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RAPID COMMUNICATION

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

Jae Sung Ko, Hye Ran Yang, Ju Young Chang, Jeong Kee Seo

Jae Sung Ko, Hye Ran Yang, Ju Young Chang, Jeong Kee Seo, Department of Pediatrics, Seoul National University College of Medicine, Seoul 110-744, Korea
Supported by grant No. 0520050040 from the Seoul National University Hospital Research Fund and by KT&G Reserach Fund
Correspondence to: Jeong Kee Seo, Department of Pediatrics, Seoul National University Children's Hospital, 28 Yongon-dong, Chongro-gu, Seoul 110-744, Korea. jkseo@snu.ac.kr
Telephone: +82-2-20723627 Fax: +82-2-7433455
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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.

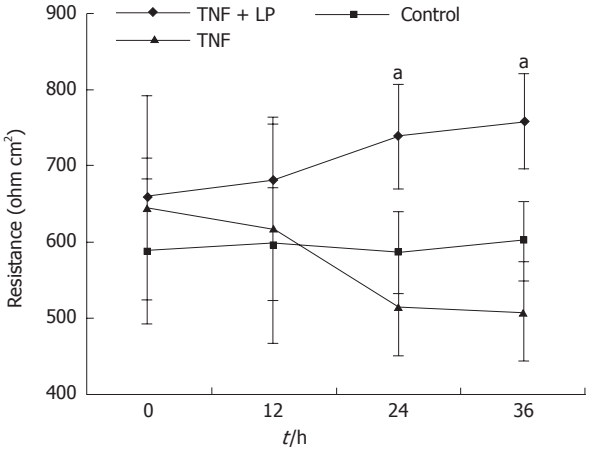

Figure 1 Effect of *L. plantarum* on transepithelial resistance. TNF-α decreased Caco-2 monolayer resistance. *L. plantarum* reversed TNF-α-induced decreases in transepithelial resistance. $^aP < 0.05$, compared with TNF-α. TER: transepithelial electrical resistance; LP: *L. plantarum*; TNF, TNF-α.

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

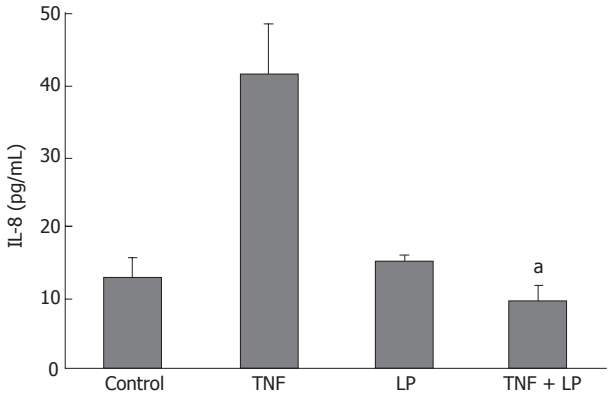

Figure 2 Effect of *L. plantarum* on TNF-α-induced IL-8 secretion by Caco-2 cells. TNF-α-induced IL-8 secretion was significantly reduced by *L. plantarum*. $^{a}P < 0.05$, compared with TNF-α. LP: *L. plantarum*; TNF, TNF-α.

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).

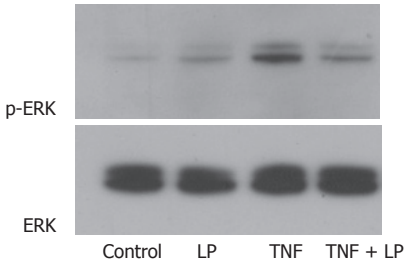

Figure 3 Effect of *L. plantarum* on the ERK pathway. Caco-2 cells were incubated with *L. plantarum*, TNF-α or *L. plantarum* plus TNF-α. Cell lysates were immunoblotted with antibodies against phosphorylated ERK and total ERK. *L. plantarum* inhibited TNF-α-induced activation of ERK-1 and -2. LP: *L.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

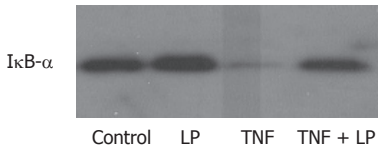

Figure 4 Effect of *L. plantarum* on IκB-α degradation. Caco-2 cells were incubated with *L. plantarum*, TNF-α or *L. plantarum* plus TNF-α. Cell lysates were immunoblotted with antibodies against IκB-α. TNF-α caused degradation of IκB-α. *L. plantarum* inhibited TNF-α-induced IκB-α degradation. LP: *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* showed that *L. GG* prevents cytokine-induced apoptosis in intestinal epithelial cells by inhibition of TNF-α-induced p38 MAP kinase activation[21]. Jijon *et al* demonstrated that VSL3 inhibits IL-8 secretion and reduces p38 MAP kinase activation[22]. 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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