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 Kataoka2 and Kiyohiko Dohi2

1Department of Cancer Research, Research Institute for Radiation Biology and Medicine, Hiroshima University and 2Second 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/Crlj 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 activities14,15 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...
dehydroepiandrosterone\(^2\) 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/Cj 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 micro-organisms, 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 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-17\(^β\) (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 (ER\(_c\)) assay. Organs were weighed, fixed in 10% phosphate-buffered formalin, and embedded in paraffin blocks. Each block was serially sectioned at 3 \(\mu\)m. 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 Sub-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.

ER\(_c\) levels were analyzed by radio-receptor assay using \([16\alpha,2\beta\]estradiol-17\(\beta\) (E\(_2\)R Assay Kit, Otsuka Pharmaceutical Co., Tokushima). The free and bound fractions were separated and measured by the dextran-coated charcoal method.\(^25\) The maximum number of binding sites (\(B\(_{\text{max}}\)) 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-Coatia Kit (bioMérieux Co., France) using \([2\beta\]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.

**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

---

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

| Treatment        | No. of rats | Body wt. (g) | Relative organ weight*  |
|------------------|-------------|--------------|-------------------------|
|                  |             |              | 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±240    | 57.8±14.4   | 20.1±2.9    |
| 10% miso+TAM     | 20          | 258±21b      | 3.1±0.3     | 148±34b    | 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; $P<0.05$.  

---

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.

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.
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 sig-
ificantly 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 \( (P<0.05) \). There were no sta-
tistically significant differences in liver, ovary, or adrenal
weight between any of the groups. The prevalence of cat-
aracts 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

Table II. Mammary Tumor Data at Termination according to Group

| Treatment            | No. of rats | Incidence\(^a\) (%) | Total no. of tumors\(^b\) | Multiplicity\(^c\) | Size\(^d\) (mm\(^2\)) | BrdU index \(^e\) (%) |
|----------------------|-------------|----------------------|---------------------------|-------------------|-----------------------|----------------------|
| Control              | 22          | 20/22                | 99                        | 4.5±3.8           | 276±278               | 7.1±2.1              |
| 10% miso             | 22          | 17/22                | 77                        | 2.4±2.3\(^d\)     | 220±331               | 5.8±2.1              |
| TAM                  | 22          | 15/22                | 68                        | 1.4±1.4\(^d\)     | 247±233               | 5.6±1.8\(^d\)       |
| 10% miso+TAM         | 20          | 2/20                 | 10\(^i\)                  | 0.2±0.7\(^k\)     | 124±39\(^j\)          | 5.2±1.3\(^o\)       |

\( ^a \) 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; \( ^e \) \( P<0.05 \), \( ^f \) \( P<0.01 \), \( ^g \) \( P<0.001 \), \( ^h \) \( P<0.0001 \) or less.  
Significantly different from TAM; \( ^i \) \( P<0.05 \), \( ^k \) \( P<0.01 \), \( ^l \) \( P<0.001 \).

Table III. E\(_2\) Level in Serum and ER\(_c\) Level in Mammary Tumors

| Treatment        | No. of samples examined | Serum E\(_2\) (pg/ml) | No. of tumors examined | \( B_{max} \) (fmol/mg protein) | \( K_d \times 10^{-10} M \) |
|------------------|-------------------------|-----------------------|------------------------|-------------------------------|-----------------------|
| Control          | 11                      | 71±26                | 8                      | 56±20                         | 2.5±1.27             |
| 10% miso         | 10                      | 46±35\(^b\)          | 7                      | 102±41\(^a\)                 | 5.8±1.98             |
| TAM              | 12                      | 56±34                | 7                      | 75±67                        | 3.0±1.48             |
| 10% miso+TAM     | 9                       | 47±28\(^b\)          | N\(^c\)                | —                             | —                    |

\( ^a \) ER\(_c\) level of mammary tumors was measured by the dextran-coated charcoal method, and the maxi-
mum 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 examined.

