6), CO-WDEIA (<0.6) and healthy groups (<0.4) (Table 5). The absorbance of HWP-IWA 15 sample exceeded the maximum measurable limit of the microplate reader at each institution, thus causing the large SD. To ensure inter-laboratory reproducibility, we practically set the maximum "unit" value at 100.0.

A total of 10 healthy subjects, 10 CO-WDEIA and 24 HWP-IWA patients were examined in the present study. The respective maximum unit values in the healthy and CO-WDEIA groups were 2.1 and 2.5 units, while the minimum unit value in the HWP-IWA group was 5.2 units. Therefore, taking between 2.5 to 5.2 the cut-off value, the diagnostic criterion was set as follows: <3.0, negative; 3.0-5.0, suspected; >5.0, positive. Results based on this criterion were consistent with available SPT results. Cases (HWP-IWA 8, 16 and 24) with negative ImmunoCAP results (class 2 and above were considered positive) were positive based on this criterion. These observations suggest that our ELISA-based GP19S-specific IgE assay is a sensitive diagnostic method.

Our findings suggest that the ELISA-based GP19S-specific IgE assay is a useful quantitative and high-throughput method for the diagnosis of HWP-IWA. This method enables the examination and diagnosis of cases nationwide using a laboratory-based, measurable criterion. Hiragun *et al.* studied wheat- and gluten-specific IgE antibodies with CAP-FEIA, and glutenin-specific IgE with histamine-release test, concluding that HWP-IWA may get better over time.[9] We believe that the results of the ELISA-based GP19S-specific IgE assay accurately reflect the clinical situation. We envisage that this method will be useful not only for patients and doctors, but also for medical researchers and cosmetics makers. Many challenges remain regarding this disease, but we expect our test method to advance the diagnosis of this condition.

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Establishment of a mast cell line, NCL-2, without *Kit* mutation, derived from NC mouse bone marrow

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ABSTRACT

Immortal mast cell lines, such as RBL-2H3 and HMC-1 cells, are commonly utilized to investigate the function of mast cells. However, they are tumor cells carrying a gain-of-function mutation of *Kit*. We established an immortal mast cell line without *Kit* mutation, NCL-2, derived from NC mouse bone marrow. NCL-2 cells could be maintained without additional growth factors and thus could respond to exogenous growth signals. Moreover, NCL-2 cells expressed FcεRI and KIT, and release histamine and LTB_4 in response to antigen stimulation. This cell line could be a useful tool to analyze proliferation, differentiation, and function of normal mast cells.

1. Introduction

Mast cells play a pivotal role in type I hypersensitivity reactions via the high affinity IgE receptors (FcεRI) and antigen-specific IgE. The crosslinking of FcεRI leads to the activation of numerous signaling molecules, SYK, LAT, PLCγ, PKC, and finally results in degranulation, and arachidonic acid and cytokine production [1,2]. These mediators are involved in the pathogenesis of allergic disorders, such as urticaria, atopic dermatitis (AD), allergic rhinitis, and allergic asthma [2].

Previous studies on the function of mast cells, especially on degranulation and on signaling pathways mediated by FcεRI crosslinking, have been conducted mostly by the use of RBL-2H3 cells [3]. RBL-2H3 cells carry a gain-of-function mutation of *Kit* [4] thus they do not need exogenous growth factors, such as stem cell factor (SCF) or interleukin (IL)-3. Investigators can maintain the cells without expensive recombinant cytokines but they cannot assess cellular responses against SCF. In addition, it is possible that active KIT protein could affect other signaling pathways such as FcεRI-mediated signaling events [5]. Moreover, Passante et al. reported that RBL-2H3 cells share similarities with basophils and thus they are an imprecise model for mast cell mediator release [6]. MC/9 cells [7], the rodent mast cells without *Kit* mutation, need IL-3 to grow. HMC-1 cells, a human mast cell line derived from mast cell leukemia, carry a gain-of-function mutation of *Kit* and lack surface expression of FcεRI [8]. Another human mast cell line, LAD2 cells, responds to SCF but grows slowly even in the presence of 100 ng/ml of recombinant human SCF [9].

Here we established NCL-2 mast cell line without a gain-of-function of *Kit* mutation, which respond to SCF and IL-3, and release various chemical mediators against several stimuli including crosslinking of FcεRI.

2. Materials and methods

2.1. Reagents

Recombinant mouse IL-3 (rmIL-3) and stem cell factor (SCF) were purchased from R&D Systems (Minneapolis, MN, USA). Human serum albumin (HSA)-conjugated dinitrophenyl (DNP), ionomycin, and adenosine triphosphate (ATP) were from Sigma (St. Louis, MO, USA). Rat DNP-specific IgE monoclonal antibody was from Biosource International (Camarillo, CA, USA).

2.2. Animals

Male NC/Kuj mice (6 weeks) were kindly donated by Dr. J. Hayakawa, Institute for Experimental Animals, School of Medicine, Kanazawa University, Japan and Balb/c nude mice were purchased from Charles River Japan (Yokohama, Japan). Mice were maintained in closed racks with free access to food and water in the Institute of Laboratory Animal Science, Hiroshima University. This study was carried out in accordance with the Guideline for Animal

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2.3. Cells

The bone marrow-derived mast cells (BMMC) from 6 weeks old male mice were generated as previously described [10]. Briefly, bone marrow cells were suspended at a density of 1×10^6 cells/ml in Minimum Essential Medium α (MEMα) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf-serum (FCS), 50 μM 2-mercaptoethanol, 2 mM L-glutamine, antibiotics (complete MEMα), and 5 ng/ml IL-3. The cells were cultured for 4 weeks, replacing half of the existing medium with fresh medium once a week. After 4 weeks, more than 98% of non-adherent cells were stained positively by alcian blue. The BMMC were subcultured about 100 times by 50-fold dilutions in the presence of IL-3. Subsequently, the cells were subcultured another 80 times by 100-fold dilutions without IL-3, and then a single clone (NCL-2) that grew without IL-3 was obtained by limiting dilution.

P815 murine mastocytoma cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% FCS and antibiotics.

2.4. Release of histamine and LTB_4

NCL-2 cells were incubated in complete MEMα supplemented with 0.5 μg/ml of DNP-specific IgE overnight. After washing twice with complete MEMα, the cells were stimulated with antigen, ionomycin or ATP at the indicated concentration for 20 min at 37 °C. The supernatants were transferred into test tubes and centrifuged at 1500×g for 5 min at 4 °C to eliminate the cell components.

