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ARTICLE

In vitro and in vivo effects of polysaccharides isolated from Korean persimmon vinegar on intestinal immunity

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Abstract The in vitro and in vivo effects of crude polysaccharide isolated from Korean persimmon vinegar (KPV-0) on the intestinal immunostimulation were examined. KPV-0 was not cytotoxic to intestinal epithelial Caco-2 cells and could be transported through a Caco-2 cell monolayer in an in vitro coculture system. For and in vitro experiment, KPV-0 treatment significantly increased IgA production by Peyer's patch (PP) cells and triggered an increase in transforming growth factor (TGF)-β1 and interleukin (IL)-6 levels. To investigate the in vivo effects of KPV-0 treatment, KPV-0 was administered to mice orally at different doses for 20 days. Oral administration of KPV-0-induced IgA and cytokines (IL-6, granulocyte macrophage-colony-stimulating factor, and TGF-β) production by PP cells and significantly increased the IgA levels in intestinal fluids and feces. These results suggested that the polysaccharides isolated from persimmon vinegar appear to modulate the intestinal immune system and could be beneficial to human health.

Keywords Immunoglobulin A · Intestinal immune system · Persimmon vinegar · Peyer's patch · Polysaccharide

Introduction

Vinegar can be prepared from various materials, such as grapes, apples, rice, and wheat, and has been widely used throughout history as a flavoring agent and preservative. Vinegar is produced via a fermentation process carried out by several microorganisms including molds, yeasts, lactic acid bacteria, and acetic acid bacteria (Rainieri and Zambonelli 2009). These organisms produce not only acetic acid but also various metabolic compounds that modify the taste and flavor of the resulting food product. Some vinegars contain antioxidants, antitumor compounds, and other bioactive metabolites, which might be responsible for the beneficial health effects attributed to vinegar (Giudici et al. 2009; Murooka et al. 2009). Persimmon vinegar, produced from persimmon fruit, contains high concentrations of various polyphenolic compounds (Matsuo and Ito 1978), such as kaki-tanin (Sakanaka et al. 2005); therefore, there is interest in evaluating the impact of persimmon vinegar on health. However, it is possible that specific polysaccharides may be selectively soluble at relatively low alcohol concentrations (5–10 %). There have been a few reports on the chemical properties and biological activities of polysaccharides isolated from persimmon vinegar (Hwang and Shin 2008; Kim and Shin 2014).

Polysaccharides are an interesting class of additives used in the food and drug industries. They have been widely studied because they have a broad spectrum of therapeutic properties, relatively low toxicities, few side effects, and unique biological, chemical, and physical properties

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(Schepetkin and Quinn 2006). The immunomodulatory, anti-inflammatory, and antitumor effects of polysaccharides have recently attracted attention in the biomedical fields (Fan et al. 2012). Many of the medicinal plants used in traditional medicine contain biologically active polysaccharides that affect the immune system, as reported for both in vitro and in vivo model systems (Paulsen and Barsett 2005; Kiyohara et al. 2010; Ramberg et al. 2010). As the most abundant class of antibodies found in the intestinal lumen of humans and in most other mammals, secretory IgA (sIgA) has long been recognized as a first line of defense against enteric pathogens and toxins in the intestinal epithelium. Production of sIgA against specific mucosal antigens is dependent on antigen sampling by Peyer's patch (PP) M cells, processing by antigen-presenting cells such as dendritic cells, T cell activation, and ultimately, B-cell class switch recombination in the gut-associated lymphoid tissue, mesenteric lymph nodes, and the neighboring lamina propria (He et al. 2007; Brandtzaeg 2010). Isolated lymphoid follicles in the small and large intestines also contribute to inducing mucosal immune responses (Newberry and Lorenz 2005). Multiple cytokines, including interleukin (IL)-4, transforming growth factor (TGF)-β, IL-5, IL-6, and IL-10 are instrumental in intestinal stimulation of SIgA production. A subset of these cytokines, notably TGF-β and IL-10, is also required for maintaining mucosally induced tolerance, thus establishing one of the many links between SIgA production, immunity, and intestinal homeostasis (Mantis et al. 2011).

In a previous study, we examined the chemical properties and innate immunostimulatory activities of crude polysaccharides isolated from vinegars manufactured with different raw materials (Kim et al. 2015). Our results indicated that the polysaccharides isolated from Korean persimmon vinegar had the most potent macrophage-stimulating and anti-complementary activities among six tested vinegars. However, the effect of polysaccharides isolated from persimmon vinegar on intestinal immunostimulatory activities such as IgA production, have not yet been evaluated. Therefore, in the present study, we examined the effects of oral administration of polysaccharides isolated from Korean persimmon vinegar on the intestinal immune system, particularly on the enhancement of SIgA, in order to evaluate the impact of persimmon vinegar as a functional dietary component.

Materials and methods

Isolation of polysaccharides from persimmon vinegar

Korean persimmon vinegar was obtained in large quantities from Sampyo Co. (Korea). Polysaccharides were isolated from persimmon vinegar as described previously (Kim and Shin 2014). Briefly, Korean persimmon vinegar was evaporated using a rotatory vacuum evaporator to remove acetic acid and flavors; crude polysaccharides were precipitated by the addition of four volumes of 95 % cold ethanol. The precipitate was dissolved in a small amount of water and dialyzed with water for 3 days using a Spectra/Por 2 membrane (MWCO 12,000–14,000; Spectrum Laboratories Inc., USA). The salt-free polysaccharide solution was lyophilized to yield the crude polysaccharide fraction of Korean persimmon vinegar (KPV-0).

