Short Communication

Diets Containing Whey Proteins or Soy Protein Isolate Protect against 7,12-Dimethylbenz(*a*)anthracene-induced Mammary Tumors in Female Rats[1]

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Abstract

A study was conducted to determine the protective effects of two common dietary proteins, soy protein isolate (soy) and bovine whey, against chemically induced mammary tumors in female Sprague Dawley rats. Rats were fed AIN-93G diets having casein, soy, or whey as the sole protein source. Rats within the same dietary groups were mated to obtain the F_1 and F_2 generations. At age 50 days, F_1 (experiment A) or F_2 (experiment B) female offspring (≥19 rats/group) were p.o. gavaged with 80 mg/kg 7,12-dimethylbenz(*a*)anthracene, and mammary glands were evaluated when 100% of the casein-fed group developed at least one palpatable tumor. Rats grew well on all three diets, but casein-fed rats gained slightly more body weight than soy- or whey-fed rats ($P < 0.05$). Vaginal opening occurred 1 day earlier in soy-fed rats than in casein- or whey-fed rats, but no other differences in reproductive and developmental parameters were observed between groups. When 50% of the casein-fed rats had at least one mammary tumor, lower tumor incidences (24–34%) were observed in the soy-fed ($P < 0.009$) and whey-fed groups ($P < 0.001$). When 100% of the casein-fed rats had at least one tumor, soy-fed rats had a lower tumor incidence (77%) in experiment B ($P < 0.002$), but not in experiment A ($P < 0.12$), and there were no differences in tumor multiplicity. Whey-fed rats had lower mammary tumor incidence (54–62%; $P < 0.002$) and multiplicity ($P < 0.007$) than casein-fed rats in both experiments. Our results indicate that diets rich in soy reduce the incidence of chemically induced mammary tumors by approximately 20%. Furthermore, whey appears to be at least twice as effective as soy in reducing both tumor incidence and multiplicity.

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Introduction

Of all environmental factors known to influence cancer, diet appears to be one of the most significant (1). A wide variety of dietary factors are thought to be important in altering cancer initiation, promotion, and progression, as well as in the prevention of cancer. Breast cancer is the most common malignant tumor among women and is the number two killer of women in the United States. Breast cancer incidence in women consuming traditional Asian diets is approximately 10% that of the general female population of the United States. Asian diets are low in red meat and fats but rich in grains such as rice and high in soybean products such as tufu and miso. Although such diets contain many components other than soy, factors found in soybeans have been reported to provide important protection against initiation, promotion, or progression of breast cancer in animal models. In addition to the protective effects of certain phytochemicals, epidemiological and experimental studies suggest that dietary bovine milk products may exert inhibitory effects on the growth of several tumor types (for a review, see Ref. 2). The antitumor activity of these dairy products has been attributed to a class of proteins that comprise approximately 20% of the total milk protein, the whey fraction (2, 3).

The present study was conducted to determine the possible preventive effects of diets containing soy protein isolate or bovine whey proteins on DMBA[3]-induced breast tumors in rats. The experiments were designed to determine the effects of long-term consumption of these proteins in diets that were formulated to meet the allowances recommended by the American Institute of Nutrition for the rat.

Materials and Methods

Experimental Design. Adult breeder female and male Sprague Dawley rats were purchased from Harlan Industries (Indianapolis, IN). They were housed individually in polycarbonate cages and allowed *ad libitum* access to water and pelleted food. Rats were randomly assigned to three groups and fed one of three semipurified diets made according to the AIN-93G diet formula (4), except that corn oil replaced soybean oil, and the protein source was either casein (New Zealand Milk Products, Santa Rosa, CA), whey (New Zealand Milk Products), or soy protein isolate (Protein Technologies International, Inc., St. Louis, MO). Diets containing soy protein isolate had 430 mg total isoflavones/kg diet, including 276 mg/kg genistein and 132 mg/kg diadzein. Amino acids were added to each diet to equalize the essential amino acids among diets.

