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IN VITRO AND ANIMAL STUDIES



Supplemental feeding of a gut microbial metabolite of linoleic acid, 10-hydroxy-*cis*-12-octadecenoic acid, alleviates spontaneous atopic dermatitis and modulates intestinal microbiota in NC/nga mice

Hiroko Kaikiri^a, Junki Miyamoto^a, Takahiro Kawakami^a, Si-Bum Park^b, Nahoko Kitamura^b, Shigenobu Kishino^b, Yasunori Yonejima^c, Keiko Hisa^c, Jun Watanabe^d, Tasuku Ogita^d, Jun Ogawa^b, Soichi Tanabe^a and Takuya Suzuki^a

^aGraduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima, Japan; ^bGraduate School of Agriculture, Kyoto University, Kyoto, Japan; ^cResearch and Development Department, Nitto Pharmaceutical Industries, Ltd, Muko, Japan; ^dNational Food Research Institute, National Agriculture and Food Research Organization, Tsukuba, Japan

ABSTRACT

The present study investigated the antiallergic and anti-inflammatory effects of 10-hydroxy-*cis*-12-octadecenoic acid (HYA), a novel gut microbial metabolite of linoleic acid, in NC/Nga mice, a model of atopic dermatitis (AD). Feeding HYA decreased the plasma immunoglobulin E level and skin infiltration of mast cells with a concomitant decrease in dermatitis score. HYA feeding decreased TNF- α and increased claudin-1, a tight junction protein, levels in the mouse skin. Cytokine expression levels in the skin and intestinal Peyer's patches cells suggested that HYA improved the Th1/Th2 balance in mice. Immunoglobulin A concentration in the feces of the HYA-fed mice was approximately four times higher than that in the control mice. Finally, denaturing gradient gel electrophoresis of the PCR-amplified 16S rRNA gene of fecal microbes indicated the modification of microbiota by HYA. Taken together, the alterations in the intestinal microbiota might be, at least in part, associated with the antiallergic effect of HYA.

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10-Hydroxy-*cis*-12-octadecenoic acid; gut microbial metabolite; atopic dermatitis; intestinal microbiota

Introduction

Functional fatty acids have attracted a good deal of attention due to their various beneficial effects on our health. Conjugated linoleic acids (CLAs), which exert anti-inflammatory and -allergic effects (Martinasso et al. 2010; Bergamo et al. 2011), can be seen as a representative of this group of fatty acids. CLAs appear to repress the activation of the nuclear factor-kappa B (NF- κ B) pathway via peroxisome proliferator-activated receptor (PPAR) γ (Zenhom et al. 2011). Recently, we have reported that gut microorganisms generate hydroxy fatty acids, oxo fatty acids, conjugated fatty acids and partially saturated (non-methylene interrupted) fatty acids from food-derived polyunsaturated fatty acids (PUFAs) via the biohydrogenation pathway (Kishino et al. 2013). Our previous study demonstrates that 10-hydroxy-*cis*-12-octadecenoic acid (HYA), one of the initial microbial metabolites of linoleic acid, protects the intestinal epithelial barrier and ameliorates colitis development (Miyamoto et al. 2015). HYA also suppresses tumor necrosis factor receptor (TNFR)

2 expression via the G protein-coupled receptor 40 pathway. Thus, the gut microbial metabolites of PUFA, including HYA, may have the potential to afford functional food ingredients; however, only limited information is currently available on their physiological functions.

An increasing number of people in industrialized countries suffer from immunoglobulin (Ig) E-mediated (type I) allergic symptoms including atopic dermatitis (AD) (Galli & Tsai 2012). AD is a chronic inflammatory skin disorder marked by elevated plasma IgE levels. The pathogenesis of AD is complex and involves a variety of immunological factors including an imbalance in helper T-cell subsets such as Th1, Th2, Th17 and Treg (Nagashio et al. 2013; Correa da Rosa et al. 2015). Classically, the disturbance of the Th1/Th2 balance has been emphasized in the AD pathogenesis as interferon (IFN)- γ secreted by Th1 cells suppresses the proliferation of Th2 cells, while interleukin (IL)-4 produced by Th2 cells blocks Th1 differentiation from naïve T cells and induces class switching to IgE. Further, a recent study showed that Th17 cells also

aggravate atopic eczema and play a pathogenic role in AD development (Suárez-Fariñas et al. 2013). In addition to the imbalance in helper T-cell subsets, abnormalities among intestinal commensal bacteria have also been suggested as one of the clinical causes of AD (Drago et al. 2012). A clinical study showed differences in gut microbiota composition between allergic and healthy children (Drell et al. 2015). In connection with these gut microbiota, intestinal IgA is associated with a reduced risk of IgE-associated allergic diseases as IgA suppresses immune activation by luminal antigens (Kukkonen et al. 2010).

The potential to ameliorate AD through dietary intervention is currently attracting a good deal of attention, as the patients increasingly wish to avoid the long-term use of topical steroids and oral anti-histamines. Our previous study demonstrated that hesperidin, a flavonoid abundant in citrus fruits, suppresses the exacerbation of AD-like symptoms and systemic immune hyper-responsiveness in part through the reduction in IgE, IL-17 and IFN- γ levels in NC/Nga mice. NC/Nga mice spontaneously develop dermatitis with associated IgE hyperproduction, in a manner very similar to that observed in human AD, when kept under conventional conditions and, therefore, are widely used for evaluating the anti-allergic activity of food components (Tanabe & Hoshi 2010; Nagashio et al. 2013). As for the amelioration of allergic disorders through the use of food components, CLAs were reported to reduce allergic airway inflammation in ovalbumin-sensitized BALB/c mice, likely via a PPAR- γ -related mechanism (Jaudszus et al. 2008).

