**Lactobacillus plantarum** induces genomic DNA-dependent and TLR9-mediated elafin secretion from Caco-2 cells

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**Abstract**

**Background:** Lactobacilli show anti-inflammatory effects in the human intestine, and their genomic DNA was identified as one of the anti-inflammatory components. Increased levels of the natural protease inhibitor elafin in the intestine plays an important role in protection against intestinal inflammation. However, there have been no previous reports regarding whether lactobacilli increase elafin levels.

**Objective:** This study was performed to investigate whether *Lactobacillus plantarum* induces elafin secretion from the human epithelial colorectal adenocarcinoma cell line, Caco-2. Moreover, we examined the roles of bacterial genomic DNA and toll-like receptor 9 (TLR9), a specific receptor of bacterial DNA, in this effect.

**Methods:** Elafin secretion from Caco-2 cells by live and heat-killed *L. plantarum* was measured. The analysis was also performed using DNase-treated *L. plantarum* and genomic DNA extracted from *L. plantarum*. We examined the role of TLR9 in elafin secretion by *L. plantarum* and its genomic DNA by suppressing TLR9 expression using RNAi in Caco-2 cells.

**Results:** Heat-killed *L. plantarum* time- and dose-dependently increased elafin secretion, whereas live *L. plantarum* had no such effect. The elafin secretion by heat-killed *L. plantarum* was partially abolished by DNase treatment of the bacterium. In addition, *L. plantarum* genomic DNA also increased elafin secretion. Furthermore, suppression of TLR9 expression partially or completely abolished elafin secretion by heat-killed *L. plantarum* and its genomic DNA.

**Conclusion:** Our results indicated that heat-killed *L. plantarum* induced genomic DNA-dependent and TLR9-mediated elafin secretion. The anti-inflammatory effects of lactobacilli may be mediated by increases in the levels of elafin in the intestine.

**Keywords:** elafin, *Lactobacillus plantarum*, genomic DNA, toll-like receptor 9, anti-inflammatory effect

**Introduction**

Lactobacilli, which are normal components of the human gut microflora, have been used as both foods and medicines because of their beneficial effects on the health of the host. For example, treatment with lactobacilli has been reported to ameliorate human intestinal inflammation in patients with inflammatory bowel disease (IBD).1 The genomic DNA, double-stranded RNA, and cell wall components of lactobacilli have been identified as components responsible for the anti-inflammatory effects.2-5 Specifically, there have been a number of reports regarding the anti-inflammatory effects of genomic DNA from lactobacilli.5-9 Rachmilewitz et al. reported that intragastric administration of probiotic medical food VSL#3* (VSL Pharmaceuticals, Inc.), which consists of eight strains of lactic acid bacteria including lactobacilli, ameliorated the severity of colitis in a dextran sodium sulfate (DSS)-induced mouse model of IBD, whereas DNase-treated VSL#3* had no effect.2 They also demonstrated that VSL#3* had no effect on the severity of DSS-induced colitis in mice deficient for...
toll-like receptor 9 (TLR9), which is a specific receptor of bacterial DNA and has been reported to play a role in one of the major pathways responsible for the anti-inflammatory effects of genomic DNA from lactobacilli. Taken together, these observations suggest that the anti-inflammatory effects of lactobacilli are mediated by their genomic DNA, and TLR9 signaling is essential for these anti-inflammatory effects.

IBD, including Crohn’s disease and ulcerative colitis, are chronic inflammatory disorders of the intestine. Although the etiology of IBD has yet to be determined, a number of mediators seem to play prominent roles in the pathogenesis of chronic inflammatory disorders. Recent studies have highlighted the roles of proteases and their endogenous inhibitors in the pathogenesis of enteritis. Elevated proteolytic activities of several proteases have been detected in the intestinal tissues of IBD patients. This increased proteolytic activity may result from decreased efficacy and expression of endogenous protease inhibitors.

