Effects of Orally-Administered *Bifidobacterium animalis* subsp. *lactis* Strain BB12 on Dextran Sodium Sulfate-Induced Colitis in Mice^S

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Inflammatory bowel disease, including Crohn's disease and ulcerative colitis (UC), is a chronically relapsing inflammatory disorder of the gastrointestinal tract. Intestinal epithelial cells (IECs) constitute barrier surfaces and play a critical role in maintaining gut health. Dysregulated immune responses and destruction of IECs disrupt intestinal balance. Dextran sodium sulfate (DSS) is the most widely used chemical for inducing colitis in animals, and its treatment induces colonic inflammation, acute diarrhea, and shortening of the intestine, with clinical and histological similarity to human UC. Current treatments for this inflammatory disorder have poor tolerability and insufficient therapeutic efficacy, and thus, alternative therapeutic approaches are required. Recently, dietary supplements with probiotics have emerged as promising interventions by alleviating disturbances in the indigenous microflora in UC. Thus, we hypothesized that the probiotic *Bifidobacterium animalis* subsp. *lactis* strain BB12 could protect against the development of colitis in a DSS-induced mouse model of UC. In the present study, oral administration of BB12 markedly ameliorated DSS-induced colitis, accompanied by reduced tumor necrosis factor- α -mediated IEC apoptosis. These findings indicate that the probiotic strain BB12 can alleviate DSS-induced colitis and suggest a novel mechanism of communication between probiotic microorganisms and intestinal epithelia, which increases intestinal cell survival by modulating pro-apoptotic cytokine expression.

Keywords: Probiotics, colitis, TNF- α , apoptosis

Introduction

Inflammatory bowel disease (IBD) in humans, including ulcerative colitis (UC) and Crohn's disease, is a complex chronic inflammatory disorder [1–3], and two common features of UC include increased cytokine production and excessive apoptosis of intestinal epithelial cells (IECs) [4, 5]. In particular, tumor necrosis factor- α (TNF- α), a major cytokine in the pathogenesis of UC, plays a central role in inducing apoptotic death in IECs, resulting in the destruction of intestinal epithelial layers [6, 7]. Moreover, the importance of TNF- α in disease pathogenesis is

underlined by pronounced clinical improvement when anti-TNF- α antibody treatment enhances mucosal healing with rapid re-epithelialization of ulcerated surfaces in UC [8].

Current IBD treatments include aminosaliclates, corticosteroids, TNF- α inhibitors, antibiotics, and immunosuppressants. However, these agents have poor tolerability and insufficient therapeutic efficacy; therefore, the need for alternative therapeutic approaches is increasing [9]. It is now clear that probiotic intervention can prevent pouchitis and has been found effective in inducing and maintaining remission in patients with UC [10, 11].

Probiotics are living microorganisms with low or no pathogenicity that exert beneficial effects on host health [9]. Modification of the gut microbiota by application of probiotics has therapeutic potential in clinical conditions associated with gut barrier dysfunction and inflamed mucosa [12]. The most commonly researched probiotic species, *Bifidobacterium*, is part of the human microflora and dominates the infant intestinal microbiota. However, their abundance decreases over time so that bifidobacteria usually account for 3% to 5% of the adult human colon microbiota [13–15].

In particular, *Bifidobacterium animalis* subsp. *lactis* BB12 is a Gram-positive, anaerobic, commensal-derived probiotic [16]. Interestingly, recent investigations have suggested that BB12 has potent anti-inflammatory effects in IBD [12, 17]. Although there are a large number of clinical and experimental studies on probiotics, neither the mechanisms of action nor the true characteristics of probiotic anti-inflammatory molecules are well understood. Thus, it is necessary to characterize the specific anti-inflammatory components of probiotics and to identify the precise mechanisms of probiotic action for its effective application in the induction and maintenance of remission in UC.

Therefore, the purpose of this study was to investigate the effects of BB12 administration on a dextran sodium sulfate (DSS)-induced mouse colitis model and provide mechanistic explanations for its probiotic effects. We show that BB12 prevents colitis development by attenuating the expression of TNF- α , followed by caspase-8-mediated apoptosis of IECs, suggesting that BB12 promotes IEC survival by regulating pro-apoptotic signal transduction pathways. These novel observations provide insights into the rationale for BB12 in the treatment of IBD, particularly colitis.

