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博士論文

Studies on the anti-allergic effects of F-fucoidan

F-フコイダンの抗アレルギー効果に関する研究

平成 28 年 2 月

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ABBREVIATIONS

BCIP, 5-bromo-4-chloro-3-indolyl phosphate

DC, dendritic cell

DC-SIGN, dendritic cell-specific intracellular adhesion molecule 3-grabbing non-integrin

IECs, intestinal epithelial cells

IFN, interferon

IgE, immunoglobulin E

IL, interleukin

MALDI-TOF-MS, matrix assisted laser desorption/ionization-time of flight mass spectrometry

NBT, nitroblue tetrazolium

OCT, optimal cutting temperature

OVA, ovalbumin

PBS, phosphate-buffered saline

PCA, passive cutaneous anaphylaxis

SDS, sodium dodecyl sulfate

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SE, standard error

TIM-3, T-cell immunoglobulin and mucin-containing protein-3; TNP, trinitrophenyl

CHAPTER 1

General Introduction

Allergic disorders are one of the major health problems in the developed country [1,2]. Allergy is defined as excessive immune response against normally harmless substances such as food, pollen or metal. Allergy is classified into four groups from type I to type IV on the basis of mediators, antigen, and effector mechanism. Especially, patients of type I allergy are increased in the world [3]. Development of type I allergy starts from phagocytosis of foreign antigens such as food, pollen or dust mites by antigen-presentation cells and presentation of the antigen to naïve T cells followed by differentiation of naïve T cells to Th2 cells, which produce Th2 cytokines such as interleukin (IL)-4 and IL-13. These cytokines induce immunoglobulin class switching to immunoglobulin E (IgE) in B cells [4], whereby blood IgE level is increased. Increased IgE binds to IgE receptors on mast cells. Subsequently, when the antigen reenters the body, the antigen bridges IgE molecules on mast cells and mast cells are activated. Activated mast cells undergo degranulation and release chemical mediators such as histamine followed by the production of eicosanoid and cytokine [5]. These substances

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induce symptoms of type I allergy including itch, rhinitis, hives, or anaphylaxis.

Through the ages, foods have been thought to modulate immune response. Actually, food factors including dietary fibers [6,7], polyphenols [8], and fatty acids [9] have been reported to exhibit immune-modulatory effects. Notably, the gut immune systems are associated with food compounds (e.g. dietary polysaccharide and *Lactobacillus*) since they pass through the gut [10,11]. In addition, it has been reported that type I allergy is suppressed by many food factors such as flavonoid [12,13], polyunsaturated fatty acid [14], *Lactobacillus* species [15-17], and polysaccharide [18]. Therefore, in this study, anti-allergic effect of F-fucoidan, which is one of the dietary polysaccharide and well-consumed in Japan, was investigated. Fucoidan is a sulfated polysaccharide found in brown sea algae. Although the structure of fucoidan from different brown sea algae varies between species, it is mainly composed of fucose and sulfated fucose [19]. The structure of fucoidan is mainly consisted from α -1,3 linked fucose and branched at C-2 or C-4 [19]. Besides these monosaccharides, other fucoidans from different brown sea algae also contain specific sugars such as glucose, galactose, xylose and uronic acid [19]. Fucoidan has various activities such as antitumor [20] and anticoagulant [21,22] activities through the modulation of immune responses. Therefore, it was supposed that fucoidan is able to suppress allergic symptoms caused by immune

disorders. Although fucoidan has been reported to have anti-allergic effects [23-25], there are few reports on the anti-allergic mechanism when fucoidan was orally administered. Fucoidan shows little or no absorption from the intestine [22,26]; It was presumed that fucoidan exhibits diverse activities against type I allergy irrespective of the administration pathway. Our laboratory previously reported that dietary F-fucoidan from *Saccharina japonica*, which consists of only fucose and sulfated fucose, exhibited anti-thrombotic effects and was eliminated without being completely absorbed [22]. This indicated that F-fucoidan can show activity without being absorbed into the body.

This study elucidated the effect of oral intake of F-fucoidan from *S. japonica* on type I allergy and its mechanism. In Chapter 2, it was investigated whether oral administration of crude F-fucoidan from *S. japonica* inhibited mast cell activation and following allergic symptoms induced by the passive cutaneous anaphylaxis (PCA) reaction, which is a simple animal model using IgE/mast cell system, and its inhibitory mechanism. Crude F-fucoidan could inhibit allergic symptoms induced by the PCA reaction only when it was through gut. The inhibitory mechanism was dependent on galectin-9, which belongs to a soluble lectin family that recognizes β -galactoside and has recently reported to prevent IgE binding to mast cells [27]. The anti-allergic properties of crude F-fucoidan were cancelled by an intravenous dose of anti-galectin-9 antibody or

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lactose, which bind competitively with galectin-9, before the PCA reaction. Moreover, crude F-fucoidan increased the expression level of galectin-9 mRNA in intestinal epithelial cells (IECs) and serum galectin-9 levels. The author demonstrated that Oral treatment with crude F-fucoidan suppressed allergic symptoms through the induction of galectin-9 in Chapter 2. Crude F-fucoidan contained three fractions. Therefore, in Chapter 3, the active form of crude F-fucoidan was clarified. Furthermore, it was examined whether the effect of F-fucoidan as shown in Chapter 2 is F-fucoidan from *S. japonica*-specific effect (Chapter 4). The results in Chapter 2 indicated that F-fucoidan might exhibit anti-allergic property even after sensitization by antigen. Therefore, in Chapter 5, the effect of F-fucoidan in an allergy model that allergy was induced by injection of allergen was demonstrated. F-fucoidan inhibited allergic symptom induced by mast cell activation but not polarization to Th2. These results indicated that F-fucoidan from *S. japonica* induced the expression of galectin-9 in IECs and secretion of galectin-9 into blood, resulting in the prevention of type I allergic symptoms by galectin-9 binding to IgE and preventing the interaction of IgE and mast cells.

CHAPTER 2

Anti-Allergic Effect of F-fucoidan Contained in *Saccharina japonica* through Induction of Galectin-9 from Small Intestinal Epithelial Cells.

2.1. Introduction

As mention in chapter 1, type I allergy is caused by the conjugation of IgE and mast cells and then the binding antigen to the complex of IgE and mast cell. These reaction are important to induce mast cell degranulation. Therefore, to investigate the inhibitory effect of F-fucoidan from *S. japonica* on mast cell activation induced by IgE, the author used PCA reaction model, which is known as a simple animal model of an IgE- and mast cell-dependent acute allergic reaction.

In this chapter, the mechanism of the effect of F-fucoidan was investigated after evaluation of the inhibitory effect of F-fucoidan on mast cell activation. Galectin-9 was particularly focused in this study because it was recently reported that galectin-9 suppressed mast cell degranulation [27]. Galectin-9, one of the galectin families which is soluble lectin that binds β -galactoside, has been reported as one of the inhibitory factor of mast cell activation [27]. Although galectin-9 was originally found as a potent

eosinophil chemoattractant, it has complicated functions in immune modulation. For example, galectin-9 has been reported to exhibit divergent activities such as preventive effects on autoimmune disease through the suppression of Th1 and Th17 cells and the induction of regulatory T cells [28] and effects on enhancement antitumor immunity by the induction of dendritic cell (DC) maturation and the promotion of Th1 immune responses [29]. Some reports suggested that galectin-9 levels increase in mouse models of allergic airway [30], food allergy [31], and atopic dermatitis [32]. However, other reports indicated that exogenous galectin-9 diminished allergic inflammation in a mouse model of allergic asthma [33]. Moreover, galectin-9 has been reported recently to interact with IgE, whereby mast cell degranulation was suppressed [27]. Thus, as it was suspected that galectin-9 involved in the inhibitory effect of F-fucoidan on mast cell activation, the author examined the contribution of galectin-9 to the effect of F-fucoidan.

2.2. Materials and Methods

2.2.1. Materials

Mouse anti-2,4,6-trinitrophenyl (TNP) monoclonal IgE was purchased from BD Biosciences (San Jose, CA, USA). 2,4,6-Trinitrochlorobenzene was purchased from

Tokyo Chemical Industry (Tokyo, Japan). Recombinant mouse galectin-9 was purchased from R&D Systems (Minneapolis, MN, USA), and an anti-mouse galectin-9 antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and R&D Systems. Anti-mouse IgE and anti-goat IgG-HRP antibodies were purchased from Bethyl Laboratories (Montgomery, AL, USA).

2.2.2. Preparation of crude F-fucoidan

Crude F-fucoidan was extracted from *S. japonica*. Powdered *S. japonica* was stirred in 20 times volume of sodium acetate buffer (pH 4.6) over night. After centrifugation (3,500 rpm, 10 min), supernatant was lyophilized. The lyophilized powder was defined crude F-fucoidan.

2.2.3. Mice

Female BALB/c mice (4 weeks old) were purchased from Japan SLC (Shizuoka, Japan). Mice were housed in an air-conditioned animal room at a temperature of $23 \pm 2^{\circ}\text{C}$ and a humidity of $55 \pm 10\%$. Mice were acclimatized with *ad libitum* supply of food and drinking water. This study was approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimentation

Regulations (permission number: 25-06-04).

2.2.4. Passive cutaneous anaphylaxis reaction

Mice were intravenously sensitized with anti-TNP IgE and then challenged with application of 1.6% 2,4,6-trinitrochlorobenzene in acetone:olive oil (1:1) as an antigen on the ear at 30 min after sensitization. Ear thickness was measured using a micrometer (Ozaki MFG., Tokyo, Japan) before and 2 h after antigen challenge, and the difference in ear thickness between before and after application of the antigen was defined as an edema. Before PCA reaction, mice were orally or intraperitoneally administered crude F-fucoidan (100, 200 or 400 µg/day) everyday for 4 days. To investigate the contribution of galectin-9 to the anti-allergic properties of F-fucoidan, mice were injected intravenously with an anti-galectin-9 antibody (50 µg) 1 h before the PCA reaction. To evaluate whether galectin-9 induced by F-fucoidan prevented type I allergy through binding IgE, mice were injected intravenously with lactose (100 mM) 10 min before the PCA reaction. Sucrose was used as a negative control in the same manners.

