博士論文（要約）

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)

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Abbreviations

BrdU: 5-Bromo-2'-deoxyuridine;

HBSS: Hanks' balanced salt solution;

SF-36: Medical Outcomes Study, 36-item Short Form;

WMS-LM1: Wechsler Memory Scale-Revised Logical Memory immediate recall;

WMS-LM2: Wechsler Memory Scale-Revised Logical Memory delayed recall;

ADASog: Alzheimer's disease Assessment Scale;

BDI: Beck Depression Inventory;

IL: interleukin;

FDR: The false discovery rate method;

PCA: principal component analysis method;

General Introduction

1. General background

1.1 Carnosine and Imidazole dipeptide

Foods that we eating on a daily basis constitutes organism. And molecules that we ingested routinely such as carbohydrates, protein and lipids constitute our body. However, foods own various functions such as primary nutritional function, secondary function that gives the "taste". In addition, food also have the effect of biological regulation function which belongs to tertiary function. In recent years, attention has been focused on the tertiary function especially.

Carnosine, a naturally occurring dipeptide composed of β-alanine and L-histidine, discovered in 1900 [1], was reported to have the effect of biological regulation function. It is not only found in skeletal muscle [2] but also in other excitable tissues such as cardiac muscle and olfactory bulb nerve where owns 50 mM carnosine sometimes [3]. Chicken and pork are the most common food provided in our meal as containing relatively high concentration of carnosine. Carnosine and related compounds are not degraded by regular (di)peptidases, but their metabolisms is characterized by its own hydrolytic enzymes, named carnosinase (CN) [4]. CN is highly present in the serum of humans, but absent from no-primate mammals, except for the Syrian golden hamster[5]. Under no-pathological conditions, serum carnosinase activity is high in the adult human, leading to almost undetectable levels of circulating carnosine in the post-absorptive state[6]. In addition, carnosine can be synthesized by carnosine synthase (CS)[7] which is mainly present in skeletal and heart muscle and certain brain regions[8]. Carnosine can be transported across the cellular membrane through a number of transporters from the proton-coupled oligopeptide transporter family[9], PEPT1.

The most common variants are the methylated analogs, anserine and ophidine (balenine), in which the imidazole ring of L-histidine is methylated at, respectively, the nitrogen atom closest to the side chain (Npi) and away from the side chain (Ntau) (see figure 1). Carnosine, anserine, and ophidine will be collectively called the histidine-containing dipeptides (HCD). As a rule, almost all mammals have both carnosine along with just one of the methylated carnosine analogs (either anserine or ophidine). The only mammalian species known not to have any of the two methylated carnosine analogs is in fact the *Homo sapiens*. Another rare exception is that some animals express all three HCD, such as the pig and the buffalo, but in these cases the methylated forms are in far minority compared with carnosine.

Of the two methylated forms, anserine is more frequently observed than ophidine. Whereas anserine is found in nearly all mammalian orders, ophidine is only found in significant amounts in marine mammals (Cetacea) and in smaller amounts in some hoofed animals: the even-toed ungulates (Artiodactyla) and odd-toed ingulates (Perissodactyla)[10]. Thus, carnosine and anserine will be called imidazole dipeptides in this study.

1.2 Chemical and biochemical properties of carnosine

It has been shown that carnosine has a wide range of physiological and pharmacological properties including antioxidant activity[11, 12] disassembling aberrant proteins[13], potent inhibitor of advanced lipoxidation end-products (ALEs) formation[2]. Its chemical properties were thoroughly researched a century ago. The protective and antioxidant effect is then active in prevention and treatment of diseases in recent years. Some studies conformed beneficial effects of carnosine on diabetic deterioration due to hyperglycemia reduction, dyslipidemia normalization, and liver damage reduction in a dose dependent manner after carnosine treatment in streptozotocin diabetic-induced model[14, 15]. And the underlying mechanism in diabetic animal models was thought to be related to the regulation effect of carnosine on autonomic nervous system or β-cells within the pancreas. Moreover, in addition to the function that reduction of ischemia/reperfusion (I/R)[16, 17] and anti-aging[18, 19] , it was reported that carnosine is also effective in reducing diabetes-related diseases. Carnosine also reported to act as an adjuvant to modulate innate immune and improve adaptive immunity *in vivo [20]*.