Materials and Methods

Animal Care and Experimental Protocols

All animal experiments were reviewed and approved by the Korea University Institutional Animal Care and Use Committee (Approval No. KUIACUC-2016-145). The experiments were performed using 8-week-old male C57BL/6J mice. The mice were housed in microisolator rodent cages at 22°C on a 12-h light/dark cycle, and food and drinking water were provided *ad libitum* throughout the experiment. Experimental colitis was induced by giving mice 3% DSS added to drinking water for 6 days. We administered 1.2×10^{10} colony forming units (CFU) of BB12 probiotics by oral gavage twice a day beginning 7 days prior to DSS treatment. Colitis development was monitored by measuring mice body weight and fecal conditions. The fecal condition was

measured on days 0, 3, and 6. Following 7 days of colitis induction, all mice were sacrificed. The mice were anesthetized with 2,2,2-tribromoethanol at 250 mg/kg body weight. Colon tissues from the cecum to rectum were collected, and the colonic lengths were measured. The isolated colons were snap-frozen and stored at –80°C.

Evaluating Colitis

We determined the disease activity index (DAI) score for colitis by scoring the loss of weight, stool consistency, and stool bleeding in accordance with a previously described method [18]. The DAI was evaluated by an observer who was unaware of the treatment groups.

Histopathologic Analysis

The colon tissues were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Histological sections cut from the paraffin blocks were stained with hematoxylin and eosin (H&E). In a blind fashion, the scoring of histological damage was divided into two categories: inflammatory cell infiltration and ulceration. Inflammatory cell infiltration was assessed in each layer of the colon, including the surface epithelium, cryptal glands, stroma, submucosa, and transmural layer and graded on a scale from 0 to 3 (0, none; 1, mild; 2, moderate; and 3, severe). We graded the severity of ulceration histologically on a scale from 0 to 4 (0, none; 1, mild and focal surface; 2, mucosal layer; 3, submucosal layer; and 4, transmural layer) [19]. We modified and established a 0–19 scoring system by summarizing all the scores.

Immunofluorescence

The following antibodies were purchased: anti-cleaved-caspase 8 (Cell Signaling Technology, USA) and anti-TNF- α (Abcam, UK). The 3 μ m tissue sections were deparaffined and rehydrated. The tissues were permeabilized with citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6) followed by microwave irradiation twice for 2 min each. The tissues were blocked with 3% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h in 25°C. After blocking, tissues were incubated with the appropriate primary antibody overnight at 4°C. The tissue sections were rinsed with PBS and incubated with the appropriate fluorophore-conjugated secondary antibody. Fluorescent antibody-stained slides were then treated with an anti-photo bleaching reagent and sealed with cover glass, and 3,3'-diaminobenzidine (DAB)-stained slides were mounted on glass slides. Both were analyzed under an Axiovert 40 CFL microscope (Carl Zeiss AG, Oberkochen, Germany).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Staining

To evaluate apoptosis, the colon tissue sections were subjected to TUNEL staining using the In Situ Cell Death Detection Kit (Roche, Basel, Switzerland) according to the manufacturer's recommended protocol. TUNEL-stained slides were lightly counterstained with 4',6-diamidino-2-phenylindole before final

mounting. The stained slides were analyzed under an Axiovert 40 CFL microscope (Carl Zeiss AG).

