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ORIGINAL PAPER

Fucoidan suppresses IgE production in peripheral blood mononuclear cells from patients with atopic dermatitis

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Abstract We previously reported that fucoidan, a dietary fiber purified from seaweed, inhibited IgE production in B cells from mice spleen in vitro and ovalbumin-sensitized mice in vivo. In this study, we examined the effect of fucoidan on IgE production in human peripheral blood mononuclear cells (PBMC) in vitro. PBMC, obtained from healthy donors or patients with atopic dermatitis (AD) with high levels of serum IgE, were cultured with IL-4 and anti-CD40 antibody in the presence or absence of fucoidan. Fucoidan significantly reduced IgE production in PBMC without affecting cell proliferation and IFN-γ production. Fucoidan also inhibited immunoglobulin germline transcripts of B cells in PBMC, and decreased the number of IgE-secreting cells. The inhibitory effects of fucoidan were similarly observed for both PBMC from patients with AD and those with healthy donors. Our findings indicate that fucoidan suppresses IgE induction by inhibiting immunoglobulin class-switching to IgE in human B cells, even after the onset of AD.

Keywords Fucoidan · IgE · Atopic dermatitis · Peripheral blood mononuclear cells · B cells · Cε germline transcript

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Abbreviations

PBMC	Peripheral blood mononuclear cells
AD	Atopic dermatitis
FcεRI	High affinity IgE receptor
mAb	Monoclonal antibody
OVA	Ovalbumin
RT-PCR	Reverse transcription-polymerase chain reaction
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

Introduction

IgE plays a critical role in immediate hypersensitivity reactions by binding to the high-affinity IgE receptor (FcεRI) expressed on mast cells and basophils. The antigen-specific IgE on FcεRI on mast cells and basophils induces the release of inflammatory mediators, including histamine, arachidonic acid metabolites, and cytokines in response to the binding of multivalent antigens, which subsequently crosslink the FcεRI and contributes to the development of allergic diseases, such as atopic dermatitis (AD), asthma, and allergic rhinitis [6, 13]. Moreover, recent studies have shown that the binding of IgE itself on FcεRI without antigen may stimulate mast cells and induce the release of such substances [8, 14]. Clinically, levels of serum IgE in approximately 80% of patients with AD are elevated [10] and correlated with the severity of cutaneous lesions of AD [4]. It is also reported that AD skin contains an increased number of IgE-bearing Langerhans cells expressing FcεRI [21], and allergens are efficiently taken up, processed and presented to T cells via IgE bound to FcεRI [11]. Moreover, epidermal Langerhans cells

activated via FcεRI induce the expression of IL-16, a chemoattractant for $CD4^+$ T cells and eosinophils, and consequently leads to inflammation in AD [17]. Recently, a recombinant humanized anti-IgE monoclonal antibody (mAb), omalizumab, which competes with FcεRI for the binding of IgE and reduces the level of circulating IgE, has been shown to be effective in the treatment of severe asthma [3]. More recently, the effect of omalizumab has also been shown in the treatment of a few cases of AD [22, 23]. However, an enormous amount of, i.e., very expensive, mAb is required to remove IgE from patients with AD, since the level of serum IgE in many patients with AD is tens or hundreds times higher than those with asthma or allergic rhinitis. Therefore, the inhibition of IgE production should be a promising and important modality in the treatment of patients with allergic disease, especially those with AD.

Fucoidan, a dietary fiber purified from seaweed, is composed of a polymer of α1 → 3-linked L-fucose with sulfate groups at the four positions on some of the fucose residues [15]. We previously reported that fucoidan has an inhibitory effect on IgE production through preventing NF-κB p52-mediated pathways in murine B cells isolated from spleen in vitro [12], and that peritoneal injection of fucoidan suppresses the increase of total and ovalbumin (OVA)-specific IgE in mouse plasma induced by OVA-sensitization in vivo [25]. However, we also observed that once cells completed immunoglobulin class-switching for IgE, IgE production of B cells was no longer affected by fucoidan. Therefore, from a clinical point of view, it is crucial to check whether fucoidan inhibits IgE production by B cells in patients with AD, especially those with hyper IgE.

