Prebiotic treatment reduced preneoplastic lesions through the downregulation of toll like receptor 4 in a chemo-induced carcinogenic model

Masanobu Fukuda,1,4 Yutaka Komiyama,2,8 Keiichi Mitsuyama,3 Akira Andoh,4 Takahiko Aoyama,1 Yoshiaki Matsumoto1 and Osamu Kanauchi1,2,4,*

1College of Pharmacy, Department of Clinical Pharmacokinetics, Niho University, 7-7-1, Narashinodai, Funabashi, Chiba 274-8555, Japan
2Central Laboratories for Frontier Technology, Kirin Holdings Co., Ltd. 1-13-5, Fukura Kanzawa-ku, Yokohama, Kanagawa 236-0004, Japan
3Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan
4Division of Mucosal Immunology, Graduate School of Medicine, Shiga University of Medical Science, Tsukinowa, Seto, Otsu, Shiga 520-2192, Japan

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Germinated barley foodstuff contains prebiotics which are reported to have anti-cancerous effects in colorectal cancer model, but the detailed mechanism remains unclear. Recent studies revealed that the role of microbiota was strongly related to the regulation of incidence and progression of colorectal cancer. The aim of this study was to examine the anti-neoplastic mechanism by prebiotics. Azoxymethane treated F344 rats were used as the sporadic cancerous model. After azoxymethane injection, either a control or germinated barley foodstuff diet was administered to the rats for another 5 weeks, and the number of abberant crypt foci, toll like receptor 4, Kirsten rat sarcoma viral oncogene homolog, adenosomatous polyposis coli tumor suppressor gene and cyclooxygenase 2 mRNA expression of colon mucosa and cercl short fatty acids were examined. The germinated barley foodstuff significantly attenuated the number of abberant crypt foci and the expression of toll like receptor 4 and cyclooxygenase 2 mRNA, compared to the control group. In addition, the cercl butyrate production in the germinated barley foodstuff group was significantly higher than that in the control. In conclusion, this prebiotic treatment for colorectal cancer may be useful without causing the adverse effects seen in either anti-cancer drugs or anti-inflammatory drugs.

Key Words: toll like receptor 4, cyclooxygenase 2, butyrate, prebiotics, microbiota

The colon is different from the other digestive organs, because it harbors an enormous number of coexisting microbiota. Dietary fiber has been shown to prevent colorectal cancer (CRC) by diluting or adsorbing fecal carcinogens (i.e. endogenous bile acid metabolite, food-derived carcinogen), thus reducing the colonic transit time, alternating the bile acids metabolism, increasing short chain fatty acids (SCFA) accompanying the change in microbiota, although this is also a controversial issue. Germinated barley foodstuff (GBF) is a heterogeneous mixture of insoluble protein and dietary fiber that functions as a prebiotic in the intestine, and is effective in patients with active ulcerative colitis. GBF has a potent effect and it increases the number of microbiotic metabolites, including SCFA and butyrate, which is an anti-inflammatory reagent and can also induce apoptosis in cancer cells. Because butyrate produced by microbiota following prebiotic treatment is stably maintained at a physiological concentration in the entire colon, this prebiotic treatment may therefore be a useful medical option for reducing the risk of CRC.

Aberrant crypt foci (ACF) are lesions consisting of a large and thick crypt in methylene blue stained colonic mucosa, which showed a loss of polarity, and the stratification of the nuclei in the crypt epithelium histologically. ACF have been reported to be precursors of adenoma and cancer, because patients with colon cancer have a higher number of ACF than control subjects. Azoxymethane (AOM) is commonly used to mimic human sporadic CRC in rodents, and pre-neoplastic lesions are detected as ACF. However, the detailed mechanism for the anti-carcinogenic effects of GBF still remains poorly understood.

Recent studies have revealed that toll like receptor 4 (TLR4) or one of the sodium-coupled monocarboxylate transporters (SLC5A8 also known as SMCT-1) was strongly related to the regulation of incidence and progression of CRC. Both parameters were related to the microbiota (TLR4) or its metabolites (SLC5A8), respectively. TLR4 has been identified as one of the regulators of the mucosal immune system to lipopolysaccharide (LPS), the pathogenic product of gram negative microbiota in the colon, and TLR4 were recently reported to show an increased expression in colon cancers, compared to the normal mucosa. Interestingly, the microbiota metabolite butyrate resulted in the downregulated TLR4 expression and IL-8 production in HT29 cells, and also stimulated the expression of SLC5A8, which induced anti-carcinogenic effects in the colon.

