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Commercial Soy Milk Enhances the Development of 7,12-Dimethylbenz(a)anthracene-induced Mammary Tumors in Rats

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Abstract. *Background: Soy milk is a major soy food in China and Japan. Isoflavones in soy food are considered to protect women again breast cancer. Materials and Methods: The effects of soy milk consumption on 7,12-dimethylbenz(a) anthracene (DMBA)-induced mammary tumors in adult female rats was investigated. Sprague-Dawley rats were given 5 mg DMBA via intragastric intubation and then assigned to receive soy milk or water in addition to a normal rodent diet. Body weights, liquid and food intake, tumor number, location and development were recorded. After 20 weeks, liver, uterus and mammary tumors were removed from the sacrificed animals and examined. Plasma 17β-estradiol concentration was also determined. Results: After 20 weeks of DMBA administration, all of the rats that drank soy milk developed mammary tumors, while the incidence in the control group was 70% ($p<0.01$). Tumor multiplicity increased in the soy milk group with borderline significance ($p=0.06$). Total tumor weight and size in the soy milk group were 1.5-fold greater than in the control group, without a significant difference ($p>0.05$). Uterine weight and plasma 17β-estradiol concentrations were similar in the two groups. Conclusion: Our results suggest that commercial soy milk enhanced the development of DMBA-induced mammary tumors in rats. Thus, careful consideration should be given when explaining the beneficial effects of soy food.*

Breast cancer is by far the most common cancer of women, comprising 23% of all female cancers, with an estimated 1.15 million new cases in 2002 (1). Although the introduction of screening programs has perturbed the preexisting trends in incidence, a steady increase in the number of cases of breast cancer continues almost everywhere (2). Incidence rates are high in the developed world, including Europe and North America. In contrast, rates are low in most African and Asian populations, despite the fact that they are also increasing (2, 3). These international differences and immigration-based studies (4) suggest that lifestyle variables, such as dietary factors, contribute to breast cancer risk.

Our previous study demonstrated that cow's milk, which is popular in developed countries, promoted the development of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in rats (5). However, the adverse effect of milk on breast cancer risk is mitigated in public by the obvious overall health benefits from milk nutrients and calcium. On the other hand, scientists are paying much attention to soy food because women in China and Japan, where breast cancer rates are low, consume much soy. Isoflavones, which are found in dietary-relevant amounts only in the soybean (6), contribute to this protective effect. They belong to a group of phytoestrogens, including genistein, daidzein, glycitein and their β-glycosides. We performed a meta-analysis based on published observational epidemiological studies and found that soy food intake was associated with a decreased risk of breast cancer through isoflavones (7). However, no clear consensus has emerged regarding the breast cancer preventive aspects of soy food and isoflavones when considering the data from animal studies and clinical studies.

Soy milk is a major soy food in China and Japan. It is recommended as an alternative milk if one has galactose intolerance or believes that cow's milk is unhealthy. Ohta *et al.* (8) found that fermented soy milk inhibited the development of mammary tumors generated by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in rats. The chemopreventive activity of fermented soy milk was partly attributed to the presence of isoflavones. Since fermented food products have the ability to reduce the risk of certain cancers by modifying pH, antagonizing pathogens and stimulating immunodulatory

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Table I. *Energy intake and body and organ weights at termination.*

	No. of rats	Body weight (g)	Energy intake (kcal/day)	Liver weight (g)	Uterine weight (g)
Control	20	330.2±15.6	52.5±3.9	10.4±0.6	0.42±0.08
Soy milk	20	319.6±17.9	54.9±5.6	10.2±0.6	0.45±0.09

Table II. *Effects of soy milk on tumor parameters at autopsy.*

	No. of rats	Incidence (%)	Multiplicity (tumor number/rat)	Total tumor weight (g)	Total tumor volume (cm^3)
Control	20	70	1.4±1.3	50.6	15.8
Soy milk	20	100[a]	2.1±1.0	81.2	26.9

[a]Significant difference form the control group ($p<0.01$).

functions (9), it is difficult to distinguish the effects of soy milk components themselves such as isoflavones from fermentation when fermented soy milk is used. In fact, using immune deficiency mice implanted with MCF-7 breast cancer cells, Chang *et al.* (10) found that the inhibitory effect of fermented soy milk was mainly due to the aqueous phase, not the isoflavone containing lipid-soluble phase. In the present study, we observed the effect of commercial soy milk on DMBA-induced mammary tumors in rats.