Cell culture

RPMI-1640 medium, Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, and Fungizone (amphotericin B) were obtained from Gibco BRL Co. (USA). Non-essential amino acids solution (NEAA) was purchased from Invitrogen (USA), and a Cell Counting Kit-8 (CCK-8) for measurement of cell cytotoxicity was purchased from Dojindo Laboratories (Japan). Lipopolysaccharide from *Escherichia coli* (LPS) was purchased from Sigma (USA). The human intestinal epithelial cell line, Caco-2, was obtained from the American Type Culture Collection (USA), and was cultured in high glucose DMEM supplemented with 1 % NEAA, 100 U penicillin, 100 μg/mL streptomycin, and 10 % heat-inactivated FBS. Caco-2 cells corresponding to passages 40 − 60 were used for experiments. The murine macrophage cell line, RAW 264.7 was obtained from the Korean Cell Line Bank (Korea) and cultured in DMEM supplemented with 100 U penicillin, 100 μg/mL streptomycin, and 10 % heat-inactivated FBS. RAW 264.7 cells corresponding to passages 10–30 were used for experiments. Both cells were cultured at 37 °C with humidified air containing 5 % CO_2.

Cell cytotoxicity

Caco-2 cells (2×10^5 cells/well) were treated with various concentrations of KPV-0 (1, 10, 100, 1000 μg/mL) in a 96-well plate (Corning Costar Corp., USA), and then incubated at 37 °C. After 24 h, 10 μL of CCK-8 solution was added, and the cells were further incubated while cytotoxic effects were evaluated. Cell cytotoxicity was determined by measuring intensity at 450 nm using a microplate reader (Molecular Devices, USA). The percent cell viability was compared to that of the negative control (NC), which corresponded to cells treated with only medium.

Coculture system

A coculture system was established as described previously (Tanoue et al. 2008), with slight modification. Caco-2 cells were seeded at 3.5×10^5 cells/well on tissue culture

polycarbonate membrane filters in 12 transwell plate (0.3 μm pore size; Corning Costar Corp., USA), and incubated for 21 days to obtain an integrated cell monolayer with a transepithelial electrical resistance value (TER) equal to 1200 Ω cm^2, which was measured using a Millicell-ERS instrument (Millipore Corporation, USA). The culture medium was changed every 3 days until the cells were fully differentiated. RAW 264.7 cells were seeded at 8.5 × 10^5 cells/well into a 6-well tissue culture plate and incubated overnight so that cells completely adhered to the bottom of the well. After exchanging culture media, the transwell insert containing Caco-2 cells was introduced into multiple plate wells preloaded with RAW 264.7 cells. To evaluate the permeability of transport across the intestinal barrier, KPV-0 (500 μL) was applied to the apical side for 24 h, and culture supernatant from the apical sides was collected for IL-6 analysis. On the other hand, to evaluate the spontaneous release of Caco-2 cells or maximum release of RAW 264.7 cells, each of these cells was individually and directly stimulated with KPV-0.

Animals

Pathogen-free, 6-week-old female C3H/He mice were purchased from Orient Bio (Korea). The mice were acclimated to their housing environment for 1 week prior to experiments. The animal room environment was controlled at a 12 h light/12 h dark cycle, 24 ± 1 °C, and 55 % humidity. A water and pellet diet was supplied ad libitum. All experimental protocols were reviewed and approved by the Kyonggi University Animal Care Committee (2013-003).

PP-mediated intestinal immunomodulation activity in vitro

After acclimatization to the housing environment for 7 days, the female C3H/He mice (now 7 weeks old) were sacrificed by cervical dislocation and the small intestine was placed into a petri dish filled with RPMI-1640 medium containing 100 U penicillin, 100 μg/mL streptomycin, and 10 % heat-inactivated FBS (FBS-RPMI). The visible PPs were carefully dissected out from the wall of the small intestine using fine scissors (6–8 PPs were obtained from each mouse), and these tissues were placed in ice cold FBS-RPMI. To obtain a single cell suspension, the PP cells were homogenized by tapping them gently with a rubber rod over a 100-gauge sterile stainless steel sieve. After filtration through a 100 μm cell strainer (BD Falcon, USA), PP cells were washed twice with FBS-RPMI. The cells were suspended at a density of 1 × 10^6 cells/mL in FBS-RPMI. For the in vitro experiment, a 180 μL-aliquot of the cell suspension was cultured with 20 μL of KPV-0 at different doses in a humidified atmosphere of 5 % CO_2 at 37 °C. After 3 days, the production of IgA and various cytokines such as TGF-β1, IL-6, and granulocyte macrophage-colony-stimulating factor (GM-CSF) in the culture supernatants was quantified by enzyme-linked immunosorbent assay (ELISA), as described below (see "ELISA" section).

In vivo experimental design

For the in vivo experiments, C3H/He mice were randomly assigned to four groups (six mice per group). After acclimation to the housing environment for 1 week, KPV-0 dissolved in saline (0.2 mL) was orally administered to the mice at different doses (50, 500, and 5000 μg/mouse/day) for 20 consecutive days. The control group received saline as an NC for the same period.

