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

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**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

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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 were 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 ($E_2$R 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 $\chi^2$ 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.

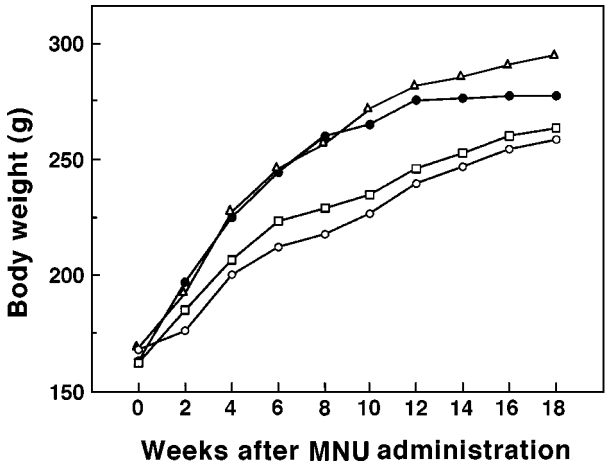


Fig. 1. Sequential changes in body weight in each group after MNU administration. ● regular diet, △ 10% miso diet, □ regular diet+TAM, ○ 10% miso diet+TAM. Each point indicates a mean.

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

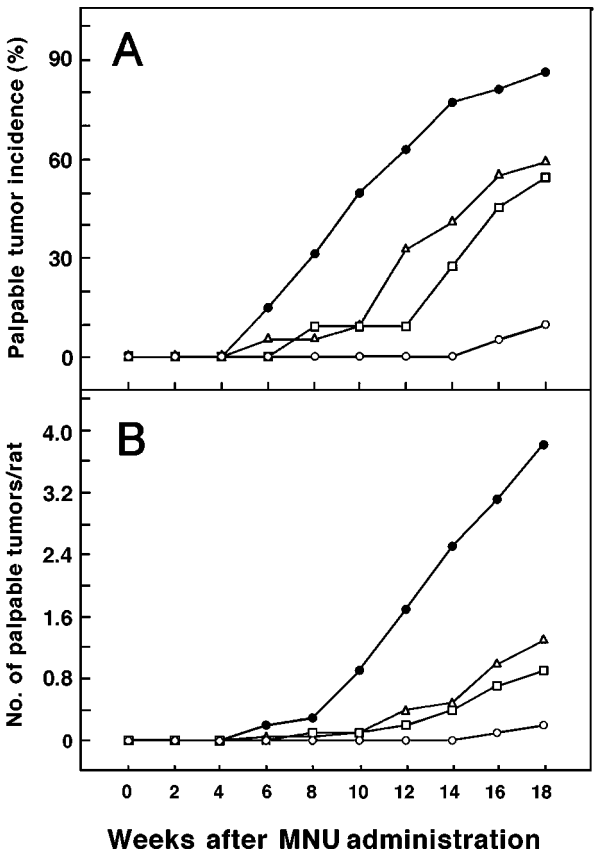


Fig. 2. Cumulative incidence (A) and multiplicity (B) of palpable mammary tumors after MNU administration. ● regular diet, △ 10% miso diet, □ regular diet+TAM, ○ 10% miso diet+TAM. Each point indicates a mean. This experiment was performed twice and yielded similar results each time.

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)](mm$^2$) | 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[f)] | 247±233 | 5.6±1.8[e)] |
| 10% miso+TAM | 20 | 2/20 | 10[i,l)] | 4 | 0.2±0.7[h,k)] | 124±39[g,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) (mm$^2$) | Final(F) (mm$^2$) | 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 (mm$^2$).
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

was 18% in the 10% miso group ($P<0.05$, data not shown) and 20% in the 10% miso+TAM group.

