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Black Raspberries Inhibit Intestinal Tumorigenesis in *Apc1638+/-* and *Muc2-/-* Mouse Models of Colorectal Cancer

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Abstract

Freeze-dried black raspberries (BRBs) produce chemopreventive effects in a rat model of colon carcinogenesis, however, the mechanisms of inhibition were not determined. Herein, we used two mouse models of human colorectal cancer to determine if dietary BRBs would inhibit colorectal tumor development and to investigate the underlying mechanisms. We found that a 12-week feeding of BRBs significantly inhibited intestinal tumor formation in both models; reducing tumor incidence by 45% and tumor multiplicity by 60% in Apc1638+/- mice and tumor incidence and multiplicity by 50% in Muc2-/- mice. Mechanistic studies revealed that BRBs inhibit tumor development in Apc1638+/- mice by suppressing β- catenin signaling and in Muc2-/- mice by reducing chronic inflammation. Intestinal cell proliferation was inhibited by BRBs in both animal models, however, the extent of mucus cell differentiation was not changed in either model. Collectively, our data suggest that BRBs are highly effective in preventing intestinal tumor development in both Apc1638+/- and Muc2-/- mice through targeting multiple signaling pathways.

Keywords

black raspberries; colon cancer; β-catenin; chronic inflammation

Introduction

Colorectal cancer (CRC) is the second-leading cause of cancer-related death in men and the third leading cause of cancer death in women in the United States (1). It is also the second most prevalent cancer worldwide. Risk factors for developing CRC include: hereditary predisposition to either familial adenomatous polyposis (FAP) or non-polyposis colon cancer (HNPCC), obesity, physical inactivity, smoking, alcohol consumption, and an inadequate intake of vegetables and fruit (2,3). An important strategy for prevention of CRC is endoscopic screening to detect and remove precursor lesions (adenomatous polyps) and to detect early stage carcinomas. Unfortunately, screening compliance is unacceptably low. Moreover, the currently used chemotherapies for metastatic disease are largely ineffective due to dose-limiting toxicity and acquired chemoresistance, thus, survival rates are poor for patients with metastatic disease.

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Chemoprevention refers to the administration of synthetic or naturally-occurring agents to block, reverse, or delay the process of carcinogenesis. For a variety of reasons, including human acceptance and low toxicity, naturally-occurring, diet-based, agents are ideal for chemopreventive interventions. Berry fruits are widely consumed in our diet and have attracted much attention due to their potential human health benefits. Multiple studies have shown that black raspberries (BRBs, *Rubus occidentalis*) exhibit diverse biological properties including antioxidant, anti-cancer, anti-neurodegenerative and anti-inflammatory activities (4-6). To determine whether BRBs could be useful for the prevention of colorectal cancer, we administered a Western-style diet containing 10% freeze-dried BRBs to two mouse models of human CRC, i.e., *Apc*1638+/- mice and Muc2 -/- mice for 12 weeks. The *Apc* mouse has one functional allele of the Apc gene that, when inactivated, leads to inappropriate signaling of the Wnt/β-catenin pathway and the spontaneous development of intestinal adenomas (7). Muc2-/- mice develop intestinal adenomas and adenocarcinomas in response to chronic inflammation (8,9). We found that BRBs inhibit intestinal tumor formation in both animal models, and that tumor inhibition was associated with a decrease in β-catenin-induced signaling in *Apc* mice and the inhibition of chronic inflammation in Muc2-/- mice.

Materials and Methods

Animals and Diets

The development of the Apc1638+/- and Muc2-/- mouse models and the methods for genotyping these mice have been described (7-10). After weaning (approximately 3–4 weeks), littermates of both mouse strains were randomized to dietary groups and fed either a Western-style (control) diet (11,12) or the Western-style diet supplemented with 10% (w/w) freeze-dried black raspberry powder (berry diet). The berry powder was mixed into the Western-style diet using a Hobart mixer as described before (13). The Western style diet was formulated on the basis of nutrient density to mimic major risk factors for colon cancer (high in fat and phosphate, and low in calcium and vitamin D). The cornstarch in the berry diet was reduced by 10% to maintain an isocaloric diet. Both the control and berry diets were stored at -20 °C before use in the experiment. All animals were housed in plastic cages with filter tops; 5 mice per cage. The animal room was controlled at 23 ± 1 °C, 50 ± 10% humidity and a 12 h light/dark cycle. Animals had free access to food and water at all times. Food cups were replenished with fresh diets twice weekly. The animals were housed and maintained in accordance with the recommendations of the UIC Animal Use Committee and the American Association of Laboratory Animal Care.