2.2.5. Sampling of tissues, RNA isolation and real-time PCR

Ears were collected after PCA reaction and homogenized using Multi-beads shocker

(Yasui Kikai, Osaka, Japan) to measure expression of IL-4 mRNA. Harvested ileum was washed in ice-cold phosphate-buffered saline (PBS) to remove feces and then cut longitudinally. IECs were isolated by scraping using a glass slide. Total RNA was extracted from the tissues using Sepasol RNA I super G (Nacalai Tesque, Kyoto, Japan) in accordance with the manufacturer's protocol. cDNA synthesis was performed using a High-Capacity cDNA Reverse Transcription kit (Life Technologies, Carlsbad, CA, USA). Quantitative PCR was performed using a 7500 Fast Real Time PCR system (Life Technologies) using TaqMan[®] Fast Universal PCR Master Mix (Life Technologies), and Gene Expression Assays for mouse IL-4, galectin-9 and β -actin (Life Technologies), in accordance with the manufacturer's protocol.

2.2.6. Histological analysis

Ears were fixed in 4% (v/v) paraformaldehyde, embedded in optimal cutting temperature (OCT) compound (Sakura Finetek Japan, Tokyo, Japan) and cut using a microtome. The sections (4 μ m thickness) were stained with 0.001% toluidine blue (pH 2.5) for 4 h to detect of granule of mast cells.

2.2.7. Immunoprecipitation, SDS-PAGE and Western blotting for detection of galectin-9

Recombinant mouse galectin-9 (250 ng) and lactose (100 mM) were mixed and left on ice for 15 min. Sucrose (100 mM) was used as a negative control. Anti-TNP IgE (50 ng) was added to each mixture solution and left on ice for additional 15 min. The mixtures were incubated with anti-mouse IgE antibodies bound to protein G Dynabeads (Life Technologies) at room temperature for 10 min. After Dynabeads were magnetically precipitated, sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl (pH 6.8), 1% SDS, 10% glycerol, 0.005% bromophenol blue, and 0.2 M 2-mercaptoethanol) was added to both of the supernatants and the precipitates, and then heated at 70°C for 10 min. Samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes (General Electric, Fairfield, CT, USA). Anti-mouse galectin-9 or anti-mouse IgE antibodies were reacted as the primary antibody at 4°C overnight, followed by anti-goat IgG-HRP antibody as the secondary antibody at 4°C for 1 h. All signals were detected by enhanced chemiluminescence using Chemi-Lumi One (Nacalai Tesque). In the case of detection of galectin-9 in serum, SDS-PAGE and Western blotting were conducted using 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/ nitroblue tetrazolium (NBT) Phosphatase Substrate and PhosphaGLO Reserve™ AP Substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) instead of

Chemi-Lumi One.

2.2.8. In-gel digestion and mass spectrometric analysis

The band after SDS-PAGE was digested by trypsin and then the digested sample was analyzed in matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS). Protein was identified using MASCOT (Matrix Science).

2.2.9. Statistical analysis

Data were expressed as means $\pm$ the SE. Statistical analysis was performed by Tukey-Kramer test. $p < 0.05$ was considered statistically significant.

2.3. Results

2.3.1. Effect of F-fucoidan on passive cutaneous anaphylaxis reaction

To investigate whether oral administration of F-fucoidan could inhibit a type I allergic reaction, PCA reaction was conducted after oral administration of crude F-fucoidan. Oral administration of crude F-fucoidan prevented ear edema induced by IgE/antigen at a concentration of more than 200 $\mu\text{g/day}$ (Fig. 2.1.A). Moreover, oral administration of

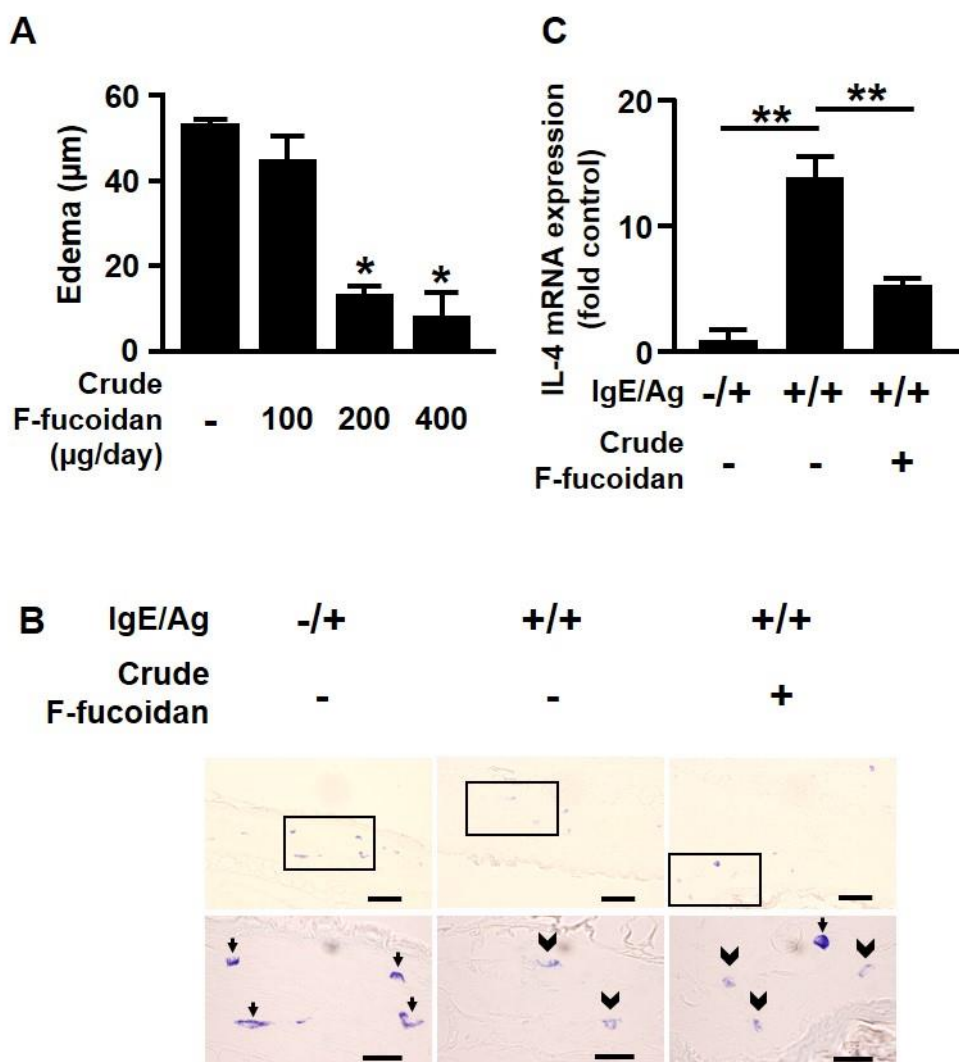


Figure 2.1. Effect of F-fucoidan on ear edema induced by the passive cutaneous anaphylaxis reaction.

Mice were administered crude F-fucoidan orally (100, 200, or 400 μg/day) for 4 days and conducted a passive cutaneous anaphylaxis (PCA) reaction. (A) Ear edema was evaluated 2 h after antigen (Ag) challenge. (B) Ears were collected after PCA reaction and ear-skin frozen sections (4 μm) were stained by toluidine blue. Boxed areas in upper side (scale bar, 50 μm) are magnified below (scale bar, 25 μm). Arrows and arrowheads indicates rest mast cell and degranulated mast cell, respectively. (C) IL-4 mRNA expression in ears after PCA reaction was measured. * $p < 0.05$, significantly different from the values of the group that did not receive crude F-fucoidan. ** $p < 0.01$. Values represent the means $\pm$ S.E. of 3 mice in each group.

crude F-fucoidan inhibited mast cell degranulation (Fig. 2.1.B) and following expression of IL-4 mRNA (Fig. 2.1.C). Many previous studies have indicated that fucoidan exhibited anti-allergic effect when fucoidan was intraperitoneally administrated [24,25]. Therefore, to investigate whether only oral administration of crude F-fucoidan can prevent ear edema induced by IgE/antigen, mice were intraperitoneally administrated crude F-fucoidan and tested PCA reaction. As a result, F-fucoidan exhibited inhibitory effect on mast cell activation only when F-fucoidan was administrated orally but not intraperitoneally (Fig. 2.2). These data suggested that F-fucoidan shows an inhibitory effect on type I allergy only the through gut.

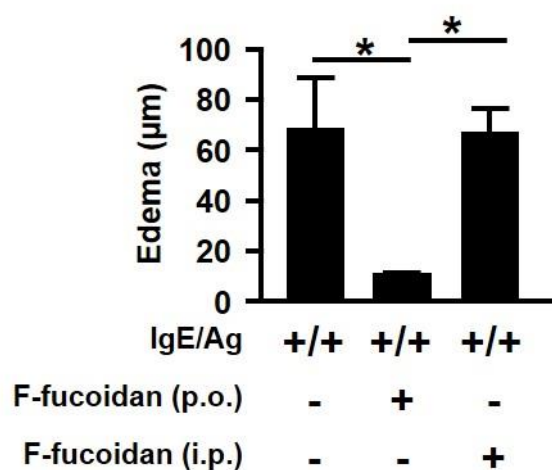


Figure 2.2. Difference in the pathway of administration of F-fucoidan.