These results suggest that the shift of protease/anti-protease balance toward a more proteolytic environment causes chronic inflammation in the intestine. Among the endogenous protease inhibitors, elafin is a natural inhibitor of the human neutrophil proteases, elastase and proteinase 3. Elafin is mainly secreted from the epithelial cells of various tissues, including the intestine, and exerts anti-inflammatory effects by protease inhibition and by restoring barrier function to damaged epithelial cells in the intestine. The expression of elafin was downregulated in the intestinal tissues of IBD patients, and this was associated with increased elastase activity. In addition, a previous study using transgenic mice expressing human elafin demonstrated that an increased level of human elafin in the intestine re-equilibrates the protease/anti-protease balance and prevents intestinal inflammation in DSS- and trinitrobenzene sulfonic acid (TNBS)-induced mouse models of IBD. These observations suggest that an increased level of elafin in the intestine may be useful in the treatment of IBD.

In the present study, we hypothesized that lactobacilli may increase elafin secretion from intestinal epithelial cells, as both lactobacilli and increased levels of elafin are involved in intestinal anti-inflammatory effects. In this context, Motta et al. engineered recombinant human elafin-expressing lactic acid bacteria, including lactobacilli, and demonstrated that oral administration of these recombinant bacteria ameliorates the symptoms of inflammation in DSS-induced murine colitis. Therefore, we used these results to suggest that an increased level of human elafin in the intestine re-equilibrates the protease/anti-protease balance and prevents intestinal inflammation in DSS- and trinitrobenzene sulfonic acid (TNBS)-induced mouse models of IBD. The expression of elafin was downregulated in the intestinal tissues of IBD patients, and this was associated with increased elastase activity.

Methods

**Bacterial strain and culture conditions**

*L. plantarum* strain D2905 was used in this study. The bacterium was grown at 27 °C, the optimal temperature for its culture, in MRS medium under static conditions. To produce heat-killed *L. plantarum*, an overnight culture was inoculated into MRS medium to an optical density at 600 nm (OD_{600}) of 0.05 and grown for 8 h. After centrifugation (7,000 × g, 15 min, 4 °C) and two washes with PBS, the bacterium was incubated for 10 min at 65 °C. The bacterium was killed by this incubation (bacterial viability: 0.001%). This heat-killed bacterial preparation was used without centrifugation and washing in the following experiments. Furthermore, DNase-treated heat-killed *L. plantarum* was prepared by incubation with 5 µg/mL DNase I (Roche) for 16 h at 37 °C.

**Preparation of genomic DNA from *L. plantarum***

Genomic DNA was extracted from an overnight culture of *L. plantarum* using a Gentra Puregene Yeast/Bact. Kit (Qiagen) according to the manufacturer’s protocol. The concentration and purity of genomic DNA preparation were confirmed by measuring OD_{260} and OD_{260/280} and OD_{260/230} ratios, respectively. Only genomic DNA with OD_{260/280} > 1.8 and OD_{260/230} > 2.0 was used for the experiments. Purified genomic DNA was tested for endotoxin contamination using Limulus Amebocyte Lysate QCL-1000 (Lonza).

**Measurement of elafin secretion by *L. plantarum***

Caco-2 cells were used as described previously. Aliquots of 2 × 10^5 Caco-2 cells were plated in each well of 24-well plates (Nunc). Cells were treated with three concentrations of live or heat-killed *L. plantarum* (1, 2, and 5 × 10^6 CFU/mL) for 12, 24, and 48 h. After incubation, cell supernatants were centrifuged at 15,000 × g for 5 min at 4 °C. The concentration of elafin in the cell supernatants was determined using a human elafin ELISA kit (R & D Systems).

**Measurement of elafin secretion by transfected *L. plantarum***

Genomic DNA of 2 × 10^5 Caco-2 cells were plated in each well of 24-well plates. Subsequently, 5 µL of *L. plantarum* genomic DNA (0.35, 0.7, and 1.7 mg/mL) was added to 245 µL of minimum essential medium (Invitrogen) containing 1% fetal bovine serum (Biowest). The genomic DNA solution was then added to 250 µL of medium containing 1.25 µL of siLentFect Lipid Reagent (Bio-Rad Laboratories) and incubated for 30 min at room temperature to produce the transfection mix. Caco-2 cells were transfected with the transfection mix for 12, 24, and 48 h. After incubation, elafin secretion was measured by ELISA as described above. As the genome size of *L. plantarum* is approximately 3 Mbp, 1, 2, and 5 × 10^5 CFU/mL of *L. plantarum* contain approximately 3.5, 7, and 17 µg/mL of genomic DNA, respectively. Therefore, we used these three concentrations of genomic DNA (3.5, 7, and 17 µg/mL) in this experiment.