**Chemopreventive effect of miso and TAM, alone and in combination** The effects of 10% miso and TAM, alone and in combination, on the incidence and multiplicity of mammary tumors are shown in Fig. 2 and Table II. In the combination group, the tumor latency was greatly reduced, and there was a significant reduction in the incidence of palpable mammary tumors during the experiment compared to the control group ($P<0.0001$ or less). The multiplicity of palpable mammary tumors in all treatment groups was significantly reduced during the experiment compared to the control group ($P<0.01$). The incidence (%) and multiplicity (mean tumors/rat) of mammary tumors at termination were 91% and 4.5 in the control group, 77% and 2.4 ($P<0.05$) in the 10% miso group, and 68% ($P<0.01$) and 1.4 ($P<0.01$) in the TAM group. Tumor incidence and multiplicity in the combination group were 10% ($P<0.0001$ or less) and 0.2 ($P<0.0001$), and were also significantly decreased compared to the values in the TAM group ($P<0.01$ and $P<0.05$, respectively). Mean tumor size in the combination group was significantly reduced compared to both the control group and the TAM group ($P<0.001$ and $P<0.05$, respectively).

The BrdU index of the mammary tumors in each group is summarized in Table II. The BrdU index of mammary tumors in the groups given TAM was significantly decreased compared to the control group ($P<0.05$). The $E_2$ levels in serum and ERc levels in mammary tumors are summarized in Table III. The groups given miso and TAM, both alone and in combination, tended to have decreased serum $E_2$ levels. The serum $E_2$ levels in the groups given the miso diet were significantly decreased compared to the control group ($P<0.05$). In the 10% miso group, the maximum number of binding sites was significantly increased in the mammary tumors when compared with the control group ($P<0.05$).

**Therapeutic effect of miso and TAM in combination** The therapeutic effects of miso in combination with TAM on the regression of palpable mammary tumors after a 6-week treatment period are summarized in Table IV. At the conclusion of the diet period, mean percent tumor size in the control and TAM group was 160% and 141% of the pretreatment value, respectively. On the other hand, the value in the combination group decreased to 85% of the pretreatment value and was significantly different from the control and the TAM group ($P<0.01$ and $P<0.05$, respectively). At the conclusion of the experiment, there were no significant differences in body weight among the groups.

**Histopathology of mammary tumors** Although fibroadenoma has quite frequently been observed in 7,12-dimethylbenz[*a*]anthracene- or radiation-induced rat mammary tumors,[26, 27] no fibroadenomas were observed in MNU-