In this study, we investigated whether fucoidan inhibits the production of IgE induced by IL-4 and CD-40 antibody in human peripheral blood mononuclear cells (PBMC) from healthy donors and AD patients with high levels of serum IgE. Moreover, we studied the number of IgE-secreting cells and the expressions of Cε germline transcript in B cells purified from PBMC to study the effect of fucoidan on B cell differentiation and immunoglobulin class-switch recombination to IgE, by ELISpot and real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR), respectively.

Materials and methods

Healthy donors and patients with AD

PBMC were obtained from 16 healthy donors (7 men and 9 women, age 31.5 ± 5.4 years, mean ± SEM) and 18 patients with AD (9 men and 9 women, age 25.9 ± 5.5 years), diagnosed according to the diagnostic criteria for AD set by the Japanese dermatological association [19]. Levels of total serum IgE in patients with AD (2376 ± 643 ng/ml) were significantly higher than those of healthy donors (188 ± 53 ng/ml) with p-value <0.01 tested by Mann–Whitney U test. This study was carried out in accordance with the Guidelines stated in the Declaration of Helsinki and was approved by the Ethical Committee of Hiroshima University Graduate School of Medicine. Written informed consent for participation was obtained from all participants.

Preparation and culture of PBMC

PBMC were obtained by means of density gradient centrifugation with Leucosep tubes (Greiner Bio-one, Frickenhausen, Germany) according to the manufacturers' protocol. PBMC were distributed into a 24-well cell culture plate at 2×10^6 cells/0.5 ml per well in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (Invitrogen), penicillin G (10 IU/ml, Invitrogen), and streptomycin (100 μg/ml, Invitrogen). For the secretion of IgE, PBMC were stimulated with 100 ng/ml of human recombinant interleukin-4 (IL-4) (R&D Systems, Minneapolis, MN, USA) and 20 μg/ml of anti-CD40 antibody (R&D) and incubated for the indicated periods at 37°C in a humidified atmosphere containing 5% CO_2 in the presence or absence of fucoidan.

Cell proliferation assay

Cell proliferation was measured using CellTiter 96 Aqueous One Solution Proliferation Assay Kit (Promega, Madison, WI, USA) according to the manufacturers' protocol. It is based on the cellular conversion of a tetrazolium salt into a soluble formazan product as a measure of proliferation. In brief, 4×10^5 PBMC were plated in each well of a 96-well cell culture plate with IL-4 and anti-CD40 antibody in the presence or absence of 100 μg/ml of fucoidan. After incubation for 3 days, the Aqueous One solution reagent was added and incubated for 4 h. The intensity of the color was measured at 490 nm using a 96-well plate reader.

Measurement of IgE, IgG, IFN-γ and IL-13

The amounts of secreted IgE, IgG, IgG1, IgG2, IFN-γ and IL-13 in the culture supernatants of PBMC after incubation for indicated periods were measured by using Human IgE Quantitative ELISA kit (Bethyl Laboratories, Montgomery, TX, USA), Human IgG Quantitative ELISA kit (Bethyl Laboratories), Human IgG subclass ELISA kit (Invitrogen), Quantikine Human IFN-γ (R&D) and Quantikine

Human IL-13 (R&D) respectively, according to the manufacturers' protocols.

ELISpot assay

The number of IgE-secreting plasma cells was determined using Human IgE ELISpot Plus kit (Mabtech AB, Nacka Strand, Sweden) following the manufactures' instruction. In brief, after pre-incubation with IL-4 and anti-CD40 antibody for 5 days, PBMC were washed to ensure the removal of secreted IgE and dispensed into the ELISpot 96-plate at 1×10^5 cells per well. After incubation for 20 h, IgE, released from B cells and captured on the bottom membrane, was stained with biotinylated anti-human IgE antibody, Streptavidin-ALP, and BCIP/NBT substrate. The number of spots was counted manually under a stereomicroscope.