2.5. Measurement of histamine

After centrifugation, 200 μl of the supernatants were mixed with equal volumes of 0.5 N perchloric acid to precipitate proteins. Both cell pellets in test tube and residual cell in culture dishes were resolved in 1 ml of 0.25 N perchloric acid to recover all residual histamine. Histamine contents in the samples were measured fluorometrically by using an automated histamine analyzing system (Tosoh Corporation, Osaka, Japan) as described previously [11]. The histamine release was expressed as percentage of total cellular histamine.

2.6. Measurement of LTB_4

The amount of leukotriene B_4 (LTB_4) in the samples was measured using enzyme-immunoassay kits (Amersham, Buckinghamshire, UK). The measurements were performed according to the manufacture's instruction. The minimum detectable concentration of LTB_4 was 6.0 pg/ml and cross-reactivity for other related substances was less than 0.03%. In order to verify the measurement of LTB_4 by enzyme immunoassay, the supernatant of reaction mixture in one experiment was fractionated by reverse phase high performance liquid chromatography (HPLC) as described before [11].

2.7. Western blot analysis

For detection of IL-3-induced tyrosine phosphorylation of Jak2, 2.5×10^6 NCL-2 cells were stimulated with 100 ng/ml of rmIL-3 for 5 min at 37 °C, then quickly centrifuged at 10,000×g and lysed in the sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 50 mM DTT, 0.1% bromophenol blue). The proteins were boiled at 95 °C for 5 min, centrifuged at 10,000×g for 10 min, electrophoresed with 5–20% polyacrylamide gel (Atto, Tokyo, Japan), and transferred to a PVDF membrane (Immobilon-P, Merck Millipore, Billerica, MA, USA). The membrane was incubated with rabbit anti-phospho-JAK2 polyclonal antibody (Biosource International, 1:1000 dilution) or anti-JAK2 antibody (Cell Signaling Technology, Beverly, MA, USA, 1:1000 dilution). After washed with Tris-buffered saline with Tween 20, the membrane was incubated with HRP-conjugated anti-rabbit IgG antibody (Cell Signaling Technology, 1:2000 dilution). The reacted antibodies were visualized by using ECL (GE Healthcare, Buckinghamshire, UK) under a luminescent image analyzer (LAS-1000 plus, Fuji Film, Tokyo, Japan).

For detection of SCF-induced tyrosine phosphorylation of KIT, NCL-2 cells (2.5×10^6) were stimulated with 100 ng/ml of rmSCF for 5 min at 37 °C and lysed in 100 μl of the lysis buffer (20 mM Tris–HCl, pH7.4, 150 mM NaCl, 10% glycerol, 1% NP-40, 1 mM phenylmethylsulphonyl fluoride, 0.15 U/ml aprotinin, 10 mM EDTA, 10 μg/ml leupeptin, 100 mM sodium fluoride, 2 mM sodium orthovanadate) at 4 °C for 30 min. Cell lysates were clarified by centrifuging for 10 min at 10,000×g at 4 °C and incubated with 30 μl of Protein G-Sepharose 4FF (GE Healthcare) to eliminate non-specific binding to Protein G. Then the samples were incubated with rat anti-mouse KIT monoclonal antibody (ACK2, GIBCO-BRL, 1:100 dilution) for 1 h at 4 °C. Protein G-Sepharose 4FF was used to collect the antigen-antibody complexes. These immunoprecipitatants were analyzed by immunoblotting using HRP-conjugated anti-phosphotyrosine antibody (4G10) (Merck Millipore, 1:2000 dilution) or anti-KIT antibody (ACK2, 1:1000 dilution).

2.8. Flow cytometric analysis of FcεRI and KIT

NCL-2 cells (2×10^5) were suspended in 100 μl of PBS(−) containing 0.125 μg of FITC-conjugated anti-mouse FcεRI antibody, APC-conjugated anti-mouse KIT antibody (eBioscience, San Diego, CA, USA), or their isotype controls for 0.5 h on ice. Cells were washed and analyzed with Attune® Acoustic focusing cytometer (Applied Biosystems, Foster City, CA, USA).

2.9. Tumor growth in mice

NCL-2 and P815 cells (both 1×10^6) were suspended in 500 μl of PBS(−) and were injected subcutaneously into the left hind flank of Balb/c nude mice. Each tumor size was expressed as a product of a longest diameter and the shortest diameter. Three mice per group were used for the experiment.

2.10. DNA sequencing analysis of Kit

A DNA fragment including aspartic acid at codon 814 of *Kit* was amplified by PCR with sense primer: 5′-CCGGAATTCGAGACG TGACTCCTGCCATC-3′ and antisense primer: 5′-CCGCTCGAGCCCAT AGGACCAGACATCAC-3′. *EcoR I* site was adapted to the sense primer and *Xho I* site was adapted to the antisense primer. *EcoR I* and *Xho I*-digested PCR products were ligated into pME18S cut with the same enzymes. DNA sequencing of the plasmids was performed with ABI PRISMTM 310 genetic analyzer (Applied Biosystems). There was no substitution of Asp814 in 26 clones.

2.11. RT-PCR analysis of mouse mast cell proteases (mMCPs)

NCL-2 cells (3×10^3) were incubated with 100 ng/ml of IL-3 or SCF for 7 days and harvested for the extraction of total RNA with RNeasy Mini Kit (Qiagen, Hilden, Germany). The reverse transcription reaction of RNA was performed with QuantiTect™ reverse transcription kit (Qiagen). PCR analysis of the expression of mMCPs in NCL-2 cells were performed with Tks Gflex™ DNA polymerase

(Takara, Shiga, Japan) and the specific primers reported previously [12].

3. Results

3.1. NCL-2 cells grew without IL-3 or SCF, and expressed FcεRI and KIT on their surface

NCL-2 cells grew independently from exogenous growth factors except FCS (Fig. 1A). The doubling time of NCL-2 cells calculated by exponential growth equation was approximately 30.1 h. On the other hand, P815 cells showed more rapid growth than NCL-2 cells (Fig. 1B), and the calculated doubling time of P815 cells was approximately 12 h. The surface expression of FcεRI and KIT of NCL-2 cells were confirmed by flow cytometric analysis (Fig 1C and D).

3.2. Degranulation and LTB_4 production of NCL-2 in response to various stimuli

Next, we investigated whether NCL-2 could release chemical mediators in response to various external stimuli including antigens or not. NCL-2 cells were sensitized with anti-DNP IgE and then simulated with various concentrations of DNP-HSA. As shown in Fig. 2A and B, NCL-2 cells secreted histamine and produced LTB_4 in a dose-dependent manner. NCL-2 cells also showed degranulation in response to ionomycin and ATP as well (Fig. 2C and D).