1.3 Effect of carnosine on gut immunity

Since carnosine was taken up in gut intestinal, it is reasonable to consider there may be some effects of carnosine on gut functions. However, the complex of carnosine with metal iron, not the carnosine dipeptide itself, received more attention in the past. One of the metal iron complex of carnosine is zinc-carnosine. Zinc is a critical component to a number of physiological processes in our bodies. Some of these functions include growth and metabolism of cells, healing of wounds, and maintenance of carbohydrate and lipid metabolism. When zinc is complexed to L-carnosine, it dissociates in the stomach at a much slower rate. This prolonged presence in the stomach allows it to maintain its gastric healing effect over a longer period of time. Zinc/L-carnosine may also help

maintain the bacterial balance of the stomach and GI tract. Studies suggest that the zinc/L-carnosine compound may have effects on certain strains of harmful bacteria, such as h. pylori, and, therefore, is able to help maintain a GI environment that is favorable to health. By supporting the bacterial balance in the stomach, it can also help maintain a healthy mucosal lining. However, whether the effect is due to carnosine or zinc-carnosine is not clear. Besides, study for the carnosine on gut immunity is rare.

1.4 Gut immunity

Structure The intestine is a part between the stomach of the gastrointestinal tract and the anus, a tube with a total length of about 7 to 9 m starting from the duodenum and ending in the anus, and in humans it can be largely divided into small intestine and large intestine due to its structure and function. The small intestine is divided into the duodenum, jejunum and ileum. The large intestine is divided into the cecum, colon, and rectum. The small intestine is about 5-7 m in length, occupies most of the abdominal cavity, digestion and absorption of food is almost done here. The large intestine is larger than the small intestine with a diameter of about 1.5 m in length, water is absorbed here and solid waste is discharged.

Role of gut There are three main functions of the gut. One is the digestion and absorption function that food will decomposed and absorbed by secreted digestive juice for absorbing it more easily. The other one is a movement function that the gut will carry food and nutrients made by decomposing. The last one, also the important one is the barrier function that protect our body from foreign subjects due to constitution of tight junction from the continuous intercellular barrier between epithelial cells. Subjects that entered from our mouth, and toxins that needless to our body, are the most likely to accumulate in the gut. By the end of digestion and absorption, the residue of food is sent to the large intestine, between the ileocecal portion and the Cannon-Boheme point, by peristaltic movement toward the anal side of the intestinal wall and reverse peristalsis toward the mouth for a certain time. Water and part of the electrolyte will be absorbed during the time.

Immunity Food with many kinds of bacteria attaching to food that may pathogenic arrive and get a close contact with the gut. To maintain the homeostasis of gut environment, the intestine possess the largest immune system which is composed of intestinal epithelial cells, intestinal epithelial lymphocytes, immune cells (T cells, B cells, dendritic cells, macrophages, etc.) present in the mucosal lamina propria. Moreover, immune cells present in the gut-associated lymphatic tissue (GALT)

composed of Peyer's patches (PPs), mesenteric lymph nodes (mLNs), and isolated lymph nodes (iLNs), interact with each other to induce immune responses, which is responsible for biological defense by innate immunity and acquired immunity. Recently, attention is also paid to its innate immune response and immune regulation function.

1.5 Immunoglobulin A

Antibodies plays an important role in immune responses. They bind to pathogens and their products to excrete them outside the body. Antibody is a protein called immunoglobulin, and there are five isotypes of IgG, IgM, IgA, IgE, IgD due to the difference in structure such as the amino acid sequence of the H chain constant region. Biological function and affinity for antigen of an antibody are determined by combination of different constant site and a variable site which is an antigen recognition site, thus exhibiting different functions in various places. B cells are responsible for the production of antibody. Immature B cells differentiated from hematopoietic stem cells in bone marrow are matured under positive selection in the spleen and circulate round the body along with blood and lymph fluid as mature B cells. During this circulation, B cells initiate somatic hypermutation (SHM) and class switch recombination (CSR) to maturation and differentiation into plasma cells by encountering antigens. Point mutations are introduced into the gene of the variable part, thus B cells with highly affinity of antibodies to antigen are produced by SHM. Class switching happens via recombination of DNA which determines the constant region of antibody. IgM, IgD, IgG, IgE, IgA constant region genes are encoded sequentially from the genes upstream, and isotype is determined by the recombination. AID (activation induced cytidine deaminase) has been reported as an enzyme essential for the initiation of CSR and SHM It is believed that AID modifies mRNA of enzyme involved in DNA cleavage during CSR and SHM, but details are not disclosed. The class switched cells then mature and become plasma cells specialized for antibody production, and produce antibodies [21, 22]. Although IgA antibodies occupy small constitutional ratio in the blood, they play an important role in defense against infection of the intestinal tract immune system. IgA antibody is mainly produced by IgA antibody plasma cells in the lamina propria of mucosa, and secreted through polymeric immunoglobulin receptor (pIgR) which is expressed in intestinal epithelial cells. IgA antibody was also reported to maintain immune homeostasis by prevention of invasion of pathogens through the mucosa and promotion of pathogens elimination, neutralization of bacterial toxins and viruses, regulation of intestinal bacteria [23-25]. IgA antibodies