Measurement of Cε, Cγ1, Cγ2 germline transcripts and IgE mRNA by real-time quantitative RT-PCR in isolated B cells

PBMC were stimulated with IL-4 and anti-CD40 antibody for 4 or 12 days, and then B cells were isolated from PBMC by auto-MACS and B cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers' instruction. B cells were negatively selected and the purity of CD19-positive cells was confirmed as >97% by FACS analysis. The purified B cells were subjected to the detection of Cε, Cγ1, Cγ2 germline transcripts and IgE mRNA. Total RNA was extracted from purified B cells by means of RNA Mini Kit (Qiagen, Tokyo, Japan), and then cDNA was generated with QuantiTect Reverse Transcription kit (Qiagen). Real-time quantitative RT-PCR was performed with the Power SYBR Green PCR Master Mix and the ABI 7300 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The specific primers for the amplification of Cε, Cγ1, Cγ2 germline transcripts, IgE mRNA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed referring to previous reports [5, 9]. The expression of GAPDH was measured as an internal control for the calibration of the gene expression.

Results

Fucoidan does not show cytotoxicity in human PBMC

We first examined whether fucoidan has a cytotoxic effect on human PBMC or not. As shown in Fig. 1, the amount of formazan products, which correlates with cell proliferation, was not suppressed, but rather enhanced especially in the culture of PBMC from patients with AD by the treatment with fucoidan.

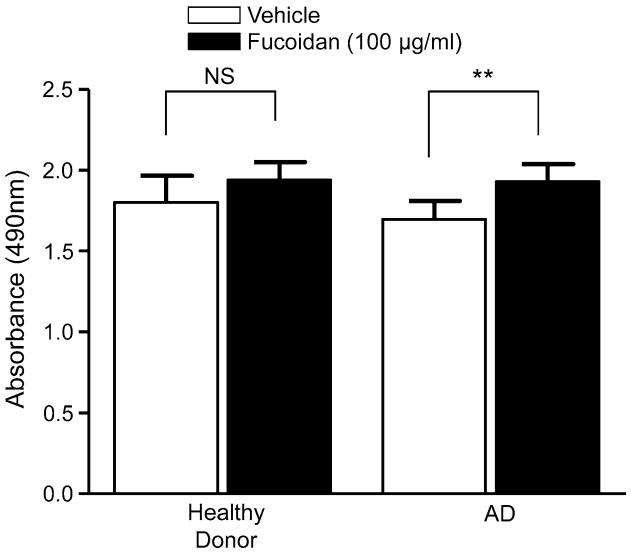

Fig. 1 Fucoidan exhibits no cytotoxicity in human PBMC. Human PBMC were cultured with IL-4 (100 ng/ml) and anti-CD40 antibody (20 μg/ml) for 3 days and then mixed with tetrazolium salt solution. The absorbance of soluble formazan products were measured at 490 nm. Data are mean ± SEM of 6 healthy donors and 11 patients with AD. $^{**}p < 0.01$; significantly different from control by paired *t* test. *NS* not significant

Time course and dose response of the inhibitory effect of fucoidan on IgE production

To investigate the role of fucoidan in the modulation of IgE production, PBMC were cultured with 100 μg/ml of fucoidan for 4, 7 or 12 days, or with various concentrations of fucoidan (0, 10, 100 and 200 μg/ml) for 12 days. In both PBMC of healthy donors and those of patients with AD, fucoidan prevented the increase of IgE production in a time- and dose-dependent manner (Fig. 2). Subsequent experiments for IgE production were performed with 12 days' incubation in the absence or presence of 100 μg/ml fucoidan.

Fucoidan suppresses IgE production of human PBMC stimulated with IL-4 and anti-CD40 antibody

Fucoidan significantly suppressed the increase of IgE production in either PBMC of healthy donors or those of patients with AD stimulated with IL-4 and anti-CD40 antibody (Fig. 3a). Fucoidan also suppressed the expression of IgE mRNA in PBMC from both groups on day 12 (Fig. 3b). There was no significant difference between healthy donors and AD patients in the production of IgE. Spontaneous release of IgE in PBMC without stimuli of IL-4 and anti-CD40 antibody was less than detection limit (10 ng/ml) of the assay regardless of the presence of fucoidan. Total IgG and IgG subclass were also suppressed by fucoidan (Fig. 4a, b).

