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Role of Metabolism by Intestinal Bacteria in Arbutin-Induced Suppression of Lymphoproliferative Response *in vitro*

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Abstract

Role of metabolism by intestinal bacteria in arbutin-induced immunotoxicity was investigated in splenocyte cultures. Following an incubation of arbutin with 5 different intestinal bacteria for 24 hr, its aglycone hydroquinone could be produced and detected in the bacterial culture media with different amounts. Toxic effects of activated arbutin by intestinal bacteria on lymphoproliferative response were tested in splenocyte cultures from normal mice. Lipopolysaccharide and concanavalin A were used as mitogens for B- and T-cells, respectively. When bacteria cultured medium with arbutin was treated into the splenocytes for 3 days, the medium cultured with bacteria producing large amounts of hydroquinone induced suppression of lymphoproliferative responses, indicating that metabolic activation by intestinal bacteria might be required in arbutin-induced toxicity. The results indicated that the present testing system might be applied for determining the possible role of metabolism by intestinal bacteria in certain chemical-induced immunotoxicity in animal cell cultures.

Key Words: *Bifidobacterium*, Arbutin, Bacterial metabolism, Immunotoxicity, Splenocytes

INTRODUCTION

In human intestine, a variety of 100-500 species of bacteria exist, so that approximately 10^{14} bacteria per g feces can be found (Gorbach *et al.*, 1967). Among them, some of intestinal bacteria are able to metabolize xenobiotics. For examples, intestinal bacteria, such as *Bifidobacterium*, *Bacteroides*, *Eubacterium*, and *Enterococcus*, have been known to deglycosylate arbutin (hydroquinone-β-D-glucopyranoside) to its aglycone hydroquinone (Blaut *et al.*, 2006). Of equal importance, because the number and species of intestinal bacteria are so different from each other that the enzymatic activities of individuals to metabolize xenobiotics in intestine are greatly different. In this matter, drugs and/or toxicants may act differently from person to person. In general, intestinal bacteria can not only produce toxic or carcinogenic metabolites but also form metabolites which would not be observed in host tissues (Sousa *et al.*, 2008). In addition, intestinal bacteria may either activate or inactivate drugs and/or toxicants through metabolism. For example, metabolism of arbutin to hydroquinone by intestinal bacteria could affect its toxicity and mutagenicity *in vitro* (Blaut *et al.*, 2006). Furthermore, intestinal bacteria can affect oral bioavailability and half-life of certain chemicals by modulating the xenobiotic metabolism in gut. As an example, the pharmacokinetics of oral hesperidin, a glycoside found in citrus fruits, could be significantly affected when animals were pre-treated orally with antibiotics (Jin *et al.*, 2010).

Recently, we have developed a toxicity testing system in which the possible role of metabolism by intestinal bacteria in xenobiotic-induced toxicity can be investigated in animal cell cultures. In this system, toxic chemicals requiring metabolic activation by intestinal bacteria are pre-incubated with bacterial cultures for 24 hr, and then the toxic potential of cultured media was tested in the mammalian cell cultures, such as human hepG2 cell lines and splenocytes prepared from untreated normal laboratory animals (Kang *et al.*, 2011). To protect the animal cell cultures from bacterial contamination, the bacteria cultured medium with certain toxicants was filter-

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sterilized prior to the addition into the animal cell cultures. Although the toxic metabolites produced by intestinal bacteria should be stable enough to be toxic in animal cell cultures, the testing method would be simple and convenient once the method of bacterial cultures is established in laboratory. Using the method, arbutin was found not only to be metabolized to hydroquinone by certain intestinal bacteria but also to be toxic in animal cells when cytotoxicity and lymphoproliferation were employed as toxic parameters (Kang *et al.*, 2011; Khanal *et al.*, 2011).

The primary objective of present study was to further develop our previous testing method as a test system for detecting toxicants that requires metabolic activation by intestinal bacteria. Due to the limited information on the application of bacterial activation to toxicity testing in animal cell cultures, ability of five intestinal bacteria to metabolize toxicants were tested as metabolic activation systems. Since the metabolic activity of intestinal bacteria would be diverse, the selection of a strain with high metabolic activity might be necessary in the process of method development, Lymphoproliferative responses of splenocytes by B- and T-cell mitogens were used as immunotoxic parameters for the present study. Because its metabolism to toxic hydroquinone and the quantitation of metabolite are well known (Blaut *et al.*, 2006), arbutin was used as a model toxicant.