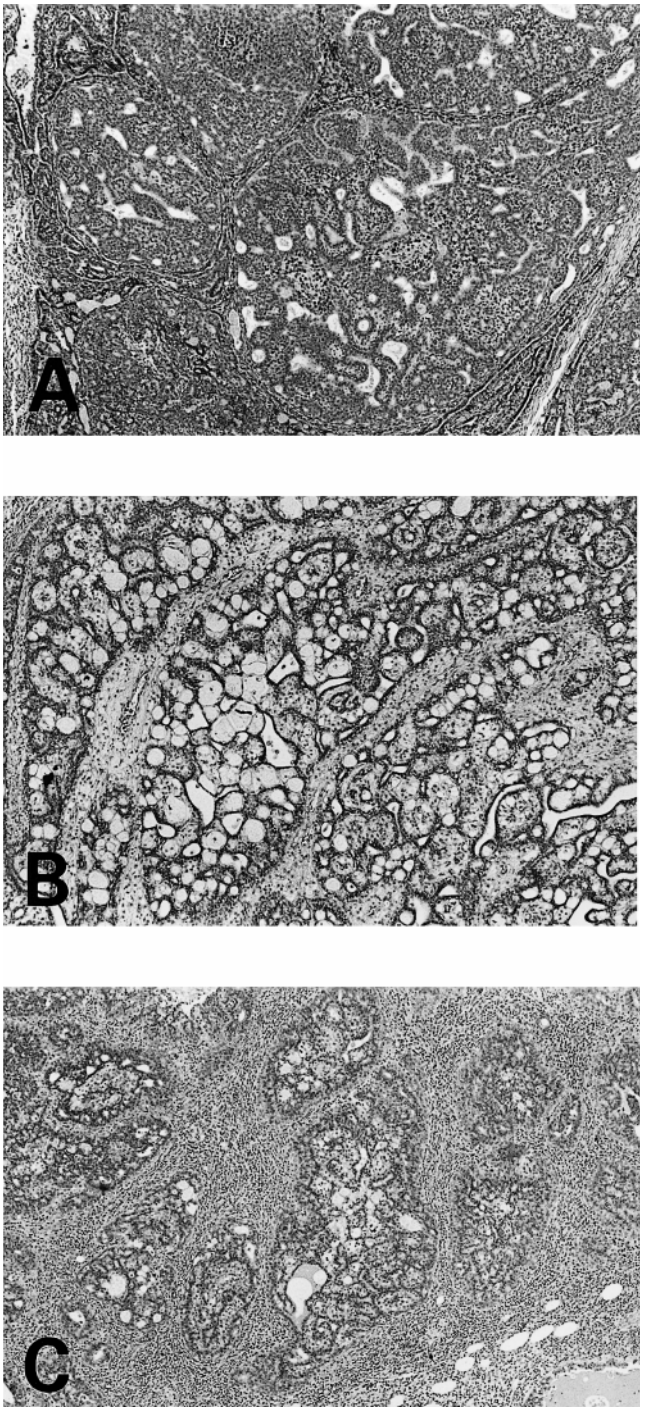


Fig. 3. Histopathology of mammary tumors in each group. All of the tumors were non-invasive papillotubular carcinoma, and photograph (A) shows a rat mammary tumor from the regular diet group. HE, ×20. Most of the neoplastic foci in the regular diet+TAM group (B) were accompanied by degenerative and vacuolated changes. HE, ×20. In the 10% miso diet+TAM group (C), heavy infiltrations of lymphoid cells were found in the stroma surrounding the tumor foci. HE, ×20.

induced rat mammary tumors in the present study. The histological appearance of the mammary tumor is shown in Fig. 3. All of the mammary tumors were non-invasive papillotubular carcinoma. The histopathology of mammary tumors in the control and the 10% miso groups was ordinary non-invasive papillotubular carcinoma (Fig. 3A), and no morphological difference between the two groups was apparent. On the other hand, most of the tumor foci in the TAM group exhibited vacuolated changes (Fig. 3B), and in the 10% miso+TAM group, heavy lymphoid cell infiltration was noted in the stroma surrounding the tumor foci (Fig. 3C).

## DISCUSSION

It has been reported that soybean products in the diet reduce the risk of cancer.[28–31] In the present study, the soybean product miso significantly reduced the multiplicity of mammary tumors, indicating that a miso diet is useful in the prevention of mammary cancer. One of the candidate cancer-preventive agents in soybeans is genistein, the most abundant isoflavone in soybeans. Genistein is a potent inhibitor of tyrosine-specific protein kinases and modulates cell proliferation and transformation.[32] It also inhibits DNA topoisomerase I and II,[33] angiogenesis,[34] and the growth of cultured human gastric cancer cell lines[35] via apoptosis,[36] and arrests the cell cycle at $G_2$-M.[37] In addition, genistein has weak phytoestrogenic activity, with a uterotropic potency of about $1\times10^{-5}$ that of diethylstilbestrol,[38] and it possesses antiestrogenic activity as well. It has been shown to compete with $E_2$ in receptor-binding assays[39, 40] and to inhibit the estrogenic effects of estrone, estradiol, and diethylstilbestrol.[41] More recently, genistein has been shown to be present at higher levels in miso than in other soybean products such as soy powder, soy milk, tofu, natto, and soy sauce.[16] In our previous study, we clearly identified the presence of genistein by high-performance liquid chromatographic analysis in the serum of rats given a miso diet, but not in the serum of rats given a regular diet.[10] Thus, it is assumed that the consumption of miso-containing foods with significant levels of genistein is one possible mechanism of the protective effect against mammary cancer.

Consumption of soybean products has been shown to reduce circulating ovarian steroids in premenopausal women.[42] Several *in vitro* studies have found that genistein inhibits the biosynthesis of progesterone in bovine granulosa cells,[43] antagonizes transforming growth factor α-induced synthesis of estrogen in granulosa and theca cells,[44] and inhibits the enzyme activity of 17β-hydroxysteroid oxidoreductase type I,[45] an enzyme that converts estrone to $E_2$. Unlike some other flavonoids, isoflavones, including genistein are generally weak inhibitors of aromatase.[46] In the present study, the serum $E_2$ levels of the rats given miso were significantly reduced compared to those of the rats not given miso. $E_2$ stimulates breast cell proliferation and may promote breast tumor growth.[47] This suggests that miso reduces the amount of $E_2$ in serum, and thereby may reduce the risk of mammary cancer.

