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Therapeutic effects of kefir grain *Lactobacillus*-derived extracellular vesicles in mice with 2,4,6-trinitrobenzene sulfonic acid-induced inflammatory bowel disease

M. K. Seo,[1] E. J. Park,[1] S. Y. Ko, E. W. Choi, and S. Kim[2,3]
Prostemics Research Institute, Seongdong-gu, Seoul 04778, South Korea

ABSTRACT

Kefir is a fermented product from yeast and lactic acid bacteria, and has been associated with various health benefits including relieving inflammatory bowel disease. Recently, it has been shown that gram-positive bacteria produce extracellular vesicles (EV). The EV could be appearing as potentially important mediators of cell to cell interaction. In this study, we explored the role of kefir grain *Lactobacillus*-derived EV in modulating inflammation responses via alleviating the production of inflammatory cytokines in tumor necrosis factor-α (TNF-α)-induced inflammation in Caco-2 cells and the 2,4,6-trinitrobenzene sulfonic acid-induced inflammatory bowel disease mouse model. Kefir-derived *Lactobacillus* EV were isolated by ultracentrifugation of the culture medium of 3 different kefir-derived strains (i.e., *Lactobacillus kefir*, *Lactobacillus kefiranofaciens*, and *Lactobacillus kefirgranum*). Nanoparticle tracking analysis showed that the size of isolated kefir-derived *Lactobacillus* EV was within 80 to 400 nm, and kefir-derived *Lactobacillus* EV uptake into recipient Caco-2 cells was confirmed by fluorescence labeling. Treatment of each kefir-derived *Lactobacillus* EV onto TNF-α-stimulated Caco-2 cells significantly reduced the level of both mRNA expression and secretion of IL-8, and Western blot analysis revealed that such an effect was related to inhibition of TNF-α signaling mediated by reducing the phosphorylation of p65, a subunit of NF-kB. Subsequent administration of kefir-derived *Lactobacillus* EV into inflammatory bowel disease-induced mice significantly alleviated the body weight loss and rectal bleeding, and enhanced stool consistency. Histological examination showed that kefir-derived *Lactobacillus* EV substantially reduced the infiltration of transmural leukocytes and loss of goblet cells within the colon, and the serum level of myeloperoxidase was significantly lower in the EV-treated group than control group. Our study demonstrates that kefir-derived *Lactobacillus* EV can be potentially used for developing innovative strategies for alleviating inflammatory bowel disease.
Key words: kefir, extracellular vesicle, functional dairy food, irritable bowel syndrome

INTRODUCTION

Kefir is a fermented milk product that originated in the Balkans, Eastern Europe, and the Caucasus (Fontán et al., 2006; Serafini et al., 2014). Kefir grains have a complex composition of microbial species; the predominant populations are lactic acid bacteria, acetic bacteria, yeasts, and fungi (Zhou et al., 2009; Pogačić et al., 2013). Some of the microorganisms isolated and identified in kefir cultures were classified using the product's name, as in *Lactobacillus kefiri*, *Lactobacillus kefiranofaciens*, *Lactobacillus kefirgranum, Lactobacillus parakefir*, and *Candida kefyr* (Wyder et al., 1999; Kwon et al., 2003; Yang et al., 2007; Taş et al., 2012). Kefir has various functions on human health due to its antimicrobial (Anselmo et al., 2010), immunoregulatory (Hong et al., 2009), antiallergenic (Hong et al., 2010), antitumoral (Gao et al., 2013), anti-inflammatory (Diniz et al., 2003), antidiabetic (Kwon et al., 2006), and antimutagenic (Guzel-Seydim et al., 2011) activities, although the mechanism of such function is not clear.

Inflammatory bowel disease (**IBD**) includes 3 entities (Crohn's disease, ulcerative colitis, and indeterminate colitis) characterized by a multifactorial pathogenesis and a chronic course. Inflammatory bowel disease is manifested by an inappropriate mucosal immune system response against intestinal luminal antigens (Farrell and LaMont, 2002; Sartor, 2004). Recently, Senol et al. (2015) demonstrated that kefir reduces the clinical disease activity index and histological colitis scores in a dextran sulfate sodium-induced colitis model, possibly via reduction of myeloperoxidase (**MPO**), tumor necrosis factor-α (**TNF-α**), and inducible nitric oxide synthase levels. A relevant study also showed that *Lac-*

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[1] These authors contributed equally to this work.
[2] Current address: Stem Cell Center, Asan Institute for Life Sciences, Asan Medical Center, Seoul 05505, South Korea.
[3] Corresponding author: skim@amc.seoul.kr

tobacillus subspecies, *L. salivarius* 433118, and *Bifidobacterium infantis* 35624 were found to attenuate colitis in IL-10 knockout mice (McCarthy et al., 2003).