3.3. Tumorigenic property of NCL-2 cells

We then estimated the tumorigenicity of NCL-2 cells in Balb/c nude mice. A mouse lymphoblast-like mastocytoma cell line, P815, showed rapid growth under the skin of nude mice and killed

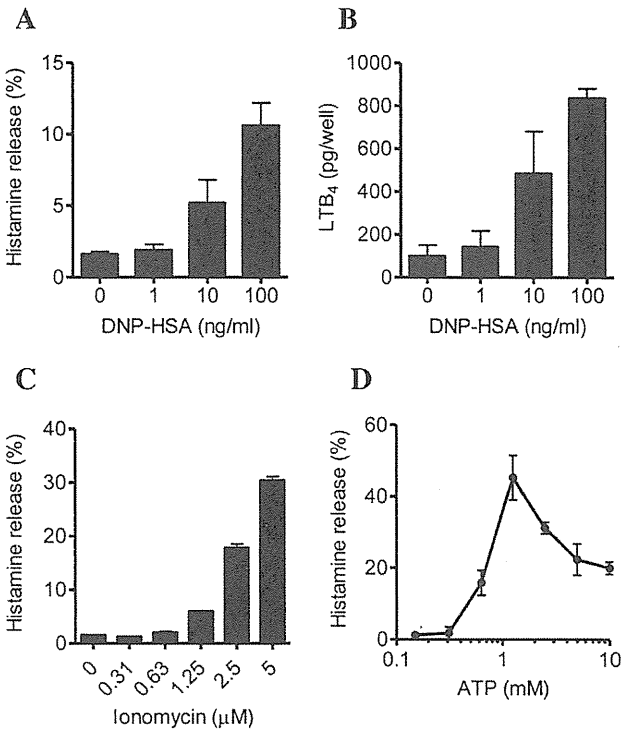

Fig. 2. NCL-2 cells release mediators in response to various stimuli. NCL-2 cells were sensitized with anti-DNP IgE, stimulated with DNP-HSA, and then the amount of histamine (A) and LTB_4 (B) released in culture supernatant were measured, respectively. NCL-2 cells were also stimulated with various concentrations of ionomycin (C) and ATP (D) and released histamine were measured. Data were expressed as mean ± SEM of three independent experiments done in duplicate.

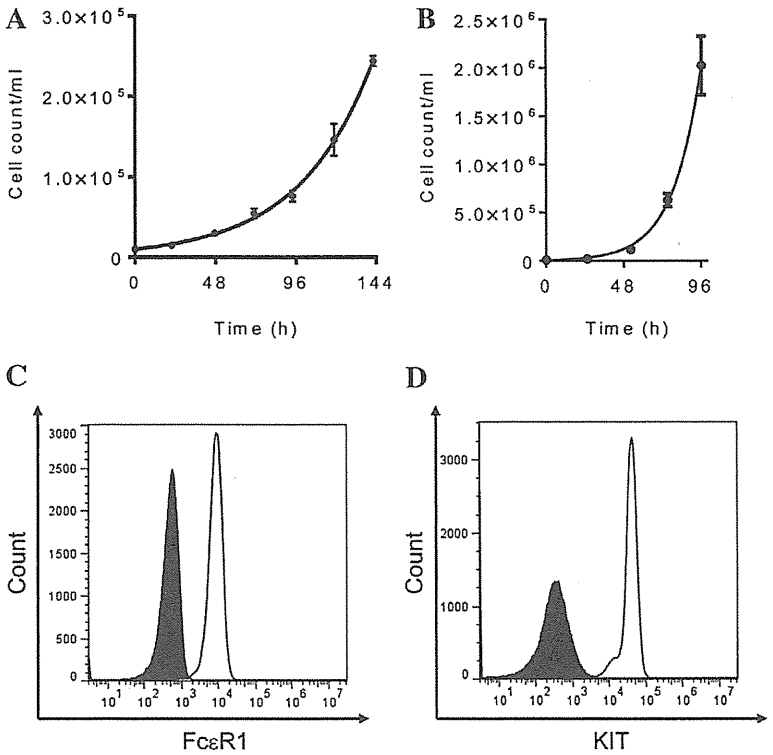

Fig. 1. NCL-2 mast cells are immortal and express FcεRI and KIT. 1×10^4/ml of NCL-2 cells (A) and P815 mastocytoma cells (B) were cultured without additional growth factors except for 10% FCS and the number of cells at each indicated time period was counted. Data were expressed as mean ± SD done in quadricate. NCL-2 cells were stained with FITC-conjugated anti-mouse FcεRI, APC-conjugated anti-mouse KIT, or their isotype controls. Surface expression of FcεRI (C) and KIT (D) of NCL-2 cells were shown as fluorescence intensity histograms (open area: specific antibodies, filled area: isotype controls).

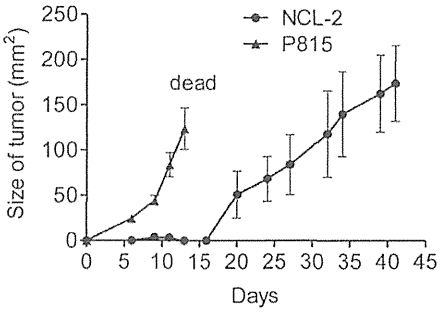

Fig. 3. Tumor formation of P815 and NCL-2 mast cell lines in nude mice. P815 and NCL-2 cells were injected subcutaneously into Balb/c nude mice. The size of each tumor at indicated time period was expressed as the product of a longest and a shortest diameter. Data were expressed as mean ± SEM of three mice of each group.

all of them within 2 weeks. Surprisingly, in contrast to P815, NCL-2 did not form tumors within 16 days. Even after inducing visible tumors, NCL-2 cells grew relatively slowly and did not kill mice at up to 40 days (Fig. 3).

3.4. Responses to exogenous growth factors, SCF and IL-3

Furthermore, we investigated whether or not NCL-2 cells could respond to exogenous growth stimuli. NCL-2 cells showed minimal tyrosine phosphorylation of KIT in quiescent state, and then showed robust tyrosine phosphorylation of KIT in response to SCF stimulation (Fig. 4A). NCL-2 cells also showed tyrosine phosphorylation of JAK2 in response to IL-3 stimulation (Fig. 4B).

3.5. The change in the expression profile of mMCPs by SCF and IL-3

NCL-2 cells were stimulated with 100 ng/ml of IL-3 and SCF for 7 days and the expression profile of mMCPs were studied. As shown in Fig. 5, NCL-2 cells, which were maintained without growth factors, already expressed mMCP-1, 4, 5, and CPA-3. Furthermore, the stimulation with IL-3 and SCF abolished the expression of mMCP-1, which connective tissue-type mast cells lack to express [13].

