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Chemoprevention of *N*-Nitroso-*N*-methylurea-induced Rat Mammary Carcinogenesis by Soy Foods or Biochanin A

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We examined the effects of soybeans, a soy product (miso) and biochanin A, an isoflavone derivative, on *N*-nitroso-*N*-methylurea (MNU)-induced rat mammary carcinogenesis. Seven-week-old female CD/Crj rats received a single i.v. dose (40 mg/kg body weight) of MNU. After administration of MNU, rats were fed diet containing 0% (control), 2% or 10% soybeans, or 10% miso as a soy-supplemented diet, or 10 or 50 mg/kg biochanin A. All rats were observed for 18 weeks after MNU administration. At 18 weeks, the multiplicity (mean tumors/rat) of palpable mammary tumors was significantly decreased in the 10% soybean (1.1) and 10% miso (1.2) diet groups compared to the control (2.2) ($P<0.05$, respectively). In the biochanin A-supplemented diet groups, the incidence (percentage of rats with tumors) was significantly decreased in the 50 mg/kg (32%) diet group compared to the control (80%) ($P<0.01$), and the multiplicity was significantly decreased in both the 10 mg/kg (0.7) and 50 mg/kg (0.5) diet groups compared to the control (2.2) ($P<0.01$ and $P<0.001$, respectively). The proliferative cell nuclear antigen labeling index of mammary tumors was significantly decreased in both biochanin A-supplemented diet groups compared to the control. The present results indicate that soybeans, miso, and biochanin A are useful for the prevention of mammary cancer.

Key words: Chemoprevention — Mammary cancer — Soybean — Miso — Biochanin A

The incidence of mammary cancer has been increasing in Japan, and this cancer is predicted to become the major cancer among females in Japan in the near future.[1)] An epidemiological study recommended that excess intake of fat, especially animal fat, and calories be avoided.[1)] Therefore, there has been an increasing public demand for information on healthy foods that may help in the primary prevention of cancers. Animal studies have revealed a protective effect of soy foods against radiation- or chemical-induced rat mammary carcinogenesis.[2, 3)] A similar conclusion was reached on the basis of epidemiological studies in Hawaii by Nomura *et al*.[4)] and in Japan by Hirayama[5)]; both studies found that the fermented soybean product, miso, appeared to have an inhibitory effect on mammary cancer in premenopausal women. We have previously shown that miso has a protective effect against radiation injuries,[6, 7)] and also reduces liver and gastric tumorigenesis in experimental animals.[8)] The optimum level of miso in the diet was 10%. Miso contains a variety of biologically active substances, including botanic proteins, vitamins, fats, enzymes, carbohydrates, saponins, isoflavones, phytosterols, and lectins.[9, 10)] Among these, two isoflavones, genistein and daidzein, are known to have various biological activities.[11, 12)]

In the present study, we investigated the effect of soybeans, miso and biochanin A on *N*-nitroso-*N*-methylurea (MNU)-induced rat mammary carcinogenesis, and at the same time, we examined cell proliferation of mammary tumors in rats given specified diets. Biochanin A is an isoflavone derivative and a precursor of genistein.

MATERIALS AND METHODS

Animals Female CD/Crj Sprague Dawley (SD) rats were purchased from Charles River Japan, Inc. (Hino) and used in the present study. Four or five rats were housed together in autoclaved cages with sterilized wood chips and kept in a room under controlled temperature (24±2°C) and humidity (55±10%), with a regular 12 h-light, 12 h-dark cycle. All rats were given food and tap water *ad libitum*. Rats were maintained under the guidelines set forth in the 'Guide for the Care and Use of Laboratory Animals' established by Hiroshima University.