In the present study, we evaluated the anti-cancerous mechanism of prebiotic GBF on the sporadic AOM-induced CRC model, particularly in the modulation of the microbiota and the changes in its metabolites, to examine the role of TLR4, a key regulator of the mucosal barrier functions.

Materials and Methods

Chemical composition and physiological characteristics of GBF. GBF consists of the aleurone and scutellum fractions of germinated barley, and is made by milling and sieving the residue of brewers’ spent grain. The dietary fiber fraction of GBF is primarily composed of low-lignified hemicellulose, which accumulates in the scutellum and aleurone fractions, as the roots and shoots of barley are produced during germination. During germination, GBF exhibits a conspicuously higher water-holding capacity than other insoluble dietary fiber types. As described in our previous study, GBF contains approximately 46% protein and 30% dietary fiber by weight.

To whom correspondence should be addressed.
E-mail: kanauchio@kirin.co.jp
Contributed equally to this work.
Animals and treatments. All animal experiments were approved by the Kirin Holdings animal experiments ethical committee and the Nihon University animal experiments ethical committee. Rats were housed individually in cages in a room kept at 20 to 25°C with 40 to 70% relative humidity on a 12-h lighting cycle (conventional conditions). The animals had free access to both food and drinking water.

Sporadic colon cancer model induced by AOM. Twenty 5-week-old male Fischer 344 rats were purchased from Charles River Japan (Kanagawa, Japan). First, the 20 rats were fed laboratory chow for 1 week during the acclimatization period. We started the experiments, and the rats were divided into two groups (n = 10/group). One group received a control diet, and the other group received the GBF diet. The total volume of protein and dietary fiber in both diets was adjusted to 14.6% protein and 3.0% dietary fiber, respectively (Table 1). After two weeks of prefeeding using the respective experimental diets, the rats were given two subcutaneous injections of AOM at a dose of 15 mg/kg body weight once per week for two continuous weeks. After the second AOM injection, the rats were administered the respective diets for more 5 weeks ad libitum, and were euthanized thereafter. Changes in the body weight and food intake are shown in Fig. 1. On the final day, rats were anesthetized with pentobarbital (i.p.), and the serum, colon and cecal contents were collected.

Histological examination. The entire colon was obtained for the histological observation of ACF. The rectum segment approximately 5 cm from the pectinate line was fixed in 4% buffered paraformaldehyde (Wako Pure Chemicals Ind., Osaka, Japan) and stained with 0.2% methylene blue. In accordance with the methods carried out in a previous report, the ACF were scored under a light microscope (Carl Zeiss Axioskop40 and AxioVision, Japan) with an area of 1 cm² of the distal colon at 100× magnification. The number of aberrant crypt foci (ACF) were counted under a light microscope (Carl Zeiss Axioskop40 and AxioVision, Japan) with an area of 1 cm² of colon being quantified at 100× magnification. Aberrant crypts appeared larger and had a thicker epithelial lining compared to normal crypts, and were gathered into a focus consisting of several crypts. *Represents significant different between Cont and GBF groups (p<0.05).

Reverse transcription-polymerase chain reaction (RT-PCR) to examine the mucosal expression of mRNA. Residual colonic mucosal specimens were evaluated for the mucosal expression of TLR4, Kirsten rat sarcoma viral oncogene homolog (KRAS), adenomatous polyposis coli tumor suppressor gene (APC) and cyclooxygenase 2 (COX2) mRNA using reverse transcription-polymerase chain reaction (RT-PCR). \(^{19,20}\) according to the methods used in our previous study. \(^{21}\) Briefly, total mucosal RNA was isolated using the Trizol reagent (Invitrogen Japan, Tokyo). First-strand cDNA was synthesized using an oligo (dT) primer and Superscript II reverse transcriptase (GIBCO BRL, Rockville, MD) and PCR was performed thereafter (10 X Taq Buffer and Taq Gold polymerase, thermal cycler; GeneAmp 2400; Perkin Elmer Cetus Co., Norwalk, CT). The PCR products were subjected to electrophoresis on 1.5% agarose gels and were stained with ethidium bromide. Primers specific for TLR4, APC, KRAS, COX2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; as housekeeping gene) were constructed according to the open software program for designing PCR primers (http://frodo.wi.mit.edu/primer3/) (Shown in Table 2).

