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RAPID COMMUNICATION

Lactobacillus plantarum inhibits epithelial barrier dysfunction and interleukin-8 secretion induced by tumor necrosis factor-α

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Abstract

AIM: To determine whether *Lactobacillus plantarum* can modify the deleterious effects of tumor necrosis factor-α (TNF-α) on intestinal epithelial cells.

METHODS: Caco-2 cells were incubated with TNF-α alone or in the presence of *L. plantarum*. Transepithelial electrical resistance was used to measure epithelial barrier function. Interleukin 8 (IL-8) secretion by intestinal epithelial cells was measured using an ELISA. Cellular lysate proteins were immunoblotted using the anti-extracellular regulated kinase (ERK), anti-phospho-ERK and anti-IκB-α.

RESULTS: A TNF-α-induced decrease in transepithelial electrical resistance was inhibited by *L. plantarum*. TNF-α-induced IL-8 secretion was reduced by *L. plantarum*. *L. plantarum* inhibited the activation of ERK and the degradation of IκB-α in TNF-α-treated Caco-2 cells.

CONCLUSION: Induction of epithelial barrier dysfunction and IL-8 secretion by TNF-α is inhibited by *L. plantarum*. Probiotics may preserve epithelial barrier function and inhibit the inflammatory response by altering the signal transduction pathway.

Key words: *Lactobacillus plantarum*; Tumor necrosis factor-α; Epithelial barrier; Interleukin-8; ERK; IκB-α

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INTRODUCTION

Probiotics are defined as living microorganisms that exert beneficial effects on human health[1]. They are effective in shortening the duration of infectious diarrhea in children, and preventing antibiotics-associated diarrhea[2,3]. Probiotics have been shown to prevent a relapse of postoperative pouchitis in ulcerative colitis[4].

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine and plays a central role in intestinal inflammation in Crohn disease. TNF-α levels in serum, stool and intestinal tissues are elevated in patients with Crohn's disease[5,6]. In Crohn's disease, the elevation in epithelial permeability of the ileal mucosa may be mediated by TNF-α[7]. Treatment with anti-TNF-α antibody is effective in cases of intractable Crohn's disease[8].

As disturbance of the intestinal microflora plays an important role in the pathogenesis of murine experimental colitis and human inflammatory bowel disease[3], probiotics have been used to modify the bacterial flora of the gut. *Lactobacillus plantarum* is isolated from Kimchi, a traditional Korean food made from fermented vegetables[9]. *L. plantarum* attenuates intestinal inflammation in the interleukin (IL) 10 gene-deficient mouse model, which spontaneously develops enterocolitis[10].

The mechanisms of action of probiotics include improvement of epithelial barrier function and immunoregulatory effects[11]. Each probiotic species may have an individual mechanism of action. The combination probiotic, VSL3 contains *L. plantarum* and enhances human intestinal epithelial barrier function[12]. Intestinal epithelial cells release potent neutrophil attractant chemokines such as IL-8 when stimulated by TNF-α. Secretion of IL-8 by epithelial cells has been suggested to be important in the pathogenesis of inflammatory bowel diseases, because IL-8 induces migration of inflammatory cells into the mucosa. Some lactobacilli inhibit the induction of IL-8 production by TNF-α in human intestinal epithelial cells[13-15]. TNF-α-stimulated IL-8 secretion by intestinal epithelial cells is mediated by extracellular signal-regulated kinase (ERK) and nuclear factor κB (NF-κB)[16].

The aim of this study was to determine whether *L. plantarum* reverses the deleterious effects of TNF-α on intestinal epithelial cells. We performed an *in vitro* study in which Caco-2 cells were treated with TNF-α alone or with TNF-α plus *L. plantarum*. We investigated the effect of

L. plantarum on TNF-α-induced alteration of epithelial barrier function, IL-8 production, and ERK/NF-κB pathway dynamics.

MATERIALS AND METHODS

Cell lines

Caco-2 cells, an established cell line model for mature differentiated enterocytes, were obtained from the American Type Culture Collection (ATCC). Cell lines were cultured in 25 mmol/L glucose-Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 1% nonessential amino acids, and 4 mmol/L glutamine. Cultures were maintained at 37℃ in an incubator containing an atmosphere of 5% CO_2. Cells were used within 14 d of seeding or within five days of confluency. The Caco-2 cell culture medium was replaced with antibiotic-free culture medium 24 h before experiments.

