Inhibition by Long-term Fermented Miso of Induction of Pulmonary Adenocarcinoma by Diisopropanolnitrosamine in Wistar Rats

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ABSTRACT

The present study was designed to investigate the effects of fermented miso in the diet on the development of lung tumors initiated by diisopropanolnitrosamine (BHP) in male Slc:Wistar rats. A total of 63 animals, 6 weeks of age, were divided into 4 groups and given BHP (2000 ppm) in their drinking water for 10 weeks. After the carcinogen treatment the rats were fed a normal control MF solid diet, or the same diet containing 10% long-term or short-term fermented miso for 12 weeks. The long-term fermented miso significantly reduced the number of lung tumors, adenocarcinomas and PCNA strongly positive tumors as compared with the short-term fermented miso. The present results thus indicate that dietary supplementation with long-term fermented miso could exert chemopreventive effects on lung carcinogenesis.

Key words: *Lung tumor inhibition, BHP, Long-term fermented miso, Wistar rats*

Miso is produced by the fermentation of soybeans, rice, wheat or oats and its major constituents are vitamins, enzymes, microorganisms, salts, minerals, plant proteins, carbohydrates and fat. It has traditionally been used in the daily diet as a flavoring for food in Japan and some other parts of Asia and is still one of the essential ingredients required for Japanese-style cooking. Recently there has been an increasing demand for so-called health foods, with primary prevention of cancer as one of the expected effects. Epidemiological studies in Okinawa[22] and Singapore[17] have indicated that fermented soybean products might have inhibitory effects on lung cancer. They have also been found to reduce the risk of liver tumors occurring spontaneously[8] or induced by neutron irradiation alone or in combination with diethylnitrosamine (DEN)[13]. Furthermore, inhibition has been reported for N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) induced gastric tumorigenesis[23,24], development of azoxymethane-induced aberrant crypt foci (ACF)[11], and dimethylbenz[a]anthracene[1]- or N-nitroso-N-methylurea (MNU)-induced rat mammary carcinogenesis[4,5]. 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 crypt after irradiation damage[14], and in decreasing the development of azoxylmethane-induced ACF[15] and also gastric tumors[16]. To determine whether soy beans themselves or the fermentation process may be the important factors, the present study was conducted to assess the effects of miso after long or short fermentation on the development of rat lung tumors after initiation with BHP.

MATERIALS AND METHODS

Animals

Sixty-three six-week-old male Slc:Wistar rats (SLC Japan In., Hamamatsu) were used in the present study. They were housed three or four to a polycarbonate cage and kept under constant conditions of temperature (24 ± 2°C) and relative humidity (55 ± 10%) with a 12hr light/12hr dark cycle. The animals were maintained under the guidelines set forth in the "Guidelines for the Care and Use of Laboratory Animals" established by Hiroshima University.

BHP (diisopropanolnitrosamine)

The carcinogen was purchased from Nacalai Tesque Co. Inc. Kyoto and dissolved in distilled water at a concentration of 2000 mg/liter just

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Fig. 1. Squamous cell carcinoma, × 100 HE staining

Fig. 2. PCNA heavy positive tumor, × 200

before use. This solution was given to rats ad *libitum* for 10 weeks from light-opaque bottles exchanged at 2 or 3 day intervals[20,21].

Diet. All rats were fed a commercial diet (MF; Oriental Yeast Co., Tokyo, Japan) alone or with added miso. Dry red miso after short- (3–4 days) or long-term fermentation (6 months) was supplemented into solid biscuits at 10% (Miso Central Institute, Tokyo, Japan). The diets were supplied with normal tap water *ad libitum* for 12 weeks.

Pathology

The animals were killed and autopsied if they became moribund, and all the remaining rats were sacrificed 22 weeks after the BHP treatment. The lungs were removed and the location and size of individual tumors were recorded. All tissues were fixed in 10% neutral formalin. Lesions in the lung were classified into five types: hyperplasia, adenomas, adenocarcinomas, papillomas and squamous cell carcinomas (Fig. 1).