Supplement of specified diets and chemicals Rats were fed a commercial control diet MF (Oriental Yeast Co., Tokyo) supplemented with soybeans, miso or biochanin A. The soybean diets were made by mixing 2% and 10% powdered soybeans provided by Hiroshima Soy Sauce Co. (Hiroshima) with 98% and 90% powdered MF, respectively, and formed into biscuits. The miso diet was similarly prepared by mixing 10% dry red miso provided by Miso Central Institute (Tokyo) with 90% powdered MF,

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and its composition was 7.69% water, 24.3% protein, 6.23% fat, 2.53% salt and a mixture of microorganisms, flavors and aromatic compounds, unsaturated fatty acid-ethylester, glycosides, isoflavones, and saponins. The number of calories was 356 kcal per 100 g. The 10% miso diet corresponds to two or three bowls of Japanese-style miso soup per day. Biochanin A (5,7-dihydroxy-4′-methoxyisoflavone) was obtained from Sigma Chemical Co., St. Louis, MO. Biochanin A was mixed into powdered MF at a dose of 10 mg/kg or 50 mg/kg and the feed was formed into biscuits. The doses of biochanin A were chosen on the basis of previous experiments in mice.[13, 14] MNU was obtained from Sigma Chemical Co., and dissolved in 0.9% NaCl solution.

Experimental procedure Under light ether anesthesia, seven-week-old female SD rats received a single dose (40 mg/kg body weight) of MNU via the right jugular vein, which was directly exposed by the cut-down method. After MNU administration, rats were divided as follows; control diet group, 2% and 10% soybean diet and 10% miso diet groups, and 10 mg/kg or 50 mg/kg biochanin A diet groups. Rats were maintained on control or specified diets until the termination of the experiment. Body weights were measured every 2 weeks. Beginning at 4 weeks after MNU administration, the location and number of palpable mammary tumors were recorded, and the size was measured with calipers under light ether anesthesia every 2 weeks until the termination of the experiment. All rats were observed up to 18 weeks and killed at 19 weeks after MNU administration. All gross palpable mammary lesions were excised, fixed in 10% phosphate-buffered neutral formalin, embedded in paraffin and serially sectioned at 3 μm. Sections were routinely stained with hematoxylin and eosin and examined. Organs were weighed and processed for histological study as described above.

For the determination of proliferating cell nuclear antigen (PCNA) in mammary tumors, 42 sections of mammary tumors that developed were randomly selected in each group, deparaffinized and incubated for one hour at room temperature with monoclonal mouse anti-PCNA (Dako-PCNA, PC10, Dakopatts A/S, Denmark) at a dilution of 1:50. Visualization of stained cells by the three-stage immunoperoxidase technique was carried out using a Histofine Sab-Po(M) Kit obtained from Nichirei Co. (Tokyo). The percentage of cells with PCNA incorporation (labeling index) was determined by counting the labeled nuclei of 1,000 cells in randomly chosen carcinoma foci. All slides were scored in a blinded manner by one person.

Statistical analysis Statistical analysis of the tumor incidences was performed by using the χ^2 test, while body and organ weights, tumor multiplicities, sizes, and PCNA index were compared by using Student's *t* test. The results were considered statistically significant if the *P* value was 0.05 or less. All *P* values reported were derived from two-sided statistical tests.

RESULTS

General observations The growth curves of rats during the observation period are shown in Fig. 1. Although the rats in the biochanin A-supplemented groups showed slightly reduced body weights compared to the control group throughout the observation period, there were no significant intergroup differences. The mean body and relative organ weights at the termination are summarized in Table I. There were no significant intergroup differences in the body, liver, uterus, ovary, or adrenal weights.

