Inhibitory Effect of Yogurt on Aberrant Crypt Foci Formation in the Rat Colon and Colorectal Tumorigenesis in RasH2 Mice

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Abstract: The inhibitory effects of yogurt consisting of milk fermented by Lactobacillus delbrueckii subsp. bulgaricus strain 2038 and Streptococcus salivarius subsp. thermophilus strain 1131 on formation of colonic aberrant crypt foci (ACF) in rats and also on development of colorectal tumors in transgenic mice harboring human prototype c-Ha-ras genes (rasH2 mice) were examined. F344 rats and rasH2 mice were fed commercial diet containing freeze-dried yogurt or starter medium (non-fermented milk). Rats were inoculated orally with heterocyclic amine 2-amino-methyl-6-phenylimidazo[4,5-b]pyridine hydrochloride (PhIP) for two weeks. The rats were necropsied 14 days after the PhIP treatment, and ACF in the colon and rectum were counted. RasH2 mice were injected with 1,2-dimethylhydrazine dihydrochloride (DMH) for 20 weeks. Three weeks after the last injection of DMH, rasH2 mice were necropsied to determine the number and the size of colorectal tumors. Yogurt supplementation in diet significantly reduced the number of ACF and aberrant crypts (ACs) in rats fed control diet (P<0.01), but not in rats fed non-fermented milk diet. On the other hand, rasH2 mice receiving the yogurt-supplemented diet had significantly reduced numbers of tumors induced by DMH compared with those fed the non-fermented milk-supplemented diet (P<0.05). These results demonstrate that the yogurt used in this study appears to have tumor-suppressing properties, and rasH2 mice are a useful model for the evaluation of antitumor activities of foods.

Key words: aberrant crypt foci, colon tumor, PhIP, rasH2 mouse, yogurt

Introduction

Yogurt is one of the most popular and traditional functional foods in many countries and cultures. There are abundant reports on the beneficial effects of yogurt on human health [1], and many investigators have suggested that the consumption of fermented dairy products, including yogurt, elicits anti-tumor effects [12, 42, 44]. However, the reported anti-tumor effects of yogurt are inconsistent, and these inconsistencies may have arisen from the different bacterial strains and culture conditions and different experimental protocols used in the studies.
2-Amino-methyl-6-phenylimidazo[4,5-b]pyridine hydrochloride (PhIP) is one of the most abundant heterocyclic amines contained in cooked meat and fish [17]. It has been reported that PhIP is metabolized to form DNA adducts [6, 14, 35] and DNA adducts of PhIP have been detected in human tissues [10]. PhIP has been reported to induce aberrant crypt foci (ACF) in animals [38], which are considered as putative pre-neoclassic lesions of the colon [3]. The chemopreventive effects of various agents have been assessed in this model.

Transgenic mice harboring human prototype c-Ha-ras genes (rasH2 mice) were developed by Saitoh et al. [34] and constitute a well-validated mouse model which has been used for the evaluation of carcinogenic potential of chemicals [19]. We reported that colonic tumors were induced by 1,2-dimethylhydrazine dihydrochloride (DMH) in a short period of time with high sensitivity in rasH2 mice [26], and that this is a good model for examining the effect of environmental factors, such as intestinal flora of the host animals [26] or the effect of functional foods [30], on the development of tumors.

In this study, we examined the effects of yogurt fermented by two bacterial strains; Lactobacillus delbrueckii subsp. bulgaricus strain 2038 and Streptococcus salivarius subsp. thermophilus strain 1131, both of which are used in commercial yogurt, on inhibition of ACF formation induced by PhIP in rats, and also on colorectal tumors induced by DMH in rasH2 mice. The results show the inhibitory effect of yogurt fermented with the two bacterial strains on colon tumorigenesis, and we propose the usefulness of the rasH2 mouse model for evaluating the anti-tumor properties of food derived components.

