The germ-free mice monocolonization with Bacteroides fragilis improves azoxymethane/dextran sulfate sodium induced colitis-associated colorectal cancer

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ABSTRACT

Objective: Inflammatory bowel disease (IBD) is generally considered as a major risk factor in the progression of colitis-associated colorectal cancer (CAC). Previous studies have indicated that the composition of gut microflora may be involved in CAC induction and progress. Bacteroides fragilis (BF) is a Gram-negative anaerobe belonging to colonic symbiotic bacteria of the host. This study was aimed to investigate the protective role of BF in a colorectal cancer (CRC) model induced by azoxymethane (AOM) and dextran sulfate sodium (DSS) in germ-free (GF) mice.

Materials and methods: Total 22 GF mice were divided into two groups: GF and BF group. Half of the GF mice were colonized with BF for 28 days before CRC induction by AOM/DSS.

Results: BF colonization increased animal survival (100%). Cecum weight and cecum/body weight ratio significantly decreased in BF/AOM/DSS group. Interestingly, there was a significant decrease in tumor number and tumor incidence in the BF/AOM/DSS group as compared to the GF/AOM/DSS group. The adenocarcinoma/adenoma incidence and histologic score were also decreased in the BF/AOM/DSS group. In addition, immunohistochemistry staining found decreased numbers of cell proliferation (PCNA) and inflammatory cell (granulocytes) infiltration in the colon mucosa of the BF group. The β-catenin staining in the BF/AOM/DSS group had fewer and weaker positive signal expressions. Taking together, the BF colonization significantly ameliorated AOM/DSS-induced CRC by suppressing the activity of cell proliferation-related molecules and reducing the number of inflammatory cells.

Conclusions: Symbiotic BF may play a pivotal role in maintaining the gastrointestinal immunophysiologic balance and regulating anti-tumorigenesis responses.

Introduction

The colorectal cancer (CRC) is one of the most common malignant neoplasms in western and Asian countries. Environmental and genetic factors influence the development of CRC, and inflammation is a critical hallmark of cancer that may arise from a variety of factors. Additionally, inflammatory bowel disease (IBD), in particular ulcerative colitis, represents a separate entity in which the risk of CRC is increased. The incidence of CRC in IBD patients, such as colitis-associated colorectal cancer (CAC), has been reported to be up to 60% higher than the general population [1]. In order to investigate the molecular pathogenesis of CRC in IBD patients, recent studies have reported that the combination of dextran sulfate sodium (DSS) with azoxymethane (AOM) as a model of CAC, which has gained popularity for its reproducibility, potency, low cost, and ease of use [2,3]. AOM has been found to be more potent and stable in solution than 1,2-dimethylhydrazine (DMH). While tumor development in other models generally requires several months, mice injected with AOM and subsequently treated with DSS develop adequate tumors in as little as 7–10 weeks [2].

The gut microbiota plays an important role in the construction of the biological barrier, which helps with nutrient absorption, energy metabolism, and immune regulation [4]. Evidence is accumulating that dysbiosis of intestinal
microbiota may greatly influence the pathogenesis of IBD and CRC in both animal models and humans [5–10]. For example, Bifidobacterium lactis and Lactobacillus casei prevent acute colitis and reduce the number of colonic neoplasms that arise in mice following administration of AOM/DSS [11,12]. It has been reported by numerous studies that oral administration of probiotics VSL#3, a commercially available probiotic cocktail that can reduce chronic inflammation and delay development of carcinoma in mouse models of CAC [9,13–15]. In contrast, the Fusobacterium nucleatum contributes to chemoresistance in CRC through modulating the network of toll-like receptors, microRNAs, and autophagy [16]. A recent study also reported that concurrent treatment with VSL#3 enhanced tumorigenesis in an AOM/Il10–/– mouse model [17]. Although the overwhelming majority of studies support the view that probiotics prevent inflammation and carcinogenesis [13,14,18]. However, the therapeutic effect of probiotics on CAC are not exactly the same.