Immunoblot Analysis

The following antibodies were purchased: anti-cleaved-PARP (Cell Signaling Technology) and anti- β -actin (Novus Biologicals, Littleton, USA). The colon tissues were homogenized and sonicated on ice in RIPA buffer with a protease inhibitor cocktail (Calbiochem, Merck Millipore, Germany) and phenylmethylsulfonyl fluoride (PMSF). Samples were centrifuged at 15,000 g for 45 min at 4°C and stored at -20°C until further use. Bradford reagent (Bio-Rad, München, Germany) was used to measure the protein concentrations in comparison with BSA standards. Proteins were denatured with sample buffer for 5 min at 100°C, separated on a 12% polyacrylamide gel, and blotted onto 0.2 mm polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 3% (w/v) BSA in 0.05% Tris-buffered saline with Tween-20 (TBS-T; 50 mM Tris, 150 mM NaCl, and 0.05% Tween-20) for 1 h at 25°C and incubated overnight at 4°C with primary antibodies. The membranes were then washed 3 times for 5 min in 0.05% TBS-T and exposed to species-specific horseradish peroxidase-labeled secondary antibodies for 1 h at 25°C. After the membranes were washed 3 times for 10 min in TBS-T, the reactions were developed using the Amersham ECL Prime Western Blotting Reagent (GE Healthcare Life Sciences, UK). We measured the signal intensities on the ImageQuant LAS 4000 (GE Healthcare Life Sciences).

Statistical Analysis

Results are expressed as the mean $\pm$ standard deviation (SD). Statistical analysis was performed using a two-tailed *t*-test. $p < 0.05$ was considered statistically significant.

Results and Discussion

Protective Effects of BB12 on DSS-Induced Colitis

A previous clinical trial suggested a mixed culture of BB12 and *Lactobacillus acidophilus* La-5 as a well-tolerated probiotic that can be used as an alternative treatment for patients with UC [20]. However, the effects of single-intervention BB12 treatment on a DSS-induced colitis model or mechanistic studies have not been reported. To test whether BB12 plays a role in preventing DSS-induced acute colitis, the mice were subjected to oral administration of BB12 for 7 days followed by DSS treatment. We added 3% DSS to the drinking water and explored the severity of colitis by monitoring the DAI value over 6 days. UC is known to cause loss of body weight, abdominal pain, and bloody diarrhea [3, 9]. We observed that the DAI score was dramatically higher in DSS-treated mice than that in control mice (Fig. 1A); however, BB12 administration led to a significant decrease in the DAI score of DSS-treated mice

Fig. 1. Effect of BB12 on DSS-induced colitis.

(A) DAI scores were evaluated for 7 consecutive days. (B) Representative images of the colon tissues at 7 days on DSS exposure. (C) Statistical analysis of colon length. (D) Representative H&E-stained distal colonic sections after 7 days of DSS exposure (magnification, $\times 100$). Control: mice treated with PBS; DSS: mice treated with 3% DSS only; DSS + BB12: mice administered with BB12 prior to DSS treatment. * $p < 0.01$, ** $p < 0.001$ compared with DSS group.

(Fig. 1A), suggesting that BB12 can alleviate susceptibility to DSS-induced colitis.

It is well known that the development of DSS-induced colitis is associated with pathological damage in the colon. For this study, after DSS exposure for 6 days, the mice were sacrificed, and the colon length was measured. After 1 week of DSS exposure, the DSS-treated mice showed a marked reduction in colon length compared with control mice; however, BB12 administration significantly attenuated the reduction of colon length in DSS-treated mice (Figs. 1B and 1C). Furthermore, histological analysis of the H&E-stained colon tissues revealed that DSS treatment resulted in colitis-associated histological alterations, such as damage to the surface epithelium, infiltration of inflammatory cells, and disruption of the cryptal glands (Fig. 1D). However, the pathological characteristics of the colon altered by DSS treatment were improved by BB12 administration (Fig. 1D), indicating its protective effect against DSS-induced colitis. Taken together, our study's findings suggested that BB12 administration resulted in reduced development of DSS-induced colitis.

Effects of BB12 on DSS-Induced IEC Apoptosis

Next, we wished to determine the point at which the

probiotics interfaced with the amelioration of DSS-induced colitis, and then, we further explored the detailed mechanisms underlying the protective effects of BB12 on DSS-induced colitis. It is well established that IECs play a pivotal role in the pathogenesis of IBD by forming a barrier to the luminal microbe. Apoptosis of IECs is a tightly regulated process in the normal intestinal epithelium, and increased cell death has been detected at inflammatory sites in both patients with IBD and a mouse model of colitis [4, 5, 21].