Materials and Methods

Forty female Sprague-Dawley (SD) rats were obtained at six weeks of age from the Shizuoka Laboratory Animal Center (Shizuoka, Japan). They were housed individually in stainless-steel wire-bottomed cages in an air-conditioned room with a 12/12 h light/dark cycle. The care and use of laboratory animals followed the Guidelines for Animal Experiments of the University of Yamanashi.

After a one-week period of adaptation, all animals were given a single dose of 5 mg of DMBA (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.5 ml of corn oil by intragastric intubation. Twenty-four hours later, the rats were randomly assigned to a soy milk group and a control group each with 20 animals. The rats in the former group were given soy milk, purchased in a local supermarket. The rats in the control group drank water. All the animals were fed a commercial rodent diet (Clea CE-2, Nippon Clea, Tokyo, Japan). Body weight and intakes of the rodent diet and soy milk were recorded weekly throughout the experiment. Rats were palpated weekly and the date that tumors appeared and the number and location of tumors were recorded (5).

At week 20 after DMBA administration, rats were decapitated between 14:00 and 16:00. Whole skin was peeled away and the location of mammary tumors attached to the skin was identified. All other organs were examined for gross abnormalities. The liver and uterus were dissected and weighed. After fixation in 10% neutral buffered formalin overnight, mammary tumors were removed from the skin and weighed. The diameter of each tumor was measured with calipers and tumor volume was calculated using the formula, largest diameter x (smallest diameter)^2 x 0.4 (11). Mammary tumors and visibly abnormal organs were embedded in paraffin, cut into sections 5-mm-thick, and stained with hematoxylin and eosin for histopathological examination. The histological diagnosis was performed according to the classification described by Konitowski *et al.* (12).

Blood plasma samples collected at autopsy were used to measure the concentration of 17β-estradiol with an EIA kit (ALPCO Diagnostics, Salem, NH, USA).

The statistical analysis of tumor incidence was performed with the Chi-squared approach. The differences in body and organ weights, food intake, tumor latency, tumor multiplicity, and 17β-estradiol concentrations between the two groups were determined by *t*-test. The volume and weight of tumors were analyzed using the Wilcoxon rank sum test. *P*-values less than 0.05 were considered significant.

Results

During the experimental period, no significant difference was observed in body weight between the control and soy milk groups. The energy intake of rats increased in the first several weeks and remained at a similar level thereafter (data not shown). Rats in the soy milk group consumed 30.5 ml±3.6 ml soy milk per day. There were no significant differences in liver weight or uterine weight between the two groups (Table I).

The results of sequential observation of mammary tumors by palpation are shown in Figure 1. Palpable tumors first appeared in week 8 after the administration of DMBA in both groups. The incidence increased with time, more rapidly in the soy milk group than in the control group. From week 14, the incidence was significantly higher in the soy milk group ($p<0.05$). At the final palpation, all rats in the soy milk group developed tumors and 14 rats in the control group had tumors (Figure 1). Tumor latency, which is defined as the lag time between the administration of DMBA and the development of the first tumor in each rat, was slightly shorter in the soy milk group (12.0±3.0 weeks) than in the control group (12.6±3.6 weeks). The pattern of increase in tumor number during the experimental period was similar with that of incidence (Figure 1). At the final palpation, there were a total of 42 tumors in the soy milk group and 28 in the control group.