Mice were orally (p.o.) or intraperitoneally (i.p.) administered crude F-fucoidan (200 μ g/day) for 4 days and induced allergic symptom by passive cutaneous anaphylaxis reaction. Ear edema was evaluated 2 h after antigen (Ag) challenge. * $p < 0.05$. Values represent the means $\pm$ S.E. of 3 mice in each group.

2.3.2. Galectin-9 content in blood of mice administered F-fucoidan

It has been reported that galectin-9 prevents mast cell degranulation and ear edema induced by PCA reaction through an interaction with IgE [27]. Therefore, to investigate the function of galectin-9 in the inhibitory effect of F-fucoidan on the PCA reaction, galectin-9 in the blood of mice administered F-fucoidan was detected by Western blotting. As shown the typical result in Fig. 2.3, a band corresponding to the molecular weight of galectin-9 was detected in serum of mice administered F-fucoidan. To confirm the identity of this band, the band after SDS-PAGE was analyzed in MALDI-TOF-MS. As a result, sequence of the band matched with the sequence of mouse galectin-9 at score of 318. It was demonstrated that the oral administration of crude F-fucoidan increased galectin-9 content in the blood.



Figure 2.3. Increase in galectin-9 in serum of mice administered F-fucoidan.

Mice were administered crude F-fucoidan orally (200 μ g/day) and blood was collected. Galectin-9 in serum was detected using an anti-mouse galectin-9 antibody.

2.3.3. Association between suppression of the passive cutaneous anaphylaxis reaction and galectin-9 induction by oral administration of F-fucoidan

It has been reported that high expression levels of galectin-9 in the intestine led to the suppression of food allergy [34]. Therefore, to examine whether expression of galectin-9 in the intestine was increased when mice were orally administered F-fucoidan, galectin-9 mRNA expression levels in IECs of mice administered F-fucoidan were measured. Galectin-9 mRNA expression levels did not show any change in response to IgE/antigen challenge compared with just an antigen challenge (Fig. 2.4.A). However, in the group administered crude F-fucoidan, the expression level of galectin-9 mRNA was significantly increased (Fig. 2.4.A). To examine the requirement for galectin-9 in the anti-allergic properties of F-fucoidan, an anti-galectin-9 antibody were intravenously injected 1 h before the PCA reaction in mice with an oral administration of crude F-fucoidan. As shown in Fig. 2.4.B, ear edema which was suppressed by oral administration of F-fucoidan were completely canceled by intravenous injection of the anti-galectin-9 antibody.

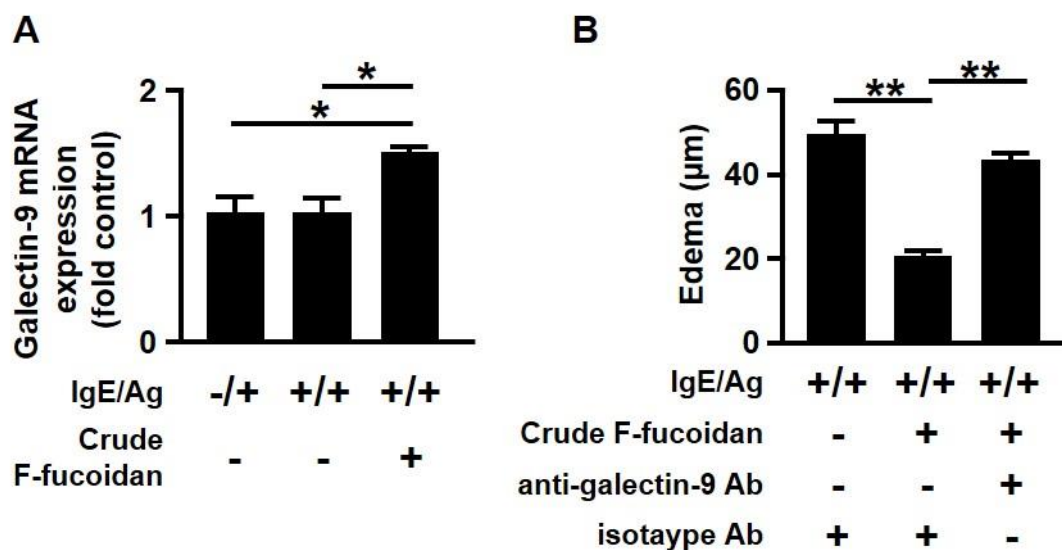


Figure 2.4. Involvement of galectin-9 in the inhibitory effect of F-fucoidan on type I allergy.

(A) Mice were administered crude F-fucoidan orally (200 $\mu\text{g/day}$) for 4 days and induced allergic symptoms by passive cutaneous anaphylaxis (PCA) reaction. Galectin-9 mRNA expression in intestinal epithelial cells after PCA reaction was measured. (B) Mice were administered crude F-fucoidan orally (200 $\mu\text{g/day}$) for 4 days and intravenously injected with anti-galectin-9 antibody (Ab) or isotype IgG 1 h before PCA reaction. Ear edema was evaluated 2 h after the antigen (Ag) challenge. * $p < 0.05$, ** $p < 0.01$. Values represent the means $\pm$ S.E. of 3 mice in each group.

2.3.4. Binding between galectin-9 and IgE in the inhibitory properties of F-fucoidan in the passive cutaneous anaphylaxis reaction

It has been reported that the interaction of galectin-9 with IgE was competitively inhibited by lactose [27]. Indeed, lactose inhibited co-immunoprecipitation of galectin-9 with IgE *in vitro* and galectin-9 was detected in the supernatant, but sucrose did not (Fig. 2.5.A).

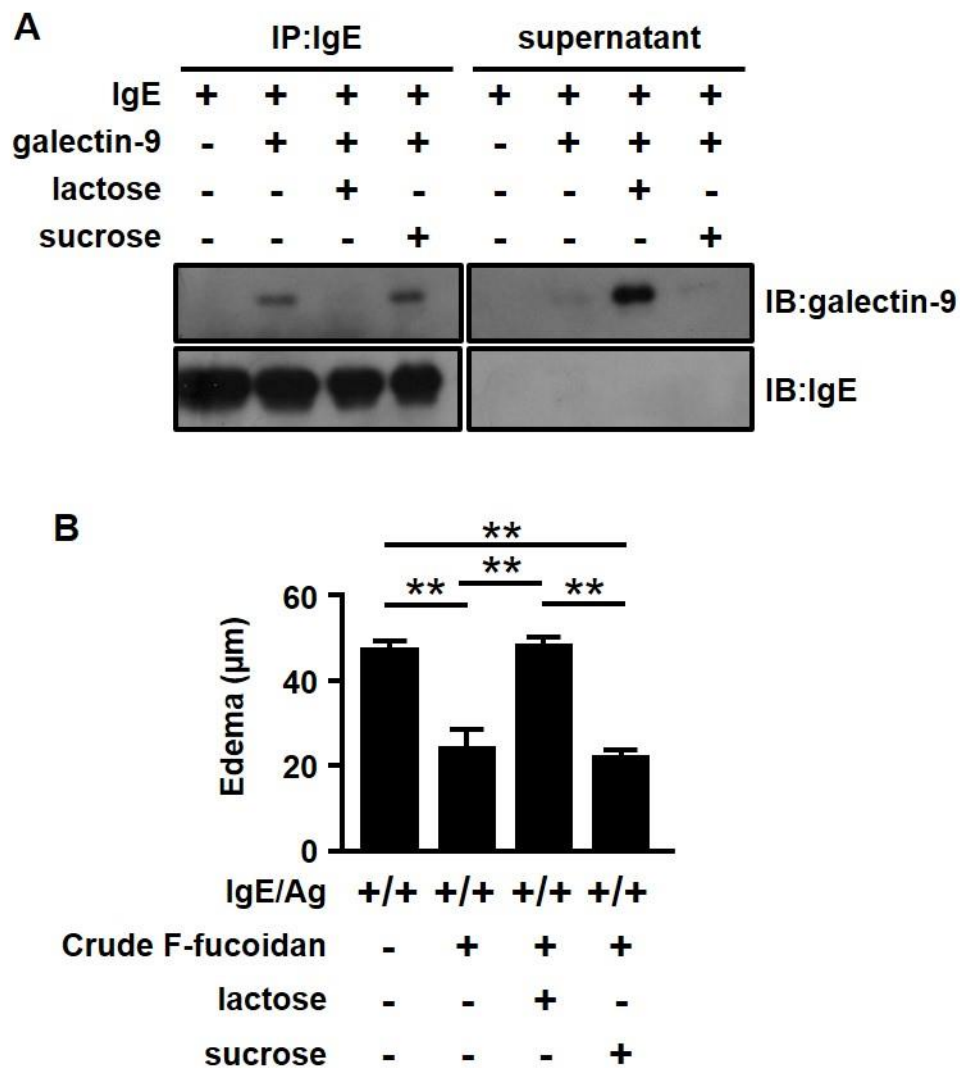


Figure 2.5. Lactose-mediated inhibition of the interaction between galectin-9 and IgE and the effect of F-fucoidan on ear edema induced by IgE/antigen.

(A) IgE and galectin-9 reacted with or without lactose, followed by immunoprecipitation with an anti-mouse IgE antibody and Western blotting with an anti-mouse galectin-9 antibody. The data are representative of triplicate experiments. (B) Mice were administered crude F-fucoidan orally (200 $\mu\text{g}/\text{day}$) for 4 days and intravenously injected with lactose or sucrose 10 min before passive cutaneous anaphylaxis reaction. Ear edema measured 2 h after antigen (Ag) challenge. * $p < 0.05$, ** $p < 0.01$. Values represent the means $\pm$ S.E. of 3 mice in each group.

