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MAIN TOPIC

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Probiotics up-regulate MUC-2 mucin gene expression in a Caco-2 cell-culture model

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Abstract Enteral probiotics such as *Lactobacillus casei GG* (LGG) have been used in the treatment of a variety of intestinal disorders in infants and children, including diarrhea, malabsorption, and *Clostridium difficile* colitis. Previous studies have identified the gene locus for mucin (MUC-2) and its expression in Caco-2 cells. Others have demonstrated that mucin, located on the surface of the intestinal epithelium, inhibits bacterial translocation (BT). We previously demonstrated that both mucin and the probiotic bacterium LGG have an inhibitory effect on BT in both an in-vitro Caco-2 cell model and a neonatal rabbit model. We hypothesized that the decline in BT by LGG is mediated by up-regulation of epithelial MUC-2. Human enterocyte Caco-2 cells were grown to confluence and incubated at 37 °C with either medium (control group) or 10^4 or 10^8 LGG for 180 min. Non-adherent LGG was washed away. Caco-2 cells were then lysed, purified, and quantified for MUC-2 protein and mRNA. The addition of LGG to the enterocyte monolayer surface resulted in significantly ($P < 0.05$) increased MUC-2 expression compared to the untreated monolayers. Protein densities for MUC-2 significantly ($P < 0.05$) increased with LGG. Density (expressed as ratio to control group) was 8.6 ± 1.3 in the low-dose group (10^4 LGG) and 15.6 ± 2.3 in the high-dose group (10^8 LGG). LGG may thus bind to specific receptor sites on the enterocyte and stimulate the up-regulation of MUC-2, resulting in increased inhibition of BT.

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Introduction

Interest in the use of live microbial agents for health maintenance and disease prevention or treatment has increased markedly over the last few years [10]. Many of these organisms, termed probiotics, are being proposed as remedies for broad number of gastrointestinal (GI) and systemic conditions [10]. Probiotics are known to enhance the intestinal microflora by replenishing suppressed nonpathogenic bacteria and inhibiting the growth of pathogenic flora [13]. Additionally, they (e.g., *Lactobacillus casei GG*, LGG) have been used in the treatment of a variety of infant and childhood intestinal disorders, including diarrhea, malabsorption, and *Clostridium difficile* colitis [1, 8, 10, 17, 22]. It has been reported that LGG adheres to the surface receptors of cultured enterocytes, resulting in inhibition of bacterial translocation (BT) of *Salmonella typhimurium*, *Entamoeba histolytica*, *Clostridium difficile*, *Escherichia coli*, and *Streptococcus dobrinus* in various in-vitro models [8, 13, 15, 16].

GI tract (GIT) mucins are large, carbohydrate-rich glycoproteins that are major components of the mucous layer of GIT epithelial surfaces [2]. These proteins are synthesized, stored, and secreted from cells on the epithelial surface and from enterocytes and goblet cells in the underlying mucosa [3]. Mucin functions, in part, by protecting the epithelial surface of the GIT from chemical, enzymatic, mechanical, and microbial damage. Studies have demonstrated that mucins play a role in inhibiting BT [12, 17, 18].

Previous studies have identified the gene locus for mucin and its expression in Caco-2 cells [3, 13]. Our laboratory has demonstrated that mucin, when added to the apical surface of an enterocyte cell-culture model, inhibits BT [12]. In addition, BT was also shown to be inhibited by the addition of LGG to Caco-2 cell culture

monolayers [21]. Based on these findings, we hypothesized that LGG-mediated inhibition of BT is controlled by up-regulation of epithelial mucin (MUC-2).

Materials and methods

LGG obtained from the American Type Culture Collection (ATCC, Manassas, VA; catalog no. 53103) was grown overnight in Ragosa SL broth (Difco, Detroit, MI), washed two times with phosphate buffered saline (PBS), and resuspended in dulbecco's modified Eagle's medium (DMEM) at a concentration of 10^6 colony-forming units (CFU)/ml. The final concentration was determined by spectrophotometry at 600 nm and the number of bacteria were verified by pour-plate assay using Ragosa agar and standard serial dilution techniques.

