Anti-inflammatory activity of probiotic *Bifidobacterium*: Enhancement of IL-10 production in peripheral blood mononuclear cells from ulcerative colitis patients and inhibition of IL-8 secretion in HT-29 cells

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Abstract

AIM: To determine the anti-inflammatory activity of probiotic *Bifidobacterium* in *Bifidobacteria*-fermented milk (BFM) which is effective against acute ulcerative colitis (UC) and exacerbations of UC, and to explore the immunoregulatory mechanisms.

METHODS: Peripheral blood mononuclear cells (PBMC) from UC patients or HT-29 cells were co-cultured with heat-killed probiotic bacteria or supernatant of *Bifidobacterium breve* strain Yakult (BbrY) or *Bifidobacterium bifidum* strain Yakult (BbiY) to estimate the amount of IL-10 or IL-8 secreted.

RESULTS: Both strains of probiotic *Bifidobacteria* contained in the BFM induced IL-10 production in PBMC from UC patients, though BbrY was more effective than BbiY. Conditioned medium (CM) and DNA of both strains inhibited IL-8 secretion in HT-29 cells stimulated with TNF-α, whereas no such effect was observed with heat-killed bacteria. The inhibitory effect of CM derived from BbiY was greater than that of CM derived from BbrY. DNAs of the two strains had a comparable inhibitory activity against the secretion of IL-8. CM of BbiY induced a repression of IL-8 gene expression with a higher expression of IL-8 mRNA 4 h after culture of HT-29 cells compared to that in the absence of CM.

CONCLUSION: Probiotic *Bifidobacterium* strains in BFM enhance IL-10 production in PBMC and inhibit IL-8 secretion in intestinal epithelial cells, suggesting that BFM has anti-inflammatory effects against ulcerative colitis.

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Key words: *Bifidobacterium*; Ulcerative colitis; Anti-inflammatory; Interleukin-10; Interleukin-8

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INTRODUCTION

Ulcerative colitis (UC) is assumed to be a result of impaired intestinal immune response to intestinal environmental antigens such as ubiquitous microflora [1-2]. There is evidence that probiotic treatment is effective against UC [3-6]. We have previously shown that treatment with bifidobacteria-fermented milk (BFM) containing live bifidobacteria is more effective than the usual treatment in inducing remission of ulcerative colitis. An improvement of the composition of intestinal flora by BFM may prevent the overgrowth of potentially pathogenic bacteria such as *Bacteroides vulgatus*, but we hypothesized that probiotic strains of bifidobacteria in BFM may directly interfere with the host signaling events that drive the intestinal inflammatory response. In inflammatory bowel disease, IL-10 is a cytokine of particular therapeutic interest since it has been shown in animal models that interleukin (IL)-10(-/-) mice spontane-
ously develop intestinal inflammation. IL-10 plays a key role in the control of inflammatory responses to enteric organisms\cite{9-12}. More recently, it has been shown in animal models that probiotic strains displaying an in vitro potential to induce higher levels of the anti-inflammatory cytokine IL-10 and lower levels of the inflammatory cytokine IL-12, offer the best protection against in vivo colitis in the model\cite{13}. A genetically engineered \textit{Lactobacillus lactis lty12} producing IL-10 ameliorated colitis in two models of experimental colitis, providing proof of principal that topically delivers IL-10, can be therapeutically efficacious\cite{14} and a recent proof-or-principle experiment using this transgenic bacterium expressing IL-10 in 10 patients with Crohn’s disease showed efficacy\cite{15}. In addition, there is an increasing amount of evidence to suggest that the potent neutrophil chemoattractant, IL-8, has an important role in the pathogenesis of inflammatory bowel disease (IBD)\cite{16-17}. Recently, a higher concentration of IL-8 was found in more histologically inflamed tissue segments from pediatric IBD patients, suggesting that IL-8-specific therapies may universally modify the inflammatory activity in IBD patients\cite{18}. In this study, we focused on the effect of probiotic strains on the secretion of IL-10 by peripheral blood mononuclear cells and also the production of IL-8 by intestinal epithelial cells.

