博士論文 (要約)

Establishment and Analysis of Mouse and Cell Models of Wheat-Dependent Exercise-Induced Anaphylaxis

（小麦依存性運動誘発アナフィラキシーのマウスおよび細胞モデルの構築と解析）

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論文の内容の要旨

Introduction

Wheat is a major staple component of food all over the world due to the special bread-making properties of the flour. With the popular consumption of wheat products, hypersensitivity caused by wheat cannot be ignored. Food-dependent exercise-induced anaphylaxis (FDEIA) is a severe form of allergy which is caused by physical exercise after ingestion of a specific food. In Japan, wheat is reported to be one of the most frequent allergenic FDEIA foods. Wheat-dependent exercise-induced anaphylaxis (WDEIA), an IgE-mediated hypersensitivity, is induced by wheat ingestion together with cofactors such as physical activity, acetylsalicylic acid (aspirin; ASA) and alcohol.

Wheat proteins are classically divided into water/salt soluble albumin and globulin, and water/salt insoluble gliadin and glutenin. Gliadin is subdivided into α-, β-, γ- and ω-gliadin. Among these wheat proteins, ω-5 gliadin has been identified as the important allergen in WDEIA. Exercise is the most important and prevalent cofactor of WDEIA. Although exercise-induced enhancement of intestinal absorption of allergens into the circulation has been implicated, only limited data are available regarding the immunological mechanisms. Researchers have been focused on the clinical manifestations in patients with WDEIA, whereas the analysis of immunological mechanism towards appropriate animal models needs to be carried out. To better understand immunological pathways, the characteristics of gliadin leading to wheat allergy and the impact of exercise on WDEIA were elucidated using a mouse model in this study.

Not only exercise, ASA and other nonsteroidal anti-inflammatory drugs (NSAIDs) have been described as cofactors of WDEIA. It has been reported that ASA induces gastric barrier loss of a gastric adenocarcinoma cell line by activating p38 MAPK pathway. The PT-GLI (peptic-tryptic digest of gliadin) stimulation in Caco-2 cells could induce pro-inflammatory reactions through NF-κB and MAPK pathways. Until now, the cell model of WDEIA has not been established. Therefore, I examined the effects of wheat proteins and ASA on MPAK pathways in an intestinal cell line (Caco-2 cells). Their effects on epithelial barrier of Caco-2 cells were examined by measurement epithelial electrical resistance (TER) and permeability in this study.

Oral immunotherapy (OIT) is applied in the treatment of patients with WDEIA. OIT involves the oral administration of allergenic proteins to the patients, and the serious adverse effects caused by OIT with intact allergen should not be ignored. Modified wheat proteins characterized by hypo-allergenicity and potential of tolerance induction have become desirable for OIT of WDEIA. In this study, gliadin was degraded by hydrolysis and deamidation. The degraded gliadin is utilized in the mouse model of WDEIA

and oral tolerance to identify the allergenicity and potential of tolerance induction of gliadin after degradation.

Chapter 1. Sensitization and elicitation of hypersensitivity by wheat gliadin

WDEIA is known as an IgE-mediated hypersensitivity. The insoluble gliadin fraction seems to hold the majority of allergens responsible for WDEIA. I firstly developed a mouse model of hypersensitivity to wheat gliadin by testing different doses of sensitization and elicitation by gliadin extract from flour. Intraperitoneal immunizations of BALB/c mice with 10, 20 and 50 μg of gliadin absorbed onto alum were performed once every 10 days. Level of anti-gliadin specific IgE was significantly higher at the 50 μg dose than the two other doses after four times immunization. It is known that IgE and IgG1 production of B cells is induced by T helper (Th) 2 cells secreting interleukin (IL)-4 and IL-13. On the other hand, IgG2a production is induced by Th1 cells secreting interferon (IFN)-γ. In the 50 μg of gliadin-immunized mice, specific IgG1 production was high, whereas IgG2a production was low. As a result, immunization of mice with 50 μg of gliadin for four times was determined for sensitization. All gliadin-immunized mice were orally administrated with 10 mg of gliadin, while control mice received the vehicle alone. As for the mechanism of IgE-mediated hypersensitivity, the cross-linking of the IgE receptors by subsequent allergen exposure on the surface of mast cells and basophils provokes immediate symptoms of allergy. The rectal temperature of sensitized mice was significantly lower than control mice after oral challenge with 10 mg of gliadin. A high level of interleukin (IL)-4 and IL-5 was observed upon *in vitro* stimulation of $CD4^+$ T cells with gliadin from spleen and mesenteric lymph nodes (MLN) in gliadin-sensitized and challenged mice. These results demonstrated that hypersensitivity was induced by high dose of gliadin sensitization in combination with oral challenge in this experimental system.

Chapter 2. Evaluation of immunological responses in MLN and the small intestine in WDEIA using a mouse model

In previous studies of WDEIA, the immunological responses, including $CD4^+$ T cell responses, in the intestine have not been fully characterized. In this study, the immune responses of $CD4^+$ T cells in the intestinal immune organ (MLN) as well as the responses in small intestinal tissue were investigated using a mouse model which gliadin as an allergen.

