Modulation of intestinal environment by prebiotic germinated barley foodstuff prevents chemo-induced colonic carcinogenesis in rats

OSAMU KANAUCHI[1], KEIICHI MITSUYAMA[2], AKIRA ANDOH[3] and TOSHIHIKO IWANAGA[4]

[1]Central Laboratories for Frontier Technology, Kirin Holdings Co. Ltd., 1-13-5 Fukuura, Kanazawa-ku, Yokohama 236-0004; [2]Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011; [3]Shiga University of Medical Science, Tsukinowa, Seta, Otsu, Shiga 520-2192; [4]Hokkaido University Graduate School of Medicine, Laboratory of Histology and Cytology, Kita 15, Nishi 7, Sapporo 060-8638, Japan

Abstract. Butyrate was shown to have a preventive effect on colon cancer *in vivo*. Germinated barley foodstuff (GBF) was in a prebiotic stage and had the potency to attenuate mucosal inflammation and to increase fecal butyrate production in colitis. This study aimed to determine whether the GBF treatment in a colon cancer model had the potency to suppress colon cancer. After a pre-feeding of either a control or a GBF diet for two weeks, male F344 rats received subcutaneous injections of azoxymethane twice, at a dose level of 15 mg/kg body weight. The injections were administered once a week for 2 weeks (n=10/group). Four weeks after that, the number of aberrant crypt foci (ACF) and heat shock protein (HSP) 25-positive cells in colonic mucosa were observed histologically. The mRNA level of slc5a8 was evaluated by *in situ* hybridization. Colonic mucosal ß-catenin was determined by Western blotting. Cecal short chain fatty acids, ß-glucosidase and ß-glucuronidase were also determined. The results showed that GBF treatment significantly decreased the number of ACF and ß-catenin formations in the colonic mucosa. GBF significantly increased the production of slc5a8, which is a tumor suppressor gene, as well as the cecal butyrate content and ß-glucosidase activity. ß-glucuronidase activity remained at the same level in GBF and control subjects. The number of HSP25-positive cells in GBF was higher than that in the control group, although it did not reach significant difference. In conclusion, GBF showed anti-tumorigenicity in the AOM rat model. Changes in the colonic environment featured through the increase of butyrate production were found. Although a more detailed study is required, this study showed the promising anti-neoplastic effects of prebiotic treatment.

Introduction

Microbiota are shown to have the potency to influence gastrointestinal disease including colorectal cancer and chronic inflammatory disorder (1-5). This means that the food, including probiotics and/or prebiotics can change the colonic microbiota and may prevent diseases such as colorectal cancer or inflammatory bowel disease (IBD) (2,3). The elaborative mechanism by which probiotics and/or prebiotics exert anti-tumorigenic effects remains unknown. However, it is postulated that modifying colonic pH, increases short chain fatty acid production, stimulating immune cells in mucosa (epithelium and lamina propria), and increasing competition for the absorption of nutrients (4).

Recently, modulating the intestinal microbiota has been attempted by the use of prebiotics or probiotics. Germinated barley foodstuff (GBF) is a heterogeneous mixture of insoluble protein and dietary fiber, which is known to function as a prebiotic in the intestine and is reported to be effective in colitis (6,7). These alterations may act beneficially, in part by causing a luminal induction of short chain fatty acids (SCFAs), which are important nutrients for the intestine, and induce an acidic environment (8). In our previous studies, GBF had a therapeutic effect on patients with active ulcerative colitis (UC) and prolonged the remission periods in inactive UC patients (6,7). It is reported that butyrate acts as an anti-tumor agent by inducing apoptosis *in vitro* (9,10). Although it is still controversial whether butyrate has a preventive effect on colon cancer *in vivo*, studies have shown that short chain fatty acids including butyrate have been effective. However, it was also reported that butyrate did not have the potency to produce cancer-preventing effects *in vivo* (11,12). Since butyrate produced by microbiota is kept in a stable physiological concentration in the entire colon, prebiotic treatment may be a useful medical option for reducing the risk of colon cancer in longstanding-colitis (13).

It was reported that the sodium/glucose co-transporter (slc5a8), a tumor suppressor gene, which codes for mono-

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Correspondence to: Dr Osamu Kanauchi, Central Laboratories for Frontier Technology, Kirin Holdings Co. Ltd., 1-13-5 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan
E-mail: kanauchio@kirin.co.jp

carboxylates including short chain fatty acids, lactate and pyruvate, was down-regulated in colon cancer (14,15). High levels of slc5a8 methylation were also observed in colon cancer. However, the coexistence of unmethylated slc5a8 in colon cancer cell lines had the potency to manifest an anti-colony forming ability (14). Changing the intestinal condition, e.g. short chain fatty acid content, may contribute to the suppression of colon cancer via slc5a8 (16).

In this study, we used the azoxymethane (AOM)-induced colonic carcinogenesis model, which is widely used and features the clinical and pathological characteristics found in human colon cancer, and which induces hyperproliferation in the premalignant phase (17-19). This study aimed to determine whether the GBF treatment in the colon cancer model had the potency to suppress and/or prevent experimental colon cancer as well as act as a modulator in the colonic environment.

