Jpn. J. Cancer Res. 89, 487–495, May 1998

Chemoprevention of *N*-Nitroso-*N*-methylurea-induced Rat Mammary Cancer by Miso and Tamoxifen, Alone and in Combination

Takahiko Gotoh,[1,3] Kazumasa Yamada,[1] Akihiro Ito,[1,4] Hong Yin,[1] Tsuyoshi Kataoka[2] and Kiyohiko Dohi[2]

[1]*Department of Cancer Research, Research Institute for Radiation Biology and Medicine, Hiroshima University and* [2]*Second Department of Surgery, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553*

We examined the effects of a Japanese fermented soybean product, miso, and tamoxifen (TAM), alone and in combination, on *N*-nitroso-*N*-methylurea (MNU)-induced rat mammary cancer. Seven-week-old female CD/Crj rats received a single i.v. dose (50 mg/kg body weight) of MNU. After administration of MNU, the rats were divided into 4 groups: regular diet (control), 10% miso diet, regular diet+TAM, and 10% miso diet+TAM. TAM was implanted s.c. in the form of pellets containing 2.5 mg at the same time as MNU was administered. All rats were observed for 18 weeks after MNU administration. Incidence (percentage of rats with tumors) and multiplicity (mean tumors/rat) of mammary tumors were 91% and 4.5 in the control, 77% and 2.4 ($P<0.05$) in the 10% miso group, 68% and 1.4 ($P<0.01$) in the TAM group, and 10% ($P<0.0001$ or less) and 0.2 ($P<0.0001$) in the 10% miso+TAM group. In the second experiment, the effect of the combination of miso and TAM on established rat mammary tumors was investigated. When the mammary tumors induced by MNU reached 10 to 25 mm, the rats were divided into 3 treatment groups: regular diet, regular diet+TAM, and 10% miso diet+TAM. At 6 weeks after the start of treatment, the mean tumor size in the control and TAM groups was 160% and 141% of the pretreatment value, but a decrease to 85% of the pretreatment value was produced by the combination of miso and TAM, and this was significantly different from both the control and TAM groups ($P<0.01$ and $P<0.05$, respectively). These results indicate that miso is useful in protecting against mammary cancer and it can be expected to have a potent antitumor effect, especially when used in combination with TAM.

Key words: Chemoprevention — Rat — Mammary cancer — Miso — Tamoxifen

Mammary cancer has been increasing in incidence in Japan and is predicted to become the leading cause of cancer death among females in the near future.[1,2] As a means of primary prevention of mammary cancer, it is recommended to avoid high risk factors such as excess intake of fat and calories, especially of animal fat.[1] Therefore, there has been an increasing public demand for information on healthy foods that may help in the primary prevention of cancers in recent years.

Epidemiological studies have suggested that women who consume a traditional diet high in soy products have a low incidence of mammary cancer.[3,4] A number of animal studies have shown an inhibitory effect of soy foods against radiation- or chemically-induced rat mammary carcinogenesis.[5,6] Recent studies in our laboratory have shown that the Japanese fermented soybean product miso has a protective effect against radiation injury,[7,8] and reduces the risk of liver, stomach, and mammary tumors, and colonic aberrant crypt foci in experimental animals.[9–11] In these studies, we have also shown that a 10% miso diet is the optimal protective dose for the primary prevention of cancer in experimental animals. Miso is mainly made by fermentation of soybeans and/or rice and contains a variety of biologically active substances including botanic proteins, vitamins, fats, enzymes, carbohydrates, saponins, isoflavones, phytosterols, and lectins.[12,13] Two of the isoflavones, genistein and daidzein, are known to have a variety of biological activities[14,15] and to be present in significant amounts in miso compared to other soy products.[16]

Tamoxifen (TAM), a synthetic nonsteroidal antiestrogen agent, competes with estrogen for binding to estrogen receptors and has been used in the treatment of human breast cancer. On the basis of a report that mammary cancer patients receiving TAM as adjuvant treatment showed a reduced risk of new primary lesions in the contralateral mammary gland,[17] a large-scale trial of chemoprevention by TAM for women at high risk of mammary cancer has been initiated in the United States and Europe.[18] Recent studies in experimental animals have shown that the combination of TAM with an aromatase inhibitor,[19] 9-*cis*-retinoic acid,[20] the somatostatin analogue octreotide,[21] or

[3] Present address: Department of Surgery, Gotoh Hospital, 1-8-20 Higashi-chuo, Kure, Hiroshima 737-0052.

