

Amelioration of the Progression of an Atopic Dermatitis-Like Skin Lesion by Silk Peptide and Identification of Functional Peptides

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The efficacy of silk peptide in treatment of atopic dermatitis was examined in a picryl chloride-induced atopic dermatitis model in NC/Nga mice. Silk peptide ameliorated the development of atopic dermatitis by lowering the serum IgE concentration. Treatment of cultured spleen cells with silk peptide reduced IgE production by enhancing the production of IFN- γ and reducing the level of IL-4. The functional peptides in the silk peptide were identified as mixture of GAGA sequences containing peptides by mass spectrometry and *in vitro* assay. Our findings indicate that silk peptide exerts an effect on atopic dermatitis by modulating the Th1/Th2 balance.

Key words: atopic dermatitis; silk; peptide; IgE; Th1/Th2

Atopic dermatitis (AD) is a common skin disease. The incidence has increased steadily in recent decades, including in children.^{1–3)} House dust mites are a cause of atopic skin lesions. They are widely distributed in the environment. AD is associated with the proliferation and differentiation of B cells into IgE-secreting plasma cells.⁴⁾

Silk is a protein polymer that is spun into fibers by lepidoptera larvae such as the silkworm *Bombyx mori*.⁵⁾ The mulberry silkworm produces fibroin protein in two salivary glands (sericterics). This silk protein is similar to collagen, elastin, keratin, and sporgin, and is an essential constituent of cocoon filament.^{5,6)} Silk protein hydrolysates have been used as biomedical suture material for centuries, and are believed to be safe for humans. They have also been used as a cellular matrix⁷⁾ in the immobilization of enzymes⁸⁾ and as an oral adjuvant.⁹⁾ Several physiological effects of silk protein have also been reported, including hypocholesterolemic effects¹⁰⁾ and anti-human immunodeficiency virus activity.¹¹⁾

In this study, silk protein hydrolysate (referred to as silk peptide) made from fibroin was fed to NC/Nga mice,¹²⁾ an inbred strain established from Japanese fancy mice, to evaluate the anti-AD function of this material. An *in vitro* experiment was also performed to evaluate

the effect of silk peptide on the immune signaling associated with IgE production. The functional peptides were identified.

Materials and Methods

Silk peptide. Silk peptide used in this study was prepared as follows: Degummed silk fiber (FINECO, Gangwon, Korea) was washed with distilled water and dried at 90 °C for 24 h and then dissolved in acidic CaCl₂ solution. The protein fiber was washed with distilled water to remove salt. Papain (Sigma, Tokyo) was added to 1% w/v in the fiber solution and the mixture was incubated at 70 °C for 1 h. The reaction was terminated by boiling for 5 min. After cooling to room temperature, the hydrolysate was mixed with active charcoal and filtrated with Whatman No. 1 filtration paper (Whatman, Tokyo). The filtrate was freeze dried and kept in a desiccator until used. To achieve the structural information, mass spectrometry was performed. The peptide, resolved in 50% methanol containing 0.1% acetic acid, was infused to IT-TOF MS (Shimadzu, Kyoto, Japan), and spectra were obtained in positive ion mode.

Animals and housing conditions. Male SPF NC/Nga Tnd mice were purchased from Charles River Japan (Osaka, Japan) at 5 weeks of age. Female Balb/cKWL mice were purchased from Kiwa Animal Laboratory (Wakayama, Japan). The animals were housed in a conventional air-conditioned room maintained at 24 °C with a relative humidity of 50%, and were given standard laboratory MF rodent chow (Oriental Yeast, Tokyo) and water *ad libitum*.

Picryl chloride-induced dermatitis. Picryl chloride (2,4,6-trinitrochlorobenzene; Nacalai Tesque, Kyoto, Japan) was used after recrystallization from ethanol. The NC/Nga mice were sensitized with 150 μ L of 5% picryl chloride dissolved in ethanol/acetone (4:1) by topical application onto shaved foot pads and abdomens. After sensitization, AD-like skin lesions were induced by repeated application of 150 μ L of 1% picryl chloride dissolved in olive oil to the ears and shaved back skin (Fig. 1). Control groups were treated with the same volume of vehicle weekly. After 3 weeks of treatment, silk peptide (100 mg/200 μ L) was administered orally daily at 9 AM. Blood was collected for IgE measurement biweekly. The severity of the dermatitis was assessed macroscopically by an AD scoring method, in which the degree of each symptom (erythema, edema, oozing, crust, excoriation, and lichenification) was scored as 0 (absent), 1 (mild), 2 (moderate), or 3 (severe). The total score for the six symptoms was recorded for each mouse. Assessment was performed biweekly in blind fashion. All animal experiments approved and were conducted under the guidelines of the Animal Experiment Committee of Osaka Prefecture University (nos. 18-074, 19-003, 20-K-007, 21-37).

