Ginger Increases ALDH1A1 Expression and Enhances Retinoic Acid Signaling in a Human Colonic Epithelial Cell Line

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Summary Aldehyde dehydrogenase 1A1 (ALDH1A1) in intestinal epithelial cells (IECs) plays a critical role in regulating immune responses through the production of retinoic acid (RA). However, little is known about its regulation by dietary components. We previously demonstrated that kakkonto, a Japanese traditional herbal medicine, and its constituent puerarin induce the expression of ALDH1A1 mRNA in colonic IECs and thereby attenuate food allergy symptoms in mice. This study aims to investigate the cellular responses of IECs to ALDH1A1 expression as a result of natural food components. The seven medicinal herbs that compose kakkonto were used to treat cultured an IEC line: Caco-2 cells. Expressions levels of ALDH1A1 were analyzed in Caco-2 cells by quantitative RT-PCR, immunocytochemistry and western blotting. Ginger increased the expression levels of ALDH1A1 mRNA and protein in Caco-2 cells. In addition, ginger significantly upregulated the gene expression of retinoic acid receptor (RAR) alpha (RARA), thereby enhancing RA signaling. Furthermore, ginger downregulated the expression of histone deacetylase (HDAC)2 and HDAC3 in Caco-2 cells. The present study suggests the possibility that food ingredients such as a ginger modulate vitamin A metabolism in the gut through the regulation of RA synthesis, which may contribute to RA-mediated regulation of immune responses and the regulation of allergic inflammation.

Key Words ginger extract, intestinal epithelial cell, retinoic acid-synthesizing enzyme, retinol metabolism, intestinal immunity, food allergy

Diet and nutrition play an important role in the development and management of intestinal immune responses. The vitamin A (retinol) metabolite retinoic acid (RA) has been shown to maintain gut immune homeostasis by inducing gut tropism of T cells and modulating T cell differentiation. In particular, regulatory T (Treg) cells play crucial roles in inducing oral tolerance to food allergens (1–3) and inhibiting inflammation in the colon (4). Thus, vitamin A deficiency in neonatal mice results in the impairment of oral tolerance induction due to insufficient induction of Treg cells (5). Likewise, in a murine dextran sulfate sodium-induced experimental colitis model, vitamin A-deficient mice exhibited more severe inflammation and slower recovery from acute inflammation (6). The bioavailability of RA is determined by the balance between RA synthesis and degradation, which is tightly regulated by multiple enzymes (7).

The enzyme of aldehyde dehydrogenase 1 family, member A (ALDH1A1) also known as retinaldehyde dehydrogenases, are responsible for RA synthesis with the enzymatic conversion of dietary vitamin A to RA. Mounting evidence suggests the potential importance of ALDH1A in the induction and function of regulatory T (Treg) cells (8–10). In gastrointestinal immunity, enhanced expression of ALDH1A enzymes is known to induce Treg cell differentiation and thereby promote immune tolerance (8, 11, 12). RA homeostasis is regulated in a tissue-specific manner, suggesting that a specific subtype of the ALDH1A family may exist for the control of RA concentrations in each target tissue (13).

Intestinal epithelial cells (IECs) strongly express ALDH1A1, which contributes to intestinal RA production (14, 15). The potential role of ALDH1A1 in inducing regulatory responses in IECs has been emerging as a possible management of immune dysfunctions, including allergies (14) and inflammatory diseases (4, 6). Consistently, we have already reported that kakkonto, a Japanese traditional herbal medicine, which is composed of seven medical herbs (kudzu, ephedra, jujube, cinnamon, peony, licorice, and ginger) upregulates ALDH1A1 in the whole colon, induces Treg cells in the colon and attenuates allergic symptoms (16). Notably, a transcriptome analysis in the colon of kakkonto-treated mice with food allergies revealed that intestinal RA metabolism was significantly modulated in the mouse, which suggests that medicinal herbs induce regulatory immune responses via enhancement of RA signaling. The human epithelial cell line Caco-2 has been widely
used as a model of the intestinal phenomena because it is difficult to collect and use human intestinal tissues for research purposes. However, Caco-2 cells originate from tumor and have been reported to differ with the several aspects (17, 18). In the human colon, ALDH1A1 is highly expressed in the epithelial cells (4). Similarly, the high expression of ALDH1A1 in Caco-2 cells has been revealed by recent studies (19, 20). Further, it has also been shown that the production of RA via the activity of ALDH1A1 in Caco-2 cells induces CD103+ dendritic cells (DCs) and Foxp3+ Treg cells, thereby regulating immune responses (19, 20). Here, to investigate the responses of IECs to the herbs, we examined the effects of the constituent herbs of kakkonto on an IEC line (Caco-2 cells).

