Dietary prevention of azoxymethane-induced colon carcinogenesis with rice-germ in F344 rats

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The modifying effect of dietary administration of defatted rice-germ and γ-aminobutyric acid (GABA)-enriched defatted rice-germ on azoxymethane (AOM)-induced colon carcinogenesis was investigated in two experiments with male F344 rats. In the first experiment (the pilot study), the effects of the defatted rice-germ, the GABA-enriched defatted rice-germ and rice-germ on AOM-induced (15 mg/kg body wt once a week for 3 weeks) formation of aberrant crypt foci (ACF) were examined. The latter two preparations (2.5% in the diet) significantly inhibited ACF formation (P < 0.005). In the second experiment, a long-term study of the effects of rice-germ was done. One group was treated with AOM alone, four groups received the carcinogen and were fed the diets containing 2.5% rice-germ or 2.5% GABA-enriched defatted rice-germ for 5 (initiation phase) or 30 weeks (post-initiation phase), two groups were treated with rice-germ or GABA-enriched defatted rice-germ alone and one group was kept on the basal diet. At the termination of the study, dietary exposure to rice-germ during the initiation phase significantly reduced the incidence of colonic adenocarcinoma (71 versus 29%, P < 0.01), GABA-enriched defatted rice-germ or rice-germ during the post-initiation phase also decreased the frequency of colonic adenocarcinoma (71 versus 20%, GABA-enriched defatted rice-germ feeding, P < 0.01; 27%, rice-germ feeding, P < 0.01). These data suggest that constituents of rice-germ are possible dietary preventatives for human colon cancers.

Introduction

Dietary factors play an important role in prevention of human diseases, including cancers (1,2). Experimental and epidemiological evidence suggests that increased dietary fiber is associated with a decreased risk of colon cancer, which is the third most malignant neoplasm in the world (3) and the second leading cause of cancer deaths in the USA. In Japan, the progressive introduction of Western dietary habits, especially an increasing fat intake and decreasing carbohydrate and dietary fiber intake, has increased colon cancer incidence (4).

Rice is the main cereal food as well as the staple food for the populations of Asian countries. It has been reported that rice components have several roles in prevention of disease. Rice seeds are known to contain antioxidative components, such as ferulic acid, phytic acid, tocopherols and oryzanols. Rice bran and rice-germ are major constituents of rice seeds (5,6). In spite of the assumption of a disease preventive potential of rice seeds, there has been no clear evidence for a cancer preventive potential of rice seed itself or the rice-germ. Saikura et al. (7) determined the distribution of free amino acids within the rice kernel and examined the effects of soaking in water on their contents for the purposes of better understanding the chemical nature of the rice kernel. They found that the γ-aminobutyric acid (GABA) content of rice-germ increases remarkably with soaking under slightly acidic conditions. GABA has been localized to endocrine-like cells of the rat antrum, small intestine and colon (8,9). However, the role of GABA in mucosal physiology is unclear. GABA has been found that the baclofen-sensitive GABA B sites. It has been reported that (15 mg/kg body wt once a week for 3 weeks) formation of formation of aberrant crypt foci (ACF) may be related to the gut via interaction with the role of GABA in mucosal physiology. 

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; GABA, γ-aminobutyric acid; PCNA, proliferating cell nuclear antigen.
Materials and methods

Animals and diet

Male F344 rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan), 4 weeks old, were used. All animals were housed in wire cages (3 or 4 rats/cage) with free access to drinking water and CE-2 basal diet (CLEA Japan Inc., Tokyo, Japan) under controlled conditions of humidity (50 ± 10%), lighting (12 h light/dark cycle) and temperature (23 ± 2°C). They were quarantined for 2 weeks and randomized into experimental and control groups for the pilot study and the long-term bioassay. Powdered CE-2 diet (345.2 g) was used as the basal diet throughout the study.

Chemicals

AOM was obtained from Sigma Chemical Co. (St. Louis, MO). Defatted rice-germ, rice-germ and GABA-enriched defatted rice-germ were supplied by Oryza Oil and Fat Chemical Co. (Ichinomiya, Japan). Samples of 100 g of the rice-germ, defatted rice-germ and GABA-enriched defatted rice-germ contained ~14, 32 and 335 mg GABA, respectively. AOM (15 mg/kg body wt) was given by s.c. injection between 10:00 and 11:00 a.m. The defatted rice-germ, rice-germ and GABA-enriched defatted rice-germ were mixed in the powdered basal diet (CE-2) at a concentration of 2.5%. These experimental diets were prepared weekly and stored in a cold room (<4°C) until use.

Experimental procedure

In the first experiment (the pilot study), 69 rats were divided into eight groups as shown in Figure 1a. Groups 1–4 received three weekly s.c. injections of AOM at a dose of 15 mg/kg body wt. Rats in group 1 were fed the basal diet alone and those in groups 2–4 were given the diets containing 2.5% defatted rice-germ, 2.5% GABA-enriched defatted rice-germ and 2.5% rice-germ, respectively, for 5 weeks, starting 1 week before the first dosing with AOM. Groups 5–7 were given the experimental diets alone. Group 8 was an untreated control. At week 5, all animals were killed and the colons were fixed in 10% buffered formalin and stained with a 0.2% methylene blue solution for analysis of ACF.

