Note

The Effect of Wasabi Rhizome Extract on Atopic Dermatitis-Like Symptoms in HR-1 Hairless Mice

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Summary We investigated the effect of wasabi rhizome extract on atopic dermatitis (AD) model mice. The wasabi extract was fed to the HR-1 hairless mice, which develop AD-like symptoms with a special diet (HR-AD diet). The extract was expected to reduce the symptoms induced. Wasabi rhizome-containing HR-AD diet (5% and 10%) reduced the scratching behavior, and the 10% wasabi rhizome HR-AD diet significantly reduced scratching behavior on days 28, 35 and 42. Plasma components (histamine, eotaxin, IgE and thymus and activation-regulated chemokine (TARC)) were decreased in the 10% wasabi rhizome HR-AD diet. In histopathological examinations (toluidine blue (T.B.), major basic protein (MBP), CD4, IL-4, IL-5, eotaxin, TARC and IgE), the wasabi rhizome-containing HR-AD diet (5% and 10%) significantly reduced the number of positive stained cells. These results suggested that the wasabi rhizome extract improved the AD-like symptoms of HR-1 hairless mice.

Key Words wasabi, 6-methylsulfinylhexyl isothiocyanate, atopic dermatitis, HR-1 hairless mice

Atopic dermatitis (AD) is a chronic dermatologic disease characterized by itchy skin (1). Histopathologically, AD exhibits an inflammation with thickened epidermis, and infiltration of a lymphocyte, eosinophil and mast cell to the corium. For a diagnosis of AD, several markers were studied. Serum IgE and histamine levels are elevated in patients with AD (2, 3). But the relationship between IgE and clinical disease is not always exclusive. It was reported that the density of the mast cells of skin increased in AD-model animals (4, 5). Thymus and activation-regulated chemokine (TARC) is the CC chemokine that is expressed in keratinocytes. The induction of TARC production plays an important role in the lesional skin through the chemokine receptor CCR4⁺/cutaneous lymphocyte-associated antigen (CLA)⁺ Th2-type lymphocytes in AD (6). Dworzak et al. reported that patients with severe AD had a significantly expanded proportion of CLA⁺/CD4⁺ T cells compared with control subjects (7). Eotaxin is chemotactic factor that is produced by a stimulus of IL-4 from fibroblasts (8). Skin lesions are caused by IL-5 or eotaxin-mediated eosinophilic infiltration into the skin (9). Major basic protein (MBP) is the basic protein in eosinophil granules, and possesses cellular cytotoxicity (10). Eosinophils are involved in the inflammatory response in AD by the release of MBP. In patients with AD, chemical mediators, such as TARC, eotaxin and MBP, exhibit a high value, and are well correlated with clinical presentation (11–14).

Wasabi (*Wasabia japonica* (Miq) Matsumura) is a plant

of Japanese origin, and it belongs to the Brassicaceae family. Wasabi has been used in Japanese life as a spice from ancient times. It is known that wasabi inhibits proliferation of bacteria, and this has been studied for many years (15, 16). It has been reported that wasabi extract and fragrance ingredient (6-methylsulfinylhexyl isothiocyanate; 6-MSITC) had a repressive effect on diabetic nephropathy in type 2 diabetic mice (17), and a platelet aggregation-inhibitory activity in human platelets (18). In a recent study, Uto et al. reported that 6-MSITC suppressed inducible nitric oxide synthase (iNOS) production of RAW264 cells, and the suppressive effects depended on inhibition in the MAPK signaling pathways (19). The expression of iNOS was upregulated in the dermal lesions of AD-model mice (20). Taniuchi et al. reported that the disease severity in patients with AD was significantly correlated with serum nitrate levels (21). These results suggested that wasabi ingredients had the possibility to improve AD.

In this study, the wasabi extract was fed to the HR-1 hairless mice, which develop AD-like symptoms with a special diet. The repressing effect on scratching behavior and the reduction effects on the histopathological index were investigated.