Inflammation is increasingly recognized for its role in colorectal tumor initiation and promotion [19]. During the process of inflammation, reactive oxygen species (ROS) and pro-inflammatory cytokines are produced by activated macrophages and neutrophil, causing DNA damage and mutations of the colon epithelial cells. These events promote mutagenesis required for tumor initiation. Inflammation reaction contributes to tumor promotion by enhancing cancer cell proliferation and survival via various possible pathways (e.g. Akt, NF-kB, Wnt/b-catenin) [19]. β-catenin is a component of the Wingless/Wnt signaling pathway. Dysfunction of the Wnt signaling pathway is important in colorectal carcinogenesis and results in the nuclear accumulation of β-catenin [20]. Taherian-Esfahani et al. demonstrated that the Wnt/β-catenin pathway has been decreased following Lactobacillus rhamnosus treatment in colorectal cancer cell lines (HT-29). In addition, the expression of SFRP2, an antagonist of Wnt pathway, has been increased in cell line HT-29 following Lactobacillus rhamnosus treatment [21].

IBD is an important risk factor for the development of colon cancer. The molecular mechanisms by which inflammation promotes cancer development are still being uncovered and may differ between CAC and other forms of colorectal cancer. Our previous studies showed that symbiotics, such as Bacteroides fragilis (BF), have anti-inflammatory and immune regulatory functions. The strain NCTC 9343 produces a symbiosis factor (polysaccharide A) that signals through Toll-like receptor 2 to suppress the immune response [22,23]. Therefore, in order to directly evaluate the role of BF in CAC, we conducted using monoclonized gnotobiotic technics to examine AOM/DSS-induced tumorigenesis and related pathogenesis.

Materials and methods

Animals

A total of 22 GF male C57BL/6JNarl mice were used (7–8 weeks old, National Laboratory Animal Center, Taipei, Taiwan). Mice were maintained in a vinyl isolator in a room kept at a constant temperature (21 ± 1°C) and humidity (55–65%) with a 12 h/12-h light/dark schedule. Mice were fed a commercial diet (5010 LabDiet, Purina Mills, St. Louis, MO) and sterile water ad libitum. To confirm GF status, microbiological assays were performed on a monthly basis by culturing feces, bedding, and drinking water in thioglycollate medium (DIFCO, Camarillo, CA). All studies were approved by the Institutional Animal Care and Use Committee (number IACUC 2012M02).

Bacterial culture and monoclonization in mice

BF strain NCTC 9343 was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Bacteria cultures were freshly grown overnight in thioglycollate medium (DIFCO, Camarillo, CA) at 37°C on a shaker. Following, cultures were collected by centrifugation (3 min at 2000 g) and washed thrice with phosphate buffered saline. Pellets were resuspended in 20 ml sterile saline. BF was colonized into mice by oral gavage with 5 x 10^7 colony-formation units (CFUs) in saline. After 28 days of colonization, the BF CFUs were determined by a plate count from stool samples and calculated per gram feces. Control mice were treated with the same volume of sterile saline. At the end of experiment, we confirm only BF was detected in the stool samples of the BF monoclonization mice and no other microbial contamination.

Induction of experimental CAC in mice

All animals were housed in plastic cages in the isolator with free access to drinking water and diets. A colonic carcinogen...
AOM/DSS-induced colorectal cancer in the GF and BF mice.

Table 1. General observation and tumor incidence of Bacteroides fragilis on AOM/DSS-induced colorectal cancer in the GF and BF mice.

|                      | GF/AOM/DSS | BF/AOM/DSS |
|----------------------|------------|------------|
| General observations |            |            |
| Body weight (g)      | 34.37 ± 2.5| 32.05 ± 2.1|
| Spleen (g)           | 0.13 ± 0.11| 0.085 ± 0.02|
| Cecum (g)            | 7.00 ± 3.1 | 3.78 ± 0.6* |
| Spleen (% of body weight) | 0.37 ± 0.28| 0.27 ± 0.05 |
| Cecum (% of body weight) | 20.2 ± 5.5 | 11.8 ± 1.6* |
| Colon length (cm)    | 10.12 ± 3.3| 9.86 ± 0.9 |
| Survival rate (%)    | 9/11 (81.8%)| 11/11 (100%)|
| Tumor assessment     |            |            |
| Total of tumors      | 56         | 16         |
| No. of tumors/mice   | 6.22 ± 2.67| 1.78 ± 2.44*|
| Small tumor (<3 mm)  | 3.60 ± 1.14| 0.67 ± 0.58*|
| Large tumor (>3 mm)  | 3.0 ± 1.41 | 0.67 ± 1.15 |
| Tumor incidence      | 9/9 (100%) | 5/11 (45.6%)|
| Adenoma              | 6/9 (66.7%)| 5/11 (45.6%)|
| adenocarcinoma       | 3/9 (33.3%)| 0/11 (0%)  |
| Histologic score     | 3.3 ± 0.71 | 1.9 ± 1.27*|

*p < .05 compared with GF/AOM/DSS.