Rats were allowed to breed, and the offspring were weaned to the same diet as their mothers. Offspring from

[3] The abbreviations used are: DMBA, 7,12-dimethylbenz(*a*)anthracene; GSH, glutathione.

different parents within a diet group were selected at random and mated to form the F_2 generation. Offspring from both F_1 and F_2 were fed their respective diets throughout their entire lives. Two generations were selected for studies to simulate populations of people consuming the same basic diet for several generations. At 50 days of age, female offspring from the F_1 generation (experiment A) and the F_2 generation (experiment B) were p.o. gavaged with sesame seed oil (Sigma, St. Louis, MO) containing 80 mg/kg DMBA (Sigma), a chemical procarcinogen used widely to produce mammary adenocarcinoma in rats. This dose was selected based on studies reported by Barnes *et al.* (5) and Lamartiniere *et al.* (6). Rats were weighed weekly, and, beginning 3 weeks after DMBA treatment, each mammary gland was palpated twice weekly. The detection date and location of each mammary mass were recorded. When at least one palpatable mammary mass was present in 100% of the casein-fed rats, all rats were killed, and all mammary glands from each rat were visually examined for tumors. However, rats in which tumor masses exceeded 3 cm in diameter were killed early for humane reasons according to our institutional animal care and use committee-approved animal protocol. All tumors were weighed and measured for volume, and a section of the tumor or the entire mass, depending on the size, was fixed in buffered formalin. Sections (5 μm) of the paraffin-embedded tumors were stained with H&E for histological analysis.

Pathology. An American College of Pathology-certified pathologist (S. K.) evaluated tumors in a blinded protocol and classified them as follows: (*a*) benign (mammary tissue minimal proliferation); (*b*) intraductal proliferation; or (*c*) adenocarcinoma (ductal carcinoma *in situ* or invasive ductal and lobular carcinoma).

Statistical Analysis. For each experiment, a mixed model ANOVA (7) was used to analyze adult body weight after injection with DMBA. Because there were multiple measurements per animal over time, day was included in the model as a with-in-animal effect to account for the correlation of measurements from the same animal. The effect of diet and the interaction between diet and day were also included in the model. A Kaplan-Meier analysis of tumor latency was performed (8). Product limit survival estimates were used to determine the median tumor latency time and confidence limits. A generalized Wilcoxon test (9) was used to compare median tumor-free times. To examine the prevalence of tumors according to breast location, a generalized estimating equation logistic regression model (10) was used to account for the clustering of tumor findings on each animal, adjusting for diet. Fisher's exact test (8) was used to compare the percentage of animals with tumors in each treatment group for each experiment. The median numbers of tumors per tumor-bearing rat (multiplicity) for each diet were compared using the nonparametric Kruskal-Wallis test. Statistical significance was set at $P < 0.05$, and all *P*s were not adjusted for multiple comparisons.

Results

Body and Organ Weights. All rats gained weight during the course of each experiment (Fig. 1). Total body weight gains were similar until approximately 5 weeks of age, when casein-fed rats began to gain slightly more weight than either soy-fed or whey-fed rats. By 8 weeks, body weights of casein-fed rats were slightly greater than those of soy-fed or whey-fed rats ($P < 0.01$) in both experiments; however, body weights did not differ between soy-fed and whey-fed rats. When development and reproductive parameters of the casein-fed group were compared with those of the soy-fed or whey-fed groups, a 1-day advancement in the age (day 37 *versus* day 38) of the vaginal opening of soy-fed rats ($P \leq 0.05$) was the only significant difference observed during the two-generation period. No differences were noted in relative organ weights, estrous cycle, breeding success rate, sex ratio, or number of offspring/litter (data not shown).