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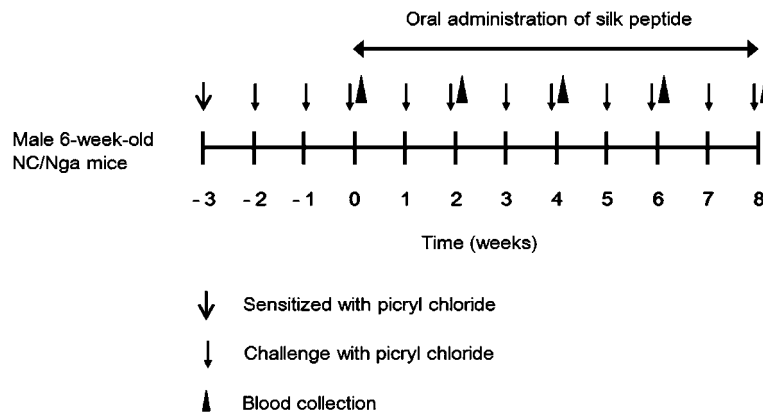


Fig. 1. Experimental Schedule for Examination of the Effects of Silk Peptide in a Picryl Chloride-Induced Model of Atopic Dermatitis in NC/Nga Mice.

Culture of spleen cells from the BALB/c mice. BALB/c mice were challenged intraperitoneally with 50 μ L of 0.1% ovalbumin (OVA; Sigma, Tokyo) emulsified in complete Freund Adjuvant (Wako Pure Chemicals, Osaka, Japan). After challenge for a week, spleen cells were obtained and cultured in RPMI 1640 medium (Wako) containing 10% fetal bovine serum, 50 μ M 2-mercaptoethanol, 100 units/mL of penicillin, and 100 mg/mL of streptomycin. The cells were treated with 100 μ g of OVA with and without silk peptide for 3 d, and the IgE, IL-4, and IFN- γ levels in the medium were quantified using BD OptEIA™ mouse ELISA kits (BD, Tokyo).

Statistical analysis. Results are expressed as mean $\pm$ SEM. Differences between experimental groups were evaluated by analysis of variance. Significance ($p < 0.05$) was obtained by Student's *t*-test.

Results

Picryl chloride-induced AD symptoms ameliorated by silk peptide

The mice received picryl chloride treatment to induce AD symptoms during the experimental period. The growth curve for the mice given silk peptide was similar to that for the control group (Fig. 2), indicating that silk peptide intake did not affect growth. There was no observation of abnormality at the autopsy held after final blood collection. The scores for AD symptoms in the control mice increased with the application of picryl chloride throughout the experimental period (Fig. 3A). In contrast, the scores for AD symptoms in the silk peptide-fed mice increased until 4 weeks, but then started to decrease. The AD symptoms in these mice resolved at 8 weeks, based on macroscopic investigation. These results indicate that silk peptide from fibroin has an anti-AD effect in NC/Nga mice.

Effect of silk peptide on serum IgE concentration

The serum IgE level is a biomarker for AD symptoms in NC/Nga mice. It was evaluated biweekly during the experimental period (Fig. 3B). The IgE level increased in the control mice throughout this period, while the level in the silk peptide-fed mice reached a plateau after 4 weeks. These results agree well with the time course of the AD symptom scores, indicating that silk peptide ameliorated the development of picryl chloride-induced AD symptoms by lowering the serum IgE concentration.

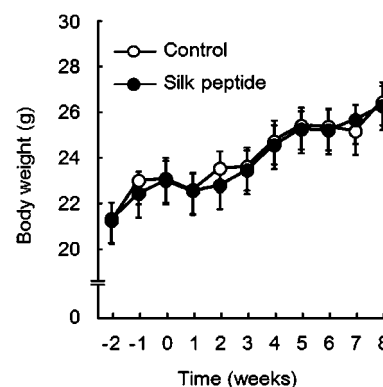


Fig. 2. Effects of Silk Peptide on Growth of NC/Nga Mice.

