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Wakame Seaweed Suppresses the Proliferation of 7,12-Dimethylbenz(*a*)-anthracene-induced Mammary Tumors in Rats

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We examined the anti-tumor proliferation effects of wakame seaweed on 7,12-dimethylbenz(*a*)-anthracene (DMBA)-induced rat mammary tumor. DMBA was administered to 8-week-old female Sprague-Dawley rats, and rats which developed mammary tumors were assigned randomly to three groups. Commercial rat feed was used in a control group (group I-A), and two feed mixtures were prepared, which contained commercial rat feed blended with wakame at 1.0% (group I-B) and 5.0% (group I-C) by weight. The respective feeds were given to each group for 8 weeks, and changes in mammary tumor size were compared. At the end of the experiment, mammary tumors and thyroid glands were resected to compare their weights. Serum total iodine and thyroxin (T4) levels were measured. Immunohistochemical studies for bromodeoxyuridine (BrdU) labeling, transforming growth factor (TGF)-β, and apoptosis were carried out in the resected tumor. Significant suppression of tumor growth was observed in groups I-B and I-C compared with I-A. In groups I-B and I-C, the weights of resected mammary tumors were significantly lower and serum total iodine concentration was significantly higher than in I-A. BrdU indices were significantly lower in groups I-B and I-C, compared with I-A. TGF-β and apoptotic index were inversely related to BrdU. These results suggest that iodine is transported from the serum into mammary tissues and induces apoptosis through the expression of TGF-β. In conclusion, wakame suppressed the proliferation of DMBA-induced mammary tumors.

Key words: DMBA-induced rat mammary tumor — Wakame — Immunohistochemistry — TGF-β — Apoptosis

Since Huggins *et al.* reported the 7,12-dimethylbenz-(*a*)anthracene (DMBA)-induced rat mammary cancer model,[1] it has been used by numerous researchers.[2] We have previously reported suppression of mammary tumor growth by inorganic iodine in this model.[3] Some reports have suggested that intake of certain seaweeds may delay the onset of cancers.[4] In the present study, therefore, we evaluated the effects of wakame, the most popular edible seaweed in Japan, on DMBA-induced rat mammary tumors. Suppression of tumor growth and lowering of the bromodeoxyuridine (BrdU) labeling index, a molecular marker of tumor proliferation, by wakame feed were studied. The effect of wakame on extent of apoptosis, which influences the suppression of breast tumor proliferation[5] and transforming growth factor (TGF)-β, a negative growth factor in breast tumor cells,[6] was also studied.

MATERIALS AND METHODS

Animals and experimental protocol Eight-week-old female Sprague-Dawley (SD) rats (Japan SLC, Inc., Shizuoka) weighing 180 to 200 g were used. A single dose of 20 mg/body of DMBA (Wako Junyaku Kogyo, Tokyo) dissolved in sesame oil was administered by gastric intubation to 150 such rats. All rats were fed with commercial rat feed, CE-2 (CLEA Japan, Inc., Tokyo). After 10 weeks, 33 rats whose mammary tumors measured about 1 cm in greatest dimension were selected and were divided into 3 groups randomly. One group was designated the control group (I-A) and received only CE-2 feed. The other 2 groups (I-B and I-C) were given CE-2 feed in which wakame seaweed (Shimakaze, Kyowa Hakko, Tokyo) accounted for 1.0% and 5.0%, respectively, of diet weight. CE-2 contained only a little iodine and 100 g of the wakame used in this experiment contained 5717 μg of iodine. Each of the 3 groups was continued on their respective feed for 8 weeks. Changes in the weight of rats and tumor size measured with a caliper in cm^2 (product of greatest perpendicular diameters) were recorded weekly and expressed as a percentage of the initial value.