The present study investigated the anti-allergic and anti-inflammatory effects of HYA in NC/Nga mice with a particular focus on the regulation of the helper T-cell balance in Peyer's patches as well as fecal IgA and microbiota composition in these mice.

Materials and methods

Reagents

HYA was synthesized according to the method published previously (Kishino et al. 2013) and was provided by Nitto Pharmaceutical Industries, Ltd (Muko, Japan). The chemical structure of HYA is shown in Figure 1. Rabbit antiphospho-NF- κ B p65 (Cell Signaling Technology, Danvers, MA), rabbit anti-claudin-1 (Thermo Fisher Scientific, Waltham, MA), goat horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG (Dako, Glostrup, Denmark) and goat fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Thermo Fisher Scientific) were used for

immunoblot and immunostaining analyses. All other reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Mice and experimental design

All animal studies were preapproved by the Hiroshima University Animal Committee, and mice were maintained in accordance with the Hiroshima University guidelines for the care and use of laboratory animals. Four-week-old female NC/Nga mice (16–18 g) were purchased from Japan SLC (Shizuoka, Japan) and housed in a room with controlled temperature ($22^{\circ}\text{C} \pm 2$) and a 12-h light/12-h dark cycle under conventional conditions. Animals were given free access to diets and water. Mice were divided into two groups: the control and HYA groups ($n=5$ each). The control group was fed an AIN-93 G formula diet, while the HYA group received an AIN-93 G diet supplemented with 0.01% (w/w) HYA. All mice were maintained on their respective diets for 6 weeks during which their scratching behavior and clinical symptoms were evaluated as described below. Once every alternate week, blood samples were collected from the tail veins of the mice, and plasma obtained by centrifugation. Total plasma IgE levels were measured using a sandwich ELISA kit (Bethyl laboratories, Inc., Montgomery, TX) according to the manufacturer's instructions. At the end of the 6-week experimental period (at 10 weeks of age), mice were sacrificed by cervical dislocation, and the skin and Peyer's patches of the small intestinal mucosa were collected for subsequent analyses as described below. Paraffin sections (7 μm thick) of the skin samples were stained with hematoxylin and eosin for histological analysis and with toluidine blue for the detection of mast cells. Fresh fecal samples were collected from each mouse one day before sacrifice for the analysis of IgA and intestinal microbiota as described below.

Evaluation of scratching behavior and clinical symptoms

The scratching behavior and clinical symptoms were evaluated as described previously with a small modification (Tanabe & Hoshi 2010; Nagashio et al. 2013). Briefly, the severity of dermatitis was assessed by assigning a score for each of five symptom items (scratching behavior, hemorrhage, edema, excoriation/erosion and xerosis/dryness) according to the method described by Matsuda et al. (1997). The scale used was as follows: 0 (no symptoms), 1 (mild), 2 (moderate) and 3 (severe). For scratching behavior, mice were habituated in the testing box for 3 min and the

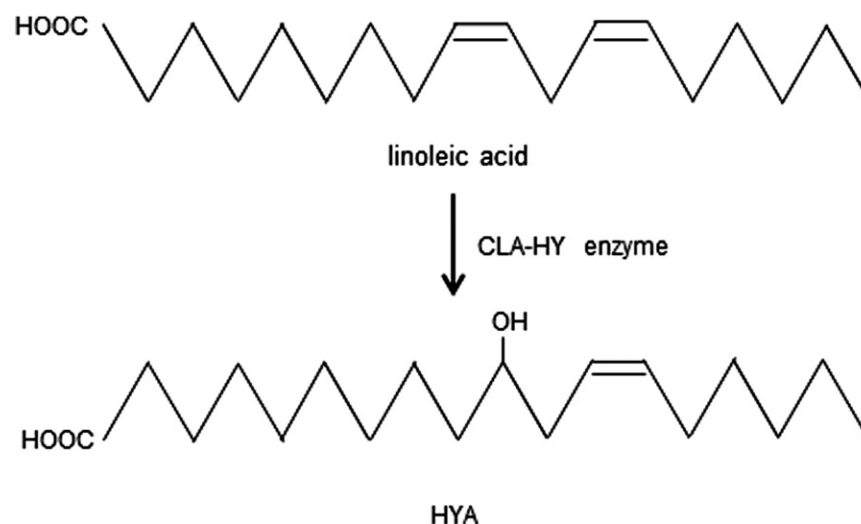


Figure 1. Chemical structures of linoleic acid and HYA. HYA is metabolized and synthesized from linoleic acid by CLA-HY enzyme.

number of scratching incidents in 3 min was assessed and converted to a score ranging from 0 to 3 for summation. The total score was expressed as the sum of scores for the five items.

Real-time RT-PCR

Total RNA was extracted from the skin and Peyer's patch cells using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The reverse-transcription (RT) reaction was performed using a high-capacity cDNA reverse-transcription kit (Thermo Fisher Scientific) at 25°C for 10 min and at 37°C for 120 min. The reaction was terminated by heating to 85°C for 5 min followed by cooling at 4°C. Real-time PCR was performed using FastStart Essential DNA Green Master (Roche Diagnostics GmbH, Mannheim, Germany). Primers were designed using the Primer3Plus Program (Untergasser et al. 2007) and the sequences are shown in Table 1. The PCR was performed at 95°C for 2 min, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. The dissociation stage was analyzed at 95°C for 20 s, followed by 1 cycle of 60°C for 20 s and 95°C for 20 s. The fluorescence of the green dye was determined as a function of the PCR cycle number, giving the threshold cycle number at which amplification reached a significant threshold. Data were analyzed by the $\Delta\Delta C_t$ method and presented as fold changes in gene expression from the unstimulated level, after normalization against the GAPDH gene.