Silk peptide was administered orally, and changes in body weight were recorded once per week. Values are mean $\pm$ SEM for five mice.

Effects of silk peptide on IFN- γ and IL-4 in cultured spleen cells

Cytokines are involved in the development of AD symptoms through regulation of IgE production. IFN- γ and IL-4 are key factors regulating IgE production in B cells. Measurement of cytokine production in spleen cells (Fig. 4) showed that the IgE and IL-4 levels in the silk peptide-treated cells were lower than those in the controls, whereas the IFN- γ level was higher in the silk peptide-treated cells. This indicates that silk peptide treatment suppresses IgE production thus improving AD symptoms *via* modification of the cytokine profile.

Identification of functional peptides suppressing IgE production

The silk peptide mixture was analyzed by direct infusion electrospray mass spectrometry. The spectra indicated that it was composed of major peptides, of m/z 547.28, 495.24, 431.25, 419.21, 403.20, and 367.18 (Fig. 5). The ions were identified as SGAGAGAG, YGAGAG, VGAGAG, GAGAGS, GAGAGA, and YGAG, respectively by monoisotopic mass values and MS/MS analysis. The common sequence among the peptides was a GA repeat. We synthesized the following peptides: GAGAGAGS, GAGAGS, GAGAGA, and GAGA. We examined their effects on IgE production in the cultured spleen cells. The effect of the peptides was stronger than that of the silk peptide, and the effects on IFN- γ and IL-4 was similar to those of the silk peptide (Fig. 6).

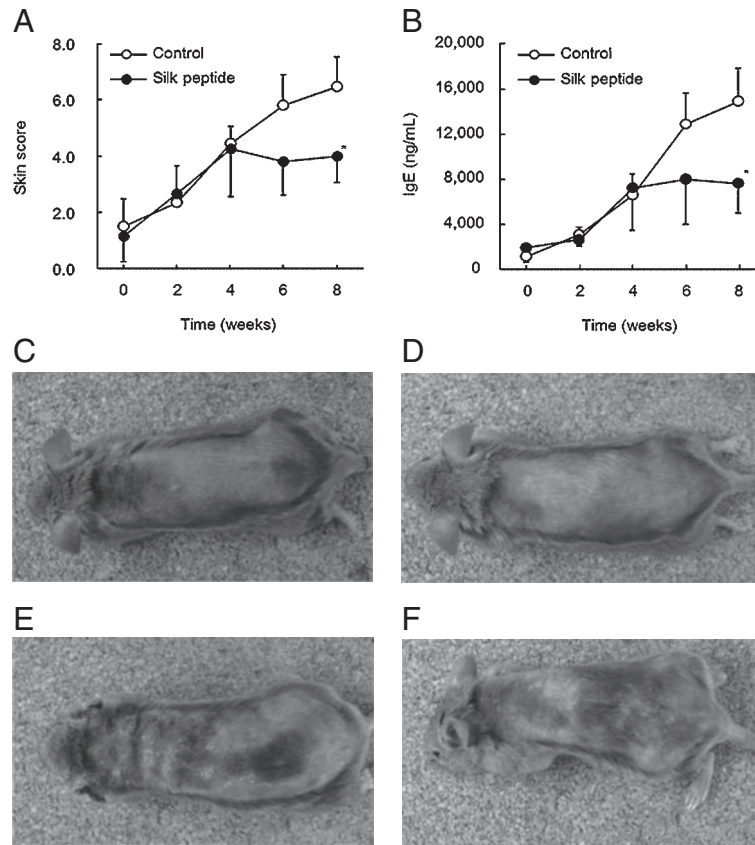


Fig. 3. Effects of Silk Peptide Administration on Skin Scores and Serum IgE Concentrations in Picryl Chloride-Induced Atopic Dermatitis.

Skin scores (A) and IgE (B) was measured once every 2 weeks as described in "Materials and Methods." Values are mean $\pm$ SEM for five mice. * $p < 0.05$ vs. Control by Tukey test. C, D, E, and F are typical appearances of NC/Nga mice. C and D were taken before picryl chloride-treatment for control and silk peptide-fed mice respectively. E and F were taken before autopsy for control and silk peptide-fed mice respectively.