To confirm the effect of lactose *in vivo*, mice administered crude F-fucoidan were

intravenously injected lactose. As shown in Fig. 2.5.B, ear edema was recovered by the intravenous administration of lactose to equal levels to mice challenged with IgE/antigen despite of oral administration of crude F-fucoidan. However, in mice administered sucrose which did not contain galactose, ear edema was suppressed to the same level as mice administered crude F-fucoidan. These data indicated that oral treatment with F-fucoidan might increase galectin-9 content in the blood and induced galectin-9 prevents the interaction of IgE and mast cells.

2.4. Discussion

In previous reports on the anti-allergic effects of fucoidan, it inhibits allergic symptoms through modulating the polarized immune response and inhibiting the production of IgE [25,35]. Briefly, fucoidan has been thought to mainly exert an inhibitory effect on allergy by modulation of the Th1/Th2 balance. However, in this study, F-fucoidan inhibited mast cell activation followed by ear edema formation, which was caused by mast cell degranulation. (Fig. 2.1). This anti-allergic mechanism of F-fucoidan is a novel and different mechanism from that was previously reported as an effect of fucoidan.

In this study, intravenous administration of an anti-galectin-9 antibody prevented the inhibitory effect of F-fucoidan on ear edema (Fig. 2.4.B). It was ascertained that the inhibitory effect of F-fucoidan on ear edema induced by IgE/antigen reaction depended on galectin-9. Galectin-9 possesses the high affinity to IgE and prevents binding of IgE to IgE receptor FcεRI, resulting in the inhibition of mast cell activation [27]. Moreover, administration of F-fucoidan increased the expression level of the galectin-9 mRNA in IECs (Fig. 2.4.A) and the content of galectin-9 in serum (Fig. 2.3). The inhibitory effect of F-fucoidan on ear edema was shown only when F-fucoidan was administered orally (Fig. 2.2). Taking into consideration these results, it was predicated that oral administration of F-fucoidan influenced IECs and promoted the expression and secretion of galectin-9 into blood. As shown in Fig. 2.5, the inhibitory effect of F-fucoidan on ear edema was also prevented by intravenous administration of lactose, which galectin-9 and suppresses the interaction of galectin-9 with IgE [27]. These results suggested that secreted galectin-9 by F-fucoidan bound to IgE in blood and prevented ear edema.

Galectin-9 has been reported to have the inhibitory properties against allergy [27,33,36] and various autoimmune diseases [28,37-39]. However, in these studies mice were administered exogenous galectin-9 or overexpressed galectin-9. The substances that can induce galectin-9 are less understood, although there was a report that combination

of oligosaccharides in human milk with bifidobacteria can increase galectin-9 in IECs and blood, whereby food allergy was suppressed [34]. In this study, we demonstrated that F-fucoidan from *S. japonica* possessed an ability to increase expression of galectin-9 in IECs (Fig. 2.4.A). Furthermore, administration of F-fucoidan increased the amounts of galectin-9 in blood (Fig. 2.3). These data indicated that F-fucoidan is a novel inducer of galectin-9. Moreover, the inhibitory effect of F-fucoidan on type I allergy through the induction of galectin-9 was observed only when F-fucoidan was administered orally (Fig. 2.2). This suggested that dietary intake of *S. japonica* could be useful for the induction of galectin-9, followed by preventive effects on allergic symptoms.

CHAPTER 3

Identification of the Active Form of F-fucoidan from *S. japonica*.

3.1. Introduction

In Chapter 2, the author exhibited that crude F-fucoidan from *S. japonica* has anti-allergic effect due to induction of galectin-9. However, crude F-fucoidan included not only F-fucoidan but also alginic acid [22,40]. Alginic acid is a polysaccharide and consisted of β -D-mannuronic acid and α -L-guluronic acid [41]. Alginic acid has been reported to exhibit anti-allergic effect through the suppression of mast cell activation [42] and the inhibition of IgE production [43]. Therefore, the author thought that the effect of crude F-fucoidan in Chapter 2 might be due to alginic acid. Furthermore, it was reported that crude F-fucoidan includes fucoidan of various molecular weights [22]. In this chapter, the author investigated which polysaccharide possessed the activity in crude F-fucoidan.

3.2. Materials and Methods

3.2.1. Reagents

Mouse anti-2,4,6-trinitrophenyl (TNP) monoclonal IgE was purchased from BD Biosciences (San Jose, CA, USA). 2,4,6-trinitrochlorobenzene was purchased from Tokyo Chemical Industry (Tokyo, Japan). 0.5% (w/v) Methyl cellulose was purchased from Wako Pure Chemical Industries (Osaka, Japan).

3.2.2. Preparation of high and low molecular weight of F-fucoidan and alginic acid

Crude F-fucoidan was prepared as described in Chapter 2. Then, crude F-fucoidan was dissolved in distilled water and applied to a Toyopearl-DEAE 650M column (2.0 × 20 cm; TOSOH, Tokyo, Japan). The each fraction was eluted by stepwise gradient of 0.5M and 1.0 M NaCl.

3.2.3. Passive cutaneous anaphylaxis reaction

Mice were purchased and preliminary bred as described in Chapter 2. The PCA reaction was conducted as described in Chapter 2. Before the PCA reaction, mice were orally administered each fraction prepared in section 3.2.2. with ratio of recovery weight from crude F-fucoidan everyday for 4 days. To investigate the influence of viscosity of crude

F-fucoidan, 100 μ l of 0.5% (w/v) methyl cellulose was administered for 4 days before the PCA reaction.

3.2.4. Sampling of tissues, RNA isolation and real-time PCR

IECs were prepared and the expression of galectin-9 and β -actin in IECs was measured as described in Chapter 2.

3.2.5. Statistical analysis

Data were expressed as means $\pm$ the SE. Statistical analysis was performed by Tukey-Kramer test. $p < 0.05$ was considered statistically significant.

3.3. Results

3.3.1. Isolation of the fraction in crude F-fucoidan

In ion-exchange chromatography, three main peaks were observed. The first fraction was non-absorbed fraction on the column and this fraction was identified as high molecular weight (30 to 50 kDa) of F-fucoidan. The fraction eluted with 0.5 M NaCl was identified as alginic acid. The fraction eluted with 1.0 M NaCl was identified as low molecular

weight (less than 20 kDa) of *F-fucoidan* (Fig. 3.1). Crude *F-fucoidan* contained high and low molecular weight of *F-fucoidan* and alginic acid at a ratio of 30%, 30%, and 23%, respectively.

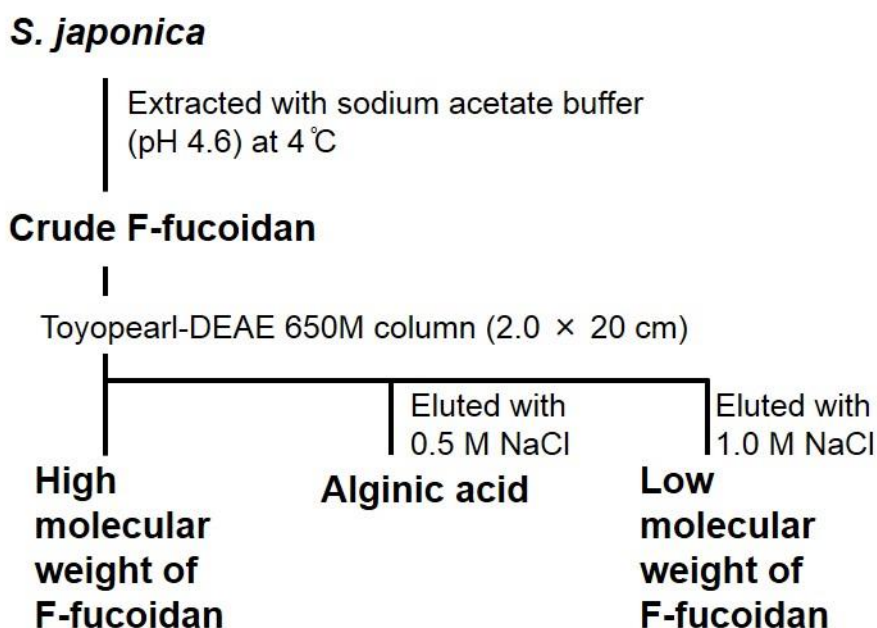


Figure 3.1. Separation of crude *F-fucoidan*.

Powdered *S. japonica* was stirred in 20 times volume of sodium acetate buffer (pH 4.6) over night. Then, crude *F-fucoidan* was applied to a Toyopearl-DEAE 650M column (2.0 × 20 cm). The fraction not adsorbed by the column was identified as high molecular weight of *F-fucoidan*. The fraction eluted with 0.5 M NaCl was alginic acid and the fraction eluted with 1.0 M NaCl was identified as low molecular weight of *F-fucoidan*.