MATERIALS AND METHODS

Materials

Arbutin and hydroquinone were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), 2-mercaptoethanol (2-ME), penicillin G, streptomycin, L-glutamine, RPMI 1640 media, lipopolysaccharide (LPS), and concanavalin A (Con A) were obtained from Invitrogen (Carlsbad, CA, USA). The assay kit for cell proliferation was purchased from Promega (Madison, WI, USA). Other reagents used in this study were of reagent grade commercially available and used as received.

Animals

Specific pathogen-free female BALB/c mice at 4-5 weeks of age were purchased from the Orient (Seoul, Korea). The animals were housed five per cage and acclimated for at least 2 weeks prior to use. Animals were freely provided with pelleted LabDiet® (Purina Mills, MO, USA) and tap water. Healthy animals at 6-7 weeks of age (20 ± 2 g) were employed for isolation of splenocytes. Animals were housed in a room at the conditions of 23 ± 3°C temperature and 50 ± 10% relative humidity. A light and dark cycle of 12-hr with the light intensity of 150-300 Lux was maintained in the animal room. This study was performed with the permission of Institutional Animal Care and Use Committee of Yeungnam University College of Pharmacy, based on the recommended Guiding Principles in the Use of Animals in Toxicology by the Society of Toxicology (Reston, VA, USA).

Bacterial cultures

Five bacteria were used in this study: *Bifidobacterium longum* HY81, *Bifidobacterium longum* HY82, *Bifidobacterium longum* HY84, *Bifidobacterium adolescentis* and *Bacteroides fragilis*. Each 10^7 *Bifidobacterium* was initially inoculated and anaerobically cultured at 37°C in BL broth without shaking in a 15-ml glass tube (Kang *et al.*, 2011). 10^7 *Bacteroides* was anaerobically cultured at 37°C in cooked meat medium (Difco, USA) containing 5 g/L yeast extract, 5 g/L potassium phosphate, 1 mg/ml resazurin, and 0.5 g/L cysteine hydrochloride in 3% initially. Arbutin was added to either BL broth or cooked meat media at the beginning of bacterial cultures. Twenty four hr later, the medium was removed for assaying the production of metabolites and the immunotoxic potential. Prior to the addition into splenocyte cultures, the medium was filter-sterilized through a 0.2 μm membrane filter.

HPLC analysis of hydroquinone produced

A chromatographic system LC-20AD (Shimadzu, Kyoto, Japan) was used for the determination of hydroquinone produced in the bacterial cultures. The analytical conditions were described in our previous report (Kang *et al.*, 2011).

Lymphoproliferative response

Aseptically isolated splenocytes from female BALB/c mice were cultured in RPMI 1640 medium containing 5% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 5×10^{-5} M 2-ME. 1.25×10^6 spleen cells/ml, sterile bacterial culture media with arbutin, and either a B-cell mitogen, LPS (40 μg/ml), or T-cell mitogen, Con A (2 μg/ml), were mixed and cultured for 72 hr in a 37°C CO_2 incubator. For determination of cell proliferation, CellTiter 96® AQueous Non-Radioactive Cell Proliferation Kit (Promega) was used. The procedure in detail was described in our previous report (Kang *et al.*, 2011).

Statistics

The statistical differences between testing groups and control were determined by one-way analysis of variance followed by Dunnett's *t*-test (SPSS program, ver. 10.0). The significance was set at $p<0.05$ with an asterisk.