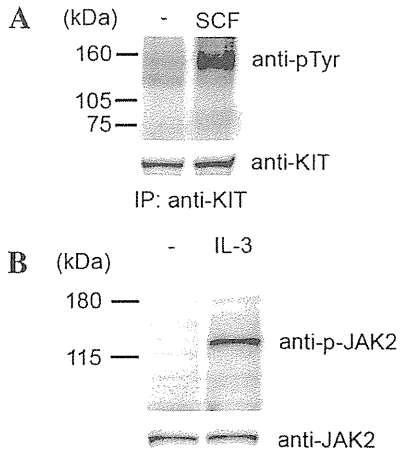

Fig. 4. NCL-2 cells respond to exogenous growth factors. (A) NCL-2 cells were stimulated with 100 ng/ml of SCF for 5 min and lysed. Cell lysates were immunoprecipitated with anti-KIT antibody. The immunocomplex was electrophoresed and blotted with anti-phosphotyrosine (anti-pTyr) or anti-KIT antibody. (B) NCL-2 cells were stimulated with 100 ng/ml of IL-3 for 5 min and lysed. Cell lysates were electrophoresed and blotted with anti-phosphorylated JAK2 or anti-total JAK2 antibody. A representative data of three independent experiments was shown for each panel.

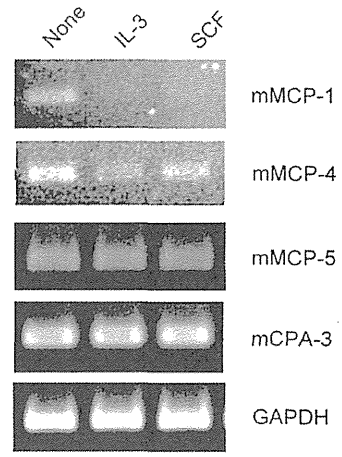

Fig. 5. The expression profiles of mMCPs in NCL-2 cells. NCL-2 cells (3×10^3) were incubated with 100 ng/ml of IL-3 or SCF for 7 days and harvested for RT-PCR analysis of the expression of mMCPs. A representative data of three independent experiments was shown.

4. Discussion

In this study, we established an immortal mast cell line, NCL-2, which can grow in the absence of SCF or IL-3. NCL-2 cells express KIT and functional FcεRI, and release histamine and LTB_4 in response to antigen stimulation. NCL-2 cells do not have a constitutively active mutation of *Kit*, thus show slow proliferation *in vitro* and substantially indolent tumor growth *in vivo* as compared with P815 mastocytoma cells. Moreover, stimulation with SCF and IL-3 induced tyrosine phosphorylation of KIT and JAK2, respectively.

NC mice were established as an inbred strain from Japanese fancy mice by Kondo in 1955. These mice spontaneously develop eczema and elevation of serum IgE similar to human AD in the conventional condition and thus are regarded as a mouse model of AD [14]. We previously reported that BMMC derived from NC/Kuj mice possess higher histamine content, higher adhesive ability, and especially, lack of apoptosis upon growth factor deprivation [15]. We, therefore, suspect that a certain population of BMMC from NC mice may survive without additional growth factors. In the process of establishing NCL-2 cells, we also obtained another immortal cell line, NCL-1, by the same long-term culture of BMMC of NC/Kuj mice. In contrast to NCL-2 cells, NCL-1 cells possessed an active mutation of *Kit* (D814F) on the single allele (data not shown). The mechanisms of immortality of NCL-2 remained unclear. However, we should keep in mind that BMMC of NC/Kuj has a potential to acquire a *de novo* active mutation of *Kit* in long-term cultures.

NCL-2 cells contained alcian-blue positive granules but not safranin-positive granules. Neither IL-3 nor SCF stimulation changed staining properties of granules even when NCL-2 cells were co-cultured with Swiss 3T3 cells for 2 weeks (data not shown). In general, BMMC express mMCP-5 and mCPA-3 without SCF, and express mMCP-4 with SCF [16]. On the other hands, NCL-2 cells express mMCP-4 without SCF stimulation, implying that NCL-2 cells might be partially differentiated mast cells. Furthermore, mucosal type mast cells (MMC) but not connective-tissue type mast cells (CTMC) express mMCP-1 [13]. IL-3 and SCF stimulation abolished the expression of mMCP-1, implying that these cytokines might shift NCL-2 cells towards CTMC-like cells.

In conclusion, NCL-2 cells do not need exogenous growth factors except for FCS to grow and are a useful tool to analyze both physiological and pathological functions of mast cells without the influence of constitutively active KIT.

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ORIGINAL ARTICLE ANAPHYLAXIS

Epidemiological link between wheat allergy and exposure to hydrolyzed wheat protein in facial soap

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Abstract

Background: Recent studies have highlighted the importance of extra-intestinal routes of sensitization to food-related allergens as the cause of epidemics of food allergy. Instances of Japanese women developing food allergy to wheat after exposure to hydrolyzed wheat protein (HWP) present in facial soap have been reported. However, the epidemiologic impact of these ingredients as a cause of food allergy has not been well studied.

Methods: To clarify the epidemiological relationship between food allergy to wheat and contact exposure to HWP, a case–control study of Japanese women aged 20–54 years with self-reported wheat allergy (WA) (cases, $n = 157$) and age-matched control subjects without WA (controls, $n = 449$) was performed using a large-scale Web-based research panel. Subjects answered a Web-based questionnaire regarding the use of skin and hair care products, as well as other possible risk factors.

Results: Current use of an HWP-containing facial soap (Cha no Shizuku; Yuka) was significantly associated with an increased risk of WA (adjusted odds ratio, 2.6; 95% confidence interval, 1.2–5.7; frequencies of current use in cases and controls; 11% and 6%, respectively). Use of Cha no Shizuku was more common in subjects with more recent-onset WA, implying that this soap may have contributed to the recent epidemic of WA.

Conclusions: An epidemiological relationship between WA and contact exposure to HWP has been documented. This study implicates a possible role of contact exposure to food-derived protein hydrolysates as a risk factor for the development of food allergy manifesting itself as anaphylaxis.

The burden of food allergy is an emerging public health problem worldwide (1, 2). The prevalence of adult-onset food allergy as well as childhood allergy has increased in recent decades (3). However, little is known about the cause of this increase, especially in adults. Recently, some studies have highlighted the importance of an extra-intestinal route of sensitization to food-related allergens, that is, environmental allergens, as the cause of food allergy (4–6). Birch pollen-related food allergy (7–9) and latex–fruit allergy syndrome (10, 11) are well-studied and characterized examples of this.