Materials and methods

Composition of GBF and reagents. GBF mainly consists of the aleurone and scutellum fractions of germinated barley obtained by milling and sieving. The dietary fiber fraction of GBF contains primarily low-lignified hemicellulose rich fiber (35% by weight) and insoluble glutamine-rich protein (48% by weight). During germination, GBF exhibits a conspicuously high capacity for holding water (20).

The protein assay kit was purchased from Pierce (BCA™ protein assay kit; Rockford IL, USA). All dietary components were purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan). Experimental reagents were purchased from Sigma Aldrich Japan (Tokyo, Japan) and Wako Pure Chemicals (Osaka, Japan).

Animals and diets. Twenty 5-week-old male Fischer 344 rats were purchased from Charles River Japan (Kanagawa, Japan). The rats were housed individually in cages in a room kept at 20-25°C and at a level of 40-70% relative humidity with a 12-h lighting cycle (20). The rats were allowed free access to food and drinking water. The experiment was approved by the Kirin Holdings ethics committee for animal experimentation. The 20 rats were fed laboratory chow for 1 week during their acclimatization period. Then we started the experiments, and the rats were divided into two groups (n=10/group). One was a controlled diet group, and the other was the GBF diet group. The total volume of protein and dietary fiber in the two diets was adjusted to 14.6% protein and 3.0% dietary fiber, respectively (20,21). The composition of the two diets is shown in Table I.

Experiment 1. After two weeks of pre-feeding using the respective experimental diets, the rats were administered subcutaneous injections of AOM twice, at a dosage level of 15 mg/kg body weight once a week for two consecutive weeks (17). After the second AOM injection, the rats were given their respective diets for 4 more weeks *ad libitum* and were sacrificed. Changes in their body weight and food intake are shown in Fig. 1.

ACF analysis. The entire colon was obtained for histological observation including the counting of aberrant crypt foci (ACF). Mucosal manifestations of Slc5a8 were carried out. The rectum at ~3 cm from the pectinate line was fixed

Table I. Composition of respective diets.

	Control	GBF
	(g/kg diet)	
Casein	146.0	100.0
Vitamin mixture[a]	10.0	10.0
Mineral mixture[a]	35.0	35.0
Choline chloride	2.0	2.0
Cellulose	30.0	
GBF		100.0
Corn oil	50.0	50.0
Corn starch	727.0	703.0

[a]AIN 93G formula (20).

with 4% buffered formalin and stained with 0.2% methylene blue. According to the methods from the previous report, the ACF were scored under a light microscope (Axioskop40 and AxioVision, Japan) with an area of 2 cm² of colon being quantified at x100 magnification (17). ACF were distinguished from normal crypts by their increased size, more prominent epithelial cells and their increased pericryptal area.

Organic acid analysis of cecal content. The organic acid content of cecal content in rats fed with the respective diets was determined and modified according to Araki *et al* (22). Briefly, 0.2 g of cecal content was weighed, 1.0 ml of Milli-Q water was added and the content was then incubated at 4°C for 30 min. After being centrifuged at 12000 rpm, at 4°C, for 10 min, supernatant was obtained, and continuously filtered by using a 0.22 μm filter. The organic acids were separated with a Shim-pack SPR-H 250L (Shimadzu Co. Ltd., Kyoto, Japan) The mobile phase was 4 mM of p-Toluene sulfonic acid. The detector was electric conductivity (Shimadzu CDD-6A, Kyoto Japan).

Detection of colonic slc5a8 expression. According to a previous study by Iwanaga *et al*, *in situ* hybridization was performed. Briefly, three non-overlapping 45-mer antisense oligonucleotide probes, complementary to the sequences: 358-402, 1491-1535 and 2993-3337 of sodium-dependent monocarboxylate transporter mRNA, were synthesized and labeled with ³³P-dATP. Fresh-frozen sections (10-μm) were fixed with 4% paraformaldehyde in a 0.1 M phosphate buffer for 15 min, and acetylated with 0.25% acetic anhydride in 0.1 M of triethanolamine-HCl (pH 8.0) for 10 min. Hybridization was performed at 42°C for 10 h by adding 10000 cpm/μl ³³P-labeled oligonucleotide probes. After being washed, sections were exposed to BioMax MR (Kodak, Rochester, NY) for 10 days (23,24). A detection of intensity was performed by using an Image Gauge (LAS1000, Fujifilm, Tokyo Japan). The intensity of slc5a8 was expressed as the value of the densitometry units.