The Western-style diet was purchased from Research Diets, Inc. (New Jersey), and the freeze-dried BRB powder was supplied by G. Stoner of the Ohio State University Comprehensive Cancer Center. The protocol for procurement, freeze-drying, chemical and microbial characterization, and storage of BRB powder has been described in detail (14).

Histopathology

After 12 weeks of consuming either the control or berry diet, all animals of both mouse strains were sacrificed by CO_2 inhalation followed by cervical dislocation. The entire intestinal tract was removed and opened longitudinally. The contents of the intestine were removed by washing with cold PBS. The full length of the intestinal tract was immediately examined for neoplastic lesions under a dissecting microscope. Tumor location (small intestine or large intestine), incidence (percentage of mice with tumor), multiplicity (number of tumors per mouse) and size (tumor volume) were recorded. Tumors were fixed in 10% buffered formalin. Two fragments, each 0.8-1.0 cm in length, of normal appearing tissue from both the duodenum and colon were placed separately into 10% buffered formalin, or

snap frozen in liquid nitrogen. Formalin-fixed paraffin-embedded tissues were used for histopathologic (H&E staining) and immunohistochemical staining. Frozen tissues were used to prepare frozen sections for histopathology or for biochemical studies.

Proliferative cell nuclear antigen (PCNA) staining was used to evaluate cell proliferation. PCNA-stained and unstained epithelial cells from about 25 well-oriented crypts per intestine per mouse in both diet groups were counted, and the percentage of PCNA positive cells was calculated. Alcian blue staining was performed to evaluate goblet cell differentiation. The percentage of Alcian blue positive cells in the intestinal crypts of each mouse was determined in the same manner as for PCNA-positive cells. All procedures, including evaluation of tumorigenesis, PCNA and Alcian blue staining and scoring, have been standardized in our laboratory as described previously (10,15-19).

Intestinal epithelial cell isolation and quantitative real-time RT-PCR—Intestinal epithelial cells from both mouse strains were isolated by incubating opened mouse intestine from colon or from the combination of duodenum and jejunum, respectively, in 15 mM EDTA buffer at 37°C for 30 min as described previously (15). Total RNA was extracted from epithelial cell pellets obtained from three individual mice per strain per diet group. The quality and quantity of RNA was determined using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE). Quantitative real-time PCR analysis was performed using the ABI Prism 7900-HT sequence detection system (96 well, Applied Biosystems, Foster City, CA) as described (15). The following primers were designed for mouse cytokine analysis and were synthesized by Sigma Oligo (St. Louis, MO): COX-2; forward 3′-TGAGCAACTATTCCAAA CCAGC-5′ and reverse 3′-GCACGTAGTCTTCGATCACTATC-5′; TNF-α, forward 3′ CCCTCACACTCAGATCATCTTCT-5′ and reverse 3′-GCTACGACGTGGGCTACAG-5′; interleukin 1 (IL-1), forward 3′- GCAACTGTTCCTGAACTCAACT-5′ and reverse 3′-ATCTTTTGGGGTCCGTCAACT-5′; interleukin 6 (IL-6), forward 3′- TAGTC CTTCCTACCCCAATTTCC-5′ and reverse 3′- TTGGTCCTTAGCCACTCCTTC-5′; interleukin 10 (IL-10), forward 3′- GCTCTTACTGACTGGCATGAG-5′ and reverse 3′-CGCAGCTCTAGGAGCATGTG-5′. β-actin was used as internal control (15).

Western blotting analyses and immunohistochemical staining

In addition to RNA, protein was extracted from each of the above-described intestinal epithelial cell pellets. In brief, the pellets were washed twice with ice-cold PBS and incubated on ice for 15 min in cell lysis buffer (Cell Signaling, Beverly, MA). After brief sonication, the cell lysate was centrifuged at 13, 500 rpm for 15 min at 4°C. The supernatant was fractionated by electrophoresis in a 12% sodium dodecyl sulfate–polyacrylamide gel and transferred to a nitrocellulose membrane. The following primary antibodies were utilized for immunoblotting: anti-p21 (1:500), anti-p27 (1:1000), anti-β-catenin(1:1000), anti-E-cadherin(1:1000), anti-c-myc(1:1000), anti-cdk4 (1:1000) and anti-cyclin D1(1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-actin(1:10000) (Sigma, St Louis, MO). Signals were detected by an enhanced chemiluminescence technique (Amersham Life Science, Piscataway, NJ).