Tumor incidence of CAC

Colons were harvested, and tumor masses were measured with a caliper. Colonic neoplasms were observed to calculate tumor incidence in each group. Tumor incidence was defined as the number of mice with tumors/total living mice in each group. According to the previous study, the sizes of tumors were separated as large = over 3 mm and small = less than 3 mm. This decision was based and slightly modified from the Zhan study (3).

Histopathologic evaluation of CAC severity

At the end of the experiments, mice were sacrificed by 95% CO₂ asphyxiation, and the distal segments of the colon were excised longitudinally along the mesenteric margin. Colorectal tissues were collected and fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned at 4 μm, stained with hematoxylin-eosin (H&E), and examined by light microscopy. The histology score consisted of a composite scale that indicated the overall degree of inflammation and hyperplasia (0 = absence of significant inflammation or dysplasia, 1 = mild inflammation without significant dysplasia, 2 = moderate inflammation with hyperplasia, 3 = severe inflammation with adenoma, 4 = severe inflammation with adenocarcinoma). Histological score was determined using a BX51 Olympus microscope in a blinded reading by a senior veterinarian.

Immunohistochemistry of PCNA, β-catenin, Ly6G, and COX-2

The 4-μm formalin-fixed, paraffin-embedded colorectal sections were subjected to deparaffinization and hydration prior to quenching of endogenous peroxidase activity (3% H₂O₂ in methanol for 10 min). For antigen retrieval, slides were submerged in 10 mM citrate buffer (pH 6.0) until boiling. The sections were incubated overnight with the primary anti-PCNA mouse monoclonal antibody (Invitrogen Co., Camarillo, CA) at a 1:200 dilution, primary anti-Ly6G rabbit polyclonal antibody (Santa Cruz Biotechnology, Dallas, TX) at a 1:100 dilution, primary anti-COX-2 rabbit monoclonal antibody (Thermo Scientific Co., Kalamazo, MI) at a 1:100 dilution, and primary anti-β-catenin rabbit polyclonal antibody (Thermo Scientific Co., Kalamazo, MI) at a 1:100 dilution. The slides were subsequently treated with Picture™ HRP Polymer conjugate at room temperature for 20 min. HRP localization was visualized using a Single Solution aminoethyl carbazole (AEC) kit. The number of PCNA, Ly6G, and COX2-positive cells was determined by at least 1000 cells were counted in 400 × microscopic fields. This method was similarly based on Wang et al.’s report [24]. The β-catenin staining intensity was graded using a scale of 0–3 as follows: degree 0 = negative reaction, 1 = weak positive signal, 2 = moderate positive signal, and 3 = strong positive signal. This method was based on Gao et al. [25]. Quantification of immunohistochemically positive cells was performed using the open source Fiji (ImageJ) software (version 1.51).

Statistical analysis

Data were presented as mean ± SD. The significance was analyzed by Student’s t test. p values less than .05 were considered significant. All statistical analyses were performed in Prism version 6.0 (GraphPad Software, San Diego, CA).

Results

BF improved AOM/DSS-induced disease symptoms

During the entire period of the experiment, there were no signs of toxicity or other adverse conditions that would suggest no adverse effects caused by BF administration. The general observations data of GF and BF mice without AOM/DSS were showed in Table S1. In groups of BF/AOM/DSS, all mice (survival rate: n = 11, 100%) survived to the end of the experiment; however, two mice died in the GF/AOM/DSS group (survival rate: n = 9, 81.8%). As shown in Table 1, the body weight was not significant differences in AOM/DSS-treated mice with or without BF monocolonization. The body weight, spleen weight, spleen/body weight ratio, and colon length were not significant differences between the GF/AOM/DSS and BF/AOM/DSS mice. The mean cecum weight of the GF/AOM/DSS group at the end of the test period was significantly lower than the GF/AOM/DSS group (p < .05). The cecum/body weight ratio of the BF/AOM/DSS group at the end of the test period was significantly lower than the GF/AOM/DSS group (p < .05).
BF attenuated AOM/DSS-induced CAC

Table 1 showed that GF/AOM/DSS-treated mice (9/9, 100% incidence) developed CAC, whereas those treated with BF/AOM/DSS (5/11, 45.6% incidence) were found to have significantly fewer CAC. In the BF/AOM/DSS group, treatment of mice with BF caused a significant decrease in the mean number of CRC per mouse, as compared to the GF mice (6.22 ± 2.67 versus 1.78 ± 2.44, \( p < .05 \)). The GF/AOM/DSS group mice developed significantly more and larger adenomatous tumors than BF/AOM/DSS mice. These results indicate that BF significantly inhibited CAC formation by AOM/DSS treatment.