**Transfection of Caco-2 cells with TLR9-siRNA**

TLR9 expression in Caco-2 cells was suppressed by RNAi, as described previously. Briefly, aliquots of 2 × 10^5 Caco-2 cells plated in 24-well plates were transfected with 50 nM TLR9
-siRNA or scramble TLR9-siRNA (Bonac Corp.) as a negative control using siLentFect Lipid Reagent. After incubation for 24 h, the cells were treated with heat-killed L. plantarum (5 × 10⁹ CFU/ml) or transfected with L. plantarum genomic DNA (17 µg/mL) for 48 h as described above. The cell supernatants were collected, and the cellular protein extracts from Caco-2 cells were prepared with PRO-PREP Protein Extraction Solution (iNtRON Biotechnology) according to the manufacturer's protocol. The concentration of elafin in the cell supernatants was determined by ELISA. Suppression of TLR9 expression in the protein extracts of cells transfected with TLR9-siRNA, but not scramble TLR9-siRNA, was confirmed by Western blotting using an antibody to TLR9 as described previously.⁶

Detection of intracellular translocated genomic DNA

Aliquots of 2 × 10⁵ Caco-2 cells were plated in each well of 24-well plates and treated with heat-killed L. plantarum (5 × 10⁹ CFU/ml) or L. plantarum genomic DNA (17 µg/mL) for various times (0, 0.5, 1, 3, 6, 24, and 48 h). After treatment, Caco-2 cells were trypsinized using trypsin-EDTA (Invitrogen) at 37 °C for 10 min. The cells were then collected and centrifuged at 1,000 × g for 5 min and washed five times with PBS. DNA was extracted from Caco-2 cells using ISOGEN (Nippon Gene) according to the manufacturer's protocol. Detection of intracellular genomic DNA was performed by PCR based on the L. plantarum-specific 16S rRNA gene.⁶ PCR was performed using KOD FX Neo (Toyobo) in 30-µL reaction mixtures containing 1 µg of DNA preparation and the following primers: sense 5'-TGGTATTGATTGGTGCTTGCA-3' and anti-sense 5'-CCACCTTCCTCCGGTTTGCA-3'. PCR products were subjected to 1.5% agarose gel electrophoresis.

Statistical analysis

Data are presented as means ± standard deviations. Statistical analyses were performed using Origin Pro 8.1 (OriginLab). In all analyses, P < 0.05 was taken to indicate significance.

Results

Increase in elafin secretion by heat-killed L. plantarum

Treatment with heat-killed L. plantarum (5 × 10⁹ CFU/ml) significantly increased elafin secretion from Caco-2 cells, reaching 0.84 ng/mL after 48 h of treatment (P < 0.01 vs. medium alone by two-way ANOVA followed by Tukey's test) (Figure 1A). In contrast, elafin secretion remained close to the background level after 48 h of treatment with live L. plantarum. Therefore, heat-killed L. plantarum was used in the following experiments. As shown in Figure 1B, treatment for 48 h with heat-killed L. plantarum at 2 and 5 × 10⁹ CFU/mL significantly increased elafin secretion from Caco-2 cells compared to medium alone (0.38 and 0.84 ng/mL vs. 0.10 ng/mL, respectively; P < 0.01, one-way ANOVA followed by Tukey's test). In contrast, there was no significant difference in elafin secretion between cells treated with 1 × 10⁹ CFU/mL heat-killed L. plantarum and medium alone. These results indicated that heat-killed L. plantarum increased elafin secretion in a time- and dose-dependent manner.