Immunoblot analysis

Skin tissue (10 mg) from the mice was homogenized in 1 mL RIPA lysis buffer containing protease and

phosphatase inhibitors (Atto corporation, Tokyo, Japan) using a polytron-type homogenizer. Protein concentrations were measured using the BCA method (Thermo Fisher Scientific). Tissue extracts were mixed with a one-half volume of Laemmli sample buffer (3× concentrated; containing 4% SDS, 10% glycerol, 10% β-mercaptoethanol and 0.04% bromophenol blue in 125 mM Tris HCl, pH 6.8) and heated 95°C for 10 min. The proteins (20 μg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P PVDF, Merck KGaA, Darmstadt, Land Hessen, Germany). Membranes were blotted for claudin-1 and β-actin using specific antibodies in combination with HRP-conjugated anti-rabbit IgG antibodies. The blots were developed using the ECL chemiluminescence method (PerkinElmer Life Sciences, Waltham, MA). Quantification was performed by densitometric analysis of specific bands on the immunoblots using Image J software (National Institutes of Health, Bethesda, MD).

Immunofluorescence analysis

Mouse skin tissues were embedded in OCT compound (Sakura Finetek, Tokyo, Japan), and frozen sections (7 μm thick) were prepared on glass slides. The sections were fixed with 3% (w/v) paraformaldehyde for 10 min and washed with PBS. The sections were blocked in 5% normal goat serum (Wako Pure Chemical Industries) and incubated with rabbit polyclonal anti-p-NF-κB p65 at 4°C for 16 h, followed by incubation for 1 h with goat FITC-conjugated anti-rabbit IgG for the detection of p-NF-κB p65. The specimens were preserved in a mounting medium,

Table 1. Primer sequences used for experiments.

Gene		Sequence
GAPDH	Forward	5'-TCAAGAAGGTGGTGAAGCAG-3'
	Reverse	5'-AAGGTGGAAGAGTGGGAGTTG-3'
Claudin-1	Forward	5'-GATGTGGATGGCTGTCATTG-3'
	Reverse	5'-CCTGGCCAAATTCATACCTG-3'
IL-4	Forward	5'-AGAGAGTGAGCTCGTCTGTAGG-3'
	Reverse	5'-CTTCTCAAGCATGGAGTTTC-3'
IL-5	Forward	5'-TGCAAGAGTTCCTTGGTGTG-3'
	Reverse	5'-GCACAGTTTTGTGGGTTTT-3'
IL-6	Forward	5'-CTGATGCTGGTGACAACAC-3'
	Reverse	5'-TCCACGATTTCCAGAGAAC-3'
IL-33	Forward	5'-CTTCTCGCTGATTCCAGAG-3'
	Reverse	5'-TGAAGCTGAACAGAACCTGTG-3'
TNF- α	Forward	5'-TCGTAGCAACACCAAGTAC-3'
	Reverse	5'-CTTTGAGATCCATGCCGTTG-3'
Mcp1	Forward	5'-TGTAATCCCTTGCTGGTC-3'
	Reverse	5'-TCAGGGTTTCTGAGGTAGGTTT-3'
Tbet	Forward	5'-ACCAGAACGACAGATCACTCA-3'
	Reverse	5'-CAAATCTCCCGGAATCCTT-3'
Gata3	Forward	5'-CATCCCTGAGCCACATCTCT-3'
	Reverse	5'-AGGGCTCTGCCTCTAACC-3'
Roryt	Forward	5'-GCAAATACGGTGGTGTGGA-3'
	Reverse	5'-CGCTGAGGAAGTGGGAAA-3'
Foxp3	Forward	5'-CGAAAGTGGCAGAGAGGATTG-3'
	Reverse	5'-TGAAGGCAGAGTCAGGAGAAG-3'

and fluorescence was visualized using a fluorescence microscope (ECLIPSE E6000, Nikon, Tokyo, Japan).

Peyer's patch cells

The Peyer's patch cells collected from the small intestinal mucosa were prepared as described previously (Kikuchi et al. 2015) with a small modification. The intestinal Peyer's patch cells were finely cut on a petri dish and were brayed on a 100- μ m mesh nylon cell strainer (BD Biosciences, San Jose, CA). The cells, suspended in advanced RPMI 1640 medium (Thermo Fisher Scientific) containing 1% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), were centrifuged at 350 $\times$ g at room temperature for 5 min and incubated with 1.5 mg/mL dispase (Thermo Fisher Scientific) at 37°C for 10 min. After washing with PBS, the Peyer's patch cell population was obtained. The cells were plated at 1×10^6 cells per well in a 96-well plate and cultured in advanced RPMI 1640 medium containing 1% fetal bovine serum, 2 mM of L-glutamine, 50 μ M 2-mercaptoethanol, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 5 U/mL penicillin and 5 μ g/mL streptomycin at 37°C. After incubation for 6 h, total RNA was extracted from the Peyer's patch cells, and the real-time RT-PCR was performed as described above. After incubation for 72 h, the cell culture medium was harvested for the determination of cytokines (IFN- γ , IL-4, IL-10 and IL-17 A) and IgA concentration using sandwich ELISA kits (DuoSet ELISA development system, R&D Systems, Abingdon, UK and mouse IgA ELISA quantitation kit, Bethyl laboratories, Montgomery, TX, respectively).

Determination of fecal IgA

Feces (10 mg) were homogenized with sea sand in PBS (1.5 mL each) and centrifuged at 830 $\times$ g at 4°C for 30 min to remove debris. IgA levels in the supernatants were determined using a mouse IgA ELISA quantitation kit (Bethyl Laboratories).

