Decrease in size of azoxymethane induced colon carcinoma in F344 rats by 180-day fermented miso

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Abstract. The present study was designed to investigate the effects of fermented miso (fermented soybean paste) on the induction of colon tumors by azoxymethane (AOM) in male F344 rats. A total of 91 rats, 6 weeks of age, were divided into 5 groups and given weekly subcutaneous injections of AOM (15 mg/kg body wt) for 3 weeks. The animals were placed on diets one week before the first AOM dose: commercial normal control MF diet or a diet containing 10% 2-year, 180-day fermented, or 3-4-day fermented miso. There were no differences in body and organ weights, and no aberrant crypt foci (ACF) among carcinogen-treated groups at week 25. The rates of tumor incidence were 45%, 85%, 75% and 60% with the 2-year, 180-day, and 3-4-day fermented miso and MF, respectively, and those for colon tumors were 34%, 55%, 60% and 55%, respectively. The size of well-differentiated adenocarcinomas and total (well differentiated and signet ring cell) adenocarcinomas in the 180-day fermented miso group was significantly smaller than that in the 2-year fermented miso and MF+AOM groups. Nuclear staining of ß-catenin in colon tumors was increased for the 3-4-day fermented miso compared to the 180-day fermented miso. Cdx2 staining tendency was decreased in colon tumors and adenocarcinomas compared to normal mucosa and ACF, which stained in 100% of cases. In addition, the PCNA index was significantly reduced in the 180-day group compared with those groups receiving the 3-4-day fermented miso and MF diet. The germinal region was also decreased. The present results indicate that dietary supplementation with 180-day fermented dietary miso could act as a chemopreventive agent for colon carcinogenesis.

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Introduction

The incidence of breast and prostate cancer in Asian countries with a high consumption of soy and soy-based products is lower than the United States. However, the rates for these cancers have shown an increase in the second generation of families that migrate to America from these countries as their diet becomes westernized (1). Thus, environment (lifestyle) may have a greater influence than genetic background. Miso, a traditional ingredient of the Japanese diet, is fermented from soybeans, rice, wheat or oats, and its major constituents are vitamins, microorganisms, salt, minerals, plant proteins, carbohydrates, and fat. It is used on a daily basis to flavor food in Japan and other parts of Asia, and remains an essential ingredient for Japanese style cooking. We have described chemopreventive effects of miso against intestinal injury in X-irradiated mice (2), and it has also been reported to reduce the occurrence of liver (3,4) and gastric tumors (5), as well as the development of aberrant crypt foci (ACF) in experimental animals in a dose-dependent manner (6). However, the effects of soy beans and related foodstuffs on cancer risk are complex (7). Recently, our experimental studies provided evidence that long-term fermented miso is quite effective in aiding the recovery of stem cells in the small intestinal crypts after irradiation (8), and decreasing the development of azoxymethane (AOM)-induced ACF (9), gastric tumors induced by *N*-methyl-*N*'-nitro-*N*-nitroso-guanidine (10) and pulmonary adenocarcinomas induced by dipropanolnitrosamine (11). To determine whether soy beans alone or fermentation processes have a role, the present study was performed to assess the chemopreventive potential of samples after different periods of fermentation.

Materials and methods

Animals. Male F344/DuCrj rats, 5 weeks of age at commencement, were purchased from Charles River and housed five to a polycarbonate cage under a constant temperature of 24±2˚C and relative humidity of 55±10%, with a 12:12-h light-dark cycle. The animals were maintained according to the Guide for the Care and Use of Laboratory Animals established by Hiroshima University. All were given free access to a

commercial diet (MF; Oriental Yeast Co., Tokyo, Japan) alone or with added miso. Dry red miso after 2 years, 180 days or 3-4-days fermentation was supplemented using biscuits at 10% w/w (Miso Central Institute, Tokyo, Japan). Normal tap water was also provided *ad libitum*.

Carcinogen. AOM was purchased from Sigma Chemical Co. (St. Louis, MO).

Experimental procedure. A total of 91 rats were divided into 5 groups. Starting at 6 weeks of age, the initiated groups were given weekly subcutaneous injections of AOM (15 mg/kg body weight) for 3 weeks to induce colon tumors. One week before the first AOM dose, the rats were fed test and control diets, then sacrificed 25 weeks after the last AOM injection. Autopsy was performed under ether anesthesia at which time the body and major organ weights were measured.