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

| Treatment        | No. of rats | No. of tumors | 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\(^c\) |

\( ^a \) 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\(^2\)).  
\( ^b \) F/I was expressed as the percentage of the initial size measured on day 0.  
\( ^c \) Significantly different from control; \( P<0.05 \).  
\( ^d \) Significantly different from TAM; \( P<0.05 \).
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 $E_Rc$ 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-
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\(_1\)-M.\(^{37}\) In addition, genistein has weak phytoestrogenic activity, with a uterotrophic potency of about 1\(\times\)10\(^{-5}\) 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.\(^{42}\) 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.\(^{43}\) 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.\(^{44}\) Several \(\text{in vitro}\) studies have found that genistein inhibits the biosynthesis of progesterone in bovine granulosa cells,\(^{45}\) antagonizes transforming growth factor-\(\alpha\)-induced synthesis of estrogen in granulosa and theca cells,\(^{46}\) and inhibits the enzyme activity of 17\(\beta\)-hydroxysteroid oxidoreductase type I,\(^{47}\) an enzyme that converts estrone to E\(_2\). Unlike some other flavonoids, isoflavones, including genistein are generally weak inhibitors of aromatase.\(^{48}\) 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.\(^{49}\) 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.\(^{50}\) Cataract is also one of the toxic effects of long-term administration of high doses of TAM in rats and humans.\(^{51,52}\) 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.\(^{49,53}\) 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.\(^{54}\)

It is documented that dietary restriction inhibits tumorigenesis in rodents.\(^{55-58}\) 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.\(^{59}\) 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 \textit{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 \(\text{[H]}\)thymidine uptake in the cells of preneoplastic lesions in the MNU-induced mammary carcinogenesis model.\(^{51}\) \textit{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 E2 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 tumor is the first investigation of the chemopreventive potential of miso and TAM in combination. The increase in ERc 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)

REFERENCES

1) Tominaga, T. and Kuroishi, T. Epidemiology of breast cancer in Japan. Breast Cancer, 2, 1–7 (1995).
2) Kuroishi, T., Hirose, K., Tominaga, S., Ogawa, H. and Tajima, K. Prediction of future cancer mortality in Japan. Jpn. J. Clin. Oncol., 22, 365–369 (1992).
3) Nomura, A., Henderson, B. and Lee, J. Breast cancer and diet among the Japanese in Hawaii. Am. J. Clin. Nutr., 31, 2020–2025 (1978).
4) Hirayama, T. A large scale cohort study on cancer risks by diet—with special reference to the risk reducing effects of green-yellow vegetable consumption. In "Diet, Nutrition and Cancer," ed. Y. Hayashi, M. Nagao, T. Sugimura, S. Takayama, L. Tomatis, L. W. Wattenberg and G. N. Wogan, pp. 41–53 (1986). Japan Scientific Societies Press, Tokyo.
5) Troll, W., Wiesner, R., Shellabarger, C. J., Holtzman, S. and Stone, J. P. Soybean diet lowers breast tumor incidence in irradiated rats. Carcinogenesis, 1, 469–472 (1980).
6) Barnes, S., Grubbs, C., Setchell, K. D. R. and Carlson, J. Soybeans inhibit mammary tumors in models of breast cancer. In "Mutagen and Carcinogens in the Diet," ed. M. W. Pariza, H.-U. Aeschbacher, J. S. Felton and S. Sato, pp. 239–253 (1990). Wiley-Liss, New York.
7) Ito, A. Is miso diet effective for radiation injuries? Miso Sci. Technol., 39, 71–84 (1991) (in Japanese).
8) Watanabe, H., Takahashi, T. and Ishimoto, T. The effect of miso diet on small intestinal damage in mice irradiated by X-ray. Miso Sci. Technol., 39, 29–32 (1991) (in Japanese).
9) Watanabe, H., Masaoka, Y., Gotou, T., Fujimoto, N. and Ito, A. Effects of miso in reducing risk of liver and gastric tumors in experimental animals. In "Food Factors for Cancer Prevention," ed. H. Ohigashi, T. Osawa, J. Terao, S. Watanabe and T. Yoshikawa, pp. 351–354 (1997). Springer-Verlag, Tokyo.
10) Gotou, T., Yamada, K., Yin, H., Ito, A., Kataoka, T. and Dohi, K. Chemoprevention of N-nitroso-N-methylurea-induced rat mammary carcinogenesis by soy foods or biochanin A. Jpn. J. Cancer Res., 89, 137–142 (1998).
11) Masaoka, Y., Watanabe, H., Tanizaki, M., Ando, Y., Yamada, K., Gotoh, T., Fujimoto, N. and Ito, A. Effect of a miso diet on colonic aberrant crypt foci in F344 rats exposed to azoxymethane. In "Recent Advances in Gastroenterological Carcinogenesis I," ed. E. Tahara, K. Sugimachi and T. Oohara, pp. 1181–1185 (1996). Mondazzi-Editore, Bologna.
12) Ito, A. and Watanabe, H. Recent topics on miso in the aspect of biological role and primary prevention of cancer. Hiroshima J. Med. Sci., 47, 5–9 (1994) (in Japanese).
13) Messina, M. and Barnes, S. The role of soy products in reducing risk of cancer. J. Natl. Cancer Inst., 83, 541–546 (1991).
14) Wang, H. J. and Murphy, P. A. Isoflavone content in commercial soybean foods. J. Agric. Food Chem., 42, 1666–1673 (1994).
15) Coward, L., Barnes, N. C., Setchell, K. D. R. and Barnes, S. The antitumor isoflavone, genistein and daidzein, in soybean foods of American and Asian diets. J. Agric. Food Chem., 41, 1961–1967 (1994).
16) Fukutake, M., Takahashi, M., Ishida, K., Kawamura, H., Sugimura, T. and Wakabayashi, K. Quantiﬁcation of genistein and genistin in soybeans and soybean products. Food Chem. Toxicol., 34, 457–461 (1996).
17) Hortobagyi, G. N. Overview of new treatments for breast cancer. Breast Cancer Res. Treat., 21, 3–13 (1992).
18) Costa, A. Breast cancer chemoprevention. Eur. J. Cancer, 29A, 589–592 (1993).