are also produced as pathogen-specific antibodies as induced upon infection but most of them are produced in normal conditions continuously. While being exposed to enormous kinds of foreign matter, the diversity of antibodies produced is limited, and with low specificity correspond to a wide range of antigens[26]. It was reported that Peyer's patches and isolated lymphoid follicles (ILFs) play important roles in IgA antibody production in the intestinal tract[27]. In the epithelial layer covering the Peyer's patch, M cells specialized for uptake of antigens from the luminal side. So antigens in the lumen are directly incorporated into Peyer's patches. Incorporated antigens are captured by dendritic cells specialized for antigen presentation. Activated T cells, B cells then induce different immune responses. Class switching occurs after activation of a mature B cell. Activated antigen-specific T and B cells proliferate and differentiate, enter the systemic blood circulation from the thoracic duct via the mesenteric lymph nodes, homing to the mucosal lamina propria. And, immune response such as antibody production is performed in the lamina propria. It has also been suggested that B cell class switching is induced in the T cell independent manner in the mucosal lamina propria[28]. It is also becoming clear that the production of IgA antibodies is regulated by interactions of various immune cell and different cytokines. IFN-γ, IL-2, IL-10, IL-21 produced by T cells and IL-5, IL-6, TNF-α, APRIL, BAFF, retinoic acid dendritic cells act on B cells to promote IgA antibody production [29-34]. To date, it has been showed that Clostridia can promote IgA producing cells in the small intestine and $CD8^+$ αβ T cell receptor intraepithelial lymphocytes (IEL) in large intestine [35].

1.6 Gut inflammation and human disease

Cytokine Inflammation is caused by immune reactions. When a cell is damaged for some reason, it has a mechanism to eliminate the cause of the injury and to remove the debris of the cell that died due to the injury. Such a process is called "inflammation". Cytokines, a protein secreted from the cells of the immune system to transmit information, play an important role in this process. For example, pro-inflammatory cytokines stimulate macrophages, strengthen their functions, or cause cell death by acting on abnormal cells. What matters here is that "cytokines are those that promote and suppress inflammation". On the other hand, multiple cytokines, including IL-4, TGF-β, IL-5, IL-6, and IL-10 are instrumental in intestinal stimulating S-IgA production. A subset of these cytokines, notably TGF-β and IL-10, are also required for maintaining mucosal tolerance, thus establishing one of the many links between S-IgA production, immunity and intestinal homeostasis [36, 37]. In other words, if cytokine

secretion is abnormal or cytokine does not work properly, abnormal immune reaction is caused. And in inflammatory bowel disease, inflammation has been chronically followed in the gastrointestinal tract where there is no need for inflammation if it is normal.

Diseases Enormous immune cells gathered in the mucous membranes of the intestine has a big influence on the immune function, which may cause allergies, enteritis and colon cancer. Intestinal inflammation causes various symptoms. Inflammatory bowel disease is a disease in which chronic inflammation continues in the gastrointestinal tract. An ulcer (the mucosa inflamed and peeling off) occurs in the gastrointestinal tract, and symptoms such as abdominal pain, diarrhea, and bloody stools appear. Crohn's disease and ulcerative colitis, are corresponded to this inflammatory bowel disease. The cause of the onset of these diseases has not been elucidated, and no cures have been established. Therefore, it is designated as a specific disease from the Ministry of Health, Labor and Welfare in Japan. Infectious enteritis is a disease in which pathogens such as bacteria, viruses, and parasites infect the intestines causing various gastrointestinal symptoms. Pathogens enter the body orally through food and drinking water in many cases, but some infections are also from pets and humans. Infections mediated by food and drinking water are often found in outbreaks, which is called food poisoning. The causative bacteria invade the body and cause inflammation in the intestines, so that symptoms accompanied by unpleasant symptoms and pain such as vomiting, diarrhea, fever and nausea appear. As for bacteria, *Salmonella, Campylobacter, Vibrio parahaemolyticus, pathogenic Escherichia coli etc*. are well known as pathogen. As for virus, norovirus in adults, rotavirus in children are well known. As for the parasites, *dysentery ameba*, *giardia lamblia caterpillar* are well known. *Vibrio parahaemolyticus* (in Fishes and shellfishes) and *enterohemorrhagic E. coli* (in beef and undiluted milk) are the main sources of infection. Rout of infection is known as oral infection via food and water, vomiting, contact infection via feces (secondary infection). Latency: *Norovirus* (1 to 3 days), *rotavirus* (1 to 3 days), *Campylobacter* (2 to 11 days), pathogenic *E. coli* (12 to 72 hours), *Salmonella* (12 to 36 hours). *Norovirus* has been said to be the most frequent cause of food poisoning in winter in recent years due to infection from raw oysters. Recently, human - human secondary infection from feces and vomit has become a problem. If intestinal inflammation is suspected, it is also important to try not to become a source of infection. In addition, symptoms of diarrhea often appear due to the occurrence of inflammation in the intestines, but the main reason is that the actions of digestion, absorption and discharge are slow due to inflammation of the intestines. "Bowel aging" is considered as one of the

reason, termed immunosencence. This decline with age affects both innate and adaptive immunity and decreased ability to generate tolerance to harmless antigens, which coincides with age-related increases in the incidence and severity of gastrointestinal infections, tumors and inflammatory diseases.

