Immunomodulatory and antitumor effects in vivo by the cytoplasmic fraction of Lactobacillus casei and Bifidobacterium longum

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The immunomodulatory and antitumor effects of lactic acid bacteria (LABs) were investigated. Cytoplasmic fraction of Lactobacillus acidophilus, Lactobacillus casei and Bifidobacterium longum were tested for the antiproliferative activity in vitro to SNUC2A, SNU1, NIH/3T3 and Jurkat cell lines by crystal violet assay. All cytoplasmic fraction suppressed proliferation of tumor cells, though L. casei and B. longum were more effective.

From these results, cytoplasmic fraction of L. casei and B. longum were chosen for further study of immunomodulation and antitumor activity in tumor-bearing mice with long-term feeding.

Key words: Lactobacillus, Bifidobacterium, immunophenotyping, in vivo, survival rate

Introduction

The enhancement of the gut mucosal barrier may prevent the invasion of pathogens and assist in handling antigens. Lactic acid bacteria (LABs), a gram-positive and non pathogenic organism, produce of lactic acid [1]. Many studies have shown the beneficial therapeutic effects of probiotic LABs. They can prevent or ameliorate diarrhea through their effects on the immune system. Moreover, They may protect infection because they compete with pathogenic viruses or bacteria for binding sites on epithelial cells [2,3,4,5] and induce systemic immune response including secretion of cytokines by directly action to mucosal lymphocytes [6,7].

Among its many therapeutic attributes, LABs have antitumor activity and inhibit metastasis [8,9,10]. LABs such as Lactobacillus acidophilus [9,11], L. casei [12,13], and Bifidobacterium longum [14,15,16] inhibit the growth of both implantable and chemically induced tumor cells in rodents. Lactobacillus has mitogenic activity, adjuvanticity and shows activating macrophages in vivo including cytostatic activity [17-22]. Perdigon et al. [33] reported that enhanced macrophage and lymphocyte activity in mice after oral administration of L. acidophilus and L. casei. Increased NK cell activity is known in mice injected with L. casei [19], L. rhamnosus [23], and yogurt containing live LAB [24].

Furthermore, whole cells, heat-killed cells, cell wall, and cytoplasmic fractions of LABs can show various functions in many works. However, most reports on antitumor activity and immunomodulatory effects of LABs, have been focused on whole cells or its membrane component, peptidoglycans, though the effect of soluble materials in food applications can be different from that of insoluble ones. As little attention has been paid for the soluble fractions, the importance of cytoplasmic fraction of LABs in vivo has been overlooked.

In this study cytoplasmic fraction of L. acidophilus, L. casei and B. longum were compared with their antiproliferative activity to tumor cells in vitro. From this preliminary results, the cytoplasmic fraction of L. casei and B. longum, and Y400 were chosen for further study of immunomodulation and antitumor activity in tumor-bearing mice with long-term feeding.

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Materials and Methods

Experimental animals
Male Balb/c mice, 6 wks old, purchased from Seoul National University, were housed in plastic cages in an air conditioned room (22±2°C, humidity 55±10%), and given food and water freely.

Preparation of LABs
L. acidophilus SNUL, L. casei YIT9029, and B. longum HY8001 were obtained from Hankuk Yakult Institute (Y ongin, Korea). Anaerobic culture condition in anaerobic jar (BBL) with catalysts (DIFCO) was described in Table 1. After cultivation, the cells were harvested by centrifugation and washed and resuspended in distilled water for disruption with French Press at 2,000 g · s · cm⁻². Cytoplasmic fractions were the supernatant of ultracentrifugation at 70000 × g for 30 min. Samples were sterilized with a 0.2-µm filter and kept at −80°C.

Tumor cells
SNU1 (human gastric cancer cells), SNUC2A (human colorectal carcinoma cells), NIH/3T3 (mouse embryo fibroblast cells), F9 (teratocarcinoma cells), and Jurkat (human acute T cell leukemia cells) were obtained from KCLB (Korean Cell Line Bank, Seoul, Korea) and maintained in RPMI medium containing 10% (v/v) fetal bovine serum (FBS, GibcoBRL, Grand Island, NY) in a humidified atmosphere with 5% CO₂ at 37°C.

Measurement of antiproliferative activity in vitro
To evaluate the antiproliferative activity of samples, cytotoxicity assay was performed with crystal violet dye for the quantitative analysis of cell numbers as a total protein amount. Cells were plated at a density of 5 × 10⁴ cells per well for NIH/3T3, SNU1, and SNUC2A, and of 10⁴ cells per well for Jurkat cells in 96-well plates. Six hrs later, serial dilution of cytoplasmic fraction of LABs from 250 µg/ml was added to wells, and incubated for 72 hrs. Washing with phosphate-buffered saline (PBS, pH 7.2), plates were fixed with 1% glutaraldehyde (Wako), and stained with 0.2% crystal violet solution (Merck). Five min later, wells were washed with tap water and 1% SDS (Sigma) was added. Absorbance was measured by ELISA plate reader (BIO-RAD model 550) at 540 nm for the cytotoxicity calculation.