Immunohistochemistry. Paraffin-embedded sections were deparaffinized in xylene, and rehydrated through graded alcohols. A 0.05M PBS buffer was used to prepare solutions and for washes between the various steps. Incubations were performed in a humidified chamber. Three-μm-thick sections were treated for 30 min at room temperature with 2% BSA and incubated with primary antibodies against β-catenin (diluted 1:100; Transduction Laboratories, BD 610153, KY) or monoclonal mouse anti-proliferating cell nuclear antigen antibody (Dako-PCNA, PC 10, code No. M 879) for 1 hr at room temperature. For each case, negative controls were performed on serial sections whereby incubation with the primary antibody was omitted. All slides were then exposed to the secondary antibody, biotinylated horse anti-universal-monkey IgG (Vectastain Universal Quick Kit, Vector Laboratories, Ca, Catalog No PK-8800) and peroxidase conjugated streptoavidin complexes. Peroxidase activity was visualized by
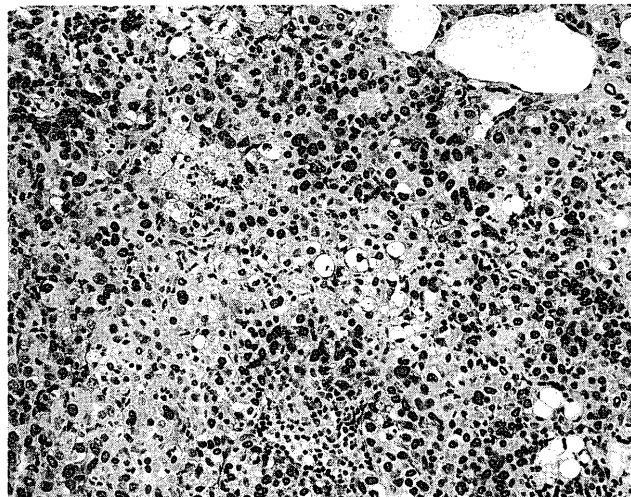
treatment with H_2O_2 and diaminobenzidine for 5 min. At the final step, the sections were counterstained with hematoxylin for 1 min. β-catenin-positive nuclei were counted and divided by the total epithelial cells in tumors to give an index for nuclear accumulation. The PCNA-strongly positive tumor cells were also counted (Fig.2).

Statistical significance

Statistical significance was determined with the Dunnett method for multiple comparisons, X^2 and Student t tests.

RESULTS

Intake of diet in the long-term miso group was significantly decreased as compared with the control value (Table 1). Body weight curves are shown in Fig. 3. Control body weights were significantly greater than in the BHP groups. The value for the short-term miso group was significantly decreased as compared with the BHP+MF group at 120 days and the day of autopsy (p < 0.05).

Only three animals died before the scheduled sacrifice. Liver, testis, and adrenal weights in the BHP groups and kidney weights in the miso groups were significantly decreased as compared with the control value (Table 2). Relative weights of liver and testis in the BHP groups were also significantly decreased, while those for kidney in the BHP and BHP+short cases and spleen in the

Table 1. Intake of diet

Group	Diet (g/day/rat)
Control	18.4 ± 0.8
BHP	16.6 ± 0.3
BHP+Short	17.0 ± 2.8
BHP+Long	16.0 ± 1.3*

mean ± SD
*: Significantly different from Control value (p < 0.05)

BHP+short group were significantly increased as compared with the control values (Table 3). There were no significant differences among the BHP treated groups.

Macroscopically, all animals had many lung tumors. The number in the long-term miso group was significantly decreased as compared to other groups (Table 4). Microscopically, all animals had

hyperplasia, adenomas and adenocarcinomas, and papillomas or squamous cell carcinomas were also evident in 13%, 27% and 29% of the animals in the BHP, BHP+short and the BHP+long groups, respectively (Table 5). Numbers of adenocarcinomas in the BHP+long and PCNA strongly positive tumors in the miso groups were significantly decreased as compared to the BHP alone group

Table 2. Organ weight (g)

Group	No.	B.W.	Organ weight				
			Liver	Kidney	Testis	Adrenal	Spleen
Control	23	495.2 ± 26.2	13.7 ± 1.1	2.4 ± 0.2	3.3 ± 0.3	0.043 ± 0.005	0.65 ± 0.09
BHP	8	448.3 ± 28.1**	11.2 ± 0.8**	2.4 ± 0.2	1.5 ± 0.4**	0.036 ± 0.004**	0.66 ± 0.07
BHP+Short	14	419.4 ± 28.7**,b	11.0 ± 0.6**	2.3 ± 0.1*	1.5 ± 0.5**	0.036 ± 0.004**	0.62 ± 0.10
BHP+Long	15	435.5 ± 33.3**	10.4 ± 1.1**	2.2 ± 0.2**,a	1.3 ± 0.2**	0.034 ± 0.005**	0.58 ± 0.06*

mean ± SD
**: Significantly different from Control value ($p < 0.01$)
*: Significantly different from Control value ($p < 0.05$)
a: Significantly different from BHP value ($p < 0.01$)
b: Significantly different from BHP value ($p < 0.05$)

Table 3. Relative organ weight (Organ weight/Body weight × 1000)

Group	No.	Liver	Kidney	Testis	Adrenal	Spleen
Control	23	27.7 ± 1.8	4.9 ± 0.4b	6.7 ± 0.6	0.086 ± 0.009	1.3 ± 0.2a
BHP	8	25.1 ± 1.4**	5.4 ± 0.4*	3.3 ± 1.0**	0.081 ± 0.010	1.5 ± 0.2
BHP+Short	14	26.3 ± 1.6*	5.5 ± 0.5**	3.6 ± 1.2**	0.087 ± 0.009	1.5 ± 0.2*
BHP+Long	15	24.0 ± 1.3**	5.0 ± 0.4	3.0 ± 0.4**	0.077 ± 0.010*	1.3 ± 0.1

