

ORIGINAL ARTICLE

Oral administration of β -carotene or lycopene prevents atopic dermatitis-like dermatitis in HR-1 mice

Makiko HIRAGUN,¹ Takaaki HIRAGUN,¹ Ikumi OSETO,¹ Kazue UCHIDA,¹ Yuhki YANASE,¹ Akio TANAKA,¹ Tomoka OKAME,² Shingo ISHIKAWA,³ Shoji MIHARA,¹ Michihiro HIDE¹

¹Department of Dermatology, Integrated Health Sciences, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima,

²Innovation Division, Kagome, Tochigi, ³Production and Purchasing Division, Kagome, Tokyo, Japan

ABSTRACT

Atopic dermatitis (AD) is a chronic relapsing eczematous skin disease. Certain populations of patients are resistant to standard therapies with topical steroids and/or calcineurin inhibitors, and require systemic medication, such as immunosuppressants. Recently, several reports have shed light on the anti-allergic effects of carotenoids. Therefore, we investigated the effect of p.o. administration of β -carotene or lycopene on AD-like symptoms of HR-1 hairless mice fed with a low zinc/magnesium diet. Mice were divided into four groups: (i) fed with a standard diet (Co group); (ii) low zinc/magnesium diet (HR group); (iii) low zinc/magnesium and β -carotene diet (HR-C group); and (iv) low zinc/magnesium and lycopene diet (HR-L group). They were then fed these diets for 8 weeks. Severities of dermatitis were assessed by their appearance, and histopathological and hematological observations. Mice in the HR group developed AD-like dermatitis both clinically and histologically. HR-C and HR-L group mice also developed xerosis and wrinkle-like skin changes, but they were milder than those of HR group mice. Histological analysis revealed that epidermis thickening and inflammatory cell infiltration in the skin of the HR-C and HR-L groups were both statistically less than those of the HR group. The concentration of thymus and activation regulated chemokine in the skin of the HR-L group and the concentration of CCL27 in the skin of the HR-C group were significantly lower than those of the HR group, respectively. In conclusion, p.o. administration of β -carotene or lycopene prevents AD-like symptoms in association with a suppression of T-helper 2 chemokines in a murine model. Ingestion of carotenoids may be beneficial for patients with AD.

Key words: atopic dermatitis, CCL27, lycopene, thymus and activation regulated chemokine, β -carotene.

INTRODUCTION

Atopic dermatitis (AD) arises through a combination of genetic and environmental factors and is characterized by itchy and long-lasting eczema with repeated periods of exacerbation and remission.¹ Skin dysfunction in barrier and immunity, and inflammation caused and/or enhanced by various allergic reactions are important in the pathophysiology of AD.² The pathogenesis of allergic inflammation in lesional skin of AD is governed by T-helper (Th)2 cell-related cytokines such as interleukin (IL)-4 and IL-13, and chemokines such as thymus and activation regulated chemokine (TARC)/CCL17, macrophage-derived chemokine/CCL22, cutaneous T-cell attracting chemokine/CCL27 and eotaxin.^{3,4} TARC and CCL27 are known to be expressed in keratinocytes exclusively and be correlated with the severity of AD.^{5,6} The guidelines published by the Japanese Society of Allergology for general physicians proposed three

mainstays as the basis of treatment of AD: (i) investigation and countermeasures of causes and exacerbating factors; (ii) skin care; and (iii) pharmacotherapy.² Topical steroids and tacrolimus are the main drug for pharmacotherapy of AD. However, certain populations of patients are resistant to standard therapies even when they adhere strictly to the guidelines, and may require cumbersome and/or less safe systemic therapies, such as phototherapies and oral immunosuppressants. Therefore, a new treatment with another mechanism to improve or prevent AD which is less of a burden for patients is needed.