% Cytotoxicity = \frac{\text{Control O.D} - \text{Sample O.D}}{\text{Control O.D}} \times 100

Experimental design for in vivo study
The mice, assigned to 14 groups (Table 2), were fed with cytoplasmic fraction of L. casei (100 mg/kg/day), B. longum (100 mg/kg/day), and whole cells of Y400 (2.6 ml/kg/day, HanKuk Yakurt Institute) as the control sample. Distilled water (D.W.) was used to substitute LABs in adjusting feeding condition of experimental groups. To evaluate the antitumor effects, F9 teratocarcinoma cells (1 × 10⁶ cells/mouse) were inoculated i.p. at the day of 14th. The change of body weights was measured at the intervals of 5 days, and survival rate was assessed. Statistics were done with Student’s t-test.

Immunophenotyping by flow cytometry analysis
Blood was collected from tail vein of mice. The PBMCs were obtained from each blood sample with 0.5 µM EDTA (Sigma) in PBS by centrifugation. The red blood cells were removed by BCL buffer. A total of 1 × 10⁶ cells were incubated with each of FITC-conjugated rat anti-mouse CD3, mouse anti-mouse Ly-49A B60 (PharMingen, BD Biosciences), mouse anti-mouse MHC class II I-Aᵇ/I-Aᵈ (Serotec, Inc., Raleigh, NC) for 30 min at 4°C. For two-color flow cytometry analysis, a rat anti-mouse CD4-CyChrome and a rat anti-mouse CD8-RPE (Serotec) were used. Flow cytometry analysis was performed in a FACSCalibur with CellQuest program (Becton Dickinson).

Table 1. List of lactic acid bacteria for the in vitro cytotoxicity assay

| Strain                  | Time for culture | Final cell numberb |
|-------------------------|------------------|--------------------|
| Lactobacillus acidophilus SNUL | 11 hr            | 2.0 × 10⁹          |
| Lactobacillus casei YIT9029 | 18 hr            | 1.1 × 10⁹          |
| Bifidobacterium longum HY8001 | 18 hr with 0.05 % cystein | 8.0 × 10⁹ |

a In MRS broth at 37°C in anaerobic condition
b Cells per milliliter

Table 2. Summary of experimental groups

| Group | Treatment                  |
|-------|----------------------------|
| Control | D.W. for 4 wks             |
| B2     | Bifidobacterium longum for 2 wks and D.W. 2 wks |
| B4     | Bifidobacterium longum for 4 wks |
| L2     | Lactobacillus casei for 2 wks and D.W. 2 wks |
| L4     | Lactobacillus casei for 4 wks |
| Y2     | Y400 for 2 wks and D.W. 2 wks |
| Y4     | Y400 for 4 wks             |

a Five mice in each group.

b Oral administration of cytoplasmic fraction of B. longum (B) and L. casei (L) at 100 mg/kg/day, and Y400 (Y) at 2.6 ml/kg/day as a dietary supplement.
Results

Antiproliferative activity of cytoplasmic fraction of LABs in vitro

All cytoplasmic fraction of LABs showed strong antiproliferative effect to tumor cells (Fig. 1). In particular, the cytoplasmic fractions of *L. casei* and *B. longum* were more effective with inhibition rates around 50% at 50 µg/ml (Table 3). These strains were selected for further study in vivo.

Increased CD3+ cells, NK cells and MHC class II+ cells in PBMCs

To access the effect of cytoplasmic fraction of LABs on cellular immunity, preparation of LABs was administered as a dietary supplement for 2 and 4 wks. Four wks later, increased CD3+ T cells were observed in all groups. Longer intake of LABs showed more effects as the all of 4 wks groups showed above increase of 70% (Fig. 2). NK cells and MHC class II+ cells which are antigen presenting cells like dendritic cells, activated macrophages and some of B

cells also showed similar increment (Fig. 3 and 4).

Increased CD8+ T cells in two color analysis of flow cytometry

The change of T cell subsets was observed after oral administration of cytoplasmic fraction of LABs. Four wks later, we found the proportion of CD4+CD8− T cells and double positive T cells were increased (Fig. 5). Interestingly, no changes was observed in CD4+CD8− T cells, resulting decreased CD4+/CD8− ratio. The summary of numbers was shown in Table 4.

Change of body weight in F9-bearing mice

During administration of cytoplasmic fraction of LABs for 4 wks, F9 was inoculated into mice at 2 wks. Though the body weight of mice before tumor inoculation were same in all groups, it was decreased by time in tumor-bearing groups with the treatment of cytoplasmic fraction of LABs. There was no difference between 2 and 4 wks feeding groups (Fig. 6).
The mice in control group began to die from 26th days after inoculation of F9 cells and died all on 44th days. Significant prolonged survival was observed in LABs treated groups. Mean survival rate of *B. longum* group for 4 wks, and Y400 for both 2 wks and 4 wks were remarkably increased as shown to be 80%, 60% and 80% on 44th days (Fig. 7).