Human colonic carcinoma (Caco-2) cells were obtained (ATCC no. HTB 37). Cell passages 25–37 were grown in DMEM supplemented with 10% fetal bovine serum (Difco), 1% non-essential amino-acid solution (Gibco, Grand Island, NY), 1% sodium pyruvate (Gibco), penicillin G (100 IU/ml), and streptomycin (100 μg/ml) (Gibco) in a 5% CO_2 atmosphere at 37 °C. Cells were grown to 70% confluence and harvested following trypsinization with trypsin-EDTA (Gibco), washed, resuspended in DMEM, and seeded at a density of 1×10^5 per well onto collagen-coated porous filters in the apical chamber of a two-chamber cell-culture system. Collagen coating of the plates was accomplished by incubating in 30 μl of 1.0 mg/ml rat-tail type I collagen (Sigma, St Louis, MO). The cells were grown for 14 days to allow them to reach confluence and fully differentiate. Media were changed every other day.

E. coli C 25, a mild-to-moderately virulent, streptomycin-resistant strain originally isolated from a neonate with necrotizing enterocolitis (provided by Dr. Henri R. Ford, Pittsburgh), was grown overnight in brain-heart infusion medium (BBL, Cockeysville, MD), washed two times with PBS, and resuspended in DMEM (Fisher Scientific, Pittsburgh, PA) at a concentration of 10^5 CFU/ml. The initial concentration of the bacteria was determined by spectrophotometry at 650 nm and the number of bacteria were verified by pour-plate assay using MacConkey's agar (Difco) and standard serial dilution techniques. LGG was grown overnight in Ragosa SL broth, washed two times with PBS, and resuspended in DMEM at a concentration of 10^6 CFU/ml. The initial LGG concentration was determined by spectrophotometry at 600 nm and the numbers of bacteria were verified by pour-plate assay using Ragosa agar and standard serial dilution techniques.

Human enterocyte Caco-2 cells were grown to confluence and incubated at 37 °C with 10^4 or 10^8 LGG concentration for 180 min. Non-adherent LGG was removed from the culture with three washes of DMEM. Caco-2 cells were then lysed with 1 ml Ripa buffer. Total cell protein was isolated following microcentrifugation at 10,000 × g for 10 min at 4 °C. A sample of the supernatant was analyzed with a spectrophotometer at 595 nm.

Aliquots (30 μg) of equal quantities of homogenated protein were solubilized in an equal volume of loading buffer (8.0 ml deionized H_2O, 2.0 ml 0.5 M Tris–HCl PH 6.8, 1.6 ml glycerol, 3.2 ml 10% sodium dodecyl sulfate, 0.8 ml 2-mercaptoethanol, and 0.8 ml 1% bromophenol blue) followed by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After 2 h of electrophoresis, the protein was transferred from the gel to BioBlot-NC nitrocellulose membranes (Bio-Rad, Hercules, CA) using a wet tank. Membranes were quenched with 5% nonfat dry milk in 1% blocking solution (Boehringer Mannheim, Indianapolis, IN) and incubated with anti-MUC-2 antibody (mouse immunoglobulin IgG1; Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:100 dilution for 1 h. After incubation, the nitrocellulose membranes were washed and antibody binding was visualized after 1-h exposure to 1:1,000 dilution of goat anti-mouse antibody IgG1-HRP conjugate (Santa Cruz Biotechnology).

The membranes were prepared for film development as follows: 0.0625 ml/cm^2 Western blot luminol reagent was pipetted into a microcentrifuge tube, hand-mixed, and added to the nitrocellulose membrane, which was placed into a sealed meal bag for 60 s. The membrane was then exposed to a radiograph film (Kodak Scintific Imaging Film, Rochester, NY) for 1 to 15 s. The resulting signal expression was quantified by densitometric analysis using NIH imaging software (Scion, Frederick, MD).

MUC-2 messenger RNA (mRNA) was detected and assayed in Caco-2 cells by reverse-transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from each cell line by adding 1 ml Trizol reagent (Gibco) to a 3.5-cm-diameter dish. The homogenized sample was incubated for 5 min at room temperature; 200 μl of chloroform per ml Trizol was added and the tube was mixed for 15 s and incubated for 3 min at room temperature. The sample was centrifuged at 12,000 × g for 15 min at 4 °C. Following centrifugation, the sample separated into three phases; the aqueous phase (60% of total volume) containing the RNA was pipetted into a separate RNase-free tube. Quantification of the isolated total RNA was performed by spectrophotometric analysis at 260 nm.