Carotenoids are natural pigments biosynthesized by bacteria, fungi and plants.^{7,8} In recent years, there has been considerable interest in dietary carotenoids due to their provitamin A activity, high antioxidant potential and ability to prevent the onset of certain cancers.⁸ Moreover, epidemiological evidence suggests that carotenoids may prevent allergic diseases such as asthma, allergic rhinitis and AD.^{9,10} Rühl *et al.*¹¹ reported

Correspondence: Michihiro Hide, M.D., Ph.D., Department of Dermatology, Integrated Health Sciences, Institute of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. Email: ed1h-w1de-road@hiroshima-u.ac.jp

Received 16 December 2015; accepted 31 January 2016.

that the serum concentrations of non-provitamin A carotenoids and lycopene were significantly lower in atopic children compared with non-atopic children. Oh *et al.*¹² reported that the intake of antioxidant nutrients, such as α -tocopherol and β -carotene, were likely to reduce the risk of AD. The effect of carotenoids on immune function also has been revealed by *in vitro* and *in vivo* assays.⁹ However, the anti-allergic effect of carotenoids on skin has not been evaluated yet. In this study, we investigated whether or not p.o. administration of representative carotenoids, β -carotene or lycopene, prevents AD-like symptoms of HR-1 hairless mice when they are fed with a low zinc and magnesium diet.^{13–15}

METHODS

Mice

Male, 4-week-old HR-1 hairless mice, which develop AD-like dermatitis when fed with a low mineral diet,^{13–15} were purchased from Hoshino Test Animal Breeder (Saitama, Japan) and maintained under specific pathogen-free conditions at the Animal Facilities of Hiroshima University. The experiments using mice were approved by the Committee of Hiroshima University on Laboratory Animals and performed in accordance with the Guidelines for the Management of Laboratory Animals in Hiroshima University.

Foods

The control mice were fed with a standard solid diet with normal concentration of zinc (67 mg/100 g diet) and magnesium (0.25 mg/100 g diet). The low mineral powder diet (HR-AD) was purchased from Shimizu Laboratory Supplies (Kyoto, Japan). Zinc (34 mg/100 g diet) and magnesium (0.14 mg/100 g diet) concentrations of HR-AD were 50% lower than those of standard solid diet. β -Carotene or lycopene were mixed with HR-AD diet in a concentration of 1 mg/g diet. The estimated dose of β -carotene and lycopene was 3–5 mg/day. β -Carotene and lycopene were provided by Kagome.

Feeding protocol

The mice were divided into four groups: (i) standard diet (Co group); (ii) HR-AD diet (HR group); (iii) HR-AD with β -carotene diet (HR-C group); and (iv) HR-AD with lycopene diet (HR-L group). They were fed with each diet for 8 weeks.

Assessment of skin

Changes in the appearance of the skin were monitored. The water content of the skin on the back of the mice was measured by using a Multiprobe MPA5 corneometer (Courage + Khazaka Electronic, Cologne, Germany) at 8 weeks after starting the special diet. The amount of transepidermal water loss (TEWL) was measured by using a Multiprobe MPA5 tewameter at 8 weeks after starting the special diet as well.

Histological examination of skin

Mice were killed at 8 weeks after the start of the special diet. Dorsal skin of the mice were dissected, fixed with 10% neutral buffered formalin, embedded in paraffin, and stained with

hematoxylin–eosin and toluidine blue. For statistical analysis, we measured epidermal thicknesses and number of inflammatory cells ($/0.06 \text{ mm}^2$) in hematoxylin–eosin-stained samples and also counted mast cells ($/0.06 \text{ mm}^2$) in toluidine blue-stained samples.

Measurement of serum immunoglobulin (IgE)

Blood samples were taken from mice at 8 weeks after the start of the special diet. Serum levels of IgE were measured by using a Mouse IgE ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, AL, USA).

Measurement of skin chemokines

Aliquots of 0.1 g mouse skin were homogenized by phosphate-buffered saline (PBS) with 0.1% nonidet P-40 and 1 mmol/L phenylmethylsulfonyl fluoride and centrifuged at 15 000 g for 10 min. The supernatants were filtered (0.22 μm) and used for the measurement of CCL27. For the measurement of CCL27, rat antimouse CCL27 capture antibody, biotinylated goat antimouse CCL27 antibody (2 $\mu\text{g}/\text{mL}$; R&D Systems, Minneapolis, MN, USA) and recombinant CCL27 standards (PeproTech, Rocky Hill, NJ, USA) were used as described previously.⁴

For the measurement of TARC, 0.1 g of mouse skin was homogenized by PBS with protease inhibitor and phosphatase inhibitor, centrifuged at 15 000 g for 60 min and then the supernatants were measured with a Mouse CCL17/TARC Quantikine ELISA kit (R&D Systems) according to the manufacturer's instructions.

Statistical analysis

Bonferroni's multiple comparison tests were used to analyze the results, and $P > 0.05$ was considered statistically significant. Analyses were performed by the use of GRAPHPAD PRISM version 5.04 (GraphPad Software, San Diego, CA, USA). Data were expressed as mean $\pm$ standard deviation.

