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Dietary Seaweed (*Laminaria*) and Mammary Carcinogenesis in Rats[1]

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ABSTRACT

To test the potential *in vivo* antitumor effect of dietary seaweed, we induced mammary tumors in female Sprague-Dawley rats with the carcinogen 7,12-dimethylbenz(*a*)anthracene. Twenty-one-day-old rats ($n = 108$) were divided into two groups. Controls were fed a standard semipurified diet, and experimental rats received the control diet with 5% *Laminaria*, a brown seaweed, replacing 5% alphacel. At 55 days of age, each rat received 5 mg 7,12-dimethylbenz(*a*)anthracene intragastrically. Rats were palpated for mammary tumors and weighed weekly for 26 weeks. Complete autopsies were then done on all rats. The seaweed diet did not alter weight gain or weights of body organs at autopsy. Experimental rats had a significant delay in the time to tumor ($p = 0.007$); median time until tumor was 19 weeks in experimental rats and 11 weeks in control animals. Among mammary adenocarcinoma tumor-bearing animals, experimental rats had fewer adenocarcinomas/individual ($p < 0.05$). There was also an overall 13% reduction in the number of experimental rats with histologically confirmed adenocarcinomas (76% among the control rats compared to 63% among the experimental rats). Components of *Laminaria* which might account for the observed difference in mammary tumor growth are varied and include the sulfated polysaccharide fucoidan. Rats in the top row of cages had a significant ($p = 0.01$) delay in time to tumor compared to rats in the lower four rows. In each row, the seaweed-fed rats had a longer time to tumor than did the control rats.

INTRODUCTION

Cross-cultural comparisons of cancer death rates show several fascinating anomalies. Breast cancer is associated with one of the most extreme variations in rates. For example, breast cancer incidence is 3 times lower in premenopausal Japanese women than in American women (14). Diet is thought to be an important variable (14). Although there are many differences between the diet of most Japanese women and most American women, a preference for brown seaweed (kelp) seems to be confined to the Asian population. Several studies have shown that seaweed extracts can prevent the growth of tumors transplanted to laboratory animals. We were interested in whether the regular intake of dietary seaweed could be prophylactic for carcinogen-induced mammary tumors in rats.

Previous studies *in vitro* and *in vivo* have shown that hot water extracts of seaweed administered i.p. inhibit the growth of certain transplantable cancers (18, 24, 27, 28). For example, tumor transplantation studies by Yamamoto and coworkers (18, 27, 28) have used aqueous seaweed fractions as chemotherapeutic agents to treat Sarcoma 180, Meth-A, B-16 melanoma, and L1210 leukemia in mice. For some of these experiments, the seaweed was dried, milled, boiled for several hr, lyophilized, and reconstituted with distilled water. Daily i.p. injections with seaweed extract started 24 hr after tumor cell inoculation resulted in tumor growth inhibition and increased life span of the mice. In all these studies, a sulfated polysaccharide component of the extract was thought to contain the active ingredient. *Laminaria angustata*, a brown kelp, was the most active of the edible seaweeds tested.

Our experiment was designed to test the potential *in vivo* antitumor effect of dietary *L. angustata* in an animal model which closely approximated human breast cancer, Sprague-Dawley rats treated with DMBA.[3] Like human breast cancers, the DMBA-induced mammary tumors in rats appear to arise in the ductal system of the mammary gland (15). The tumors are hormone dependent and provide an appropriate model for estrogen receptor-positive breast cancer in humans (2). Sprague-Dawley rats are suitable for testing the effects of DMBA because spontaneous mammary tumors are rare in rats less than 1 year old (4, 17). Although the incidence of tumors after any given dose of DMBA depends on the fat content of the diet, we estimated from other studies in the literature that a diet of approximately 10% fat would result in 60 to 80% of female rats developing tumors by 180 days after receiving 5 mg DMBA i.g. In our study, we compared the development of DMBA-induced mammary tumors of rats fed either a seaweed-supplemented diet or an Alphacel (nonnutritive cellulose fiber)-supplemented diet. Thus, this study differed from the seaweed experiments of others because whole seaweed, rather than extracts, was used in a carcinogen-induced, rather than a tumor transplant, animal model.