Decrease in heat-killed L. plantarum-induced elafin secretion by DNase treatment

Treatment with DNase-treated and non-treated heat-killed L. plantarum significantly increased the level of elafin secretion from Caco-2 cells compared with medium alone (1.18 and 0.54 ng/mL vs. 0.20 ng/mL, respectively; P < 0.01, one-way ANOVA followed by Tukey's test). However, the level of elafin secretion in cells treated with DNase-treated heat-killed L. plantarum was significantly lower than that in cells treated with non-treated heat-killed L. plantarum (P < 0.05) (Figure 2). These observations indicated that DNase treatment partially abolished the increase in elafin secretion by heat-killed L. plantarum, although we could not exclude the possibility that DNase caused the decrease in elafin secretion by heat-killed L. plantarum regardless of the DNase activity.

Figure 1. Time- and dose-dependent increases in elafin secretion by heat-killed Lactobacillus plantarum.

Caco-2 cells were treated with live or heat-killed L. plantarum (5×10⁹ CFU/ml) for 12, 24, and 48 h (A), or with three concentrations of the heat-killed L. plantarum (1, 2, and 5×10⁹ CFU/ml) for 48 h (B). The elafin concentrations in the cell supernatants were determined by ELISA. Data are expressed as ng/ml and represent the means ± standard deviations of three separate experiments performed in triplicate. *P < 0.05, **P < 0.01 compared with medium alone.
Figure 2. Influence of DNase treatment on elafin secretion by heat-killed Lactobacillus plantarum.
Caco-2 cells were treated with DNase-treated (DNase (+)) or non-treated (DNase (−)) heat-killed L. plantarum (5×10⁹ CFU/ml) for 48 h. The elafin concentrations in the cell supernatants were determined by ELISA. Data are expressed as ng/ml and represent the means ± standard deviations of three separate experiments performed in triplicate. **P < 0.01 compared with medium alone, #P < 0.05 compared with DNase non-treated heat-killed L. plantarum.

Increase in elafin secretion by L. plantarum genomic DNA
Transfection with L. plantarum genomic DNA (17 µg/mL) significantly increased elafin secretion from Caco-2 cells, reaching 0.33 ng/mL after 48 h of treatment (P < 0.01 vs. medium alone, two-way ANOVA followed by Tukey's test) (Figure 3A). As shown in Figure 3B, transfection with L. plantarum genomic DNA at 7 and 17 µg/mL for 48 h significantly increased elafin secretion from Caco-2 cells compared with medium alone (0.17 and 0.33 ng/mL vs. 0.07 ng/mL, respectively; P < 0.01, one-way ANOVA followed by Tukey's test). In contrast, no significant change in elafin secretion was observed in those transfected with 3.5 µg/mL of genomic DNA compared to controls. These results indicated that L. plantarum genomic DNA increased elafin secretion in a time- and dose-dependent manner.

Mediation of TLR9 on elafin secretion by heat-killed L. plantarum and its genomic DNA
TLR9-siRNA-transfected Caco-2 cells showed decreased TLR9 expression compared to siRNA non-transfected and scramble TLR9-siRNA-transfected cells as a negative control (Figure 4A). This observation confirmed that TLR9-siRNA suppressed TLR9 expression in Caco-2 cells. Treatment with heat-killed L. plantarum significantly increased the level of elafin secretion in non-transfected cells, scramble TLR9-siRNA-transfected cells, and TLR9-siRNA-transfected cells (0.74, 0.80, and 0.39 ng/mL, respectively). On the other hand, treatment with L. plantarum genomic DNA significantly increased elafin secretion in non-transfected cells and scramble TLR9-siRNA-transfected cells (0.33 and 0.35 ng/mL, respectively), but the genomic DNA had no such effect in TLR9-siRNA-transfected cells (0.09 ng/mL). In addition, the levels of elafin secretion in TLR9-siRNA-transfected cells treated with heat-killed L. plantarum and L. plantarum genomic DNA were significantly lower than those in non-transfected cells and scramble TLR9-siRNA-transfected cells (P < 0.05 and P < 0.01, respectively; two-way ANOVA followed by Tukey's test) (Figure 4B). These observations indicated that suppression of TLR9 expression partially or completely abolished elafin secretion by heat-killed L. plantarum and its genomic DNA, respectively.