Experiment 2. As described in Experiment 1, after two weeks of the pre-feeding of the respective diets, the rats received subcutaneous injections of AOM twice, at a dosage level of 15 mg/kg body weight once a week for two consecutive weeks. After the second AOM injection, the rats were given
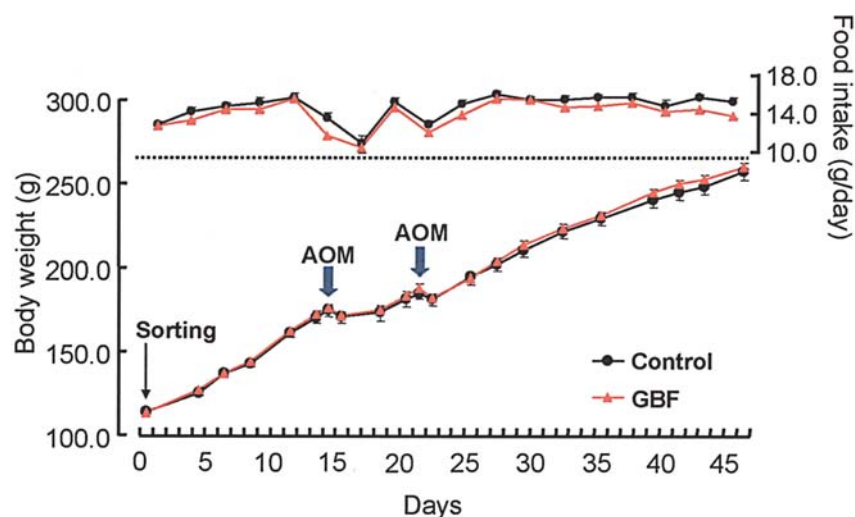
Figure 1. Change in body weight and food intake in rats fed either the control or the GBF diet (Experiment 1). The arrows show the injection of azoxymethane (AOM) s.c. The data are expressed as mean changes ± SE. No significant differences in body weight and food intake were detected between the two groups during the experimental period.

their respective diets for 4 more weeks *ad libitum*. Then, 5 rats in each respective group were sacrificed and the remaining rats were euthanized an additional week later.

ACF analysis. Using 5 rats from each of the respective groups, the entire colon was obtained for ACF counting. The colon was fixed with 4% buffered formalin and stained with 0.2% methylene blue. As in the previous report, the number of ACF in the entire colon was counted under a light microscope at x100 magnification. The ACF were distinguished from normal crypts by their increased size, more prominent epithelial cells and their increased pericryptal area. The number of ACF and the number of aberrant crypts (ACs) per rat were quantified (17).

Western blotting of ß-catenin. For the assaying of the Western blotting of ß-catenin of the colonic mucosa, the colon was cut into 2-mm samples and washed with ice-chilled PBS. The cytoplasmic fraction of colonic mucosa was extracted according to the method of Fujise *et al* (25). After being centrifuged (136000 g x 30 min), the supernatant was analyzed through immunoblotting. Equal quantities (25 μg protein/ml) of protein were electrophoresed in an SDS-PAGE. Thereafter, they were electroblotted onto a polyvinyliden difluoride membrane (Immobilon-P, Millipore, MA, USA). After being blocked with Blockace (Dainippon Sumitomo Pharma, Osaka, Japan), the membrane was incubated with primary antibodies. The monoclonal anti-ß-catenin antibody (610153, BD Biosciences Japan, Tokyo, Japan), the anti-ß-actin antibody (A5441, Sigma, MO, USA), and polyclonal goat anti-mouse Ig/HRP (P0447, Dakocytomation, Denmark) were used. The detection kit (ECL Plus Western blotting detection system) was purchased from GE Healthcare (Buckinghamshire, UK). The detection of chemiluminescence was performed by using an Image Gauge (LAS1000, Fujifilm, Tokyo Japan). The intensity of ß-catenin was expressed as the value of the adjusted signal intensity with ß-actin densitometry units. Results are shown as the relative value of the control group.

ß-glucuronidase and ß-glucosidase activity of cecal content. Cecal content (0.3 g) was diluted with 1.0 ml of ice-chilled PBS (pH 7.4) and shaken (TS-100, Thermal Kagaku Sangyo Co., Tokyo, Japan) for 30 min at 4˚C. After shaking, it was centrifuged (15000 rpm, 10 min, 4˚C) and the supernatant was immediately filtered by using an Ultra free-MC (at 10000 rpm, 10 min, 4˚C, Millipore, MA, USA). The activity of ß-glucuronidase and ß-glucosidase was determined by a modified method measuring the release rate of p-nitrophenols from p-nitrophenylglucuronide (Sigma) and p-nitrophenyl-glucopyranoside (Sigma), respectively (26). The data were measured in the units: μmol/min/g cecal content.