Effects of soybeans, miso and biochanin A on the occurrence of mammary tumors The cumulative incidence and multiplicity of palpable mammary tumors are shown

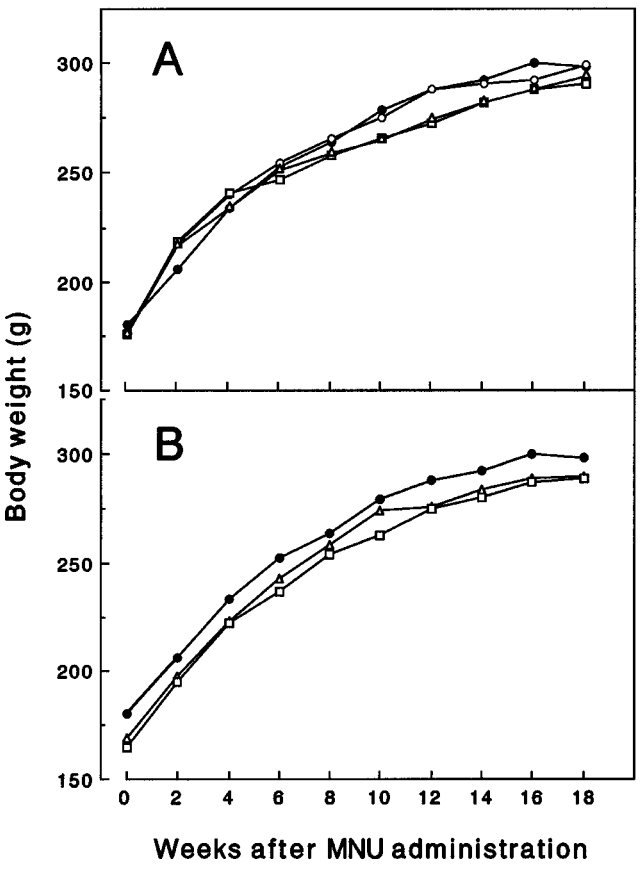

Fig. 1. Sequential changes in body weights after MNU administration. A, Soy-supplemented groups. ● Control diet, ○ 2% soybean diet, □ 10% soybean diet, △ 10% miso diet. B, Biochanin A-supplemented groups. ● Control diet, △ 10 mg/kg diet, □ 50 mg/kg diet. Each point is a mean value.

Table I. Body Weights and Relative Organ Weights[a)] in Each Group

Group	Effective no. of rats	Body wt. (g)	Liver wt. (g/100 g BW)	Uterus wt. (mg/100 g BW)	Ovary wt. (mg/100 g BW)	Adrenal wt. (mg/100 g BW)
Control	20	298±35[b)]	3.0±0.4	205±68	54.6±16.9	20.6±6.4
Soy foods						
2% soybean	18	299±26	2.9±0.2	203±64	51.3±12.0	21.6±2.9
10% soybean	18	292±32	3.0±0.3	204±82	52.6±9.30	18.8±4.1
10% miso	20	294±29	3.1±0.3	181±48	56.0±17.5	20.6±5.9
Biochanin A						
10 mg/kg	19	291±30	3.0±0.7	202±74	45.1±19.3	21.5±7.4
50 mg/kg	19	289±28	2.9±0.3	230±68	47.0±19.5	19.4±2.9

BW: Body weight.
a) Relative organ weights were calculated per 100 g of body weight.
b) Values are mean±SD.