Protein expression in the intestine was also analyzed by immunohistochemistry, as we described previously (15). Briefly, formalin-fixed and paraffin-embedded mouse intestinal tissues were sectioned, de-paraffinized and dehydrated. Five animals were analyzed from each of the control and berry diet groups. To block endogenous peroxidase, the sections were incubated in H_2O_2 (0.3%) /methanol for 20 min. Sections were then incubated with 10% normal goat serum to block non-specific antibody binding. To expose epitopes for immuno-detection, sections were treated in a steamer for 20 min in citrate buffer (pH 6.1).

The procedure for PCNA staining was as described by the manufacturer, and the percentage of PCNA stained cells determined as described above.

Statistical analysis—Chi-square was used for tumor incidence analysis, and the Student's *t* test was used for tumor multiplicity and other quantification (including PCNA scoring and qRT-PCR) analysis. $P<0.05$ indicated significant difference.

Results

Dietary BRBs inhibit intestinal tumorigenesis in Apc1638+/- mice

After 12 weeks of treatment with the control and BRB diets, all Apc1638+/- mice were sacrificed and their intestinal tumors were quantitated. 100% (11/11) of the Apc1638+/- mice fed the Western-style control diet developed small intestinal tumors with an average of 2.33 tumors per mouse (Fig. 1). However, only 55% (6/11) of mice fed the 10% BRB-supplemented diet developed intestinal tumors, and the tumor multiplicity was reduced significantly ($p<0.01$) to 0.91 tumors per mouse (Fig. 1). The tumor sizes were slightly reduced by BRBs in the Apc1638+/- mice, but the reduction was not significant (data not shown), which could be due to short-term (12 weeks) feeding.

Dietary BRBs inhibit tumor formation in Muc2-/- mice

Unlike Apc1638+/- mice, Muc2-/- mice developed tumors throughout the entire intestine, principally in the colon and rectum. As shown in Figure 2, Muc2-/- mice fed the control diet, 44% (8/18) developed small intestinal tumors and 100% (18/18) developed colon and rectal tumors. However, feeding the BRB diet reduced the incidence of small intestinal tumors about 30% (44% in Western-style diet vs 14% (3/21) in BRB-diet) and the multiplicity from 0.67 to 0.14 tumors per mouse ($p<0.05$). Similarly, the colon tumor incidence was decreased by 24% (100% vs 76% (16/21)), and the multiplicity was reduced remarkably from 3.83 to 1.60 tumors per mouse ($p<0.001$). The multiplicity of rectal tumors was reduced dramatically from 1.0 to 0.37 per mouse ($p<0.001$), and the rectal tumor incidence by about 62%, from 100% (18/18) to 38% (8/21). The difference of tumor sizes in the control and BRBs groups were not significantly either in the Muc2-/- mice (data not shown).

Tumor inhibition by BRBs is linked to reduced intestinal cell proliferation and not differentiation

We investigated the effects of BRBs on intestinal cell proliferation in both mouse strains. The duodenum, the principal site of tumor formation in Apc1638+/- mice, and the colon, the major site of tumor formation in Muc2-/- mice were stained for PCNA. BRBs significantly inhibited intestinal cell proliferation in the duodenum of Apc1638+/- mice (Fig. 3A) and in the colon of Muc2-/- mice (Fig. 3B). However, the berry diet did not alter intestinal cell differentiation as measured by Alcian Blue staining for goblet cell identification (data not shown).

BRB-induced tumor inhibition in Apc1638+/- mice is associated with reduced β-catenin signaling and a modest suppression of cell cytokines

Aberrant β-catenin expression is a common feature of APC mutation-induced tumorigenesis in the small intestine of Apc+/- mice and in human colorectal cancer (20-22). We determined, therefore, the expression levels of β-catenin and its downstream targets in normal intestinal mucosa of Apc**1638**+/- mice fed either the control or 10% BRB diet. Proteins extracted from mouse intestinal epithelial cells were evaluated by Western blotting. The level of β -catenin was dramatically decreased, and c-Myc and cyclin D1, both downstream of β-catenin, were modestly decreased in BRB-fed mice. In contrast, the cyclin-

dependent kinase inhibitor, p27kip1, was increased in BRB-fed mice when compared to mice fed the control diet (Fig. 4 A).