Heat shock protein 25 immunostaining assay. The rat and murine forms of HSP27 are slightly smaller than the human form, and for that reason we described their HSP as HSP25 in this study (27). When using the other 5 rats from the respective groups, the entire colon was removed, cut and rinsed with ice-chilled PBS. The distal colon, 8 cm upward from anus, was then cut off. The colorectum was longitudinally cut into two equal parts. One was mounted on cardboard and fixed in buffered formalin for the HSP25 immunostaining analysis and the other was weighed and immediately used for Western blotting of the ß-catenin in the colonic mucosa. Colon sections were embedded in paraffin and 4 μm cross-sections were cut. According to the methods described by Ren *et al* (27), sections were stained for HSP25. The LSAB2 kit (K0609, Dakocytomation) and primary antibody, rabbit polyclonal antibody HSP27, which was purchased from Abcam (ab5579, Abcam Japan, Tokyo, Japan), were used. The number of stained cells per view was counted under a light microscope at x200 magnification for 6 different areas (2 views were made in the distal, middle and proximal part of the colon, respectively), and the results are shown as the mean value per view.

Statistical analysis. Values are presented as the mean ± SEM. Comparisons of data were made by Student's t-test or the χ^2 analysis. Differences were considered significant at $P < 0.05$.

Table II. The number of aberrant crypt foci in colonic mucosa.

	Incidence	1 AC[a]/ACF	2 ACs/ACF	3ACs/ACF	>4ACs/ACF[b]	Total ACs/ACF[c]
Control	(5/5)	57.60±6.10	64.60±6.10	26.80±1.11	17.80±3.02	2.04±0.06
GBF	(5/5)	57.60±6.10	64.60±6.10	26.80±1.11	17.80±3.02	2.04±0.06
P-value		0.037	0.006	0.089	0.013	0.119

[a]Aberrant crypt, [b]counted the number of aberrant crypts/ACF as 4 and [c]mean number of aberrant crypt/one focus.
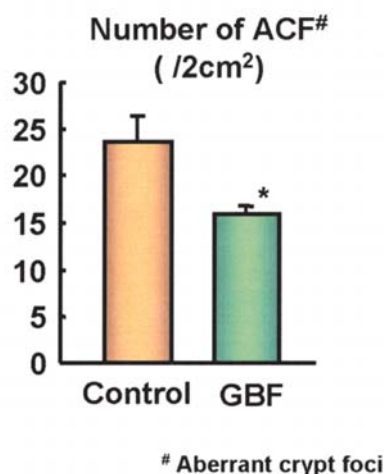

Figure 2. The number of abberant crypt foci (ACF) were counted under a light microscope, measured per 2 cm^2 of colon and were quantified at x100 magnification. The data are expressed as mean changes ± SE. ACF in the GBF group were significantly lower than that in the control group. *P<0.05 for the difference between the control and GBF.
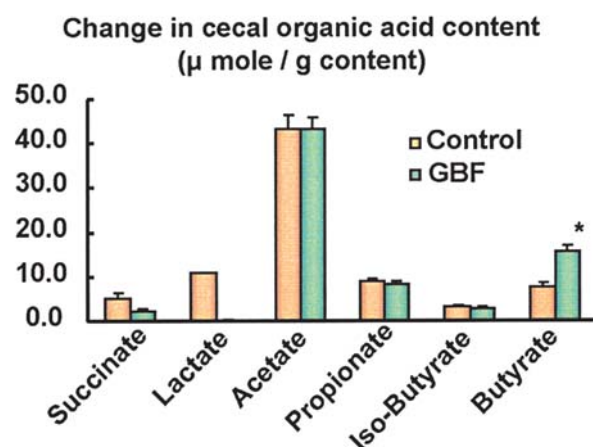

Figure 3. Concentrations of cecal organic acids in rats fed with the control or the GBF diet (Experiment 1). The data are expressed as mean changes ± SE. Butyrate content was significantly higher in the GBF group than that of the control group, and the succinate content was lower in the GBF group than that of the control group (p=0.057). *P<0.05 for the difference between the control and GBF.

Results

Experiment 1. Fig. 1 shows the changes in the body weight, food intake and schedule of AOM administrations. During the experimental period, there were no significant differences in body weight and food intake between the two groups. The number of ACF in the 2 cm^2 parts of the colon is shown in Fig. 2. After the GBF treatment, the number of ACF was significantly lower than that of the control group. Fig. 3 indicates the change in the cecal organic acid content of the two groups. The GBF treatment showed a significant decrease in the production of succinate and an increase in the amount of butyrate, compared to the control group. Slc5a8 was detected in the distal colon in the two groups. However, the amount of slc5a8 in the GBF group was significantly higher than that of the control group (Fig. 4).
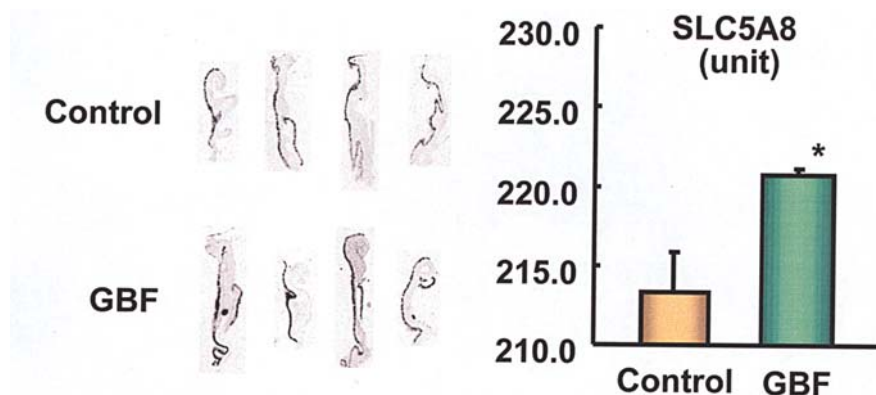