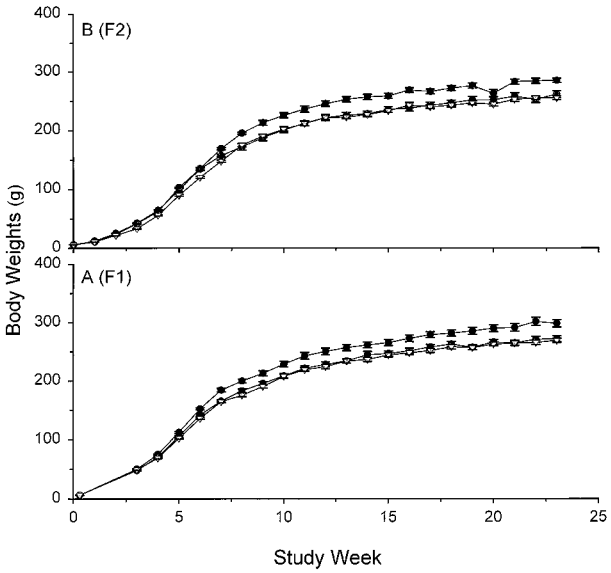

Fig. 1. Body weights of female rats. P.O. gavage of 80 mg/kg DMBA at age 50 days. In experiment A (*top panel*), F_1 rats were fed either casein diets ($n = 19$; ●), soy diet ($n = 25$; ○), or whey diet ($n = 24$; ▲). In experiment B (*bottom panel*), F_2 rats were fed either casein diets ($n = 39$), soy diet ($n = 31$), or whey diet ($n = 35$). Data are presented as means ± SEM.

Time Course for Tumor Formation. The time course of palpatable breast mass appearance is shown in Fig. 2, and data concerning these tumors are presented in Table 1. At the time when 100% of the casein-fed rats developed at least one breast mass, relatively fewer rats fed whey protein had tumors compared to casein-fed rats in both experiments ($P < 0.002$); whereas soy-fed rats had relatively fewer tumors compared to casein-fed rats ($P < 0.008$) only in experiment B.

Tumor Latency. The tumor latency (postinjection time before the appearance of the first breast mass) was observed to be greater in soy-fed and whey-fed rats than in the casein-fed group, but this was variable (Table 1). However, another representative measure of tumor latency is the posttreatment time at which the probability of developing a tumor is 50%. Using the day at which 50% of the rats developed a tumor as a measure of latency for rats from either the F_1 or F_2 experiments, whey-fed rats had a 31–35-day (60–64%) longer latency period than casein-fed rats ($P < 0.002$), and soy-fed rats had a 13–14-day (25–26%) longer latency period than the casein group ($P < 0.009$). Furthermore, whey delayed this onset more than soy in experiment B ($P < 0.05$), but not in experiment A ($P < 0.197$).

Tumor Characteristics. Ninety-five percent (experiment A) and 85% (experiment B) of the rats bearing at least one tumor had an adenocarcinoma in the casein-fed group, whereas 90% (experiment A) and 79% (experiment B) of soy-treated rats and 86% (experiment A) and 63% (experiment B) of whey-treated rats had at least one adenocarcinoma, respectively (Table 1). There were no statistically significant differences between groups in the mean tumor volume (Table 1).

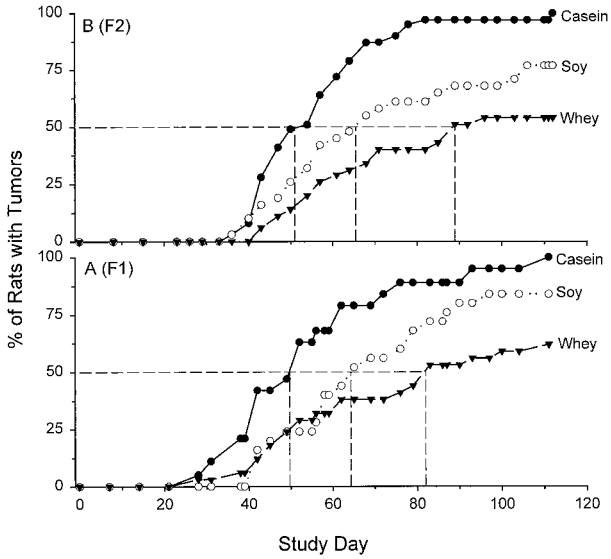

Fig. 2. Mammary tumor incidence (percentage of rats with tumors) of female rats depicted in Fig. 1. *Dashed line* indicates the post-DMBA day at which 50% of the casein-fed rats developed at least one mammary tumor. Statistical analyses of these data appear in Table 1.

Tumor Multiplicity and Location. Of the tumor-bearing rats, the median number of mammary tumors/tumor-bearing rat (*i.e.*, multiplicity) was lower ($P < 0.007$) in each experiment for whey-fed rats than for casein-fed rats (Table 1), whereas multiplicity for soy-fed rats did not differ significantly from that of the casein-fed group. Tumor location was studied, and cervical and thoracic regions were at higher risk of developing tumors (data not shown).

