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Differential immune-stimulatory effects of LTAs from different lactic acid bacteria via MAPK signaling pathway in RAW 264.7 cells

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ABSTRACT

Objective: Lipoteichoic acid (LTA) is an immune-stimulatory component found in the cell wall of lactic acid bacteria, which are a major group of Gram-positive bacteria known to have beneficial health effects in humans. In this study, we evaluated the stimulatory effects of LTAs isolated from different lactobacilli species with mouse macrophage RAW 264.7 cells.
Methods: RAW 264.7 cells were stimulated with pLTA (isolated from *Lactobacillus plantarum* K8), rLTA (isolated from *Lactobacillus rhamnosus*), dLTA (isolated from *Lactobacillus delbreukii*), and sLTA (isolated from *Lactobacillus sakei* K101). Tumor necrosis factor (TNF)-α and interleukin (IL)-10 production were examined by ELISA, and nitric oxide (NO) production was assayed using Griess reaction. The mRNA and protein expression levels of inducible nitric oxide synthase (iNOS) was examined by reverse transcriptase polymerase chain reaction (RT-PCR) and Western blotting. Signaling molecules were also examined by Western blotting.
Results: pLTA and rLTA moderately induced TNF-α, IL-10, and NO production compared with stimulation of RAW 264.7 cells with dLTA and sLTA. Similar results were obtained for the mRNA and protein expression levels of iNOS. Western blot analysis showed that treatment of cells with pLTA or rLTA resulted in minimal phosphorylation of ERK, JNK and p38 MAPK while, dLTA and sLTA were strong activators of MAPK signaling. In addition, the glycolipid structure of LTAs was found to be composed of different fatty acid chain groups and lengths. Taken together, these results suggest that the differential immunostimulatory effects of LTAs isolated from different lactobacillus species may be related to their different ability to activate the MAPK signaling pathway, which are modulated by a unique glycolipid structure of LTA.

Introduction

Lactic acid bacteria (LAB), a group of Gram-positive bacteria, are known for their human health-promoting effects such as modulation of the immune system, improvement of defense against infections, prevention of allergies, and antitumor effects (Meydani and Ha, 2000; Schabussova and Wiedermann, 2008; de Vrese et al., 2006; Abd el-Gawad et al., 2004). Several strains of LAB also have effects upon the production of cytokines, such as IL-10, IL-4, IL-12, TNF-α, IL-6, TGF-β, IL-8, and on T-cell proliferation (Mohamadzadeh et al., 2005; Christensen et al., 2002; Hart et al., 2004; Wallace et al., 2003). Previous studies have demonstrated that *Lactobacillus* species can be used as probiotics (Brashears et al., 2003; Parvez et al., 2006). Probiotics have been used successfully to treat various clinical conditions, such as atopic disease, inflammatory bowel disease (IBD), irritable bowel syndrome, and cardiovascular diseases (Miraglia del Giudice et al., 2006; McFarland and Dublin, 2008; Vanderpool et al., 2008; Rajiv et al., 2010). For example, *L. plantarum* reduces serum cholesterol,

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LDL-cholesterol, and triglyceride levels (Xie et al., 2011), and attenuates inflammatory bowel disease (IBD) (Schultz et al., 2002). *L. rhamnosus* suppresses allergic sensitization and airway inflammation (Feleszko et al., 2007). *L. delbrueckii* can protect against *Listeria monocytogenes* (dos Santos et al., 2011), while *L. sakei* has been suggested to have beneficial effects in children with atopic eczema-dermatitis syndrome (AEDS) (Woo et al., 2010). The immuno-stimulating potency of lactobacilli appears to be species-specific (Ryu et al., 2009). For example, a previous study reported that *L. rhamnosus* and *L. plantarum* moderately induced the expression of co-stimulatory molecules and stimulated low-level production of cytokines and chemokines (Veckman et al., 2004). In contrast, *L. delbrueckii* was reported to stimulate the production of high levels of the pro-, and anti-inflammatory cytokines TNF-α, IL-10, respectively, as well as NO in the mucosal immune system (dos Santos et al., 2011).

Among the various Gram-positive bacteria cell wall components, lipoteichoic acid (LTA) is considered to be analogous to the lipopolysaccharide (LPS) of Gram-negative bacteria endotoxins, due to similar biochemical and physiological properties (Ginsburg, 2002). However, the LTAs of *L. plantarum* and *Bacillus subtilis* have differential immuno-stimulatory effects; they induce the production of cytokines and stimulation of TLR2 to different degrees (Ryu et al., 2009). Interestingly, LTA isolated from *L. plantarum* has been shown to have an inhibitory effect on LPS-induced TNF-α production (Kim et al., 2008). Therefore, in this study, we isolated highly pure LTAs from *L. plantarum* K8 (pLTA), *L. rhamnosus* (rLTA), *L. delbreukii* (dLTA), and *L. sakei* K101 (sLTA) to investigate if they had differential immuno-stimulatory effects. Many recent studies have shown that activation of MAPKs, such as ERK, JNK, and p38, is generally involved in LPS-induced TNF-α production (Kraatz et al., 1999). Interestingly, pLTA has been shown to suppress LPS-activated signaling (Kim et al., 2008). In this study, we therefore examined the ability of LTAs isolated from various lactobacilli species to induce cytokine production and activate the MAPK signaling pathway in RAW 264.7 cells. The results indicate that LTAs are responsible for the species-specific immuno-stimulatory effects and it is due to their differential ability to activate the MAPK signaling pathway.