We did not find any significant difference in any parameter other than tumor incidence at autopsy (Table II). Multiplicity (tumor number/rat) showed a borderline significant increase in the soy milk group ($p=0.06$). The tumor number per tumor-bearing rat was almost the same in the two groups. Because of the wide variance in tumor number and volume and their non-normal distribution, total tumor weight and volume are shown in Table II. Although the tumors in the soy milk group were much heavier and larger than those in the

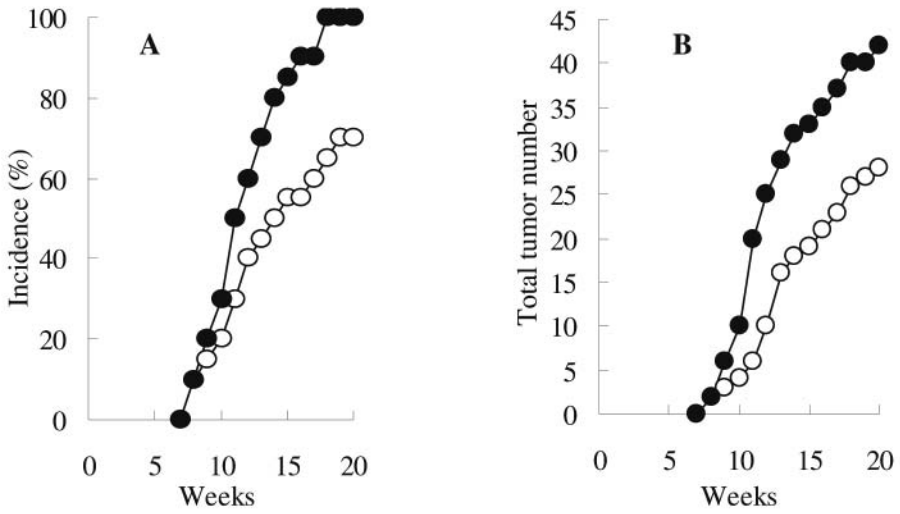

Figure 1. *Time course of the number of palpable mammary tumors in rats after DMBA administration. Open circles: control group; closed circles: soy milk group. From week 14, the incidence was significantly higher in the soy milk group than in the control group.*

control group, there were no significant differences between the two groups using the non-parametric test. Histopathological examination revealed that almost all of the mammary tumors were adenocarcinomas.

At autopsy, the plasma 17β-estradiol concentration was 43.0 pg/ml±6.4 pg/ml in the control group and 46.6 pg/ml±6.9 pg/ml in the soy milk group, a difference that was not significant (Figure 2).

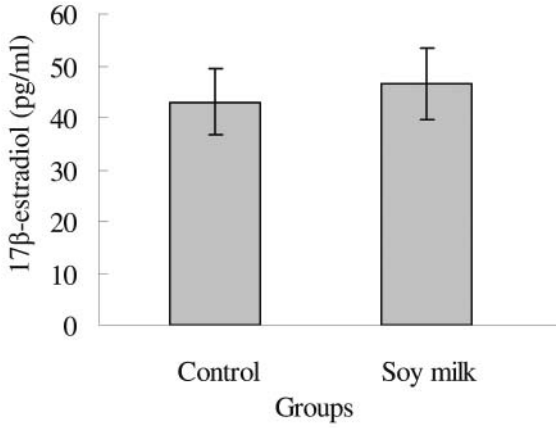

Figure 2. *The plasma concentration of 17β-estradiol at autopsy.*

Discussion

In the present study, we found that soy milk promoted the development of mammary tumors generated by DMBA in rats. This seems to contradict most results from observational epidemiological studies and our meta-analysis (7). In fact, the US Food and Drug Administration (FDA), while recognizing the claim that soy has a favorable cardiovascular effect, has not accepted the recommendation proposed by the American Soybean Association that the consumption of soy has a protective effect against certain types of cancer (13). The possible reason for this is the inconsistent results from laboratory studies.