**MATERIALS AND METHODS**

**Cell culture.** Caco-2 cells were obtained from RIKEN Cell Bank (Yokohama, Japan) and were cultured in DMEM (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37°C in a humidified 5% CO2 atmosphere. The dried extracts of all constituent medicinal herbs of kakkonto (Tsumura & Co., Tokyo, Japan) were dissolved in water (1% as final concentration) and were added to Caco-2 cells for 24 h. Concentrations indicated in this study are all expressed as the final concentration.

**Mice.** DO11.10 ovalbumin (OVA)-specific TCR transgenic mice (DO11.10 mice) were maintained under specific pathogen-free conditions in the animal facility of University of Toyama. All animal experiments were approved by the Animal Experiment Committee of the University of Toyama (approval numbers: A2018 INM-4 and A2015 INM-3).

**Quantitative PCR.** Cells were lysed in TRIzol, and total RNA was isolated following the manufacturer’s instructions (Invitrogen, Waltham, MA, USA). cDNA was synthesized using an RT reagent kit (Toyobo, Tokyo, Japan). Transcription levels were measured by real-time PCR using a Mx3005P system (Stratagene, La Jolla, CA, USA) and were normalized to GAPDH. The sequences of the primers are listed in Table S1 (Supplemental Online Material).

**Immunohistochemistry.** Cells were cultured with/without ginger extract for 48 h and were immediately fixed with 4% paraformaldehyde. Anti-ALDH1A1 antibody (1 μg/mL) (Proteintech, Rosemont, IL, USA) and fluorescent (Alexa Fluor 488)-conjugated secondary antibody (1 μg/mL) (Invitrogen) were used for immunostaining. Images were captured using an LSM710 confocal microscope (Carl Zeiss, Jena, Germany).

**Western blotting.** Cells were harvested and lysed in lysis buffer containing 2% SDS. Anti-ALDH1A1 antibody (Santa Cruz Biotechnology, Dallas, TX, USA), anti-Actin (ACTB) antibody (Santa Cruz Biotechnology), Alexa Fluor 680-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) and IRDye 800-conjugated secondary antibody (Jackson ImmunoResearch) were used to visualize the protein bands by Licor Odyssey scanner (LI-COR Bioscience, Lincoln, NE, USA).

**Flow cytometry.** DO11.10 mice were orally exposed to OVA (100 mg/kg/d) with/without ginger (300 mg/kg/d) for 5 d. OVA was dissolved in sterilized water. Ginger was suspended in 0.5% methylcellulose solution and administrated 1 h before OVA administration. One day after the final OVA administration, colonic lamina propria cells were isolated from the mice and stained with PE-conjugated anti-mouse CD4 (clone RM4-5; BD Biosciences, CA, USA) and APC-conjugated secondary antibody (Jackson Immunoresearch) to visualize the protein bands by LicoR Odyssey scanner (LI-COR Biosciences, Lincoln, NE, USA).

**Statistical analysis.** Statistical differences were deter-
RESULTS

Ginger induces ALDH1A1 transcription in Caco-2 cells

We have already demonstrated that kakkonto administration modulates intestinal RA metabolism in an oral immunotherapy-treated food allergy model (16), subsequently inducing Treg cells in the colon and suppressing food allergy symptoms (21, 22). In addition, at dose of 100 μg/mL kakkonto, ALDH1A1 in Caco-2 cells showed a tendency of increased expression (Fig. 1A). These results suggest that any of the constituent medicinal herbs in kakkonto could stimulate IECs to produce ALDH1A1. To evaluate the cellular and molecular mechanisms underlying the regulation of ALDH1A1, the herbs were directly applied to Caco-2 cells. Among these seven herbs, ginger significantly enhanced ALDH1A1 mRNA expression in Caco-2 cells (Fig. 1B).

Furthermore, ginger enhanced ALDH1A1 mRNA expression in a dose-dependent manner (Fig. 1C).

The upregulated expression of ALDH1A1 mRNA coincided with elevated levels of its protein, as shown by immunocytochemistry (Fig. 2A) and western blotting (Fig. 2B). Taken together, IEC has an increased ability to produce RA in response to ginger treatment.

Retinoic acid receptor (RAR) expression in ginger-treated Caco-2 cells

To investigate RA signaling in a ginger-stimulated culture system, we evaluated the expression of subtypes of retinoic acid receptors (RARs). Transcriptional activations of RAR genes were analyzed by quantitative RT-PCR. After ginger treatment, mRNA expression of RARα (RARA), but not RARβ (RARB) or RARγ (RARG), was significantly increased in Caco-2 cells (Fig. 3), indicating that the enhanced RA signaling in the ginger-treated Caco-2 cells occurred via RARα.
Histone deacetylase (HDAC) expression in ginger-treated Caco-2 cells

Intestinal epithelial expressions of ALDH1A family members are known to be regulated by histone deacetylase (HDAC) signaling (14, 23). To gain insight into the mechanism underlying ginger-induced ALDH1A1 expression in Caco-2 cells, mRNA expression levels of HDAC family member subtypes were analyzed in the cells. HDAC2 and HDAC3 were reduced at the transcriptional level in the ginger-treated cells (Fig. 4), indicating potential inhibitory effects of ginger on HDAC enzyme activities.