Measurement of in vivo IgA and cytokine production by PPs

Mice receiving oral doses of KPV-0 were sacrificed on day 21. PP cell suspensions (1 × 10^6 cells/mL) were prepared using the method described above (see "PP-mediated intestinal immunomodulation activity in vitro" section). A 200 μL-aliquot of cell suspension was incubated at 37 °C in humidified air containing 5 % CO_2, and after 3 days, IgA, TGF-β1, IL-6, and GM-CSF levels in the culture supernatants were quantified using ELISA.

Measurement of IgA and cytokines in small intestinal fluid and feces

After removing PPs from the mice administered with KPV-0, the remainder of the small intestine tissue was carefully collected to evaluate IgA levels in the small intestinal fluid. Furthermore, feces excreted by the mice were collected and dried to evaluate fecal IgA levels on day 21. The fecal pellet (100 mg) or the small intestinal tissue (100 mg) was resuspended in 1 mL (10 volumes, w/v) of Tris-HCl buffer (pH 8.0). Each mixture was allowed to stand for 12 h in an ice-water bath, and then centrifuged at 17,000×*g* for 15 min at 4 °C. Each supernatant was diluted (as necessary) with Tris-HCl buffer, and IgA levels were quantified using ELISA.

ELISA

IgA levels in culture medium, intestinal fluid, and feces were measured using a Mouse IgA Assay Kit (Bethyl Laboratory, USA). Samples were diluted in Tris-HCl buffer containing 1 % bovine serum albumin (Sigma) and 0.05 % Tween 20 (Sigma). Detection of TGF-β1 (eBioscience Inc., USA) requires its liberation from a large complex where it is present in an inactive form. To activate

latent TGF-β1 to an immunoreactive form, 100 μL of culture media from PP cells was acidified by addition of 1 N HCl (20 μL) for 10 min at room temperature, followed by neutralization with 1 N NaOH (20 μL). IL-6 and GM-CSF ELISA kits from BD Biosciences Co. (USA) were used according to the manufacturer's instructions. ELISA data were acquired at 450 nm using a microplate reader (Molecular Devices), and concentrations were calculated using an appropriate standard curve.

Statistical analysis

All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 12.0 (SPSS Inc., USA). Differences among groups were evaluated by one-way analysis of variance (ANOVA) and Duncan's multiple range test, and were considered significant at $p < 0.05$. All data are presented as mean ± standard deviation (SD).

Results

Isolation of polysaccharides from Korean persimmon vinegar

Polyphenols exist in a multitude of foods, including vegetables, fruits, teas, wines, juices, and vinegars, and are known to exert diverse physiological effects that include countering lipid peroxidation, hypertension, hyperlipidemia, inflammation, DNA damage, and cancer (Osada et al. 2006; Liu et al. 2012). Some of these foods also contain high molecular compounds such as polysaccharides and glycoproteins that could also exert physiologically relevant or pharmacological effects. Here, we have focused on the immunomodulatory effects of polysaccharides isolated from persimmon vinegar. The high molecular weight mixture was isolated from Korean persimmon vinegar by ethanol precipitation after the evaporation of acetic acid and flavor compounds. The precipitate was dissolved in a small amount of water, and dialyzed for 3 days to remove low molecular weight components. The resulting precipitate was finally lyophilized to yield the KPV-0.

KPV-0 transported across a Caco-2 cell monolayer affects the production of IL-6 in RAW 264.7 cells

The present study aimed to characterize the effects of KPV-0 on the intestinal immune system. Caco-2 cell cytotoxicity with respect to KPV-0 was measured for doses of KPV-0 ranging from 1 to 1000 μg/mL, with a 24 h incubation period. The results indicated that cell viability was greater than 100 % for all doses tested in comparison to the NC. Moreover, the cell viability observed for doses

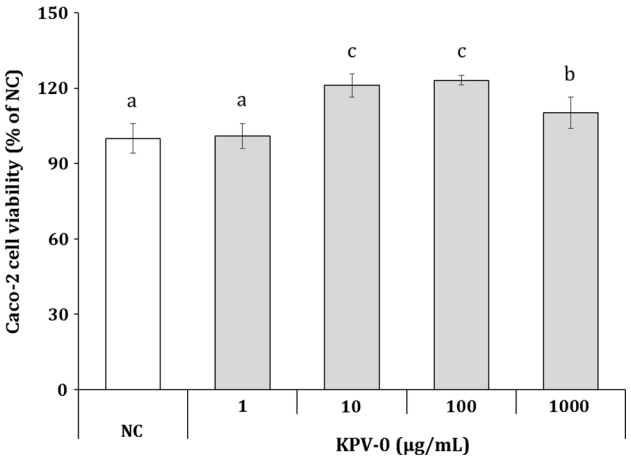

Fig. 1 Cytotoxic effect of KPV-0 on Caco-2 human epithelial cells. Caco-2 cells in 96-well plates were treated with various concentrations of KPV-0 (1, 10, 100, and 1000 μg/mL), and then incubated for 24 h. Cell cytotoxicity was measured by a water soluble tetrazolium salts (WST) assay. The cells incubated with only medium was used as a negative control (NC), and incubated with only LPS (10 μg/mL) was used as a positive control (PC). Results are expressed as mean ± SD of triplicate samples, and are displayed as relative viability (%) in comparison to the NC. One-way ANOVA was applied using Duncan's multiple range test, and different superscripts indicate significant difference ($p < 0.05$). KPV-0, crude polysaccharide isolated from fermented Korean persimmon vinegar