The animals were killed at the end of the 8 weeks (none had died), and all mammary tumors that were histologically confirmed mammary cancers, thyroid glands and

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other endocrine-related organs (ovaries and adrenal glands) were removed and weighed. Venous blood samples were obtained from each rat at this time for measurement of serum total iodine concentration and serum thyroxin (T4) levels, which might have some direct or indirect effect on tumor growth. Total serum iodine was measured with a Technicon Autoanalyzer, and serum T4 levels by radioimmunoassay. BrdU and TGF-β labeling indices and the extent of apoptosis were determined in the resected mammary tumor tissues.

Histology of removed mammary tumors stained with hematoxylin-eosin (HE) All mammary tumors were histologically stained with HE and findings were compared among the three groups.

Immunohistochemistry and determination of labeling index for BrdU The mammary tumors were removed 30 min after subcutaneous injection of 150 μg/body of BrdUrd (Sigma Inc., St. Louis, MO). A monoclonal mouse anti-BrdU antibody (DAKO Corp., Kyoto) diluted 20-fold was used as the primary antibody. Specimens were stained by the streptavidin-biotin-peroxidase complex method using a Histofine SAB-PO Kit (Nichirei Corp., Tokyo). One thousand cells were counted in a visual field, and the average of four fields was used to determine the labeling index (LI).

Immunohistochemistry and determination of LI for TGF-β For the primary antibody, 2.5 μg/ml of a rabbit anti-TGF-β1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used. Specimens were stained by the streptavidin-biotin-peroxidase complex method as above. Negative controls were run by replacing the primary antibody with phosphate-buffered saline (PBS). One thousand cells were counted in a visual field at random, and the average of four fields was used to determine LI.

TUNEL (TdT-mediated dUTP-biotin nick-end labeling) method and assessment of apoptotic cells labeled by TUNEL The extent of apoptosis was defined by TUNEL assay using an *in-situ* cell death detection kit (Boehringer Mannheim, Mannheim, Germany) as described by Kesari *et al*.[7] One thousand cells were counted in a visual field at random, and the average of four fields was used to determine the apoptotic index (AI).

Subchronic toxicity test in a completely independent experiment Fifteen normal 4-week-old female SD rats were prepared and assigned to three groups as above which were given the same feed as the I-A, I-B and I-C groups, respectively. They were raised without further intervention for 36 weeks, and weight changes were determined weekly. At the end of the 36-week experiment, weights of thyroid gland and venous serum T4 levels were compared among the three groups.

Environment for animals Throughout the experimental period the animals were housed in stainless steel cages in a controlled environment at a temperature of 22.5°C and 50 to 55% humidity. The animals were exposed to fluorescent light from 9 a.m. to 9 p.m. under 24-h central control and their cages were equipped with automated tap water supply and automated water-flush cleaning.

Data analysis The significance of differences was assessed using JMP statistical software for Macintosh (SAS Institute Inc., Cary, NC). ANOVA and Tukey-Kramer HDA (honestly significant difference) were used to compare all pairs. A P value of less than 0.05 was taken as indicative of a significant difference.

RESULTS

Changes in rat body weight No statistically significant differences in the rats' body weights were observed among the three groups over the 8-week period (Fig. 1).

Changes of tumor size In group I-A the tumor size increased by more than 450% during the 8 weeks. In contrast, the tumor growth rate was suppressed significantly ($P<0.01$) at each week in groups I-B and I-C. In group I-C tumor size showed almost no change through the 8-week period. A close relationship existed between the extent of suppression and the concentration of wakame in the feed (Fig. 2).

Weight of mammary tumors, thyroid glands and other endocrine-related organs The combined weight of all mammary tumors of each rat in group I-A was approximately 20 g at the end of the experiment. The total weight was significantly lower ($P<0.01$) in groups I-B and I-C. Weights of thyroid glands did not differ significantly among the three groups (Fig. 3), and this was also the case for ovaries and adrenal glands (data not shown).