To determine whether BRBs produced anti-inflammatory effects in Apc**1638**+/- mice, we used quantitative RT-PCR to evaluate changes in a set of inflammatory biomarkers in intestinal epithelial cells. The mRNA levels of cyclooxygenase-2 (COX-2) and of the pro-inflammatory cytokines: tumor necrosis factor-alpha, interleukin-6 and interleukin-10 were modestly decreased, whereas IL-1 was slightly increased (Fig. 4B). None of these changes were significant.

BRB-induced tumor inhibition in Muc2-/- mice is associated with inhibition of chronic inflammation but not with β-catenin signaling

We have reported that tumor formation in Muc2-/- mice is associated with chronic inflammation; β-catenin is not involved (8,9). To evaluate the effects of BRBs on inflammation in Muc2-/- mice, we determined the mRNA expression levels of cytokines in the supernatants of whole colonic epithelial cells. COX-2 and all pro-inflammatory cytokines, i.e., tumor necrosis factor-alpha, and interleukins-1, 6 and 10, were significantly decreased by BRBs ($p<0.05$ or $p<0.01$) (Fig. 5A).

We also investigated changes in proteins involved in β-catenin signaling in both the small intestine and colon of Muc2-/- mice. Unlike in Apc**1638**+/- mice, the β-catenin signaling pathway in the small intestine of Muc2-/- mice was not influenced by berry treatment (Fig. 5B). However, E-cadherin and another cyclin-dependent kinase inhibitor, $p21^{WAF1/Cip1}$, were induced by BRBs in the colon of Muc2-/- mice (Fig. 5C).

Discussion

Black raspberries are known to contain multiple compounds with chemopreventive potential (4,5,13,14). Previous studies have demonstrated the protective effects of dietary freeze-dried BRBs on the occurrence of chemically-induced tumors in rodents, including tumors of the colon (4,14,23). The molecular events involved in the inhibition by BRBs of chemically-induced tumorigenesis in the rat esophagus have been investigated in detail (4,5,24-28) however, there is very little mechanistic information at the molecular level for BRBs in the rodent colon (23). Using two distinctive mouse models, Apc1638+/- and Muc2-/- mice, each of which has its unique molecular events for study, we found that dietary BRBs were effective in inhibiting intestinal tumorigenesis. In Apc1638+/- mice, this inhibition likely occurred through effects of the berries on aberrant β-catenin signaling, whereas, in Muc2-/- mice, it was linked to inhibition of chronic inflammation and increases in the expression of E-cadherin and p21. Collectively, these data suggest that BRBs produce a broad range of protective effects in the intestine, colon and rectum. This is in agreement with our previous investigations in which dietary BRBs were shown to produce a genome-wide protective effect on the expression of genes associated with the development of tumors in carcinogen-treated rat esophagus (24,28).

Mutations in the Apc gene are frequently observed in human colorectal cancers and β-catenin activation is known to be an early event of APC mutation-initiated colorectal carcinogenesis. Apc mutations in the mouse lead to the accumulation of cytoplasmic β-catenin, its subsequent nuclear translocation, and the resultant activation of aberrant TCF4-c-Myc signaling. Thus, Wnt/β-catenin signaling is a promising target for chemoprevention and chemotherapy of colorectal cancer (29,30). Our previous studies have demonstrated that sulindac, a non-steroidal anti-inflammatory drug, and the naturally-occurring compound, 20(S)-25-OCH3-PPD, derived from the leaves of *Panax notoginseng*, both inhibit human colorectal cancer cell proliferation and promote apoptosis by targeting the Wnt/β-catenin

signaling pathway (31,32). Here, we demonstrate that BRBs also target β-catenin signaling in Apc1638+/- mice, intriguingly, as shown in Fig.4A, β-catenin protein levels in non-tumor epithelial cells were upregulated by the Western high-risk diet (9,33) and were driven back to normal levels by BRBs. Our data suggest that suppression of the Wnt signaling pathway is another mechanism by which BRBs inhibit tumorigenesis.