Probiotics

L. plantarum (ATCC 8014) was incubated in Lactobacillus MRS broth at 37℃ for 24 h, then diluted in MRS broth to a density of 0.5 absorbance units at a wavelength of 600 nm. Then, 1×10^7 colony-forming units of *L. plantarum* per mL were added at a multiplicity of 10:1 to the Caco-2 cells. Untreated cells were used as controls in all experiments.

Electrical resistance measurements

Caco-2 cells were grown as polarized monolayers on 6.5 mm transwell plates (0.4 μm pores; Corning Incorporated, Acton, MA, USA). Caco-2 monolayers with epithelial resistance greater than 500 Ωcm^2 were used, and *L. plantarum* was added apically to the polarized monolayers. TNF-α (10 ng/mL) was simultaneously added to the basolateral side of the cell monolayers. Electrical resistance across the monolayers was measured at various times using an epithelial volt-ohm meter (World Precision Instruments, Sarasota, FL, USA). Measurements were expressed in Ωcm^2 after subtracting mean values for resistance obtained from cell-free inserts.

ELISA for IL-8 measurement

TNF-α (10 ng/mL) and *L. plantarum* were added simultaneously to Caco-2 cells and incubated for 5 h. Culture medium was collected and centrifuged for 10 min to pellet residual bacteria. The supernatant was collected for determination of IL-8 concentration using an ELISA (Pierce, Rockford, IL, USA). Cytokine concentrations were determined using 96-well plates as described by the manufacturer.

Western blotting

TNF-α (10 ng/mL) and *L. plantarum* were added simultaneously to Caco-2 cells. The treated and untreated cells were washed with PBS and scraped into cell lysis buffer (20 mmol/L HEPES, 0.1% SDS, 1% Triton X-100, phosphatase inhibitor and protease inhibitor cocktail). Thirty minutes after treatment, the lysate was centrifuged at 15000 r/min for 15 min at 4℃. The protein content of the supernatant was determined using Bio-Rad DC reagents (Bio-Rad, Hercules, CA, USA). For western blotting, equal amounts of cellular lysate protein were mixed with Laemmli sample buffer and separated by SDS-PAGE. Separated proteins were transferred to PVDF membranes, which were blocked and then immunoblotted with anti-phospho-ERK, anti-ERK and anti-IκB-α (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blot was then developed using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence.

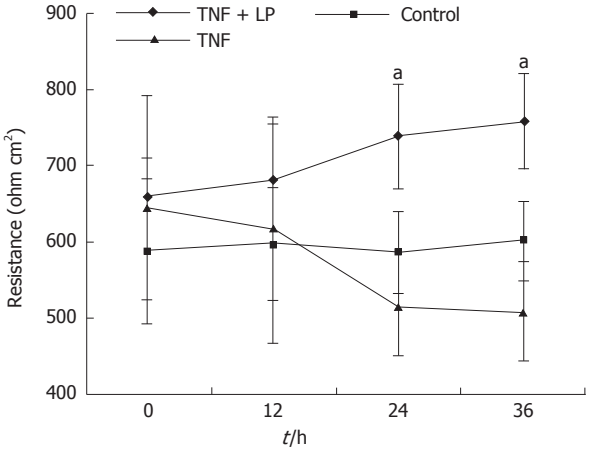

Figure 1 Effect of *L. plantarum* on transepithelial resistance. TNF-α decreased Caco-2 monolayer resistance. *L. plantarum* reversed TNF-α-induced decreases in transepithelial resistance. $^aP < 0.05$, compared with TNF-α. TER: transepithelial electrical resistance; LP: *L. plantarum*; TNF, TNF-α.

Statistical analysis

All data are expressed as means ± SD. Data comparisons were made with Student's *t* test. Differences were considered significant at $P < 0.05$.

RESULTS

Transepithelial electrical resistance

To determine the effect of *L. plantarum* on TNF-α-induced epithelial barrier dysfunction, Caco-2 cells were basolaterally incubated with TNF-α alone or with TNF-α plus *L. plantarum*, which was administered apically. Transepithelial electrical resistance was monitored for 36 h. The monolayer resistance of TNF-α treated cells did not change until 12 h had elapsed. TNF-α caused a decline in transepithelial resistance 24 h after treatment. *L. plantarum* inhibited TNF-α-induced decrease in transepithelial electrical resistance at 24 h and 36 h after treatment ($P < 0.05$) (Figure 1). The epithelial barrier function of TNF-α-stimulated Caco-2 cells was thus preserved by *L. plantarum*.