Based on Chapter 1, sensitized mice were forced to run on a treadmill (exercise group) or put in cages (rest group) for 30 min after oral administration with 1 mg/ml of gliadin. The unsensitized mice were orally administrated with vehicle alone as control followed by exercise and rest. Both the gliadin-exercise and gliadin-rest group showed a great decrease in rectal temperature compared with corresponding control group. The MLN $CD4^+$ T cells stimulated by gliadin showed remarkably higher production of IL-4 in sensitized mice compared to those in unsensitized mice. However, there was no difference in the production of IL-4 between gliadin-exercise group and gliadin-rest group. The IL-17-producing $CD4^+$ T

helper cells, Th17 cells, are linked to inflammation. The production of IL-17 tended to be higher in gliadin-exercise group than gliadin-rest group, implying the tendency of inducing inflammatory responses is higher in gliadin-exercise group than in gliadin-rest group. Intestinal immune responses were evaluated by measuring mRNA expression levels of cytokines and inflammation factors as well as the release of histamine in the jejunum. Gliadin-exercise group expressed a high level of genes related to exercise (IL-6 and tissue transglutaminase; tTG) and mast cells (mouse mast cell protease-1; mMCP-1 and IL-9) compared with the control-exercise group and the gliadin-rest group. The production of histamine in the gliadin-exercise group was significantly higher than that in the control-exercise group. These results suggested that oral administration with 1 mg of gliadin could cause anaphylaxis with or without exercise, but the allergic responses were induced in the small intestine by exercise. In order to further confirm the role of oral challenge in the induction of intestinal allergic responses, the gliadin-sensitized mice were orally administrated with vehicle alone (without gliadin) followed by exercise as sensitized-control exercise group. The expressions of IL-9 and mMCP-1 as well as release of histamine were significantly higher in gliadin-exercise group than sensitized-control exercise group. Therefore, allergic responses were induced by the subsequent gliadin exposure in the small intestine followed by exercise in sensitized mice.

Chapter 3. Evaluation of reduced allergenicity of degraded gliadins in WDEIA model and oral tolerance model

In order to prepare modified gliadin with hypo-allergenicity for OIT, the peptic digest of gliadin (peptic-GLI) and peptic-tryptic digest of gliadin (PT-GLI) were prepared, and deamidation was carried out by dispersing PT-GLI in HCl to give HCl-PT GLI.

The degraded gliadins (PT-GLI and HCl-PT GLI) had decreases in IgE-binding capacity and Th2 cell activation. The IgE-binding capacity of peptic-GLI was reduced, but Th2 cell reactivity was maintained. Therefore, cross-linking of IgE bounded to mast cells might be weakened by peptic-GLI, PT-GLI and HCl-PT GLI. Epitopes of gliadin recognized by Th2 cells might be partially degraded by hydrolysis and deamidation in the case of PT-GLI and HCl-PT GLI. I chose the HCl-PT GLI to replace gliadin in the elicitation phase of WDEIA model. The symptoms and allergic responses induced by gliadin were not elicited by HCl-PT GLI, which further indicated that the cross-linking of the IgE was weakened by HCl-PT GLI. The different characteristics of degraded gliadins in activation of mast cells and T cells make them good candidates for oral tolerance analysis. Oral administration of gliadin before sensitization could suppress the production of gliadin-specific IgE in serum and IL-4 in spleen and MLN. Oral administration of degraded gliadin could only suppress the production of IL-4, while the serum specific IgE level was not affected. The weak suppression of degraded gliadin in Th2 responses of spleen may relate to the high IgE level in sera. Th2 cytokine responses were suppressed by oral administration with degraded gliadin before sensitization, implying that degraded gliadin may have the potential to enervate Th2 cells and be applied in OIT for patients with wheat allergy.

Chapter 4. Effect of ASA and gliadin on epithelial barrier in Caco-2 cell model of WDEIA

The possible pathway of exercise and ASA acting in WDEIA is that they may cause damage to the gastrointestinal mucosa, which could lead to increased permeability and facilitate wheat allergen absorption. ASA, instead of exercise, is a good candidate for *in vitro* experiment to investigate the change of epithelial barrier. I tried to establish a cell model of ASA in combination with gliadin or PT-GLI to evaluate their roles in inducing WDEIA. The fully differentiated Caco-2 cells were applied to mimic *in vivo* intestinal epithelium in humans.

I found that treatment with 15 mM of ASA for 12 h did not induce apoptosis of Caco-2 cells, while this reduced the TER. The activation of p38 MAPK and JNK induced by ASA which is related to the decrease in TER was confirmed in Caco-2 cells. Gliadin and PT-GLI had no effect on TER and activation of MAPK pathways, suggesting that ASA rather than gliadin (or PT-GLI) dominates the damage of epithelia barrier and the ASA-induced permeation of wheat proteins may be involved in the allergic reactions of WDEIA. In order to confirm the permeability caused by ASA, mass spectrometry and enzyme-linked immunosorbent assay (ELISA) were performed to detect the transport of four IgE-binding epitopes of ω-5 gliadin as well as gliadin from the apical chamber to the basolateral chamber, respectively. Small epitopes could pass through epithelial barrier with or without ASA. The IgG1-binding capacity of culture medium showed that ASA enhanced the permeation of intact gliadin through epithelium.

Conclusions

In conclusion, hypersensitivity and WDEIA mouse models were established using gliadin in this study. The role of exercise in inducing allergic responses in the small intestine was highlighted in the mouse model of WDEIA. The effects of exercise on potential activation of mast cell and induction of inflammatory responses were further confirmed in this model. In the cell model of WDEIA, the role of ASA in induction of intestinal epithelial damage and activation of MAPK pathways were confirmed by using Caco-2 cells.

Oral administration with degraded gliadins before sensitization could suppress the Th2 cytokine responses. Although the IgE level was not suppressed, the low IgE-binding capacity of them may weaken allergic reactions in wheat hypersensitivity or WDEIA.

In this study, the mouse model and cell model elucidated the role of exercise and ASA in inducing WDEIA, respectively. These models provide a good way for the understanding of immunological pathways of WEDIA. The degraded gliadin with underlying hypo-allergenicity may become a useful tool in immunoprophylaxis and treatment for patients with wheat allergy.