Discussion

Cancer rates in countries with high consumption of soybeans are lower than those in the United States, where less soy products are consumed (1), and cancer rates increase in the second generation of families that migrate to the United States from these countries as their diet becomes Westernized (11). Data from the present study substantiate the breast cancer prevention claims for experimental diets containing soy protein isolate as reported by others (5, 12). Furthermore, these data extend our knowledge on soy protein-containing diets and add new information on another dietary factor of animal origin, whey protein, with respect to chemically induced breast cancer. Results from our work demonstrate clearly that diets containing isolated soy protein or whey protein can significantly increase the age of onset of DMBA-induced tumors and reduce the percentages of rats that develop tumors. Moreover, rats fed diets containing whey also had decreased tumor multiplicity, whereas soy treatment did not appear to alter multiplicity. There were no detectable dietary effects on tumor volume.

To our knowledge, this is the first demonstration that rats fed whey protein-containing diets develop fewer DMBA-induced breast tumors than rats fed either casein- or soy-based diets. The whey protein diet delayed the age of onset of DMBA-induced tumors, reduced the percentages of rats that develop tumors, and attenuated tumor multiplicity compared with soy protein isolate or casein protein diets.

The mechanisms by which whey protein could alter carcinogenesis are unknown. Speculation has focused primarily on increases in tissue GSH levels observed during whey consumption (2). Greater GSH concentrations would tend to be protective because of: (*a*) the well-known xenobiotic detoxification pathway involving GSH and glutathione *S*-transferases; (*b*) GSH free radical detoxification; and (*c*) improved immune responses (13). Other possible mechanisms could include bioactive peptides that are either fragments of whey hydrolysis or contaminants of whey protein, such as insulin-like growth factor I, which could act on one of many cellular processes to reduce tumor incidence. Studies are currently underway in our laboratory to determine the mechanisms by which whey protein consumption prevents DMBA-induced breast cancer.

Although the mechanisms by which soy protein isolate prevents such cancers are still unknown, factors that are physically bound to or associated with the isolated protein, especially the isoflavones, have been implicated as being important (5). Genistein is one such isoflavone present in soybeans as genistein glucoside or the 6′-*O*-malonylglucoside. Lamartiniere *et al.* (6) demonstrated that rat pups receiving high doses of pure genistein aglycone at ages 2, 4, and 6 days developed fewer DMBA-induced breast tumors. This work has been substantiated in unpublished studies in our laboratory.[4] However, it should be noted that isoflavones have not been reported to be uniformly protective against chemically induced mammary tumor models in animals. For example, Hilakivi-Clarke *et al.* (14) reported a doubling of chemically induced mammary tumors in the offspring of mothers treated with genistein during pregnancy. Hsieh *et al.* (15) reported that dietary genistein enhanced the growth of MCF-7 tumors that were implanted s.c. in ovariectomized athymic mice. It should be pointed out that these two studies used genistein aglycone rather than diets made with soybean meal, soybean flour, or soy protein isolates. The disparity between laboratories using various animal models may reflect differences in species, developmental timing of genistein treatment, differences in endocrine status (*e.g.*, intact *versus* ovariectomized athymic females), or the chemical form of the isoflavone (pure aglycone, glucoside, or protein-bound form) and points to the need for further careful research on the conditions under which dietary factors affect cancer risk.

In the current study, soy-fed rats received a diet in which the entire protein source was the soy protein isolate used in the majority of commercially available soy-based infant formulas (Protein Technologies International, Inc.). A 333-g rat in our study consumed approximately 25 g/day of diet formulated with 20% soy protein (w/w). Because the genistein content was 1.36 mg/g protein, the genistein intake was approximately 6.8 mg/day (20.4 mg/kg/day). This is the highest dose of isolated soy protein possible within the AIN-93G diet formula. This genistein intake compares with the approximately 11 mg/day reported for 4-month-old infants who consume soy-based infant formula (16). Dose-response and time course studies for soy isolated protein effects and its cancer-preventive actions remain to be determined.