[4] To whom correspondence should be addressed.

dehydroepiandrosterone[22] is useful for chemoprevention of mammary cancer.

In the present study, we investigated the chemopreventive effect of miso and TAM, both alone and in combination, on *N*-nitroso-*N*-methylurea (MNU)-induced rat mammary carcinogenesis by determining the incidence and multiplicity of mammary tumors. In addition, the therapeutic effect of the same regime was also investigated in rats with established mammary tumors.

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 with controlled temperature (24±2°C) and humidity (55±10%) under a regular 12-h light, 12-h dark cycle. All rats were given food and tap water *ad libitum*. They 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 diet and chemicals Rats were fed a commercial regular diet MF (Oriental Yeast Co., Tokyo) with or without miso. Miso was made into biscuits by combining 10% dry red miso provided by the Miso Central Institute (Tokyo) with 90% regular powdered MF. Its composition was 7.69% water, 24.3% protein, 6.23% fat, 2.53% salt plus a mixture of microorganisms, flavors and aromatic compounds, unsaturated fatty acid-ethylester, glycosides, isoflavones, and saponins. Total caloric content per 100 g was 356 kcal. MNU was obtained from Sigma Chemical Co., St. Louis, Mo. and dissolved in 0.9% NaCl solution at a concentration of 50 mg/kg body weight. TAM (Sigma Chemical Co.) was fused with cholesterol powder under heating and converted to pellets. Each pellet was weighed and cut into smaller pellets each containing 2.5 mg of TAM and 7.5 mg of cholesterol.

Chemoprevention by miso and TAM, alone and in combination Under light ether anesthesia, 7-week-old female SD rats were given a single dose (50 mg/kg body weight) of MNU via the right jugular vein, which had been directly exposed by the cut-down method. After MNU administration, the rats were divided into 4 groups, which were given regular diet (control group), 10% miso diet (10% miso group), regular diet+TAM (TAM group) and a 10% miso diet+TAM (10% miso+TAM group). Each group consisted of about 20 rats and was maintained on a regular diet or miso diet until the conclusion of the experiment. A TAM pellet, prepared as described above, was implanted s.c. on the back at the same time as MNU was administered. TAM pellets were renewed at 5 weeks after the first implantation. The rats were weighed every 2 weeks, and, beginning 4 weeks after MNU administration, the location and number of palpable mammary tumors were recorded, and their sizes were measured with a caliper under light ether anesthesia every 2 weeks until the conclusion of the experiment. All rats were observed up to 18 weeks and killed at 19 weeks after MNU administration.

One hour before being killed, the rats were weighed and injected i.p. with 20 mg/kg body weight of 5-bromo-2'-deoxyuridine (BrdU) obtained from Sigma for BrdU incorporation assay in the mammary tumors. The animals were killed by exsanguination from the abdominal aorta under light ether anesthesia. The serum was obtained and kept at −20°C until used for estradiol-17β (E_2) assay. All gross palpable and nonpalpable mammary tumors we excised and weighed, and the tumor size was measured with calipers. Excised mammary tumors >20 mm in diameter were divided into half: one half was fixed in 10% phosphate-buffered formalin and embedded in a paraffin block for histological and immunohistochemical study, and the other half was frozen in liquid nitrogen and stored at −70°C until used for cytosolic estrogen receptor (ERc) assay. Organs were weighed, fixed in 10% phosphate-buffered formalin, and embedded in paraffin blocks. Each block was serially sectioned at 3 μm. Sections were routinely stained with HE.

Sections of 28 mammary tumors that developed in each group were randomly selected for BrdU incorporation assay, deparaffinized, and incubated with monoclonal mouse anti-bromodeoxyuridine (Dako-BrdUrd, Bu20a, DAKO A/S, Denmark) at a dilution of 1:20 for 1 h at ambient temperature. 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 BrdU index was determined as the percentage of nuclei showing BrdU incorporation by counting 1,000 nuclei in tumor foci. All slides were blinded and scored by one person.