In summary, the soybean product miso is a useful agent for chemoprevention of MNU-induced rat mammary cancer, and it 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.
19) Tominaga, T., Yoshida, K., Shimozuma, K., Hayashi, K. and Kosai, G. Effect of CGS 16949A plus tamoxifen on induced mammary tumors in rats. *Eur. J. Cancer*, 26, 600–603 (1990).

20) Anzano, M. A., Byers, S. W., Smith, J. M., Peer, C. W., Mullen, L. T., Brown, C. C., Roberts, A. B. and Sporn, M. B. Prevention of breast cancer in the rat with 9-cis-retinoic acid as a single agent and in combination with tamoxifen. *Cancer Res.*, 54, 4614–4617 (1994).

21) Weckbecker, G., Tolevsai, L., Stolz, B., Pollak, M. and Bruns, C. Somatostatin analogue octreotide enhances the antineoplastic effects of tamoxifen and ovariectomy on 7,12-dimethylbenz(a)-anthracene-induced rat mammary carcinomas. *Cancer Res.*, 54, 6334–6337 (1994).

22) McCormick, D. L., Rao, K. V. N., Johnson, W. D., Bowman-Gram, T. A., Steele, V. E., Luett, R. A. and Kellogg, G. J. Exceptional chemopreventive activity of low-dose dehydroepiandrosterone in the rat mammary gland. *Cancer Res.*, 56, 1724–1726 (1996).

23) Hochberg, R. B. and Rosner, W. Interaction of 16α-t[131]Iodo-estradiol with estrogen receptor and other steroid-binding proteins. *Proc. Natl. Acad. Sci. USA.*, 77, 328–332 (1980).

24) Tominaga, T., Nomura, Y., Kobayashi, S., Yayoi, E., Wada, T., Enomoto, K., Iino, Y. and Mori, I. Estrogen receptor assay in human breast cancer tissue using 16α-t[125]I-estradiol-17β. *Prog. Med.*, 3, 399–404 (1983).