Figure 4. Left, colonic mucosal hybridization of slc5a8 labeled as ^{33}P-dATP. Right, the intensity of slc5a8, and the expression of slc5a8 in the GBF group being significantly higher than that of the control group. The data are expressed as mean changes ± SE. *P<0.05 for the difference between the control and GBF.
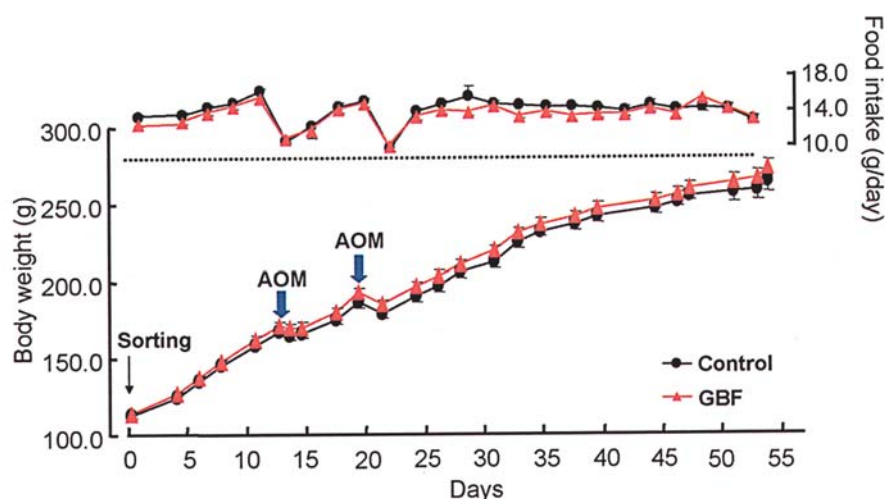
Figure 5. Changes in body weight and food intake in rats fed either the control or the GBF diet (Experiment 2). The arrows show the injection of azoxymethane (AOM) s.c. The data are expressed as mean changes ± SE. No significant differences in body weight and food intake were detected between the two groups during the experimental period.
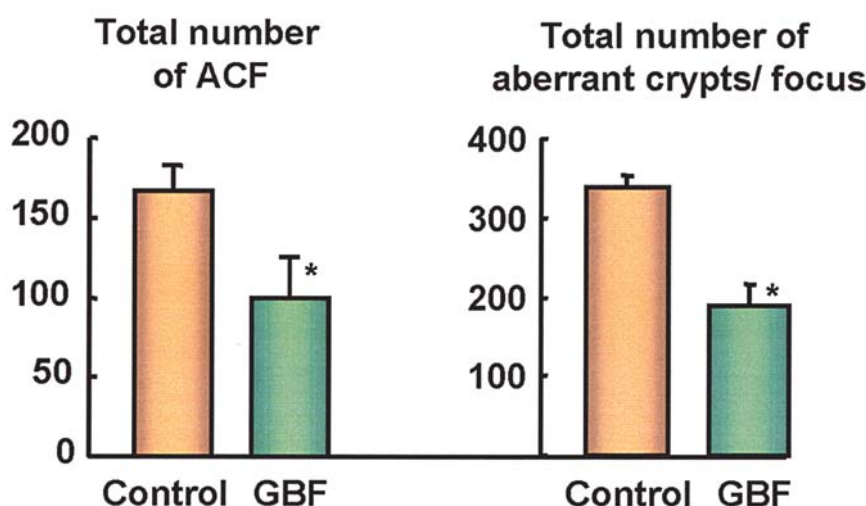

Figure 6. The number of abberant crypt foci (ACF) were counted under a light microscope in the entire colon and the total number of aberrant crypts per focus were quantified at x100 magnification. The data are expressed as mean changes ± SE. ACF and the total number of aberrant crypts per focus in the GBF group were significantly lower than that of the control group. *P<0.05 for the difference between the control and GBF.

Experiment 2. Fig. 5 shows changes in the body weight, food intake and schedule of AOM administrations. During the experimental period, there were no significant differences in body weight and food intake between the two groups, which was also the case in Experiment 1. The number of ACF in the entire colon (n=5/group) is shown in Fig. 6 and Table II. ACF were caused to form in the rats, and the ACs per ACF were lower in the GBF treatment group than in the control group. Although the number of ACs per ACF was high in the control group, it did not reach a significant difference. The total number of ACF and ACs was significantly lower in the GBF treatment in comparison with the control group levels. Fig. 7 shows the typical appearance of a normal crypt and ACF.

The manifestation of colonic mucosal ß-catenin is shown in Fig. 8, along with the significant decrease of ß-catenin in the GBF treatment compared with that of the control group.