For the second experiment (the long-term study), a total of 102 rats were randomly divided into eight groups. Groups 1–5 received three weekly s.c. injections of AOM (15 mg/kg body wt). Rats in groups 2 and 3 were fed the diets containing 2.5% GABA-enriched defatted rice-germ and 2.5% rice-germ, respectively, starting at 5 weeks of age and continued until 2 weeks after the last injection of AOM. Groups 4 and 5 were fed the basal diet mixed with 2.5% GABA-enriched defatted rice-germ and 2.5% rice-germ, respectively, for 30 weeks during the post-initiation phase, starting 2 weeks after the last injection of AOM. Groups 6 and 7 did not receive AOM and were fed diets mixed with the rice-germ preparations during the study (35 weeks). Group 8 served as an untreated control. The experimental diets were stored in a dark cold room (<4°C) until used and provided in food pots. All rats were carefully observed daily and weighed weekly until they reached 14 weeks of age and then every 4 weeks. Consumption of the experimental diets was also recorded to estimate intake of test compounds. The experiment was terminated at 35 weeks after the start and all animals were killed by an ether overdose to assess the incidences of preneoplastic and neoplastic lesions in all organs, including large bowel.

At autopsy, the intestine was excised, opened longitudinally, flushed clean with saline and examined for the presence of tumors. Abnormal lesions of other organs were also examined histologically. Colon, after fixation in 10% buffered formalin for at least 24 h. Fixed colon sections were dipped in a 0.2% solution of methylene blue in distilled water for 30 s, then briefly washed with distilled water. Using a light microscope at a magnification of ×40, ACF were distinguished by their increased size, their more prominent epithelial cells and their increased...
Inhibition of colon cancer by rice-germ

Table I. Effect of various types of rice-germ on AOM-induced ACF formation in male F344 rats (a pilot study)

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment</th>
<th>No. of animals</th>
<th>Body weight (g)</th>
<th>Liver weight (g)</th>
<th>Total no. ACF/colon</th>
<th>Total no. ACF/cm²</th>
<th>Total no. ACs/colon</th>
<th>Total no. ACs/focus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AOM alone</td>
<td>14</td>
<td>206 ± 11</td>
<td>9.8 ± 0.8</td>
<td>93 ± 20</td>
<td>8.0 ± 1.8</td>
<td>2.10 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>AOM + defatted rice-germ</td>
<td>13</td>
<td>206 ± 10</td>
<td>10.2 ± 0.7</td>
<td>74 ± 13</td>
<td>6.1 ± 1.4</td>
<td>150 ± 23</td>
<td>2.03 ± 0.12</td>
</tr>
<tr>
<td>3</td>
<td>AOM + GABA-enriched defatted rice-germ</td>
<td>13</td>
<td>209 ± 13</td>
<td>10.5 ± 1.0</td>
<td>63 ± 19b</td>
<td>4.8 ± 1.3b</td>
<td>133 ± 40b</td>
<td>2.02 ± 0.16</td>
</tr>
<tr>
<td>4</td>
<td>AOM + rice-germ</td>
<td>13</td>
<td>208 ± 11</td>
<td>10.7 ± 0.9</td>
<td>40 ± 5b</td>
<td>2.9 ± 0.3b</td>
<td>83 ± 9b</td>
<td>1.85 ± 0.17b</td>
</tr>
<tr>
<td>5</td>
<td>Defatted rice-germ</td>
<td>4</td>
<td>209 ± 6</td>
<td>10.3 ± 0.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>GABA-enriched defatted rice-germ</td>
<td>4</td>
<td>214 ± 11</td>
<td>10.6 ± 1.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>Rice-germ</td>
<td>4</td>
<td>217 ± 11</td>
<td>11.5 ± 0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>No treatment</td>
<td>4</td>
<td>205 ± 7</td>
<td>10.3 ± 0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

ACs, aberrant crypts.

Mean ± SD.

Significantly different from group 1 (bP < 0.001 and cP < 0.005).

pericryptal space compared with surrounding normal crypts. The number of ACF observed per colon, the number of aberrant crypts observed in each focus and the location of each focus were recorded. After scoring, colons were processed for measurement of the PCNA-positive cell index and for histological examination.

PCNA immunohistochemistry

Anti-PCNA antibody (Dako Co., Kyoto, Japan) was used with the avidin–biotin complex method. Immunohistochemical staining was performed according to the method in a previous paper (23). 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 then incubated with 10% normal horse serum at room temperature for 30 min to block background staining and then stained with anti-PCNA antibody. To determine the PCNA-positive index, 15 full-length crypts were examined. The numbers and positions of PCNA positively stained nuclei in each crypt column were recorded. The cell position was determined by dividing the crypt into left and right segments. The first basal cells in each segment was considered ‘position 1’. Numbers of positively stained nuclei were counted and divided by the total number of nuclei to give the PCNA-positive index (%). The scorer was unaware of the group to which the specimens belonged.