Serum iodine and T4 levels Serum iodine concentration was significantly higher ($P<0.01$) in groups I-B and I-C compared to group I-A. The serum iodine concentration had a positive relationship with the concentration of

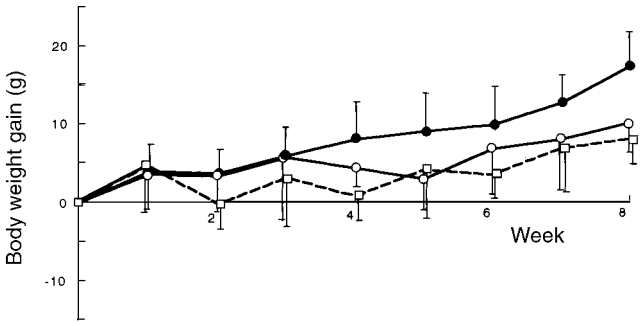

Fig. 1. Weekly changes in rat body weight (mean±SD). □, I-A: received CE-2 feed; ●, I-B: received CE-2+1% wakame; ○, I-C: received CE-2+5% wakame. n=11 in each group. No group significantly differed from the control group.

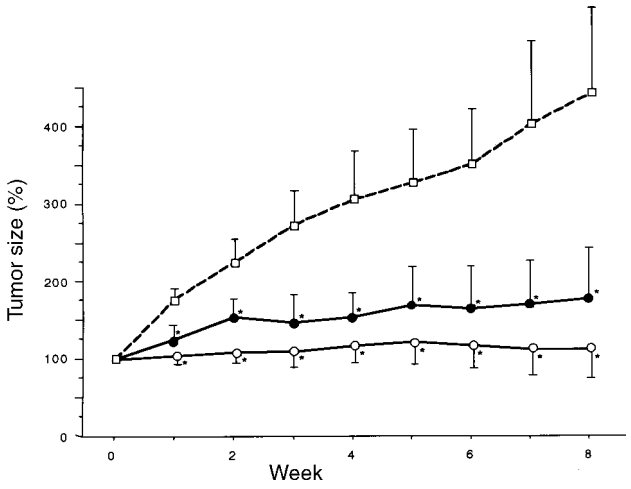

Fig. 2. Weekly changes in tumor size expressed as a percentage of the size measured on day 0 (mean±SD). □, I-A; ●, I-B; ○, I-C. n=11 in each group. Groups I-B and I-C were significantly different from the control group (P<0.05) throughout the 8-week period.

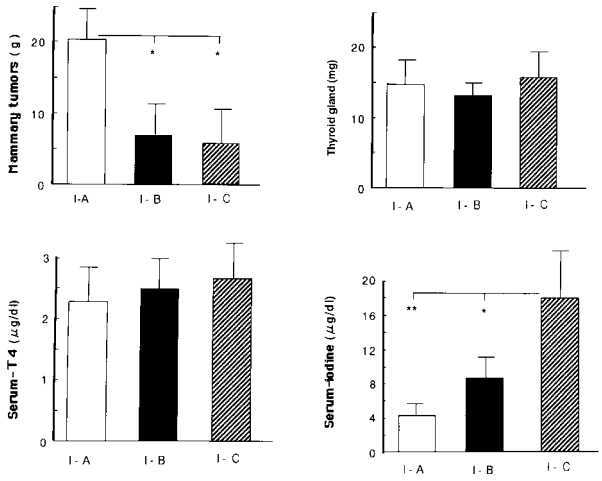

Fig. 3. Combined weight of all mammary tumors, thyroid gland weight, and serum concentrations of iodine and T4 were compared at the end of 8 weeks (mean±SD, n=11) in each group. The weights of mammary tumors and serum iodine concentration in groups I-B and I-C were significantly different (P<0.05) from those in I-A.

wakame in the diet. Serum T4 levels showed no significant differences among the three groups (Fig. 3).