Organic acids analysis of cecal content. An organic acid analysis of the cecal contents in rats fed the respective diets was performed. Briefly, 0.2 g of the cecal contents were weighed, then 1.0 ml of Milli-Q water was added and the mixture was incubated at 4°C for 30 min. After centrifugation at 12,000 rpm at 4°C, for 10 min, the supernatant was obtained, and continuously filtered using a 0.22 μm filter. The organic acids were separated with the Shim-pack SPR-H 250L (Shimadzu Co. Ltd., Kyoto, Japan). The mobile phase was 4 mM of p-toluene sulfonic acid, and the detector was electric conductivity (Shimadzu CDD-6A, Kyoto, Japan). \(^{16,21}\) Statistical analysis. All values are presented as the mean ± SD. Comparisons of data were made using Student’s t test or the χ² test. Differences were considered to be statistically significant for p values of less than 0.05.

Table 1. Composition of the experimental diets

|          | Control | GBF*  |
|----------|---------|-------|
|          | (g/kg diet) |       |
| Casein   | 146     | 100   |
| Vitamin Mix** | 10   | 10    |
| Mineral Mix** | 35   | 35    |
| Choline chloride | 2    | 2     |
| Cellulose | 30     | 0     |
| GBF      | 0       | 100   |
| Corn oil | 50      | 50    |
| Corn starch | 727  | 703   |

*Germinated Barley Foodstuff (GBF) (Protein; 46.3%, Dietary fiber; 34.0%). ** According to AIN 93G formula.
The expression of TLR4 mRNA was not significantly different between the groups. The APC gene is considered to be a gatekeeper gene which maintains the stability of the colonic epithelium. It was also reported that carcinogenic treatment in rats resulted in decreased APC expression.\(^{(25)}\) APC mutations were present at a relatively low level, and APC mutations did not contribute to the initiation of ACF.\(^{(24)}\) Although not statistically significant, the expression of APC mRNA in the GBF group tended to be higher than in the control group.

**Discussion**

It has previously been reported that sporadic colorectal carcinoma has an adenoma-carcinoma sequence, in which genomic instability and the loss of key tumor suppressor genes such as APC result in the loss of p53 gene function, thereby driving the adenoma to a carcinoma.\(^{(25)}\) ACF have been detected in the colonic mucosa in a rodent model as well as in human colon cancers, and ACF, particularly large and dysplastic appearance foci, were reported to be useful and identifiable precursors of adenoma and carcinoma.\(^{(8)}\) The number of ACF was significantly inhibited in the GBF group compared to the control group. Prebiotic GBF had anti-cancer effects in the present rodent model. In addition, patients with colon cancer were found to have a greater number of ACF than patients with noncancerous lesions, which was accompanied by a high rate of KRAS mutations.\(^{(8, 26)}\) However, we found that the change in KRAS expression, instead of KRAS mutations, was less important in the present study, and we were unable to detect any differences in KRAS expression between the two groups.

The expression of KRAS mRNA was not significantly different between the groups. The APC gene is considered to be one of the key component of the β-catenin signaling pathway, which plays an important role
in colon carcinogenesis. APC mutations are reported to be responsible for the accumulation of β-catenin in the nucleus, and the constitutive activating transcription of T-cell factor or lymphoid enhancer factor. Therefore, APC mutations activated the oncogene β-catenin signaling pathway. However, APC mutations have been reported to be present at relatively low levels, and the APC mutations did not contribute to the development of ACF. GFB may therefore reinforce the role of the gatekeeper genes in the present experiment, because carcinogen-treated rats have been reported to cause a decrease in the APC mRNA expression. In the present study, the expression of APC gene was slightly higher in the GFB group than in the control group, although this trend did not reach statistical significance (p = 0.20). In the future, we will evaluate the detailed mutation profiles of the APC and KRAS genes in this colon cancer model to confirm the anti-carcinogenic mechanism of GFB treatment.

