博士論文の要約

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学位論文題名

Modulation of inflammatory responses by megalo-type isomaltosaccharides

（イソマルトメガロ糖による炎症反応の調節作用）

There is an emerging concern worldwide in “non-communicable disease (NCD)”, which is various types of chronic diseases including obesity, diabetes, and cardiovascular diseases, etc. The risk factors for NCDs are considered to be unhealthy diet, insufficient exercise, alcohol consumption, and smoking. WHO reported that the global death rate by NCDs reached 52% under the age 70 years. There are also socioeconomic impacts by NCD especially on socially disadvantage population having limited access to medical care. Thus, prevention of NCDs is another option for those people. Because dietary habit is closely involved in the development of NCDs, dietary intervention is expected to be available to prevent NCDs. A possible way to prevent NCDs is to control energy consumption and dietary composition in the dietary intervention. Food science is able to provide several options to struggle such problems through modulation of dietary composition. One of the promising ingredients is non-digestible saccharides (NDSs).

NDSs are hardly digestible in small intestine and some of them are utilized by bacteria in large intestine. They are abundant in grains, fruits, and vegetables. Physicochemical property is diverse depending on the type of NDSs in terms of solubility, water-holding capacity, viscosity, and fermentability, and the properties are involved in their physiological functions such as improvement of glucose and lipid metabolism, excretion of harmful substances, production of fatty acids, regulation of immune responses, and so on.

Also, it is reported that NDSs can prevent or ameliorate chronic diseases, such as diabetes, cardiovascular disease, and cancer.

NDSs can be classified with solubility and viscosity. Viscous water-soluble NDS slows down the digestion and absorption of glucose and consequently suppresses elevation of blood glucose level after meal and secretion of insulin, which participate in treatment and prevention of diabetes. Also, water-soluble NDS holding a large amount of water has strong viscosity and interact with bile acids and cholesterol, which contributes to excretion of these molecules into feces. As a result, cholesterol is utilized to synthesize bile acids in the liver, accompanied by decrease in the low-density lipoprotein cholesterol, suggesting that NDS prevent cardiovascular disease. Insoluble NDSs are possible to increase the amount of defecation. Also, insoluble NDSs promote intestinal peristaltic movement, thereby shortening transit time in the large intestine, which improves constipation and also contributes to prevention of diverticular disease. Some of the NDSs can be utilized by intestinal bacteria, leading to growth of beneficial bacteria in the colon and production of short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate. The SCFAs contribute to gut barrier function, mineral bioavailability, and reduction of secondary bile acid.

NDSs influence several functions of immune system in human studies. Ingestion of whole-wheat meal reduces postprandial serum interleukin 18 (IL18) concentrations both in diabetic and non-diabetic subjects. The National Health and Nutrition Examination Survey Data in the United States demonstrated that NDS consumption is negatively associated with serum C-reactive protein (CRP). Increase in serum CRP concentration is frequently found in acute and chronic inflammation, suggesting that ingestion of NDS attenuates development of acute and chronic inflammation.

Recently, mechanisms of the influence of NDS on immune responses have been partially clarified in experimental animals. Fructo-oligosaccharides promote IgA production in mice and isolated Peyer's patch cells. Pro-inflammatory cytokines and NF-κB expression in Caco-2 cells was diminished by α3-sialyllactose or fructo-oligosaccharides, raising the possibility that the oligosaccharides retards inflammation by activation of peroxisome proliferator-activated receptor alpha and peptidoglycan recognition protein 3. Another study reported that cytokine productions from dendritic cells in response to NDSs depend on the chain length and sugar composition. These NDSs also modulate polarization of T cells via crosstalk between intestinal epithelial cells and dendritic cells depending on their structure. These studies suggest that NDSs regulate various immune cells including B cells, dendritic cells, and T cells as well as epithelial cells, which modulates symptoms in immune-related diseases.

The interaction between innate and adaptive immunity influences health maintenance. Macrophages and neutrophils in innate immunity are able to incorporate exogenous substances, and microbes. Also, they process such substances into small pieces and present them as antigens to the adaptive immune cells, which classifies these cells as antigen present cells. The fundamental roles of innate immunity are to provide an immediate defense against infection and effective induction of adaptive immunity. On the other hands, adaptive immunity participates antigen-specific responses including antibody production and elimination of cancer cells or virus-infected cells. The cooperation between innate and adaptive immunity is required to maintain homeostasis and host defense form pathogens.

Macrophages are divided into two phenotypes, and the balance of the phenotypes is involved in inflammation, disease development, maintaining homeostasis and tissue repair. The classically-activated types of macrophages (M1 macrophages) stimulated by

lipopolysaccharide (LPS) and Interferon gamma (IFNγ). M1 macrophages are fighting against microorganisms and produce pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), IL1, and IL6, which sometimes induce tissue damages. In contrast, the alternatively-activated types of macrophages (M2 macrophages) are involved in tissue remodeling, clearance of parasites, and produce anti-inflammatory cytokines such as IL4, IL10, and IL13. The balance of the M1 and M2 types determines direction of the fate, for example tissue injury or repair.

"Megalosaccharide" is one of the categories in carbohydrates and indicates a saccharide containing 10 to 100 monosaccharide units. Isomaltosaccharides (IMs) have α-1,6-glycosidic linkages in the chain of the glucosaccharides. Previously, we investigated whether megalo-type isomaltosaccharides (M-IMs) influences solubility and intestinal absorption of quercetin-3-glucoside and found that M-IMs enhance absorption of quercetin in a small intestine via the promotion of the solubilization in the small intestinal contents. Also, M-IMs display delayed absorption and may stay relatively long time in the gastrointestinal tract, which enables them to encounter host immune cells. There is almost no information on the regulation of immune responses to IMs, especially for the responses depending on the degree of polymerization. We expected that the innate immune system might have the chance to encounter IMs. In this study, we investigated whether M-IMs influence cytokine productions in primary macrophages isolated from rats and the ingestion of M-IMs affects antigen-specific antibody productions and acute hepatitis in an animal model.