25) Ratcliffe, W. A., Carter, G. D., Dowsett, M., Hillier, S. G., Middle, J. D. and Reed, M. J. Oestradiol assays: applications and guidelines for the provision of clinical biochemistry service. *Ann. Clin. Biochem.*, 25, 466–483 (1988).

26) Shinha, D. K., Pazik, J. E. and Dao, T. L. Progression of rat mammary development with age and its relationship to carcinogenesis by a chemical carcinogen. *Int. J. Cancer*, 31, 321–327 (1983).

27) Cronkite, E. P., Shellabarger, C. J., Bond, V. P. and Lippincott, S. W. Studies on radiation-induced mammary gland neoplasia in the rat. *Radioisotopes*, 12, 81–93 (1960).

28) Hirayama, T. Epidemiology of stomach cancer in Japan with special reference to the strategy for the primary prevention. *Jpn. J. Cancer Res.*, 14, 159–168 (1984).

29) Messina, M. J., Persky, V., Setchell, K. D. R. and Barnes, S. Soy intake and cancer risk: a review of in vitro and in vivo data. *Nutr. Cancer*, 21, 113–131 (1994).

30) Adlercreutz, H., Honjo, H., Higashi, A., Fotsis, T., Hamalainen, E., Hasegawa, T. and Okada, H. Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet. *Am. J. Clin. Nutr.*, 54, 1093–1100 (1991).

31) Wu, A. H., Ziegler, R. G., Horn-Ross, P. L., Nomura, A. M. Y., West, D. W., Kolonel, L. N., Rosenthal, J. F., Hoover, R. N. and Pike, M. C. Tofu and risk of breast cancer in Asian-Americans. *Cancer Epidemiol. Biomarkers Prev.*, 5, 901–906 (1996).

32) Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M. and Fukami, Y. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.*, 262, 5592–5595 (1987).

33) Okura, A., Arakawa, H., Oka, H., Yoshinari, T. and Monden, Y. Effect of genistein on topoisomerase activity and the growth of [VAL 12]H-ras-transformed NIH3T3 cells. *Biochem. Biophys. Res. Commun.*, 157, 183–189 (1988).

34) Fotsis, T., Pepper, M., Adlercreutz, H., Hase, T. A., Montesano, R. and Schweigerer, L. Genistein, a dietary ingested isoflavone, inhibits cell proliferation and in vitro angiogenesis. *J. Nutr.*, 125, 790–797 (1995).

35) Yanagihara, K., Ito, A., Toge, T. and Numoto, M. Anti-proliferative effects of isoflavones on human cancer cell lines established from the gastrointestinal tract. *Cancer Res.*, 53, 5815–5821 (1993).

36) Yanagihara, K., Numoto, M., Taucchi, H., Akama, Y., Yokozaki, H., Tahara, E., Kamiya, K. and Seito, T. Genetic status of p53 and induction of apoptosis by radiation or isoflavones in human gastric carcinoma cell lines. *Int. J. Oncol.*, 9, 95–102 (1996).

37) Matsuoka, Y., Maruni, N., Sakai, T., Satomi, Y., Yoshida, Y., Matsumoto, K., Nishino, H. and Aonke, A. Genistein arrests cell cycle progression at G-M. *Cancer Res.*, 53, 1328–1331 (1993).

38) Martin, P. M., Horwitz, K. B., Ryan, D. S. and McGuire, W. L. Phytoestrogen interaction with estrogen receptors in human breast cancer. *Endocrinology*, 103, 1860–1867 (1978).

39) Shutt, D. A. and Cox, R. I. Steroid and phyto-estrogen binding to sheep uterine receptors in vitro. *J. Endocrinol.*, 52, 299–310 (1972).

40) Mathieson, R. A. and Kitts, W. D. Binding of phyto-estrogens and estradiol-17β by cytoplasmic receptors in the pituitary gland and hypothalamus of the ewe. *J. Endocrinol.*, 85, 317–325 (1980).

41) Foulman, Y. and Pope, G. S. The interaction in the immature mouse of potent oestrogens with coumestrol, genistein and other utero-vaginotrophic compounds of low potency. *J. Endocrinol.*, 43, 215–225 (1966).