mean ± SD
**: Significantly different from Control value ($p < 0.01$)
*: Significantly different from Control value ($p < 0.05$)
a: Significantly different from BHP value ($p < 0.01$)
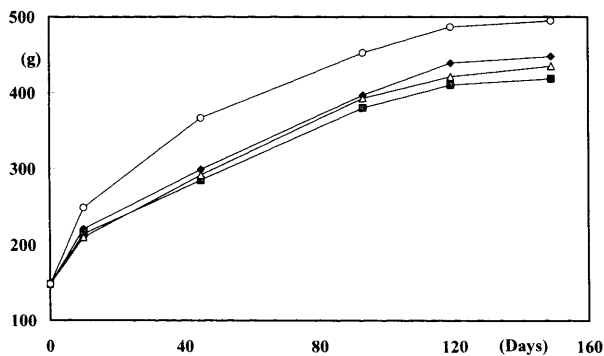b: Significantly different from BHP value ($p < 0.05$)

Fig. 3. Body weight
—◆— BHP
—□— BHP+Short
—▲— BHP+Long
—○— Control

Table 4. Number and size of tumors in macroscopic observation

Group	No.	Number	Size (mm)
BHP	8	148.5 ± 56.1	1.35 ± 0.26
BHP+Short	14	116.2 ± 32.0	1.45 ± 0.22
BHP+Long	15	106.5 ± 41.1*	1.47 ± 0.36

mean ± SD
*: Significantly different from BHP value ($p < 0.05$)

Table 5. Incidence of tumors (%)

Group	No.	Lung			Thyroid	Liver
		Adenoma	Adenocarcinoma	Papilloma/Squamous cell carcinoma		
BHP	8	8 (100)	8 (100)	1 (13)	8 (100)	0
BHP+Short	14	14 (100)	14 (100)	4 (29)	11 (78)	1 (7)
BHP+Long	15	15 (100)	15 (100)	4 (27)	10 (67)	0

Table 6. Number and size of lung tumors

Group	No.	No. of adenoma	No. of adeno-carcinoma	No of PCNA heavy positive tumors	Size of PCNA heavy positive tumors (μm)
BHP	8	62.63 ± 13.90	6.50 ± 3.46	21.00 ± 7.52	955 ± 609
BHP+Short	14	61.60 ± 19.00	6.33 ± 2.74	15.40 ± 6.60*	946 ± 668
BHP+Long	15	57.79 ± 24.88	2.93 ± 1.67**	11.71 ± 4.71**	822 ± 574

mean ± SD
*: Significantly different from BHP value (p<0.05)
**: Significantly different from BHP value (p<0.01)

(Table 6). A smaller size (0.5–1 mm) of tumor was significantly evident in the miso group as compared to the BHP alone group. However, the size of PCNA positive tumors was not significantly different among the groups. There was no β-catenin staining of nuclei or cytoplasm in any lung tumor.

Thyroid tumors in the miso groups showed a reduced tendency as compared to the BHP alone group. One liver tumor developed in a BHP+short animal.

DISCUSSION

In the present experiment, while tumor incidences did not significantly differ among the groups, lung tumor size and numbers of adenocarcinomas and PCNA strongly positive tumors in the animals treated with long-term fermented miso were significantly decreased. Soy foods contain significant amounts of the isoflavone, genistein, which has various biological and antitumorigenic effects[10,12], as well as antiestrogenic activities[3,18]. Koo et al reported a significant inverse association between tofu/soy intake and lung cancer in non-smokers in Hong Kong after adjustment for age, number of live births and schooling[9]. Significant protective effects for tofu were also reported by Swanson and colleagues in a study conducted in a mining community in Yunnan Province (China), which showed a dose-dependent inverse relationship with the risk of lung cancer[19]. Ershow et al also reported inverse associations of legumes, fruit, staple grains, and animal protein with lung cancer risk in a case-control study in China[2]. Changes in the isoflavone constitution during the fermentation of miso, i.e. conversion of glycosides to unconjugated forms, which might be expected to reduce their anti-carcinogenic effects, has been reported[7]. However, we found that prolonged fermentation might be very important for protection against radiation, being associated with the prolongation of animal survival, a decrease in toxicity to small intestinal crypts[14] and a decrease rate of ACF[15] and gastric tumors[16]. Therefore, the effective substances may not be isoflavones. To our knowledge there have been no reports regarding the effective substances in miso of different fermented stages and further study is now needed for their elucidation.

In the present experiment, papillomas or squamous cell carcinomas demonstrated a tendency to increase with miso treatment. Wakui et al concluded from their study that miso soup consumption was positively associated with the risk of squamous cell carcinoma in males[22], but Hirayama reported a lack of any association with lung cancer risk in Japan[6]. In the study by Wakui[22], risk alteration seemed to be large for squamous cell carcinoma, which is not consistent with Koo's findings[9]. Thus it is considered that miso may protect against adenocarcinomas of the lung but not against squamous cell carcinomas.

In conclusion, the results of the present study indicate that supplementation of the diet with fermented miso might be useful for lung tumor prevention. Further experiments are required to evaluate the effects of individual components, including minerals, in miso.

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