The activity of ß-glucosidase and ß-glucuronidase in cecal content is shown in Fig. 9. ß-glucuronidase activity in

the two groups was at the same level, but ß-glucosidase activity in the GBF group was significantly higher than that of the control group. The cecal organic acid content in the two groups is shown in Fig. 10. The GBF treatment significantly increased acetate and butyrate content, in comparison with the control group. Fig. 11 shows a microscopic view of HSP25-positive cells in the colonic mucosa (x200) and the number thereof. Although the number of HSP25-positive cells per view did not reach a significant difference between the two groups, the GBF treatment showed an increase in the number of cells comparatively.

Discussion

GBF is a safe prebiotic for UC patients and it may be useful to continue ingestion in order to reduce the risk of colorectal cancer without causing adverse effects produced by anti-cancer or anti-inflammatory drugs. GBF administration induced
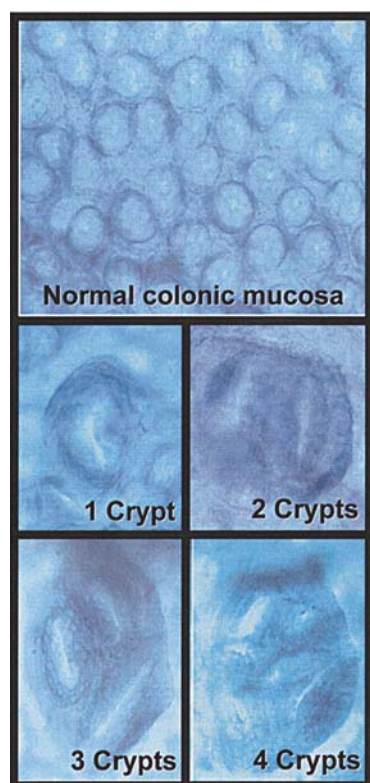
Figure 7. Typical aberrant crypts. ACF were distinguished from normal crypts by their increased size, more prominent epithelial cells and their increased pericryptal area.

Figure 8. ß-catenin expression in colonic mucosa. The intensity of ß-catenin in the GBF group was significantly lower than that of the control group. The data are expressed as mean changes ± SE. *P<0.05 for the difference between the control and GBF.

an increase in the luminal short chain fatty acid concentration, especially butyrate, which is produced abundantly in the colon by bacterial fermentation of residual food fractions (e.g. dietary fiber) (28,29). One of the promising mechanisms of GBF causing the anti-colitis effect may be due to the increased level of butyrate. The butyrate from GBF significantly suppressed the NFκB activation and phosphorylation of STAT3, which sequentially reduced IL-6 production in the
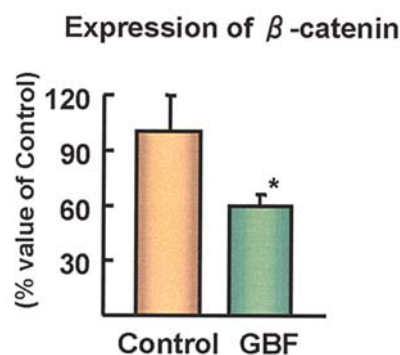
colitis model (30). It was also reported that butyrate possesses the potency to act as an energy source for the epithelium and cause an increase of cell differentiation while suppressing the proliferation of tumor cells through the induction of apoptosis (31,32). Thus, it is considered that the increase of luminal short chain fatty acids may contribute to the reinforcement of epithelial integrity which suppresses mucosal damage.

The incidence of ACF in colonic mucosa in the two groups was exactly the same. However, the total number of ACF and ACs was significantly lower in the GBF group compared to that of the control group. In general, colon cancer was understood to arise in a multi-step process of histopathological and molecular changes that transform a normal colonic epithelium into invasive colorectal cancer, accompanied by the processes of hyperproliferative epithelium (aberrant crypt foci), small adenoma, large adenoma and colon carcinoma (33). Although more detailed studies are needed, our data suggest that GBF affects the early stages of the pathogenesis of colon cancer, and facilitates the prevention of transforming hyperproliferative epithelia. It was reported
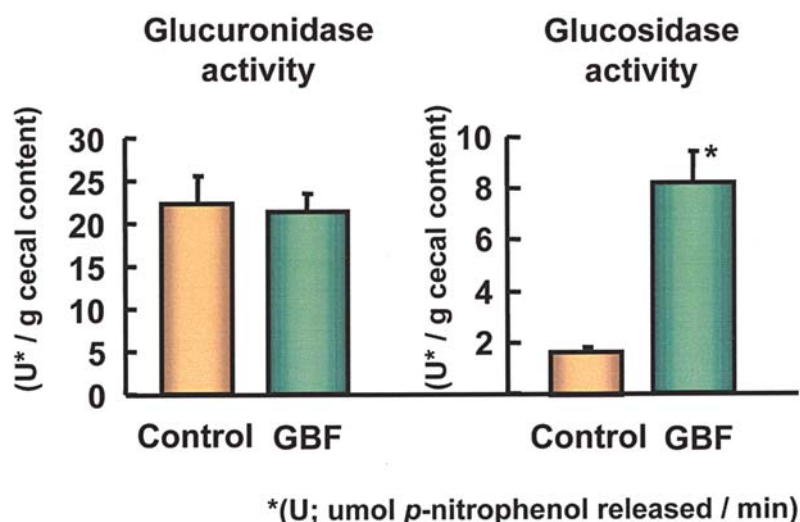