42) Lu, L-J. W., Anderson, K. E., Grady, J. J. and Nagamani, M. Effect of soya consumption for one month on steroid hormones in premenopausal woman: implication for breast cancer risk reduction. *Cancer Epidemiol. Biomarkers Prev.*, 5, 63–70 (1996).

43) Kaplanski, O., Shemesh, M. and Berman, A. Effects of phyto-estrogens on progesterone synthesis by isolated bovine granulosa cells. *J. Endocrinol.*, 89, 343–348 (1981).

44) Gangrade, B. K., Davis, J. S. and May, J. V. A novel mechanism for the induction of aromatase in ovarian cells in vitro: role of transforming growth factor α-induced protein tyrosine kinase. *Endocrinology*, 129, 2790–2792 (1991).

45) Makela, S., Davis, V. L., Tally, W. C., Korkman, J., Salo, L., Vihko, R., Santti, R. and Korach, K. S. Dietary estrogens act through estrogen receptor-mediated processes and...
show no antiestrogenicity in culture breast cancer cells. *Environ. Health Perspect.*, 102, 572–578 (1994).

46) Campbell, D. R. and Kurzer, M. S. Flavonoid inhibition of aromatase enzyme activity in human preadipocytes. *J. Steroid Biochem. Mol. Biol.*, 46, 381–388 (1993).

47) Pike, M. C., Spicer, D. V., Dahmoush, L. and Press, M. F. Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. *Epidemiol. Rev.*, 15, 17–35 (1993).

48) Lazenby, C., Westwood, F. R. and Greaves, P. Crescentic cataracts in Alderley Park rats. *Vet. Pathol.*, 30, 70–74 (1993).

49) Greaves, P., Goonetilleke, R., Nunn, G., Topham, J. and Orton, T. Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. *Cancer Res.*, 53, 3919–3924 (1993).

50) Jaiyesimi, J. A., Buzdar, A. U., Decker, D. A. and Hortobagyi, G. N. Use of tamoxifen for breast cancer: twenty-eight years later. *J. Clin. Oncol.*, 13, 513–520 (1995).

51) Jordan, V. C. Effect of tamoxifen (ICI 46,474) on initiation and growth of DMBA-induced rat mammary carcinoma. *Eur. J. Cancer*, 12, 419–424 (1976).

52) Tucker, M. J. The effect of long-term food restriction on tumors in rodents. *Int. J. Cancer*, 23, 803–807 (1979).

53) Birt, D. F., Pelling, J. C., White, L. T., Dimitroff, K. and Barnett, T. Influence of diet and calorie restriction on the initiation and promotion of skin carcinogenesis in the Seneca mouse model. *Cancer Res.*, 51, 1851–1854 (1991).

54) Gillette, C. A., Zhu, Z., Westerlind, K. C., Melby, C. L., Wolfe, P. and Thompson, H. J. Energy availability and mammary carcinogenesis: effects of calorie restriction and exercise. *Carcinogenesis*, 18, 1183–1188 (1997).

55) Osborne, M. P., Ruperto, J. F., Crowe, J. P., Rosen, P. P. and Telang, N. T. Effect of tamoxifen on proneoplastic cell proliferation in N-nitroso-N-methylurea-induced mammary carcinogenesis. *Cancer Res.*, 52, 1477–1480 (1992).

56) Taylor, I. W., Hodson, P. J., Green, M. D. and Sutherland, R. L. Effects of tamoxifen on cell cycle progression of synchronous MCF-7 human mammary carcinoma cells. *Cancer Res.*, 43, 4007–4010 (1983).

57) Sutherland, R. L., Green, M. D., Hall, R. E., Reddel, R. R. and Taylor, I. W. Tamoxifen induces accumulation of MCF-7 human mammary carcinoma cells in the G1/G0 phase of the cell cycle. *Eur. J. Cancer Clin. Oncol.*, 19, 615–621 (1983).

58) Osborne, C. K., Boldt, D. H., Clark, G. M. and Trent, J. M. Effect of tamoxifen on human breast cancer cell cycle kinetics: accumulation of cells in early G1 phase. *Cancer Res.*, 43, 3583–3586 (1983).