**Materials and Methods**

**Bacterial strains and preparation of fermented milk**

*Lactobacillus delbrueckii* subsp. *bulgaricus* strain 2038 and *Streptococcus salivarius* subsp. *thermophilus* strain 1131, both of which are used in commercial yogurt, were maintained at the Central Institute of Meiji Milk Products Co., Ltd. (Tokyo, Japan). Each of the two strains was separately pre-cultured in a broth containing 10% (w/v) of skimmed milk and 0.1% (w/v) of yeast extract at 37°C for 15 h. Then, the cultures were inoculated into starter medium consisting of 87.5% (v/v) milk, 2.5% skimmed milk and 10% (v/v) distilled water at a concentration of 2%, and incubated at 42°C for 3 h. The fermented product was lyophilized and used as a yogurt powder. The starter medium without bacteria was lyophilized and used as non-fermented milk.

**Experimental diet**

Three defined high-fat, low-calcium diets were used in the ACF study in rats. The control diet consisted of a modification of the AIN 93G diet [33] with high fat (12.3%) and a low level of calcium (113 mg %). The yogurt diet contained lyophilized yogurt powder at a concentration of 10.4646%. Non-fermented starter medium in place of yogurt was added to the diet as non-fermented milk. For the tumorigenicity study in transgenic rasH2 mice, the commercial CMF diet (Oriental Yeast Co., Ltd., Tokyo, Japan) supplemented with 10% (w/w) of either yogurt powder or non-fermented starter medium was used as the yogurt diet or non-fermented milk diet, respectively.

**Chemicals**

PhIP was obtained from the Nard Institute (Osaka, Japan). DMH was purchased from Sigma (St. Louis, MO, USA).

**Animals**

Four-week-old male SPF F344 rats were purchased from Japan SLC Co., Ltd. (Shizuoka, Japan). Transgenic male and female SPF CB6F1-Tg rasH2 mice, a F1 hybrid of male transgenic (Tg C57BL/6J) and inbred BALB/cByJ female, were transferred from the Central Institute for Experimental Animals (Kawasaki, Japan) to the University of Tokyo for the tumorigenesis study. The original characteristics of rasH2 mice were described by Saitoh *et al.* [34]. All animals were housed in a clean rack in a conventional animal room on a 12:12 light cycle at 24 ± 1°C with a relative humidity of 55 ± 5%.

The study was approved by the Laboratory Animal Use and Care Committee of the Faculty of Agriculture, the University of Tokyo.

**Experiment schedule**

**Experiment 1:** A total of 29 four-week-old male F344
rats were randomly divided into three groups and experimental diet feeding was started at 14 days before treatment with PhIP and feeding continued throughout the study. PhIP was dissolved in water at a dose of 20 mg/ml, and rats were orally inoculated daily by intragastric gavage with PhIP solution at a dose of 75 mg/kg body weight, five times a week for 2 weeks without anesthesia [27]. Fourteen days after the last inoculation of PhIP, rats were euthanized by ether inhalation and the colons were excised, washed with PBS and opened flat. They were fixed with 10% phosphate-buffered formalin and stained with 0.2% methylene blue (w/v) dissolved in PBS for 10 min [2]. The numbers of AC and ACF were counted under a microscope at a magnification of 40× or 100×. ACF were identified as lesions with aberrant crypts (ACs) increased in size with a thicker epithelial lining according to previously reported criteria [2].

**Experiment 2:** Male and female rasH2 mice were fed the experimental diet from 8 weeks of age for 3 weeks. DMH was suspended in PBS (pH 7.0) at a concentration of 4 mg/ml and subcutaneously inoculated into mice at a dose of 20 mg/kg body weight without anesthesia. Mice were injected with DMH weekly for 20 weeks. Three weeks after the last injection of DMH, the mice were euthanized by ether inhalation and necropsied to determine the number and size of the colorectal tumors [26]. All tumors were divided into three groups according to size assessed by the average diameters of the short and long axes of the tumor masses (L: longer than 5 mm, M: from 2 to 5 mm, S: shorter than 2 mm), and were assigned five points for L, three points for M, and one point for S. The total number of the points per mouse colon was calculated as the tumor score, and the mean tumor scores were compared between the two groups.