ERc levels were analyzed by radio-receptor assay using [16α-^{125}I]estradiol-17β (E_2R Assay Kit, Otsuka Pharmaceutical Co., Tokushima). The free and bound fractions were separated and measured by the dextran-coated charcoal method.[23,24] The maximum number of binding sites (B_{max}) and the dissociation constant (K_d) values for the receptors were determined by a Scatchard plot analysis. Serum E_2 levels were measured with an Estradiol-Coatria Kit (bioMérieux Co., France) using [^{125}I]estradiol.[25]

Therapeutic study of miso and TAM in combination Seven-week-old female SD rats were given a single dose (50 mg/kg body weight) of MNU via the right jugular vein. When the tumor size reached between 10 and 25 mm in the largest dimension, the rats were divided into 3 treatment groups (day 0): regular diet (control group; n=7), regular diet+TAM (TAM group; n=7), and 10%

miso diet+TAM (10% miso+TAM group; n=10). TAM was converted to a 2.5 mg pellet as described above and implanted s.c. on the back at the start of treatment (day 0). Existing tumors were monitored throughout the experiment. At 6 weeks after the start of treatment, monitored tumor size was calculated as the product of the largest dimension and the maximum orthogonal diameter, and expressed as a percentage of the initial size measured on day 0. The rats were then killed and weighed.

Statistical analysis Data are shown as means±SD. Statistical analysis of the incidence of tumors was performed by using the χ^2 test, and the data for tumor multiplicity and size, the data for body and organ weight, the biochemical data, and the BrdU index were compared among groups by using Student's t test. The results were considered statistically significant when the P value was 0.05 or less. All P values reported were derived from two-sided statistical tests.

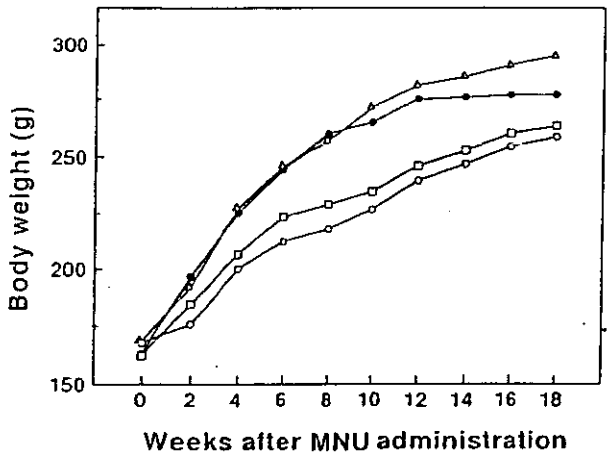

Fig. 1. Sequential changes in body weight in each group after MNU administration. ● regular diet, △ 10% miso diet, □ regular diet+TAM, ○ 10% miso diet+TAM. Each point indicates a mean.

RESULTS

General observations The time course of body weight gain in each group is shown in Fig. 1. The body weight of the control group increased up to 12 weeks after MNU

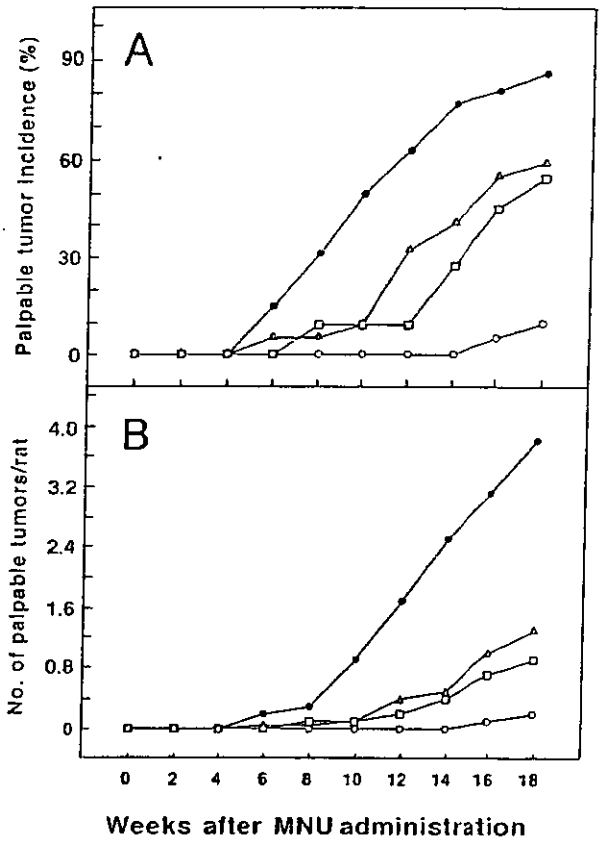

Fig. 2. Cumulative incidence (A) and multiplicity (B) of palpable mammary tumors after MNU administration. ● regular diet, △ 10% miso diet, □ regular diet+TAM, ○ 10% miso diet+TAM. Each point indicates a mean. This experiment was performed twice and yielded similar results each time.