of 10 and 100 μg/mL suggested increased cell proliferation (1.21- and 1.23-fold increase, respectively, relative to the NC). KPV-0 was not cytotoxic to cells for the doses tested (Fig. 1). Next, small intestinal transport of KPV-0 was examined using a two-compartment transwell Caco-2 monolayer system as a pharmacologically predictive in vitro model of the small intestinal epithelium. An in vitro intestinal model was established using a coculture system of intestinal epithelial Caco-2 and RAW 264.7 cells. When KPV-0 was applied apically to the Caco-2 cell layer without RAW 264.7 cells in the basal compartment, there is profoundly no production of IL-6 (data not shown). As shown in Fig. 2, IL-6 production of RAW 264.7 cells (basal side) via transepithelial transport of KPV-0 increased dose-dependently. IL-6 production mediated via Caco-2 transport was 574.57 pg/mL (61.4 %), but was 935.79 pg/mL (100 %) when RAW 264.7 cells were directly treated with KPV-0 at a dose of 1000 μg/mL.

Effects of KPV-0 on the in vitro production of IgA and IgA-related cytokines by PPs

The effect of KPV-0 on IgA production by PP cells from C3H/He mice was examined in vitro. PP cells were cultured in FBS-RPMI medium supplemented with KPV-0 at different doses for 3 days. As shown in Fig. 3A, Stimulation of PP cells with KPV-0 in vitro facilitated the production of IgA in a dose-dependent manner. For a KPV-0

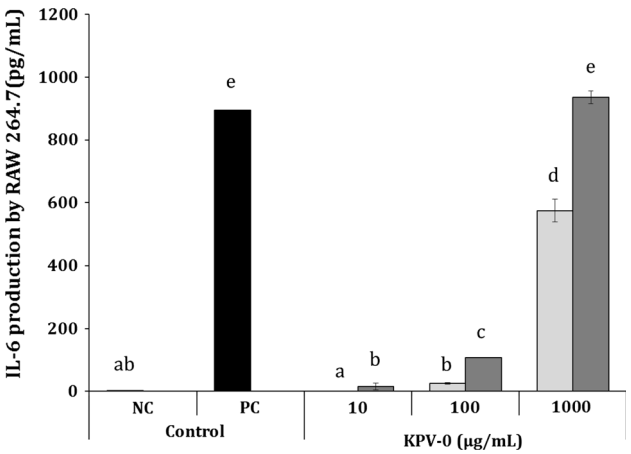

Fig. 2 Effect of KPV-0 on the production of IL-6 in a coculture system of Caco-2 and RAW 264.7 cells. The Caco-2 cells in apical side were incubated with the RAW 264.7 cells in basolateral side, and the coculture system was incubated for 24 h. Levels of IL-6 in culture supernatants from both the apical and basolateral sides were quantified by ELISA. Various concentrations of KPV-0 were applied to the apical side (*filled square*) to evaluate the permeability of transport across the Caco-2 cells. Alternatively, KPV-0 was applied to the basolateral side (*filled square*) to evaluate the maximum release of IL-6 by direct treatment of KPV-0 to RAW 264.7 cells. The RAW 264.7 cells treated with medium was used as the negative control (NC), and treated with LPS (10 μg/mL) was used as a positive control (PC). One-way ANOVA was applied using Duncan's multiple range test, and different superscripts indicate significant difference ($p < 0.05$). KPV-0, crude polysaccharide isolated from fermented Korean persimmon vinegar; LPS, lipopolysaccharide from *Escherichia coli*

dose of 1000 μg/mL, IgA production by PP cells was remarkably enhanced to 2.92-fold of that by the NC. To investigate the relationship between IgA production and cytokines such as TGF-β1, IL-6, and GM-CSF, the levels of these cytokines in the culture supernatants of PP cells was quantified. TGF-β1 production in PP cells stimulated with KPV-0 increased dramatically in a dose-dependent manner (Fig. 3B), increasing 19.3-fold relative to that for the NC at a dose of 1000 μg/mL, similar to the pattern observed for IgA production. In contrast, KPV-0 slightly, but significantly ($p < 0.05$) triggered IL-6 production in the PP cells in all tested doses (Fig. 3C). This enhancement (6.3-fold) peaked at a dose of 100 μg/mL. However, KPV-0 had no effect on the production of the hematopoietic growth factor GM-CSF in PP cells (data not shown).

Effects of oral administration of KPV-0 on the in vivo production of IgA and IgA-related cytokines