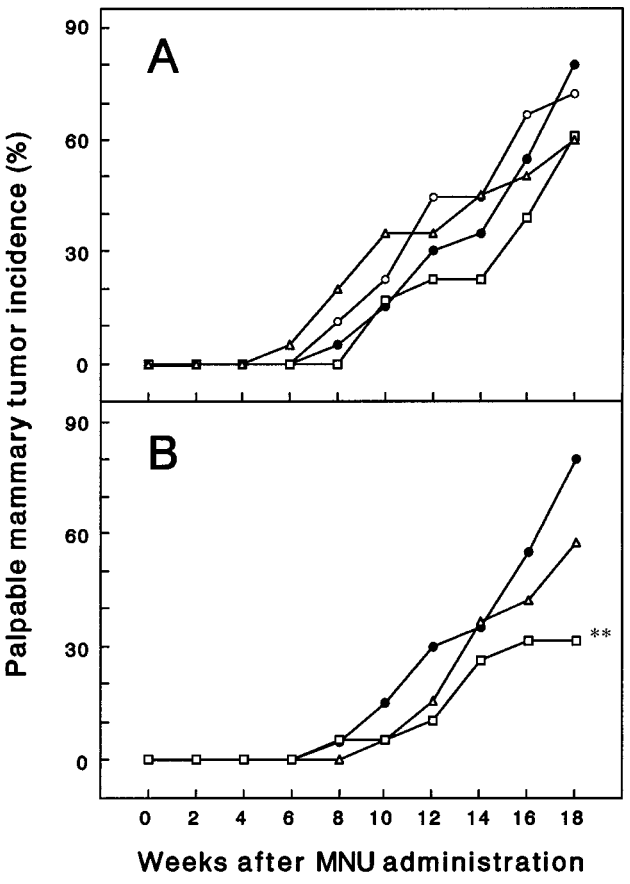

Fig. 2. Cumulative incidence of palpable mammary tumors after MNU administration. A, Soy-supplemented groups. ● Control diet, ○ 2% soybean diet, □ 10% soybean diet, △ 10% miso diet. B, Biochanin A-supplemented groups. ● Control diet, △ 10 mg/kg diet, □ 50 mg/kg diet. Each point is a mean percent incidence. ** Significantly different from the control at $P<0.01$.

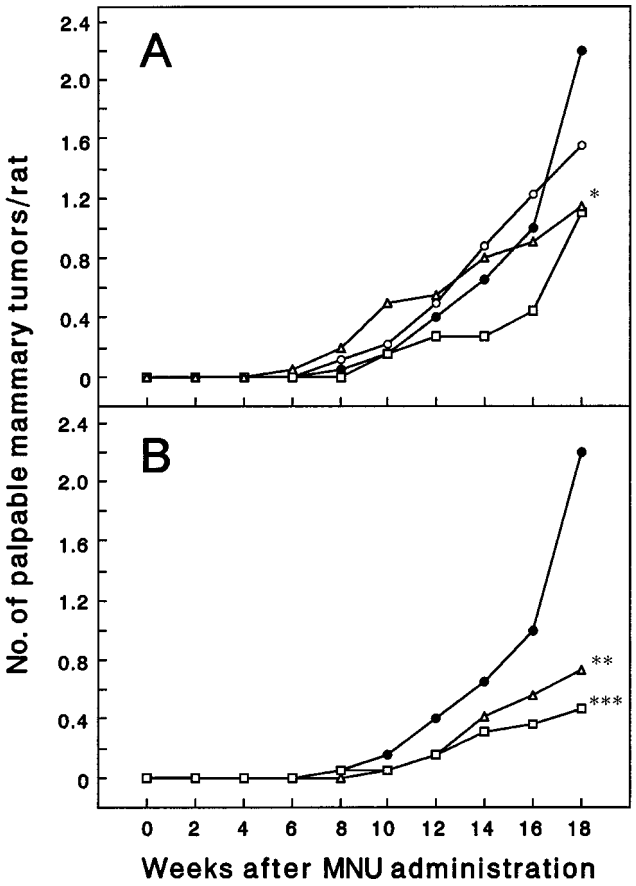

Fig. 3. Cumulative multiplicity of palpable mammary tumors after MNU administration. A, Soy-supplemented groups. ● Control diet, ○ 2% soybean diet, □ 10% soybean diet, △ 10% miso diet. B, Biochanin A-supplemented groups. ● Control diet, △ 10 mg/kg diet, □ 50 mg/kg diet. Each point is a mean value. *, **, *** Significantly different from the control at $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