Experimental groups. Animals in carcinogen-initiated groups 1-4 were fed diets containing 10% miso fermented for 2 years, 180 days and 3-4-days and MF diet, respectively, while control group 5 received only the MF diet.

Visualization and histological examination. Upon termination of the studies, each colon was removed, flushed with saline, longitudinally slit open from cecum to anus, placed on a paper towel and fixed in 10% buffered formalin for 24 h. Following the protocol cited by Magnuson and Bird (12), the fixed colons were stained with 0.5% methylene blue for 15-30 min, then placed on a glass slide with the luminal side up. Viewing the stained colons under a light microscope at a magnification of x20-30, the presence of ACF was assessed to determine the number of ACFs per colon, ACs per colon, and ACs per focus. Histological evaluation was performed using routine procedures with H&E staining. Tumors were classified into three types: microadenomas, adenomas and

Table I. Dietary intake and drinking water per day per rat.

Group	Diet (g/day/rat)	Water (ml/day/rat)
2-year fermented miso+AOM	12.2±0.7[c]	24.4±3.0[a,c]
180-day fermented miso+AOM	12.5±0.9[c]	23.1±3.2[a,c]
3-4-day fermented miso+AOM	12.4±0.9[c]	21.7±2.2[a,c]
MF+AOM	12.7±0.6[b]	16.4±2.5
MF control	13.4±1.0	17.2±3.1

Mean ± SD. [a]Significantly different from MF+AOM value ($P<0.01$). [b]Significantly different from MF value ($P<0.05$). [c]Significantly different from MF value ($P<0.01$).

adenocarcinomas, with the latter featuring displastic cells invading the muscularis mucosa or beyond (13).

Immunohistochemistry. An anti-PCNA antibody (Dako Co., Kyoto, Japan) was used with the avidin-biotin complex method for assessment of cellular proliferation. Tissue sections were deparaffinized with xylene, hydrated through a graded ethanol series and incubated with 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. They were incubated with 10% normal house serum at room temperature for 30 min to block background staining and with primary antibodies against ß-catenin (diluted 1:100; Transduction Laboratories, BD 610153, KY), Cdx2 (diluted 1:50; Biogenex CDX2-88) (14) or monoconolal mouse anti-proliferating cell nuclear antigen antibody (diluted 1:200; Dako-PCNA, PC 10, code no. M 879) for 1 h at room temperature. The locations of the nearest and farthest PCNA-positive cells from the bed of the crypt were defined as the base, and top, and the distance between them was measured as the germinal region (15). Cell numbers observed in single

Table II. Body and organ weights (relative weights).

Group	No. of rats	Body weight (g)	Liver (g)	Kidney (g)	Testis (g)	Spleen (g)
2-year fermented miso+AOM	20	360.7±19.00[b]	9.1±0.8[b] (2.52±0.13[b])	2.10±0.14 (0.58±0.03)	3.32±0.59 (0.92±0.16)	0.680±0.043 (0.19±0.01)
180-day fermented miso+AOM	20	371.7±26.62[a]	9.8±0.9[b] (2.64±0.16)	2.10±0.17 (0.57±0.03)	3.29±0.12 (0.89±0.06)	0.734±0.204 (0.20±0.07)
3-4-day fermented miso+AOM	20	373.1±21.80[a]	9.5±0.8[b] (2.56±0.15[b])	2.02±0.13[b] (0.54±0.03)	3.26±0.11 (0.88±0.05)	0.705±0.057 (0.19±0.02)
MF+AOM	20	365.8±17.30[b]	9.1±0.6[b] (2.49±0.15[b])	2.05±0.14[b] (0.56±0.03)	3.22±0.25 (0.88±0.07)	0.057±0.780 (0.24±0.21)
MF control	11	390.6±17.10	10.7±0.7 (2.74±0.12)	2.22±0.17 (0.57±0.04)	3.38±0.11 (0.87±0.04)	0.688±0.052 (0.18±0.01)

Mean ± SD. There are not significantly different among AOM groups. [a]Significantly different from MF value ($P<0.05$). [b]Significantly different from MF value ($P<0.01$).

Table III. Number of ACFs in macroscopic findings.