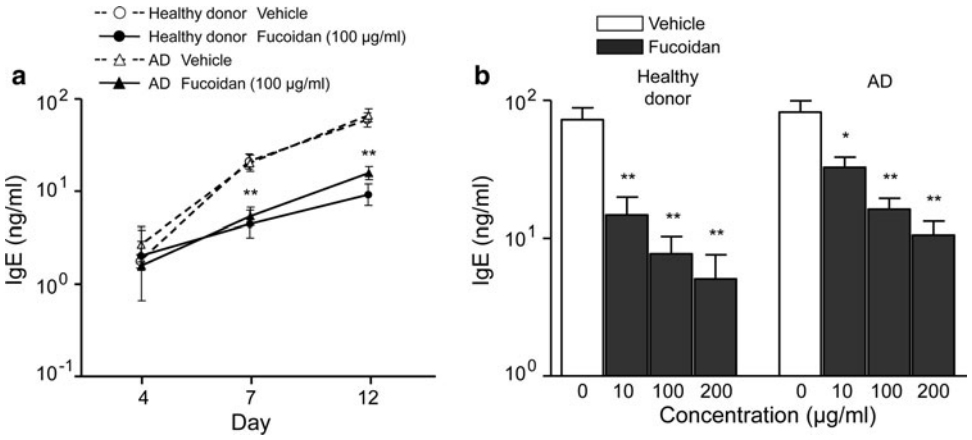

Fig. 2 Fucoidan prevents the increase of IgE production in a time- and dose-dependent manner. Human PBMC are cultured with IL-4 (100 ng/ml) and anti-CD40 antibody (20 μg/ml). The amounts of IgE in culture supernatants were measured by sandwich ELISA for human IgE. Data are mean ± SEM of six healthy donors and six patients with AD, being normalized by logarithmic transformation. The statistical difference was determined by paired *t* test. $^{**}p < 0.01$, $^{*}p < 0.05$; significantly different from control. **a** Human PBMC were cultured with 100 μg/ml of fucoidan for 4, 7 or 12 days. **b** Human PBMC were cultured with various concentrations of fucoidan (0, 10, 100 and 200 μg/ml) for 12 days

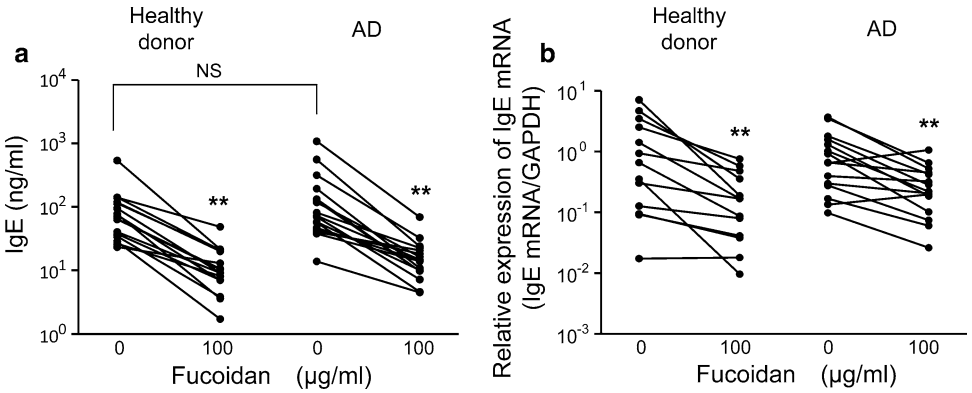

Fig. 3 Fucoidan suppresses the production of IgE in human PBMC at levels of protein and mRNA. Human PBMC were cultured with IL-4 (100 ng/ml) and anti-CD40 antibody (20 μg/ml) for 12 days. Data are logarithmically transformed for the statistical analysis. **a** The amounts of IgE in culture supernatants from 16 healthy donors and 18 patients with AD were measured by sandwich ELISA for human IgE. **b** B cells were isolated from the cultured PBMC by using magnetic cell separation. The expression of IgE mRNA from 13 healthy donors and 15 patients with AD was measured by using real-time quantitative RT-PCR. $^{**}p < 0.01$; significantly different from control by paired *t* test. *NS* not significant by Student's *t* test

Fucoidan does not alter the production of IFN-γ but enhances the production of IL-13 from human PBMC

Since IFN-γ plays a key role to inhibit the development of T_H2 cells which promote IgE production, we measured the amount of IFN-γ in culture supernatants after the incubation of human PBMC in the presence and the absence of fucoidan for 12 days. As shown in Fig. 5a, the production of IFN-γ was not affected by the treatment with fucoidan both in PBMC of healthy donors and in those of patients with AD. On the other hand, the production of IL-13, a T_H2 cytokine with functional similarity to IL-4, was significantly enhanced by the treatment with fucoidan in either PBMC of healthy donors or those of patients with AD (Fig. 5b).