Several mechanisms have been proposed for the anticarcinogenic activity of isoflavones, including: (*a*) inhibition of proteases; (*b*) antioxidant activity; (*c*) increased synthesis and decreased degradation of steroid hormone binding globulin synthesis; (*d*) weak estrogenic agonist/antagonist activity through estrogen receptor α; (*e*) estrogen receptor β-mediated actions; (*f*) altered hormone production, metabolism, or action;

[4] T. M. Badger, unpublished observations.

Table 1 Onset, incidence, type, and multiplicity of DMBA-induced mammary tumors in female rats fed diets containing casein, soy, or whey proteins

	Experiment A (F_1)			Experiment B (F_2)		
	Casein	Soy	Whey	Casein	Soy	Whey
No. of Rats[a]	19	25	34	39	31	35
Tumor onset						
Day of first tumor[b]	28	42	28	36	36	43
Day at 50% tumors[c]	52	65	83	54	68	89
P[d]	0.007 (C-S)	0.197 (S-W)	0.001 (C-W)	0.009 (C-S)	0.05 (S-W)	0.001 (C-W)
Tumor incidence						
% Rats with tumors[e]	100	84	62	100	77	54
P[f]	0.12 (C-S)	0.084 (S-W)	0.002 (C-W)	0.002 (C-S)	0.07 (S-W)	0.001 (C-W)
Tumor type[g]						
Adeno-CA[h]	95	90	86	85	79	63
IDP[i]	5	5	5	13	21	32
Multiplicity[j]	5 (2–12)	3 (1–12)	2 (1–22)	3 (1–7)	3 (1–10)	2 (1–7)
P[k]	0.131 (C-S)	0.42 (S-W)	0.001 (C-W)	0.77 (C-S)	0.026 (S-W)	0.007 (C-W)
Tumor volume (cm^3)[j]	1.9 (0.2–12.7)	1.5 (0.1–5.3)	0.6 (0.1–15.4)	3.0 (0.02–14.2)	1.8 (0.02–15.4)	0.8 (0.1–16.2)
P[k]	0.89 (C-S)	0.69 (S-W)	0.49 (C-W)	0.79 (C-S)	0.55 (S-W)	0.48 (C-W)

[a] The number of rats per group per experiment. Some rats were diagnosed by our institutional veterinary staff with DMBA toxicity (severe anemia) between 12 and 15 days after gavage. These rats were euthanized. In experiment A, 0 casein-treated rats, 1 whey-treated rat, and 0 soy-treated rats developed toxicity, and in experiment B, 3 casein-treated rats, 7 whey-treated rats, and 6 soy-treated rats developed toxicity. The numbers of rats per group reflect those rats not developing DMBA toxicity.
[b] Post-DMBA day at which the first breast mass was detectable by palpation.
[c] Post-DMBA day at which the probability of tumor development is 50%.
[d] *P* based on generalized Wilcoxon test. Parentheses contain diet treatment comparisons for casein (C), soy (S), or whey (W).
[e] Percentage of rats with at least one breast mass on day when 100% of casein-fed rats had at least one tumor.
[f] *P* based on Fisher's exact test. Parentheses below contain diet treatment comparisons for casein (C), soy (S), or whey (W).
[g] Exact χ^2 test for independence detected no significant differences in incidences of tumor types across the three diets ($P = 0.95$ for experiment A, and $P = 0.37$ for experiment B).
[h] Percentage of tumor-bearing rats that had at least one adenocarcinoma (Adeno-CA).
[i] Percentage of tumor-bearing rats with at least one tumor being rated as intraductal proliferation (IDP).
[j] Median tumor numbers in tumor-bearing rats with the minimum and maximum numbers in parentheses.
[k] Pairwise comparison based on Dunn's procedure. Three groups were tested simultaneously based on the nonparametric Kruskal-Wallis test. Parentheses contain comparisons where C = casein, S = soy, and W = whey. Median values were presented because the data were skewed according to Ref. 25.

and (*g*) induction of Phase II detoxification systems (for a review, see Ref. 17). However, in addition to genistein, there are several other biologically active factors associated with soy protein. For example, other isoflavones such as diadzien and other factors such as saponins, phytosterols, protease inhibitors, and inositol hexaphosphate that have also been implicated in cancer prevention are present (1).