**Statistical analyses**

Data were expressed as means ± SD. Statistical testing was performed using the Mann-Whitney *U*-test for non-parametric data or one-way analysis of variance (ANOVA) followed by Schiffe’s test for parametric data. The level of significance was *P*<0.05. All data handling and testing were performed using the statistical software package StatView 5.0 for Windows, SAS Institute Inc. (Cary, NC, USA).

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**Results**

**Experiment 1:** Inhibition of aberrant crypt foci in rats

Male Fischer 344 rats were given PhIP orally at a dose of 75 mg/kg five times a week for 2 weeks. The rats were divided into three diet groups: the control, non-fermented milk and yogurt groups. Each group was fed experimental diet from 14 days before the start of PhIP inoculation until 2 weeks after the last inoculation of PhIP. There was no significant difference in body weight among the three groups during the experimental period (Fig. 1). The incidence of ACF in all groups was 100%. ACF were frequently observed in the middle-colon area. Most ACF consisted of one or two aberrant crypts. As shown in Table 1, the number of PhIP-induced ACF per colon was significantly smaller in the yogurt diet group than in the control group (*P*<0.01). Yogurt ingestion reduced the number of ACF to 54.3% of that of the control. The number of ACs per colon after 2 weeks of treatment was also reduced to 52.8% of the respective control value (*P*<0.01) (Table 1). Non-fermented milk diet showed a reduction in the number of ACF and ACs, but the differences were not statistically significant. Mean numbers of ACs/focus were not significantly different among the three groups.

**Experiment 2:** Inhibitory effect of yogurt on colon tumors in rasH2 mice

RasH2 mice were injected with DMH once a week for 20 weeks. They were fed either yogurt or non-fermented milk diet for three weeks before injection of DMH and the diets were continued until the end of the study. Mean body weights of mice of the same sex during the experimental period did not differ between the two diet groups both for males and females (Fig. 2). Survival rate of rasH2 mice in each diet group was 80 to 90%, and there were no differences in survival rates between the non-fermented milk group and yogurt group. The cause of death in those animals was unknown. The incidence of colorectal tumors three weeks after the last injection of DMH was 100% in both non-fermented milk and yogurt groups. As shown in Table 2, the yogurt diet significantly reduced the number of colorectal tumors induced by DMH in male rasH2 mice (*P*<0.05). Although the statistical difference was not significant, the
tumor score was higher in the non-fermented milk group than in the yogurt group (Table 2).

**Discussion**

The beneficial effects of lactic acid bacteria and milk fermented by lactic acid bacteria have been reported starting with Metchnikoff’s *Prolongation of Life: Optimistic Studies* first published in 1908 [22]. Beneficial bacterial strains include *Lactobacillus acidophilus* [11], *L. casei* [15], *Bifidobacterium longum* [37], *B. infantis* [16] and some other lactic acid bacteria. Two bacterial species, *L. bulgaricus* and *Streptococcus thermophilus*, are used as a starter culture for the fermentation of milk to make yogurt, and various reports regarding the anti-tumor effects of these two lactic acid bacteria have been published: the anti-mutagenic activity of yogurt [4], the protective effect of *L. bulgaricus* against DNA damage [12, 42, 44], and the preventive role of yogurt against colon cancer [7, 36]. Of note, Shackeford et al. reported that drinking milk fermented by *L. bulgaricus* and *S. thermophilus* altered the metabolism of DMH and shifted the colon tumor distribution toward the anus [36]. However, the results of those studies were inconsistent, which seemed to be related to the different bacterial strains and experimental conditions used.