3.3.2. Identification of the active polysaccharides in crude *F-fucoidan* on mast cell activation

To identify the active polysaccharides in crude *F-fucoidan*, mice were administrated three

fractions at the dose corresponding to weigh ratio of crude F-fucoidan (200 µg/day), high and low molecular weight of F-fucoidan (60 µg/day, respectively), or alginic acid (46 µg/day), and tested the PCA reaction. As shown in Fig. 3.2.A, high molecular weight of F-fucoidan prevented ear edema induced by IgE/antigen. In contrast, low molecular weight of F-fucoidan or alginic acid did not inhibit ear edema. Especially, alginic acid did not prevent ear edema at all. It was understood that high molecular weight of F-fucoidan was unstable at more than 50°C and changed to low molecular weight of F-fucoidan. To confirm whether the heat degradation of high molecular weight of F-fucoidan affected the inhibitory effect on ear edema, mice were orally administrated high molecular weight of F-fucoidan heated at 50°C for 10 min and tested the PCA reaction. The heat degradation of high molecular weight of F-fucoidan did not inhibit ear edema (Fig. 3.2.A). This suggested that high molecular weight of F-fucoidan *per se* exhibit the inhibitory effect on ear edema induced by mast cell activation. Moreover, as it was demonstrated that the inhibitory effect of crude F-fucoidan on mast cell was due to induction of galectin-9 in Chapter 2, the expression of galectin-9 in IECs from mice administrated each fraction in crude F-fucoidan was measured. In IECs from mice administrated crude or high molecular weight of F-fucoidan, galectin-9 mRNA expression was increased, whereas low molecular weight of F-fucoidan and alginic acid did not affect the expression

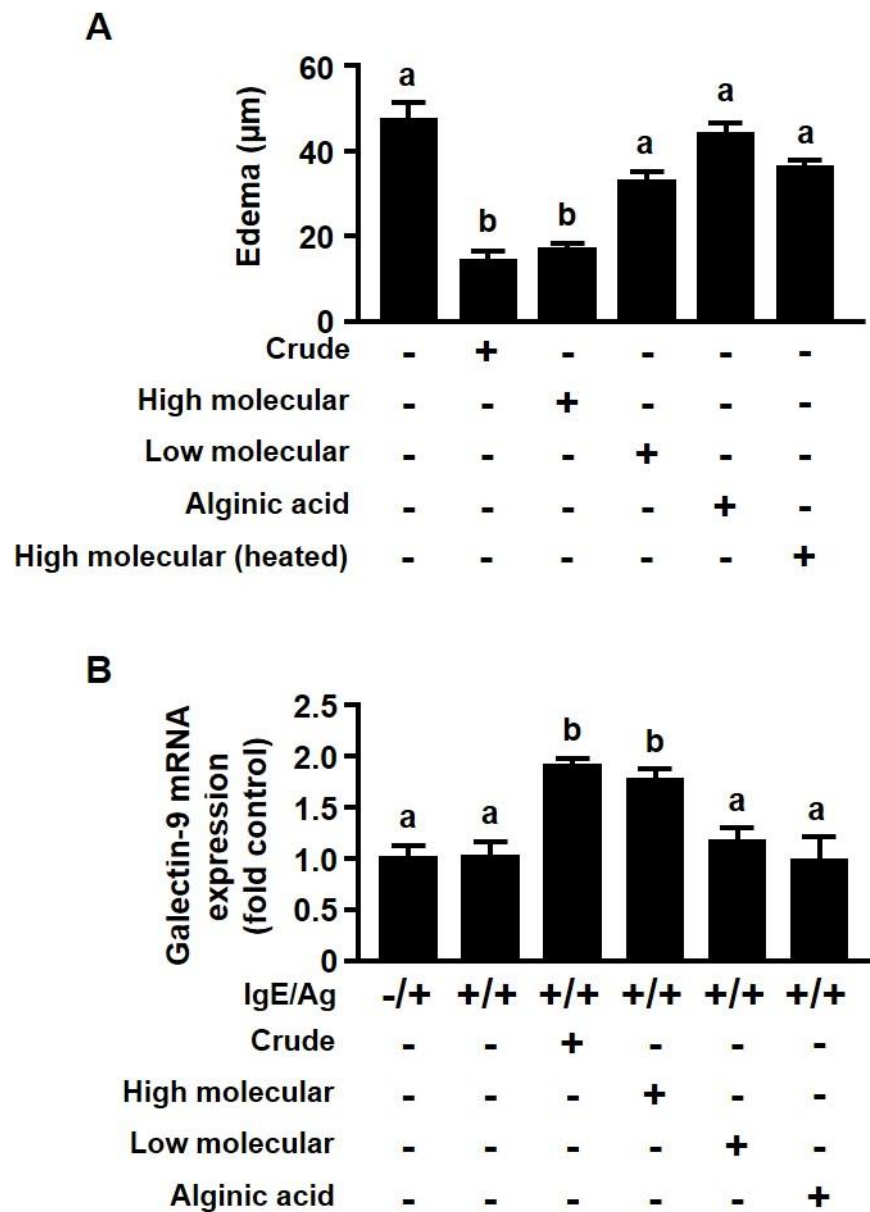


Figure 3.2. The effect of the fractions in crude F-fucoidan on ear edema induced by the passive cutaneous anaphylaxis reaction and galectin-9 mRNA expression.

Mice were administered crude F-fucoidan, high and low molecular weight of F-fucoidan, or alginic acid orally (200, 60, 60, 46 $\mu\text{g/day}$, respectively) for 4 days and tested for the passive cutaneous anaphylaxis (PCA) reaction. (A) Ear edema was evaluated 2 h after the antigen (Ag) challenge. (B) Galectin-9 mRNA expression in intestinal epithelial cells after PCA reaction was measured. Different alphabet indicates a significant difference.

of galectin-9 mRNA (Fig. 3.2.B). This result was similar to the result of ear edema. Therefore, it was considered that the active form of crude F-fucoidan, which exhibited the inhibitory effect on mast cell activation through galectin-9 induction, was high molecular weight of F-fucoidan.

3.3.3. The contribution of viscosity in crude F-fucoidan to the inhibitory effect on mast cell activation

Fractions of crude and high molecular weight of F-fucoidan, which exhibited the inhibitory effect on mast cell activation, have viscosity. The heat degradation of high molecular weight of F-fucoidan which did not inhibit ear edema in section 3.3.2. lost viscosity. Moreover, it has been reported that high viscous polysaccharide from *Kjellmaniella crassifolia* could modulate immune responses [44]. To examine whether the effect of F-fucoidan on ear edema induced by IgE/antigen was dependent on viscosity or molecular weight, mice were administered methyl cellulose which is hydrosoluble polysaccharide and has viscosity, and conducted the PCA reaction. Ear edema in mice administered methyl cellulose was no change compared with that in mice stimulated by IgE/antigen (Fig. 3.3). This result suggested that crude and high molecular weight of F-fucoidan exhibited the inhibitory effect on mast cell activation regardless of the viscosity.

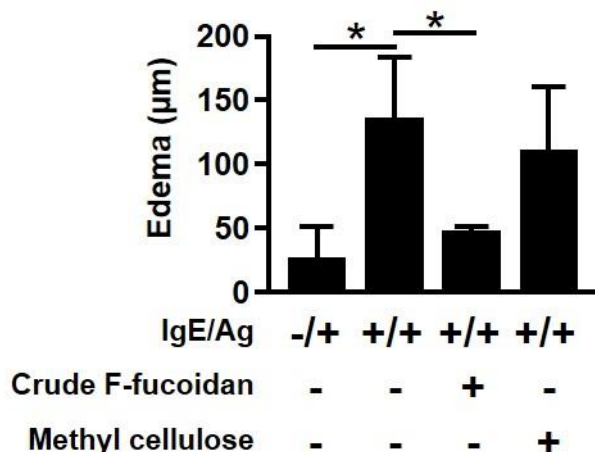


Figure 3.3. The inhibitory effect of viscosity on ear edema induced by the passive cutaneous anaphylaxis reaction.

Mice were administered crude F-fucoidan (200 $\mu\text{g/day}$), or 100 μl of 0.5% (w/v) methyl cellulose was administered for 4 days and tested for the passive cutaneous anaphylaxis reaction. Ear edema was evaluated 2 h after the antigen (Ag) challenge. * $p < 0.05$. Values represent the means $\pm$ S.E. of 3 mice in each group.

3.4. Discussion

Crude F-fucoidan was divided into three fractions of high and low molecular weight of F-fucoidan and alginic acid in DEAE-chromatography. Although the crude F-fucoidan possessed the inhibitory effect on mast cell activation by secretion of galectin-9 as shown in Chapter 2, it was not clarified which fractions of crude F-fucoidan exerted the effect. Therefore, each fraction was administrated at the dose contained in effective amount of crude F-fucoidan (200 $\mu\text{g/day}$) and conducted the PCA reaction. It was revealed that only high molecular weight of F-fucoidan can inhibit ear edema and increase

expression of galectin-9 mRNA in IECs (Fig. 3.2). Low molecular weight of F-fucoidan might inhibit ear edema if it was carried out at higher dose, because it was seemed that low molecular weight of F-fucoidan slightly inhibit ear edema (Fig. 3.2.A). However, only high molecular weight of F-fucoidan could inhibit ear edema and increase galectin-9 mRNA expression at the dose contained in effective amount of crude F-fucoidan. Therefore, it was eliminated that low molecular weight of F-fucoidan inhibited by the different mechanism with crude and high molecular weight of F-fucoidan if low molecular weight of F-fucoidan at more dose can inhibit ear edema.

In this study, alginic acid did not inhibit ear edema in contradiction to previous reports that alginic acid has anti-allergic effects [42,43]. Mice were administered alginic acid at considerably less dose, 2.3 mg/kg body weight, compared with previous reports that alginic acid was administered at 0.5-1 g/kg body weight. Therefore, it was presumed that alginic acid cannot inhibit ear edema in this study.

In this chapter, the author identified the active form possessing the inhibitory effect on mast cell activation as a high molecular weight fraction in crude F-fucoidan. Moreover, the effect of F-fucoidan was not due to viscosity but molecular weight. Therefore, it was demonstrated the caution that *S. japonica* should cook less than at 50°C to exert its anti-allergic activity.

CHAPTER 4

Inhibitory Effects of Fucoidan from Various Brown Sea Algae on Mast Cell Activation.

4.1. Introduction

In Chapter 2 and 3, the author showed the anti-allergic mechanism and the active form of fucoidan from *S. japonica*. However, it is well-known that fucoidan is contained in various brown sea algae such as Kombu, Wakame, and Hijiki [45], and fucoidan from various sea algae has been reported to possess anti-allergic effect. For example, fucoidan from *Undaria pinnatifida* sporophylls suppressed Th2 immune response [46], and fucoidan from *Fucus vesiculosus* inhibited ear swelling induced by contact hypersensitivity [47]. Although administration of fucoidan in these reports was intraperitoneally injection, fucoidan from various sea algae has possibility having the inhibitory effect on ear edema induced by IgE/antigen even with oral administration. In this chapter, the author investigated whether the inhibitory effect of fucoidan from *S. japonica* on mast cell activation was specific.

