Enhancement of Antimicrobial Peptide Induction in Both Oral and Intestinal Mucosa by Fermented Daily Products

Kazumune Arikawa,¹ Ryoki Kobayashi,² Ryoko Kurisu,³ Chieko Taguchi,¹ Toshikazu Uchiyama,⁴ Keiko Kamachi,⁵ Tomoko Ochiai,² and Ikuo Nasu¹

Departments of ¹Preventive and Public Oral Health, ²Oral Immunology, ³Undergraduate school student, ⁴Renascent Dentistry, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan

⁵Kagawa Nutrition University, Sakado, Saitama, 350-0288, Japan

Article History

Received 21 January 2014

Accepted 13 February 2014

Keywords :

fermented food,

Natto, antimicrobial peptide,

cathelici

Abstract

Antimicrobial peptides (AMPs), including defensins and cathelicidins, are widely distributed in cells and tissues as the host defense mechanism to prevent microbial invasion. Various barriers and secretory epithelial cells produce AMPs either constitutively or inducibly. It is widely accepted that defensins and cathelicidins are expressed in various mucosal tissues within highly regulated processes, which pathogenic and commensal agents might affect in a different way.

In this study, we examined the production of AMPs in both genetic and protein levels in mice, which were orally administered with suspended natto, a traditional Japanese fermented soybean product. A significant induction of the murine cathelicidin (cathelin-related antimicrobial peptide; CRAMP)-specific mRNA was detected in not only the small intestine but also oral mucosa in mice fed a natto suspension. In addition, the expression of CRAMP in plasma was also confirmed by ELISA. Our result suggests that, when mice were administered natto, the fermentation product might have activated the oral immune system through the intestines, resulting in the production of AMPs in oral mucosa.

Introduction

In 2013, UNESCO placed “washoku,” or traditional Japanese cuisine, on their Intangible Cultural Heritage of Humanity list. Over the past 50 years, the percentage of elderly people in Japan has increase four-fold from 5.7% in 1960 to 23.1% in 2010. Japan will face a super-aged society, in which 40% of the population will be over 65 years of age(1). Likewise, a marked expansion of home-based care and/or local-care enhances the difficulty of maintaining healthy lives for elderly people. Furthermore, the long lifespan of the Japanese has been attributed in part to traditional Japanese food(2). These suggestions prompted the passage of the Shokuiku (food and nutrition) Basic Law in 2005(3), which promotes well-balanced meals based on traditional Japanese cuisine, especially based on the fermented food known as “natto.”

Probiotics, according to the definition of FAO and WHO, are live microorganisms that, when administered in adequate amounts, confer health benefits to the host(4). Orally administered probiotics exhibit widespread effects on gut homeostasis and immunomodulation of both mucosal and systemic immunity. Probiotics may counter aggressive enteric commensals in the gut and reinforce the barrier function of the epithelium and can also contribute to the regulation of innate and adaptive immune responses of the host under healthy or pathogenic conditions(5). These were originally used for the production of natto, a traditional Japanese staple made from fermented soybean. Natto has multiple probiotic functions, such as proliferation of natural killer cell (NK cell) activity(6), and its bacterial component, *Bacillus subtilis* var. *natto*, can stimulate lymphocyte proliferation(7, 8).

Antimicrobial peptides (AMPs) in the gastrointestinal tract function within a specialized microenvironment that includes the mucous layer overlying the surface epithelium.

Correspondence to :

Kazumune Arikawa

E-mail : arikawa.kazumune@nihon-u.ac.jp

The defensins, a type of AMP, are produced by epithelial cells on the mucosal surface and by neutrophils (9, 10). Cathelicidins comprise another group of mammalian antimicrobial proteins (11–13), one of them being the 19 kDa cationic antibacterial protein (hCAP18), in which C-terminal 37 amino acid peptide is termed LL-37. The LL-37 has been shown to act as an antibacterial against Gram-positive and Gram-negative bacteria (9, 14). Although probiotics have been suggested to enhance natural and acquired immunity, it is not clear whether their effects are associated with murine cathelicidin (cathelin-related antimicrobial peptide; CRAMP) expression. In the host defense mechanism, little is known about the role of AMPs produced by mucosal epithelia, including gastric and oral systems. To define the involvement of dietary foods in the host defense system, we examined the effect of the traditional Japanese fermented food, “natto,” on the expression of AMPs in the oral mucosa of mice.