MATERIALS AND METHODS

One hundred sixteen weanling (21-day-old) female Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Wilmington, MA). To control for any possible genetic factors, 4 littermates from 29 different litters were used. The sets of littermates were weighed on arrival and, balancing for weight, 2 from each litter were assigned to either control or seaweed group. The rats were housed individually and were assigned to a rack, column, and row, using an incomplete Latin square design. Four racks, each having 5 rows and 6 columns with randomly spaced empty cells, were utilized. With this arrangement, we hoped to be able to control for environmental differences that have been associated with cage assignment (13).

Sun-dried *L. angustata*, obtained from Japan (Mitoku Co., Tokyo, Japan), was milled and then incorporated into a normal rat diet, replacing alphacel, the nonnutritive cellulose fiber (Solka-Floc; Brown and Co., Berlin, NH). The base diet consisted of 20% casein, 26.3% dextrin,

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[3] The abbreviations used are: DMBA, 7,12-dimethylbenz(a)anthracene; i.g. intragastric(ally).

32.7% sucrose, 4% corn oil, 4% lard, 4% AIN-76 mineral mix, 1% AIN-76 vitamin mix, 0.65% choline bitartrate, 0.3% DL-methionine, 0.05% inositol, and either 5% seaweed or 5% nonnutritive alphacel. The test animals were fed the *Laminaria*-enriched diet, and the control group animals were fed the normal alphacel diet. The rats began their respective diets at the time of their arrival and were fed approximately 15 g of the food per day for the entire period of the study. The nutritional analysis of the semipurified diet is presented in Table 1. With 8% fat, this diet basically replicated the percentage of daily calories derived from fat in Japan (10).

The pellets were made at Bioserve, Inc. (Frenchtown, NJ). Care was taken to avoid the use of heat and water, since other studies of vitamin composition of seaweed meal have found a deterioration of both vitamin E and β-carotene when the seaweed was exposed to heat and moisture (8). Agar, a normal binding agent, was not used because it is derived from a red seaweed. Instead, the food was mixed and pelleted under pressure. Four rats from each dietary group wre sacrificed when they reached 50 days of age to look for any possible gross abnormalities associated with seaweed consumption. None was found. At 55 days of age, the remaining 108 rats were given DMBA. To ensure the absence of food in the stomach at the time of DMBA, the rats were fasted overnight. A single gavager, blind to dietary assignment, administered 5 mg DMBA (Sigma) dissolved in 0.25 ml corn oil.

For the next 26 weeks (182 days post-DMBA), the rats were weighed and palpated weekly. All palpations were done by the same person who was blind to dietary assignment. A tumor was defined as a discrete palpable mass that was recorded for at least 2 consecutive weeks. Sites and approximate size of mammary tumors were recorded. If any tumor became large and ulcerated, or the animal became critically ill, the rat was sacrificed early. All the surviving rats were killed 181 to 188 days after DMBA.

At autopsy, all mammary tumors were removed and fixed in 10% phosphate-buffered formalin. The location and size of each tumor were recorded. Tumor tissue was stained with hematoxylin and eosin for histological examination. Other tissues were carefully examined grossly for metastases or other lesions. Body organs (heart, liver, kidneys, spleen, and brain) were weighed to allow comparisons between the 2 groups of animals.

The Wilcoxon test (25) was used to assess the difference in time to first palpable tumor and to compare number of histologically confirmed tumors, and weight gain in the 2 groups. Fisher's exact test (5) evaluated the differences in incidence; the Cox proportional hazards model (3) assessed the relative effect of diet, initial weight, rack, column, and row of cage placement. Plots of time to first tumor were obtained using the modified Kaplan-Meier method (11). All tests were 2 sided.

RESULTS

The mean body weights of control and seaweed-fed rats are shown in Chart 1. Rats were included for this analysis only until they developed a tumor. There was no significant difference in weight gain between the 2 groups at any week.

Plots of time to first palpable tumor are presented in Chart 2.