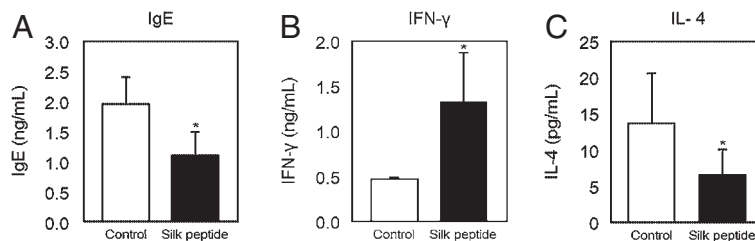


Fig. 4. Effect of Silk Peptide on Production of IgE and Cytokines in Cultured Spleen Cells Obtained from OVA-Sensitized Mice.

IgE (A), IFN- γ (B), and IL-4 (C) in the medium were measured after OVA treatment. Values are mean $\pm$ SEM for five mice. *Significantly different from the control group by Student's t -test ($p < 0.05$).

Discussion

Atopic dermatitis is the most common skin disease. It is characterized by erythema, edema, excoriation, and scaling.¹³⁾ The incidence of AD continues to increase in industrialized countries.¹⁴⁾ Topical steroid therapy is used to treat AD, but long-term steroid administration cannot be performed due to side effects. Hence there is a requirement for new treatments for AD.

Silk and related materials, including silk water extract, have long been used in traditional Chinese medicine. Silk is composed of two major polypeptides, sericin and fibroin. Sericin is the major constituent of cocoon proteins. It is mostly removed from the cocoon and disposed of when the cocoon is used to make silk textiles.¹⁵⁾ Fibroin is a core silk protein composed of 18 natural amino acids. It has a molecular weight of

350–360 kDa.¹⁶⁾ The main components of fibroin are glycine, alanine, serine, and tyrosine.¹⁷⁾ Silk hydrolysates made from the cocoon or sericin and fibroin as raw materials have been found to be bioactive. Sericin reduces the levels of serum lipids¹⁸⁾ and fibroin has a protective effect against high glucose-induced apoptosis.¹⁹⁾ Low molecular weight peptides from fibroin also stimulate the differentiation of 3T3 cells to adipocytes.²⁰⁾ Hence in this study, we examined the anti-atopic effect of silk peptide from fibroin.

Oral administration of silk peptide markedly reduced the development of picryl chloride-induced AD in NC/Nga mice under normal conditions without affecting growth, and no abnormality was found at autopsy. A high serum IgE concentration is a major characteristic of AD, with a strong correlation between clinical observations and serum IgE concentrations in patients.²¹⁾ Hence

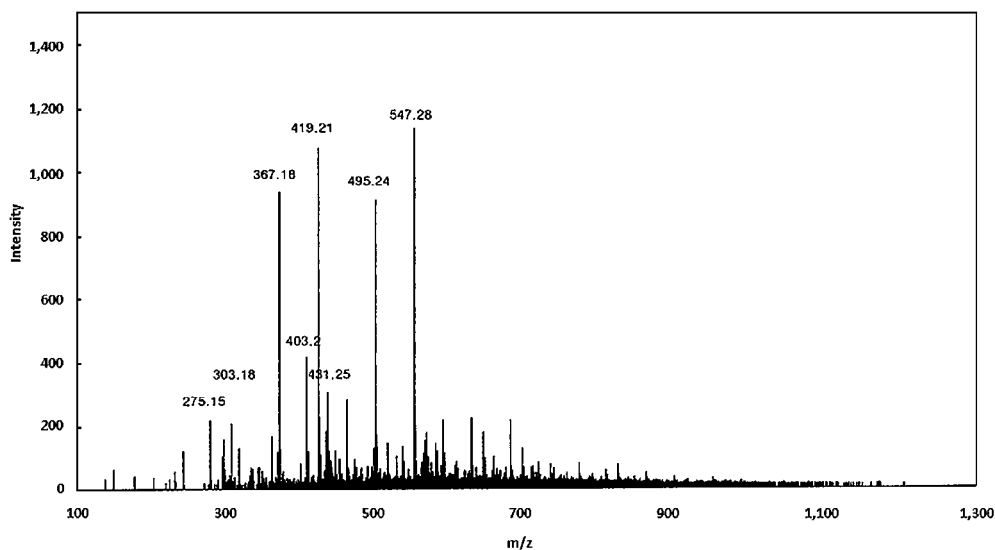


Fig. 5. Mass Spectrum of Silk Peptide.