Materials and Methods

Mice

Six-to-eight-week-old female BALB/c mice were purchased from Sankyo Laboratory Service (Tokyo, Japan) and maintained under pathogen-free conditions in the experimental facility of Nihon University School of Dentistry at Matsudo. These mice received sterile food and water. All animals were maintained and used in accordance with the Guidelines for the Care and Use of Laboratory Animals of Nihon University, School of Dentistry at Matsudo (AP11MD012).

Oral administration of fermented foods

Mice were orally administered suspended fermented soybeans (natto) twice a day for 7 consecutive days by oral gavage with 200 μ l of PBS.

Sample collection and detection of CRAMP-specific mRNA

To determine the CRAMP-specific mRNA transcripts, mucosal components were isolated as follows. One day after the final administration, the submandibular gland (SMG), small intestine, and gum were collected and total mRNAs were purified from several tissues using a TRIzol reagent (Invitrogen, Tokyo, Japan) in accordance with the manufacturer's protocol. Superscript II reverse transcript (Invitrogen, Carlsbad, CA) was used to generate cDNA for purified total RNA obtained from each mouse. Quantitative

real-time RT-PCR analyses were performed using a Thermal Cycler Dice real-time PCR system (Takara, Shiga, Japan), with two sets of primers; CRAMP; 5'-GCCGCTGATTCTTTTGACAT-3' and 5'-ATTCTTCTCCCCACCTTTGC-3' GAPDH; 5'-TGTGTCCGTCGTGGATCTGA-3' and 5'-TTGCTGTTGAAGTCGCAGGAG-3' (Castaneda-Delgado 2010). Target RNA levels were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA.

To examine the expression of CRAMP in plasma, ELISA was performed utilizing the mouse Cramp ELISA kit (Cat. CSB-E15061m: CUSABIO BIOTECH, Wuhan, P.R.China). Briefly, plasma of mice was collected one day after the last administration of natto. All samples were assayed in duplicate under the manufacturer's instruction.

Statistical analysis

The statistical significance of differences between groups was determined by the unpaired Student t test. Values of $p < 0.05$ were considered to be significant.

Results

Mouse body weight

Body weights of mice administered natto showed no significant differences within the experimental period when compared to the PBS-administered group (data not shown). This indicated that no differences in either stress levels or nutritive value were observed between the two.

Detection of CRAMP-specific mRNA in mucosal components

To determine the induction of CRAMP in mucosa of mice administered natto, RT-PCR was carried out to detect CRAMP-specific mRNA in mucosal components. Total RNAs were purified from the small intestine, SMG, and gum. As shown in Fig. 1, significant induction of CRAMP-specific mRNAs was detected in the small intestine. Conversely, levels of CRAMP-specific mRNA in the gum were markedly decreased. In addition, these transcripts were detected on SMG but were not significant when compared with the control group.

Identification of CRAMP protein in plasma

Finally, we conducted the ELISA experiment to confirm the presence of CRAMP protein in plasma. One day after the administration, plasma was collected and subjected to

Fig.1 Comparisons of the expression of the antimicrobial peptide-specific transcript in mucosal components of mice fed natto. Total RNAs were purified from several tissues. Target CRAMP-specific mRNA transcripts were quantitatively determined by real-time PCR. Results are the mean $\pm$ 95%CI; n=3 per group, * $P < 0.05$ compared with PBS-fed mice.

Fig.2 Protein levels of CRAMP were determined one day after the last administration by ELISA. The values shown are the mean $\pm$ 95%CI taken from three mice in each experimental group. * $p < 0.05$; when compared to mice administered with PBS.

ELISA. As shown in Fig. 2, an elevated level of protein expression was confirmed in mice fed the natto suspension.

Discussion

We examined the AMP expressions in mucosal components of mice, which were orally administered natto. Interestingly, our data suggested that a significant expression of CRAMP-specific mRNA was detected in both the small intestine and gums compared to the control group. These observations are the first results to show that daily consumption of natto could induce AMPs not only in the

small intestine but also in the gums via the gut and/or oral immune system.