Table 1
Nutritional analysis of diets

Nutritional component	% of component	
	Seaweed	Control
Moisture	7.67	7.12
Protein	17.70	17.80
Fat	8.43	8.58
Fiber	2.86	3.40
Ash	4.05	3.99
Carbohydrate	59.29	59.11
Total	100.00	100.00

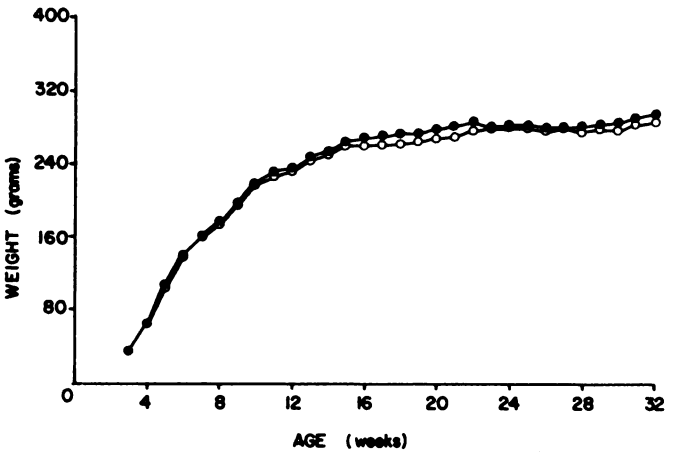

Chart 1. Mean body weights of non-tumor-bearing rats compared by dietary groups. Control rats (O) and seaweed-fed rats (●).

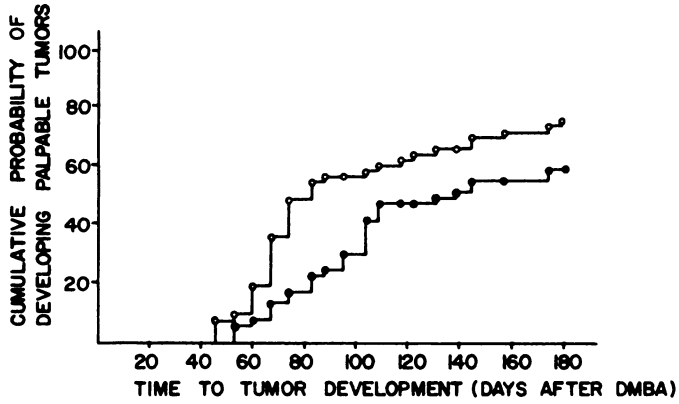

Chart 2. Cumulative probability of developing palpable mammary tumors in rats fed control diet (O) and seaweed diet (●).

There was a significant delay in the time to first palpable tumor in the seaweed-fed rats ($p = 0.007$). The median time from DMBA until first palpable tumor was 11.0 weeks (77 days) for the control rats and 19.8 weeks (139 days) for the seaweed-fed rats.

During the course of the study, 13 rats (8 control and 5 experimental) were sacrificed between 74 and 170 days post-DMBA. Ten of these rats had developed large (approximately 4 cm in diameter) mammary tumors. In addition, 2 rats developed malignant lymphomas, and one other rat developed a large necrotic ear gland tumor (Zymbal's gland carcinoma). There were no intercurrent deaths.

At the time of autopsy, 12 tumor-free rats (6 control and 6 experimental) were found to have small nonpalpable mammary masses; 11 of these were found histologically to be adenocarcinomas and one, an adenoma. Ninety-three % of all the tumors found in the mammary gland region at autopsy were adenocarcinomas. Included in this adenocarcinoma group were 5 tumors which were predominantly fibroadenoma but which had focal proliferations of malignant epithelial cells. The other tumors consisted of 7 fibroadenomas, 5 adenomas, 3 epidermal inclusion cysts, and one adenocarcinoma of sebaceous glands.

Although transient swelling of the salivary glands characteristic of sialodacryoadenitis viral infection had been noted in 6 rats (3 controls and 3 experimental), no abnormalities were found at autopsy. These palpable enlargements (approximately 1 cm) had

appeared between the 13th and 15th weeks post-DMBA and lasted 1 to 2 weeks. Unilateral Zymbal's gland carcinomas were found in 6 rats (3 control and 3 experimental). Marked splenomegaly as a result of extramedullary hematopoiesis was found in 2 rats (one control and one experimental) with large, necrotic mammary tumors. No other significant gross abnormalities were found in any of the rats. There were no differences in average organ weights between the 2 dietary groups.