Histological findings of mammary tumors stained with HE Mammary tumors were cystic adenocarcinomas, and tumors of wakame groups revealed a decreased density of epithelial cells, increased lymphatic invasions and fibrosis (Fig. 4).

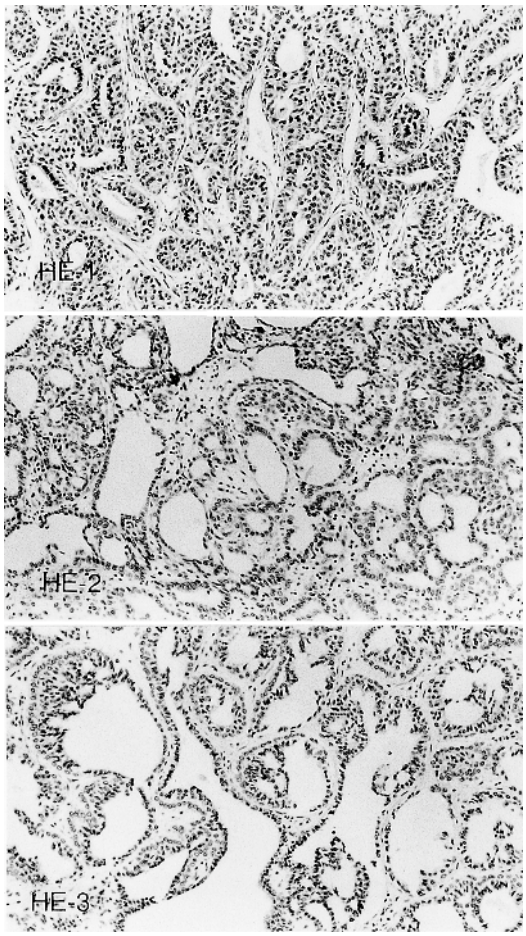

Fig. 4. Histopathological findings in breast mammary tumors stained with HE. ×50. Wakame groups revealed a decreased density of epithelial cells, increased lymphatic invasion and fibrosis. HE-1, I-A group; HE-2, I-B group; HE-3, I-C group.

Immunohistochemistry for BrdU and TGF-β and TUNEL assay In tumors resected at 8 weeks, more cells stained positively for BrdU in group I-A than in groups I-B and I-C. TGF-β-positive cells and apoptotic cells were clearly more numerous in groups I-B and I-C than in group I-A (Fig. 5).

LI of BrdU and TGF-β and AI In groups I-B and I-C, LI of BrdU was significantly lower, while LI of TGF-β was significantly higher in a dose-dependent manner, compared with group I-A (P<0.001). AI was significantly higher in groups I-B and I-C, compared with group I-A (P<0.001) and was dose-dependent. The LI of TGF-β showed an inverse relationship with LI of BrdU and a positive relationship with AI (Fig. 6).

Subchronic toxicity test In the 15 rats in the toxicity test group, no significantly different changes in rat body weight as compared with groups I-A, I-B and I-C were