Recently, it has been shown that AMPs have direct antimicrobial activity against various microorganisms, including bacteria, fungi, certain parasites, and viruses, and are considered to be important effector molecules of innate immune defense (10, 15). The cathelicidins, one of the major mammalian AMPs, contain a highly conserved signal sequence. The cathelin domain, however, shows substantial heterogeneity in the C-terminal domain, which is responsible for the structure in antibacterial activity (16). The CRAMP, which closely resembles the human LL-37 (17), is first synthesized as an inactive prepro-protein (16), and then processed to form an active mature peptide to kill bacteria (18). Cathelicidins possess a broad antibacterial spectrum ranging from Gram-positive to Gram-negative bacteria, as well as from fungi to envelope viruses (9, 18-20). Our data indicated that ingestion of natto might induce AMPs not only in the intestines but also in the oral mucosa of mice.

According to a public announcement, the lifespan in Japan is the highest in the world. In 2008, US health expenditures could account for 16% of the nation's gross domestic product (GDP), twice the Japanese rate. Compared with other countries, Japanese health expenditures accounted for two-thirds more of the GDP than those of France and Germany, suggesting that the Japanese have the most cost-

effective healthcare system. In addition, the annual cost of health care has been approximately 670,000 yen per elderly person for the past 10 years. However, the aging of the population is expected to have an impact on future spending growth (21). In 2005, programs associated with the Shokuiku Act (food and nutrition education that was promoted in the national project Healthy Japan21 by the Ministry of Health, Labour and Welfare) integrated nutrition education into the home/family, school, and local community (government, non-profit organizations, companies, etc.). This integration is believed to be essential to achieve significant behavioral changes on an individual and social level. Based on these issues, we employed the traditional Japanese food, natto, in order to examine its beneficial effects towards enhancement of the immune response in the gut and other mucosal compartments, including the oral cavity.

Fermentation is one of the major processes used in the production of food, and natto is created from growing *Bacillus subtilis* var. *natto*, on the surface of cooked soybean (22). Various types of fermented soybean foods are consumed in Asian countries, such as Japan, Korea, China, Indonesia, and Vietnam. Natto, the most common Japanese fermented soybean product, contains large amounts of vitamin K and is a traditional food in eastern Japan. Many reports have suggested that natto intake may help promote bone formation in menopausal women (23) as well as prevent postmenopausal bone loss (24), and that natto extracts have anti-thrombotic effects (25). Moreover, recently, our data have suggested that significant induction of the NK cell was detected in intestines with enhanced cytotoxicity (6). This suggests that ingestion of natto might improve health conditions. Notably, our data indicated that daily consumption of natto was effective for oral health. As described above, an increase in a highly aged society with higher medical expenses has been predicted worldwide. Taking probiotics might be a key to the prevention of diseases and to the maintenance of the quality of life. Therefore, we would like to propose natto as an effective Japan-derived probiotic.