Materials and Methods

Wasabi rhizome extract. Freeze-crushed wasabi rhizome (8.0 kg) and distilled water (8.0 kg) were mixed and incubated for 3 h in 37°C. The mixture was moved to a closed container and volatile isothiocyanates were removed by decompression processing. Fifty percent ethanol (40 kg) was added to the mixture and it was

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Table 1. Ingredients of the wasabi rhizome extract.

Ingredients (per kg)	
Energy	14,500 kJ
Moisture	55 g
Crude protein	144 g
Crude fat	5 g
Carbohydrate	713 g
Crude ash	83 g
Na	3,990 mg
Mg	3,680 mg
Zn	25.8 mg
6-MSITC	1,400 mg
Allyl isothiocyanate	0 mg

Analysis method of each ingredient is as follows. Energy: protein (g) $\times$ 16.7 kJ+fat (g) $\times$ 37.7 kJ+carbohydrate (g) $\times$ 16.7 kJ, moisture: air oven method, crude protein: Kjeldahl method, crude fat: acid hydrolysis method, carbohydrate: 1,000-(moisture (g)+protein (g)+fat (g)+crude ash (g)), crude ash: direct ashing method, Na: atomic absorption spectrometry, Mg and Zn: inductively coupled plasma spectrometry, 6-MSITC and allyl isothiocyanate: gas chromatography.

stirred at room temperature for 1 h. After centrifuge separation, it was filtered using diatomite and 39 kg of extractive liquid was obtained. The extractive liquid was concentrated using a rotary evaporator, and spray dried using 370 g of dextrin (Matsutani Chemical Industry Co., Ltd., Hyogo, Japan). Finally, 650 g of powdered wasabi rhizome extract was obtained. The ingredients of the wasabi rhizome extract are shown in Table 1. 6-MSITC was detected in the wasabi rhizome extract, but other isothiocyanates weren't detected.

Animals. Male HR-1 hairless mice (4 wk old) were obtained from Hoshino Laboratory Animals (Saitama, Japan). HR-1 hairless mice were fed a special diet to develop AD-like symptoms. The detailed ingredients of the diet have been described (22). The experiment obeyed the "Guidelines for proper conduct of animal experiments" of the Science Council of Japan, and was performed ethically.

Care conditions. Male HR-1 hairless mice (5 wk old) were sorted by stratified randomization based on body weight before day one of the feeding. Mice were housed in a conventional animal room, under controlled temperature ($22\pm 3^\circ\text{C}$), humidity ($50\pm 20\%$) and ventilation (13–17/h) with lights on from 08:00 to 20:00. They were housed in polycarbonate cages separately, and given tap water freely. They were fed a normal diet (Labo MR stock, Nosan Corp., Yokohama, Japan) or a HR-AD diet (HR-AD manufactured diet, Nosan Corp.) or a diet with wasabi rhizome extract (5% or 10%) added to the HR-AD manufactured diet, with free feeding. The wasabi rhizome extract and the HR-AD diet were mixed once a week. About 83% of 6-MSITC in HR-AD+wasabi diet remained after 1 wk. On day 42, plasma samples were taken for hematological examination, and dorsal skin samples were isolated and fixed by

10% neutral formalin. The energy content of each diet was calculated by the value indicated on the catalog of the diet and the measurement of wasabi rhizome extract.

Measurement of scratching behavior. The scratching behavior of the mice was analyzed and recognized at three time points: 28, 35 and 42 d after starting the special diet. Mice were videotaped for 30 min, and the frequency of scratching was counted retrospectively at sequential 5-min intervals (23). Scratching frequency was defined as scratching the neck with the hind limbs. The cumulative scratching frequency every 5 min was calculated.