\section*{MATERIALS AND METHODS}

\subsection*{Bacteria and related preparations}
\textit{Bifidobacterium bifidum} strain Yakult (BbiY) and \textit{Bifidobacterium breve} strain Yakult (BbrY) were grown in MRS broth (Becton, Dickinson and Company, Sparks, MD). Heat-killed BbiY or BbrY was prepared by heating bacteria re-suspended in distilled water at 100°C for 30 min, and then lyophilized\cite{23}. For the preparation of conditioned medium (CM), bacteria grown in MRS broth were collected by centrifugation and cultured over 16 h in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO) containing 10% fetal calf serum (FCS) and 2% lactose, then centrifuged\cite{23}. The supernatant was filtered on a 0.22 μm membrane and neutralized with sodium hydroxide. To characterize the active component in CM, it was separated into fractions of more than and less than 3 kDa through Centricon YM-3 (Millipore, Bedford, MA), adjusted to the initial volume, and then to heat treatment at 100°C for 15 min\cite{23}. DNA was isolated using the method of Yuki\cite{23} with a slight modification. Briefly, bacterial cells were suspended in Tris-EDTA buffer (pH 8.0) containing 0.5 mol/L sucrose and treated with N-acetylmyramidase SG (Seikagaku Corp., Tokyo, Japan) and lysozyme (Sigma-Aldrich) at 37°C for 1 h. The cells were lyed by addition of sodium dodecyl sulfate and protease K (Sigma-Aldrich) followed by a 60-min incubation at 65°C. Deproteinization was done by extraction with Tris-saturated phenol and phenol/chloroform/isoamyl alcohol (25:24:1). Finally, DNA was precipitated by ethanol.

\subsection*{Peripheral blood mononuclear cells}
Peripheral blood mononuclear cells (PBMCN) were isolated from peripheral blood of UC patients by Ficoll-Conrad (Lymphosepae I; Immuno-Biological Laboratories, Takasaki, Japan) density gradient centrifugation. Table 1 summarizes the patient characteristics. All 9 patients (outpatient) had active UC which was moderate or mild (1 mild, 8 moderate) according to the criteria of Truelove & Witts\cite{20}. All patients received a standard therapeutic regimen consisting of oral 5-ASA (mesalazine) and five of the 9 patients with active UC took a low dose of oral predonine. Cells (2 × 10⁶) were cultured with heat-killed bacteria (10 μg/mL) in 200 μL of AIM-V medium (Invitrogen Corp., Carlsbad, CA) in a flat-bottomed 96-well culture plate (Nunc, Roskilde, Denmark) for 48 h. Supernatant was collected and frozen until cytokine levels were quantified. In each assay, a positive control with lipopolysaccharide (LPS, 10 μg/mL) added to PBMCN and a negative control with no stimuli were included.

\section*{Quantification of cytokine levels in culture supernatant}
IL-10 and IL-8 levels in the culture supernatant were determined by sandwich enzyme-linked immunosorbent assay (ELISA). IL-10 was detected using anti-human IL-10 antibody (51-26171E, BD Biosciences PharMingen, Franklin Lakes, NJ) and biotinylated antibody (51-26172E, BD Biosciences PharMingen). IL-8 was detected using anti-human IL-8 antibody (AHC0932, Invitrogen BioSource) and biotinylated anti-human IL-8 antibody (AHC0789, Invitrogen BioSource).

\subsection*{HT-29 cell culture}
HT-29 cells were cultured at 37°C in an atmosphere containing 50 mL/L CO₂ in RPMI-1640 medium containing 10% FCS, 1 mmol/L sodium pyruvate (Invitrogen) and 10 mmol/L 7-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Confluent monolayers were incubated in 96-well or 24-well tissue culture plates with human TNF-α (10 ng/mL, PeproTech, London, UK).

\subsection*{Quantitative RT-PCR}
Total RNA was isolated from HT-29 cells using the TRizol reagent (Invitrogen) according to the manufacturer’s instructions. The RNA was reverse transcribed into cDNA using a SuperScript First-Strand Synthesis System kit (Invitrogen). Real-time quantitative PCR was performed in an ABI prism 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) with the SYBR Premix Ex Taq (Takara Bio, Shiga, Japan). The following primers were used to amplify cDNA fragments: IL-8: forward: 5'-ACACTGCGCAAACACAGAAGTA-3', reverse: 5'-TTTGCTTGGAGTTCCTGACATC-3'; GAPDH: forward: 5'-GCACCGTCAAGGCTGAGAAC-3', reverse: 5'-ATGGTGTTGAGAAGCCAGGT-3'; β-actin: forward: 5'-ACGGCACAACAGTACCCGAG-3'; reverse: 5'-CT-CAGGACGCAACAGCACAAGATC-3'. All results were finally determined after correction with GAPDH expression.

\section*{Statistical analysis}
Results were expressed as mean ± SD. Differences in mean values between groups were analyzed with Student’s t-test.