**Survival rate in F9-bearing mice**

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Increased survival rate of tumor-bearing mice by cytoplasm of lactic acid bacteria

**Fig. 4.** Expansion of MHC class II$^\text{d}$ cells after administration of LABs. The mice were orally administered cytoplasmic fraction of *B. longum* (B) and *L. casei* (L), and Y400 (Y) for 2 or 4 wks. PBMCs of mice were analyzed for the cell surface MHC class II expression by flow cytometry. The percentage of increase compared to control that was not given LABs was shown.

**Fig. 5.** Double staining of PBMC for CD4 and CD8 after administration of LABs. The cells were taken from mice that were given cytoplasmic fraction of *B. longum* (B) and *L. casei* (L), and Y400 (Y) as dietary supplement for 2 or 4 wks. Control was PBMC from a group that was not given LABs.
Discussion

To examine direct antiproliferative effect of cytoplasmic fraction of *L. acidophilus*, *L. casei* and *B. longum*, we conducted cytotoxicity assay on colon cancer, gastric cancer, and acute T cell leukemia cells with NIH/3T3, a fibroblast cell line used on general cytotoxicity assay. The cytoplasmic fractions of LABs were found to have anti-proliferative effect in vitro on tumor cells. In particular, the cell fraction of both *L. casei* and *B. longum* showed high activity on all tumor cells, and led us in vivo antitumor study with these strains. Our data showing different antiproliferative activity in these strains consist with the result of Pessi et al. [25].

Despite of an immune change with the challenge can be measured more than 2 wks, most of previous in vivo studies was done for one week or less [11]. Feeding mice with LABs in long period is necessary for the evaluation of cellular immunity by probiotics. By feeding of LABs before and after F9 tumor inoculation, the body weight was decreased compared to control suggesting tumor growth was controlled and restrained by potentiated host immunity. This observation was sustained by the increased survival rate in 4-wks feeding group than 2-wks. Similar results was reported by other researchers with the direct intraperitoneal injection of *L. casei* 9018 against the sarcoma-180 [19,27].

For the antitumor activity of LABs in vivo, the increased specific tumor immunity in probiotic treated mice was from activated immune cells, not by direct killing on tumor cells, in the study with *Streptococcus thermophilus* on chemically induced tumor [28]. In our experiment, increased CD8+ T cells was observed in long-term feeding groups with a profound change of other immune cells, indicating feeding of preparation of LABs modify cellular immunity. The increased CD4+/CD8+ T cells may suggest the stage of body against immune stimulation before maturation as the single positive T cells, though its exact nature is not clear. As

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### Table 4. Change of T cell subsets with feeding of LABs

| Group | CD4 CD8 | CD4+CD8+ | CD4+CD8- | CD4-CD8+ |
|-------|---------|----------|----------|----------|
| Control | 65.3 ± 1.7* | 15.5 ± 0.4 | 1.7 ± 0.2 | 17.6 ± 2.4 |
| B2     | 24.5 ± 10.1* | 12.0 ± 0.9 | 7.0 ± 0.2* | 56.5 ± 9.5* |
| B4     | 29.0 ± 5.1* | 12.3 ± 0.7 | 6.9 ± 1.7* | 51.8 ± 3.0* |
| L2     | 35.7 ± 10.5* | 10.2 ± 0.0 | 10.8 ± 5.9* | 43.3 ± 9.3* |
| L4     | 22.4 ± 8.5* | 11.0 ± 0.8 | 12.0 ± 0.8* | 54.6 ± 8.6* |
| Y2     | 16.3 ± 2.0* | 11.3 ± 0.5 | 17.1 ± 3.9* | 55.4 ± 1.6* |
| Y4     | 10.1 ± 4.9* | 11.3 ± 0.3 | 25.3 ± 7.1* | 53.2 ± 2.9* |

*Double stained PBMC for CD4 and CD8 were analyzed by flow cytometry with Cell Quest program.

Mean ± S.D. (n=5, *P ≤ 0.05)

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Fig. 6. Effect of LABs on body weight change in F9-bearing mice. Cytoplasmic fraction of *B. longum* (B) and *L. casei* (L), and Y400 (Y) were administrated as dietary supplement for 2 or 4 wks. F9 cells were inoculated on day 14. Control was not given LABs. The values are expressed as the mean±SD (n = 5).
In this study, oral administration of cytoplasmic preparation of LABs as a dietary supplement is found to have antitumor effects in vivo with the modulation of cellular immunity, suggesting that both *L. casei* and *B. longum* in intestinal microflora can activate immune system to prevent diseases including tumors.

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