References

1. Arai, H, Y. Ouchi M, Yokode H, Ito H, Uematsu F, Eto S, Oshima K, Ota Y, Saito H, Sasaki K, Tsubota H, Fukuyama Y, Honda A, Iguchi K, Toba T, Hosoi T, Kita M. o. S. f. Aging: Toward the realization of a better aged society: messages from gerontology and geriatrics. *Geriatr Gerontol Int*, 12: 16-22, 2012.
2. Cockerham WC, Hattori H, Yamori Y: The social gradient in life expectancy: the contrary case of Okinawa in Japan. *Soc Sci Med*, 51: 115-122, 2000.
3. Annual Health, Labour and Welfare Report 2007-2008. In Part 2.
4. Reid G, Sanders ME, Gaskins HR, Gibson GR, Mercenier A, Rastall R, Roberfroid M, Rowland I, Cherbut C, Klaenhammer TR: New scientific paradigms for probiotics and prebiotics. *J Clin Gastroenterol*, 37: 105-118, 2003.
5. Boirivant, M, W Strober: The mechanism of action of probiotics. *Curr Opin Gastroenterol*, 23: 679-692, 2007.
6. Kobayashi R, Arikawa K, Ichikawa K, Taguchi C, Utsunomiya T, Iijima M, Uchiyama T, Kamachi K, Nasu I, Fukumoto M, Kawai Y, Ochiai T: Traditional Japanese Fermented Food *Natto* Enhances NK Cell Activity in Intestine. *Int J Oral-Med Sci* 12: 90-94, 2013.
7. Hosoi T, Ametani A, Kiuchi K, Kaminogawa S: Improved growth and viability of lactobacilli in the presence of *Bacillus subtilis* (natto), catalase, or subtilisin. *Can J Microbiol*, 46: 892-897, 2000.
8. Hosoi T, Ametani A, Kiuchi K, Kaminogawa S: Changes in fecal microflora induced by intubation of mice with *Bacillus subtilis* (natto) spores are dependent upon dietary components. *Can J Microbiol*, 45: 59-66, 1999.
9. Bals R, Wang X, Zasloff M, Wilson J M: The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. *Proc Natl Acad Sci U S A*, 95: 9541-9546, 1998.
10. Boman HG: Antibacterial peptides: basic facts and emerging concepts. *J Intern Med*, 254: 197-215, 2003.
11. Zaiou M, Gallo RL: Cathelicidins, essential gene-encoded mammalian antibiotics. *J Mol Med (Berl)*, 80: 549-561, 2002.
12. Lehrer RI, Ganz T: Cathelicidins: a family of endogenous antimicrobial peptides. *Curr Opin Hematol*, 9: 18-22, 2002.
13. Gennaro R, Zanetti M: Structural features and biological activities of the cathelicidin-derived antimicrobial peptides. *Biopolymers*, 55: 31-49, 2000.
14. Travis SM, Anderson NN, Forsyth WR, Espiritu C, Conway BD, Greenberg EP, McCray PB, Lehrer RI, Welsh MJ, Tack BF: Bactericidal activity of mammalian cathelicidin-derived peptides. *Infect Immun*, 68: 2748-2755, 2000.
15. Ganz T, Lehrer RI: Antimicrobial peptides of leukocytes. *Curr Opin Hematol*, 4: 53-58, 1997.
16. Zanetti M, Gennaro R, Romeo D: Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. *FEBS Lett*, 374: 1-5, 1995.
17. Gallo RL, Kim KJ, Bernfield M, Kozak CA, Zanetti M, Merluzzi L, Gennaro R: Identification of CRAMP, a cathelin-related antimicrobial peptide expressed in the embryonic and adult mouse. *J Biol Chem*, 272: 13088-13093, 1997.

18. Dorschner, RA, Pestonjamas VK, Tamakuwala S, Ohtake T, Rudisill J, Nizet V, Agerberth B, Gudmundsson GH, Gallo RL: Cutaneous injury induces the release of cathelicidin antimicrobial peptides active against group A Streptococcus. *J Invest Dermatol*, 117: 91-97, 2001.
19. Johansson J, Gudmundsson GH, Rottenberg ME, Berndt KD, Agerberth B: Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. *J Biol Chem*, 273: 3718-3724, 1998.
20. Turner J, Cho Y, Dinh NN, Waring AJ, Lehrer RI: Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils. *Antimicrob Agents Chemother*, 42: 2206-2214, 1998.
21. Arai F, Miyamoto T, Ohneda O, Inada T, Sudo T, Brasel K, Miyata T, Anderson D M, Suda T: Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor kappaB (RANK) receptors. *J Exp Med*, 190: 1741-1754, 1999.
22. Ikeda H, Doi Y: A vitamin-K2-binding factor secreted from *Bacillus subtilis*. *Eur J Biochem*, 192: 219-224, 1990.
23. Katsuyama H, Ideguchi S, Fukunaga M, Fukunaga T, Saijoh K, Sunami S: Promotion of bone formation by fermented soybean (Natto) intake in premenopausal women. *J Nutr Sci Vitaminol (Tokyo)*, 50: 114-120, 2004.
24. Ikeda Y, Iki M, Morita A, Kajita E, Kagamimori S, Kagawa Y, H Yoneshima: Intake of fermented soybeans, natto, is associated with reduced bone loss in postmenopausal women: Japanese Population-Based Osteoporosis (JPOS) Study. *J Nutr*, 136: 1323-1328, 2006.
25. Omura K, Hitosugi M, Kaketani K, Zhu X, Maeda H, Tokudome S: Fibrinolytic and anti-thrombotic effect of NKCP, the protein layer from *Bacillus subtilis* (natto). *Biofactors*, 22: 185-187, 2004.