PCR-denaturing gradient gel electrophoresis analysis of fecal microbiota

Microbial DNA was extracted from the feces using a Power Fecal DNA Isolation Kit (MO Bio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. The V2-V3 region of the 16S rRNA gene from the DNA samples was amplified with universal primers, HDA1 with the GC clamp (5'-CGCCCGGG-GCGCGCCCCGGGGCGGGGGCGGGGGCACGGGGGG-CTCCTACGGGAGG-3') and HDA2 (5'-GTATTA-CCGCGGCTGCTGGCAC-3') using the TaKaRa Ex Taq Hot Start Version (Takara Bio, Shiga, Japan). The cycling conditions were as follows: initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min and final elongation at 72°C for 7 min. The PCR products were then subjected to denaturing gradient gel electrophoresis (DGGE). The DGGE was performed using 8% polyacrylamide gel containing 30% to 55% denaturant (a 100% denaturing solution contained 40% formamide and 7 M urea). The gel was run at 80 V for 16 h 60°C in TAE buffer using a DCode system (Bio-Rad, Hercules, CA). The gel was silver-stained with Bio-Rad silver stain (Bio-Rad) and densitograms of the DGGE bands were created by Image J software (Rasband 1997–2012). Results of the hierarchical cluster analysis of the DGGE band patterns were displayed as dendrograms. The squared distances between clusters were measured by Ward's method (Ward 1963).

Statistical analysis

All values were expressed as mean $\pm$ SEM. Statistical analysis was performed using Tukey's post-hoc test with the Statcel 3 (OMS publishing, Saitama, Japan) program (Figures 2–7). A difference with a $p < .05$ was considered significant.

Results

Feeding HYA alleviated AD-like skin symptoms in NC/nga mice

In the control mice, AD-like lesions gradually developed from 8 weeks of age, with dermatitis particularly prominent around the ears and dorsal skin at 10 weeks

(Figure 2(A)). In contrast, no obvious skin lesions were observed on the HYA-fed mice. As shown in Figure 2(B), an increase in the dermatitis score was evident from 8 to 10 weeks of age in the control mice, with the scores at 9 and 10 weeks significantly higher than the initial score at 4 weeks (Figure 2(B)). The HYA group did not show any significant changes in scores throughout the experiment. The dermatitis score in the HYA mice at 10 weeks of age tended to be lower than in the control mice (Figure 2(B)). Total plasma IgE levels in both groups gradually increased up to 10 weeks of age. However, the levels in the HYA-fed group were constantly lower than those in the control group with significant differences observed at 6 and 10 weeks of ages (Figure 2(C)). There were no significant differences in body weight between the two groups throughout the experimental period (data not shown).

Feeding HYA suppressed skin inflammation and restored the skin barrier

Histological analysis demonstrated that the supplemental HYA attenuated the thickening of the

epidermis and dermal edema in the dorsal skin. As a result of measuring the thickness of the epidermis, a significant difference was observed (Figures 3(A,C)). The number of mast cells in the HYA-fed mice was lower than that in the control mice (Figure 3(B,D)). The TNF- α mRNA expression in the dorsal skin of the HYA group was also lower than that in the control group. The expression levels of IL-33 (a marker of mast cell activation) and mast cell protease-1 (Mcp1, a marker of mast cells) tended to be lower in the HYA-fed mice than in the control mice. The expression levels of IL-4 and IL-5 were not different between the two groups (Figure 4(A)). The protein expression of p-NF- κ B p65 also tended to be lower in the HYA-fed mice than in the control mice (Figures 4(B,C)). The mRNA expression of claudin-1 in the HYA-fed mice was higher than that in the control mice (Figure 5(A)). The protein level of claudin-1 tended to be higher in the HYA-fed mice than in the control mice (Figure 5(B)), indicating the restoration of the skin barrier. Immunofluorescent microscopy confirmed the increase in the protein level of claudin-1 in the HYA-fed mice (Figure 5(C)).