Figure 9. Bacterial glycolytic enzyme activities (ß-D-glucuronidase and ß-D-glucosidase) in cecal content of rats fed the control and the GBF diet. Glucosidase activity in the GBF group was significantly higher than that of the control group, and glucuronidase activity of the respective groups was at the same level. The data are expressed as mean changes ± SE. *P<0.05 for the difference between the control and GBF.
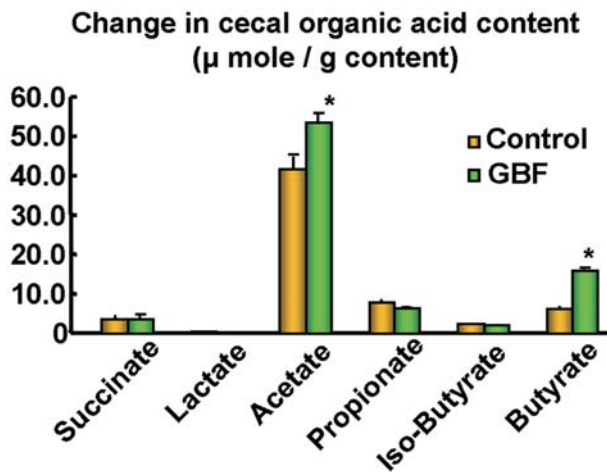
Figure 10. Concentrations of cecal organic acids in rats fed with the control or the GBF diet (Experiment 2). The data are expressed as mean changes ± SE. The butyrate and acetate content was significantly higher in the GBF group than that of the control. *P<0.05 for the difference between the control and GBF.

that GBF had the potency to cause anti-colitis effects in HLAB27 transgenic rats which showed mild colitis along with hyperplasia in their colonic mucosa (34). Interestingly, GBF increased the mucosal barrier function in healthy animal specimens without hyperplasia. This effect of GBF may definitely be different between healthy subjects and those with certain pathogenic conditions.

The present study shows that prebiotic treatment (GBF) increased cecal ß-glucosidase activity, while its ß-glucuronidase level remained the same as in the control group.

Although ß-glucosidase activity is controversial, it was reported that inulin or the mixture of inulin and bifidobacterium longum significantly increases the activity in AOM-treated rats (35). In addition, some oligosaccharides have been shown to increase ß-glucosidase activity in gut flora due to an increase in the amount of lactobacillus (36). This activity is reported to induce butyrate production, but its effect is unclear as this same activity has been reinforced in the generation of toxin and bacterial protective metabolites against chemically induced cancer.

The ß-glucuronidase activity is reported to be closely related to carcinogenesis of AOM in the colon. Briefly, AOM is converted to methylazoxymethanol (MAM) by cytochrome P450 in the liver and is delivered to the colon, or MAM is conjugated with glucuronic acid in bile acid, and sequentially, it is hydrolyzed by bacterial ß-glucuronidase and is absorbed in colon (37). In the two cases, MAM is further metabolized to a reactive compound to create the methylation of DNA. It was postulated that evidence of the same activity of ß-glucuronidase between the two groups indicated that the active compound of carcinogenesis of AOM in the colon did not change its concentration. Thus, the preventive effect of GBF upon the incidence of preneoplastic action was not caused by the difference of MAM concentration in colon.