BF attenuated AOM/DSS-induced CAC by gross and histopathologic findings

At necropsy, macroscopically nodular and polypoid-like tumors mass were observed in the distal colon. For the GF/AOM/DSS group, colonic tumors were found in all mice (9/9, 100%), while the incidence in BF/AOM/DSS mice was 45.6% (5/11). In the BF/AOM/DSS group, tumor numbers and sizes were significantly fewer and relatively smaller, respectively (Table 1). This result suggests that inhibition of colorectal tumor development by BF was due to a reduction in the number and size of tumors. Figure 2 shows representative H&E staining of histological sections of these groups. In colonic tissue, multifocal adenomatous lesions were observed without invasion into the submucosa. In histopathological findings, the colonic tissues belonged to benign hyperplasia and tubular adenoma (5/11, 45.6%) in the BF/AOM/DSS group (Figure 2(D)). However, the well-/moderately differentiated tubular adenocarcinoma (3/9, 33.3%) and hyperplasia to tubular adenoma (6/9, 66.7%) were found in the GF/AOM/DSS group (Figure 2(C)). In addition, the mucosa revealed tightly packed glands with a normal number of goblet cells; however, crypt architecture remained normal in the BF/AOM/DSS group. The BF monocolonization groups revealed a marked reduction of these CAC morphologic changes.

Decreased cell proliferation and inflammatory responses in BF/AOM/DSS

The cellular composition of the colorectal carcinogenesis and inflammation was analyzed by immunohistochemistry staining. Expression levels of proliferating cell nuclear antigen (PCNA), \( \beta \)-catenin, cyclooxygenase 2 (COX2), and lymphocyte antigen 6 complex locus G6D (Ly6G) in the lamina propria and submucosa were assessed. The immunohistochemistry data of GF and BF mice without AOM/DSS were showed in Figure S1. Epithelial cell proliferation in colons of the GF/AOM/DSS group compared to the BF/AOM/DSS group was significantly increased (GF/AOM/DSS: 543 ± 10 vs. BF/AOM/DSS: 184 ± 18, \( p < .05 \)). Strong \( \beta \)-catenin expression was seen in the adenocarcinoma cells of GF/AOM/DSS mice (Figure 3(D)). Although the intensity was relatively weaker than carcinoma cells, adenoma cells showed positivity for \( \beta \)-catenin in the BF/AOM/DSS group (Figure 3(E)). In inflammatory cells analysis, large numbers of Ly6G-positive neutrophils and COX2-positive macrophages were...
found in the lamina propria, indicating a significant inflammatory response in GF/AOM/DSS mice (Figure 3(G, J)). In contrast with the BF/AOM/DSS group, Ly6G- (GF/AOM/DSS: 34 ± 8 versus BF/AOM/DSS: 6 ± 4, \( p < .05 \)) and COX2- (GF/AOM/DSS: 112 ± 8 versus BF/AOM/DSS: 87 ± 13, \( p < .05 \)) positive cells decreased in the colon tissue of the BF/AOM/DSS group (Figure 3(H, K)). Taken together, these data suggest that the BF microbiota reduced colonic epithelial cell proliferation and improved inflammation in BF/AOM/DSS mice.

Discussion

In this study, we demonstrate the anti-inflammatory and anti-tumorigenic effects of BF on CAC development by GF and mono-gnotobiotic mice (different microbial condition). The results showed that the anti-tumorigenic effect of BF was supported by the decreased number and size of the tumor masses, histological dysplasia, as well as suppressed PCNA and \( \beta \)-catenin expression in the colon tumor tissue. Interestingly, the adenocarcinoma were found three mice in the GF/AOM/DSS group (3/9, 33%) and no occurrence in BF/AOM/DSS group (0/11, 0). In previous studies, Tanaka and Ward [26,27] have reported that these tumors can be definition to tubular adenoma or well differentiated tubular adenocarcinoma. The incidences of tubular adenoma and adenocarcinoma at the 20th week was 38% and 100%, respectively. In this study, we found the 100% tumor incidence and ratio of adenoma and adenocarcinoma were 66.7% and 33.3% in the GF/AOM/DSS group. However, only adenoma was observed in the BF/AOM/DSS group (45.6%). This data shows that BF may reduce the occurrence of adenocarcinoma. The previous study demonstrated AOM/DSS induced CAC were only 80% incidence in C57BL/6 genetic background SPF mice [28]. Although the tumor incidence appeared to be some differences in the same strain mice at different microbial condition. Therefore, we suggest this mice strain is feasible and susceptible used for AOM/DSS-induced CAC in GF condition.