Interestingly, the level of COX2 mRNA of colonic mucosa in patients with colorectal cancer has been reported to be markedly elevated, compared with that of healthy controls and thus reducing the COX2 expression by green tea may contribute to the attenuation of colonic neoplastic lesions in rats. In our experiment, the colonic mucosal mRNA expression of COX2 was significantly lower in the prebiotic GFB group than in the control.

The present study suggested the modulation of microbiota by the prebiotic GFB, which was comprised of an increase in butyrate in the lower intestinal tract. Butyrate has been reported to have anti-neoplastic effects under physiological concentrations (2–5 mM) due to cell cycle regulation (increased apoptosis in colonic tumor cell lines). Interestingly, butyrate, a metabolite of microbiota induced by anaerobic fermentation in the lower intestine, has been shown to suppress TLR4 gene expression. The ligand of TLR4 is well-known to be LPS, and LPS induces the degradation of IκBα, which results in the transmigration of nuclear factor κB (NF-κB) to the nucleus, where it binds to DNA and induces the production of pro-inflammatory cytokines. Although the detailed mechanism remains unclear, LPS stimulated the induction of IL-8 in the HT-29 cell line, and butyrate has been reported to attenuate LPS-induced IL-8 production, and this anti-inflammatory action is considered to cause a downregulation of the TLR4 expression. In addition, TLR4 was upregulated in ulcerative colitis patients, and TLR4 was reported to detect the gram negative microbiota and to activate the innate immune system. Furthermore, a recent study revealed that TLR4 had the ability to regulate the COX2 expression in chronic colon cancer model via NF-κB expression. In the present study, GFB decreased the TLR4 expression through an increase in butyrate production and decrease of COX2 expression, compared with that of the control group. In our previous study, butyrate and short chain fatty acids also dramatically attenuated the proinflammatory cytokine IL-6 production as well as the TLR4 mRNA expression in the T84 cell line.

Increased butyrate in the present study may therefore have contributed to the increased tumor suppressor action, as described by other researchers. Coleman et al. reported the inverse correlation between butyrate concentration and the number of ACF to suggest a pivotal mechanism of protection against neoplasia. These actions have been reported and include the upregulation of carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1; increase of anoikis), the death receptor DR5 (increase of sensitivity to TNF-related apoptosis-inducing ligand) and Fas-mediated cytotoxicity, and the downregulation of COX2 and glutathione (increased resistance to oxidative stress). Anti-inflammatory agents, including NSAIDs, are also known to have anti-tumorigenic effects due to the inhibition of NFκB activation. In particular, butyrate has been reported to have anti-inflammatory effects and it has thus been used for the treatment of patients with chronic inflammatory bowel disease, including ulcerative colitis, as an enema solution. GFB has also been reported to have an attenuated NFκB activity in a colitis model, and such anti-inflammatory effects may therefore play a pivotal role in the progression of colon cancer. Prebiotics, and GFB in particular, may deliver the natural anti-inflammatory agent butyrate to the entire colon at physiological concentrations without any adverse effects. These may play an important role in the inhibition of CRC to control mild or undetectable inflammation, as well as to monitor the invasion of pathogenic microbiota by TLR signals in the colon.

Considering the above factors, the appropriate improvement of the microbiota in the AOM-induced sporadic colon cancer model by prebiotics may contribute to the attenuation of low-grade inflammation, and the reduced incidence of CRC. In conclusion, GFB demonstrated a preventive effect on tumorigenicity in the AOM-induced CRC model. Although more detailed GFB studies concerning the mechanism of the onset of CRC, particularly regarding the changes in the microbiota and its metabolites on mucosal barrier function, are still required, the nutraceutical preventative treatments of GFB for CRC may be useful without causing any of the adverse effects seen in treatments with either anti-cancer drugs or anti-inflammatory drugs.

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Abbreviations

ACF aberrant crypt foci
AOM azoxymethane
APC adenomatous polyposis coli tumor suppressor gene
COX2 cyclooxygenase 2
CRC colorectal cancer
GAPDH glyceraldehyde-3-phosphate dehydrogenase
GBF germinated barley foodstuff
KRAS Kirsten rat sarcoma viral oncogene homolog
LPS lipopolysaccharide
RT-PCR reverse transcription-polymerase chain reaction
SCFA short chain fatty acids
SLC5A8 or SMCT-1 sodium-coupled monocarboxylate transporters
TLR4 toll like receptor 4

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