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Adhesion of Lactic Acid Bacteria Can Modulate the Secretion of Cytokines on HT-29 Colon Adenocarcinoma Cells

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Lactic acid bacteria (LAB) has been emphasized to provide beneficial function to human gastrointestinal (GI) ecosystem as a probiotic. It has not been well-documented that LAB could modulate immune system in GI tract. The purpose of this study is to determine whether adhesion of three LAB (*Lactobacillus casei* 3260, *Lac. casei* 3109 and *Lac. GG*) on HT29 colon adenocarcinoma cells could change cytokine production pattern (Th1/Th2 profiles) *in vitro*. Our findings revealed that binding of *Lac. casei* 3260 and *Lac.GG* to HT-29 cell induced the increased level of Th1 type cytokine, IL-2 and IFN-γ while it decreased Th2 type cytokines, IL-1β, IL-6, and TNF-α, but not IL-4, implicating that binding of LAB on HT-29 normalized Th1/Th2 profile. In conclusion, as a probiotic, LAB may give a good effect on colon cancer by balancing the Th1/Th2 cytokine profile, but it is critically dependent on binding capacity of LAB to tumor/cancer cells and thus the pattern of cytokine production due to LAB adhesion may be bacterial strain-specific.

Key Words: Lactic acid bacteria, Adhesion, Colon cancer, Cytokines, Th1/Th2 profile

INTRODUCTION

During the past decade, the vast evidence has supported the potential benefit of lactic acid bacteria in health promotion as a probiotics. It includes improvement of gastrointestinal microfloral ecosystems, reduction of serum cholesterol, stimulation of immunological system, removal of oxidative stress, and anticarcinogenic activities. The anticarcinogenicity of LAB has been extensively studied by Burns and Rowland.[1)] According to their reports, LAB supplementation has been shown to have protective effects against a broad range of events such as induction of DNA damage in the colonic mucosa, formation of aberrant crypt foci, tumor incidence, and alteration of bacterial enzyme activities thereby reducing the levels of enzymes involved in carcinogen formation.[2~4)] In gastrointestinal tract, there might be many factors to influence the initiation and promotion of tumor cells, such as change of intestinal cytokine pattern and binding of LAB on the intestinal cells.

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Cytokines comprise a large family of intracellular communicating molecules that play important roles in immunity, inflammation and repair, as well as general tissue homeostasis. In every tissue, there is a cytokine network where the production of individual cytokines stimulates and inhibits production of, and response to, other cytokines. Therefore, it is well accepted that cytokines are key molecules controlling autocrine or paracrine communications within and between the individual cell types. Because of different patterns of receptor expression, cytokines influence a growth and survival of tumor cells. Wilson and Balkwill have highlightened the evidence for the tumor activity of endogenous TNF, IL-1 and a range of chemokines that are related to chronic inflammation and increased susceptibility to cancers.[5] However, over-expression of, or treatment with, the some cytokines can provoke a powerful anti-tumor response.[6]

It has been suggested that LAB, as a probiotic, could modulate or stimulate gastrointestinal immune system. One of the important criteria for a good probiotic strain is believed to be its ability to adhere to mucosal surfaces of the human gastrointestinal tract.[7] Adhesion of probiotic LAB has been reported to be species specific and temporarily colonize the intestine, which beneficially influence the microbial balance of the host. For a last few years, some strains of LAB such as *Lac. acidophilus* LA1, *Lac.* GG, and *Lac. casei* Shirota have been proved to have an higher ability to adhere to *in vitro* intestinal cell lines, and their binding efficacy and characteristics have been defined in many studies.[8] Despite many studies of LAB binding on colonic cancer cells, there are little reports concerning the cytokine secretion in the intestine. The aim of this study is to investigate that adhesion of three representative LAB on colonic adenocarcinoma cell (HT-29) could modulate the pattern of cytokine secretion after lipopolysaccharide (LPS) treatment.

MATERIALS AND METHODS

1) Bacterial Strains, culture, and counts

Lac. casei KCTC 3260, *Lac. casei* 3109, and *Lac. GG* were obtained from the frozen stock culture collection of KCTC (Korean Culture Type Collection). All strains were cultured for 24 hr in deMan-Rogosa-Sharpe (MRS) broth (Oxoid, Hampshire, United Kindom) under aerobic conditions (37°C, 10% CO_2). All strains were serially transferred at least three times prior to use in studies. Bacterial counts were determined by flow cytometry using a FACS Calibur equipped with an air-cooled 488-nm argon-ion laser at 15 mV. Direct counts were enumerated by using Fluoresbrite beads (2.0 um, Polysciences Inc.) as an internal calibration. Viability of bacterial populations was assessed by using SYTOX green nucleic acid stain (Molecular Probes, S-7020) at 1μM/10^6～10^7 bacteria to detect non-viable bacteria. A band pass filter of 525 nm was used to collect the emission for green SYTOX. To support the precise bacterial counts, the colony forming unit (CFU) was determined by plating serial 10-fold dilutions of bacterial suspension with MRS agar plates. For adhesion on HT-29 cells, all strains have been adjusted to 2×10^9 CFU/ml, respectively.