Slc5a8 is reported to be a solute carrier transporter which regulates the uptake of monocarboxylates in epithelium (15). Although ~60% of short chain fatty acids (monocarboxylates) was absorbed by simple diffusion across the cell membranes, the residual 40% was absorbed by the ionized SCFA involving the co-transportation of inorganic protons, such as Na^+, K^+ and H^+ (23,24). In addition, it was reported that the high frequency of slc5a8 methylation observed in colon cancer and the
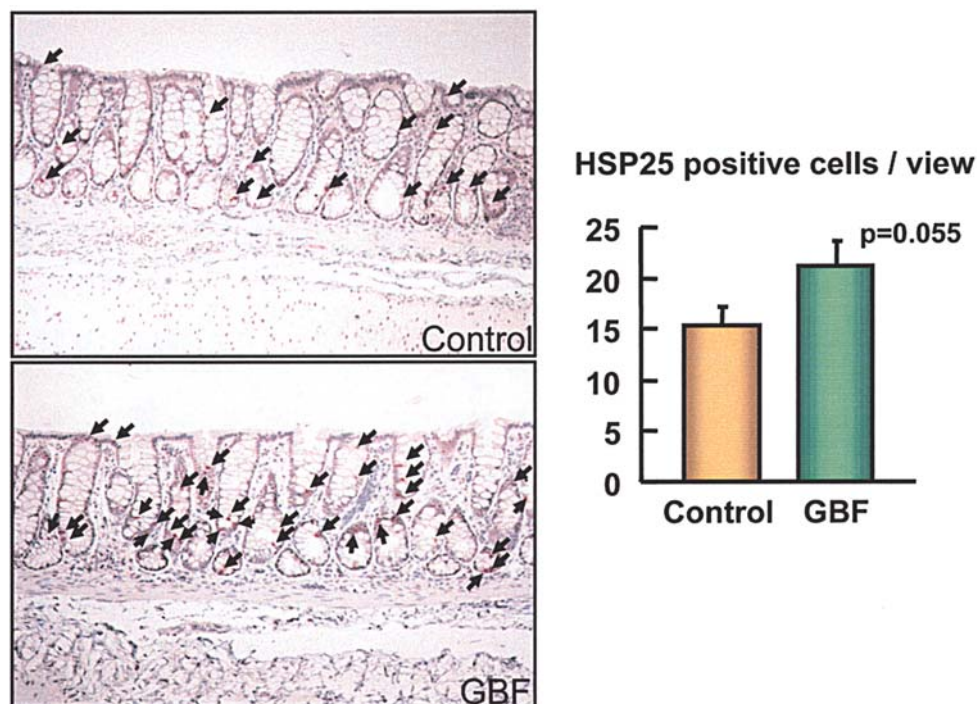
Figure 11. The arrows in the left panel show heat shock protein 25 (HSP25)-positive cells (at x200 magnification). The right panel shows the number of stained cells per view counted from 6 different areas (2 views in the distal, middle and proximal parts of the colon, respectively). The number of HSP25 in the GBF group was higher than that of the control group, although it did not reach a significant difference (p=0.055). The data are expressed as mean changes ± SE.

reconstruction of unmethylated slc5a8 in colon cancer cells suppressed the colony forming ability (14). In the present study, the increase of slc5a8 was observed in the colonic epithelium in the GBF group, although it did not reach a significant difference. Although more detailed studies are required, prebiotic treatment modulated the intestinal environment, and as a result, increased the butyrate content in the lower intestinal tract. Simultaneously, this change may produce an increased utilization of monocarboxylate in the epithelium and suppress the incidence of carcinogenesis through activation of the tumor suppressor gene slc5a8.

ß-catenin, acts as a scaffolding protein at cell-cell adherent junctions and as a transcriptional activator mediating Wnt signal transduction (38). Studies of ß-catenin have also reported that frequent mutations were observed in AOM-induced colon cancer in rodent models and showed increased expression in colonic cancer models and in humans (39). In this study, the GBF treatment significantly attenuated the expression of ß-catenin in colonic mucosa, in comparison with the control group. Kim *et al* suggested that a reduction of ß-catenin by food alternation caused a decrease in the transcriptional activity stimulating ß-catenin/T cell factor 4 responsive oncogenes such as c-*myc* (8). Although the complex effects of GBF on ß-catenin are still unknown, when considering the evidence along with the data showing a decrease of ACF in GBF treatments, it can be postulated that GBF plays a pivotal role in the prevention of colon carcinogenesis development in the early stages.

Small heat shock protein, especially HSP25, has been suggested to confer a degree of mucosal protection against a number of injurious events (hyperthermia, ionizing radiation, and oxidative stress). HSP25 is also inducible by a variety of stimuli, such as cytokines, change in microbiota, and SCFA (27). It was reported that dietary-resistant starch prevented tumor induction concomitant in the case of increased colonic epithelium HSP25 formations in rats (40). A controversial report stated that HSP25 enhanced the tumorigenicity in immunogenic cell clones due to an increase of resistance to the apoptotic effect (41). In this study, GBF increased the formation of HSP25 and butyrate production in AOM-treated rats. The increase of HSP25 was considered to be induced by a change in the intestinal environment (butyrate). Furthermore, HSP25 may act to protect against the injurious damage caused by AOM. AOM treatment caused transient body weight loss in the two groups, making it evident that this treatment caused relatively severe stress to rats. Further *in vivo* studies regarding the role HSP25 plays concerning the pathogenesis of colon cancer are needed.

It is known that the risk of colorectal cancer is higher in patients with UC, compared to the general population (42,43). However, not all UC patients have the same risk; a greater risk is observed in UC patients with more extensive colorectal inflammation which continues for longer periods. It is well known that UC is required for long-term medical and nutritional treatment to sustain the remission periods of patients. In conclusion, GBF showed a preventive effect on tumorigenicity in the AOM rat model. This result will contribute to the reduction of the risk of contracting colonic cancer in patients with IBD as well as in healthy humans (44). The nutraceutical preventive treatment of using GBF for colon cancer may prove useful without causing the adverse effects seen in treatments with anti-cancer or anti-inflammatory drugs.

Acknowledgements

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