MRNA (poly-A positive) MUC-2 was reversed transcribed into cDNA by adding 2 μg/ml total cellular mRNA to the following reaction mixture: PCR nucleotide mix (Boehringer-Mannheim) 10 mM; M-MLV RT (Moloney murine leukemia virus reverse transcription, Gibco) at 200 IU/μl; Oligo (dT) primer (New England Biolabs, Beverly, MA) at 40 μM, and RNase inhibitor (Roche Diagnostic, Mannheim, Germany) at 40 U/μl. Diethyl pyrocarbonate-treated water was added to yield an appropriate final concentration of 100 μl. The samples (100 μl) were incubated at 40 °C for 70 min and 95 °C for 3 min, and the reaction mixture was stored at 4 °C until assay.

PCR was performed with oligomers designed for human MUC-2 (Accession no. AF007194) using an optimizing program (OLIGO 4.1, National Bioscience, Plymouth, MN). The oligonucleotide primers were sense: 5′-CTC CAA GCC ACA CTG CCC-3′ and antisense: 5′-TGC TCC CCA AAC TAT CTG-3′. Primer pairs for β-actin (GeneBank accession no. M12481) were sense: 5′-GAG GGA AAT CGT GCG TGA CAT-3′ and antisense: 5′-AGA AGG AAG GCT GGA AAA GAG-3′. This generated a 520-base-pair cDNA product.

RT product (2 μl), forward and reverse specific oligomers (5 μM, 1 μl), 6 μl PCR buffer (Roche), 0.2 μl 25 mM $MgCl_2$ (Roche), and 0.8 μl (5 IU/μl) Taq polymerase (Perkin-Elmer, Foster City, CA) was pipetted into microcentrifuge tubes with double distilled water added to a total final volume of 10 μl. The product was run in a thermal cycler for 32 cycles using PCR program DHT 5 as follows: Step 1: 94 °C for 2 min; step 2: 94 °C for 15 s, 55 °C for 15 s, 72 °C for 1 min for 32 cycles; step 3: 72 °C for 5 min; and step 4: storage at 4 °C. The PCR product was run on 2% agarose gel containing ethidium bromide for 1 h at 160 V. Signal expression was quantified by densitometric analysis using Kodak image analysis software. Statistical analysis was by ANOVA with $P < 0.05$ considered significant.

Results

Bacterial translocation

E. coli translocation across Caco-2 monolayers was inhibited by LGG in a dose-dependent manner as follows: 21.7% for 10^4 LGG, 28.8% for 10^5 LGG, 36.6% for 10^6 LGG, and 50.2% for 10^7 LGG concentrations. The highest concentration of LGG tested (10^8) resulted in complete inhibition of *E. coli* translocation ($P < 0.05$, Fig. 1). These data suggest that the observed effect of LGG may occur through a receptor mechanism that stimulates a cellular function that inhibits BT.

Immunoassay for MUC-2

The addition of LGG to the enterocyte monolayer surface resulted in increased MUC-2 expression compared to the untreated monolayers. The molecular weight of the MUC-2 was more than 200 kDa as expected. Protein densities (expressed as ratio of MUC-2 protein from cells treated with LGG vs no LGG) was 8.6 ± 1.3 in the low-dose (10^4 LGG) and 15.6 ± 2.3 in the high-dose (10^8 LGG) group. This led to a significant ($P < 0.05$) elevation of MUC-2 expression in both low- and high-dose groups compared to the control group (Fig. 2).

MRNA expression of MUC-2

MUC-2 gene expression in Caco-2 cells was evaluated through RT-PCR. The expression of MUC-2 was based on the relative expression compared to β-actin. Figure 3 shows the MUC-2 expression, β-actin for inter assay control, and PCR 123 base-pair ladder. The mean band density of MUC-2 mRNA for the control group was 1.05 ± 0.09 versus 1.48 ± 0.02 for the LGG-treated group and was statistically significant ($P < 0.05$) (Fig. 4).