RESULTS

Appearances of HR-1 mice

In the group of mice fed with the HR-AD diet (HR group), the skin became xerotic and showed wrinkle-like changes (Fig. 1b), as compared with the skin of mice in the standard diet group (Co group) (Fig. 1a). In the group of mice fed with the HR-AD diet and β -carotene (HR-C) or lycopene (HR-L), slight changes of the skin with xerosis and wrinkle-like appearance were observed, but were milder than those in the HR group (Fig. 1c,d). Orange and slightly pinkish pigmentations of skin due to depositions of carotenoids were observed in the mice of the HR-C and HR-L groups, respectively (Fig. 1c,d).

Assessments of skin condition

Skin moisture levels of the HR-C and HR-L groups were statistically higher than that of the HR group (Fig. 2a). Moreover, TEWL of HR-L group, but not HR-C group, was statistically lower than that of HR group (Fig. 2b).

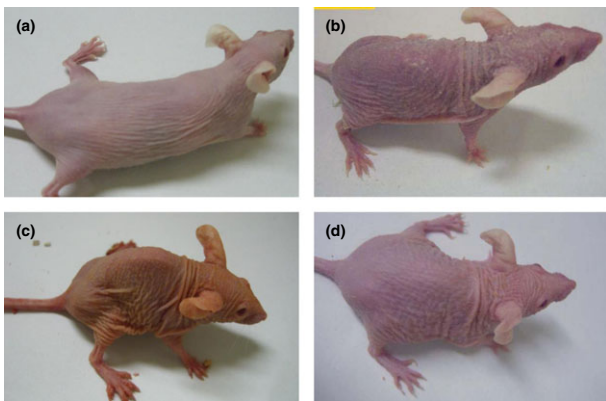


Figure 1. Representative macroscopic findings in mice fed with (a) standard diet, (b) low zinc and magnesium (HR-AD) diet, (c) HR-AD diet and β -carotene, and (d) HR-AD diet and lycopene for 8 weeks.

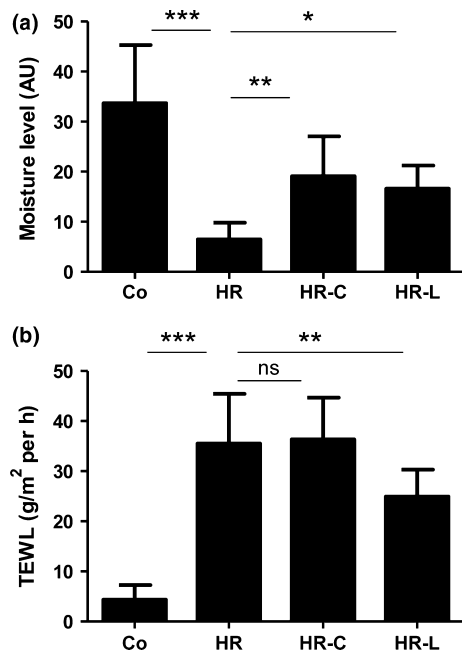


Figure 2. Comparison of (a) water content in corner layer and (b) transepidermal water loss (TEWL) between mice fed with each diet for 8 weeks. Co, group fed with standard diet ($n = 6$); HR, group fed with low zinc and magnesium (HR-AD) diet ($n = 6$); HR-C, group fed with HR-AD diet and β -carotene ($n = 6$); HR-L, group fed with HR-AD diet and lycopene ($n = 6$). Data are expressed as mean $\pm$ standard deviation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Histological examination

Histological examination of skin specimens with hematoxylin-eosin staining showed thickening of the epidermis and dermis, with inflammatory cell infiltration into the dermis of the mice in the HR group, as compared with the Co group (Fig. 3, left).