Intracellular translocation of L. plantarum genomic DNA
Intracellular translocation of the genomic DNA was a prerequisite for recognition by TLR9, because TLR9 is localized in
Elafin secretion by L. plantarum

Figure 5. Intracellular translocation of genomic DNA from Lactobacillus plantarum in Caco-2 cells.
Caco-2 cells were treated with heat-killed L. plantarum (5×10⁹ CFU/ml) or L. plantarum genomic DNA (17 µg/ml) for various times (0 – 48 h). Intracellular translocation of the genomic DNA was detected by PCR based on the coding sequence of the L. plantarum-specific 16S rRNA gene. Data are representative of three separate experiments. M; 100 bp DNA ladder marker.

Discussion
The results of the present study indicated that treatment with heat-killed L. plantarum increased the level of elafin secretion from Caco-2 cells, which was partially dependent on the bacterial genomic DNA. Moreover, TLR9 was shown to be the primary mediator of elafin secretion by heat-killed L. plantarum and its genomic DNA. To our knowledge, this is the first report regarding induction of elafin secretion from intestinal epithelial cells by lactobacilli and the molecular mechanisms underlying this effect.

Over the past several decades, lactobacilli have been reported to show anti-inflammatory effects and have been used...
to treat patients with IBD. Increased elafin levels have been shown to play an important role in intestinal anti-inflammatory effects. However, there have been no previous reports of increases in elafin levels induced by lactobacilli in the intestine. As elafin production has not been characterized in rodents, we examined whether lactobacilli could increase the level of elafin in the intestine using Caco-2 cells, and our results indicated that heat-killed L. plantarum increased elafin secretion from Caco-2 cells (Figure 1). These observations suggested that orally administered lactobacilli may increase elafin levels in the intestines of IBD patients. As the protease/anti-protease imbalance due to downregulation of elafin was shown to be involved in chronic inflammation in the intestine of IBD patients, the increase in elafin levels by lactobacilli in the intestine may lead to re-equilibration of the protease/anti-protease balance and subsequently ameliorate inflammation in these patients. On the other hand, live L. plantarum had no effect on elafin secretion (Figure 1), although live lactobacilli therapy has been reported to ameliorate human IBD. In general, orally administered live lactobacilli cannot survive in the low pH gastric environment, and the secretion of lysozymes, bile acid, and pancreatic fluid in the duodenum also influence the viability of the lactobacilli. Consequently, the lactobacilli would be killed by these conditions prior to reaching the intestine. Thus, it is possible that orally administered live lactobacilli are killed, and then increase elafin secretion from human intestinal epithelial cells.

Genomic DNA has been identified as the principle anti-inflammatory component of lactobacilli. Therefore, we hypothesized that the increase in elafin secretion by heat-killed L. plantarum depends on its genomic DNA. In the present study, DNase treatment partially abolished the increase in elafin secretion by heat-killed L. plantarum (Figure 2). Moreover, L. plantarum genomic DNA increased elafin secretion from Caco-2 cells, although to a lesser extent than heat-killed L. plantarum (Figures 1 and 3). These results indicated that the increase in elafin secretion by L. plantarum is partially mediated by its own genomic DNA and suggested that lactobacilli may exert anti-inflammatory effects through genomic DNA-dependent elafin secretion from epithelial cells in the human intestine. These observations also suggested that bacterial components other than genomic DNA may also be involved in the increase in elafin secretion by heat-killed L. plantarum. Previous studies showed that dsRNA and cell wall components are also anti-inflammatory components of lactobacilli. Therefore, the increase in elafin secretion by heat-killed L. plantarum may be related not only genomic DNA but also to these other bacterial components.

The anti-inflammatory effects of lactobacilli genomic DNA are primarily mediated by TLR9 signaling. Consistent with this, suppression of TLR9 completely abolished the increase in elafin secretion by L. plantarum genomic DNA in the present study (Figure 4). These observations indicated that TLR9 signaling is a major pathway responsible for the induction of elafin secretion by L. plantarum genomic DNA. However, suppression of TLR9 only partially abolished the increase in elafin secretion by heat-killed L. plantarum, suggesting that TLR9 is one, but not the only, mediator of elafin secretion by heat-killed L. plantarum. As bacterial components other than genomic DNA, including dsRNA and cell wall components, may be involved in the upregulation of elafin secretion by heat-killed L. plantarum, the receptors for these bacterial components (i.e., TLR3 and TLR2) may also partially regulate these effects. Further studies are required to fully elucidate the molecular mechanism of elafin secretion by heat-killed L. plantarum.