Reflecting the worldwide trend toward the use of 'natural materials' as ingredients of skin and hair care products including facial soaps, body soaps, cosmetics, shampoo, hair treatments and hair conditioner, food-derived protein hydrolysates is becoming increasingly commonly used as moisturizers or emulsifiers. Considering the potential importance of the extra-intestinal route of sensitization, it can be hypothesized that skin and/or rhinoconjunctival exposure to these ingredients may be a cause of the development of food allergy (12–16). However, epidemiological evidence supporting this hypothesis has been limited so far.

In late 2010, a small number of cases of Japanese women with wheat-dependent exercise-induced anaphylaxis (WDEIA)

Abbreviations
AD, atopic dermatitis; AR, allergic rhinitis; BA, bronchial asthma; CIs, confidence intervals; HWP, hydrolyzed wheat protein; OR, odds ratio; WA, wheat allergy; WDEIA, wheat-dependent exercise-induced anaphylaxis.

associated with skin and/or rhinoconjunctival contact exposure to hydrolyzed wheat protein (HWP) in facial soap were reported (17, 18). After the publication of these papers, numerous similar cases have been reported from various institutions in Japan (19–23). This suggests that primary sensitization to HWP contained in skin and hair care products resulting in the development of food allergy to wheat may be relatively common in Japanese women. It is hypothesized that HWP contained in these products might be contributing to the epidemic of wheat allergy (WA) in women.

The aim of this study was to elucidate the epidemiological link between WA and exposure to HWP contained in skin and hair care products. Because WA is relatively rare in the general population, a case–control design was chosen. We used a large-scale Web-based research panel to recruit wheat-allergic patients and age-matched controls. The use of skin and hair care products containing HWP was compared between the cases and controls.

Methods

Study design

A case–control study of Japanese women aged 20–54 years was performed. Both wheat-allergic subjects (cases) and control subjects were selected from the population living in Japan, which was obtained using a large-scale Web-based research panel. Characteristics of the studied Internet research monitor population are shown in the Supporting information. The ethics committee of Sagamihara National Hospital approved the study protocol (No. 7 in 2010).

Web-based survey

Figure 1 shows the protocol for the Web-based survey. First, the research company sent e-mails inviting participation in the screening survey. These were sent to an age-stratified random sample of subjects in the research panel between January 14 and January 25, 2011. No further invitations were sent once 200 000 responses had been obtained. The Web-based screening questionnaire consisted of five questions regarding WA (Q1–Q5, shown in the Appendix S1). Subjects (hereafter, cases) were considered as having self-reported WA if they met the following three criteria: (i) affirmative response to Q1, 'Do you experience any allergic symptoms after eating certain foods?'; (ii) indicating 'wheat' in the response to Q2, 'Which of the foods listed below are you allergic to?'; and (iii) indicating <2 h in their response to Q3, 'How many hours after eating wheat do your allergic symptoms appear?'. Three times as many age-matched controls were also chosen from subjects who denied food allergy in Q1. Consequently, e-mail of invitations to the secondary survey was sent to 328 cases and 984 controls.

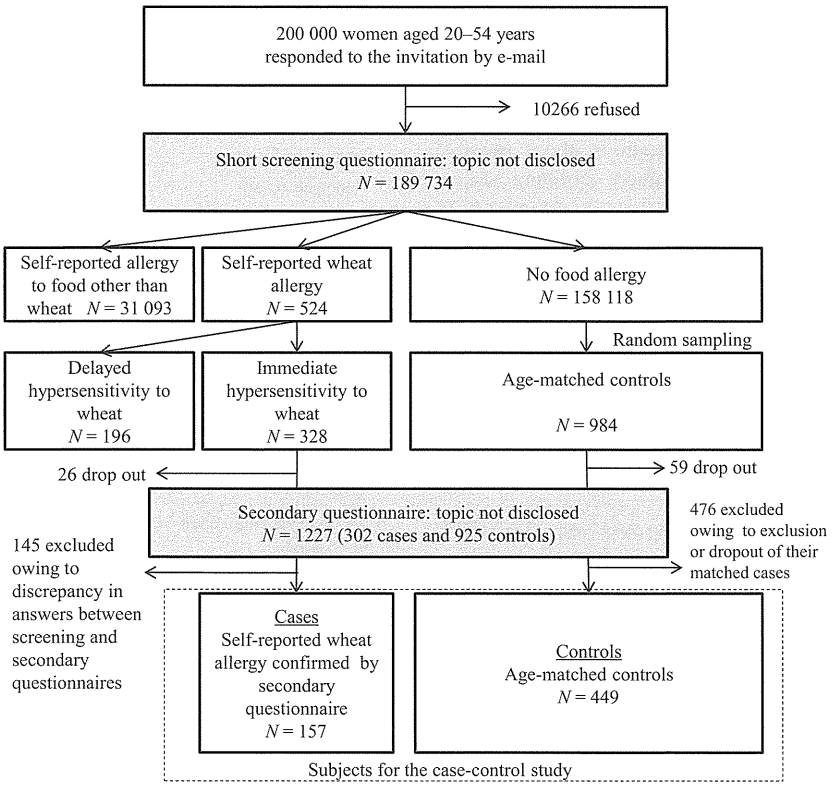

Figure 1 Protocol of Web-based survey.

The first e-mail inviting participation in the secondary survey was sent on January 28, 2011, and reminders were sent up to three times until we received answers. The secondary survey ended on February 7, 2011. The secondary questionnaire contained detailed questions regarding WA, allergic diseases other than food allergy, and use of skin and hair care products. To ensure the validity of the answers in the screening questionnaire, the secondary questionnaire included the same questions as Q1 and Q2 of the former and in addition the question 'At what age did you become allergic to wheat?', which was almost the same as Q5 of the screening questionnaire. Of the 328 cases to whom an invitation to the secondary survey was sent, 302 responded (for a response rate of 92%). We excluded 145 cases that showed discrepancies between the answers to these three questions in the screening and secondary questionnaires. Finally, data from 157 cases and 449 matched controls could be analyzed here.

Questions in the secondary questionnaire

The secondary questionnaire included questions about the use of soap, shampoo, hair treatment and conditioner, and cosmetics. All the popular brands of skin and hair care products in Japan were listed on the questionnaire, and subjects were asked to indicate which product they were 'currently using' or 'have used before' in the past 5 years. If the currently used brand was not listed, subjects were asked to provide the name of the brand they were using. If subjects gave an affirmative response for current or past use of the HWP-containing facial soap 'Cha no Shizuku' (Yuka, Fukuoka, Japan), the causal brand in most of the cases reported (17, 21), additional questions were posed on when they started to use and when they discontinued use of this brand and whether they experienced allergic symptoms after contact with the soap.

