論文の内容の要旨

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論文題名

Effect of imidazole dipeptides on gut immunity and inflammatory responses
(イミダゾールジペプチドの腸管免疫系および炎症反応に対する作用に関する研究)

Imidazole dipeptides (IDP), also known as histidine-derived dipeptides, are a generic name of peptides to which amino acids containing imidazole group are bonded. Those include carnosine (β-alanyl-L-histidine), anserine (β-alanyl-1-methyl-L-histidine), balenine (β-alanyl-3-methyl-L-histidine) and homocarnosine (γ-aminobutyryl-L-histidine). They are distributed widely in skeletal muscles and nervous system of vertebrates. As dietary sources, chicken and pork meat, tuna and bonito have the relatively high concentration provided as regular diets. Carnosine, contained as high as 200-250 mmol/kg dry muscle, is the most studied compound. Its biochemical properties include antioxidant, bivalent metal ion chelating, muscular proton buffering, and reactive carbonyl scavenger activities. Recently, anti-aging and anti-fatigue activities of IDP are attracting more attentions. However, the effect of IDP on immune responses and inflammatory responses is less studied.

The gastrointestinal tract bears roles in the digestion of food, absorption of nutrients and excretion of feces, and is constantly exposed to the invasion of pathogens. The intestine is a place where highly populated immune cells, neurons, gut microbiota and food-derived substances interact with each other to keep the homeostasis of our body. Immunoglobulin A (IgA) acts as the major defense against invasion of pathogens in the mucosa, and their production is controlled by interactions of various immune cells and gut microbiota in the intestine. Increased IgA antibody production can promote protection against pathogens in the mucosa. Inflammation is primarily a

protective response against harmful stimuli including pathogenic infection, and involves immune responses. Recently, chronic inflammation has been revealed as the underlying mechanism of lifestyle diseases and age-related dysfunction including cognitive decline. Cytokines are small secreted proteins released by cells as one of the main mediators and signals of gut-brain axes. They have a specific effect on the interactions and communications between cells to promote immune responses or inhibit inflammatory responses.

In the previous studies of our laboratory, it was shown that carnosine exerts apparently opposing actions, the enhancing effect on gut immune response and the inhibitory effect on inflammatory response. The purpose of this study is to investigate the enhancing effect of dietary carnosine on gut immune responses such as IgA production and cytokine responses. The other one is to investigate whether dietary IDP exert anti-inflammatory effect leading to improvement of cognitive function in healthy elderly people.

Chapter 1. Dietary carnosine enhances small intestinal immune responses

IgA, the most abundant immunoglobulin isotype in mucosal secretions, provides protection against antigens at intestinal surfaces. Peyer's patches (PP) have been considered to be the major inductive site for IgA antibody. However, it was reported that mesenteric lymph node (mLN) is sufficient for IgA antibody production in mice lacking PP, suggesting mLN is an alternative site for IgA induction. Among lots of cytokines, interleukin (IL)-6 and transforming growth factor (TGF)-β are required to promote IgA class switching and IgA secretion. It was also reported that IgA antibody shapes intestinal microbiota while signals induced by commensal colonization are central for regulating IgA induction.

Previous studies in our laboratory demonstrated that dietary carnosine increased IgA antibody production in mouse small intestinal mucosa. To confirm this result, BALB/c mice were fed 0.5% carnosine-containing water *ad libitum* for two weeks. The small intestinal mucosa was collected to measure the total IgA antibody by ELISA, and a significant increase of IgA was observed. Next, to investigate the effect of carnosine on cytokine production, IL-6 and TFG-β secretion in small intestinal mucosa was measured by ELISA. As a result, IL-6 secretion was increased while TFG-β secretion has no obvious change after the two-week carnosine administration.

In our preliminary study, it was suggested that the increased IgA antibody production by dietary carnosine was canceled by administering antibiotics prior to feeding carnosine. This result was confirmed by administering the mixtures of four antibiotics for two weeks before feeding 0.5% carnosine-containing water along with the antibiotics for another two weeks. The small intestinal mucosa was collected, and total IgA antibody, IL-6 and TFG-β were measured by ELISA. As for cytokine secretion, the increased IL-6 secretion was also canceled by administering the antibiotics. These results suggest that enhancing effect of carnosine on IgA antibody production and IL-6 secretion in small intestinal mucosa requires the existence of microbiota.

Our preliminary study also suggested a possibility that carnosine acts as an adjuvant to promote influenza virus-specific IgA antibody production at infectious site locally. Based on this, I hypothesized that dietary carnosine increases antigen-specific IgA antibody production in a T-cell dependent way in the small intestinal mucosa. To investigate this, 4% ovalbumin (OVA)-containing water was administered for 5-7 days in the presence or absence of 0.5% carnosine to DO11.10 mice transgenic for the T-cell receptor genes that recognize the OVA-derived peptide specifically. As a result, OVA-specific IgA antibody production in OVA-fed DO11.10 mice has no

obvious difference between the carnosine-administered and the control groups in the small intestinal mucosa, suggesting the enhancing effect of carnosine on IgA antibody production is not likely to function in a T-cell dependent way.