Materials and methods

Cell lines and stimulation

Mouse macrophage cell line, RAW 264.7 cells, was cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml of streptomycin. Cells were maintained at 37 °C with 5% CO_2 in a humidified incubator. RAW 264.7 cells were seeded onto 96-well plates or 12-well plates. After incubation for 24 h, the RAW 264.7 cells were stimulated with LTA or LPS (*Escherichia coli* 055:B5; Sigma–Aldrich, St. Louis, MO).

Preparation of LTA

Highly-pure and structurally intact LTA was isolated from *L. plantarum* K8 (KCTC10887BP), *L. sakei* K101 (KCCM11175P), *L. rhamnosus*, *L. delbrueckii*, and *S. aureus* by *n*-butanol extraction, as described previously (Han et al., 2003). The purity of the purified LTA was determined by measuring the protein and endotoxin content through conventional silver staining after PAGE and the *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD), respectively.

Cell viability assay

Cell viability was determined using the PreMix™ WST-1 Cell Proliferation Assay System (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. Cells (5×10^4 cells/ml) in 100 μl culture medium were treated with 0.1 μg/ml LTAs for 6 h at 37 °C and 5% CO_2. 10 μl of WST-1 reagent was added, and the cells were incubated for 2 h at 37 °C and 5% CO_2. Absorbance was measured using a microplate reader (Eppendorf BioPhotometer, Hauppauge, NY) at a test wavelength of 450 nm and a reference wavelength of 600 nm.

ELISA

After the RAW 264.7 cells were stimulated with LTA or LPS, cell supernatants were collected and assayed for cytokine production by standard sandwich ELISA. The production of TNF-α and IL-10 was determined using a purified anti-mouse TNF-α antibody and purified anti-mouse IL-10 antibody for capture, respectively. The biotinylated anti-mouse TNF-α antibody and the biotinylated anti-mouse IL-10 antibody (R&D systems, Minneapolis, MN) were used for detection, according to the manufacturer's instructions. The optical density of the wells was determined using a microplate reader (Eppendorf BioPhotometer, Hauppauge, NY) at a wavelength of 450 nm with correction at 540 nm.

NO measurement

The nitrite concentration in the cell culture medium was measured as a proxy for NO production using the Griess reaction (Green et al., 1982). In brief, RAW 264.7 cells (1×10^5 cells/ml) were plated and incubated with various concentrations of LTA or LPS for 48 h. After stimulation, the cell culture medium was saved for nitrite measurements. One hundred microliters of culture supernatant was mixed with the same volume of Griess reagent (0.1% N-(1-naphthyl)-ethylenediamine dihydro-chloride and 1% sulfanilamide in 2.5% phosphoric acid). The absorbance of the mixture was measured at 540 nm on an automated EL800 plate reader (BioTek instruments, Inc., Winooski, VT). The concentration of NO was calculated using sodium nitrite as a standard.

Western blot analysis

Phospho-CREB, c-Jun, and c-Fos levels were analyzed using nuclear extracts prepared as described previously (Feleszko et al., 2007). Specific anti-c-Jun, anti-c-Fos, and anti-phospho-CREB antibodies were purchased from Cell Signaling Technology (Danvers, MA). For Western blotting analysis, 20 μg of total protein was added to Proprep buffer (iNtRON Biotechnology, Seongnam, Korea), boiled for 5 min, and resolved by 10% SDS-PAGE using a Tris/glycine/SDS buffer (25 mM Tris, 250 mM glycine, 0.1% SDS), and then the proteins were blotted onto nitrocellulose membranes (100 V, 2 h, 4 °C). After blocking for 1 h in TBS-T (20 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat milk, membranes were washed three times in TBS-T and probed overnight with anti-phospho-MAP kinase Ab (Cell Signaling Technology, Danvers, MA) or anti-iNOS (Santa Cruz Biotechnology, Santa Cruz, CA) Ab in TBS-T/5% BSA. Following three washes in TBS-T, membranes were incubated with secondary HRP-conjugated donkey anti-rabbit IgG (Amersham Pharmacia Biotech) for 2 h and washed five times in TBS-T. Bands were detected using enhanced ECL reagents (GE Healthcare Bioscience, Piscataway, NJ) according to the manufacturer's instructions.