IL-8 induction

The secretion of IL-8 into culture medium was measured to determine the effect of *L. plantarum* on the inflammatory response of Caco-2 cells to TNF-α. IL-8 concentrations in media of Caco-2 cells cultured with *L. plantarum* were not significantly different from those of the controls. When TNF-α (10 ng/mL) was incubated with the cells for 5 h, IL-8 secretion was increased to

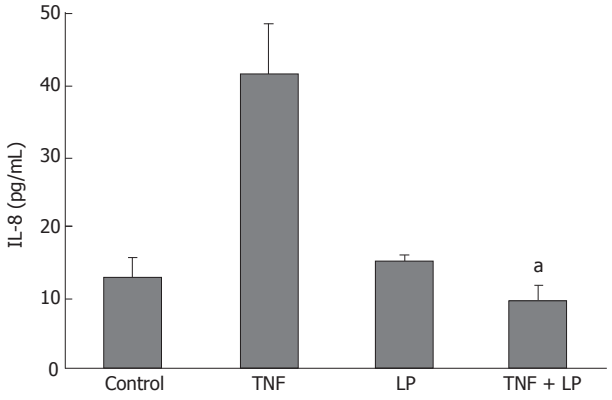

Figure 2 Effect of *L. plantarum* on TNF-α-induced IL-8 secretion by Caco-2 cells. TNF-α-induced IL-8 secretion was significantly reduced by *L. plantarum*. $^{a}P < 0.05$, compared with TNF-α. LP: *L. plantarum*; TNF, TNF-α.

41.5 ± 7.2 pg/mL. IL-8 secretion was reduced to 9.5 ± 2.1 pg/mL ($P < 0.05$) when TNF-α was cocultured with *L. plantarum* (Figure 2). These data showed that *L. plantarum* inhibited TNF-α-induced IL-8 secretion.

Western blots of ERK and IκB-α

The effect of *L. plantarum* on TNF-α–induced ERK pathway activity was investigated. Treatment of Caco-2 cells with TNF-α induced phosphorylation of ERK-1 and ERK-2. The amount of p-ERK in *L. plantarum*-treated cells was not significantly different from that of the control. Phosphorylation of ERK-1 and ERK-2 in TNF-α-treated cells was decreased by *L. plantarum*. Nonphosphorylated forms of ERK showed the presence of same amounts of these proteins. *L. plantarum* thus inhibited TNF-α-induced activation of the ERK pathway (Figure 3).

To study the effect of *L. plantarum* on the NF-κB pathway, the level of IκB-α was determined using western blotting. NF-κB activation involves the phosphorylation of IκB-α and subsequent degradation of IκB-α, resulting in the translocation of NF-κB to the nucleus. Treatment with TNF-α caused degradation of IκB-α. Coincubation with TNF-α and *L. plantarum* inhibited TNF-α-induced degradation of IκB-α (Figure 4).

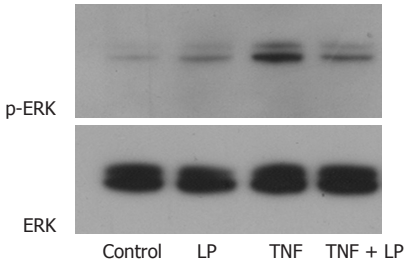

Figure 3 Effect of *L. plantarum* on the ERK pathway. Caco-2 cells were incubated with *L. plantarum*, TNF-α or *L. plantarum* plus TNF-α. Cell lysates were immunoblotted with antibodies against phosphorylated ERK and total ERK. *L. plantarum* inhibited TNF-α-induced activation of ERK-1 and -2. LP: *L. plantarum*; TNF, TNF-α.