RESULTS AND DISCUSSION

Metabolism of arbutin by intestinal bacteria

In addition to xenobiotic metabolism by enzymes expressed

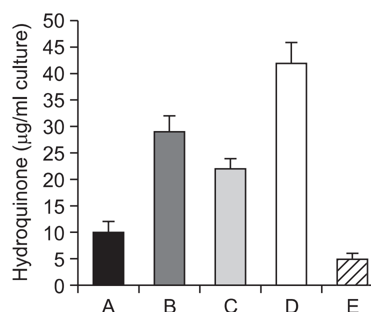

Fig. 1. Production of hydroquinone from arbutin in the culture media of intestinal bacteria. Arbutin at 10 mM was added in the culture media at the beginning of bacterial cultures. Twenty four hr later, the cultured media were subjected to analysis for hydroquinone. Each bar represents mean ± S.E. of triplicate determination. (A) *Bifidobacterium longum* HY81. (B) *Bifidobacterium longum* HY82. (C) *Bifidobacterium adolescentis*. (D) *Bifidobacterium longum* HY84. (E) *Bacteroides fragilis*.

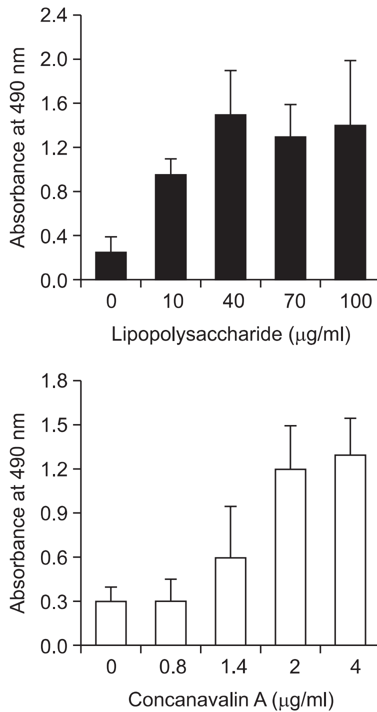

Fig. 2. Effects of lipopolysaccharide and concanavalin A on the proliferation of splenocytes prepared from normal mice. Spleen cells were prepared from untreated female BALB/c mice and cultured for 72 hr in the presence of given concentrations of either lipopolysaccharide or concanavalin A. Each bar represents mean ± S.E. of triplicate determination.

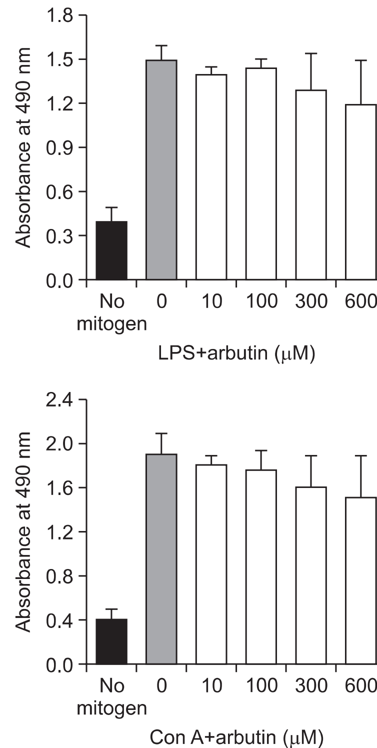

Fig. 3. Effects of arbutin on lipopolysaccharide (LPS) and concanavalin A (Con A) mitogenicity in splenocyte cultures. Spleen cells were prepared from untreated female BALB/c mice and cultured for 72 hr with arbutin in the presence of either LPS at 40 μg/ml or Con A at 2 μg/ml. Each bar represents the mean of control ± S.E. of triplicate cultures.

in host tissues, the fate of xenobiotics would also be affected by intestinal bacteria through enzymes which would not be expressed in the host tissues. Same metabolite(s) produced in host tissues would also be formed by bacterial metabolism in intestine. Among numerous bacteria in human intestine, *Bifidobacterium*, *Bacteroides*, *Eubacterium*, and *Enterococcus* have been reported to deglycosylate arbutin to hydroquinone (Blaut *et al.*, 2006). The ability of intestinal bacteria to metabolize arbutin was tested using four strains of *Bifidobacterium* and one *Bacteroides* in the present study. It was partly intended to select a strain of intestinal bacteria showing strong xenobiotic-metabolizing activities for the development of *in vitro* toxicity testing methods using intestinal bacteria as a metabolic activation system.