Group	No. of rats	ACFs	Total ACs	Mean of ACs
2-year fermented miso+AOM	19	118.5±47.6[b]	375.5±163.5[b]	3.2±0.4
180-day fermented miso+AOM	19	98.6±58.6[b]	316.3±194.8[b]	3.2±0.4
3-4-day fermented miso+AOM	19	114.6±47.6[b]	365.2±153.7[b]	3.2±0.4
MF+AOM	20	101.0±48.2[b]	298.6±148.9[b]	3.0±0.3
MF control	11	2.2±3.1	1.1±1.2	4.5±6.1

Mean ± SD. There was not significantly different among AOM groups. [a]Significantly different from MF value (P<0.05). [b]Significantly different from MF value (P<0.01).

Table IV. Incidence of tumors.

			Colon tumors in rats				
	Total no. of rats	Rats with tumors			Adenocarcinoma		
Group			Total	Adenoma	Differentiated	Signet	Duodenal tumor
2-year fermented miso+AOM	20	9 (45)	7 (35)	2 (10)	4 (20)	1 (5)	2 (10): 1 adenocarcinoma, 1 signet ring cell carcinoma
180-day fermented miso+AOM	20	17 (85)	11 (55)	5 (25)	6 (30)	0	8 (40): 1 adenoma, 6 adenocarcinoma, 1 signet ring cell carcinoma
3-4-day fermented miso+AOM	20	15 (75)	12 (60)	0	9 (45)	3 (15)	3 (15): 2 adenoma, 1 signet ring cell carcinoma
MF+AOM	20	12 (60)	11 (55)	2 (10)	7 (35)	2 (10)	2 (10): 1 adenocarcinoma, 1 signet ring cell carcinoma

half crypts, from the beds of crypts to the surface, were counted and converted into % values for bottom and top positions. Numbers of positively stained nuclei were counted and divided by the total number of nuclei to give the PCNA index (%). ß-catenin- and Cdx2-positive nuclei numbers were also counted and divided by the total epithelial cells in tumors to give indices for nuclear accumulation.

Statistics. Statistical significance was determined with the Dunnett's method for multiple comparisons using logarithmic transformation and the Student's t-test.

Results

General observations. Diet intake was significantly decreased in the AOM groups, and drinking water increased in the miso groups (Table I). Final body and organ weights are shown in Table II, with increased values for the MF group compared to the other groups. Average body and liver weights of rats in the AOM groups were significantly different compared with the MF groups. Kidney weights in the 3-day fermented miso and MF+AOM groups also differed significantly from the MF values, but no variation was evident for testis or spleen

weights. Relative liver weights (organ weight/body weight x100) in the 2-year and 3-day fermented miso and MF+AOM groups were significantly decreased compared with the MF group values. There were no differences in body and organ weights among carcinogen-treated groups at week 25.

Colonic ACF. Table III summarizes data for the mean numbers of ACF per colon, total number of AC per colon and mean number of AC per focus using surviving animals. All rats treated with AOM showed a 100% incidence. The average number of ACF per colon, and total AC number and number of AC per focus was not significant among carcinogen-treated groups.

Tumor induction. Three animals with duodenum signet cell carcinomas died 103, 149 and 163 days after the first AOM injection in miso groups. The total colon tumor incidence in the 2-year fermented miso group was decreased 45% compared with the control group, as shown in Table IV. Signet ring cell carcinomas in the large intestine were not found in the 180-day fermented miso group. Tumor size upon macroscopic observation in the 180-day fermented miso group was significantly decreased compared with the 2-year

Table V. Number of colon tumor per rat and tumor size.

Group	No. of colon tumors per rat	Mean tumor size (mm)	Differentiated adenocarcinoma size (mm)	Adenocarcinoma (differentiated + signet ring cell) size (mm)
2-year fermented miso+AOM	0.50±0.83	5.77±5.56	9.72±5.43[a]	11.40±6.37
180-day fermented miso+AOM	0.85±1.04	2.77±3.01[a,b]	3.56±2.56[a,c]	3.56±2.56[a,b]
3-4-day fermented miso+AOM	0.95±1.05	2.68±2.18[a,b]	3.66±2.45[a,c]	7.97±9.24
MF+AOM	1.10±1.30	4.32±3.81	6.86±3.38	7.26±3.51

Mean ± SD. [a]Significantly different from AOM value (P<0.05). [b]Significantly different from 2-year fermented miso+AOM value (P<0.05). [c]Significantly different from 2-year fermented miso+AOM value (P<0.01).