2) HT-29 cell culture

The HT-29 cell line (ATCC HTB39) was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in Macoy's 5A (Whitaker, USA) supplemented with 10% heat-inactivated (55°C, 30 min) fetal bovine serum (FBS, Whitaker, USA), 2 mM L-glutamine (Sigma), 100 U/ml penicillin and 100 mg/ml strptomycin (BioWhitaker, USA) at 37°C in an atmosphere of 5% CO_2. For bacterial adhesion, HT-29 monolayers were prepared on 24-well tissue culture plates with a concentration of 5×10^5 cells per well to obtain confluence, treated with 3μM methotrexate to induce secretion of mucous, and maintained for 2 weeks prior

to use. The cell culture medium was changed every other day and replaced by fresh non-supplemented Macoy's 5A at least 1 hr before the bacterial adhesion.

3) Measurement of cytokines

The production of IL-1β, IL-2, IL-4, IL-6, IFN-γ, and tumor necrosis factor-α (TNF-α) was determined by sandwich ELISA. Mouse anti-human IL-1β, IL-2, IL-4, IL-6, and TNF-α purified antibodies; rabbit anti-human IL-1β, IL-2, IL-4, IL-6, IFN-γ, and TNF-α biotinylated antibodies; and recombinant human IL-1β, IL-2, IL-4, IL-6, IFN-γ, and TNF-α were obtained from Endogen (Cambridge, MA). Briefly, HT-29 cells were treated with 10 ng/ml of LPS to induce Th2 cytokine production and maintained overnight. About 2×10^9 CFU/ml of bacterial stock in PBS has added into HT-29 monolayer cells and incubated for 6 hours at 37°C in an atmosphere of 5% CO_2. After centrifugation of 24 well plate at 800 rpm for 10 min, 500μl of supernatant was carefully transferred to 1.5 ml of eppendorf tube and stored at -80°C until immunoassay.

4) Statistics

All variables were compared using a one-way analysis of variance (ANOVA), followed by a two-tailed Student's t-test for comparison between any two groups. Differences between two groups were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

1) Th1 type cytokines

T cells could be split into two main categories, Th1 and Th2, and Th1/Th2 profile is regarded as a critical factor in initiation and promotion of cancer cells. Th1 cell predominantly produces Th1 type cytokines such as IL-2 and IFN-γ that stimulate the growth of T cells and activation of cytotoxic T cell or natural killer (NK) cells. Our data have indicated that LPS treatment lowered the secretion of both IL-2 and IFN-γ (Fig. 1, 2). It is not surprised that LPS treatment usually induces Th2 cytokines causing the shift of cytokine pattern from Th1 toward Th2 type. Interestingly, binding of LAB has increased IL-2 and IFN-γ level to near normal range. There might be two possibilities to explain the increased level of these cyto-

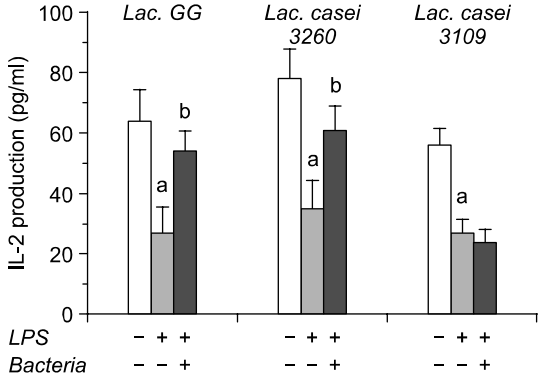

Fig. 1. Production of IL-2 from HT-29 colon adenocarcinoma cell after treatment of three different lactic acid bacteria. Data were represented by mean±SD. a: significantly different ($p<0.05$) compared to negative control (without treatment of LPS and bacteria), b: significantly different ($p<0.05$) compared to only LPS treatment.