Fucoidan suppresses the increase of IgE-secreting cells

To examine whether fucoidan inhibits the differentiation of B cells to IgE secreting phenotype, we performed ELISpot assay targeted against IgE. Spots on the bottom membranes indicate the number of IgE-secreting B cells (see the "Materials and methods"). Fucoidan significantly suppressed both the increase of IgE-secreting cells from healthy donors and those from patients with AD (Fig. 6a).

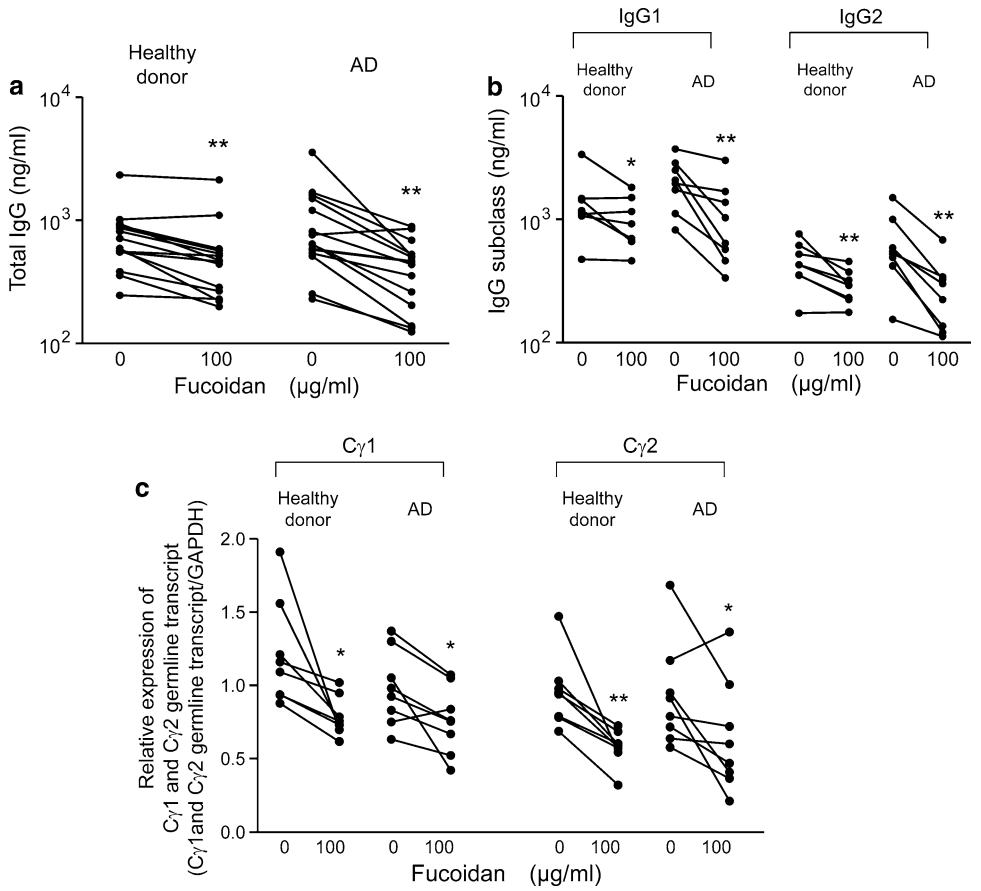

Fig. 4 Fucoidan suppresses the production of total and subclass IgG in human PBMC. Human PBMC were cultured with IL-4 (100 ng/ml) and anti-CD40 antibody (20 μg/ml) for 12 days. The data of total and subclass IgG productions are normalized by logarithmic transformation. The statistical difference was determined by paired *t* test. $^{**}p < 0.01$, $^{*}p < 0.05$; significantly different from control. **a** The amounts of total IgG in culture supernatants from 14 healthy donors and 15 patients with AD were measured by sandwich ELISA for human IgG. **b** The amounts of IgG1 and IgG2 in the culture supernatants from eight healthy donors and eight patients with AD were measured by sandwich ELISA for subclasses of human IgG. **c** B cells were isolated from PBMC, which were cultured for 4 days, by using magnetic cell separation. Levels of Cγ1 and Cγ2 germline transcripts from eight healthy donors and eight patients with AD were measured by using real-time quantitative RT-PCR