Table II. Palpable Mammary Tumor Data at 18 Weeks in Each Group

Group	Effective no. of rats	Mammary tumors				
		Incidence[a)]	%	Total no. of tumors	Multiplicity[b)]	PCNA index %
Control	20	16/20	80	43	2.2±1.8[c)]	8.7±2.1
Soy foods						
2% soybean	18	13/18	72	28	1.6±1.9	7.0±1.7
10% soybean	18	11/18	61	20	1.1±1.4[d)]	6.3±1.4
10% miso	20	12/20	60	23	1.2±1.2[d)]	7.2±0.4
Biochanin A						
10 mg/kg	19	11/19	58	14	0.7±0.8[e)]	5.6±1.6[f)]
50 mg/kg	19	6/19	32[e)]	9	0.5±0.8[f)]	4.5±2.5[f)]

a) Tumor incidence was expressed as number of rats with tumor per effective number of rats.
b) Tumor multiplicity was calculated as number of tumors per effective number of rats.
c) Values are mean±SD.
d) Significantly different from control, $P < 0.05$.
e) Significantly different from control, $P < 0.01$.
f) Significantly different from control, $P < 0.001$.

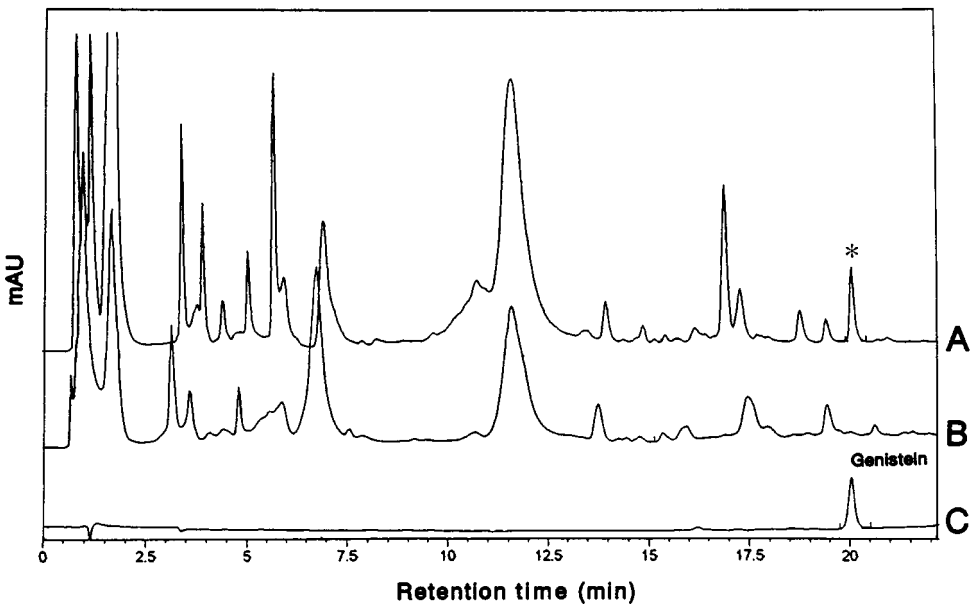

Fig. 4. High-performance liquid chromatography (HPLC) profiles of genistein in a serum sample of a rat given 10% miso diet (A), control diet (B), and a standard sample (C). The samples were injected into a packed column of C-18 (5 μm particle size, 4 mm i.d.×150 mm). The mobile phase was a gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid during 45 min, pumped at a rate of 1.5 ml/min, and the elution profile was monitored at 262 nm. All chromatographic procedures were performed at ambient temperature. A peak fraction corresponding to genistein in a standard sample was detected at the retention time of 20 min. A peak fraction (*) coinciding with the retention time of genistein was detected in a sample from a rat given 10% miso diet, but not in a sample from a rat given the control diet.

in Figs. 2 and 3, respectively, and those observed at 18 weeks are summarized in Table II. There were no significant intergroup differences in the incidence of palpable mammary tumors in the soy-supplemented groups during the observation period (Fig. 2). However, tumor multiplicity in the 10% soybean and 10% miso diet groups were significantly lower than that in the control group at 18 weeks (P<0.05, respectively). At 18 weeks, the tumor in-

cidence in the 50 mg/kg biochanin A diet group was significantly lower than that in the control group ($P<0.01$), and the tumor multiplicity in both the 10 mg/kg and 50 mg/kg biochanin A diet groups was significantly lower than that in the control group ($P<0.01$ and $P<0.001$, respectively). Inhibition of tumor multiplicity in the biochanin A-supplemented diet groups showed a dose-response relationship.