The questionnaire also included questions about possible risk factors associated with WA, namely smoking status, weight, height, allergic rhinitis (AR), atopic dermatitis (AD), and bronchial asthma (BA) comorbidities. Cases with WA were also asked whether their allergic symptoms were induced or exacerbated by exercise, whether they had been diagnosed as having WDEIA by their doctor, the frequency of WA episodes over the last 12 months, how much wheat product they consumed, and the type of symptoms induced by wheat ingestion.

Statistical analysis

The collected data were analyzed using SPSS ver. 21.0 (IBM, Tokyo, Japan). Descriptive statistics were used to characterize the cases and control subjects. Conditional logistic regression was used to compare the demographics of cases and controls and determine adjusted odds ratios (OR) and 95% confidence intervals (CIs) for the association between use of skin and hair care product and risk of WA.

The chi-square test and Fisher's exact test were used to determine whether distributions of categorical variables significantly differed from each other. Trend analysis was tested with the use of SPSS CROSSTAB command's linear-by-linear association test. A P-value of ≤ 0.05 was considered statistically significant.

Results

Characteristics of cases and controls

Demographic characteristics of 157 subjects with self-reported WA and the age-matched controls are shown in Table 1. Cases were significantly more likely to have AR, BA, and AD than control subjects. In 85% of the cases, the onset of WA was in adolescence or adulthood and 52% had their WA diagnosed by a doctor.

Table 1 Demographic characteristics of cases with self-reported WA and their age-matched control subjects

	Cases (n = 157)	Control subjects (n = 449)	P-value
Gender (=female), n (%)	157 (100)	449 (100)	N.A.
Age, years, mean ± SD	35.2 ± 7.9	35.3 ± 7.8	N.A.
Allergic diseases, n (%)			
Allergic rhinitis	119 (76)	226 (50)	<0.001
Asthma	37 (24)	23 (5)	<0.001
Atopic dermatitis	58 (37)	56 (12)	<0.001
Smoking status, n (%)			
Non-smoker	117 (75)	326 (73)	0.164
Past smoker	13 (8)	62 (14)	
Current smoker	27 (17)	61 (14)	
Body mass index, kg/m^2, mean ± SD	21.6 ± 3.6	21.0 ± 3.2	0.067
Presentation of WA			
Onset age >12 years, n (%)	133 (85)	–	
WA diagnosed by doctor, n (%)	81 (52)	–	
Exacerbation of WA symptoms by exercise, n (%)	67 (43)	–	
WDEIA diagnosed by doctor, n (%)	31 (20)	–	
Frequency of episodes of WA during the last 12 months, n (%)			
0	28 (18)	–	
1–2	46 (29)	–	
3–5	37 (24)	–	
6–10	16 (10)	–	
11–	30 (19)	–	
How much wheat have you eaten recently?			
No wheat	13 (8)	–	
A small amount of wheat	45 (29)	–	
A usual amount of wheat	90 (57)	–	
Avoid eating only before exercise	9 (6)	–	

WA, wheat allergy; WDEIA, wheat-dependent exercise-induced anaphylaxis; N.A., not assessed.

Use of skin and hair care products as risk factors for WA

Frequencies of the use of different skin and hair care products are shown in Table 2. All brands which were currently used by more than 3% of the cases are shown in the table and were analyzed. Because comorbid allergic diseases were associated with the use of some brands of skin and hair care products (data not shown), statistical analysis of the association between the use of these products and risk of WA was performed after adjustment for AR, BA, and AD. The frequency of cases currently using the HWP-containing soap, 'Cha no Shizuku', was 11%, significantly higher than in control subjects (5%, $P = 0.014$), with an adjusted OR of 2.6 (95% CIs, 1.2–5.6). As shown in Table 2, some other brands also contained HWP. However, use of no other brand showed a statistically significant association with WA. To compare the risk of WA between users of 'Cha no Shizuku' soap and users of other HWP-containing products, we performed an unconditional multivariate logistic regression analysis after adjustment for AR, BA, and AD and after limiting the subjects to those who were currently using any of the HWP-containing brands listed in Table 2. When compared with the use of other HWP-containing brands, use of 'Cha no Shizuku' was associated with an increased risk of WA with an adjusted OR of 2.3 (95%CI, 0.94–5.56) (data not shown). However, this difference did not reach statistical significance ($P = 0.07$).

Associations between the use of 'Cha no Shizuku' and WA were also evaluated using different exposure parameters. The subjects were divided into three groups according to their history of product use as follows: (i) subjects who were currently using the product (current users), (ii) those who had used it before but not currently (past users), and (iii) those who had never used it (never users). We found that only current use was associated with WA with an OR of 2.6 (1.2–5.7) (Table 3, model 1). Subjects were then divided into four groups according to the duration of use of the product. It can be seen that the duration of use ≥2 years was associated with an increased risk of WA with an OR of 4.2 (1.6–10.7) (Table 3, model 2). In an analysis of all subjects who had ever used the soap (current or past users), current users were more likely to have used it for ≥2 years ($P = 0.005$).

Of the 29 subjects who had ever used the soap (current or past users) in the allergic group, seven experienced itchiness of the face and/or eyes, sneezing, a runny nose, or

Table 2 Frequency of current use of each skin and hair care product and associations with self-reported wheat allergy