It has been reported that the release of biological cargos including nucleic acids, toxins, and enzymes is mediated by extracellular vesicles (**EV**) in gram-positive bacteria, suggesting the highly conserved cellular transport system in various species (Deatherage and Cookson, 2012; Brown et al., 2015). Functionally EV are reported to be involved in numerous biological processes including the elimination of unwanted proteins or molecules from the cell, exchange of molecular materials between cells, communication between cells, and antigen presentation (Deatherage and Cookson, 2012). Various types of gram-negative bacteria have been shown to produce outer membrane vesicles containing a polysaccharide, DNA, immunomodulatory compounds, and communication factors (Zhou et al., 1998; Schwechheimer et al., 2013). Although the EV were discovered in gram-negative bacteria decades ago (Mashburn-Warren and Whiteley, 2006; Lee et al., 2008), studies in gram-positive bacteria have been completely overlooked until recently. This can be attributed to the structural traits of gram-positive bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, and *Lactobacillus*, whose plasma membrane is surrounded by a much thicker cell wall. However, recent studies have shown that gram-positive bacteria produce biologically active EV (Rivera et al., 2010; Gurung et al., 2011).

The aim of this study was to examine whether EV derived from *Lactobacillus kefir*, *Lactobacillus kefiranofaciens*, or *Lactobacillus kefirgranum* can mediate the role of these functional bacteria in alleviating the production of inflammatory cytokine in TNF-α-induced inflammation in Caco-2 cells, and to investigate the protective effects of kefir-derived *Lactobacillus* EV in 2,4,6-trinitrobenzenesulfonic acid (**TNBS**)-induced IBD in a mouse model. Our results will provide essential information on understanding the mechanism of EV-mediated role of functional bacterium in reducing inflammation.

MATERIALS AND METHODS

Cell Culture and Treatment

Caco-2 cells were cultured at 37°C under a 5% CO_2 humidified atmosphere in Eagle's minimum essential medium containing 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 10% fetal bovine serum. Commercially available TNF-α (Sigma-Aldrich, H8916) was dissolved in 100% dimethyl sulfoxide to a final concentration of 2 μg/mL of TNF-α (20 ng/mL), which was identified as the optimal dose for stimulation experiments. The culture medium containing the TNF-α was removed after 6 h of pretreatment, and a fresh culture medium containing kefir-derived *Lactobacillus* (*L. kefir*, *L. kefiranofaciens*, and *L. kefirgranum*) EV was added for 24 h.

Preparation of Kefir-Derived Lactobacillus EV

Secreted EV were isolated from kefir culture medium by differential centrifugation essentially as previously described (Oliveira et al., 2010). *Lactobacillus kefir* KCTC 3611, *L. kefiranofaciens* KCTC 5075, and *L. kefirgranum* KCTC 5086 were obtained from the Korean Collection for Type Cultures (**KCTC**). All strains were maintained and cultured on MRS medium (Difco, Franklin Lakes, NJ) at pH 5.2 to 7.0 under anaerobic conditions unless otherwise mentioned. Conditioned medium was collected after 96 h and was centrifuged at 300 × *g* at 4°C for 10 min to eliminate bacteria. The supernatant was centrifuged at 1,200 × *g* at 4°C for 20 min and 10,000 × *g* at 4°C for 30 min. The EV pellet was collected by ultracentrifugation at 100,000 × *g* at 4°C for 70 min and suspended in PBS. All samples were filtered through a 0.22-μm syringe filter (Merck Millipore, Darmstadt, Germany).

Characterization of Kefir-Derived Lactobacillus EV

Real-time high-resolution particle detection, counting, and sizing were performed on the NanoSight NS300 following manufacturer protocols (Malvern Instruments, Malvern, UK). Particle concentration (particles/mL) of kefir-derived *Lactobacillus* EV was calculated by the NanoSight system. The Nanoparticle Tracking Analysis system was also used to compare changes in concentrations and sizes before and after ultracentrifugation of EV in kefir-derived *Lactobacillus* culture medium. For cryo-transmission electron microscopy images, grid (200 mesh R 2/2 Quantifoil holey-carbon) was vitrified using a Vitrobot Mark III (Leica, Wetzlar, Germany) at room temperature and 100% humidity. A 4-μL droplet of the vesicle suspension was applied to the grid. Excess sample was removed by blotting once between 1 and 2 s with filter paper. The blotted grid was plunged into liquid ethane and transferred under liquid nitrogen. Sample analysis was carried out under an FEI transmission cryoelectron microscope (Tecnai F20 G2, FEI Corp., Hillsboro, OR) with an acceleration voltage of 200 kV. Images were recorded with a 2k × 2k Gatan Ultrascan 1000 CCD camera (Gatan, Pleasanton, CA). Kefir-derived *Lactobacillus* EV were isolated as described above and labeled with PKH26 (Sigma-Aldrich, St. Louis, MO) for 10 min at room temperature. Labeled EV were washed twice with