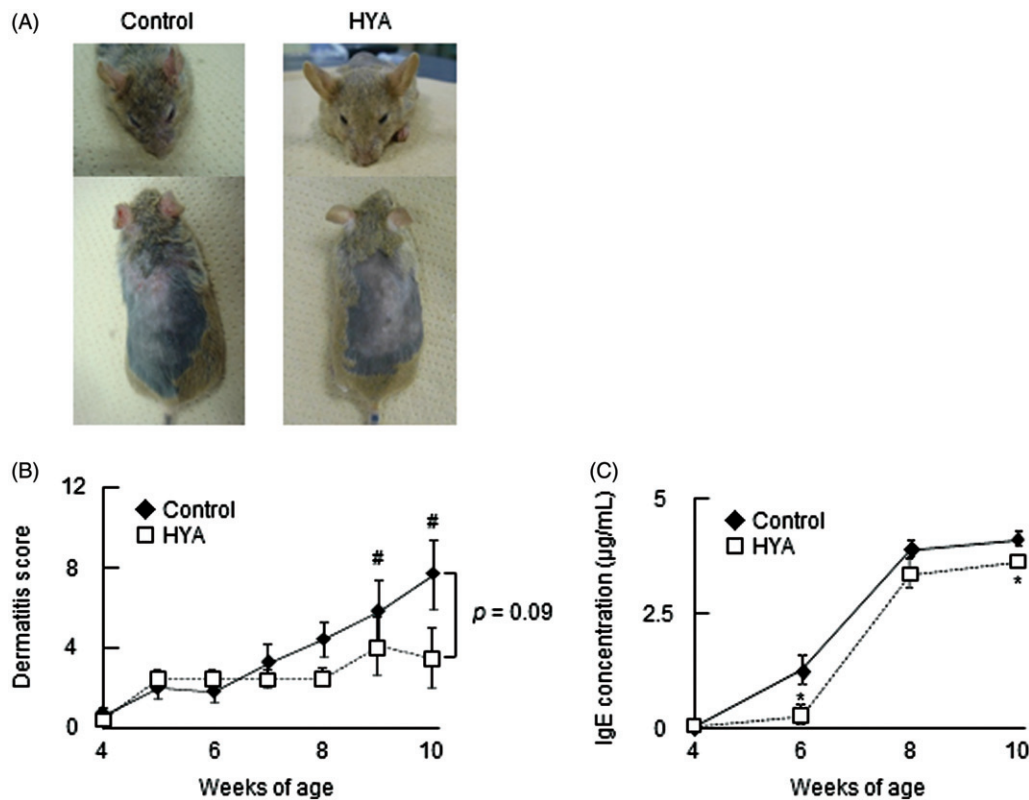


Figure 2. Inhibitory effects of HYA on skin lesions and inflammation in NC/Nga mice. (A) Representative photographs of 10-week-old mice are shown. Control mice showed severe lesions and inflammation of the dorsal skin. Feeding HYA alleviated AD-like skin lesions. (B) Clinical scores of the AD symptoms were evaluated based on five items (scratching behavior, hemorrhage, edema, excoriation/erosion and xerosis/dryness). (C) Plasma total IgE levels were measured by ELISA. Values are means $\pm$ SEM ($n = 5$). * $p < .05$ versus control group. # $p < .05$ versus each initial value at 4 weeks old.

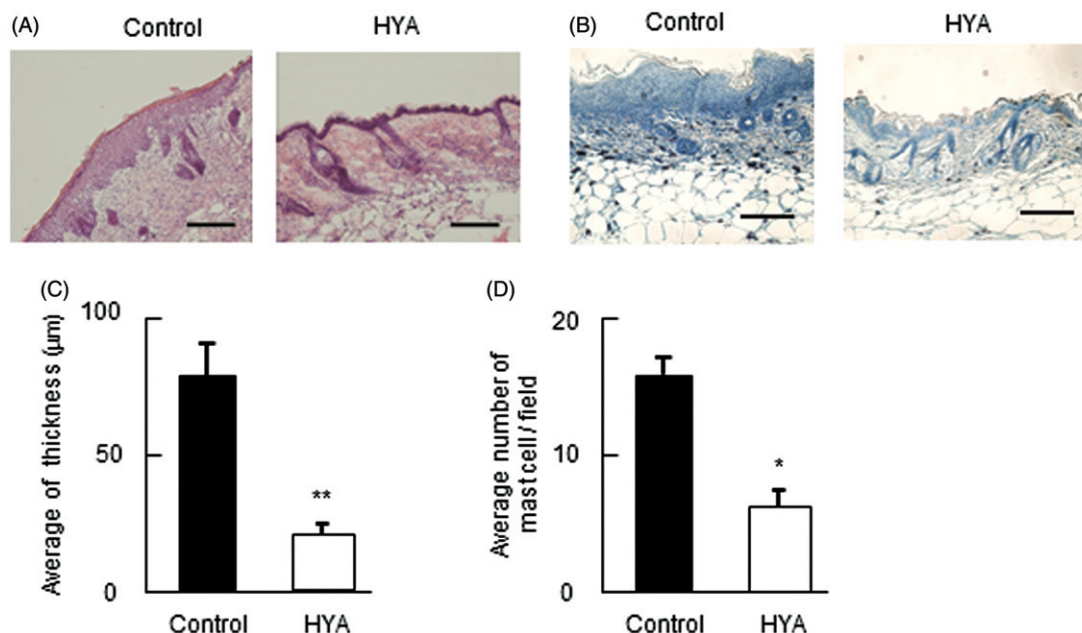


Figure 3. Anti-inflammatory effects of HYA in the dorsal skins of NC/Nga mice. (A) Dorsal skin samples of mice were stained with hematoxylin and eosin. Scale bar = 200 μm. (B) Dorsal skin samples of mice were stained with toluidine blue. Scale bar = 100 μm. (C) The thickness of epidermis was quantified. (D) The number of mast cells was quantified. Values are means ± SEM ($n = 5$). * $p < .05$ versus control group.

Th1-response was prominent in the HYA-fed mice

The production of IFN- γ , a Th1-type cytokine, from the intestinal Peyer's patch cells in the HYA-fed mice was higher than that in the control mice (Figure 6(A)). HYA feeding also tended to increase the expression of T-bet, a specific transcriptional factor to Th1 cells ($p = .06$, Figure 6(B)). On the other hand, there were no significant differences inter-group in the mRNA expression of GATA-3, ROR γ , or Foxp3, or in the production of IL-17 or IL-10 (data not shown). IL-4 production in both groups was below the detection limit.

Feeding HYA-enhanced intestinal IgA production and modulated in intestinal microflora

Feeding HYA-enhanced intestinal IgA secretion, with the fecal IgA level of the HYA-fed mice was approximately fourfold higher than that of the control mice (Figure 7(A)). A representative DGGE band profile was shown (Figure 7(B)). Cluster analysis of the DGGE profiles revealed that feeding HYA moderately modulated intestinal microflora in the mice (Figure 7(C)). The hierarchical analysis indicated two clusters; Control-A, Control-B, Control-C and Control-D belonged to the first cluster, and HYA-A, HYA-C and HYA-E and Control-E belonged to the second cluster. Although two mice (HYA-B and D) were in the first cluster, they were separated from the subcluster where four control mice belonged to. This result suggested

that HYA feeding had an impact on intestinal microflora composition.

Discussion

The prevalence of allergic disorders including AD has drastically increased in the last several decades. To our knowledge, the present study is the first to demonstrate that supplemental feeding with HYA, the initial metabolite of linoleic acid, ameliorates AD development in NC/Nga mice, a widely used model of human AD. Although the precise mechanism underlying the HYA-mediated anti-allergic effect is still unclear, the modulation of intestinal IgA and microflora might be involved, at least in part, in the regulation of the Th1/Th2 balance in NC/Nga mice.