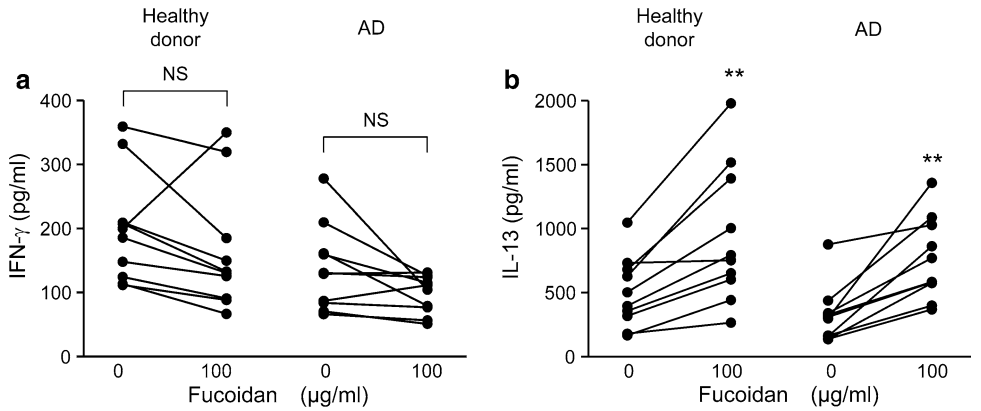

Fig. 5 Fucoidan does not enhance the production of IFN-γ but increase the production of IL-13 in human PBMC. Human PBMC from ten healthy donors and ten patients with AD were cultured with IL-4 (100 ng/ml) and anti-CD40 antibody (20 μg/ml) for 12 days. **a** The amounts of IFN-γ in supernatants were measured by ELISA. **b** The amounts of IL-13 in supernatants were measured by ELISA. *NS* not significant from control by paired *t* test. $^{**}p < 0.01$; significantly different from control by paired *t* test

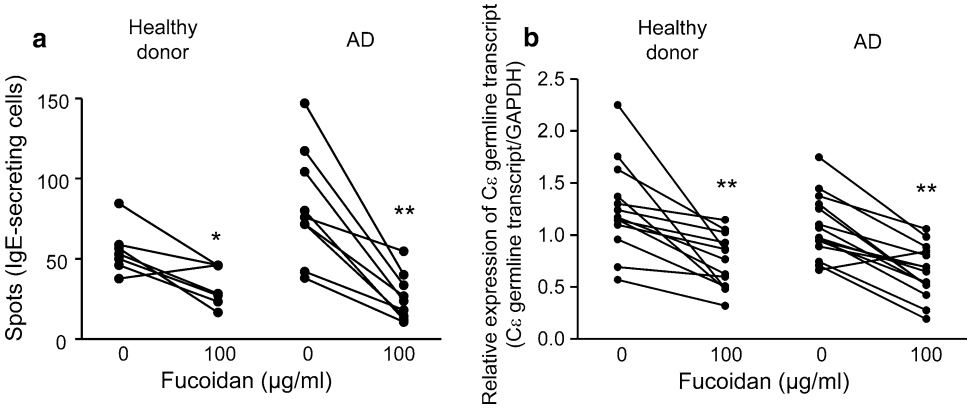

Fig. 6 Fucoidan suppresses class-switch recombination to IgE in human B cells. Human PBMC were cultured with IL-4 (100 ng/ml) and anti-CD40 antibody (20 μg/ml). **a** The number of IgE-secreting B cells from seven healthy donors and nine patients with AD was determined by means of ELISpot after the incubation for 5 days. **b** B cells were isolated from PBMC, which were cultured for 4 days, by using magnetic cell separation. Levels of Cε germline transcript from 13 healthy donors and 15 patients with AD were measured by using real-time quantitative RT-PCR. $^{**}p < 0.01$, $^{*}p < 0.05$; significantly different from control by paired *t* test