Table I. Body Weight and Relative Organ Weight according to Group

Treatment	No. of rats	Body wt. (g)	Relative organ weight[a]			
			Liver	Uterus	Ovary	Adrenal
Control	22	277±30	3.1±0.4	180±61	58.9±34.4	19.7±3.3
10% miso	22	294±29	3.3±0.4	188±40	59.5±23.1	20.2±2.9
TAM	22	263±20	3.0±0.4	137±47[b]	57.8±14.4	20.1±2.9
10% miso+TAM	20	258±21[b]	3.1±0.3	148±34[b]	53.3±29.6	21.7±3.9

a) Relative organ weight was calculated per 100 g of body weight.
b) Significantly different from control; P<0.05.

Table II. Mammary Tumor Data at Termination according to Group

Treatment	No. of rats	Mammary tumors					
		Incidence[a]	(%)	Total no. of tumors[b]	Multiplicity[c]	Size[d] (mm²)	BrdU index (%)
Control	22	20/22	91	99	4.5±3.8	276±278	7.1±2.1
10% miso	22	17/22	77	53	2.4±2.3[e]	220±331	5.8±2.1
TAM	22	15/22	68	31	1.4±1.4[g]	247±233	5.6±1.8[e]
10% miso+TAM	20	2/20	10[g,i]	4	0.2±0.7[h,k]	124±39[e,j]	5.2±1.3[e]

a) Number of rats with tumors per total number of rats.
b) Includes both palpable and nonpalpable tumors.
c) Number of tumors per rat.
d) Tumor size was expressed as a product of the largest dimension and maximum orthogonal diameter.
Significantly different from control; *e*) $P<0.05$, *f*) $P<0.01$, *g*) $P<0.001$, *h*) $P<0.0001$, *i*) $P<0.0001$ or less.
Significantly different from TAM; *j*) $P<0.05$, *k*) $P<0.01$, *l*) $P<0.001$.

Table III. E_2 Level in Serum and ERc Level in Mammary Tumors

Treatment	Serum E_2		ERc[a]		
	No. of samples examined	(pg/ml)	No. of tumors examined	B_{max} (fmol/mg protein)	K_d ($\times 10^{-10}$ M)
Control	11	71.0±26.7	8	56.9±20.6	2.54±1.27
10% miso	10	46.3±35.8[b]	7	102.8±41.2[b]	5.88±1.98
TAM	12	56.4±34.5	7	75.9±67.9	3.06±1.48
10% miso+TAM	9	47.9±28.4[b]	N[c]	—	—

a) ERc level of mammary tumors was measured by the dextran-coated charcoal method, and the maximum number of binding sites (B_{max}) and dissociation constant (K_d) values were determined by Scatchard plot analysis.
b) Significantly different from control; $P<0.05$.
c) Not examinied.

Table IV. Effect of Miso and TAM in Combination on Established Mammary Tumors

Treatment	No. of rats	No. of tumors	Mammary tumors[a]		
			Initial(I) (mm²)	Final(F) (mm²)	F/I (%)
Control	7	10	248±103	418±240	160
TAM	7	12	229±178	293±224	141
10% miso+TAM	10	10	245±152	179±80	85[b,c]

a) Initial and final sizes (6 weeks after start of treatment) were expressed as palpable mammary tumor size, and calculated as the product of two axes (mm²).
F/I was expressed as the percentage of the initial size measured on day 0.
b) Significantly different from control; $P<0.05$.
c) Significantly different from TAM; $P<0.05$.

administration and then plateaued. The body weight of the groups given TAM was significantly lower than that of the control group ($P<0.01$). Mean body and relative organ weights at the time of death are summarized in Table I. Mean body weight in the 10% miso+TAM group was significantly decreased compared to the control group ($P<0.05$). The relative weight of the uterus in the groups given TAM was significantly decreased compared to the control group ($P<0.05$, respectively). There were no statistically significant differences in liver, ovary, or adrenal weight between any of the groups. The prevalence of cataracts was similar in all treatment groups in the present study. The incidence (%) of rats with cataracts was 45% in the control group and 48% in the TAM group, while it