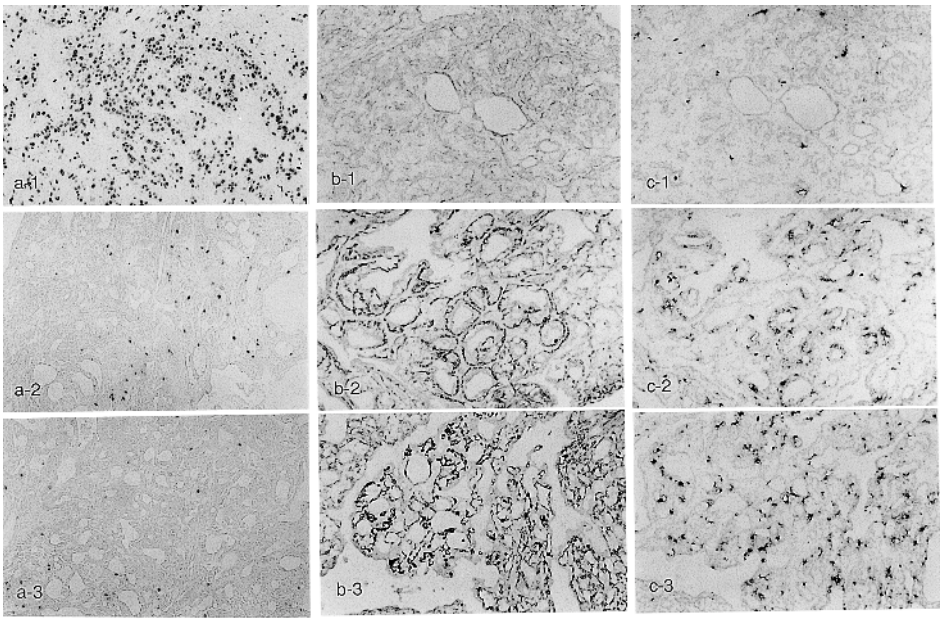

Fig. 5. Profiles of immunohistochemistry for BrdU and TGF-β and apoptotic features in the 3 groups. a, BrdU; b, TGF-β; c, apoptosis. 1, I-A group; 2, I-B group; 3, I-C group. ×50. n=11 in each group. More positive-staining cells were found in a-1 and b-3 compared with a-2, a-3, and b-1, b-2. TUNEL-reactive cells were more frequently seen in c-3 compared with c-1 and c-2.

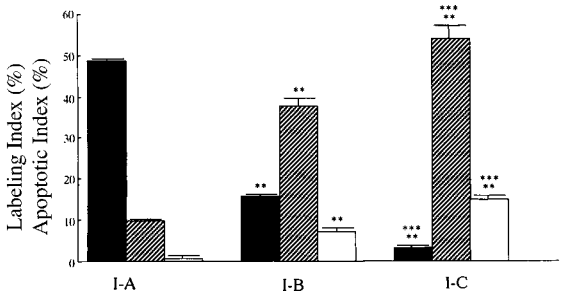

Fig. 6. Labeling indices of BrdU (■) and TGF-β (▨) and apoptotic indices (□) (n=11 in each group) (mean±SD). Groups I-B and I-C were significantly different from group I-A in each factor (** P<0.05 vs. I-A, *** P<0.001 vs. I-B group).

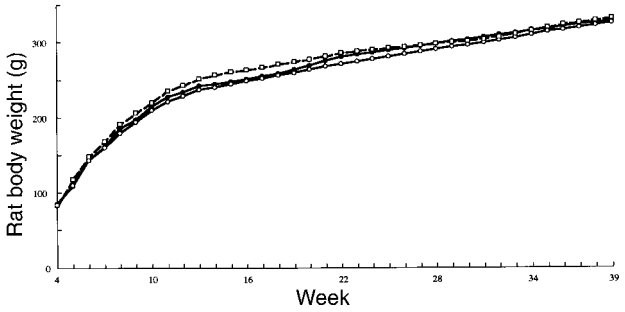

Fig. 7. Subchronic toxicity test (n=5 in each group). Body weight did not differ among the three groups through the 36 weeks.

seen through the 36-week period (Fig. 7). Weights of the thyroid gland and T4 levels at the end of the 36th experimental week showed no significant differences among three groups (data not shown).

DISCUSSION

Though several workers have examined the relationship between thyroid disease and breast cancer,[8–10] little work has been done on the relationship between iodine and breast cancer.[11] We have previously demonstrated that growth of rat mammary tumors induced by DMBA was suppressed by administration of inorganic iodine in the form of Lugol solution.[3] Our earlier studies suggest a direct suppressive effect of iodine on mammary tumor cells' growth *in vivo*[12] and *in vitro*.[13]

Epidemiological research has shown that the incidence of breast cancer and its severity are less in Japan than in Europe and the United States.[14] Some reports have suggested that certain seaweeds may suppress the onset of

various cancers.[4] Low incidence of breast cancer in Japanese women has been attributed to the seaweeds in the Japanese diet.[15–17]

In the present study we examined whether wakame, the most popular variety of seaweed in the Japanese diet (in miso soup or wakame salad), which contains organic iodine can suppress rat mammary tumor proliferation like inorganic iodine, as demonstrated earlier. Pulverized wakame at various concentrations was administered to rats with DMBA-induced mammary tumors, mixed with the commercial rat feed CE-2.