Muc2-/- mice spontaneously develop intestinal adenomas that frequently progress to invasive adenocarcinomas when the animals reach 6 months to one year in age. Tumor development in the intestine of Muc2-/- mice is associated with activation of inflammation-related pathways (8,9). Recent studies in our laboratory indicate that the Western-style diet markedly accelerates colorectal cancer formation in Muc2-/-, mice; i.e., tumors arise within 3 months (Fang and Yang et al, unpublished data). Herein, we found that BRBs significantly inhibit colorectal tumor formation in Muc2-/- mice fed the Western style diet. The berries dramatically decreased mRNA expression levels of the pro-inflammatory cytokines, IL-1, IL-6, IL-10, TNF-α and COX-2. Interestingly, E-cadherin and p21 were significantly increased in the colon, and β-catenin was not changed. The differences of BRBs inhibition of β-catenin in the two mouse models could be caused by differential interaction between BRBs and the Apc or Muc2 genes. Whether there is a gene-diet interaction or how this interaction affects the efficacy of BRB tumor inhibition is under investigation.

E-cadherin, a protein that mediates cell–cell adhesion through calcium-dependent homophilic interactions in the extracellular domain, plays an important role in epithelial–mesenchymal transition (EMT). p21 has been shown to regulate E-cadherin (34), but the mechanism(s) of its regulation are not clear. It is well known that E-cadherin binds to β-catenin, and the E-cadherin/β-catenin complex plays an important role in cell proliferation and differentiation during development (35). The observation that E-cadherin, but not β-catenin, was increased in the intestine of berry fed animals in the present study could have been due to the formation of a complex between β-catenin and E-cadherin. Such a complex could limit the translocation of β-catenin from the cell membrane into the nucleus, resulting in the modest effects we observed of the berry diet on c-Myc and cyclin D1 expression levels in the intestine.

EMT occurs at the invasive front of tumors, the same site where tumors are infiltrated by tumor associated macrophages. The decreased expression of macrophage associated TNF-α in berry fed animals could result in the stabilization of Snail, a transcription factor that represses the expression of E-cadherin (36,37). Currently, we are investigating whether the enhanced E-cadherin expression in berry fed animals is the result of a decreased stability of Snail mediated by TNF-α.

In summary, the present study shows that dietary black raspberries are effective in inhibiting intestinal tumor formation in the Apc1638+/- and Muc2-/- mouse models of intestinal and colorectal cancer. Tumor inhibition is associated with reduced cell proliferation, suppression of the β-catenin signaling and chronic inflammation pathways, and increasing cellular E-cadherin. Our data add importantly to the existing knowledge regarding the molecular mechanisms of BRB-mediated chemoprevention, and provide additional rationale for assessing the potential chemopreventive effects of BRBs for human colorectal cancer.

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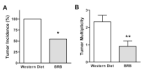

Fig. 1.

BRBs significantly inhibit intestinal tumor incidence (A) and multiplicity (B) (mean±SD) in Apc1638+/- mice at 12 weeks. (* $p<0.05$, ** $p<0.01$, compared to the mice fed a Western-style diet. 11 mice per group were studied.).

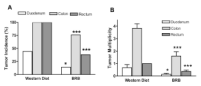

Fig. 2.

BRBs significantly inhibit intestinal tumor incidence (A) and multiplicity (B) (mean±SD) in Muc2-/- mice at 12 weeks. (*p<0.05, ***p<0.001, compared to the mice fed a Western-style diet. There were 18 mice in the Western-style diet group and 21 mice in the BRBs groups, respectively.).

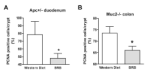

Fig. 3.
BRBs inhibit intestinal cell proliferation (assayed by PCNA staining) in the Apc1638+/- mouse small intestine (A) and the Muc2-/- mouse colon (B). (*p<0.05, compared to the mice fed a Western-style diet).