Table VI. Cell proliferation in colonic mucosa without tumors.

Group	No. of cells	No. of PCNA-positive cells	Labeling index	Lowest cell position	Highest cell position	Germinal region
2-year fermented miso+AOM	26.6±4.2[d]	5.6±2.6[d]	21.3±9.4[d]	2.8±2.4	16.1±4.7[d]	49.8±18.0[d]
180-day fermented miso+AOM	26.6±4.3[d]	4.8±2.4[d]	18.2±8.8[d]	3.2±2.4[c]	14.7±5.6[d]	42.8±22.6[d]
3-4-day fermented miso+AOM	28.3±5.9	11.2±4.7[b]	39.7±14.2[b]	2.5±2.9	20.1±4.9[b]	62.6±15.6[b]
MF+AOM	29.9±4.8[a]	10.4±3.9[b]	35.5±12.6[b]	2.3±1.7	20.2±4.4[b]	60.0±12.7[b]
MF	27.3±3.3	5.8±2.2	21.7±9.3	2.7±3.1	15.2±3.4	45.6±13.2

Mean ± SD. [a]Significantly different from MF value (P<0.05). [b]Significantly different from MF value (P<0.01). [c]Significantly different from MF+AOM value (P<0.05). [d]Significantly different from MF+AOM value (P<0.01).

Table VII. ß-catenin-positive tumors.

Group	Total cases	Negative	Nucleus	Cytoplasm	Nucleus + cytoplasm
2-year fermented miso+AOM	10	2 (20)	7 (70)	0	1 (10)
180-day fermented miso+AOM	21	11 (52)	6 (29)[a]	3 (14)	1 (5)
3-4-day fermented miso+AOM	21	8 (38)	13 (61)[a]	0	0
MF+AOM	20	9 (45)	11 (55)	0	0

[a]Significantly different (P<0.05).

fermented miso group (Table V). Upon microscopic observation, the size of differentiated adenocarcinomas and total adenocarcinomas (well-differentiated and signet ring cell) in the 180-day and 3-4-day fermented miso groups was significantly smaller than in the 2-year fermented miso and MF+AOM groups. Duodenum tumors in the 180-day fermented miso group showed a tendency to be increased.

Cell proliferation. PCNA-labeling indices for cell proliferation in the distal colon are given in Table VI. Significant variation in cell number for half crypts, numbers of PCNA-positive cells, highest position of cells and width of the germinal region in the 2-year and 180-day fermented miso groups were significantly decreased compared with the MF+AOM values.

ß-catenin staining. Some normal and tumor specimens were examined with ß-catenin immunohistochemistry. In normal colon mucosa, immunostaining revealed ß-catenin to be mainly localized at the cell membranes of surface epithelial cells. Cytoplasmic and nuclear immunostaining was observed in microadenomas, adenomas and adenocarcinomas, but not in ACFs. The cytoplasmic staining was homogeneous, while both homogeneous and heterogeneous patterns were noted for nuclei. The index or ß-catenin-positive nuclei in the 3-4-day fermented miso group was significantly larger than the 180-day fermented miso value (P<0.05) (Table VII).

Cdx2. Some normal and tumor specimens were examined with Cdx2 immunostaining. Generally, normal mucosa and

Table VIII. Cdx2 staining in colon tumors.

	Total cases	Positive	Negative
ACF	3	3 (100%)	0
Microadenoma	2	1 (50%)	1 (50%)
Adenoma	6	4 (67%)	2 (33%)
Differentiated adenocarcinoma	11	4 (36%)	7 (64%)
Signet ring cell carcinoma	4	1 (25%)	3 (75%)
Total	26	13	13

ACF were positive in all nuclei, and positive rates decreased through microadenomas and adenomas to adenocarcinomas (Table VIII). Negative staining in nuclei was elevated with the 3-4-day fermented miso (6/8) compared with the 180-day fermented miso (1/4).