Discussion

The microbiota of the human intestine influence health and well-being [4]. Whereas it has long been accepted that gut bacteria play a role in host pathogenesis, current opinion holds that certain microflora components can have beneficial affects such as resistance to gastroenteritis, improvement in blood lipids, antitumor properties, lactose tolerance, and enhanced GI immunity [4, 5]. It is postulated that in the breast-fed infant, elevated gut *Bifidobacterium* may provided health advantages in comparison with formula-fed infants [18, 24].

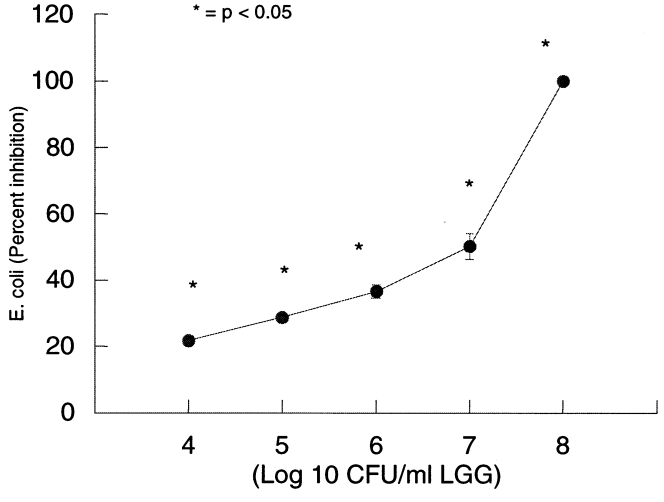

Fig. 1 Addition of *Lactobacillus casei GG* (LGG) to enterocyte monolayer surface results in significant inhibition of *E. coli* bacterial translocation. Percent inhibition was significantly different between all LGG doses tested

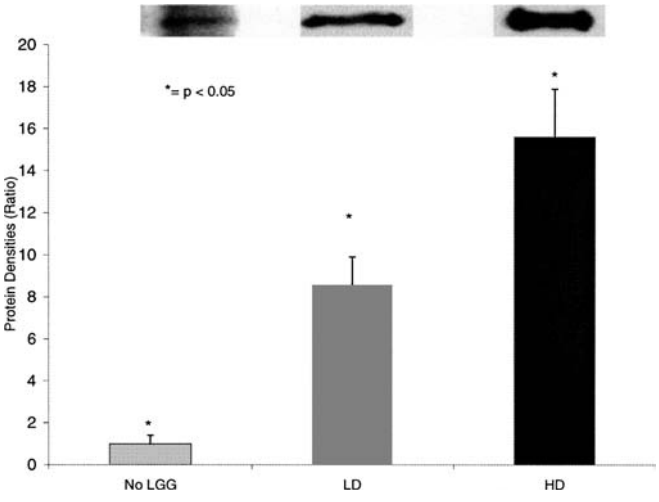

Fig. 2 Protein densities (expressed as ratio to no *Lactobacillus casei GG*, LGG): 8.6 ± 1.3 in low-dose (LD = 10^4 LGG), 15.6 ± 2.3 in high-dose (HD = 10^8 LGG). HD and LD significantly different from control and each other ($P < 0.05$)

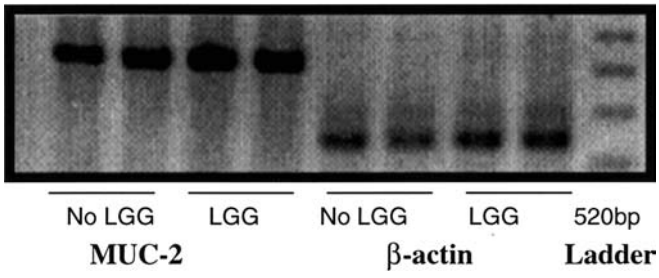

Fig. 3 RT-PCR demonstrates that confluent Caco-2 cells express mucin gene (MUC-2) in both LGG-treated and non-treated monolayers