Seventeen animals had one to 3 tumors which appeared in the course of the experiment but regressed before necropsy (5 experimental and 12 control). Only 3 of these 17 animals had no other tumors still palpable at necropsy (2 experimental and one control). On 4 other animals (2 experimental and 2 control), the time to first tumor would change between 1 and 6 weeks if the regressed tumors were ignored. If these spontaneously regressing tumors were omitted from the time to tumor analysis, the median time to tumor would have been 83 days for the control rats and 145 days for the experimental rats ($p = 0.02$). Thus, both including and excluding the data on rats with spontaneously regressing tumors yielded the same result, that the seaweed-fed rats experienced a significant delay in the time to the appearance of palpable tumors.

There was also no difference ($p = 0.27$) in the relative percentage of spontaneously regressing tumors compared to the total number of palpable tumors in each group (11% for the controls and 6% for the seaweed-fed rats). When the data were compared by number of tumor regressions per rat with palpable tumors, the difference was also nonsignificant ($p = 0.17$).

Tumor data were based on the autopsy findings. There was a nonsignificant difference ($p = 0.21$) between the 2 dietary groups in the final incidence of adenocarcinomas. Forty-one of 54 control rats (76%) were found to have one or more adenocarcinomas at autopsy, compared to 34 of 54 seaweed-fed rats (63%). If the 12 rats with nonpalpable tumors were omitted from the analysis, the difference betweeen the 2 dietary groups was again nonsignificant. With a total of 108 rats in the study, there was an 80% chance of detecting a significant ($p < 0.05$) difference in the number of rats with adenocarcinomas if the true underlying percentages associated with the 2 diets were as different as 50 and 76%.

The number of adenocarcinomas per adenocarcinoma-bearing rat refers to the number of grossly distinct tumors found at autopsy and then confirmed to be adenocarcinomas. There was a significantly lower number of adenocarcinomas per adenocarcinoma-bearing rat for the seaweed-fed group (2.2 compared to 3.3 among the controls; $p = 0.04$). This relationship was observed regardless of whether we included or excluded the rats sacrificed prior to the end of the study.

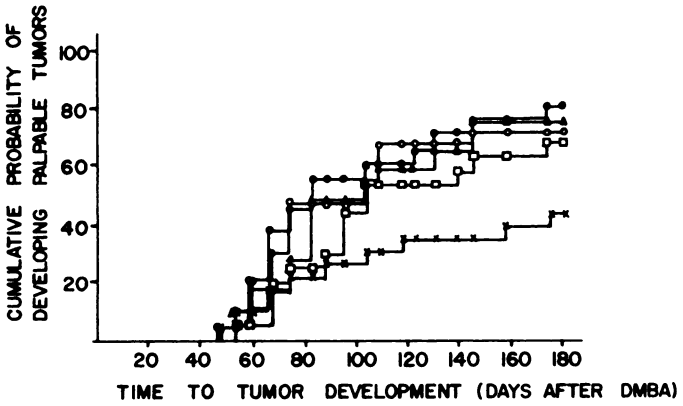

Chart 3. Differences in the cumulative probability of palpable mammary tumors associated with cage location by row, for both dietary groups combined. Rats housed in Row 1, top (×); Row 2 (○); Row 3 (●); Row 4 (△); and Row 5, bottom (□).

The variables [initial weight (3 groups: <30 g; 30 to 40 g; >40 g), row, column, rack, and diet] were tried in proportional hazards models. Only Row 1 and a seaweed diet were significantly associated with time to palpable tumor. In each row, the seaweed-fed rats had a longer time to tumor than did the control rats. Chart 3 shows the percentage of rats that developed palpable tumors compared over time by row. Top-row rats had a significant delay in time until tumor ($p = 0.01$). Rack and column location and initial weight were not associated with any significant differences in tumor incidence.