Regulatory T cells in the colon of ginger-exposed mice

We examined whether ginger could promote the production of OVA-induced antigen-specific Treg cells by inducing ALDH1A1 expression in IECs. Ginger and OVA were orally administered to DO11.10 mice to evaluate frequency of OVA specific Treg cells in the colon lamina propria. Ginger supplementation upregulated the frequency of CD4^+Foxp3^+ Treg cells in the colon lamina propria of DO11.10 mice by 1.5 times compared to vehicle (Fig. 5).

DISCUSSION

We demonstrated that ginger induces ALDH1A1 in cultured IECs and accelerates the frequency of CD4^+Foxp3^+ Treg cells in the lamina propria of mouse colon. These findings indicate that ginger enhances RA-mediated regulatory immune responses in the colon.

ALDH1A1 is highly expressed in IECs and is thought to play an important role in the mucosal immune system (14, 23). It is assumed that RA produced by ALDH1A1 in IECs induces ALDH1A2^+ CD103^+ tolerogenic DCs and that these tolerogenic DCs with sample antigens migrate to the draining mesenteric lymph node and present the antigens to naïve T cells (19). Intestinal RA-induced tolerogenic DCs accelerate TGF-β-dependent differentiation into induced Treg (iTreg) cells (24). An increase in iTreg cell number in intestinal lamina propria is essential for the oral tolerance induction (25). The tolerogenic properties of CD103^+ DCs in murine colitis models also appear to be regulated by ALDH1A1 in IECs (20). It has been reported that the expression levels of ALDH1A1 protein in IECs are reduced throughout disease progression in mice with chronic colitis and mice with colitis-associated cancer (4). Therefore, it is suggested that, in the induction of RA-mediated immune tolerance and anti-inflammatory function, ALDH1A1 in IECs may play a crucial role in the initiation of RA metabolism. In this study, we found a pivotal mechanism underlying upregulated RA production that is related to ginger. We further investigated whether ginger could promote the production of ovalbumin (OVA)-induced antigen-specific iTreg cells. This study revealed that ginger tends to accelerate the production of CD4^+Foxp3^+ iTreg cells in the colon of OVA-specific TCR transgenic mice (Fig. 5). Ginger, a popular functional food, has been widely used as an important spice and a traditional medicinal herb. This study provides another useful aspect that ginger affects RA metabolism by upregulating ALDH1A1 function in IECs, which is followed by Treg cell induction.

The pathway for the synthesis of RA first requires the oxidation of retinol to form retinal, which is followed by oxidation of retinal to produce RA (26, 27). Subsequently, RA binds to nuclear receptor RAR/retinoid X receptor (RXR) heterodimers to regulate the transcription of target genes. RAR expression has been reported to be promoted by RA signaling through a positive feedback loop (23, 28). In the present study, significant upregulation of RARA gene expression was observed in ginger-treated Caco-2 cells. In this in vitro culture system, the cell culture medium for Caco-2 cells contained retinol, which was supplied by the FBS. Therefore, it is suggested that the enhanced expression of RARA in the ginger-treated Caco-2 cells may reflect an increased concentration of RA that was converted by strongly expressed ALDH1A1 in the ginger-treated Caco-2 cells.

In the present study, we demonstrated that the gene
expression levels of HDAC2 and HDAC3 were significantly reduced by ginger treatment. It has been reported that the expression of ALDH1A1 in IECs is under the control of HDACs (14, 23). In addition, the anti-inflammatory effect of [6]-shogaol, one of the most bioactive components of ginger, is attributed to the suppression of HDACs in astrocytes (29). However, the suppression of HDAC gene expression does not necessarily correspond with the inhibition of HDAC enzymatic activity. Therefore, further studies are needed to address the involvement of HDACs in the effects of ginger.

Our previous study indicates that the increased concentration of intestinal RA is involved in the induction of intestinal Treg cells, thereby suppressing allergic symptoms in a murine model of food allergy (16). Taken together, the present study reveals that ginger could modulate RA metabolism in the gut through the regulation of RA biosynthesis and could exert an inhibitory effect on allergic inflammation.

Acknowledgments
Disclosure of state of COI

The authors have no conflict of interest regarding the contents of this article.

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Supporting information
Supplemental online material is available on J-STAGE.

REFERENCES


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