Hematology. In the plasma samples, histamine (Histamine ELISA, IBL Co., Ltd., Gunma, Japan), eotaxin (Quantikine[®] Immunoassay Eotaxin, R&D Systems, Inc., Minneapolis, USA), IgE (Mouse IgE ELISA Quantitation Kit, Bethyl Laboratories, Inc., Montgomery, USA), TARC (Quantikine[®] Immunoassay CCL17/TARC, R&D Systems, Inc.) were analyzed according to the manual of the kit.

Skin histopathology. Skin samples were stained with several methods and the positive stained cells counted in the view area of the microscope ($\times 400$). For the examination of mast cells, the tissues were stained with toluidine blue (T.B.) (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Immunohistological indexes were detected using anti-mouse antibodies. Antibodies such as MBP (Biogenesis Inc., Poole, UK), CD4 (Santa Cruz Biotechnology, Inc., California, USA), IL-4 (Santa Cruz Biotechnology), IL-5 (Santa Cruz Biotechnology), eotaxin (Chemicon International Inc., California, USA), TARC (Santa Cruz Biotechnology) and IgE (Biogenesis Inc.) were used.

Statistical analysis. Results were expressed as the mean $\pm$ SE for each group in diet intake, body weight and scratching behavior, and as the mean $\pm$ SD for each group in the plasma components and the histopathological indexes. For body weight and diet intake, the significant difference between normal diet and the HR-AD diet was determined using the Student's *t*-test if the sequence was homoscedastic, or Aspin-Welch's *t*-test when it was heteroscedastic, after doing the *F*-test. The variance between the HR-AD diet and HR-AD+5% or 10% of wasabi rhizome extract diet was tested by the Bartlett test. When it was homoscedastic, they were tested by one-way ANOVA and when heteroscedastic, they were tested by the Kruskal-Wallis test. When a variance was significant, the significant difference was determined using Tukey's test. For scratching behavior, the significant difference between the normal diet and the HR-AD diet, HR-AD diet and HR-AD+5% or 10% of wasabi rhizome extract diet was determined using Student's *t*-test when it was homoscedastic, and Aspin-Welch's *t*-test when it was heteroscedastic, after doing the *F*-test. For the hematological examination and histopathological examination, the significant difference of each diet was determined using one-way ANOVA. Values of $p<0.05$ were considered to be statistically significant in the *F*-test, Bartlett test, one-way ANOVA and

Table 2. Time-course changes in diet intake of HR-1 hairless mice (g).

	Time (d)						
	0	7	14	21	28	35	42
Normal	4.6±0.3	5.5±0.2	5.7±0.3	6.1±0.3	6.5±0.2	6.8±0.3	6.5±0.2
HR-AD	3.8±0.2	4.1±0.3	4.4±0.2	4.6±0.1	4.4±0.4	3.7±0.3	3.6±0.5
HR-AD+5% wasabi	3.9±0.1	4.3±0.2	4.8±0.2	5.0±0.2	5.7±0.3	5.2±0.3	5.2±0.5
HR-A+10% wasabi	3.6±0.2	3.9±0.1	4.3±0.1	4.5±0.2	5.4±0.2	5.2±0.3	5.1±0.6

The diet intake of the HR-1 mice was analyzed and recorded every 7 d, after starting the special diet. Values given are the mean±SE of 8 animals. *t*-test: **p*<0.05, ***p*<0.01. Tukey's test: #*p*<0.05, ##*p*<0.01.

Table 3. Time-course changes in body weight of HR-1 hairless mice (g).