AD is known to be a chronic inflammatory skin disorder; however, HYA feeding ameliorated the AD-like symptoms in NC/Nga mice as indicated by the clinical dermatitis scores and histological analysis. In association with the reduced skin lesions, pro-inflammatory TNF- α expression and NF- κ B p65 phosphorylation were lower in the HYA-fed mice. HYA feeding also suppressed mast cell infiltration, which is a major mediator of allergic inflammation, in the dorsal skin. Accumulating evidence has indicated that the skin inflammation and lesions associated with AD are closely related to increased Th2 cytokine and IgE production (Segawa et al. 2008). Our results suggested

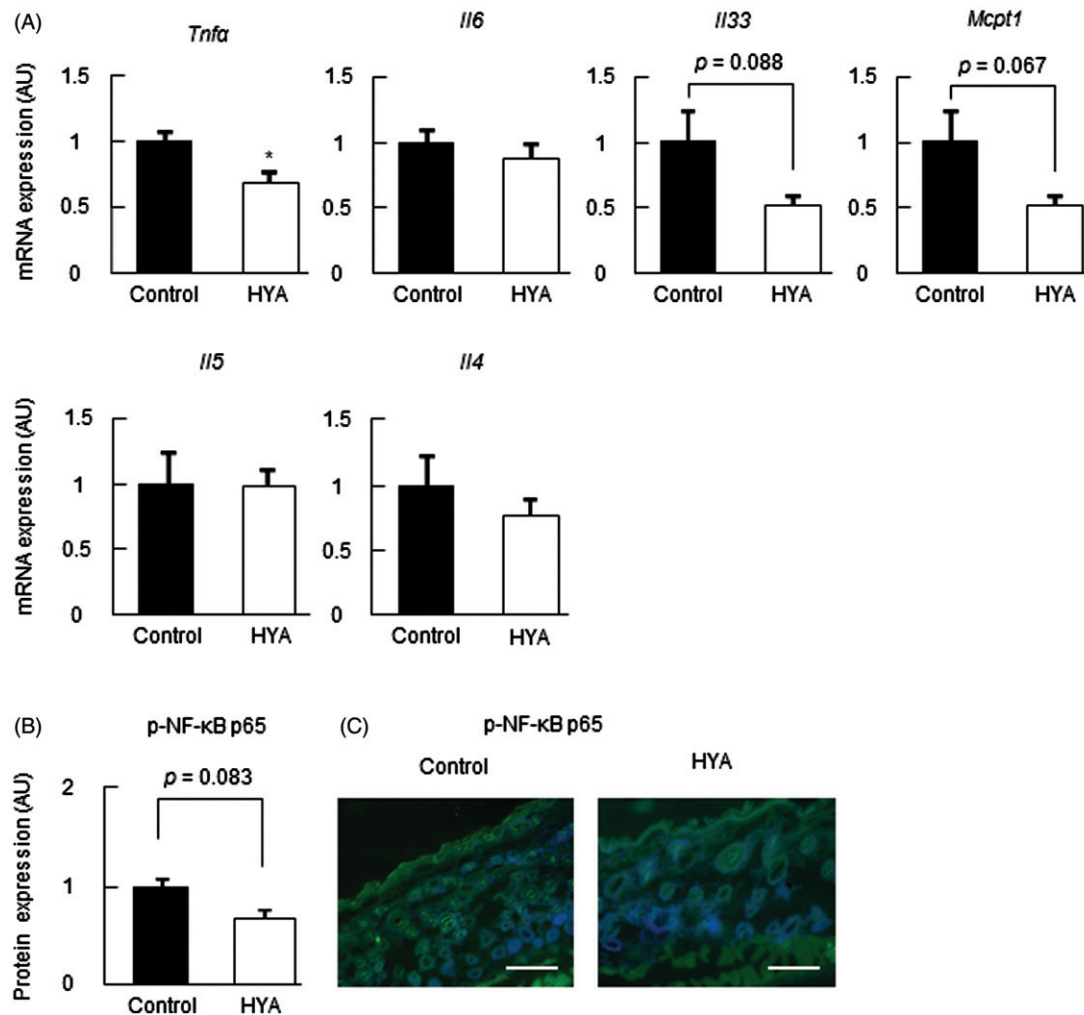


Figure 4. Cytokine expression in the dorsal skin of NC/Nga mice. (A) Total RNA was extracted from dorsal skin samples of mice, and the mRNA expression levels of TNF- α , IL-4, IL-5, IL-6, IL-33 and Mcpt1 were measured by RT-PCR. (B and C) Phospho-NF- κ B p65 in the dorsal skin samples of mice were analyzed by immunoblot (B) and immunofluorescence (C) analyses. Phospho-NF- κ B p65 (Alexa 488) and nuclei (DAPI) were stained using an immunofluorescence method. Scale bar = 200 μ m. Values are means $\pm$ SEM ($n = 5$). * $p < .05$ versus control group.

that the anti-allergic and anti-inflammatory effects of HYA occurred via decreases in serum total IgE and modulation of cytokine production associated with the Th1/Th2 balance.

Previous clinical studies demonstrated that most AD patients present higher plasma IgE levels and that the level correlates with the AD severity (Hamilton 2010; Kiiski et al. 2015). Feeding HYA slightly, but significantly, suppressed the serum IgE level. Elevated serum IgE responses in AD are known to reflect increases in Th2 cytokine responses. Although IL-4, a Th2 cytokine, produced from Peyer's patch cells was under the detection limit in both the control and HYA groups, feeding HYA increased the production of a Th1 signature cytokine, IFN- γ , which negatively regulates Th2 cells. Spergel et al. demonstrated that IFN- γ -deficient mice show reduced dermal thickening in an ovalbumin-sensitized murine model of AD

(Spergel et al. 1999). The increased Th1 response induced by HYA was also confirmed by the higher expression of T-bet, a Th1-specific transcription factor. Accordingly, feeding HYA appeared to decrease the serum IgE level through a shift in the Th1/Th2 balance toward Th1-dominant immunity.

