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

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.

Keywords

Mucin · Lactobacillus casei GG (LGG) · Caco-2 cell culture model · Bacterial translocation

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 bovis 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 initial 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% CO2 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 x 10^4 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.

Caco-2 cell monolayers were transfected with plasmid DNA (pCMV-LGG, 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 10^6 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 (Difco, Detroit, MI), 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 number of bacteria were verified by pour-plate assay using Ragosa agar and standard serial dilution techniques.

For Caco-2 cells, 10^5 bacteria/ml were grown to confluence and incubated at 37 °C with 10^5 or 10^6 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 x 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 homogenized protein were solubilized in an equal volume of loading buffer (8.0 ml deionized H2O, 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/cm² 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. Total RNA concentration was verified by measuring the absorbance at 260 nm. RNA was then mixed for 15 s and incubated for 3 min at room temperature. The sample was centrifuged at 20,000 × g for 15 min 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 CTA AGG CTT CCG GTG TGG-3' and antisense: 5'-AGA AGG TCT TGG TCC TAT AAA AGA AAG CTT-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 MgCl2 (Roche), and 0.8 μl (5 μl/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; 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 10^5 LGG, 28.8% for 10^6 LGG, 36.6% for 10^7 LGG, and 50.2% for 10^8 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.