In a recent study, Yu Zhan and colleagues reported AOM administration (10 mg/kg) followed by four cycles of 1.5% DSS in GF and SPF mice. The results showed that GF mice developed a higher tumor incidence compared to SPF mice [3]. In the present study, single-dose administration of AOM...
(10 mg/kg) and three cycles of 1% DSS in GF mice induced a higher tumor incidence and tumor number in the distal colon. In addition, two mice died in the GF/AOM/DSS group during the experimental period, although the DSS-treated dose differed slightly from previous studies. However, our results were still consistent with the previous report [29,30]. Altogether, these results strongly suggest that the lack of gut microbiota can enhance the host development of colon tumors.

DSS administration was reported to shorten colon length in GF and SPF mice [31]. In addition, shorter colon lengths of the GF/DSS group than the BF/DSS group were observed in our previous study. However, in this study, colon length showed no significant differences between the GF/AOM/DSS and BF/AOM/DSS mice. These results might due to the experimental period being longer than the previous report by Song and Ju [32,33]. In mortality, all mice survived in the BF/AOM/DSS group, and two mice died in the GF/AOM/DSS group. We suggest that repeated DSS administration might have caused serious bowel symptoms, such as hemorrhage and anemia, resulting in death of some GF mice. This result was consistent with our previous data.

β-catenin is a 92-kDa protein that binds to the cytoplasmic tail of E-cadherin. In this study, the slight β-catenin expression in hyperplastic mucosa epithelial cells was observed in the BF/AOM/DSS group. Our findings suggest that BF may contribute to β-catenin signaling inactivation to ameliorate intestinal tumorigenesis. Previous studies reported that Fusobacterium nucleatum-mediated intestinal tumorigenesis in ApcMin/− mice was due to a TLR4/p-PAK1/p-β-catenin cascade [34]. According to our finding, there may be a correlation between β-catenin signaling inactivation by BF and protection of AOM/DSS-induced CAC through the TLR4/p-PAK1/p-β-catenin pathway. However, further investigation to confirm BF involvement in this signaling regulation is needed.

Cell proliferation plays an important role in multistep carcinogenesis [35]. PCNA is a protein that exists in the nucleus and plays an important role in the regulation of cell transition from the G1-to-S phase [36]. Our results found that PCNA expression in tumor cells was significantly decreased in the BF/AOM/DSS group as compared to the GF/AOM/DSS group. These phenomena agree with the decreased epithelial cell proliferation in the former study [37,38].

In previous studies, symbiont B. fragilis protects animals from experimental colitis, including Crohn’s disease and TNBS-induced colitis [39]. The possible mechanism includes activation of CD4+ T cells into Foxp3 (+) Treg cells to induce anti-inflammatory cytokine secretion, such as IL-10, and to suppress pro-inflammatory cytokine IL-17 [39,40]). In these studies, the numbers of COX2 (macrophages) and Ly6G (neutrophils) were lower in the BF/AOM/DSS than GF/AOM/DSS group. This finding was similar to our previous DSS colitis model. Therefore, these results suggest the suppressive effects of BF on the development of colitis-related colon cancer might correlate with inhibition of inflammatory cell activation especially macrophages and neutrophils in UC-colitis. On the other hand, many studies provide evidence for the direct anti-tumorigenic effects of probiotics, including Lactobacillus casei BL23, Bifidobacterium lactis, Lactobacillus acidophilus, and Enterococcus faecalis, in AOM/DSS models [11,12,32]. Therefore, our results were consistent with previous reports, which indicated that inflammatory cell markers, such as COX-2 and Ly6G, were reduced in symbiotics, and probiotics prevented AOM/DSS-induced CAC [37,41].

IBD-associated colorectal carcinogenesis is probably promoted by chronic inflammation, but the mechanism is still unclear. This study was designed to investigate the role of BF in the non-hereditary tumor development of an AOM/ DSS-induced colorectal cancer model. We clearly demonstrated that monocolonization of BF in AOM/DSS germ-free mice could significantly prevent inflammation and tumor formation.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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