The etiology of cataract is uncertain but it is probably the result of age-related degenerative changes or metabolic factors in the lens epithelium or bow area. However, its prevalence can be modulated by alterations in sex hormone status.[48] Cataract is also one of the toxic effects of long-term administration of high doses of TAM in rats and humans.[49, 50] In the present study, there was no difference regarding the appearance of cataract between the control and TAM groups, but the groups given miso tended to show decreased appearance of cataract. This suggests that miso has a protective effect against the appearance of cataract.

Atrophic change of the uterus is another toxic effect of TAM in rats.[49, 51] In the present study, the uterine weight in the groups given TAM was significantly decreased compared to the groups not given TAM. This result should be attributable to the antiestrogenic effect of TAM on uterine tissue.[51]

It is documented that dietary restriction inhibits tumorigenesis in rodents.[52–54] In the present study, TAM-administered groups showed about 10–20% body weight reduction compared to the control, as evidenced by a suppression of the weight gain by 20–25 g. A two-year carcinogenicity study of TAM in rats showed that the growth rate was reduced in all groups treated with various doses of TAM.[49] This reduction in growth is believed to be a consequence of the pharmacological activity of TAM and related to changes in hormonal status.[49] On the other hand, our present results on the cumulative incidence of tumor-bearing rats and growth pattern in the TAM group are consistent with those found in animals given a 0.5 mg/kg diet of TAM by Anzano *et al.*,[20] using the same MNU-induced rat mammary carcinogenesis model. Thus, this systemic effect did not overtly affect mammary carcinogenesis in the present study.

TAM inhibits [$^3$H]thymidine uptake in the cells of preneoplastic lesions in the MNU-induced mammary carcinogenesis model.[55] *In vitro*, TAM inhibits the proliferation of human mammary cancer cells by preventing the transition of cells from the early $G_1$ phase to the mid-$G_1$ phase of the cell cycle, and as a result, cells accumulate in early $G_1$ phase, while the number of cells in S and $G_2$ plus M phases decreases.[56–58] Thus, TAM has a cytostatic effect. In the present case, the BrdU index of mammary tumors was significantly decreased in the groups given TAM and the values of BrdU index were comparable in all the groups given TAM. These results suggest that the antipro-

liferative activity may be mainly due to the effect of TAM on the mammary tumors.

We have successfully used the combination of miso and TAM for chemoprevention and for adjuvant therapy of established rat mammary cancer. To our knowledge, this is the first investigation of the chemopreventive potential of miso and TAM in combination. The increase in ERc levels of mammary tumors on the miso diet alone in the present study may point to another endocrine pathway mediating this potent antitumor effect, in addition to the decrease in the amount of $E_2$ in serum. The miso diet may increase the hormone dependency of mammary tumors and consequently increase the sensitivity of mammary tumors to TAM, producing a synergistic antitumor effect. Furthermore, the finding that heavy lymphoid cell infiltration was induced in the stroma surrounding the neoplastic foci may suggest another antitumor effect, involving immunomodulation rather than hormonal changes.

In summary, the soybean product miso is a useful agent for chemoprevention of MNU-induced rat mammary cancer, and is expected to have an excellent antitumor effect, especially when used in combination with TAM. Further investigation will be required to assess the usefulness and the precise mechanism of the miso and TAM in combination as a chemopreventive or therapeutic agent against mammary cancer, and to establish whether this strategy will be applicable to humans.

ACKNOWLEDGMENTS

We thank Misses Kurumi Ishimaru and Midori Tanizaki for their technical assistance. A part of this study was supported by a grant from the Miso Central Institute, Tokyo, and a Grant-in-Aid from the Ministry of Health and Welfare, Japan.

*(Received January 6, 1998/Revised March 6, 1998/Accepted March 10, 1998)*

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