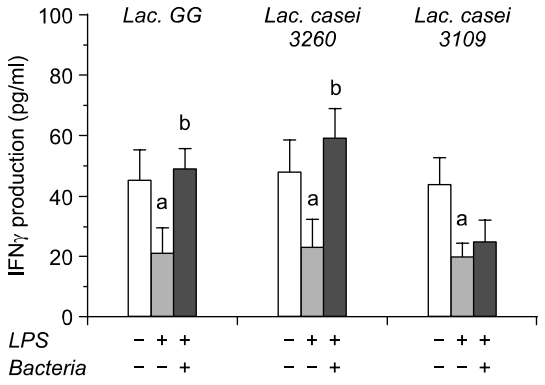

Fig. 2. Production of IFNγ from HT-29 colon adenocarcinoma cell after treatment of three different lactic acid bacteria. Data were represented by mean±SD. a: significantly different ($p<0.05$) compared to negative control (without treatment of LPS and bacteria), b: significantly different ($p<0.05$) compared to only LPS treatment.

kines. First, LAB could capture LPS to prevent induction of Th2 cytokines resulting in normalizing the level of Th1 cytokines. Although there is no direct evidence that LPS binds to surface of LAB, we assume that there is still a chance because structure of LAB cell wall partially contains LPS with similar composition and thereby interact with exogenous LPS in randomized manner. Haskard et al.[9] has reported that AFB_1 is bound noncovalently and extracellularly on LAB and suggested that cell wall polysaccharide and peptidoglycan are the two main elements responsible for the binding of mutagens to LAB. This data may indirectly support our data addressing LPS binding to LAB. Secondly, binding of LAB on HT-29 cells may regulate the gene expression, especially Th1 type cytokines in this study. This data could be supported by Servin's review indicating that binding characteristic of LAB on Caco-2 intestinal epithelial cell induced the gene expression for antibiotic proteins.[10] Among the bacterial strains, it showed the different level of IL-2 and IFN-γ production, suggesting that modulation of IL-2 and IFN-γ secretion is strain-specific. This result may be caused by different binding capacity of LAB to either LPS or HT-29 cells. There are many reports that LAB has a broad range of binding on intestinal cells by approximately 0～45%. And the most has agreed that *L. GG* has higher binding ability to both *in vivo* and *in vitro* intestinal cells.[11,12] Unpublished data from our laboratory indicated that *Lac. casei* 3260 has a similar ability on binding to intestinal cell lines as compared to *Lac. GG*, but not *Lac. casei* 3109. The pattern of Th1 cytokine secretion was accordance with binding ability of LAB to intestinal cells. Therefore we assume that the binding characteristics could influence cytokine production in HT-29 cell culture.

2) Th2 type cytokines

Th2 type cytokines involve IL-1, IL-4, IL-6 and TNF-α that stimulate the growth of B cells and mediate the inflammation response. Among them, TNF-α is a major mediator of dramatic cellular change and dynamic tissue modeling. It can be detected in malignant and/or stromal cells in human breast, ovarian and colorectal cancer often in association with IL-1, IL-6 and M-CSF. In breast cancer, infiltrating macrophages are a major source of TNF-α, which may regulate a key angiogenic enzyme (thymidine phosphorylase) in the tumor epithelium.[13] In prostate cancer, TNF-α production correlates with loss of androgen responsiveness and cachexia. And it is also associated with poor prognosis in haematologic malignancies.[14] In ovarian cancer, TNF-α mRNA is found in epithelial tumor islands, where there is a correlation with high TNF-α expression and high tumor grade.[15] There is also evidence for pro-cancer actions of TNF-α in animal models.[16] Treatment of experimental ascitic ovarian cancer xenografts promotes adhesion of free floating tumor cells to the peritoneum and solid tumor formation, and over-expression of TNF-α confers invasive properties on some tumor cells. Evidence from animal models suggests that the IL-1, especially IL-1β, may also promote tumor development and spread. In mouse models of metastases, treatment with the IL-1 receptor antagonist significantly decreased tumor development, suggesting that local production of IL-1 aids development of metastases. Moreover, mice deficient in IL-1β were resistant to the development of metastases.[17]

Our data have revealed that IL-1β, IL-6 and TNF-α production significantly reduced after LAB bound to HT-29 cells (Fig. 3, 5, 6). However, IL-4 in this study has not shown any statistical difference, implicating that activation of B cells may be not involved in gastrointestinal ecosystem change caused by LAB adhesion (Fig. 4). Referred as pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α intercommunicate each other to induce inflammatory response by mediation of nuclear factor kappa B (NF-kB). It is so far well-accepted that TNF-α stimulates the translocation of NF-kB, which results in the genetic expression of IL-1β, IL-6 and TNF-α, and further exacerbate inflammatory reaction in tissue or cells. It is of importance in pointing out that chronic inflammation in-

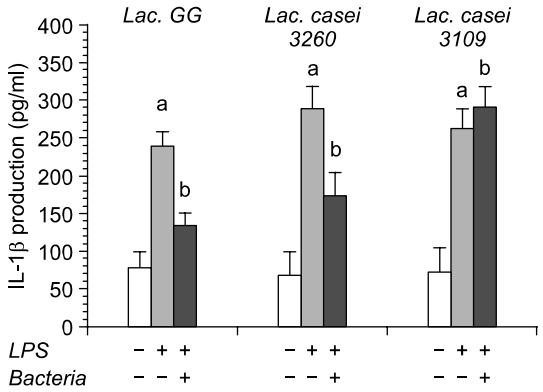

Fig. 3. Production of IL-1β from HT-29 colon adenocarcinoma cell after treatment of three different lactic acid bacteria. Data were represented by mean±SD. a: significantly different ($p<0.05$) compared to negative control (without treatment of LPS and bacteria), b: significantly different ($p<0.05$) compared to only LPS treatment.