It is generally accepted that the composition of intestinal microbiota is closely related to AD development. Watanabe et al. showed that the disturbance of microflora by oral antibiotic treatment exacerbates atopic symptoms, as indicated by clinical score and scratching behavior, in NC/Nga mice (Watanabe et al. 2010). In our study, the PCR-DGGE analysis of 16S rRNA gene profiles in the feces indicated that HYA feeding produced a moderate change in intestinal microflora composition. These findings suggest that the HYA-mediated change in the microflora is, at least in part, involved in the increased Th1 response and

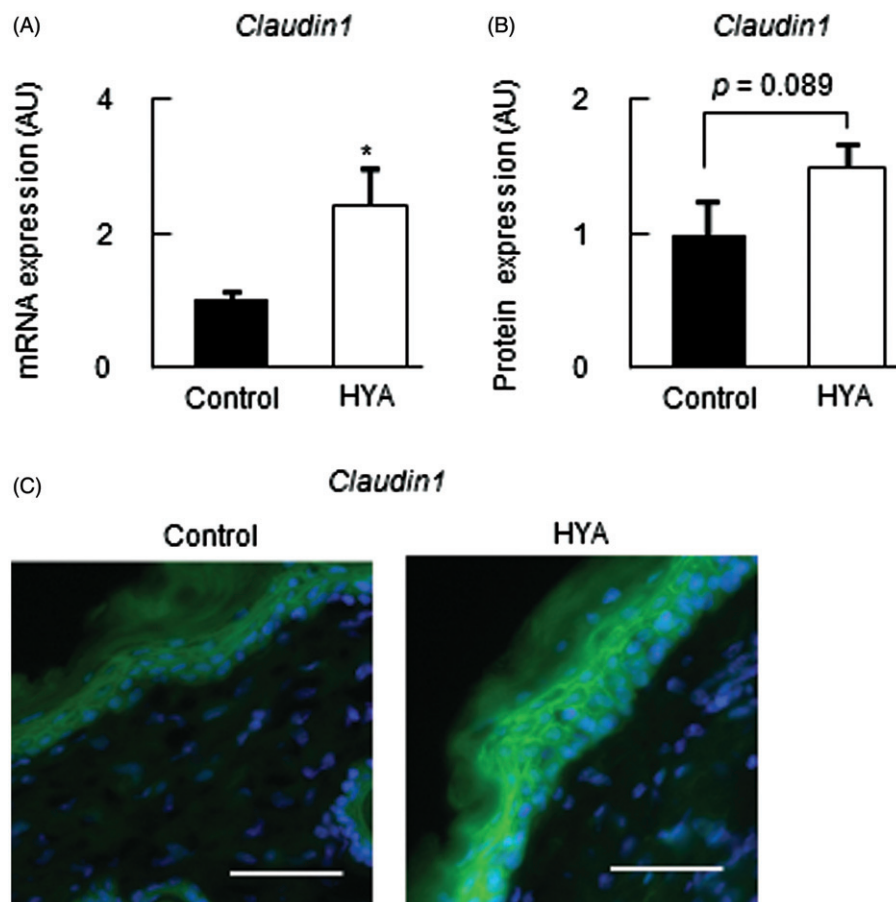


Figure 5. Tight junction protein phosphorylation of NF- κ B in the dorsal skin of NC/Nga mice. (A) The mRNA expression of claudin-1 in dorsal skin samples of mice was measured by RT-PCR. (B and C) Claudin-1 expression in the dorsal skin samples of mice were analyzed by immunoblot (B) and immunofluorescence (C) analyses. Claudin-1 (Alexa 488) and nuclei (DAPI) were stained using an immunofluorescence method. Scale bar = 50 μ m. Values are means $\pm$ SEM (n = 5). * p < .05 versus control group.

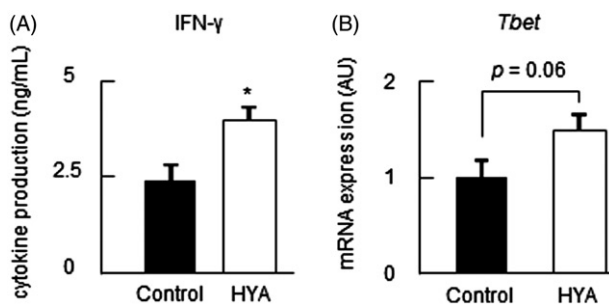


Figure 6. Induction of Th1 responses by HYA in the Peyer's patches of the small intestines of NC/Nga mice. (A) IFN- γ production from Peyer's patch cells were measured by ELISA. (B) Total RNA was extracted from Peyer's patch cells, and mRNA expression of Tbet was measured by RT-PCR. Values are means $\pm$ SEM (n = 5). * p < .05 versus control group.

anti-allergic effect. Based on the results of previous studies, intestinal microflora appear to have a role in the Th1 response. Sudo et al. reported that Th1 responses are diminished in germ-free mice compared with those in specific pathogen-free mice (Sudo et al. 1997). Our previous study also demonstrated that

feeding galactooligosaccharide, a prebiotic, modulates intestinal microflora and increases IFN- γ production from mouse splenocytes (Tanabe & Hoshi 2010). In addition, some strains of bacteria belonging to the genus *Lactobacillus* reportedly enhance Th1 responses (Pochard et al. 2002). Taken together with the results of the present study, these findings support the idea that the modulation of intestinal microbiota by food factors including HYA could afford a preventive approach against allergic diseases such as AD.