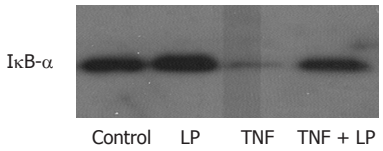

Figure 4 Effect of *L. plantarum* on IκB-α degradation. Caco-2 cells were incubated with *L. plantarum*, TNF-α or *L. plantarum* plus TNF-α. Cell lysates were immunoblotted with antibodies against IκB-α. TNF-α caused degradation of IκB-α. *L. plantarum* inhibited TNF-α-induced IκB-α degradation. LP: *L. plantarum*; TNF, TNF-α.

DISCUSSION

Ma *et al*[17] demonstrated that TNF-α decreases transepithelial electrical resistance of Caco-2 cells after 24 and 48 h. We also observed a decrease in transepithelial electrical resistance after 24 h. We showed that the TNF-α-induced decrease in transepithelial electrical resistance was inhibited by *L. plantarum*. *Saccharomyces boulardii* prevented a decrease in transepithelial electrical resistance in enteropathogenic *E. coli*-infected T84 cells[18]. Intestinal mucosal permeability is decreased by VSL3 in IL-10 gene-deficient mice[12]. All these findings support the contention that probiotics enhance epithelial barrier function.

In our study, TNF-α-induced IL-8 secretion was inhibited by *L. plantarum*. This indicates that *L. plantarum* attenuates the epithelial inflammatory response to TNF-α. McCracken *et al*[19] showed that *L. plantarum* decreased TNF-α-induced IL-8 secretion in HT-29 cells in which IL-8 mRNA levels were elevated. In contrast, *Lactobacillus reuteri* and *L. GG* inhibited TNF-α-induced IL-8 secretion and IL-8 mRNA expression[14,15]. The level of IL-8 expression is correlated with disease activity in patients with inflammatory bowel disease. A number of Lactobacillus and Bifidobacterium species, including *L. plantarum*[10], *L. reuteri*[19], *VSL3*[12], *L. salivarius* and *B. infantis*[20], attenuate experimental colitis in IL-10 knockout mice.

ERK and p38 mitogen-activated protein (MAP) kinase contribute to TNF-α-stimulated IL-8 secretion by intestinal epithelial cells *via* a posttranscriptional mechanism[16]. Yan *et al*[21] showed that *L. GG* prevents cytokine-induced apoptosis in intestinal epithelial cells by inhibition of TNF-α-induced p38 MAP kinase activation. Jijon *et al*[22] demonstrated that VSL3 inhibits IL-8 secretion and reduces p38 MAP kinase activation. The effect of *L. plantarum* on TNF-α-stimulated ERK activation had not been investigated. We demonstrated that *L. plantarum* inhibited ERK activation in TNF-α-treated intestinal epithelial cells. ERK signaling is involved in IL-8 production because ERK inhibitors attenuate IL-8 secretion induced by TNF-α[23]. In our study, *L. plantarum* inhibited TNF-α-induced ERK activation, suggesting that *L. plantarum* may inhibit IL-8 secretion, at least partially, through the ERK pathway. NF-κB regulates IL-8 transcription, and some lactobacilli have been shown to inhibit TNF-α-induced NF-κB translocation to the nucleus and IκB-α degradation[13,14]. We also showed that *L. plantarum* inhibited the degradation response of IκB-α to TNF-α. In contrast, *L. GG* did not affect TNF-α-induced ERK activation or IκB-α degradation[21]. Probiotics may exert anti-inflammatory responses

by modifying the signal transduction pathway. The mechanisms involved may depend on the species of probiotics.

Epithelial barrier functions are modulated by the NF-κB and MAP kinase pathways. A TNF-α-induced increase in intestinal tight junction permeability was shown to be mediated by NF-κB activation[17]. The increase in transepithelial resistance induced by VSL3 is mediated in part *via* the ERK pathway[24]. The effect of *L. plantarum* on monolayer resistance appears to be mediated by NF-κB and the ERK pathway. Although *in vitro* models are useful for evaluating mechanisms by which probiotics exert beneficial effects and provide a rationale for the therapeutic use of probiotics, the beneficial health effects of probiotics should also be determined by double-blinded placebo-controlled trials.

In summary, *L. plantarum* inhibits epithelial barrier dysfunction, IL-8 secretion, ERK activation, and IκB-α degradation in TNF-α-stimulated Caco-2 cells. Our findings suggest that probiotics may preserve epithelial barrier function and inhibit the inflammatory response by affecting the signal transduction pathway in human intestinal epithelium.

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