Original

Inhibitory Effect of Lactobacillus Casei Strain Shirota on Azoxymethane-Induced Intestinal Tumors in the Rat

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Abstract : The effect of oral administration of viable *Lactobacillus casei* strain Shirota (LcS) on prevention of azoxymethane (AOM)-induced intestinal tumors in the rat was investigated. Thirty rats each received a weekly subcutaneous injection of AOM at a dose of 7.4 mg/kg body weight for 10 weeks. Twenty-five weeks after the start of the carcinogen treatment, all rats were sacrificed, and the total intestine and the mesenteric lymph nodes (MLN) were removed. The incidence of tumors and lymphocyte surface markers derived from the MLN were investigated. The incidence of colon cancer was significantly lower in the rats which had consumed the LcS diet compared to the controls. The number of lymphocytes and CD8-positive lymphocytes was significantly higher in the rats given the LcS diet. LcS may therefore effectively inhibit chemically-induced colon carcinogenesis in the rat. CD8-positive T lymphocytes may play a key role in this protective effect against colon carcinogenesis.

Key words : *lactobacillus casei*, colon cancer, azoxymethane, CD8, mesenteric lymph node

Introduction

Several epidemiologic studies have suggested that the intake of dairy products is associated with a decreased risk of colon cancer. Studies from Finland and Denmark showed a decreased incidence of colon cancer in Finns who consume large quantities of fermented dairy products containing viable lactobacilli¹⁾. This effect may be mediated via an effect of the lactobacilli on the nature and numbers of intestinal bacteria²⁾.

Lactobacillus casei strain Shirota (LcS) was isolated from human feces in the 1930's. LcS is a gram-positive and nonpathogenic organism, and heat-killed LcS cells have been reported to exhibit potent antitumor activity in experimental animal models, possibly by modification of host responses³⁻⁵⁾. The aim of this study was to investigate the potential protective effect of oral administration of viable *Lactobacillus casei* strain Shirota on chemically-induced colon tumors in a rat model.

Materials and Methods

Forty male Sprague-Dawley rats 6 weeks old were obtained from Saitama Experimental Animals Supply (Saitama, Japan). The rats were housed, five per cage, in a suspended wire-bottomed cage in an animal room which had a constant temperature (23°C) and a

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Fig. 1. Experimental protocol.

12-hour light-dark cycle. The rats consumed a control diet (Type MF powder, Oriental Yeast, Chiba, Japan) for 1 week and then were assigned randomly to three groups. This control diet contained 54% carbohydrates, 23.8% proteins, 3.2% fibers, 5.1% lipids, 6.1% minerals mixture and 7.8% water. The caloric value of this diet was 3.57 Kcal/g.

The control diet was given to Groups A and B, whereas in Group C, the same diet plus 5 % LcS diet was given throughout the experiment. Rats were given food and water freely. Rat body weight was measured weekly, and food intake was recorded every three days.

LcS was provided by Yakult Honsha Co., LTD (Tokyo, Japan), as a Biolactis powder. Each gram of lyophilized *Lactobacillus casei* strain Shirota contained about 2.1×10^{10} live bacterial cells. Thirty rats (Groups B, C) each received a weekly subcutaneous injection of azoxymethane (Sigma Chemical Co, St. Louis, MO, USA) at a dose of 7.4 mg/kg of body weight for 10 weeks. Group A was a control group which received a subcutaneous injection of saline instead of AOM for 10 weeks. Twenty-five weeks after the start of carcinogen treatment, all rats were sacrificed under the diethyl ether (Fig. 1). The colon and small intestine were removed and opened longitudinally along the antimesenteric side from the anus, rinsed with ice-cold 0.9% NaCl solution, and subsequently examined for the presence of tumors. Tumor tissue was examined histologically, fixed, and stained with hematoxylin-eosin. Histological types were defined according to the criteria of the Japanese Research for Cancer of the Colon and Rectum⁶⁾.

The mesenteric lymph node was immediately removed from each rat in each groups. The specimens were minced and pressed through a stainless steel mesh (#90 μ m) with RPMI Medium 1640 (Gibco BRL, NY, USA) supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, KS, USA) to produce a single cell suspension. Lymphocytes were isolated using Lymphoprep (Nycomed Pharma, AS, Oslo, Norway), then washed twice with RPMI 1640 medium supplemented with 10% FBS. Viable cells were counted by the trypan blue exclusion method. Fluorescein isothiocyanate (FITC)-conjugated anti-CD4 and anti-CD8 antibodies, and phycoerythrin (PE)-conjugated anti-CD3 antibodies were purchased from PharMingen Inc. (San Diego, CA, USA). Lymphocytes in 100 μ l of RPMI Medium 1640 supplemented with 10% FBS were incubated with FITC-conjugated anti-CD4 and anti-CD8 antibodies, or PE-conjugated anti-CD3 antibodies for 20 minutes. After incubation, the lymphocytes were separated from erythrocytes and fixed with 2 % para-formaldehyde using Optirase B Lysing solution (Immunotech, MA, France). The staining patterns were analyzed with CONSORT 30 (Becton Dickinson, San Jose, CA,

USA) by FACScan (Becton Dickinson, San Jose, CA, USA).