In the primary cell experiment, sodium periodate or $NaIO_4$ was used to collect a large amount of primary macrophages. WKAH/Hkm Slc rats (5-6 weeks old) administered with 5 mM $NaIO_4$ in saline. At 72 hours after the administration, the macrophages were collected from the peritoneal cavity. The cell suspension was centrifuged, and the cells were seeded on plates. The adherent macrophages were cultured with IMs or LPS as a positive control. M-

IMs significantly increased the TNFα production in the primary macrophages. By contrast, there was no significant influence of oligo-type isomaltosaccharides (O-IMs) on TNFα production. Because the fundamental structure was almost the same between M-IMs and O-IMs, these results indicated that the recognition of IMs by macrophages requires a certain size. TNFα and IL6 production significantly increased in response to M-IMs as well as LPS in the macrophages. The level of nitric oxide (NO) in the M-IM-stimulated macrophages was significantly lower than that in LPS-stimulated macrophages, but both of them were significantly higher than that of the control. The treatment with LPS or M-IMs promoted cell viability, but no difference in cell viabilities was found between LPS and M-IMs. The NO production was not consistent with cell viability. The induction of inflammation-related factors can be detected by using a PCR array in response to M-IMs as well as LPS. The patterns were nearly the same between M-IMs and LPS. We selected some of the inflammation-related factors to confirm the quantitative difference. The overall gene expression pattern in response to M-IMs was quite similar to that to LPS. To investigate whether the existence of M-IMs influences the phagocytosis, we measured antigen incorporation activity of the primary macrophages by using labeled *Escherichia coli* particles. No difference was observed of phagocytic ability in the macrophages among control, LPS, and M-IMs. We investigated whether a toll like receptor 4 (TLR4) signal inhibitor (TAK-242) influences the cytokine production induced by M-IMs. TAK-242 suppressed the TNFα production induced by M-IM in the primary macrophages. These findings raise the possibility that TLR4 is responsible for TNFα production by M-IMs.

The cell experiment demonstrates that M-IMs modulated macrophage functions in terms of pro-inflammatory cytokine productions in primary culture. However, the results of this study suggested that M-IM was very weak to induce TNFα production compared with

LPS. Moreover, our previous study showed that the ingestion of M-IM does not aggravate an experimental colitis. It was predicted that M-IM does not induce inflammation and may be recognized by innate immune cells such as macrophages, which present antigen to T cell, resulting in production of antigen-specific antibody. M-IM have potentiality that can regulate not only innate immunity, but also adaptive immunity in *in-vivo*. Also, an *in-vivo* experiment by using rats, M-IMs promote flavonoids absorption. Because inflammatory reactions were observed in high concentrations of M-IMs in cell experiments, we have to evaluate the safety in order to apply M-IM as a functional food. Thus, we investigated whether a long-term ingestion of the M-IMs influence antibody production and LPS-induced acute inflammation as a part of safety assessments.

We examined the impact of M-IMs on antigen-specific antibody production by using keyhole limpet hemocyanin (KLH), which is widely used as a functional test in immuno-toxicology. Male F344/Jcl rats (5 weeks old) were fed the diet with or without 3% M-IMs for 5 weeks. KLH was administered on day 14, and the serum KLH-specific IgM was measured on day 21. Also, KLH-specific IgG was analyzed on day 32. At the end of the experimental period (day 35), the rats were subcutaneously administrated with LPS to induce acute liver injury. At 6 h after the LPS administration, the rats were euthanized with sodium pentobarbital and the blood plasma was collected from aortic vessel with heparin and aprotinin. There was no significant difference in body weight and food intake by the ingestion of M-IMs, suggesting no effect of the ingestion of M-IMs on the growth. Although serum KLH-specific antibody production tended to increase, there was no significant difference. Liver injury markers, plasma alanine aminotransferase and aspartate aminotransferase decreased significantly by the ingestion of M-IMs. It is possible that the suppression of the liver injury markers by ingestion of M-IMs was likely to be associated

with the inflammatory cytokines production. Therefore, we confirmed production of the pro-inflammatory cytokines of the ingestion of M-IM in the LPS-induced hepatitis models. As a result, the ingestion of M-IMs significantly suppressed plasma concentration of IL1β production. Also, both IL6 and caspase-1 concentrations tended to decrease in the rats fed the M-IM-supplemented diet. However, there was no significant difference in the TNFα concentration. There was no significant difference of the expressions of inflammatory cytokines in the liver between the groups. In a separate experiment, male F344/Jcl rats (5 weeks old) were fed control diet for 5 days. Then, the rats were divided into two groups and fed diets supplemented with or without M-IMs (30 g/kg diet) for 37 days. The expression of cluster of differentiation 14 (CD14) in the liver significantly decreased in the M-IM-fed rats.

In conclusion, ingestion of M-IMs ameliorated the LPS-induced acute liver injury in rats. Such reduced inflammation by the M-IMs may be associated with dysfunction of CD14 signaling. It is reported that LPS concentrations increase in various obese mice. In addition, there is a postprandial increase in blood LPS even in mice fed a normal diet, suggesting that such transient increase of LPS is involved in the onset of chronic inflammation. Also, there is no influence of the M-IM ingestion on antibody production, indicating that M-IMs influence the innate immunity rather than the adaptive immunity, which may contribute to prevention of acute inflammation induced by endotoxin. We propose that the M-IMs can be used as a functional ingredient in foods to prevent chronic inflammation as well as NCDs in clinical applications.