4.2. Materials and Methods

4.2.1. Preparation of crude F-fucoidan from brown sea algae

Crude F-fucoidan was prepared from Makombu (*S. japonica*), Gagome Kombu (*Saccharina sculpera*), Rishiri Kombu (variant species of *S. japonica*), Rausu Kombu (variant species of *S. japonica*), Naga Kombu (*Saccharina longissima*), Hidaka Kombu (*Saccharina angustata*), Atsuba Kombu (*Saccharina coriacea*), or Okinawa Mozuku (*Cladosiphon okamuranus* Tokida) by the same methods as described in Chapter 2.

4.2.2. Passive cutaneous anaphylaxis reaction

Mice were purchased and preliminary bred as described in Chapter 2. The PCA reaction was conducted as described in Chapter 2. Before the PCA reaction, mice were orally administered crude F-fucoidan from above brown sea algae (200 µg/day) everyday for 4 days.

4.2.3. Statistical analysis

Data were expressed as means $\pm$ the SE. Statistical analysis was performed by Tukey-Kramer test. $p < 0.05$ was considered statistically significant.

4.3. Results

4.3.1. Yield of fucoidan from various sea algae

Yield of crude fucoidan from various sea algae was described in table 4.1. The algae which contained the most amount of fucoidan was Atsuba Kombu and the algae which contained the fewest amount of fucoidan was Okinawa Mozuku. Surprisingly, Gagome Kombu contained different amount of fucoidan depending on origins.

Table 4.1. Yield of fucoidan from various brown sea algae

Brown sea algae	Origins	Yield (%)
Makombu (<i>S. japonica</i>)	Usujiri	5.6
Gagome Kombu (<i>S. sculpera</i>)	Sanriku	5.9
	Hakodate	3.3
Rishiri Kombu (variant species of <i>S. japonica</i>)	Wakkanai	3.8
Rausu Kombu (variant species of <i>S. japonica</i>)	Rausu	4.0
Naga Kombu (<i>S. longissima</i>)	Hakodate	3.3
Hidaka Kombu (<i>S. angustata</i>)	Samani	4.5
Atsuba Kombu (<i>S. coriacea</i>)	Akkeshi	11.4
Okinawa Mozuku (<i>C. okamuranus</i> Tokida)	Nakagami	1.5

4.3.2. Inhibitory effect of fucoidan from various sea algae on ear edema induced by

IgE/antigen

To investigate whether fucoidan fraction extracted from various algae possessed the inhibitory effect on mast cell activation, mice were administered 200 $\mu\text{g/day}$ of crude fucoidan from various sea algae for 4 days and tested the PCA reaction. Fucoidan from other sea algae did not inhibit ear edema at all (Fig. 4.1).

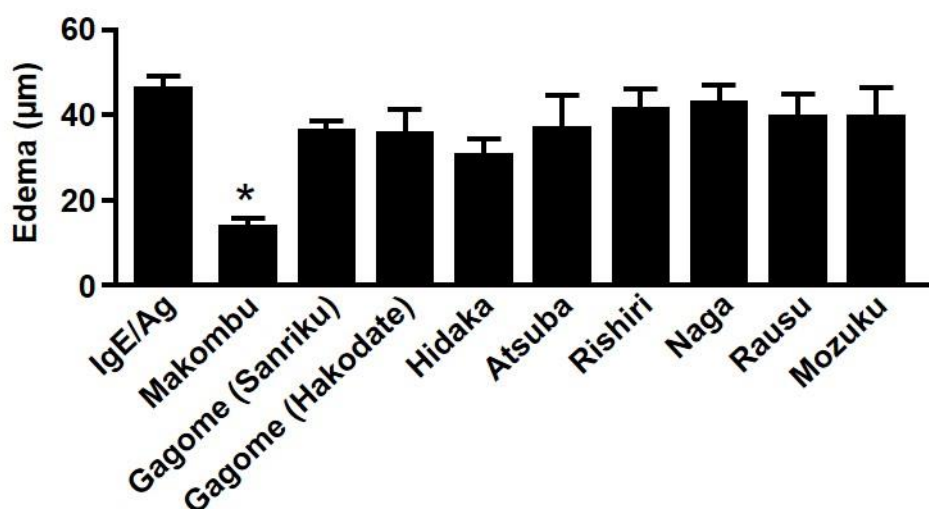


Figure 4.1. The inhibitory effect of fucoidan from various sea algae on ear edema induced by the passive cutaneous anaphylaxis reaction.

Mice were administered crude fucoidan from Makombu (*S. japonica*), Gagome Kombu (*Saccharina sculpera*), Rishiri Kombu (variant species of *S. japonica*), Rausu Kombu (variant species of *S. japonica*), Naga Kombu (*Saccharina longissima*), Hidaka Kombu (*Saccharina angustata*), Atsuba Kombu (*Saccharina coriacea*), or Okinawa Mozuku (*Cladosiphon okamuranus* Tokida) orally and tested for the passive cutaneous anaphylaxis reaction. Ear edema was evaluated 2 h after the antigen challenge. * $p < 0.05$, significantly different from the values of the group that did not receive crude fucoidan. Values represent the means $\pm$ S.E. of 3 mice in each group.

4.4. Discussion

It has been reported that the structure of fucoidan varies from species of brown sea algae [19]. For example, it was clarified that fucoidan contained in *S. japonica* is consisted of only fucose and sulfated fucose, and that in *C. okamuranus* Tokida is consisted of fucose, sulfated fucose and glucuronic acid [48]. Therefore, it was investigated whether the anti-allergic property of F-fucoidan from *S. japonica* shown in Chapter 2 was F-fucoidan-specific one. Generally, it has been known that Rishiri and Rausu Kombu are variant species of *S. japonica*. Thus, the author speculated that fucoidan from Rishiri and Rausu Kombu could inhibit ear edema induced by IgE/antigen. Unexpectedly, any fucoidan from sea algae except for Makombu (*S. japonica*) could not affect ear edema induced by IgE/antigen (Fig. 4.1). This suggested that the inhibitory effect of fucoidan on mast cell activation was fucoidan from *S. japonica*-specific effect. It was clarified that only fucoidan consisted of fucose and sulfated fucose could exhibit anti-allergic effect with the induction of galectin-9 secretion.

CHAPTER 5

The Immune-Modulatory Effect of F-fucoidan from *S. japonica*.

5.1. Introduction

In Chapter 2, the author demonstrated that F-fucoidan from *S. japonica* possessed anti-allergic effect by inhibition of mast cell activation through induction of galectin-9 which possessed the high affinity to mast cells. This was predicted that F-fucoidan might exhibit anti-allergic effect even after sensitization by antigen. To confirm this hypothesis, ovalbumin (OVA)-induced allergy model was used. OVA has been commonly used as experimental antigen for induction of type I allergy [49-51]. Administration of OVA as antigen allows observation of allergy from sensitization to pathogenesis. The author evaluated the effect of F-fucoidan on deflection to Th2 by measuring serum contents of IgE and IgG₁, and cytokine production after stimulation by OVA. The effect of F-fucoidan on mast cell activation was estimated by measurement of rectal temperature as severity of allergy after stimulation by OVA because it seems to be difficult to detect the allergy symptom in the increase of IgE contents in blood.

5.2. Materials and Methods

5.2.1. Reagents

OVA and Al(OH)₃ were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

5.2.2. Preparation of crude and high molecular weight of F-fucoidan

Crude and high molecular weight of F-fucoidan were prepared as described in Chapter 2 and 3.

5.2.3. Ovalbumin-induced allergy model

Mice were purchased and preliminary bred as described in Chapter 2. Mice were sensitized by intraperitoneal injection of 300 µl PBS containing 10 µg OVA mixed with 1 mg Al(OH)₃ adjuvant and boosted by injection of 10 µg OVA with 0.5 mg Al(OH)₃ adjuvant every 5 days for 4 weeks. Oral administration of crude or high molecular weight of F-fucoidan (200, 60 µg/day, respectively) was started on the 7th day before first injection of OVA. One day before the injection of OVA, blood samples were collected from the tail vein, and on the final day, whole blood was collected by cardiac puncture. The blood samples were centrifuged at 10,000 rpm for 10 min after incubation at room temperature for 1 h and at 4°C overnight for serum. The amount of total IgE, OVA-

specific IgE and OVA-specific IgG₁ were measured from serum by using ELISA kit (BD Biosciences, San Jose, CA, USA, DS Pharma Biomedical, Osaka, Japan, and Cayman Chemical, Ann Arbor, MI, USA, respectively). After sensitization by twice intraperitoneally injection of OVA, rectal temperature was measured every 10 min for 90 min immediately following intravenous injection of OVA (2 µg/mouse) using a rectal thermometer for mice (AD-1687; A & D, Tokyo, Japan).

5.2.4. Measurement of cytokine production from splenocytes

Splenocytes were prepared from mice which were induced allergy by OVA and administrated F-fucoidan. To evaluate OVA-induced cytokine production, the hemolyzed splenocytes (5×10^6 cells/ml) were stimulated with 25 µg/ml OVA and cultured in 96 well microplate for 72 h. Culture supernatants were collected to measure the amount of cytokine using Mouse Th1/Th2 10plex Ready-to-Use FlowCytomix Kit (Affymetrix, Santa Clara, CA, USA) according to manufacturer's protocol.

5.2.5. Sampling of tissues, RNA isolation and real-time PCR

IECs were prepared and the expression of galectin-9 and β-actin in IECs was measured as described in Chapter 2.

5.2.6. Statistical analysis

Data were expressed as means $\pm$ the SE. Statistical analysis was performed by Tukey-Kramer test. $p < 0.05$ was considered statistically significant.