1.7 Gut-brain axes

The idea that the state of the gut affects the mental state is never new, it has been said for over 100 years. In the early 19th to the early 20th century it was thought that intestinal waste products and toxins cause poisoning and cause depression, anxiety, psychosis (so-called autologous poisoning), administration of laxatives and abdominal surgery were performed. However, the association between gut and psychiatric disorders was refuted subsequently, gradually replaced by Freud's psycho-neurosis theory, etc. Very recently, as the work and importance of intestinal bacteria become clear, the relationship between the intestines and the mind has been drawing attention again in the field of psychiatry. Currently, the correlation between intestinal bacteria and intestine and brain is termed "intestinal bacteria - intestinal - brain - related axis". The gastrointestinal tract is densely innervated by noradrenergic and dopaminergic nerves, and their fibers are found in the gut mucosa, constituting part of the neuro-enteric system [38]. The intestine and intestinal bacteria are clearly closely related, the state of the intestines directly affects the intestinal bacteria, and conversely, the state of the intestinal bacteria determines the state of the intestines. Study was reported that commensal clostridia, enriched in β-glucuronidase activity, could generate free catecholamines in gut lumen to regulate homeostasis and behavior [39]. As for the relationship between intestine and brain, constipation, abdominal pain, and diarrhea are stress to brain (mental state), conversely, anxiety, impatience, and pressure causes gastrointestinal symptoms. Intestinal bacteria and brain are also involved, the state of intestinal bacteria is involved in tranquilization such as serotonin production in the brain, and intestinal bacteria produce hormones (anti-stress hormone) when stress is applied to regulate and suppresses stress. Conversely, stress in the brain stimulates sympathetic nerves, affecting digestive tract function and intestinal bacteria adversely. It was pointed out anxiety disorders, depressive symptoms, autism are reported in humans by various diseases of the intestine (inflammatory bowel disease (ulcerative colitis, Crohn's disease), irritable bowel syndrome, leaky gut syndrome, etc.).

2. Group background

2.1 Previous study

In our laboratory, previous study showed that carnosine inhibit IL-8 secretion in H_2O_2-stimulated human-derived epithelial cells (Caco-2 cells). And carnosine promoted IL-8 and IL-6 secretion in TLR-stimulated mice-derived small intestinal epithelial cells (Mos13 cells). In addition, carnosine enhanced IgA production and IgA-producing plasma cell in mice lamina propria, which is required for the presence of gut microbiota. Moreover, carnosine also showed the adjuvant ability with virus in nose. Furthermore, it was showed that the histidine structure plays an important role in regulation of immune response of carnosine. Thus, it has been suggested that carnosine has a regulatory effect of immune response on intestinal epithelial cells and enhances the immune response in the mucosa. [40, 41][42]

2.2 The purpose of this study

Previous studies in our laboratory have suggested that carnosine regulates the immune response of intestinal epithelial cells and enhances the intestinal mucosal immune response, particularly enhances IgA antibody production [42]. Increased lifestyle diseases and deterioration of health condition in young people are pointed out as a problem that modern society suffers. Improvement of dietary life to prevent diseases has been paid more attentions. On the other hand, elderly suffered from potential cognitive decline due to age-related inflammation has also been a concern of our society along with the increasing elderly population. Social demands for food ingredients that possesses biological functions for maintaining and promoting health have been stronger. Therefore, in present study, we aimed to investigate the mechanism of the enhancing effect of carnosine on intestinal mucosal immune response *in vivo* and *in vitro* in detail. And, we also trying to investigate the inhibitory effect of carnosine on elderly people.

In chapter 1, we investigated the enhancing effect of carnosine on the immune response of mouse mucosa from the sight of IgA antibody production and lymphocyte change. Production ability of prevalent IgA and antigen specific IgA antibody, as well as cytokine secretion were studied, in addition to the absence of gut microbiota state. B cells and T cells, as well as composition of them in detail, were studied in mesenteric lymph node and Peyer's patches.

In chapter 2, we trying to figure out effect of imidazole dipeptide in elderly people aged over 60 whose inflammatory response are correlated with age. Two experiment was studied, including 3-month-period (termed experiment Ⅰ) and 12-month-period (termed experiment Ⅱ). Cognitive

function was evaluated according to WMS (Wechsler Memory Scale-Revised Logical Memory delayed recall). We aimed to figure out whether cognitive improvement is correlated to variation of cytokine.

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