To our knowledge, only two studies have examined the association between natural soy food and carcinogen-induced mammary tumors in rats. Ohta *et al.* (8), as mentioned above, used fermented soy milk. The other study also used a fermented soy food, miso, which significantly decreased mammary tumor multiplicity (14). Almost all animal studies have used soy protein isolates, isoflavones or their components genistein and daidzein to explain the effects of soy food on carcinogen-induced mammary tumors.

In general, in uterine or perinatal to prepubertal exposure to isoflavones inhibits the carcinogen-induced mammary tumors through a decrease in terminal end buds and an increase in lobular structures (15). In contrast, exposure in adults has provided inconsistent results from stimulation to inhibition of tumorigenesis (8, 16-23). In 1998, Hsieh *et al.* (16) first found that genistein, a major isoflavone in soy food, stimulated the growth of mammary tumors in athymic BALB/c (nude) ovariectomized mice injected with estrogen-dependent MCF-7 human breast cancer cells. Allred *et al.* (17) further demonstrated that soy protein isolates and genistein stimulated the growth of breast cancer cells *in vivo* in a dose-dependent manner. Although we did not determine the isoflavone levels in soy

milk, their presence is an accepted fact and the isoflavone concentration is 0.28 mg/ml according to the table of nutrients provided by the producer. Thus, isoflavones in soy milk are likely to contribute to DMBA-induced mammary tumor in rats.

In another Allred study, genistein resulted in large MNU-induced mammary tumors in ovariectomized SD rats (18). These effects depended on the estrogenic activity of genistein because uterine weight (0.17 g *vs.* 0.10 g) and serum 17β-estradiol concentrations (5.5 pg/ml *vs.* 13.6 pg/ml) were significantly higher in the genistein group than in the control group. In our study, uterine weight and plasma 17β-estradiol concentrations were similar in the two groups. One explanation is that the endogenous estrogen from intact rats we used covered the effects caused by soy milk. Kijkuokool *et al.* (19) also found no differences of uterine weight or serum estradiol levels at autopsy, although genistein significantly increased the multiplicity and size of *N*-nitrosomethylurea-induced mammary tumors in intact rats.

In the present study, total tumor weight and size in the soy milk group was approximately 1.5-fold that in the control group. However, the tumors varied extensively in weight and size. One possible explanation is that the dose of DMBA (5 mg/rat) used was relatively low. Consequently, some small tumors still developed when the experiment approached its end. The low dose of DMBA also resulted in the low multiplicity in the present study. Constantinou *et al.* (22) gave rats 15 mg of DMBA by intragastric intubation and all groups had a high incidence (≥90%). Given the significant decrease in multiplicity (8.0±1.0 *vs.* 5.5±0.9), the authors concluded that the soy protein isolate had chemopreventive effects on DMBA-induced mammary tumors. Interestingly, when isoflavones were removed from the soy protein isolate, the multiplicity decreased further to 4.0±0.7. Thus, the popular belief that isoflavones prevent breast cancer was not supported by that study.

In the present study, the mechanism by which soy milk promoted the development of DMBA-induced mammary tumors in rats is not clear. We cannot confirm the estrogenic actions of isoflavones in soy milk because of interference from endogenous estrogens. Thus, we will perform a further study using ovariectomized rats and observe more parameters such as the expression of estrogen receptors. In addition, rats in the present study drank about 30 ml of soy milk per day. This equates to a human equivalent of 6 liters when calculated on the basis of body weight (60 kg), an impossible amount for humans to consume. In addition, the metabolism of isoflavones differs between rodents and humans because the bacteria in the rodent gut effectively convert genistein and daidzein to the metabolite equol, whereas only 30%-50% of humans carry bacteria with this metabolic capacity (24). Therefore, we must note that the outcomes from carcinogen-induced mammary tumors in rats cannot be directly extrapolated to the development of spontaneous breast cancer in humans. However, this issue is worth investigating further due to the high incidence of breast cancer and increasing consumption of soy food worldwide.

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