DISCUSSION

Similar weight gain of rats in both groups and similar organ weights at autopsy suggest that seaweed supplementation did not disturb body growth. This conclusion is based on the observations that seaweed did not alter the growth of the seaweed-fed group of rats between 21 and 55 days of age and that, subsequent to the carcinogen ingestion, weight gain did not differ between the 2 groups. In addition, no clinical abnormalities during the course of the experiment and no lesions, other than those induced by DMBA, were detected in any of the rats. It is therefore probable that the addition of 5% seaweed was not harmful to these rats. In other contexts, brown seaweeds have been used safely as mineral and vitamin supplements for poultry and dairy cows (9, 26). The use of seaweed as a normal food in Japan indicates that it is well tolerated by humans. The estimated national average per capita daily intake of seaweed in Japan ranges from 4.9 g (10) to 7.3 g (23). This is probably an underestimate, as actual seaweed intake is hard to measure. For example, seaweed is used as a condiment with most meals, as a soup base, and is added to flour, salads, and entrees for extra flavor. *L. angustata*, the seaweed used in this experiment, is most commonly used in the preparation of miso soup.

The magnitude of protection (ratio of hazards = ratio of medians = 1.67 in our study) afforded by the seaweed diet is within the range of other tumor inhibitors: *Bacillus Calmette-Guérin*, 1.58 to 2.0 (12); selenium, 1.12 to 1.71 (7); L-arginine, 1.40 to 1.56 (19); and retinyl acetate, 1.35 to 3.00 (6). Since some of these other studies used very high doses of additive, whereas our study tried to approximate normal dietary intake, brown seaweed appears to be a good candidate for further investigation.

Cage placement by row significantly affected tumor incidence, but column and rack placement did not. Rats fed seaweed took longer to develop tumors and got fewer tumors than rats not fed seaweed, both within Row 1 and within Rows 2 to 5. The row effect is similar to that reported by Lagakos and Mosteller (13), who found that the incidence of chemically induced tumor deaths was different in the top row. There were no differences among the tumor incidences seen in the other 4 rows. Cage assignment of column and rack were not associated with large differences in tumor incidence. The row effect does not lend itself easily to biological explanation, and we report it here as an important variable to consider in cage assignment.

One explanation for the observed delay in tumor onset among the seaweed-fed rats might be that the seaweed in some way

altered the susceptibility of the rats by directly affecting mammary gland development. Russo and Russo (15, 16) have decribed the importance of the relative proportion of proliferating mammary cells in determining susceptibility to carcinogenic exposures. Although this possibility cannot be overlooked, it seems unlikely. The seaweed-fed rats, rather than remaining tumor free as the older virgin and parous rats do when challenged with DMBA, developed a similar number of adenocarcinomas by the end of the experiment. This argues against seaweed affecting the development of the mammary glands or inhibiting the initial metabolism of the DMBA. It seems more likely that seaweed modulated the development of the DMBA tumors, affecting the promotion of tumor growth.

Reasons for the possible effects of dietary seaweed on tumor growth have been suggested elsewhere (20, 21) and include seaweed as a source of nondigestible fiber, minerals, vitamins, antibacterial activity against fecal flora, stimulation of the immune response, and cytotoxic activity of its storage carbohydrate laminarian, which is a highly branched 1,3-β-glucan.

Perhaps another important mechanism of action is the effect of the cell wall polysaccharide sulfate ester, fucoidan, and its component fucose (1). The antitumor activity reported for brown seaweed by Yamamoto and others is based on an extraction method utilized in the manufacture of fucoidan and α-L-fucose. Fucoidan, unique to brown seaweed, may affect tumor growth. Usui *et al.* (24) have specifically tried fucoidan in a small pilot study of 5 animals with Sarcoma 180. A dose of 50 mg/kg/day produced 30% tumor inhibition and complete regression in 2 of 5 animals followed for 45 days.

Seaweed has shown consistent antitumor activity in several *in vivo* animal tests. In extrapolating these results to the Japanese population, seaweed may be an important factor in explaining the low rates of certain cancers in Japan. Breast cancer shows a 3-fold-lower rate among premenopausal Japanese women and a 9-fold-lower rate among postmenopausal women in Japan than reported for women in the United States (14). Epidemiological data are compatible with a role for brown seaweed in breast cancer prevention. Since low levels of exposure to some toxic substances have been shown to be carcinogenic, then it may be that low levels of daily intake of food with antitumor properties may reduce cancer incidence.

The results of this experiment suggest that a diet containing 5% brown seaweed was effective in delaying the time to DMBA-induced tumor development in rats. The mechanism for this activity remains to be elucidated.

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