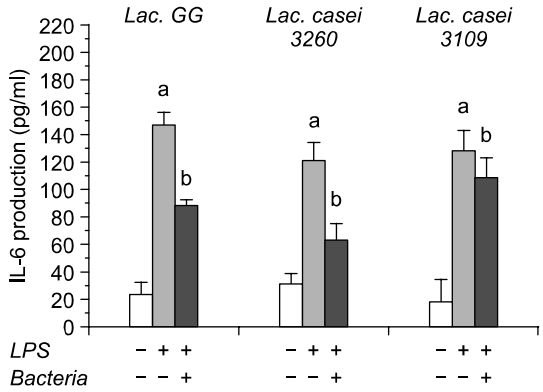

Fig. 5. Production of IL-6 from HT-29 colon adenocarcinoma cell after treatment of three different lactic acid bacteria. Data were represented by mean±SD. a: significantly different ($p<0.05$) compared to negative control (without treatment of LPS and bacteria), b: significantly different ($p<0.05$) compared to only LPS treatment.

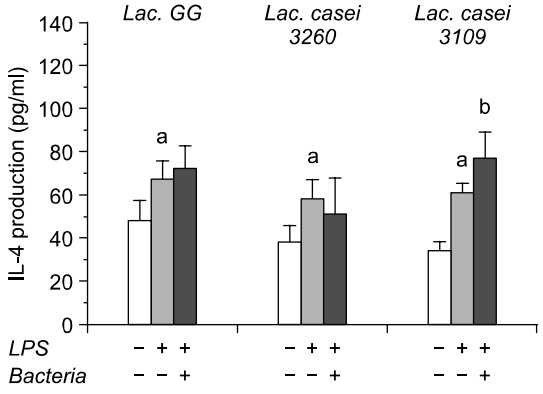

Fig. 4. Production of IL-4 from HT-29 colon adenocarcinoma cell after treatment of three different lactic acid bacteria. Data were represented by mean±SD. a: significantly different ($p<0.05$) compared to negative control (without treatment of LPS and bacteria), b: significantly different ($p<0.05$) compared to only LPS treatment.

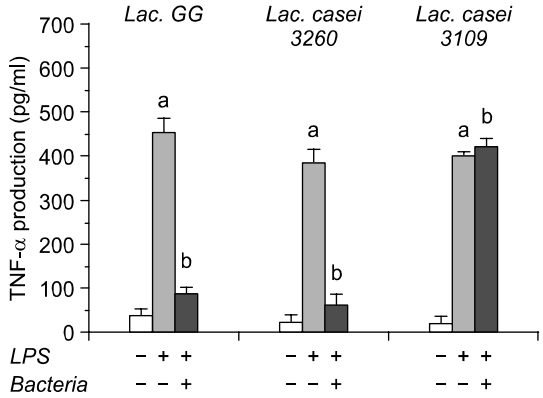

Fig. 6. Production of TNF-α from HT-29 colon adenocarcinoma cell after treatment of three different lactic acid bacteria. Data were represented by mean SD. a: significantly different ($p<0.05$) compared to negative control (without treatment of LPS and bacteria), b: significantly different ($p<0.05$) compared to only LPS treatment.

creases the risk of tumor development.[5] Therefore, the data in this study are in accordance with this concept and further represent possible evidence for the association of inflammatory cytokine production with tumor development.

3) Conclusion

LAB has been emphasized to provide beneficial function to human gastrointestinal ecosystem. However, it has not been well-documented that LAB could modulate immune system in GI tract. Thus our study aimed to determine whether adhesion of LAB on HT-29

colon adenocarcinoma cells could change cytokine production pattern (Th1/Th2 profiles) *in vitro*. LPS has been treated in at least amount (10 ng/ml) to activate TNF-α production and thereby resulted in the shift of cytokine pattern from Th1 to Th2 type. We found that, after binding of LAB on HT- 29 cells, Th1 type cytokines were increased and Th2 type cytokines except IL-4 were decreased, indicating the normalization of Th1/Th2 profile. In conclusion, as a probiotic, LAB may give a good effect on colon cancer by balancing the Th1/Th2 cytokine profile, but it is critically dependent on binding capacity of LAB to tumor/cancer cells and thus the pattern of cytokine production due to LAB adhesion may be bacterial strain-specific.

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