	No. of current users (%)			
Name of brand*	Cases ($n = 157$)	Controls ($n = 449$)	Adjusted OR† (95%CI)	*P*-value
Facial soaps and body soaps				
K.B.	31 (19.7)	118 (26.3)	0.7 (0.4–1.1)	0.111
U.D.	18 (11.5)	60 (13.4)	0.8 (0.4–1.5)	0.506
Cha no Shizuku‡	17 (10.8)	26 (5.8)	2.6 (1.2–5.6)	0.014
G.C.	15 (9.6)	56 (12.5)	0.9 (0.5–1.9)	0.866
D.C.	10 (6.4)	16 (3.6)	2.2 (0.2–23.1)	0.519
U.L.	5 (3.2)	11 (2.4)	1.2 (0.4–3.7)	0.817
Shampoo				
U.L.	19 (12.1)	70 (15.6)	1.1 (0.8–1.5)	0.607
P.P.	13 (8.3)	67 (14.9)	0.7 (0.3–1.3)	0.207
S.W.‡	13 (8.3)	17 (3.8)	1.8 (0.8–4.3)	0.184
S.R.	10 (6.4)	34 (7.6)	0.8 (0.4–1.8)	0.577
C.I.‡	8 (5.1)	24 (5.3)	1.2 (0.5–3.0)	0.736
P.H.	8 (5.1)	21 (4.7)	1.3 (0.5–3.4)	0.542
S.G.	5 (3.2)	12 (2.7)	1.6 (0.5–5.1)	0.416
Hair treatment				
U.L.	17 (10.8)	66 (14.7)	0.9 (0.5–1.7)	0.755
P.P.	16 (10.2)	73 (16.3)	0.8 (0.4–1.4)	0.392
S.W.‡	12 (7.6)	17 (3.8)	1.5 (0.6–3.6)	0.416
S.R.	9 (5.7)	32 (7.1)	0.8 (0.4–1.9)	0.678
P.H.	8 (5.1)	21 (4.7)	1.5 (0.6–3.7)	0.430
C.I.‡	7 (4.5)	28 (6.2)	0.9 (0.4–2.3)	0.844
K.E.	6 (3.8)	23 (5.1)	0.5 (0.2–1.5)	0.246
K.A.	6 (3.8)	23 (5.1)	0.6 (0.2–1.6)	0.295
S.G.	5 (3.2)	11 (2.4)	1.6 (0.5–5.2)	0.404
P.V.	5 (3.2)	9 (2.0)	1.1 (0.3–4.6)	0.844
Cosmetics				
R.H.	18 (11.5)	62 (13.8)	0.8 (0.4–1.5)	0.531
D.S.‡	17 (10.8)	28 (6.2)	1.7 (0.8–3.7)	0.136
D.C.	12 (7.6)	34 (7.6)	1.2 (0.6–2.5)	0.610
F.N.	8 (5.1)	16 (3.6)	1.0 (0.4–2.6)	0.996
O.R.	7 (4.5)	24 (5.3)	0.8 (0.3–2.0)	0.581
S.E.	7 (4.5)	16 (3.6)	0.9 (0.3–2.8)	0.903
A.L.	5 (3.2)	10 (2.2)	1.3 (0.4–4.7)	0.667

OR, odds ratio.
*Names of brands other than 'Cha no Shizuku' (Yuka) are indicated by their initials.
†Adjusted for allergic rhinitis, atopic dermatitis, and asthma.
‡Some products of this brand contain hydrolyzed wheat protein.

Table 3 Association between use of the facial soap 'Cha no Shizuku' containing HWP and self-reported wheat allergy

Facial soap containing HWP, 'Cha no Shizuku'	Cases ($n = 157$) No. (%)	Controls ($n = 449$) No. (%)	Adjusted OR* (95% CI)
Model 1			
History of use			
Never used	128 (82)	383 (85)	1
Ever used	12 (8)	40 (9)	1.0 (0.4–2.1)
Currently using	17 (11)	26 (6)	2.6 (1.2–5.7)
Model 2			
Duration of use			
Never used	128 (82)	383 (85)	1
<1 year	12 (8)	41 (9)	0.9 (0.4–2.1)
1 year	4 (3)	14 (3)	1.0 (0.3–3.5)
≥2 years	13 (8)	11 (2)	4.2 (1.6–10.7)

OR, odds ratio; 95% CI, 95% confidence interval; HWP, hydrolyzed wheat protein.
*Adjusted for allergic rhinitis, atopic dermatitis, and asthma.

swelling of the eyelids after using it (24%). This was significantly higher than in the control group (5%, 3/66) ($P = 0.008$).

Presentation of WA in relation to the use of 'Cha no Shizuku'

To further elucidate the relationship between the use of the soap and WA, we assessed associations between history of use and the presentation of WA. Figure 2 shows the frequency of current users and past users relative to the time of onset of WA. Frequencies of current users were higher in more recent-onset WA (P for trend = 0.002), suggesting that the use of the soap is associated with the recent epidemic of WA. Compared with the frequency of current users in the controls, a significantly higher frequency was observed only in cases who had developed WA within the last 4 years ($P = 0.001$). This might be explained by the fact that this facial soap started to be sold in 2004, namely 7 years before this study.

Associations between history of soap use and presentation with WA are shown in Table 4. Current use of the soap was associated with doctor-diagnosed WA. Although the overall frequency of diagnosed WA was 52%, it was as high as 76% among the current users. Additionally, eye and nose symptoms after wheat ingestion were more common among current users than never users, and about half of the current users were also diagnosed as having WDEIA by their doctor. These presentations of WA were compatible with those of cases who were clinically diagnosed as suffering from WDEIA induced by this soap (17, 21).

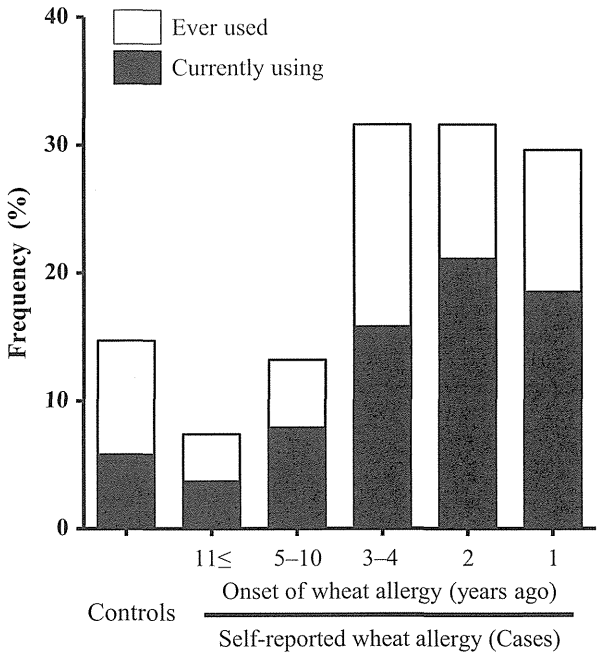

Figure 2 Frequencies of subjects who were currently using or had ever used the facial soap 'Cha no Shizuku' containing hydrolyzed wheat protein according to time of onset of wheat allergy.

Discussion

This Web-based case–control study explored epidemiological relationships between the use of the HWP-containing facial soap 'Cha no Shizuku' and risk of WA. Current use of the soap was significantly associated with increased risk of WA with an adjusted OR of 2.6. Additionally, current use of this soap was more common in cases with more recent-onset WA, implying that it may have contributed to the recent epidemic of WA. To the best of our knowledge, this is the first study that shows the epidemiological impact of contact exposure to food-derived protein in skin and hair care products on the epidemic of adult-onset food allergy.