	Group sorting	Time (d)						
		0	7	14	21	28	35	42
Normal	19.4±0.4	19.8±0.4	23.9±0.3	26.0±0.3	27.3±0.4	28.5±0.4	29.2±0.4	29.9±0.4
HR-AD	19.2±0.4	18.1±0.5	22.7±0.5	24.8±0.6	26.4±0.6	26.6±1.1	27.6±0.7	27.5±0.9
HR-AD+5% wasabi	19.1±0.4	18.6±0.5	23.3±0.5	25.8±0.6	27.8±0.7	29.4±0.8	29.9±0.8	30.4±0.7
HR-A+10% wasabi	19.1±0.4	18.2±0.3	22.6±0.3	24.9±0.4	26.9±0.4	28.5±0.4	29.5±0.3	29.8±0.3

The body weight of the mice was analyzed and recorded every 7 d, after starting the special diet. Mice were sorted by stratified randomization based on body weight before day one of the feeding. Values given are the mean±SE of 8 animals. *t*-test: *

p<0.05. Tukey's test: #*p*<0.05.

Kruskal-Wallis test. Values of *p*<0.05 and *p*<0.01 were considered to be statistically significant in the Student's *t*-test and Tukey's test.

Results and Discussion

Diet intake and body weight

The intake of normal diet-fed mice exhibited an increase over the time-course (Table 2). The intake of HR-AD-fed mice was significantly less than that of normal diet-fed mice. The intake of HR-AD+5% wasabi-fed mice exhibited a significantly high value on days 28 and 35 compared with that of the HR-AD diet-fed mice. The intake of HR-AD+10% wasabi-fed mice exhibited a significantly high value on day 35 compared with that of the HR-AD diet-fed mice. Throughout the measurement period, no statistically significant difference was seen between normal diet-fed mice and HR-AD+wasabi-fed mice.

The body weight was increased smoothly in normal diet-fed mice (Table 3). The body weight of HR-AD-fed mice was less than that of the normal diet-fed mice, especially on days 0, 7 and 42. There was no significant difference in body weight among normal diet-fed mice and HR-AD+5% wasabi-fed mice. On day 42, the body weight of HR-AD+5% wasabi-fed mice indicated a high value compared with that of HR-AD-fed mice. There

Fig. 1. Effects of wasabi rhizome extract on the scratching behavior in normal diet-fed mice, HR-AD diet-fed mice, HR-AD+5% wasabi diet-fed mice and HR-AD+10% wasabi diet-fed mice. Values given are the mean±SE of 8 animals. One-way ANOVA: **p*<0.05, ***p*<0.01.

was no significant difference in body weight among normal diet-fed mice and HR-AD+10% wasabi-fed mice.

The energy contents of the normal diet, HR-AD diet, HR-AD+5% wasabi diet and HR-AD+10% wasabi diet were estimated at 10,850, 14,810, 14,800 and 14,780 kJ/kg, respectively. The accumulations of energy intake on measurement days (days 0, 7, 14, 21, 28, 35 and

Fig. 2. Hematological examination of plasma of HR-1 mice fed a normal diet, HR-AD diet, HR-AD+5% wasabi diet and HR-AD+10% wasabi diet, on day 42. Charts show histamine (A), eotaxin (B), IgE (C) and TARC (D). Values given are the mean $\pm$ SD of 8 animals. One-way ANOVA: * $p < 0.05$.

Fig. 3. Histopathological examination of skin from HR-1 mice fed a normal diet, HR-AD diet, HR-AD+5% wasabi diet and HR-AD+10% wasabi diet, on day 42. Charts show T.B. (A), MBP (B), CD4 (C), IL-4 (D), IL-5 (E), Eotaxin (F), TARC (G) and IgE (H). Values given are the mean $\pm$ SD of 8 animals. One-way ANOVA: * $p < 0.05$.

42) were estimated at 453.7 ± 15.0 kJ on the normal diet, 423.6 ± 15.9 kJ on the HR-AD diet, 502.3 ± 20.2 kJ on the HR-AD+5% wasabi diet and 473.5 ± 15.1 kJ on the HR-AD+10% wasabi diet (mean $\pm$ SE). The respective HR-AD+wasabi diet had an energy intake higher than the HR-AD diet ($p < 0.05$). The results suggested that the major cause of HR-

AD+wasabi diet increase over the HR-AD diet was the increase in energy intake.