Since KPV-0 stimulated IgA, TGF-β, and IL-6 production in cultured PP cells in vitro, we next examined whether these results could be recapitulated in vivo in the intestinal immune system. Mice received 50, 500, or 5000 μg of KPV-0 per day via oral administration for 20 days. PP cells were isolated and cultured in the absence of additional KPV-0, and after 3 days, the levels of IgA and various cytokines in the culture supernatants were quantified. As shown in Fig. 4A, oral administration of KPV-0 elicits the production of IgA by PPs in a dose-dependent manner. Mice administered 5000 μg KPV-0 per day for 20 days showed the most potent increase in IgA (7.95 ng/mL, 3.7-fold increase over the NC). IgA production by stimulation with KPV-0 in vivo produced a pattern similar to that observed in vitro. Levels of IgA in fecal extracts and in small intestinal fluid for all KPV-0-administered groups were higher than those in the NC (Fig. 4B, C) and produced a pattern similar to that of IgA production in PPs. To examine which cytokines were induced by KPV-0 in vivo, PP cells isolated from mice receiving KPV-0 were cultivated for 3 days, and the levels of IL-6, GM-CSF, and TGF-β1 in the culture supernatants were quantified. At doses of 500 and 5000 μg per day, KPV-0 treatment significantly induced the production of IL-6 (1.33- and 2.23-fold increase, respectively, Fig. 5A) and GM-CSF (1.71- and 5.33-fold increase, respectively, Fig. 5B). TGF-β1 levels slightly, but significantly, increased after oral administration of KPV-0 at 500 and 5000 μg per day (1.14- and 1.24-fold, respectively, Fig. 5C). In general, the levels of all cytokines were not significantly enhanced at a KPV-0 dose of 50 μg per day. Together, our results suggest that oral administration of KPV-0 not only stimulates intestinal IgA, TGF-β, and IL-6 production but also increases localized GM-CSF production in PP cells.

Discussion

Since ancient times, persimmon vinegar has been used as a natural flavoring and preservative agent. Most biomedical studies on persimmon vinegar have focused on antioxidant and anticancer activities associated with polyphenols or organic acids. However, little information exists on the physiological activities of the polysaccharides in vinegars. KPV-0, crude polysaccharide isolated from Korean persimmon vinegar possessed potent macrophage stimulatory and anti-complementary activity, which composed mainly of neutral sugar (78.4 %) including rhamnose, arabinose, galactose, and glucose (24.8, 18.6, 13.1, and 10.1 %, respectively), in addition to 21.6 % of uronic acid (Kim et al. 2015). High contents of rhamnose, arabinose, and galactose in KPV-0 indicated that KPV-0 mainly comprised arabinogalactan side chain of rhamnogalacturonan-I in which a part of pectic polysaccharide among several plant polysaccharide (Ridley et al. 2001). On the basis of these results, the current study was conducted to elucidate the effects of KPV-0 on the intestinal immune system both in vitro and in vivo.