Fucoidan suppresses the immunoglobulin class-switch recombination in purified human B cells

To study the effect of fucoidan on class-switch recombination to IgE, we purified B cells from PBMC incubated for 4 days with IL-4 and anti-CD40 antibody, and measured the expressions of Cε germline transcript with real-time quantitative RT-PCR. Fucoidan suppressed the induction of Cε germline transcript in the purified B cells from healthy donors and those from patients with AD (Fig. 6b). Cγ1 and Cγ2 germline transcripts were also suppressed by the treatment with fucoidan (Fig. 4c).

Discussion

In this study, we demonstrated that fucoidan, a sulfate polysaccharide contained in seaweed, inhibited IgE production in human PBMC induced by IL-4 and anti-CD40 antibody. Fucoidan also inhibited the Cε germline transcript in B cells and decreased the number of IgE-secreting cells. The effects of fucoidan were similarly observed in PBMC from patients with AD and high serum IgE, and in those from healthy donors.

IgE synthesis is predominantly regulated by the balance of T_H1 and T_H2 cytokines. Namely, IL-4 or IL-13 induces the expression of Cε germline transcript and subsequently up regulates class-switch recombination to IgE in B cells in concert with CD40 ligand [7]. Conversely, IFN-γ suppresses the development of T_H2 cells and the production of IL-4, leading to the inhibition of IgE production. Fucoidan did not alter the production of IFN-γ and enhanced the production of IL-13; therefore, fucoidan could inhibit IgE production even under the condition that PBMC were stimulated by sufficient Th2 cytokines. Moreover, fucoidan also suppressed the increase of total IgG, but did not show cytotoxicity in the culture of human PBMC. These results suggest that fucoidan inhibits immunoglobulin class-switching in B cells as observed in the culture of mouse splenocytes and B cells. Identification of a molecular target for fucoidan on B cells or possibly on other cells in PBMC should be a subject for further studies. We confirmed that several ligands for scavenger receptors suppressed IgE production by murine spleen cells (data not shown). Since fucoidan binds to several scavenger receptors [16], the effect of fucoidan observed in this study may be related to the activation of such scavenger receptors.

We previously have demonstrated that fucoidan inhibits NF-κB p52-mediated pathway in murine B cells, but the direct target on human B cells is still not clear. In this study, we stimulated and cultured B cells in human PBMC, because we could not detect a sufficient amount of IgE by ELISA in the supernatants of purified human B cells even in those cultured with IL-4 and anti-CD40 antibody (data not shown). Therefore, we cannot exclude a possibility of an indirect effect of fucoidan on B cells through other type cells in PBMC. In fact, Avery et al. [1] have reported that IgE production of human B cells induced by IL-4 and anti-CD40 antibody was enhanced in the presence of IL-21.

We have also reported that the effect of fucoidan was not observed if B cells were pre-stimulated with IL-4 and anti-CD40 antibody in vitro [12], or mice were sensitized by OVA in vivo [25] before the administration of fucoidan. These observations suggest that fucoidan may not prevent a further increase of IgE in patients who have already developed allergic diseases and high levels of serum IgE. However, we demonstrated that new induction of IgE in PBMC of patients with AD is effectively suppressed in the

presence of fucoidan by inhibiting immunoglobulin class-switching in B cells in peripheral blood.

For the treatment of IgE-mediated diseases, such as asthma, allergic rhinitis and atopic dermatitis, several modalities targeting IgE production have been tried. Among them, the administration of soluble IL-4R [20] and IFN-γ [2] to inhibit the development of T_H2 and promote T_H1 functions did not sufficiently block the increase of IgE. However, a recently developed humanized monoclonal antibody against CD23, the low-affinity receptor for IgE, blocks class-switch recombination to IgE, effectively inhibits IgE production from human PBMC [24] and showed efficacy in a Phase I clinical trial for the treatment of asthma [18]. Further studies of fucoidan focusing on appropriate modes of administration and more details of its molecular target may allow us to treat AD more easily by reducing new production of IgE by pre-differentiated B cells.

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