\section*{RESULTS}

\subsection*{Effect of probiotic bacteria on IL-10 secretion in PBMCN}
The secretion of IL-10 increased in all the cultures of
PBMNC isolated from the nine UC patients with a historical record of the treatment as shown in Table 1 in the presence of heat-killed BbrY or BbiY, compared to the basal secretion in the absence of probiotic bacteria (Figure 1). BbrY induced significantly higher levels of IL-10 than BbiY in 8 out of the 9 PBMNC preparations. Incubation with BbrY also elicited significantly higher levels of IL-10 than that with LPS (10 μg/mL) in 6 out of the 9 PBMNC preparations.

Table 1  Characteristics of the patients with ulcerative colitis

| Patient No. | Age | Sex | Disease extent | Clinical pattern | Disease duration (yr) | Disease severity | Treatment |
|-------------|-----|-----|----------------|-----------------|----------------------|-----------------|-----------|
| 1           | 23  | Male| Total          | Relapsing       | 6                    | Moderate        | +         |
| 2           | 38  | Female| Total        | Relapsing       | 2                    | Moderate        | +         |
| 3           | 32  | Male| Total          | Relapsing       | 2                    | Moderate        | +         |
| 4           | 36  | Female| Proctitis     | Relapsing       | 15                   | Mild            | +         |
| 5           | 23  | Male| Total          | First           | 3                    | Moderate        | +         |
| 6           | 20  | Male| Total          | Relapsing       | 2                    | Moderate        | +         |
| 7           | 25  | Male| Total          | First           | 0.5                  | Moderate        | +         |
| 8           | 42  | Male| Left-sided     | Chronic         | 7                    | Moderate        | +         |
| 9           | 36  | Male| Left-sided     | Relapsing       | 14                   | Moderate        | +         |

SASP: Salazosulphapyridine.

Effect of probiotic Bifidobacterium on IL-8 secretion in TNF-α-stimulated HT-29 cells

HT-29 cells were incubated with TNF-α for six hours in the presence or absence of heat-killed BbiY and BbrY. Neither of the heat-killed probiotic bacteria had an effect on the secretion of IL-8 at the concentration ranging from 1 μg/mL-100 μg/mL (Figure 2).

Next, we examined the effect of conditioned medium (CM) on IL-8 secretion by HT-29 cells. Both CMs of BbiY and BbrY inhibited TNF-α-induced secretion of IL-8 in a dose-dependent manner (Figure 3). Concentrations of BbiY and BbrY cultured over 16 h in RPMI-1640 medium were 3.6 × 10^8 CFU/mL and 2.2 × 10^8 CFU/mL, respectively.

Effect of probiotic Bifidobacterium on IL-8 secretion in probiotic-derived CM

HT-29 cells were incubated with TNF-α for six hours in the presence or absence of heat-killed BbiY and BbrY. Neither of the heat-killed probiotic bacteria had an effect on the secretion of IL-8 at the concentration ranging from 1 μg/mL-100 μg/mL (Figure 2).

Next, we examined the effect of conditioned medium (CM) on IL-8 secretion by HT-29 cells. Both CMs of BbiY and BbrY inhibited TNF-α-induced secretion of IL-8 in a dose-dependent manner (Figure 3). Concentrations of BbiY and BbrY cultured over 16 h in RPMI-1640 medium were 3.6 × 10^8 CFU/mL and 2.2 × 10^8 CFU/mL, respectively.

Nature of the inhibitory effect on IL-8 secretion in probiotic-derived CM

To investigate the nature of this soluble factor, BbiY-derived CM was subjected to molecular sieve and heat treatment. The fractions of less than and more than 3 kDa both retained the inhibitory activity toward the secretion of IL-8 in HT-29 cells, though the former fraction was greater than the latter one (Figure 4). The inhibitory effect of the latter fraction but not the former one was diminished by heat-treatment (data not shown). We checked the effect of acetic and lactic acid with their major constituents in less than 3 kDa fraction. Acetic acid but not lactic acid inhibited the IL-8 secretion in HT-29 cells, when added at the same
The efficacy of bifidobacteria-fermented milk (BFM) in the treatment of ulcerative colitis has been reported elsewhere. To determine the anti-inflammatory activity of probiotic Bifidobacterium strains in the BFM, we firstly investigated the effects of these strains on the production of anti-inflammatory cytokine IL-10 by PBMNC isolated from UC patients in vitro. IL-10 down regulates the TNF-α secretion by macrophages. IL-10 knockout mice develop IBD under conventional conditions, indicating the importance of IL-10 in the control of intestinal inflammation. It was reported that intragastric administration of IL-10-secreting Lactococcus lactis causes a 50% reduction in colitis in mice treated with dextran sulfate sodium and BFM containing BbiY and BbrY ameliorates IBD in SAMP1/Yit mice with an up-regulation of IL-10 synthesis and down-regulation of IFN-γ production in mesenteric lymph node cells. In this study, the two heat-killed probiotic bacterial strains in BFM induced the secretion of IL-10 in PBMNC isolated from UC patients.