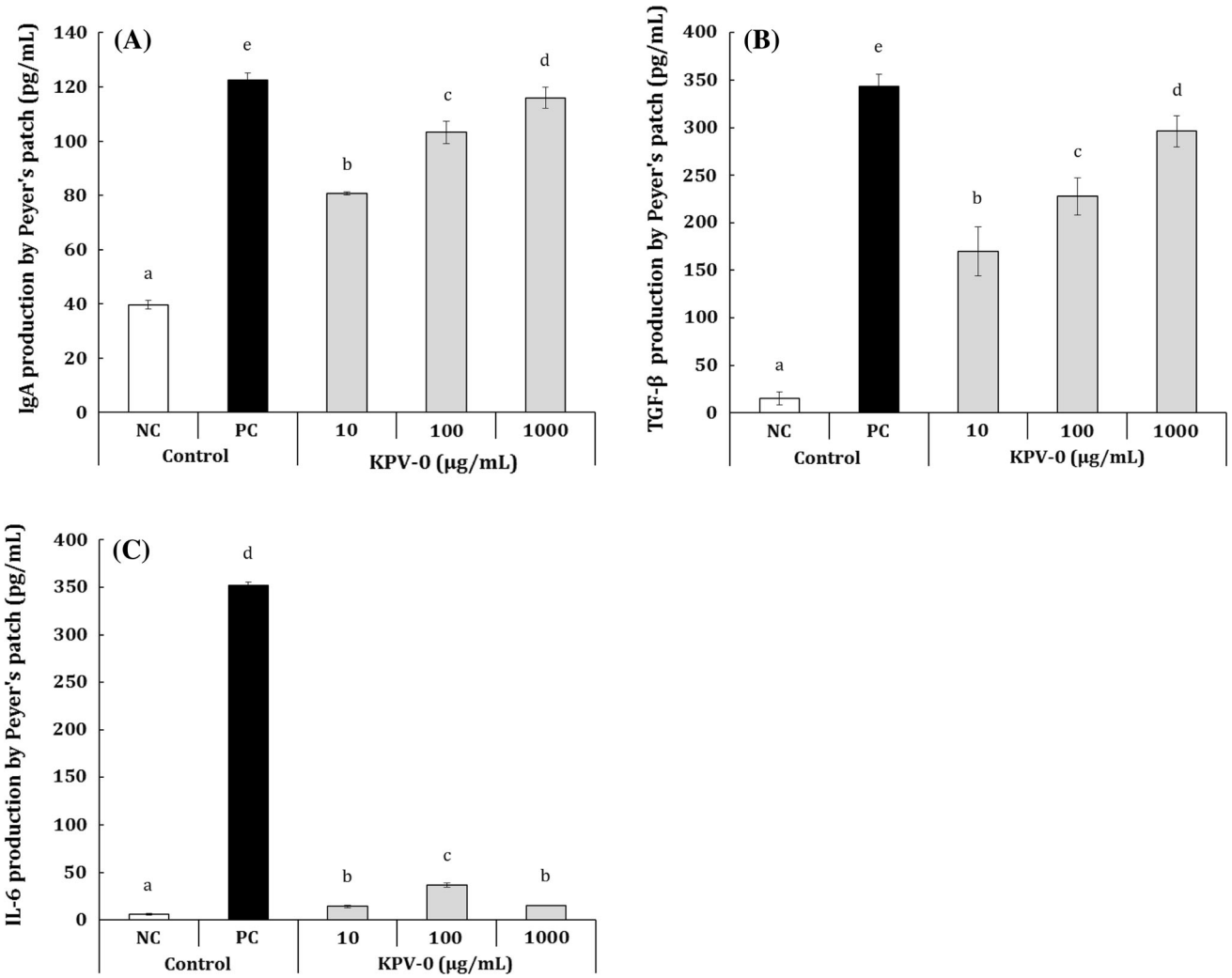

Fig. 3 In vitro effects of KPV-0 on the production of intestinal IgA (**A**), TGF-β1 (**B**), and IL-6 (**C**) by Peyer's patches. Peyer's patch cell suspension from C3H/He mice was incubated with various concentration of KPV-0 (20 μL). After 3 days, the levels of IgA, TGF-β1, and IL-6 in culture supernatants were determined by ELISA. The cells incubated with only medium was used as a negative control (NC), and incubated with only LPS (10 μg/mL) was used as a positive control (PC). One-way ANOVA was applied using Duncan's multiple range test, and different superscripts indicate significant difference ($p < 0.05$). KPV-0, crude polysaccharide isolated from fermented Korean persimmon vinegar; LPS, lipopolysaccharide from *Escherichia coli*

An in vitro intestinal model was established using a coculture system of intestinal epithelial-like Caco-2 and murine RAW 264.7 macrophage cells. These cells did not contact each other in the system. Therefore, the response of RAW 264.7 cells was mediated only via the transport of Caco-2 cells. IL-6 can be secreted by macrophages in response to specific microbial molecules, referred to as pathogen-associated molecular patterns (PAMPs). These PAMPs bind to an important group of detection molecules of the innate immune system called pattern recognition receptors, including Toll-like receptors. These are present on the cell surface and intracellular compartments and induce intracellular signaling cascades that give rise to cytokine production. To investigate whether KPV-0 affects the induction of IL-6 in the coculture model, IL-6 was detected following treatment of Caco-2 cells (apical side) with various concentrations of KPV-0 in this model. KPV-0 was not cytotoxic to Caco-2 cells for all the tested doses, but dose-dependently induced IL-6 production from RAW 264.7 cells across the Caco-2 cell barrier. Taken together, these results suggest that KPV-0 is capable of being absorbed by cells without prior degradation associated with digestion following oral consumption. Similarly, Courts (2013) reported that commercially available dietary supplements, such as modified citrus pectin, are directly absorbed following oral consumption.

The in vitro effects of KPV-0 on the intestinal immune system were investigated using PP cells isolated from C3H/He mice. A key intestinal strategy to generate immune protection in a non-inflammatory manner is the production