The mechanisms underlying the modulation of intestinal microflora by HYA remain unclear. It is unlikely that the ingested HYA reaches the large intestines, where microbes colonize, to directly influence microflora composition as HYA is thought to be readily absorbed in the small intestines. Accordingly, we speculate that the intestinal IgA production induced by HYA may have a role in microflora modulation. A previous study using IgA-deficient mice revealed the importance of IgA in the regulation of intestinal microflora (Blutt et al. 2012). They showed that the lack of IgA increases anaerobic bacteria, including

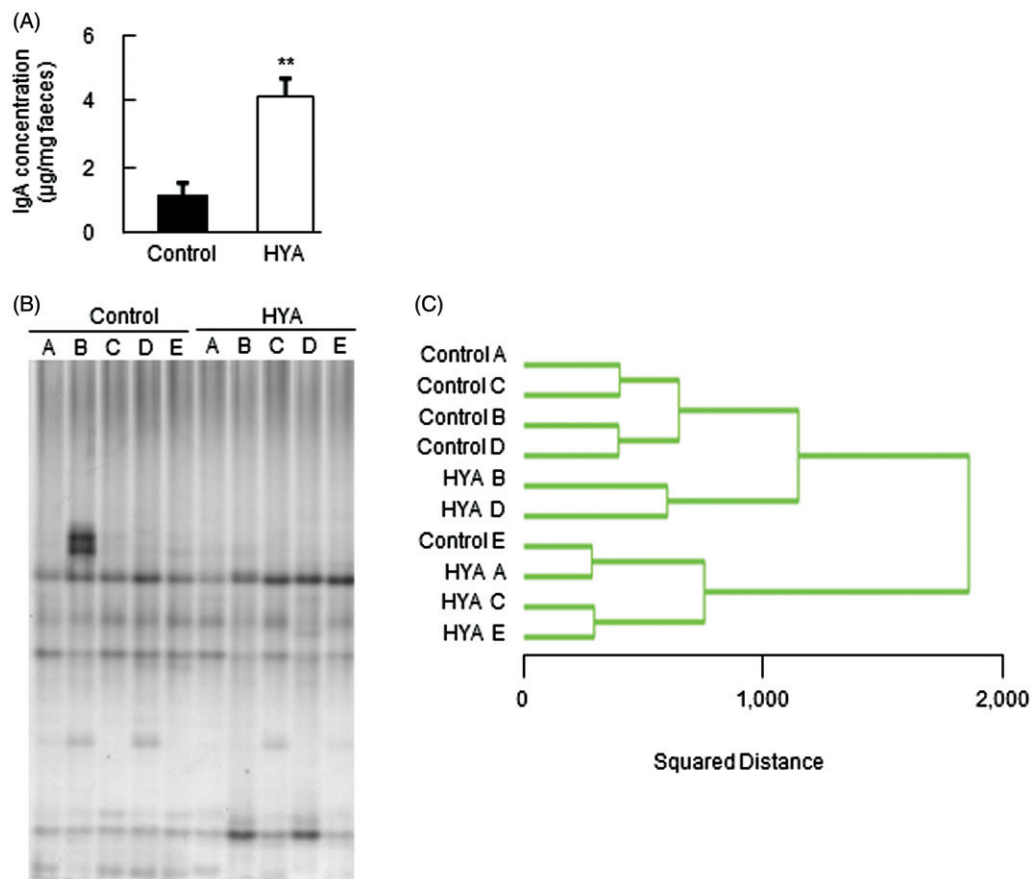


Figure 7. Fecal IgA level and PCR-DGGE analysis of 16S rRNA gene profiles in the feces of NC/Nga mice. (A) Fresh fecal samples were collected before sacrifice and IgA concentration was measured by ELISA. Values are means $\pm$ SEM ($n = 5$). ** $p < .01$ versus control group. (B) Silver staining of PCR products separated by PCR-DGGE. (C) Hierarchical cluster analysis of PCR-DGGE 16S rRNA gene profiles in fecal microbiota. The squared distances between 2 clusters were measured by Ward's method.

segmented filamentous bacteria. Furthermore, intestinal IgA is known to provide a primarily immunological defense against microbial and food antigens, which would promote Th2 responses (Furuya et al. 2013). Inoue et al. showed that the reduction in skin lesions resulting from feeding *Lactobacillus johnsonii* NCC533 is accompanied with higher intestinal IgA production (Inoue et al. 2007). A clinical study also demonstrated a lower incidence of atopic diseases in infants with higher fecal IgA levels (Viljanen et al. 2005). Thus, the HYA-induced IgA production could have a role in the anti-allergic effect via a number of different pathways.

Another possible mechanism for the anti-allergic effect of HYA involves the maintenance of the epidermal barrier as HYA feeding increased claudin-1 expression in the dorsal skin of mice. A significant body of evidence indicates that the skin symptoms associated with AD are closely related with epidermal barrier defects that allow increased stimulation by antigens. A recent study showed that the impairment of epidermal TJ function, which is mediated by a

reduction in claudin-1, has a role in the development of barrier defects in AD patients (De Benedetto et al. 2011). However, the increase in claudin-1 may be a distal event to the other mechanisms, including intestinal IgA production and microflora modification. Whereas linoleic acid and the metabolites were markedly decreased in plasma of another AD mouse model and the AD-like symptoms including skin barrier defect were completely prevented by supplementation with linoleic acid. This may suggest microbial metabolites of linoleic acid such as HYA directly regulates the skin barrier in mice (Fujii et al. 2013). Further studies are required to determine the precise mechanisms underlying the HYA-mediated claudin-1 expression in the skin.

Conclusions

The present study showed that supplemental feeding with HYA alleviated AD-like skin lesions and the elevation of plasma IgE in NC/Nga mice. In association with the anti-allergic effects, feeding HYA shifted the

Th1/Th2 balance toward a Th1-dominant condition. Our results suggested that the HYA-mediated effects occurred through the modulation of intestinal microflora composition and IgA production. This study also indicated that HYA has potential as a novel functional food factor for the prevention of allergic diseases such as AD.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this manuscript.

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ORCID

Takuya Suzuki  <http://orcid.org/0000-0003-3709-543X>

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