All of the MNU-induced rat mammary tumors in the present study were histologically non-invasive papillotubular carcinomas. The PCNA index in mammary tumors was significantly decreased in the biochanin A-supplemented groups compared to the control ($P<0.001$, respectively). There were no significant differences in PCNA index between control and soy-supplemented groups (Table II).

DISCUSSION

We have previously reported that the soy product miso reduced spontaneous or fission neutron-induced liver tumorigenesis in mice,[15] *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine-induced gastric tumorigenesis in rats,[16] and azoxymethane-induced colonic aberrant crypt foci in rats.[17] Several animal studies have also shown an inhibitory effect of soy foods on spontaneous and chemically induced mammary carcinogenesis.[18] Soy foods contain significant amounts of two isoflavones, genistein and daidzein, which have various biological activities and antitumorigenic effects. These isoflavones, especially genistein, have been proposed to inhibit tyrosine-specific protein kinases,[19] DNA topoisomerases I and II,[20] angiogenesis,[21] the growth of cultured human gastric cancer cell lines,[13] and cell cycle arrest at G_2-M.[22] Recently, it was reported that genistein was found at higher levels in miso than in other soybean-related products, such as soy powder, soy milk, tofu, natto, and soy sauce.[23] Likewise, in our preliminary experiment, we clearly identified the presence of genistein in serum of rats given 10% miso diet, but not in serum of rats given control diet (Fig. 4), by high-performance liquid chromatography (HPLC). Genistein has an antiestrogenic activity. It competes with 17β-estradiol in receptor-binding assays,[24, 25] and inhibits the estrogenic effects of estrone, estradiol and diethylstilbestrol.[25–27] The role of estrogen in the initiation and promotion of mammary cancer has long been recognized. The growth of mammary cancer can also be inhibited by the deprivation of estrogens via blockade of synthesis of estradiol or its precursors.[28] Thus, it is likely that the consumption of soy foods containing genistein may provide some protection against mammary cancer. In the present study, the multiplicity of mammary tumors was significantly inhibited by a 10% soybean or 10% miso diet, indicating that soy-supplemented diet does have a preventive effect against mammary cancer.

Biochanin A is a genistein precursor, that is not present in soy foods. Structurally, it differs from genistein only in a single methoxy group at the 4′ position, but it inhibits tyrosine-specific protein kinase activity 30-fold less effectively than genistein.[19] On the other hand, we have previously reported that biochanin A and genistein inhibited human gastric cancer cell growth approximately equally in cell culture, but *in vivo*, when equal doses of biochanin A and genistein were administered to BALB/c athymic nude mice into which human gastric cancer cells had been transplanted, biochanin A significantly inhibited tumor growth, whereas genistein did not.[13] In addition, biochanin A was reported to decrease the incidence and multiplicity of benzo[*a*]pyrene-induced lung tumors in mice.[29] In the present study, administration of biochanin A significantly decreased the incidence of mammary tumors at a dose of 50 mg/kg, and the multiplicity of tumors at the dose of either 10 or 50 mg/kg, and it also significantly decreased the mammary tumor PCNA index at both doses. The inhibitory effect of biochanin A on tumor multiplicity was dose-dependent. These results suggest that biochanin A could be a useful chemopreventive substance for mammary cancer, and may also be carcinostatic for mammary cancer.

In summary, the results of the present study indicate that soybeans, miso, and biochanin A are all useful for mammary cancer prevention. Further investigation will be required to evaluate the usefulness and mechanism of biochanin A as a carcinostatic drug for mammary cancer treatment.

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