'Cha no Shizuku' was a popular facial soap in Japan. The company (Yuka) sold more than 40 million bars to more than 4 million customers up to 2010. This product contained an acid-HWP named Glupearl 19S (Katayama Chemical, Inc, Osaka, Japan) included for its moisturizing effect and emulsification function. The soap started to be sold in 2004, and sales increased with time. A minority of allergists and dermatologists began to become aware of the potential role of HWP in this soap as an inducer of WA in 2009–2010. However, this was not recognized by the general allergist and the public until the company started a voluntary recall of the HWP-containing soap on May 25, 2011. After the voluntary recall, the allergy problem induced by this soap was publicized by the media and in the meantime has grown to be recognized as a major public health problem in Japan. Therefore, the current study was performed before the general recognition of the allergy problem. In October 2011, the Japanese Society of Allergology (JSA) determined the diagnostic criteria for the immediate WA induced by exposure to Glupearl 19S contained in this soap (21). To date, more than 2000 patients meeting these criteria have been reported to the JSA.

Among the skin and hair care products containing HWP, a statistically significant association with WA was observed only for 'Cha no Shizuku' soap. The reason for this cannot be determined from the present study. Differences in the amount of HWP in the different products may explain this, because 'Cha no Shizuku' contained the relatively high level of 0.3% of HWP. Another possibility may relate to the high volume of sales of this soap, resulting in a sufficient sample size for reliable statistical analysis. One more explanation may relate to the difference in biochemical properties between Glupearl 19S and the HWPs in other products. HWPs produced by different companies have been used in skin and hair care products in Japan. A strong sensitizing capacity of Glupearl 19S has been shown in animal models, although not in a direct comparison with other HWPs (24). Glupearl 19S contains higher molecular weight proteins, which may increase its allergenicity (25, 26). More recently, studies have shown that the deamidation of glutamine residues in gluten by acid-heat treatment also contributes to the

Table 4 Use of the facial soap 'Cha no Shizuku' containing hydrolyzed wheat protein and the presentation of WA

	History of use			
	Currently using (n = 17)	Ever used (n = 12)	Never used (n = 128)	P-value
Onset age >12 years	17 (100)	11 (92)	102 (80)	0.079
WA diagnosed by doctor	13 (76)*	6 (50)	62 (48)	0.094
Exacerbation of WA symptoms by exercise	10 (59)	5 (42)	52 (41)	0.361
WDEIA diagnosed by doctor	8 (47)*	2 (17)	21 (16)	0.011
Frequency of episodes during the last 12 months				
0	3 (18)	2 (17)	23 (18)	0.679
1–2	3 (18)	4 (33)	39 (30)	
3–5	7 (41)	1 (8)	29 (23)	
6–10	2 (12)	2 (17)	12 (9)	
11–	2 (12)	3 (25)	25 (20)	
How much wheat have you eaten recently?				
No wheat	0 (0)	1 (8)	12 (9)	0.170
A small amount of wheat	9 (53)	2 (17)	34 (27)	
A usual amount of wheat	6 (35)	8 (67)	76 (59)	
Avoid eating only before the exercise	1 (12)*	1 (8)	6 (5)	
Self-reported symptoms of WA				
Swelling of the eyelid	9 (53)*	4 (33)	17 (13)	<0.001
Itchiness of the eyes	9 (53)*	4 (33)	25 (20)	0.008
Red swelling of the face	2 (12)	2 (17)	15 (12)	0.880
Itchiness of the face	6 (35)	2 (17)	31 (24)	0.484
Sneezing/runny nose/ stuffy nose	5 (29)*	3 (25)	14 (11)	0.062
Swelling of the lips	2 (12)	2 (17)	20 (16)	0.909
Generalized itchiness	9 (53)	4 (33)	39 (30)	0.181
Generalized urticaria	8 (47)	3 (25)	32 (25)	0.157
Generalized redness	4 (24)	2 (17)	15 (12)	0.381
Discomfort or itchiness of the throat	6 (35)	3 (25)	31 (24)	0.615
Dyspnea	4 (24)	1 (8)	19 (15)	0.507
Cough	3 (18)	1 (8)	14 (11)	0.673
Wheeze/stridor	2 (12)	0 (0)	14 (11)	0.476
Abdominal pain	4 (24)*	1 (8)	8 (6)	0.052
Nausea and vomiting	3 (18)	1 (8)	9 (7)	0.328
Diarrhea	4 (24)	1 (8)	10 (8)	0.116
Blurring of vision	0 (0)	0 (0)	6 (5)	0.493
Weakness	0 (0)	0 (0)	8 (6)	0.385
Loss of consciousness	0 (0)	0 (0)	3 (2)	0.707
Palpitation	2 (12)	1 (8)	12 (9)	0.941
Headache	1 (6)	0 (0)	7 (5)	0.704
Feeling sluggish or drowsy	1 (6)	0 (0)	14 (11)	0.403

WA, wheat allergy; WDEIA, wheat-dependent exercise-induced anaphylaxis.
Data are expressed as number (%).
*$P < 0.05$ when compared with never used group.

allergenicity of acid-HWP (27–30). Additionally, the possibility remains that ingredients other than HWP in 'Cha no Shizuku' soap caused allergic contact dermatitis to disrupt the skin barrier and facilitated the penetration of higher molecular weight proteins of Glupearl 19S into an inflammatory milieu (31, 32). Indeed, the soap contained more than 10 kinds of ingredients including phenoxyethanol and 1,3-butylene glycol, which are known contact sensitizers (33, 34).

The main limitation of this study relates to the definition of WA, which was self-reported, not doctor-diagnosed. It is generally recognized that diagnosis by an allergist after provocation testing is more reliable than the definition of diseases by self-report. However, especially in the case of WDEIA, the sensitivity of the provocation test is not always very good. Using patients diagnosed by provocation test as cases may not be suitable for epidemiological studies that estimate the effect of exposure on the general population, because such patients are different from the general set of patients with WA. This study was a Web-based survey; such surveys have the advantage that both cases and controls can be recruited from the same cohort of the general population, and patients with rare diseases are identifiable in large-scale cohorts.

In conclusion, this Web-based case–control study documents an epidemiological relationship between WA and contact exposure to HWP in Japanese women. These findings imply a possible role of contact exposure to food-derived protein hydrolysates in skin and hair care products in the epidemic of adult-onset food allergy. We urge that more attention should be paid to the possible role of food-derived protein in skin and hair care products in the induction of food allergy.

Author contributions

YF designed the study and wrote the manuscript. MT, HN, and KA contributed to the critical revision of the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Distribution of the population of 20–54 year-old women in Japan according to the national population census (A), the number of women aged 20–54 years who were registered to MACROMILL research panel in 2011 (B), and number of studied subjects in the screening survey (C).

Appendix S1. Screening questionnaire.

Data S1. Characteristics of the studied internet research monitor population.

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