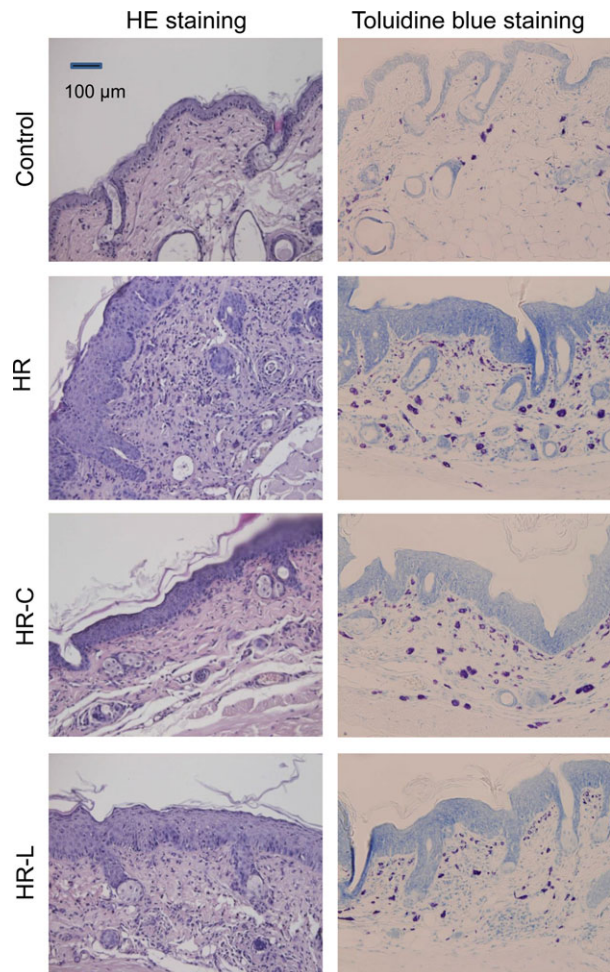


Figure 3. Histopathological findings of the mouse skin after feeding mice with each diet for 8 weeks. Skin specimens of mice were evaluated by hematoxylin-eosin (HE) and toluidine blue staining.

Both the thickening of the epidermis and inflammatory cell infiltration were observed in the skin of the HR-C and HR-L groups, but were statistically milder than those of the HR group (Fig. 4a,b). Toluidine blue staining (Fig. 3, right) revealed that the number of mast cells was significantly increased in the HR group when compared with the Co group. The number of mast cells in the HR-L group, but not the HR-C group, was statistically lower than that of the HR group (Fig. 4c).

Serum IgE and skin chemokines

The serum IgE level of the HR group was significantly higher than that of the Co group. However, there were no significant differences among the HR group and HR-C or HR-L groups (Fig. 5a). The amounts of skin TARC and CCL27 in the HR group were significantly higher than those in the Co group (Figs. 5b,c). The amount of skin TARC in the HR-L group, but not the HR-C group, was statistically lower than that in the HR group (Fig. 5b). In contrast to skin TARC, the amount of skin

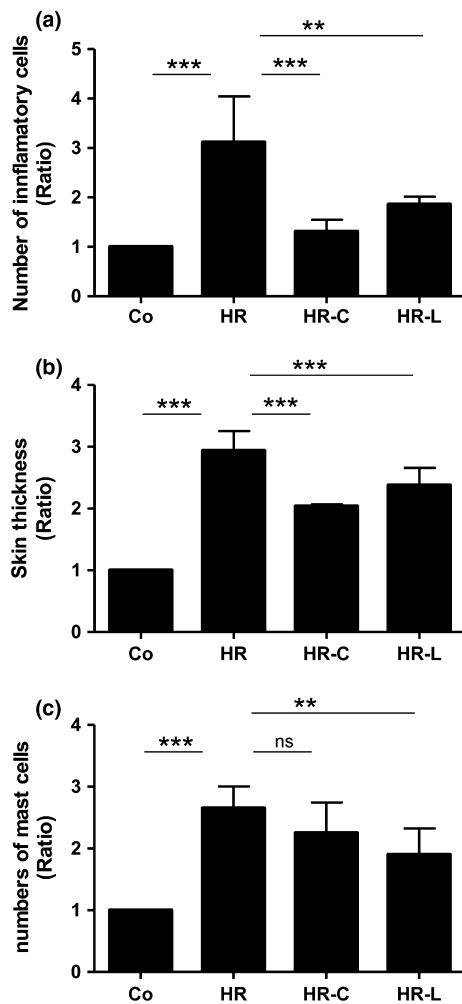


Figure 4. Statistical analysis of histopathological findings in Fig. 3. (a) $n = 20$, (b) $n = 6$, (c) $n = 10$. Data are expressed as mean $\pm$ standard deviation. ** $P < 0.01$, *** $P < 0.001$. Co, standard diet; HR, low zinc and magnesium (HR-AD) diet; HR-C, HR-AD diet and β -carotene; HR-L, HR-AD diet and lycopene.