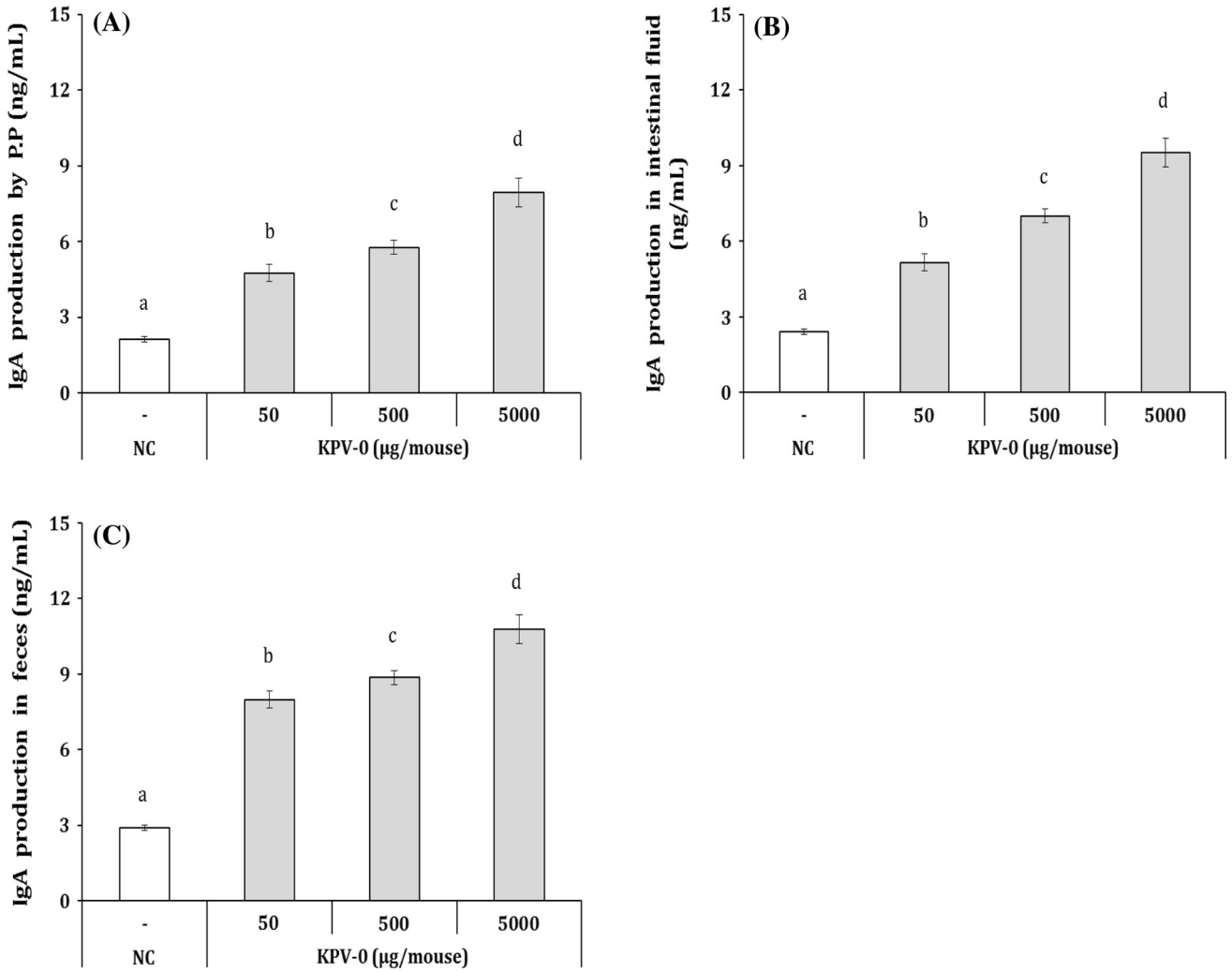

Fig. 4 In vivo effects of KPV-0 on IgA levels produced by Peyer's patches (**A**), in small intestinal fluid (**B**), and in feces (**C**). Peyer's patch cell suspension was prepared from C3H/He mice (six mice per group) administered KPV-0 at 50, 500, and 5000 μg per day for 20 days, and was then incubated in the absence of KPV-0 for 3 days. The levels of IgA in the culture supernatants were determined by ELISA. Residual small intestine tissue and feces excreted from the mice were individually extracted with Tris-HCl buffer, and then centrifuged to recover clear supernatant. The level of IgA in each supernatant was measured by ELISA. Saline administration served as the negative control (NC). One-way ANOVA was applied using Duncan's multiple range test, and different superscripts indicate significant difference ($p < 0.05$). KPV-0, crude polysaccharide isolated from fermented Korean persimmon vinegar

of IgA, the most abundant antibody isotype produced in our body (Macpherson et al. 2008). PPs are critical to initiating an antigen-specific immune response to pathogens capable of penetrating M cells (Cerutti and Rescigno 2008). As shown in Fig. 3A, when PP cells were directly treated with KPV-0, IgA production significantly increased in a dose-dependent manner. TGF-β1 production similarly increased with KPV-0 treatment. TGF-β1, an essential cytokine that directs IgA switching, is required for maintaining mucosally induced tolerance, thus establishing one of the many links between IgA production, immunity, and intestinal homeostasis (Mantis et al. 2011). KPV-0 slightly, but significantly, triggered IL-6 production in PP cells. IL-6, similar to TGF-β1, enhances intestinal IgA production by promoting the differentiation of IgA-expressing B cells into plasma cells (Sato et al. 2003). However, GM-CSF production by PP cells was unaffected by KPV-0 treatment in vitro (data not shown). Taken together, these results suggest that KPV-0 may elicit the production of IgA via TGF-β1 and IL-6.

To clarify the effects of KPV-0 in vivo, various concentrations of KPV-0 (50, 500, and 5000 μg per day) were administered to 7-week-old female C3H/He mice for 20 days. We demonstrated that KPV-0 significantly and dose-dependently elicited IgA production by PPs isolated from KPV-0-treated mice at all the doses tested. The IgA concentrations in fecal extracts and in the small intestinal fluid produced a pattern similar to that for the PPs. KPV-0