Values are expressed as means $\pm$ standard deviation (SD). Statistical analyses were performed using Stat View 4.5 (Abacus Concepts, Berkeley, CA). One-factor ANOVA with Fisher's PLSD correction was used to examine all the experimental data except for tumor incidence which was analyzed by Fisher's exact test.

Results

There were no significant differences in body weight or food intake between the three groups (Table 1). No tumors were observed in the non-carcinogen group (Group A). The incidence of tumor and the number of tumors in each treatment group are shown in Table 2. A total of 14 (93.3%) Group B and 8 (53.3%) Group C rats had colon tumors; this difference was significant. Group B rats also had a significantly higher total number of tumors per rat, either in the colon or total intestine, than Group C rats. The mean number of colon tumors per rat, was 1.9 and 0.8 in Groups B and C, respectively. These tumors were all examined histologically and confirmed to be malignant, and included two lesions of carcinoma in adenoma (Figs. 2, 3). The incidence of well, moderately, poorly differenti-

Table 1. Final body weight and food intake

Group	No. of Rat	Body weight (g)	Food intake (g/dayRat)
A	10	606 $\pm$ 44	22.2 $\pm$ 3
B	15	581 $\pm$ 48	21.9 $\pm$ 3
C	15	573 $\pm$ 38	21.9 $\pm$ 3

Value is expressed as the mean $\pm$ SD

Table 2. Inhibitory effect of LcS on AOM-induced intestinal tumors

Group	No. of Rats	Rats with Tumors	No. of Tumors	Tumors/ Rat	Tumors/ bearing Rat
Colon					
A	10	0	0	0	0
B	15	14 (93.3)*	29	1.9**	2.1
C	15	8 (53.3)	12	0.8	1.5
Small intestine					
A	10	0	0	0	0
B	15	5 (33.3)	6	0.4	1.2
C	15	1 (6.7)	1	0.1	1
Total intestine					
A	10	0	0	0	0
B	15	14 (93.3)	35	2.3**	2.5
C	15	9 (60)	13	0.9	1.4

Numbers in parentheses, percentage.

Statistical significance as follows.

*, $p < 0.02$ versus Group C**, $p < 0.01$ versus Group C.

Fig. 2. Macroscopic view of azoxymethane-induced colon cancer.
(a) Elevated lesion. (b) Depressed lesion.

ated, mucinous, and signet-ring cell adenocarcinomas was 62.5%, 18.8%, 6.2%, 4.2% and 8.3%, respectively. There was no significant difference in the level of differentiation of tumors, the tumor diameter or depth of invasion between the groups (Table 3).

There were no significant differences in the numbers of lymphocytes derived from the mesenteric lymph nodes between the three groups. However, the number of lymphocytes in Group C was greater than that in Group B. While there were no significant differences in the percentage of CD3- or CD4-positive lymphocytes among the three groups, the percentage of CD8 lymphocytes in Group C was significantly higher than that in Group B (Table 4).

Discussion

Human colorectal cancer is one of the most common malignant tumors. Survival following surgical treatment with or without various adjuvant therapy for advanced colorectal cancer has remained unimproved in recent years. Prevention of the development of colorectal cancer is therefore likely to be a more effective strategy. Chemoprevention of human colorectal cancer has been reported in various studies, where fiber, vitamins A, C, D, E, calcium, low fat and nonsteroidal anti-inflammatory drugs (NSAIDs) have all been shown to affect the incidence of this disease⁷⁻¹⁰⁾.

Fig. 3. Histological findings of azoxymethane-induced colon cancer.
 (c) Well and moderately differentiated adenocarcinoma.
 (d) Signet-ring cell carcinoma.
 (Hematoxylin-eosin $\times 200$)

Table 3. Pathological findings of AOM-induced intestinal tumors

Group	Tumor Diameter (mm)	Histological Type					Depth of Invasion	
		well	moderately	poorly	signet-ring	mucinous	m, sm	$\geq$ mp
B	5.97 ± 2.9	21 (60)	7 (20)	2 (5.7)	3 (8.6)	2 (5.7)	26 (74.3)	9 (25.7)
C	8.77 ± 13.2	9 (69.2)	2 (15.4)	1 (7.7)	1 (7.7)	0 (0)	11 (84.6)	2 (15.4)

Numbers in parentheses, percentage.