CCL27 in the HR-C group, but not the HR-L group, was statistically lower than that in the HR group (Fig. 5c).

DISCUSSION

In this study, we have revealed that carotenoids (β -carotene or lycopene) ameliorate symptoms of AD-like dermatitis in a murine model. AD-like symptoms, including visual appearances, moisture levels of the skin, inflammatory cells in the dermis and skin thickness, of HR-1 mice fed with a low zinc and magnesium diet were significantly reduced by the intake of β -carotene or lycopene.

As reported previously,^{13–15} HR-1 mice fed with a low mineral diet (HR group) developed AD-like lesions both clinically and histopathologically. Mice of the HR-C and HR-L groups developed xerosis and wrinkle-like changes of the skin and showed scratching behavior, but they were clearly milder than

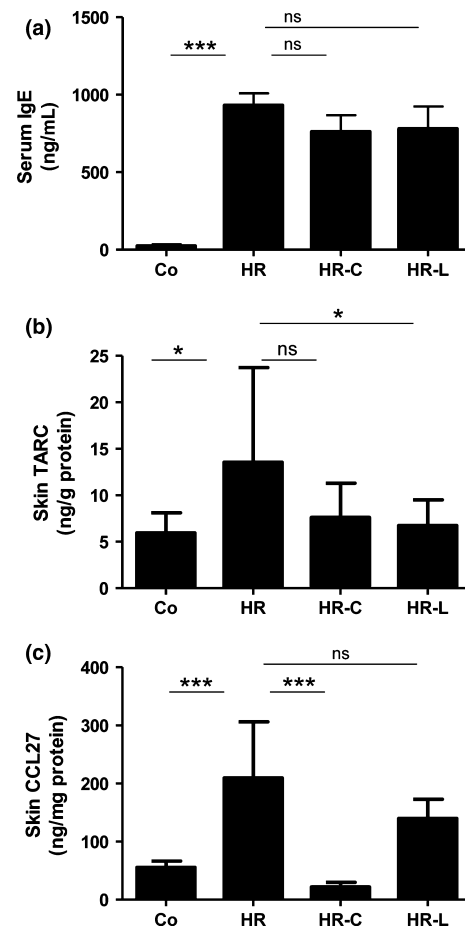


Figure 5. (a) Serum immunoglobulin E levels of mice fed with each diet for 8 weeks were measured. Co, $n = 6$; HR, $n = 4$; HR-C, $n = 4$; HR-L, $n = 6$. Skin samples of mice fed with each diet for 8 weeks were taken and skin concentrations of (b) thymus and activation regulated chemokine (TARC) and (c) CCL27 in the skin were measured by enzyme-linked immunoassay. Co, $n = 6$; HR, $n = 6$; HR-C, $n = 4$; HR-L, $n = 6$. Data are expressed as mean $\pm$ standard deviation. * $P < 0.05$, *** $P < 0.001$. Co, standard diet; HR, low zinc and magnesium (HR-AD) diet; HR-C, HR-AD diet and β -carotene; HR-L, HR-AD diet and lycopene.

those observed in mice of the HR group. One unique change observed in mice fed with carotenoids was the change in skin color. Skin color of mice in the HR-C group changed to orange and that in the HR-L group changed to light pink. Aurantiasis cutis is a well-known symptom due to excess ingestion of carotenoids. The concentration of carotenoids of the skin was higher than those of other organs, such as the lungs and colon.¹⁶ However, the accumulation of carotenoid itself is harmless and reversible. Therefore, β -carotene and lycopene likely exerted a direct effect on the skin.

The evaluation of skin condition by using an MPA5 multiprobe analyzer and by histopathological analysis revealed that mice in the HR-L group showed significantly better skin condition than

those in the HR group on all evaluation criteria including moisture level of the skin, TEWL, skin thickness, and number of inflammatory cells and mast cells. On the other hand, β -carotene showed weaker effects on TEWL and the number of mast cells. These results suggest that the anti-allergic effect exhibited by lycopene on skin may be stronger than that of β -carotene.