When 10 mM arbutin was incubated with five different strains of human intestinal bacteria for 24 hr, hydroquinone could be produced with different extents. As shown in Fig. 1, *Bifidobacterium* strains could produce more hydroquinone than *Bacteroides*. In addition, hydroquinone was produced differently among the strains of *Bifidobacterium* tested. *B. longum* HY84 produced hydroquinone most abundantly among five intestinal bacteria tested during the culture interval of 24 hr. The results clearly indicated that all strains selected in the present study might have xenobiotic metabolizing activities to metabolize arbutin to hydroquinone in the present culture condition. Because all strains could produce hydroquinone, all five strains were tested their metabolic potential to cause arbutin-induced immunotoxicity in splenocyte cultures.

Toxicity of bacteria cultured media with arbutin in splenocyte cultures

Initially, effects of LPS and Con A on lymphoproliferative responses were tested in splenocyte cultures isolated from normal mice to optimize the testing method (Fig. 2). When LPS and Con A were treated into the culture media for splenocytes, the splenocytes showed maximum proliferation at 40 μg/ml and 2 μg/ml of LPS and Con A, respectively. Therefore, the concentrations of LPS and Con A in subsequent experiments were set at the above concentrations. Next, effects of arbutin on LPS and Con A mitogenicity were tested to select testing concentrations of arbutin that might not affect the proliferation of splenocytes. As shown in Fig. 3, arbutin did not affect both mitogenicity tests up to 600 μM, which might be consistent with the result obtained in our previous report (Kang *et al.*, 2011).

Subsequently, the medium containing metabolically activated arbutin by bacterial cultures was applied in LPS and Con A-induced B- and T-cell mitogenicity in splenocyte cultures from normal mice, respectively (Figs. 4 and 5). For the study, each bacterium was anaerobically cultured in the presence of arbutin for 24 hr, followed by a filter sterilization of cultured medium. Then the immunotoxic effects of filtered medium were tested. When the bacteria cultured medium with arbutin was treated directly into the splenocyte cultures isolated from untreated normal mice in the presence of either LPS or Con A for 72 hr, lymphoproliferative responses of splenocytes

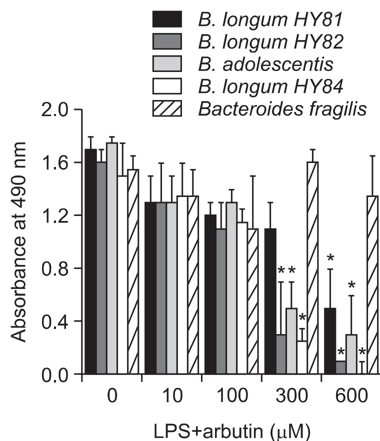

Fig. 4. Effects of arbutin metabolism by intestinal bacteria on lipopolysaccharide (LPS) mitogenicity in splenocyte cultures. Individual bacteria were cultured with arbutin for 24 hr, and then the cultured media were treated into the splenocyte cultures in the presence of 40 μg/ml LPS for additional 72 hr following the filter sterilization. Each bar represents the mean ± S.E. of triplicate cultures. The asterisk indicates the value significantly different from each control at $p<0.05$ (*).

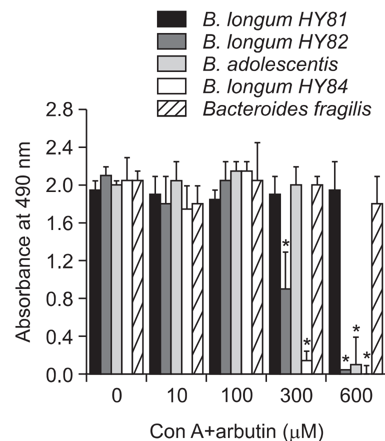

Fig. 5. Effects of arbutin metabolism by intestinal bacteria on concanavalin A (Con A) mitogenicity in splenocyte cultures. Individual bacteria were cultured with arbutin for 24 hr, and then the cultured media were treated into the splenocyte cultures in the presence of 2 μg/ml Con A for additional 72 hr following the filter sterilization. Each bar represents the mean ± S.E. of triplicate cultures. The asterisk indicates the value significantly different from each control at $p<0.05$ (*).