Although no overt effects of soy or whey diets were observed on sexual development (other than the a 1-day advance in vaginal opening), reproductive organ weights, or reproductive performance, enhanced breast differentiation represents a potential mechanism underlying the protective effects of these diets. This would be more likely in rats fed soy diets because of the well known estrogenic isoflavones than in those fed whey diets, in which no such estrogenic factors have ever been reported. Other potential mechanisms might involve altered DMBA metabolism, DNA repair, or proliferative/apoptotic responses to DMBA-induced damage. These possibilities are currently under investigation in our laboratory.

Studies involving dietary prevention of tumors often use casein as the "control" protein source and compare all dietary effects to the results of groups fed casein. One possibility not often discussed is that casein-fed rats could be at greater risk of developing DMBA-induced mammary tumors than rats fed diets containing whey or soy protein isolate. If that were true, our data would suggest that factors in casein and soy protein isolate could promote tumor formation compared with whey. This is an area requiring further research.

Several studies have demonstrated that calorie restriction (30–40%) in rats resulted in reduced tumor formation (see Ref. 18 for a review). Rats fed either soy or whey in the present study had a tendency to gain weight at a slightly but significantly lower level than rats fed casein diets. Thus, whether the reduced DMBA-induced tumors in soy-fed or whey-fed rats were related to the lower body weight gain could become an issue. However, rats in calorie restriction studies had severe diet restriction (30%) and a much greater reduction in weight gains (30–60%). The situation in the present study differs substantially from calorie restriction studies on several counts. For example, the rats in the present study were not diet-restricted, and although food intake in the current studies was not monitored, it has been monitored in subsequent and similar studies using the same diets and rats of the same age and has been found not to differ significantly between casein, whey, and soy diets. Thus, because food consumption was equivalent in all three dietary groups, neither reduced caloric intake nor lower protein intake or quality could account for the protective effects of soy or whey diets as compared with casein diets. Previously published studies comparing casein-fed and soy protein-fed rats demonstrated similar slight reductions in body weight gains in rats consuming soy-based diets, although food intake was the same (19–21). The slightly lower body weight gains of soy-fed rats could be associated with the recently reported estrogenic effects on lowering body weight gains demonstrated by addition of estradiol to rat diets (22). Furthermore, soy-fed and whey-fed rats had excellent weight gains that were just slightly lower than those in casein-fed rats. Thus, it is not clear why rats gain slightly less weight on diets made with the same formula and components except for the source of protein. However, it is clear that: (*a*) rats in the present study were not calorie-restricted; (*b*) the weight gains in each group were excellent; and (*c*) it is highly unlikely that reduced tumor development was related to this very slight reduction in body weight gains. Furthermore, it should be emphasized that there were no dif-

ferences between the weight gains of soy-fed rats and whey-fed rats, but there were significant differences between tumor formation, demonstrating clear dietary effects at the same rate of weight gain. This latter point illustrates a disconnection between prevention of DMBA-induced breast tumors and the small differences in body weight gain in the present study.

It should be noted that chemical induction of breast tumors (*i.e.*, the chemical induction model of carcinogenesis) using agents such as DMBA is the most widely used animal model for studying breast cancer. The relevance of this model to human breast cancer undergoes constant scientific debate, including the perspective that human breast cancer may not be caused by a single bolus of a carcinogen such DMBA and/or that inactivation or detoxification of carcinogens such as DMBA may have little application to humans. On the other hand, DMBA is a polycyclic aromatic hydrocarbon. Polycyclic aromatic hydrocarbons generated by cooking and in cigarette smoke are considered to be major human dietary and environmental carcinogens. Increased Phase I and II metabolism (detoxification) of such compounds (exogenous and endogenous) is also considered to result in chemoprotection and reduced cancer risk (23, 24). Thus, results presented herein could have significant relevance to prevention of human breast cancer. Further research into the mechanisms discussed above is required to answer these important questions.

DMBA-induced mammary tumors were studied in female rats fed diets formulated with single protein sources. Rats fed diets with the same soy protein isolate used in the majority of soy-based infant formulas had lower mammary tumor incidence than rats fed diets made with casein. Diets formulated with whey protein provided significantly more protection against DMBA-induced mammary tumors than casein- or soy-based diets. These results suggest diets containing these commonly consumed protein sources have great potential in reducing the risks of breast cancer. Our data further suggest that whey protein may be one of the most potent dietary sources of cancer prevention identified to date.

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