In our murine model, β -carotene and lycopene did not suppress total serum IgE. Sato *et al.*¹⁷ reported that β -carotene improves the Th1–Th2 balance, inhibiting ovalbumin (OVA)-specific IgE and IgG1 production and antigen-induced anaphylactic response in an OVA conjugated BALB/c murine model. In contrast, Bando *et al.*¹⁸ reported that the supplementation with β -carotene alone had no effect on OVA-specific IgE or total IgE production in the same OVA conjugated BALB/c mouse model. Taken together, β -carotene and lycopene may directly act on the skin rather than on B cells in spleen. Thus, we assessed the effect of carotenoids on chemokines especially related to keratinocytes and revealed that skin CCL27 in the HR-C group and TARC in the HR-L group were significantly lower than those of HR group. The anti-allergic effect of β -carotene and lycopene were different on TEWL and mast cell number *in vivo*, and skin chemokines *in vitro*. It may be due to differences in their structures (bicyclic vs acyclic), the existence of provitamin A activity⁸ and their metabolism *in vivo*.

We could not demonstrate a direct anti-inflammatory effect of carotenoids on human keratinocyte cells *in vitro*, using HaCaT cells, a cell line of human keratinocytes, stimulated by interferon (IFN)- γ and IL-10. However, the production of TARC from HaCaT cells tended to be suppressed by β -carotene and lycopene (see Supporting Information). Moreover, several reports have revealed that serum carotenoid levels among pediatric patients with AD are lower than that of normal children.^{11,12} Therefore, prospective cohort or intervention studies are needed to assess the real effects of carotenoids on human AD.

In conclusion, we revealed that the p.o. intake of β -carotene or lycopene ameliorated AD-like symptoms, possibly through suppressing the production of cutaneous chemokines in HR-1 mice. Oral intake of β -carotene or lycopene has the potential to prevent or alleviate symptoms of AD.

ACKNOWLEDGMENTS: We thank for Dr Faiz Kermani and Mr Satoshi Sunabori for their critical review of the manuscript.

CONFLICT OF INTEREST: This study was funded in part by Kagome.

REFERENCES

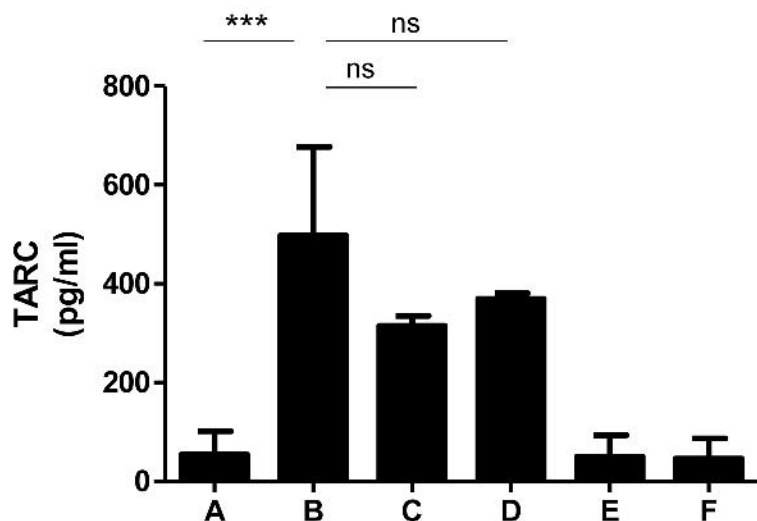
- 1 Saeki H, Furue M, Furukawa F *et al.* Guidelines for management of atopic dermatitis. *J Dermatol* 2009; **36**: 563–577.
- 2 Katayama I, Kohno Y, Akiyama K *et al.* Japanese Guideline for Atopic Dermatitis 2014. *Allergol Int* 2014; **63**: 377–398.
- 3 Bieber T. Atopic dermatitis. *N Engl J Med* 2008; **358**: 1483–1494.
- 4 Chen L, Lin SX, Agha-Majzoub R, Overbergh L, Mathieu C, Chan LS. CCL27 is a critical factor for the development of atopic dermatitis in the keratin-14 IL-4 transgenic mouse model. *Int Immunol* 2006; **18**: 1233–1242.
- 5 Morales J, Homey B, Vicari AP *et al.* CTACK, a skin-associated chemokine that preferentially attracts skin-homing memory T cells. *Proc Natl Acad Sci U S A* 1999; **96**: 14470–14475.
- 6 Machura E, Rusek-Zychma M, Jachimowicz M, Wrzask M, Mazur B, Kasperska-Zajac A. Serum TARC and CTACK concentrations in children with atopic dermatitis, allergic asthma, and urticaria. *Pediatr Allergy Immunol* 2012; **23**: 278–284.
- 7 Armstrong GA, Hearst JE. Carotenoids 2: genetics and molecular biology of carotenoid pigment biosynthesis. *FASEB J* 1996; **10**: 228–237.
- 8 Namitha KK, Negi PS. Chemistry and biotechnology of carotenoids. *Crit Rev Food Sci Nutr* 2010; **50**: 728–760.
- 9 Akiyama H, Katayama S, Kanda T *et al.* Prevention of allergic disease development and symptoms by food factors. *Curr Pharm Des* 2014; **20**: 892–905.
- 10 Rosenlund H, Magnusson J, Kull I *et al.* Antioxidant intake and allergic disease in children. *Clin Exp Allergy* 2012; **42**: 1491–1500.
- 11 Rühl R, Taner C, Schweigert FJ, Wahn U, Grüber C. Serum carotenoids and atopy among children of different ethnic origin living in Germany. *Pediatr Allergy Immunol* 2010; **21**: 1072–1075.
- 12 Oh SY, Chung J, Kim MK, Kwon SO, Cho BH. Antioxidant nutrient intakes and corresponding biomarkers associated with the risk of atopic dermatitis in young children. *Eur J Clin Nutr* 2010; **64**: 245–252.
- 13 Makiura M, Akamatsu H, Akita H *et al.* Atopic dermatitis-like symptoms in HR-1 hairless mice fed a diet low in magnesium and zinc. *J Int Med Res* 2004; **32**: 392–399.
- 14 Fujii M, Nakashima H, Tomozawa J *et al.* Deficiency of n-6 polyunsaturated fatty acids is mainly responsible for atopic dermatitis-like pruritic skin inflammation in special diet-fed hairless mice. *Exp Dermatol* 2013; **22**: 272–277.
- 15 Fujii M, Shimazaki Y, Muto Y, Kohno S, Ohya S, Nabe T. Dietary deficiencies of unsaturated fatty acids and starch cause atopic dermatitis-like pruritus in hairless mice. *Exp Dermatol* 2015; **24**: 108–113.
- 16 Nierenberg DW, Nann SL. A method for determining concentrations of retinol, tocopherol, and five carotenoids in human plasma and tissue samples. *Am J Clin Nutr* 1992; **56**: 417–426.
- 17 Sato Y, Akiyama H, Suganuma H *et al.* The feeding of beta-carotene down-regulates serum IgE levels and inhibits the type I allergic response in mice. *Biol Pharm Bull* 2004; **27**: 978–984.
- 18 Bando N, Yamanishi R, Terao J. Inhibition of immunoglobulin E production in allergic model mice by supplementation with vitamin E and beta-carotene. *Biosci Biotechnol Biochem* 2003; **67**: 2176–2182.