Discussion

In the present study, dietary administration of 180-day fermented miso, but not 2-year or 3-4-day fermented miso, inhibited the development of AOM-induced colon adeno-carcinomas in the rat colon. In epidemiological studies, Watanabe et al reported that rectal tumors were significantly decreased by both intake of soy beans and tofu, but some authors have described no inhibition or even an increase associated with miso intake (16). Poole et al found rectal tumors to be decreased by tofu and soybean in the U.S. (17), while Hu et al reported reduced rectal, but not colon tumors in China (18). LeMarchard et al presented evidence of a gender-dependence, with tofu intake in Hawaii being linked to decreased colon tumors in females, but not in males (19). Adenoma of the sigmoid colon was decreased by miso soup, and colon tumors were decreased dose dependently by tofu and soy products in the U.S. or by isoflavone and soy protein in both males and females in Japan (19). Tuyns et al reported soybeans to be clearly protective against colon rectal cancers on the basis of a case control study in Belgium (20).

In animal experiments, however, Davies et al found no protection when 6-week-old male Fisher 344 rats were fed diets high in fat and low in calcium with soy protein with varying isoflavone contents one week before to 31 weeks post-AOM treatment, and soy high in isoflavones did not protect against the development of colon tumors in AOM-treated rats fed a high fat, high risk diet (21). Similarly, development of intestinal tumors was not prevented in the Min mouse model fed a Western diet (high fat, low fiber and calcium) with low and high isoflavones (22). In fact, Rao et al showed an increase in non-invasive and total adeno-carcinoma multiplicity in AOM-treated rats fed genistein in a casein-based diet (22), and McIntosh et al reported that the number of intestinal tumors was increased with diets containing soy protein compared to casein (23). Gee et al observed no suppression of ACF by genistein in the DMH model of colorectal carcinogenesis (24). However, the data of Hakkak et al (25) and Thiagarajan et al provide evidence

that soybeans and soy products can reduce the development of early stages of colon cancer in rats (26). Pereira et al also found purified genistein to inhibit induction of ACF (27). Several components of soy other than isoflavones have been previously reported to reduce chemically induced colon cancer, but the results are again contradictory. Isoflavones have been proposed as the most effective constituents for inhibition of mammary cancer, also possessing antiestrogenic activity (28-30). Gotoh and associates demonstrated that the administration of biochanin A, a genistein precursor, inhibits the development of rat mammary tumors (31). However, we previously found that it did not reduce colonic ACF induced by AOM in rats (32). Thus, inhibitory effects of miso against colorectal and mammary cancers may be due to different constituents.

We earlier found that prolonged fermentation might be important for protection against radiation, being associated with the prolongation of animal survival and decrease in toxicity to small intestinal crypts (8). It also was associated with decreased development of ACF (9), lung adenocarcinomas and gastric tumors (10) and pulmonary adenocarcinomas (11). Therefore, the fermentation process may produce cancer-protective substances. Yamamoto et al reported that the consumption of miso soup but not soyfood, such as soy beans, tofu, deep fried tofu and nattou, was inversely associated with the risk of breast cancer in women (33). Changes in isoflavone constitution during the fermentation of miso, i.e. conversion of glycosides to unconjugated forms that might be expected to reduce their anti-carcinogenic effects, have in fact been reported (34,35). Therefore, the effective substances may not be just isoflavones.

In the present study, 180-day fermented miso appeared linked to a significant decrease of the PCNA-positive index, which is in line with an earlier report that the BrdU labeling index with 10% miso supplementation was significantly lower than that of a normal diet group in AOM-treated rats, and the length of the germinal region and PCNA-positive index were reduced by a water soluble extract from cultured medium of *Ganoderma lucidum* (Rei-Shi) mycelia (15). Like calcium, which has been hypothesized to be a regulator of cell proliferation in the colon and inhibitor of experimental carcino-genesis (36), ingredients of miso after 180 days fermentation could act as preventive agents for colon carcinogenesis by suppressing proliferation.

On the other hand, miso has been revealed to contain nitrosamine precursors (37-39) that might result in endogenous formation of *N*-nitroso compounds in humans. Indeed, in another study it showed considerable mutagenicity after nitrite treatment (38,39). Home-made miso is frequently consumed, and people commonly store their products for long periods, often for ≥2 years (40), and this could conceivably increase nitorosamine precursors. Other unknown ingredients of miso could also naturally be related to cancer risk, and further comparative studies of ingredients after different periods of fermentation are clearly warranted.

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