were significantly suppressed in different extents. As shown in Fig. 4, LPS mitogenicity was mostly suppressed when arbutin was pre-cultured with *B. longum* HY82 and HY84 and *B. adolescentis*. In case of *B. longum* HY81, arbutin was immunosuppressive only at the highest concentration. In addition, arbutin was not immunosuppressive when pre-cultured with *B. fragilis*. These results were so consistent with Fig. 1 that the suppression of LPS mitogenicity was proportional to the ability of individual bacteria to produce hydroquinone in the bacterial cultures. Likewise, similar results were observed in Con A mitogenicity with bacteria cultured media (Fig. 5). From the results, *B. longum* HY84 would be the best strain that can be used as a metabolic activation system to test immunotoxic compounds requiring metabolism by intestinal bacteria, at least in case of arbutin-containing test materials. Once again, the results supported that intestinal bacterial activation might be required in arbutin-induced immunotoxicity.

Although arbutin was selected as a model toxic compound in the present study, it might be possibly exposed to human body. Exposure to arbutin or hydroquinone would be achieved by oral intake of arbutin-rich foods. Arbutin is found in a variety of food plants or their products, such as wine, coffee, wheat products, broccoli and certain fruits. As an example, pears reportedly contain up to 4.8 mg hydroquinone per portion (Deisinger *et al.*, 1996). Because arbutin in pears is matrix-bound, it would be possible that a considerable proportion of dietary arbutin may reach the distal part of human intestinal tract, where it can act as a potential substrate for intestinal bacteria (Blaut *et al.*, 2006; Kang *et al.*, 2011).

Toxic mechanism of hydroquinone has well been studied. It is capable of producing reactive oxygen species and causing oxidative stress (Rubio *et al.*, 2011). Once hydroquinone is produced from arbutin, it enters in redox cycle and produces reactive oxygen species, including superoxide anion, hydrogen peroxide, and highly reactive hydroxyl radical (Zhao-Yang *et al.*, 2008). In addition, hydroquinone might be a pro-oxidant that can cause cytotoxicity and apoptosis (Robertson and Orrenius, 2000). Therefore, metabolism of arbutin to hydroquinone would cause generation of reactive oxygen species which mediate cellular adverse effects, including oxidative DNA damage and cell death (Terasaka *et al.*, 2005). In this regard, arbutin exposed by an oral route can be metabolically activated by intestinal bacteria to hydroquinone. Our previous reports and the present results suggested that some intestinal bacteria might possess such arbutin-metabolizing activities, and that the metabolic activity of intestinal bacteria would be very diverse (Kang *et al.*, 2011). Moreover, our recent reports indicated that human intestinal microflora is capable of metabolizing arbutin to hydroquinone which can induce apoptosis in HepG2 cells (Khanal *et al.*, 2011). In the report, following the incubation of arbutin with human fecal preparation, the reaction mixture induced cytotoxicity and apoptosis in HepG2 cells. In addition, the mixture considerably inhibited the expression of anti-apoptotic Bcl-2 in association with an increase in proapoptotic Bax expression. Likewise, the incubation stimulated the cleavage of caspase-3 and the production of reactive oxygen species. Taken together, all these findings suggest that arbutin might require metabolic activation to hydroquinone for exerting its toxicity including apoptotic cell death.

Since a report indicated that the oral route is the most preferred way of drug administration among pharmaceutical products (Lennernäs and Abrahamsson, 2005), considerable attention must be paid to the stability of drug in the intestinal lumen. In fact, the environments for xenobiotic metabolism in host tissues and in intestine would be so different that most of hepatic metabolism can be oxidation and conjugation, whereas the intestinal bacteria can conduct reductive and hydrolytic reactions. So the intestinal tract would be a distinct site for biotransformation of xenobiotics when compared with host tissues like liver (Shamat, 1993). Therefore, extensive consideration is necessary in drug development processes to assess the possible roles of metabolism by intestinal bacteria in the fate of xenobiotics.

The present study was to further develop our previous

method to detect toxicants that require metabolic activation by intestinal bacteria in animal cell cultures. We could select a bacterial strain showing a potent metabolic capacity, at least in case of arbutin, which could be applied to the immunotoxicity testing in splenocyte cultures. The results suggested that metabolism by intestinal bacteria would be a critical factor to be considered in arbutin-induced immunotoxicity, although further optimization is required for the method development in the future.

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