Compared to the control group animals, rats fed with wakame-containing diets had significantly lower tumor growth. In group I-C, in which rats were given 5% wakame in the diet, almost no tumor growth occurred (Fig. 2). Most rats had more than one DMBA-induced mammary tumor, and so the combined weight of all tumors resected from a rat was used as another indicator of tumor growth. Total tumor weight in the rats receiving wakame-containing diet was significantly lower as compared to rats receiving commercial diet alone, at the end of 8 weeks. However, the weight of thyroid glands was not affected (Fig. 3). Though DMBA-induced mammary tumor is estrogen-dependent, the weights of the ovaries did not differ among the three groups.

BrdU is a widely accepted marker of cell proliferation. Comparison of LI values of BrdU in the resected mammary tumors in the three groups confirmed that the suppression of tumor growth was actually due to suppression of tumor cell proliferation, with rats fed with wakame showing significantly lower LI than the control group (Fig. 5). In order to study the mechanism of suppression of tumor cell proliferation by wakame, we examined the AI in the resected tumors using the TUNEL assay.[5, 18] An inverse relationship between the LI of BrdU and AI was seen. TGF-β, which is thought to be one of the factors inducing apoptosis[6] was investigated by immunohistochemistry and was found to show a positive relationship with AI (Fig. 6). Knabbe *et al*. reported that, *in vitro*, TGF-β was a hormonally regulated growth inhibitor with possible autocrine and paracrine functions in breast cancer cells.[6] Kesari *et al*. found that TGF-β acted as a paracrine growth factor inhibiting endothelial proliferation and thereby downregulating angiogenesis, and noted an inverse association between apoptosis and angiogenesis.[7] These findings may suggest that some components of wakame cause apoptosis by inducing expression of the negative growth factor TGF-β, thus inhibiting tumor cell proliferation. Serum iodine concentrations were higher in rats fed with a higher concentration of wakame. In our previous experiments, inorganic iodine applied directly to breast cancer cells *in vitro* suppressed their proliferation.[13] *In vivo*, the iodine content of breast tumors whose growth was strongly suppressed by the administration of inorganic iodine was significantly higher than that of tumors whose proliferation was not suppressed, suggesting a direct effect of iodine on mammary tumor proliferation. Thus, we may infer that higher serum iodine levels due to wakame administration may have resulted in higher iodine concentration in the mammary tumor cells, thereby inducing apoptosis via induction of the expression of TGF-β.

However, wakame contains not only iodine, but also other components such as carbohydrates, vitamins, carotin, etc. Welsh reported that carotin had a chemopreventive effect on rat mammary cancer,[2] and fucoidan also shows activity.[19] Our future research should include a detailed analysis of wakame components to characterize their mechanism of action.

Wakame has been a part of the Japanese diet for a long time, and has no known toxicity or other ill-effects. However, when used in high concentrations, as in our experiment, its safety is not well established. Therefore, we felt it necessary to investigate its toxicity in rats. In the safety assurance component of our study, 5% wakame was found to be nontoxic to rats in terms of changes of body weight, thyroid gland weight and serum T4 level during 36 weeks of administration.

In conclusion, wakame had a strongly suppressive effect on the proliferation of DMBA-induced mammary tumor in rats. These results were presented at the UICC Symposium on Familial Cancer and Prevention (1997, Kobe, Japan).

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