Overproduction of inflammatory cytokines results in the development of inflammation in the intestine. In fact, the levels of the inflammatory cytokine interleukin (IL)-8 have been shown to be increased in the intestine of IBD patients. Therefore, it is important to examine IL-8 secretion in assessment of the level of inflammation in the intestine. Our previous study indicated that genomic DNA of lactobacilli decreased H$_2$O$_2$-induced IL-8 secretion from Caco-2 cells. In addition, we identified an oligodeoxynucleotide (ODN), which markedly decreased H$_2$O$_2$-induced IL-8 secretion, from the genomic DNA of L. casei, and demonstrated that oral administration of this anti-inflammatory ODN to mice ameliorated DSS-induced murine colitis. Therefore, suppression of IL-8 secretion in vitro is an important factor for ameliorating colitis in this in vivo model of IBD. In our preliminary study, we found that heat-killed L. plantarum and its genomic DNA decreased H$_2$O$_2$-induced IL-8 secretion from Caco-2 cells without inducing a pro-inflammatory response (data not shown). These observations suggested the possibility of an anti-inflammatory effect in an in vivo model of IBD by heat-killed L. plantarum and its genomic DNA. On the other hand, we also showed previously that suppression of TLR9 expression by RNAi abolished the decrease in H$_2$O$_2$-induced IL-8 secretion by lactobacilli genomic DNA and the anti-inflammatory ODN. These observations indicated that the TLR9 signaling pathway plays a major role in mediating the decrease in IL-8 secretion. However, at present, we cannot explain the mechanism by which activation of the TLR9 signaling pathway results in the observed decrease in IL-8 secretion. The results of the present study indicated that L. plantarum genomic DNA increased elafin secretion from Caco-2 cells through TLR9 (Figures 3 and 4). Therefore, the increase in elafin levels may play important roles in the reduction of IL-8 secretion by lactobacilli genomic DNA, likely due to inhibition of pro-inflammatory proteases and restoration of barrier function to damaged epithelial cells. Further studies of the increase in elafin levels may elucidate the mechanism by which the TLR9 signaling pathway induces the observed anti-inflammatory effects.

TLR9 is expressed in human epithelial cells, including Caco-2 cells, and is localized in the endosome and lysosome. The bacterial genomic DNA must undergo translocation into Caco-2 cells to be recognized by TLR9. However, bacterial genomic DNA cannot translocate into mammalian cells because of its high molecular weight and hydrophilicity. Therefore, translocation of DNA into eukaryotic cells has generally been performed using transfection reagents, such as liposomes. In the present study, we confirmed that L. plantarum genomic DNA translocated into Caco-2 cells in the presence (Figure 5), but not in the absence (data not shown), of transfection reagent. However, translocation of genomic DNA was also observed in Caco-2 cells treated with heat-killed L. plantarum. At present, we cannot explain these observations.
studies showed that *L. plantarum* produces a major polyanime, putrescine, and that polyanimes form a complex with and stabilize the DNA. This complex subsequently forms nanoparticles and is subsequently readily translocated into eukaryotic cells. One possible explanation for the translocation of genomic DNA into Caco-2 cells treated with heat-killed *L. plantarum* is that the genomic DNA and polyanime contained in heat-killed *L. plantarum* may form such a complex, thus facilitating translocation of the genomic DNA into Caco-2 cells due to the transfection effect of the polyanime.

**Conclusion**

The results of the present study indicated that heat-killed *L. plantarum* increased the level of elafin secretion from Caco-2 cells, and the bacterial genomic DNA and TLR9 play crucial roles in this effect. Lactobacilli therapy is useful for the treatment of IBD, although the underlying mechanisms are not fully understood. Our observations suggest that one possible mechanism for the efficacy of lactobacilli therapy may involve an increase in elafin levels in the intestine. Further studies of the elafin induction associated with lactobacilli and their genomic DNA may lead to the development of novel therapeutic approaches to ameliorate intestinal inflammation.

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