5.3. Results

5.3.1. The effect of F-fucoidan on increase of serum IgE and IgG₁ contents induced by ovalbumin

To confirm whether F-fucoidan affected the Th1/Th2 balance, the amount of serum IgE and IgG₁ were measured. Oral administration of crude or high molecular weight of F-fucoidan shows no changes in serum total IgE content increased by OVA injection (Fig. 5.1.A). Moreover, the increase in OVA-specific IgE and IgG₁ in serum at end of experiment was not suppressed by administration of crude or high molecular weight of F-fucoidan (Fig. 5.1.B, C). These data suggested that F-fucoidan from *S. japonica* could not modulate immune response, particularly Th2 dominant, in contrast with previous reports about fucoidan function [23-25,46].

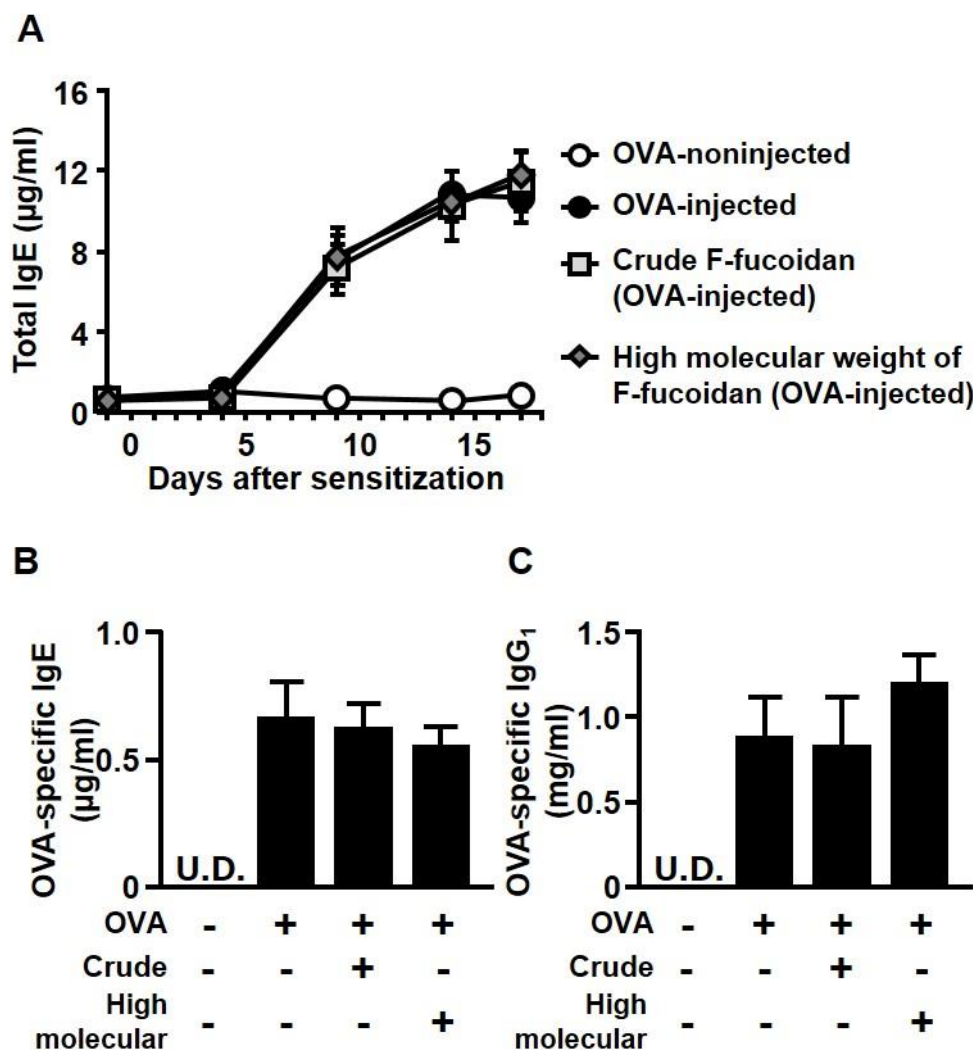


Figure 5.1. The effect of F-fucoidan on the increase in IgE and IgG₁ induced by ovalbumin injection.

Mice were administered crude or high molecular weight of F-fucoidan orally (200, 60 $\mu\text{g/day}$, respectively) and immunized with ovalbumin (OVA) as described in the Materials and Methods. (A) Amount of total IgE ($\mu\text{g/ml}$) in serum collected from the tail vein 1 day before injection OVA was measured. Serum OVA-specific IgE ($\mu\text{g/ml}$) (B) and OVA-specific IgG₁ (mg/ml) (C) content on the final day were measured. Values represent the means $\pm$ S.E. of 6–7 mice in each group. U.D. indicates under detection.

5.3.2. The effect of F-fucoidan on ovalbumin-induced cytokine production

To further evaluate systemic Th1/Th2 balance, cytokine production from splenocytes

which were prepared from OVA-induced allergy model mouse. Interferon (IFN)- γ and IL-4 were used as marker of typical Th1 and Th2 cytokines, respectively. When splenocytes were re-stimulated with OVA, splenocytes from mice induced allergy by OVA increased IL-4 production (Fig. 5.2.A) and decreased IFN- γ production (Fig. 5.2.B). Administration of crude or high molecular weight of F-fucoidan did not affect the increase of IL-4 and decrease of IFN- γ induced by OVA stimulation.

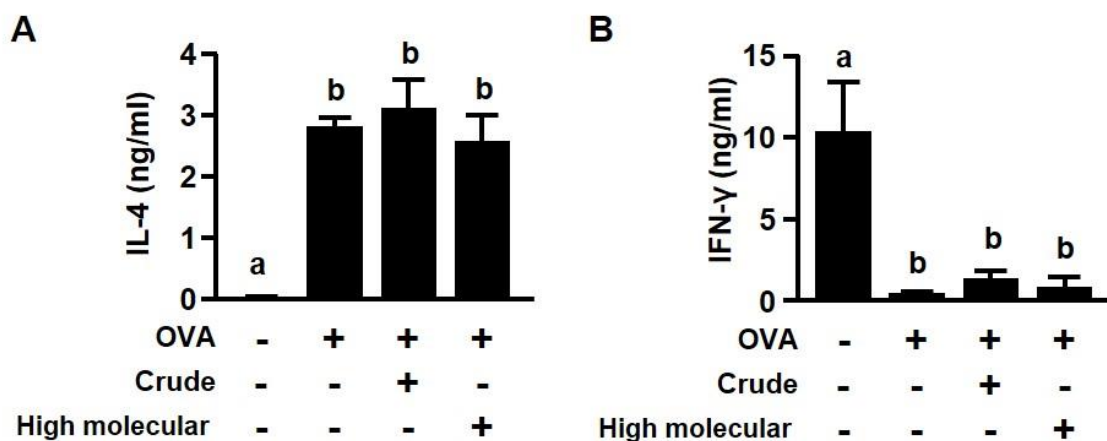


Figure 5.2. The effect of F-fucoidan on cytokine production induced by ovalbumin injection.

Mice were administered crude or high molecular weight of F-fucoidan orally (200, 60 μ g/day, respectively) and immunized with ovalbumin (OVA) as described in the Materials and Methods. Splenocytes (5×10^6 cells/ml) were prepared and re-stimulated by OVA (25 μ g/ml) as described in the Materials and Methods. Culture supernatants were collected and the amount of IL-4 (ng/ml) (A) and IFN- γ (ng/ml) (B) were measured. Different alphabet indicates a significant difference. Values represent the means $\pm$ S.E. of 3 mice in each group.

5.3.3. The inhibitory effect of F-fucoidan on allergic symptom in OVA-induced allergy model

It was demonstrated in Figs. 5.1. and 5.2. that F-fucoidan did not exhibit the immune modulatory effects. However, F-fucoidan can inhibit mast cell activation and following allergic symptom as shown in Chapter 2. Therefore, the author presumed that F-fucoidan could inhibit type I allergic symptom induced by OVA injection even though IgE and IgG₁ contents were no changes. Therefore, rectal temperature was measured after intravenous injection of OVA to evaluate the effect of F-fucoidan on allergic symptom. It has been reported that mast cell released chemical mediator such as histamine by mast cell activation and this mediator induced vessel permeability and vasodilatation [52]. Vasodilation has been known to induce hypotension, followed by decrease of body temperature. Thus, decrease of rectal temperature has been commonly used as a marker of systemic allergic symptom [49,53,54]. Rectal temperature decreased 5.6°C at 40 min after intravenous injection of OVA and resumed at 90 min after injection of OVA in the OVA-control group (Fig. 5.3). In contrast, its decrease in mice administrated crude or high molecular weight of F-fucoidan was moderate. Rectal temperature in mice administrated crude or high molecular weight of F-fucoidan decreased 3.8 and 3.7°C, respectively at 30 min after injection of OVA and almost resumed at 80 min after injection

of OVA (Fig. 5.3). This result indicated that F-fucoidan can inhibit allergic symptom without modulating Th1/Th2 balance.

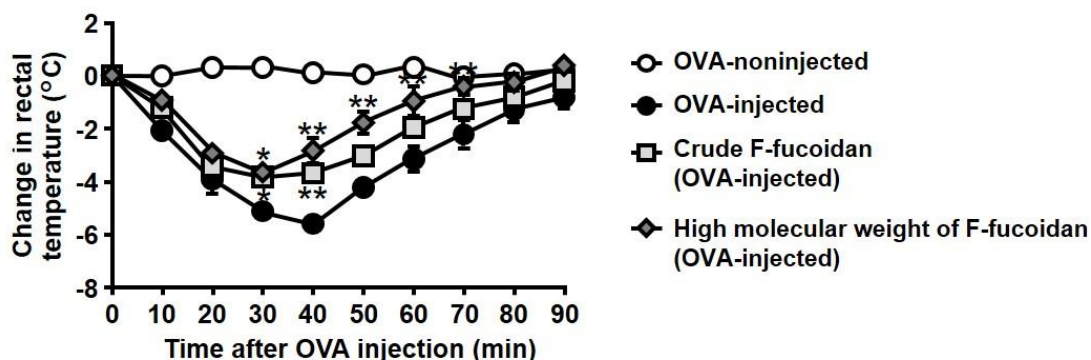


Figure 5.3. The effect of F-fucoidan on decrease of rectal temperature induced by ovalbumin injection.