Value is expressed as the mean $\pm$ SD.

m, mucosa sm submucosa mp, muscularis propria

Lactobacilli may be one of the most potent chemopreventive organisms. Goldin and Gorbach showed that dietary supplements of *Lactobacillus acidophilus* and *Lactobacillus GG* suppressed the incidence of 1,2-dimethylhydrazine-induced colon cancer in rats^{11, 12}. Although LcS has been shown to have anti-tumor activity in many experimental and clinical studies, there have been no reports on the chemopreventive effect of oral administration of viable LcS on colon carcinogenesis. The results of this study demonstrated that oral

Table 4. Number and percentage of cell surface marker of lymphocytes derived from the MLN

Group	No. of MLN lymphocytes ($\times 10^6/\text{ml}$)	percentage of CD3 percentage of CD4 percentage of CD8		
A	4.86 ± 2.9	52 ± 8.6	38 ± 6.8	$9.6 \pm 2.1^*$
B	3.17 ± 2.4	46 ± 8.8	35 ± 7.7	$6.1 \pm 1.2^{**}$
C	5.95 ± 2.7	49 ± 4.8	36 ± 3.7	8.9 ± 0.6

Value is expressed as the mean $\pm$ SD.

*, $p < 0.0001$ versus Group B. **, $p < 0.05$ versus Group C.

administration of viable LcS inhibited the initiation and early phase of promotion in AOM-induced colon carcinogenesis in the rat. The incidence and number of colon tumors were reduced by 40% with oral administration of LcS.

The flow cytometric method is simple and easy, and exhibits high sensitivity and reproducibility. Lim et al suggested that dietary fiber moderately modified the proportions of CD4- positive and CD8-positive T cells in the MLN lymphocytes¹³⁾. Takagi *et al.* demonstrated that spleen lymphocyte populations (CD3, 4, 8) in 3-methylcholanthrene-induced subcutaneous carcinogenesis in mice could be recovered by oral administration of heat-killed LcS¹⁴⁾. In the first study of the effects of oral probiotics on MLN in a chemically-induced tumor model, we found that the oral administration of viable LcS increased the number of lymphocytes derived from MLN and significantly increased the numbers of CD8-positive lymphocytes to the same levels as the control group. It is not certain whether this recovery was partly due to the lower incidence of colon cancer in Group C compared with that in Group B. An assessment of the changes in number of and surface markers on lymphocytes derived from MLN with time in rats given LcS may help address this question. Matsuzaki et al demonstrated that intrapleural administration of LcS was effective in prolonging the survival of Meth A fibrosarcoma (Meth-A)-bearing BALB/c mice¹⁵⁾. The effect of LcS was in part dependent on T cells, because no effect was found when LcS was administrated in either nude mice or severe combined immunodeficient (SCID) mice. In addition, the anti-CD4 monoclonal antibody did not affect the survival of the mice and the effect of LcS was in part diminished by treatment with an anti-CD8 monoclonal antibody. CD8- positive T cells may play an important role in the action of the anti-tumor effect of LcS¹⁶⁾.

The mechanism of inhibition of colon carcinogenesis by orally-administrated LcS was reported to be via modification of the host immune system via gut-associated lymphoid tissue (GALT). LcS inoculated into the intestine were initially taken up into M cells of the Peyer' patches and transferred to lymphocytes and macrophages. Thereafter, lymphocytes leave the mucosa and migrate through the MLN and the thoracic duct to reach the systemic circulation^{17, 18)}. However, LcS is not likely to directly modify T-cell function. Kato et al suggested that the oral administration of LcS leads to a dominant state of cellular immunity via macrophage activation and the subsequent enhancement of Th-1 cells functions in a host. Macrophages were stimulated by LcS to produce interleukin (IL)-12, which in turn stimulated cytotoxic T cells and natural killer (NK) cells, increasing expression of Th-1 cytokines which play an important role in anti-tumor activity¹⁹⁻²¹⁾.

In clinical studies, intra pleural instillation of heat-killed LcS was recommended for the treatment of malignant pleural effusion²²⁾. In addition, heat-killed LcS, when used in combination with radiotherapy for cervical cancer, increases the early effects on tumor reduction and histological changes²³⁾. Aso *et al.* suggested that the oral administration of viable LcS may prevent recurrence of superficial bladder cancer²⁴⁾.

The results of the present study suggest that *Lactobacillus casei* strain Shirota may be a useful prophylactic organism against colon carcinogenesis. In the near future, the results of the present study may be confirmed in a clinical study.

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