**DISCUSSION**

The efficacy of bifidobacteria-fermented milk (BFM) in the treatment of ulcerative colitis has been reported elsewhere. To determine the anti-inflammatory activity of probiotic Bifidobacterium strains in the BFM, we firstly investigated the effects of these strains on the production of anti-inflammatory cytokine IL-10 by PBMNC isolated from UC patients in vitro. IL-10 down regulates the TNF-α secretion by macrophages. IL-10 knockout mice develop IBD under conventional conditions, indicating the importance of IL-10 in the control of intestinal inflammation. It was reported that intragastric administration of IL-10-secreting Lactococcus lactis causes a 50% reduction in colitis in mice treated with dextran sulfate sodium and BFM containing BbiY and BbrY ameliorates IBD in SAMP1/Yit mice with an up-regulation of IL-10 synthesis and down-regulation of IFN-γ production in mesenteric lymph node cells. In this study, the two heat-killed probiotic bacterial strains in BFM induced the secretion of IL-10 in PBMNC isolated from UC patients.
It has been found that the degree of local inflammation and tissue damage in patients with IBD is dependent on the local expression of specific chemokines within affected tissues. IL-8 and IL-2 are elevated in ileal tissue and lymph nodes of patients with IBD. The exact role of these cytokines in the pathogenesis of IBD is not fully understood. However, there is evidence that these cytokines may contribute to the development of inflammation and tissue damage in IBD.

**Innovations and breakthroughs**

This study has shown that Bifidobacterium strains proved to be beneficial in inducing and maintaining remission of ulcerative colitis exhibit regulatory activities that contribute to the control of intestinal inflammation, thus unravelling the biological basis of the beneficial effects of probiotics in human disease.

**Applications**

Anti-inflammatory activity of probiotic bifidobacteria shown in this study supports the beneficial effects of BFM on in clinical trials. Therapeutic applications of BFM in the treatment of UC are promising.

**Peer review**

Probiotic Bifidobacterium strains in BFM that proved to be beneficial in inducing and maintaining remission of UC enhanced IL-10 production in PBMNC and inhibited TNF-α-induced secretion of IL-8 by HT-29 cells. This study has shown that Bifidobacterium strains that proved to be beneficial in inducing and maintaining remission of ulcerative colitis exhibit regulatory activities that contribute to the control of intestinal inflammation, thus unravelling the biological basis of the beneficial effects of probiotics in human disease.

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**COMMENTS**

**Background**

We have previously shown that treatment with bifidobacteria-fermented milk (BFM) containing live bifidobacteria is more effective than usual treatment in inducing and maintaining remission of ulcerative colitis (UC). We hypothesized that probiotic strains of Bifidobacteria in BFM may interfere with the intestinal inflammatory response as well as prevention of the overgrowth of potentially pathogenic bacteria such as Bacteroides vulgatus. In this study, we focused on the effect of probiotic strains on the secretion of IL-10 by peripheral blood mononuclear cells (PBMNC) and also the production of IL-8 by intestinal epithelial cells. No single factor produced by the probiotic strain seems to be responsible, because the fractions of less than and more than 3 kDa, differing in heat-sensitivity, were found to have inhibitory effects on IL-8 secretion in this study. Acetic acid but not lactic acid, a major component of CM, was likely to largely contribute to the inhibitory activity of the less than 3 kDa fraction. Components responsible for the inhibition of IL-8 in the more than 3 kDa fraction remain to be investigated.

**Research frontiers**

There is evidence that probiotic treatment is effective in patients with UC. The precise mechanisms by which probiotic microorganisms used in clinical trials exert their beneficial effect have not been well defined yet.

**Innovations and breakthroughs**

This study has shown that Bifidobacterium strains that proved to be beneficial in inducing and maintaining remission of ulcerative colitis exhibit regulatory activities that contribute to the control of intestinal inflammation, thus unravelling the biological basis of the beneficial effects of probiotics in human disease.
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