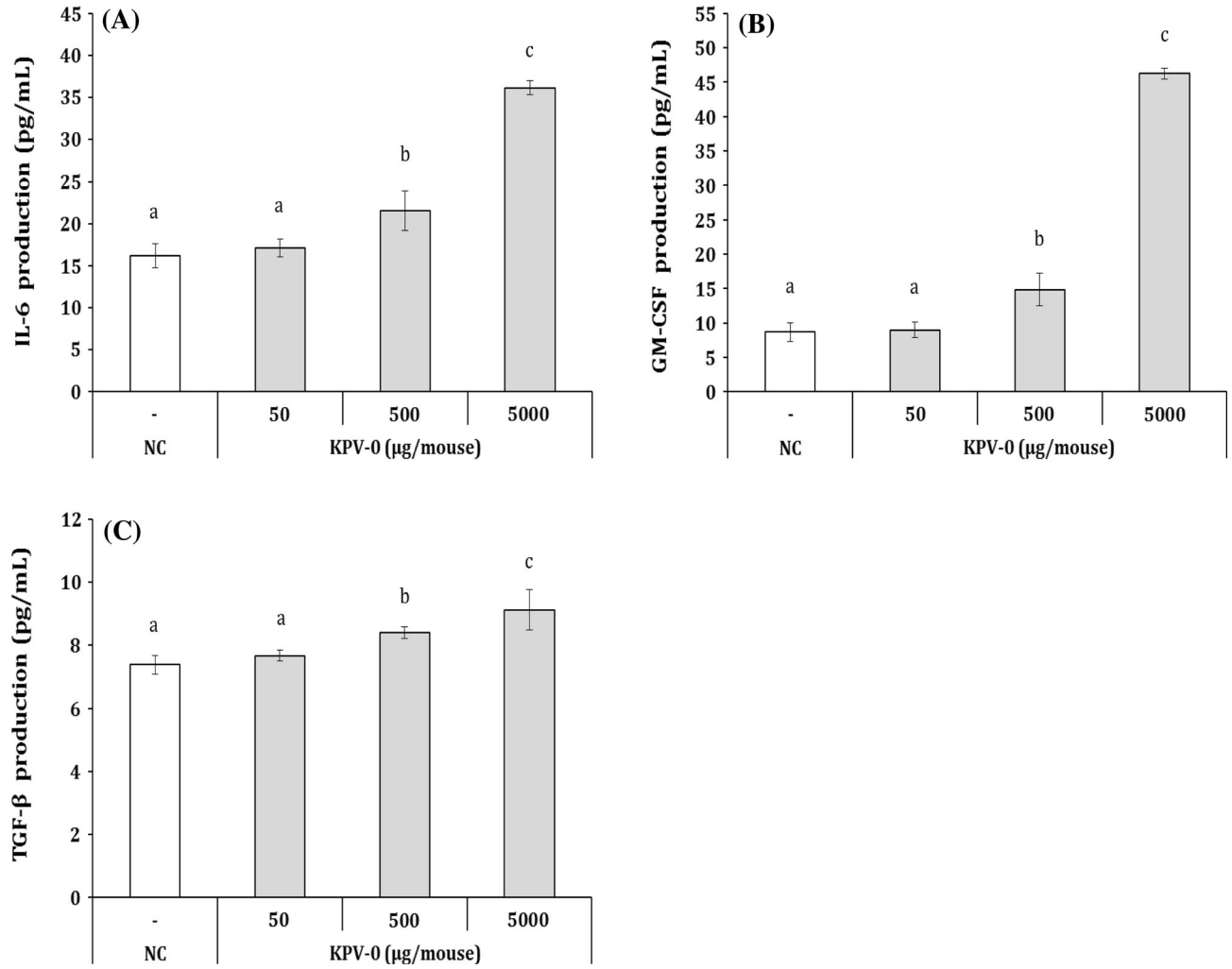

Fig. 5 In vivo effects of KPV-0 on the production of IL-6 (**A**), GM-CSF (**B**), and TGF-β1 (**C**) by Peyer's patches. Peyer's patch cell suspension was prepared from C3H/He mice (six mice per group) administered KPV-0 at 50, 500, and 5000 μg per day for 20 days, and was then incubated in the absence of KPV-0 for 3 days. The levels of IL-6, GM-CSF, and TGF-β1 in the culture supernatants were determined by ELISA. Saline administration served as the negative control (NC). One-way ANOVA was applied using Duncan's multiple range test, and different superscripts indicate significant difference ($p < 0.05$). KPV-0, crude polysaccharide isolated from fermented Korean persimmon vinegar

particularly enhances IgA production, both in the small intestinal fluid and feces, as well as in lymphocytes from PPs. PPs are critical for initiating an antigen-specific immune response to pathogens involving IgA. Cerutti and Rescigno (2008) have reviewed that SIgA reflects the long time needed for B cells to get activated, undergo affinity maturation, leave the PPs, recirculate through the thoracic duct, and reach the final gut destination. In this respect, our results suggest that KPV-0 modulates not only the mucosal immune system but also the systemic immune system through PPs. However, further studies are warranted to clarify which kinds of lymphocytes are stimulated by KPV-0 and through what mechanism.

To examine which cytokines were induced by KPV-0 in vivo, PP cells isolated from mice receiving KPV-0 were cultivated for 3 days, and the levels of IL-6, GM-CSF, and TGF-β1 in the culture supernatants were quantified. Indeed, PPs are rich in cytokines with IgA-inducing functions, including TGF-β (Gonnella et al. 1998). In addition to TGF-β, PPs contain IL-4, IL-6, and IL-10, which facilitate the expansion of IgA-expressing B cells and their differentiation to IgA-secreting plasma cells (Defrance et al. 1992; Okahashi et al. 1996; Fayette et al. 1997; Sato et al. 2003). KPV-0 treatment caused a dose-dependent increase in the levels of IL-6, GM-CSF, and TGF-β1 at doses of 500 and 5000 μg per day, but there was no significant increase at 50 μg per day. There was a substantial difference in the level of GM-CSF production detected in vivo and in vitro.

GM-CSF is an important hematopoietic growth factor and immune modulator that has profound effects on the

functional activities of various circulating leukocytes and is produced locally because it acts in a paracrine manner to recruit circulating neutrophils, monocytes, and lymphocytes to enhance their functions in host defense (Shi et al. 2006). Since PPs are mainly composed of T and B cells and T cells are known as a source of CSFs and various cytokines, T cells activated by oral administration of KPV-0 may contribute to the secretion of hematopoietic growth factors from PPs. The secreted IL-6 and GM-CSF act on cells participating in systemic immunity (Peters et al. 2001; Vacek et al. 2007). Taken together, oral administration of KPV-0 stimulates not only IgA production via TGF-β and IL-6 production but also stimulates the production of hematopoietic growth factors (GM-CSF and IL-6) of the systemic immune system through PPs. We conclude that a crude polysaccharides isolated from Korean persimmon vinegar appear to modulate the intestinal immune system and could be beneficial to human health.

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