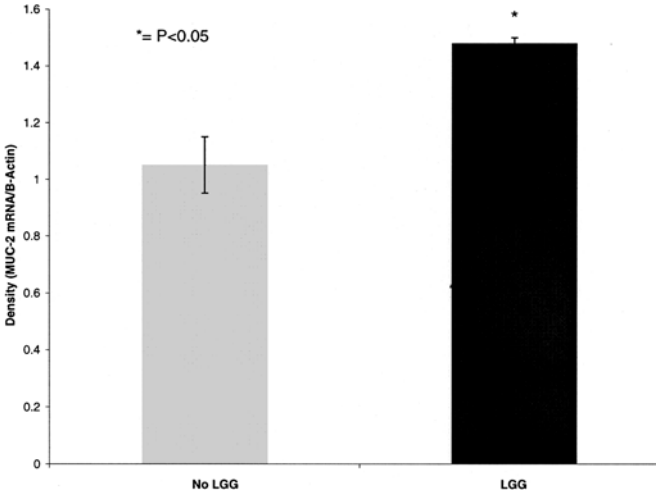

Fig. 4 Mean band density of mucin gene mRNA for control group 1.05 ± 0.09 versus 1.48 ± 0.02 for LGG-treated group ($P < 0.05$)

Probiotics are known to enhance the intestinal microflora by replenishing suppressed nonpathogenic bacteria and inhibiting the growth of pathogenic flora [13]. Additionally, they have been used in the treatment of a variety of intestinal disorders in infants and children, including diarrhea, malabsorption, and *C. difficile* colitis [8, 10, 17, 22].

Some interesting data have arisen from the use of probiotics to reduce diarrhea and gastroenteritis in infants. Mechanisms that have been suggested to explain these beneficial effects include: (1) reduced gut luminal pH through stimulation of the lactic acid–producing microflora [18]; (2) direct antagonistic effects on pathogens [7–11]; (3) competition for pathogen-binding and receptor sites [9]; and (4) improved immune function and stimulation of appropriate immunomodulatory cells [6, 14, 23].

The intestinal mucosa forms a crucial barrier between the host and the environment. The intestinal mucous gel layer is thought to be a vital component of the gut barrier and is composed, in part, of a family of glycoproteins known as mucins [4], high-molecular-weight glycoproteins synthesized and secreted by the intestinal epithelium. In a previous study, two strains of lactobacillus (*L. plantarum* 299v and *L. rhamnosus GG*) were found to inhibit enteropathogenic (diarrheogenic) *E. coli* by inducing MUC-2 gene expression using a HT-29 cell line (colonic adenocarcinoma cells) [20]. Other studies have shown that the intestinal mucin gene MUC-2 was constitutively expressed by the confluent Caco-2 and LS174T cell lines [13].

We previously demonstrated that the addition of either mucin or *LGG* to Caco-2 cell cultures in vitro inhibits BT [12, 16, 19]. In a separate study with a different cell line (fetal I-407 cells), however, mucin had less of an inhibitory effect [12].

In this study, we investigated the effects of LGG on the Caco-2 cell line using an in-vitro model. We hypothesized that LGG, which binds to the epithelial surface [20], induces up-regulation of epithelial mucin and that this increased mucin inhibits BT. Caco-2 cells grown in DMEM express significant levels of MUC-2. These cells can be induced to express higher concentrations of MUC-2 by adding the probiotic LGG. We demonstrated that the addition of LGG to the Caco-2 cells induced MUC-2 expression that correlated with LGG dosage [21]. In a previous study by Mack et al. [20], two strains of lactobacillus (*L. plantarum* 299v and *L rhamnosus GG*), using cell line HT-29 and *E coli* strain E3248/69, were shown to inhibit bacterial binding. In a previous study, we demonstrated that LGG application to a Caco-2 cell monolayer induced significant inhibition of *E. coli* C25 isolated from a neonate with necrotizing enterocolitis [21]. Thus, increased mucin production may explain the mechanism of the inhibitory effect of LGG on BT in this cell-culture line.

From this study, we surmise that LGG may bind to specific receptor sites on the enterocyte and stimulate the up-regulation of MUC-2, resulting in increased inhibition of BT. These results are in accord with our previous studies with both mucin and LGG addition to the Caco-2 cell line in-vitro model [12, 20].

The issue of the safety of probiotics needs a comment. Nonpathogenic bacteria used as probiotics are members of the normal intestinal flora. The few studies available on patients in high-risk categories, such as immunocompromised patients, have confirmed the safety of tested strains [6, 23]. Lactic acid-producing bacteria have a long history of safe use in foods and in other products tested in clinical studies. Therefore, the risk of probiotics usage in the clinical setting appears small [24]. However, it is important to stringently evaluate new probiotics and their effects in experimental animal studies and cell-culture studies.

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