Effects of wasabi rhizome extract on scratching behavior

In HR-AD diet-fed HR-1 mice, nerve fibers markedly increased in the epidermis (24). The increase in intraepidermal nerve fibers, as well as the skin barrier dysfunction, plays a crucial role in the increased

scratching behavior. We investigated the effects of intake of wasabi rhizome extract on scratching behavior (Fig. 1). HR-AD-fed mice indicated a significantly high value compared with that of normal diet-fed mice on days 28, 35 and 42. On the other hand, HR-AD+10% wasabi diet-fed mice indicated significantly lower values compared with that of HR-AD-fed mice on days 28, 35 and 42, and compared with that of HR-AD+5% wasabi diet-fed mice on day 35. Thus, the wasabi-containing diet was highly effective in preventing scratching behavior in HR-1 hairless mice.

Effects of wasabi rhizome extract on the plasma components and histopathological indexes

In four of the plasma components (histamine, eotaxin, IgE and TARC), HR-AD diet-fed mice were significantly higher than normal diet-fed mice (Fig. 2). On the other hand, HR-AD+10% wasabi diet-fed mice were significantly lower than HR-AD diet-fed mice in four of the plasma components. HR-AD+5% wasabi diet-fed mice indicated decreasing but not significant tendency. In all of the histopathological indexes (T.B., MBP, CD4, IL-4, IL-5, eotaxin, TARC and IgE), HR-AD diet-fed mice were significantly higher than normal diet-fed mice (Fig. 3). In all of histopathological indexes, HR-AD+5% and 10% wasabi diet-fed mice were significantly lower than HR-AD diet-fed mice. The wasabi rhizome extract-containing diet used in this study repressed scratching behavior and reduced the level of chemical mediators. It was considered that feeding of a wasabi rhizome extract suppressed cross talk between cells which participate in skin injury such as Th2 cells, B cells, mast cells and fibroblasts at several points of signaling. But the molecular target of the substance included in the wasabi rhizome extract was not clear.

In our study, the substance which was responsible for the improvement of the AD-like symptom was not clear. HR-1 hairless mice fed a diet with a reduced magnesium level developed AD-like symptom (22). The magnesium content of each diet was estimated at 2,700 mg/kg in the normal diet, 200 mg/kg in the HR-AD diet, 370 mg/kg in the HR-AD+5% wasabi diet and 560 mg/kg in the HR-AD+10% wasabi diet. The accumulation of magnesium intake on measurement day (day 0, 7, 14, 21, 28, 35 and 42) was estimated at 112.9 ± 3.7 mg in the normal diet-fed mice, 5.7 ± 0.2 mg in the HR-AD diet-fed mice, 12.6 ± 0.5 mg in the HR-AD+5% wasabi diet-fed mice and 17.9 ± 0.6 mg in the HR-AD+10% wasabi diet-fed mice (mean $\pm$ SE). The magnesium intake of HR-AD diet-fed mice and wasabi diet-fed mice were significantly lower than that of normal diet-fed mice. There was statistical significance between HR-AD-fed mice and wasabi diet-fed mice. Akamatsu et al. made AD-like model mice using a special diet including magnesium of 1,400 mg/kg (23, 25). In our study, magnesium in wasabi rhizome extract is low; it appeared that magnesium derived from wasabi was not a key substance involved in the improvement of the AD-like symptoms.

Morimitsu et al. reported that a part of 6-MSITC entered the circulatory system as its glutathione conju-

gate (26). Uto et al. reported that 6-MSITC inhibited MAPK signaling pathways in inflammation (19, 27). It has also been reported that expressions of TARC and eotaxin are controlled via MAPK signaling pathways (28, 29). 6-MSITC might adjust chemical mediators through MAPK signaling pathways. There is a possibility that 6-MSITC is the key substance which improved the AD-like symptoms in HR-1 hairless mice.

This study showed that wasabi rhizome extract might be efficacious in the treatment of human AD.

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