In the present study, we first evaluated the effect of yogurt fermented by *L. delbrueckii* subsp. *bulgaricus*...
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strain 2038 (*L. bulgaricus* 2038) and *S. salivarius* subsp. *thermophilus* strain 1131 (*S. thermophilus* 1131) on ACF formation induced by PhIP in rats. PhIP has been reported as one of the most abundant heterocyclic amines contained in cooked meat and fish [17], and not only induces ACF [38], but also induces colonic cancer in male rats when orally administered [13]. ACF are generally considered to be putative preneoplastic lesions of the colon [3]. Ochiai et al. reported that progression from dysplastic ACF to colon cancer may be one of the major routes involved in the development of colon cancer [29]. Based on this background, the animal model of ACF induced by PhIP has been widely used for the evaluation of anti-tumor effects of various chemopreventive agents. Weisburger et al. reported that the number of ACF in the rat colon increases when they are fed a high fat diet [41]. It is also reported that the protective effect of lactic acid bacteria on colon cancer was shown in animals given high fat diets [5]. In the present study, we evaluated the effect of yogurt on the formation of ACF in F344 rats by feeding modified AIN93G diet with a high fat and low calcium concentration, which mimicked the level of fat typical of a Western diet, and observed the inhibitory effect of yogurt on ACF formation in rats. The number of ACs and ACF were significantly smaller in the yogurt diet-fed rats compared with the control diet-fed group. Interestingly, in the non-fermented milk-fed group, reduced numbers of ACF and ACs were also observed. This result is consistent with previous work, in which milk itself showed a protective effect.
against the carcinogenesis process induced by a mixture of three heterocyclic amines [39].

RasH2 mice are highly sensitive to various carcinogens [43] and they are recognized to be an appropriate animal model for the alternative short-term carcinogenicity bioassay [19]. Recent reports have further confirmed the conformity of rasH2 mice derived from different breeding facilities [20]. Recently, we reported that DMH induced colorectal tumors in rasH2 mice were strongly affected by intestinal flora [26] and by the ingestion of an apple pectin and culture condensate of *Bifidobacterium longum* [30]. In this study, the addition of yogurt fermented by *S. thermophilus* 1131 and *L. bulgaricus* 2038 to the diet significantly reduced the number of the colorectal tumors induced by DMH in rasH2 mice. These results indicate that rasH2 mice are a suitable model animal for the evaluation of tumor-preventive effects by environmental factors such as food-derived components as well as chemopreventive agents [24].

The precise mechanisms by which yogurt has an effect on colon carcinogenesis are still unknown. Among the various beneficial effects of yogurt, there have been many reports that indicate the effect of fermented milk on the host’s immune response [23], which may be one of the potential mechanisms of the inhibitory effect of yogurt against tumorigenesis. The starter bacteria *L. bulgaricus* and *S. thermophilus* are considered to be acid-intolerant and the survival of these bacteria in the gut is still controversial [8, 9, 21, 31]. However, the active components of lactic acid bacteria responsible for immunomodulation in the host are generally considered to be bacterial cell wall, cytoplasmic extract or bacterial DNA [23]. Thus, it is not mandatory for the bacteria to be alive in order to exert an immunoregulatory activity in the gut after ingestion. Nagafuchi *et al.* screened the immunopotentiating activity of 90 *L. bulgaricus* strains and found that the beneficial effects of lactic acid bacteria on the systemic and mucosal immune system differed among strains [25], which may explain the discrepancies in anti-tumor effects of lactic acid bacteria among various reports.

The other possible mechanism responsible for carcinogenesis is oxidative damage to the cells [28]. The anti-oxidative effect of lactic acid bacteria has been reported [18]. Both of the bacterial strains used in this study, *S. thermophilus* 1131 and *L. bulgaricus* 2038, showed production of radical scavengers and inhibited oxidation of erythrocyte membranes and low-density lipoprotein in vitro [40]. Although further studies are needed on the mechanism of the anti-oxidative effect of these organisms in vivo, the radical scavenging ability of these two strains might be responsible for the anti-tumor effects of yogurt.

Other mechanisms such as the suppression of harmful intestinal bacteria, sequestration of potential mutagens, production of anti-mutagenic compounds, reduction of pH in the colon or alteration of other physiologic conditions as reviewed by Rafter [32] must be clarified in order to determine beneficial effects of yogurt on the host.

In conclusion, supplementation of yogurt fermented by *S. thermophilus* 1131 and *L. bulgaricus* 2038 in the diet exhibits anti-tumor activity in rasH2 mice. RasH2 mice, confirmed to be sensitive to both genotoxic and non-genotoxic carcinogens in short-term assays, should be a useful animal model for evaluating the inhibitory effects of food on tumor development, which have milder effects compared with medication.

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