Finally, it was examined whether dietary carnosine affect the cellular composition in intestinal immune tissues. B cells and T cells were collected from mLN and PP in BALB/c mice fed 0.5% carnosine-containing water for two weeks. Cells were analyzed by flow cytometry. The whole cell number of mLN was found to be increased significantly while there was no obvious change in the cells of PP. In addition, the percentage of the cells expressing B220 molecules, often used as a B-cell marker, was increased significantly in both PP and mLN. Moreover, the total cell number of $B220^+$ B cells and IgA plasma cells were also increased in mLN while total $CD8^+$ T cells were increased in PP. These results suggest the possible source for increased IgA-producing cells may come from mLN.

Chapter 2. Anti-inflammatory effect of dietary IDP on healthy elderly people

Age-associated diseases, especially cognitive dysfunction, are increasing globally. Although the mechanism of age-associated cognitive decline is still unclear, an age-related increase of chronic inflammation is likely to be involved. Recent evidence has implicated chemokines in many neurobiological processes potentially relevant to psychiatric disorders. Some studies reported the anti-oxidant effect of carnosine and anserine in the brain. In addition, our previous study reported that the inhibitory effect of carnosine on proinflammatory cytokine IL-8 production in H_2O_2/TNF-α-stimulated human intestinal epithelial Caco-2 cells. Therefore, I hypothesized that dietary IDP intake may play an inhibitory role in the inflammatory state. Also, new insights from preclinical and clinical research may give rise to novel biomarkers for age-related cognitive decline.

In Experiment 1, a total of 60 healthy people aged 40 or more (mean 63.37 years) participated. Thirty-nine of them aged 60 or more were picked up and divided into the active group and the placebo group. The test formula was a powder containing anserine and carnosine (3:1) derived from chicken meat. The active group was requested to take a twice-daily dose of the IDP formula (500 mg/dose), and the placebo group received a formula contained an equivalent amount of essential amino acids as in the test formula but no IDP. Along with a cognitive tests, 28 serum cytokines from blood samples were measured over a three-month-period test. The concentration of all cytokines in the participants' sera were measured by a Luminex-based multiplex beads array assay, except for CXCL12 with ELISA. First, the blood cytokines at the baseline were found to be divided into several groups with correlation of the serum levels with each other. After the three-month intake, five proinflammatory cytokines (IL-8, IL-5, CCL2, CCL4, and G-CSF) were significantly decreased only in the active group, while the placebo group showed no obvious difference in cytokines. As we expected, cognitive function was also improved in the active group at the end of the test, suggesting dietary IDP intake decreased proinflammatory cytokines to improve cognitive function in healthy elderly people.

Next, I performed another stratified analysis where participants within the active group were divided into two groups according to a score gain during the test for Wechsler Memory Scale-Logical Memory (WMS-LM), an evaluation of cognitive memory for delayed memory index, which was obtained by collaborators. Participants whose score gain over zero termed "Responder", and the others termed "Nonresponder". As a result, the decrease of a kind of chemokine at the follow-up compared to the baseline in the Responder group was significantly greater

than that in the Nonresponder group. Moreover, three cytokines were significantly decreased in Responders at the follow-up, while no obvious difference was observed in Nonresponders.

In Experiment 2, a total of 88 healthy elderly people aged 60 or more were participated in the 12-month period test. Forty-five cytokines in serum were measured with the Luminex-based multiplex beads array assay. The formula containing IDP and the placebo were the same as Experiment 1. At the baseline, a positive correlation was found between the age and the amounts of CCL27, CXCL9, IL-8, and TNF-α. In addition, the serum levels of some of the cytokines were found to have a positive correlation with each other.

Next, the change in cytokine levels between the baseline and the follow-up at 12 months in all participants was analyzed. As a result, six cytokines were decreased significantly only in the active group individually, whereas other two cytokines were increased. To investigate whether there is relevance between the change in cytokine levels and the improved cognitive function, the data of participants aged 65 or more (mean = 71.09 years) were analyzed, since it was found by the collaborators that the WMS-LM score gain of participants aged 65 or more at the 12-month follow-up was significantly higher in the active group compared to the placebo group. As a result, I found seven cytokines were decreased, while another cytokine was increased significantly in the active group individually, suggesting the possibility that these changes in cytokine levels correlate with the improved cognitive function. In another stratified analysis with “Responders” and “Nonresponder” within the whole active group participants, two chemokines were decreased significantly in Responder individually.

Collectively, it was found that the serum level of a chemokine was decreased after dietary IDP intake in both Exp.1 and Exp.2. Also, the levels of two chemokines were found to be decreased only in the Responder group in both experiments. These results suggest that dietary IDP intake caused the decrease in the serum levels of some proinflammatory cytokines, which may result in the improvement or preservation of cognitive function in elderly people.

Conclusions

Previous studies of carnosine has been mainly focused on its biochemical characteristics such as anti-oxidative function, little was studied on the aspect of immunity. In this study, I found that dietary carnosine enhanced IgA antibody production to activate immune response which is likely to occur in a T-cell independent way in a non-inflammatory state of mice. It is possible that the increased B cells in mLN may result in increasing IgA-producing cells. On the other hand, I found dietary IDP may act as an anti-inflammatory agent. Dietary IDP down-regulated some kinds of proinflammatory cytokines, which may result in the improved cognitive function in elderly people who have a chronic inflammation. These cytokines may be promising candidate biomarkers working as potential targets for therapeutic modulation to optimize learning and memory function or as diagnostic markers for cognitive status.