Silk peptide was analyzed by direct infusion electrospray mass spectrometry as described in "Materials and Methods."

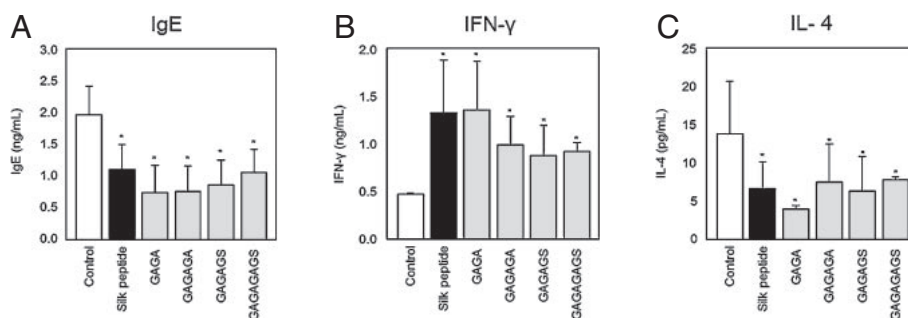


Fig. 6. Effect of Synthetic Peptide on Production of IgE and Cytokines in Cultured Spleen Cells Obtained from OVA-Sensitized Mice.

IgE (A), IFN- γ (B), and IL-4 (C) in the medium were measured after OVA treatment. Values are mean $\pm$ SEM for five mice. *Significantly different from the control group by Student's *t*-test ($p < 0.05$).

IgE production is an important target for therapeutic agents for AD. The silk peptide reduced both the skin severity of AD and the total plasma IgE level. It took at least 4 weeks to ameliorate AD symptoms. It is necessary to examine digestion and absorption of functional silk peptide to explain the time lag, but our findings indicate that oral administration of silk peptide is effective in the preventing development of AD by reducing IgE production in NC/Nga mice.

IgE production is regulated by a Th2 reaction.²²⁾ Th2 cells produce cytokines such as IL-4, which is implicated mainly in the production of IgE in B cells, since both Th2 cytokines, including IL-4 and Th1 cytokines such as IFN- γ , play important roles in inflammation and the development of AD.²³⁾ We examined the effect of silk peptide on production of IL-4 and IFN- γ in OVA-sensitized spleen cell culture. Treatment with silk peptide reduced the production of IgE and IL-4, but increased IFN- γ production. In human DND39 B cells, the expression of ϵ germline transcripts (ϵ GT) *via* STAT6 activation is induced by IL-4-induced IgE class switching,²⁴⁾ and the polyphenol strictinin inhibits IgE production through inhibition of IL-4-mediated ϵ GT expression.²⁵⁾ In the silk peptide-treated DND39 cells, IL-4-induced ϵ GT expression was not inhibited (data not shown). These findings indicate that silk peptide activated Th1 and suppressed Th2 reactions in the spleen cell culture, suggesting that the anti-AD function

of silk peptide occurs through modulation of the Th1/Th2 balance.

Mass spectrometric analysis revealed that the silk peptide was composed of major peptides identified as SGAGAGAG, YGAGAG, VGAGAG, GAGAGS, GAGAGA, and YGAG (Fig. 5). The result conforms well to the presence of the estimated proteolytic products of the fibroin protein, whose amino acid sequence is composed of GAGA repeat.⁵⁾ Synthetic peptides GAGAGAGS, GAGAGS, GAGAGA, and GAGA suppressed IgE production in the cultured spleen cells. The GAGA peptide was the strongest of the peptides. These results indicate that the functional peptides have a GAGA sequence, and that silk peptide is a mixture of the functional peptides. It is now necessary to establish the molecular mechanism of the anti-AD function of silk peptide.

In conclusion, the results of this study indicate that silk peptide can suppress the development of picryl chloride-induced AD by reducing IgE production in NC/Nga mice. Silk peptide is a mixture of functional peptides containing the GAGA sequence. The suppression of IgE production might be due to the ability of silk peptide to modulate the Th1/Th2 balance. Further studies are needed to elucidate the mechanism of modulation of the Th1/Th2 balance. The results described here might contribute to the development of therapeutic agents against AD.

Acknowledgments

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