SUPPORTING INFORMATION

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

Figure S1. To examine the influence of carotenoids on the production of thymus and activation regulated chemokine (TARC), HaCaT cells were stimulated by the combination of interferon (IFN)- γ and interleukin (IL)-10 in the presence or absence of carotenoids for 48 h.

Appendix S1. A method of Figure S1.



Supplementary Figure

To examine the influence of carotenoids on the production of TARC, HaCaT cells were stimulated by the combination of IFN- γ and IL-10 in the presence or absence of carotenoids for 48 hours. The concentrations of TARC in the culture supernatants were measured by ELISA. Data were mean of 3 experiments and expressed as mean $\pm$ SD.

A: no stimulation, B: 10 ng/ml IFN- γ and 100 ng/ml IL-10, C: B+ 5 μ M β -carotene, D: B+ 5 μ M lycopene, E: 5 μ M β -carotene alone, F: 5 μ M lycopene alone. *** p <0.001

Supplementary

Methods

HaCaT cells were maintained in Dulbecco's modified eagle medium (DMEM) containing 10% fetal calf serum (FCS) and antibiotics. The cells were trypsinized and 0.5 ml of 1×10^5 /ml cells were seeded onto 24 well plates. After reaching confluence, culture medium was replaced with 0.1% FCS-DMEM and cells were incubated for an additional 12 hrs. Subsequently, cells were stimulated by the combination of IFN- γ and IL-10 in the presence or absence of carotenoids for 48 hrs and then culture supernatants were collected for the measurement of TARC by Quantikine[®] ELISA Human CCL17/TARC Immunoassay (R&D systems, Minneapolis, MN). Recombinant human IFN- γ and IL-10 were purchased from R&D systems. Beta-carotene and lycopene were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).