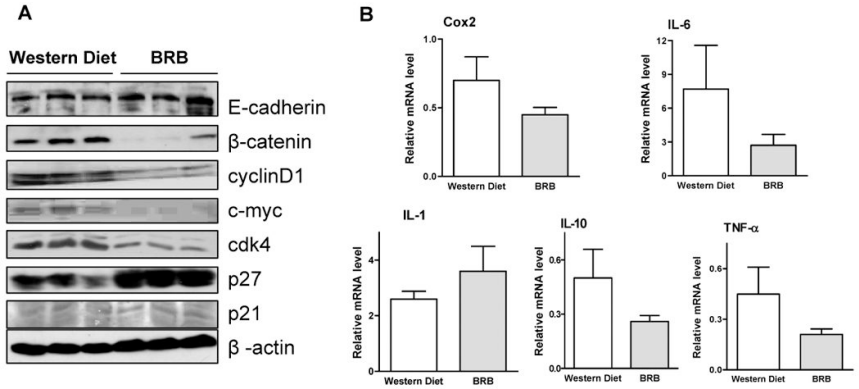

Fig. 4.

Alterations in β-catenin signaling (A) and inflammatory factors (B) (mean±SD) in Apc+/- mice by BRBs as assayed by Western blotting and real-time RT-PCR, respectively. Protein and RNA were extracted from intestinal epithelial cells from three individual mice per group.

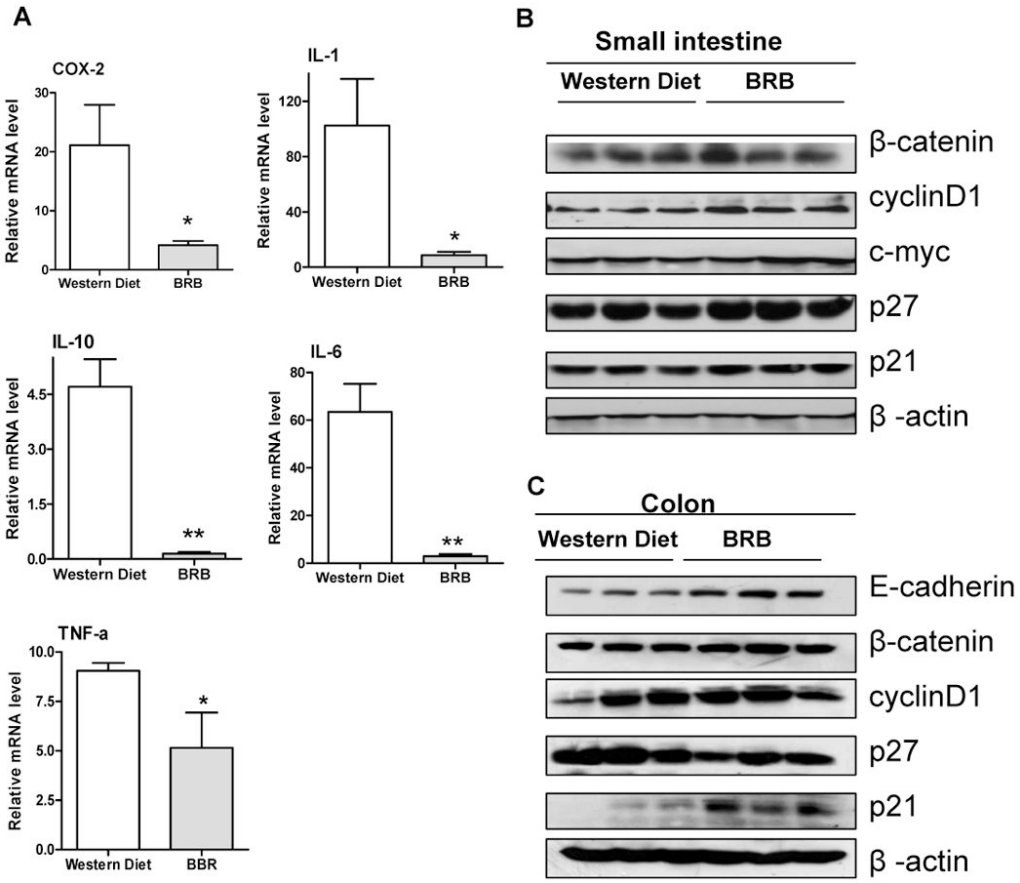

Fig. 5.

Alterations in inflammatory factors (A) (mean±SD) and molecular changes (B,C) by BRBs in Muc2-/- mice analyzed by real-time RT-PCR and Western blotting, respectively. RNA was extracted from colon tissues, and the protein for Western-blotting was extracted from small intestinal epithelial cells (B) and colonic epithelial cells (C) from three individual mice of each group. (*p<0.05, **p<0.01, compared to the mice fed a Western-style diet).