Mice were administered crude or high molecular weight of F-fucoidan orally (200, 60 $\mu\text{g/day}$, respectively) and immunized by intraperitoneal injection of ovalbumin (OVA) as described in the Materials and Methods. Rectal temperature was measured every 10 min for 90 min immediately following intravenous injection of OVA (2 $\mu\text{g}/\text{mouse}$). * $p < 0.05$, ** $p < 0.01$, significantly different from the values of the group that was not administrated F-fucoidan and injected OVA at the same time point. Values represent the means $\pm$ S.E. of 4 mice in each group.

5.3.4. Association between the anti-allergic effect of F-fucoidan in ovalbumin-induced allergy model and galectin-9 induction

To confirm that the anti-allergic effect of F-fucoidan in OVA-induced allergy model was dependent on galectin-9, galectin-9 mRNA expression in IECs of mice after measurement of rectal temperature was determined. Galectin-9 mRNA expression in IECs of mice injected OVA without F-fucoidan did not show any change compared with IECs of OVA-

noninjected mice (Fig. 5.4). In contrast, administration of crude or high molecular weight of F-fucoidan increased expression of galectin-9 mRNA in IECs (Fig. 5.4). This indicated that the inhibitory effect of F-fucoidan on the decrease of rectal temperature which induced by mast cell activation was due to galectin-9.

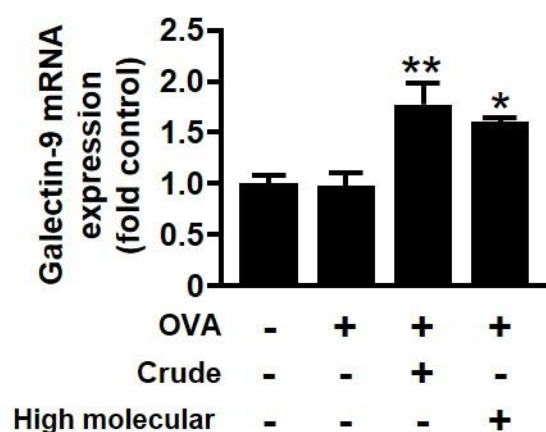


Figure 5.4. Expression of galectin-9 mRNA in intestinal epithelial cells in ovalbumin-induced allergy model.

Mice were administered crude or high molecular weight of F-fucoidan orally (200, 60 μ g/day, respectively) and immunized by intraperitoneal injection of ovalbumin (OVA) as described in the Materials and Methods. Galectin-9 mRNA expression in intestinal epithelial cells of mice after measurement of rectal temperature was measured. * $p < 0.05$, ** $p < 0.01$, significantly different from the values of the group that was not injected OVA. Values represent the means $\pm$ S.E. of 4 mice in each group.

5.4. Discussion

It has been reported that fucoidan exhibits immune modulatory effects including

anti-allergic effect [23-25,46,55]. However, F-fucoidan from *S. japonica* exerted anti-allergic property by inhibition of mast cell activation in dependent on galectin-9 as shown in Chapter 2. Galectin-9 has been reported not to affect serum IgE and IgG₁ contents [27], suggesting that galectin-9 does not influence immune responses. The author presumed that F-fucoidan exhibits anti-allergic effect by inhibiting mast cell activation dependently on galectin-9 but not affecting immune systems. The effect of F-fucoidan in an animal model that allergy was induced by sensitization of antigen was investigated. F-fucoidan from *S. japonica* did not affect the increase in serum IgE and IgG₁ contents (Fig. 5.1) and production of Th1 and Th2 cytokines (Fig. 5.2) induced by OVA. In contrast, F-fucoidan inhibited allergic symptoms which was caused by mast cell degranulation such as decrease of body temperature (Fig. 5.3). The effect of F-fucoidan was dependent on galectin-9 in OVA-induced allergy model (Fig. 5.4), as is the results in the PCA reaction in Chapter 2. These results suggested that F-fucoidan could inhibit mast cell activation but not polarization to Th2 which was previously reported as an effect of fucoidan [23-25,46] and that F-fucoidan could suppress type I allergy even after sensitization by antigen.

CHAPTER 6

General Discussion and Conclusion

It has been known that type I allergic symptoms are induced by increased IgE in blood and thereby mast cell activation. Therefore, there are mainly two therapeutic approaches in allergy that suppress polarization to Th2 and increase of IgE in blood, and that directly prevent mast cell activation [5]. Among the food factors having the anti-allergic effect, dietary fiber and probiotic foods, which are barely absorbed into the body, have been frequently reported to exhibit the effect of the former [15-18,56]. In contrast, carotenoids, flavonoids, and other small components of food, which are absorbed into the body, have been reported to have the effect of the latter [12-14,57], although some of them also have the immune modulatory effects. Fucoidan, which is a dietary polysaccharide focused in this study, has mainly been reported to have the effect of the former [23-25,46], since it is poorly absorbed into the body [22,26]. However, in this study, it was elucidated that F-fucoidan (only high molecular weight of F-fucoidan) from *S. japonica* exhibited the inhibitory effect on mast cell activation through gut (Chapter 2, 3). The inhibitory mechanism was a novel mechanism that F-fucoidan induced galectin-

9 expression and secretion into blood, which has not been reported previously. This effect was shown by only fucoidan from *S. japonica* (Makombu) not by fucoidan from other sea algae (Chapter 4). Furthermore, in contrast with previous reports, it was eliminated that F-fucoidan from *S. japonica* inhibited allergic symptom induced by mast cell activation without affecting deflection to Th2 (Chapter 5).

The result that F-fucoidan inhibited ear edema and the decrease of rectal temperature which were caused by mast cell activation, but did not suppress the increase of serum IgE and IgG₁ contents and the disproportion of Th1/Th2 balance (Chapter 2, 5) was consistent with a previous report that the exogenous galectin-9 could suppress ear swelling induced by IgE/antigen, although it did not affect the serum contents of IgE and IgG₁ [27]. Besides, while it has been reported that mast cells expressed T-cell immunoglobulin and mucin-containing protein-3 (TIM-3), which is one of the major targets of galectin-9 [58,59], activation of TIM-3 on mast cells did not affect mast cell degranulation induced by IgE/antigen [59]. These reports and the results in this study suggested that galectin-9 induced by F-fucoidan interacted with IgE but not TIM-3 and prevented allergic symptoms.

In Chapter 2, it was demonstrated that 200 µg of crude F-fucoidan could inhibit type I allergic symptom induced by mast cell activation. It was clarified that crude F-

fucoïdan was contained 5% in dried *S. japonica* as shown in Chapter 4. Therefore, this effective dose is converted to 12 g of dried *S. japonica* in 60 kg of human body weight. This dose is the amount which can be eaten effortlessly. In addition, it was elucidated that high molecular weight of F-fucoïdan exhibited the anti-allergic effect through induction galectin-9 but not low molecular weight of F-fucoïdan (Chapter 3). This suggested that *S. japonica* was taken unheated *S. japonica* for getting the anti-allergic effect of F-fucoïdan.

Our laboratory previously reported that oral administration of F-fucoïdan was not completely absorbed into the body and was excreted in the feces [22]. Therefore, it was assumed that F-fucoïdan interacts with factors on cells in small intestine and promotes the expression and secretion of galectin-9. Fucoïdan has been reported to interact with several lectins [60-63], and IECs express various lectins [64-66]. Thus, fucoïdan could be recognized through one of the lectins on IECs. In addition, DCs have been reported to pass their dendrites between IECs and have DC-specific intracellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), which recognizes fucose [67]. It has been known that DCs can protrude dendrites to the luminal side, and DC-SIGN is also expressed on protruding dendrites [68]. Thus, F-fucoïdan might be recognized by DCs using intraepithelial dendrites but not by IECs. However, the receptor that recognizes F-fucoïdan has not yet been identified, although there are some candidates.

In the future, to clarify further mechanism of the effect of F-fucoidan, the culture system mimicked *in vivo* is needed. Our laboratory previously developed the co-culture system with Caco-2 cells (apical side) as IECs and RBL-2H3 cells (basolateral side) as mast cells [69]. Although this system could evaluate the anti-allergic effect of prebiotics or polyphenols, the effect of F-fucoidan *in vivo* was not mimicked. It has been reported that Caco-2 cells did not express galectin-9 generally but Caco-2 cells express galectin-9 only when Caco-2 cells were induced differentiation to M cells [70]. It was estimated that the effect of F-fucoidan *in vivo* was not observed *in vitro* co-culture system, since the effect of F-fucoidan *in vivo* was dependent on galectin-9. T84 and HT-29 cells, which are known as human IEC cell lines, have been reported to express galectin-9 and secret galectin-9 into basolateral side [71]. Therefore, the construction of a novel co-culture system using these cell lines is needed. If a novel co-culture system is constructed, further mechanisms of the anti-allergic effect of F-fucoidan such as receptor for F-fucoidan and following signaling will be clarified.

The author concluded that oral treatment with F-fucoidan from *S. japonica* prevented type I allergy through the inhibition of mast cell activation but not the inhibition of Th2 polarization. This anti-allergic mechanism of F-fucoidan suggested that F-fucoidan may be an effective inhibitor that can prevent symptoms in type I allergic

patients even after sensitization to allergen.

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