To examine the effects of BB12 administration on DSS-induced IEC apoptosis, we performed TUNEL staining using the colon tissue of mice with DSS-induced colitis. As shown in Figs. 2A and 2B, TUNEL staining of the mice colon tissues showed that the number of apoptotic IECs was significantly higher in DSS-treated mice than that in control mice, and more importantly, this increased sensitivity to apoptosis was markedly reduced by BB12 administration.

To further confirm the effect of BB12 administration on suppression of apoptosis, we analyzed the expression of cleaved PARP (c-PARP), another marker of apoptosis, in isolated colon epithelial cells using immunoblot analysis. Consistent with the TUNEL staining results, DSS treatment induced apoptosis of IECs as indicated by increased c-PARP expression; however, the increased apoptotic activity seen

Fig. 2. Effect of BB12 on DSS-induced apoptosis of IECs.

(A) TUNEL assay for apoptosis (magnification, $\times 200$). (B) The percentage of TUNEL-positive cells. (C) Immunoblot analysis and (D) densitometric quantification of colonic mucosal c-PARP. Results are expressed as mean $\pm$ SD of three independent experiments. $n = 7$ mice per group. Control: mice treated with PBS; DSS: mice treated with 3% DSS only; DSS + BB12: mice administered with BB12 prior to DSS treatment. * $p < 0.05$, ** $p < 0.01$ compared with DSS group.

in IECs from DSS-treated mice was drastically reduced with BB12 administration (Figs. 2C and 2D). These results indicate that BB12 protects against colitis-induced apoptosis of IECs, which is reflected in the reduced susceptibility to colitis.

Effects of BB12 on TNF- α -Induced Cell Death in IECs

It is reported that DSS-induced colitis is associated with increased levels of pro-inflammatory cytokines, including interleukin (IL)-6, IL-10, and TNF- α [22]. In particular, TNF- α is a major cytokine that promotes intestinal mucosal inflammation and damage in IBD. Numerous studies have shown that TNF- α expression is increased in patients with IBD, and TNF- α -mediated signaling plays an important role in inducing apoptosis in the small intestine and colon tissues [23, 24].

Thus, we determined whether BB12 is involved in regulating TNF- α -induced programmed cell death in IECs. To analyze the influence of BB12 administration on TNF- α production in the colon during colitis, we checked for the presence of TNF- α in the colon tissue by immunohistochemical analysis. Consistent with the levels of colitis observed in this study, the level of TNF- α was markedly increased in the colons of DSS-treated mice compared with that in the

colons of control mice; however, the extent to which TNF- α level was significantly reduced by BB12 administration differed (Fig. 3A). These results suggested that the decrease in TNF- α levels induced by BB12 administration inhibits DSS-induced apoptosis in IECs.

Of note, TNF- α promotes IEC apoptosis by death receptor-dependent caspase-8 activation [25]. Thus, we measured caspase-8 activity by evaluating TNF- α production in the colon tissues of DSS-induced colitis in the presence or absence of BB12. As indicated by the increase in TNF- α level, caspase-8 activity in the colon tissues of DSS-treated mice was higher than that in the colon tissues of control mice (Figs. 3B and 3C). However, the increase in expression of the key proteins involved in TNF- α -mediated IEC apoptosis was significantly reduced following BB12 administration, as compared with control (Figs. 3B and 3C). Collectively, these results demonstrated that BB12 is capable of inhibiting DSS-induced apoptosis of IECs by down-regulating TNF- α production.

In summary, in this study, we showed that BB12 administration alleviates susceptibility to DSS-induced colitis with a concomitant decrease in the apoptotic death of IECs, presumably by suppressing TNF- α production (Fig. 3D; proposed model). The present study supports the

Fig. 3. Effect of BB12 on TNF- α -mediated IEC apoptosis.

(A) Representative immunohistochemical staining for TNF- α in the colon tissues of DSS-induced colitis mice (magnification, $\times 200$). (B) Graphical representation of the number of TNF- α -positive cells in the colon. (C) Immunohistochemical analysis of caspase-8 expression in the colon sections (magnification, $\times 200$). (D) Graphical representation of the fold change of caspase-8-positive cells in the colon. Data are shown as mean $\pm$ SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$ compared with DSS group.

rationale for BB12 as a potential therapeutic agent in the treatment of IBD, particularly UC.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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