Polyamine levels

In the pilot study, polyamine levels in the colonic mucosa were assayed by the method of Koide et al. (24). At autopsy, the colons for measurement of polyamine level were immediately removed, slit open longitudinally and freed of all contents. The colonic mucosa was scraped with a bladed knife and stored at −70°C.

Statistical methods

Fisher’s exact probability test, Student’s unpaired t-test or Welch’s t-test was used for statistical analyses. A value of P < 0.05 was considered significant.

Results

The pilot study

In the pilot study there were no significant differences in body weight gains in all groups. The mean liver weights did not significantly differ among the groups. The daily intakes of diets with or without rice-germ preparations were between 15.1 and 15.4 g/rat. During the study (5 weeks) no clinical signs of toxicity were observed in any group. Histologically, there were no toxic changes in liver, kidney, lung and heart of the rats in all groups. As shown in Table I, colonic ACF were recognized only in rats treated with AOM (groups 1–4). The total number of ACF/colon, number of ACF/cm² colon and total number of aberrant crypts (ACs)/colon of groups 3 or 4 were significantly smaller than of group 1. The number of AC/Focus of group 4 was also significantly smaller than of group 1 (P < 0.005 and P < 0.001, respectively). In this experiment, polyamine levels of colonic tissues in groups 3 and 4 tended to be lower than of group 1, although the difference (Figure 2) was not significant.

General observations in the long-term study

Food intake of groups 2–7 did not differ from that of groups 1 and 8, which were fed the basal diet without the rice-germ preparations (data not shown). In this study, dietary administration of two rice-germ preparations caused no clinical signs of toxicity, low survival, poor condition or histological changes suggesting toxicity in the liver, kidney and lung. The body weight gains of rats treated with AOM and fed the rice-germ preparations (groups 2 and 3) were comparable throughout the experimental period (Figure 3). The body weight gains of rats fed the experimental diets after AOM treatment (groups 4 and 5) were slightly lower than those fed basal diet.

Incidence, multiplicity and distribution of intestinal neoplasms

Macroscopically, most tumors developed in the large intestine (mainly in the middle and distal colon) although some did form in the small intestine of rats in groups 1–5. They were sessile or pedunculated tumors and histologically tubular adenomas, adenocarcinomas or signet ring cell carcinomas, with a higher incidence of adenocarcinoma. A few rats had...
Table II. Incidence of intestinal neoplasms of rats in each group (a long-term study)

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment</th>
<th>No. of animals</th>
<th>Entire intestine</th>
<th>Small intestine</th>
<th>Large intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total AD ADC</td>
<td>Total AD ADC</td>
<td>Total AD ADC</td>
</tr>
<tr>
<td>1</td>
<td>AOM alone</td>
<td>20</td>
<td>16(80)</td>
<td>4(20)</td>
<td>15(75)</td>
</tr>
<tr>
<td>2</td>
<td>AOM + GABA-enriched defatted rice-germ</td>
<td>14</td>
<td>8(57)</td>
<td>4(29)</td>
<td>6(43)</td>
</tr>
<tr>
<td>3</td>
<td>AOM + rice-germ</td>
<td>14</td>
<td>6(43)</td>
<td>2(14)</td>
<td>4(29)</td>
</tr>
<tr>
<td>4</td>
<td>AOM → GABA-enriched defatted rice-germ</td>
<td>15</td>
<td>6(40)</td>
<td>3(20)</td>
<td>6(40)</td>
</tr>
<tr>
<td>5</td>
<td>AOM → rice-germ</td>
<td>15</td>
<td>3(20)</td>
<td>1(7)</td>
<td>5(33)</td>
</tr>
<tr>
<td>6</td>
<td>GABA-enriched defatted rice-germ</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>Rice-germ</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>No treatment</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

AD, adenoma; ADC, adenocarcinoma.

a No. of rats with neoplasms (%).
b,c,d Significantly different from group 1 by Fisher’s exact probability (b \( P < 0.05 \), c \( P < 0.01 \), d \( P < 0.02 \)).

Mean Body Weight (g)

Fig. 3. Body weight gains of each group in the long-term study.

renal mesenchymal tumors and/or altered hepatocellular foci in groups 1–5, but these lesions were not found in other groups. Animals in groups 6–8 did not have any neoplasms in the organs examined, including the intestine. The incidences and multiplicities of intestinal neoplasms are shown in Tables II and III. In general, the values of groups 2–5 were small compared with those of group 1. Statistically, the incidences of tumors in the entire intestine of groups 3 and 4 were significantly lower than of group 1 (\( P < 0.05 \) and \( P < 0.02 \), respectively). Furthermore, the incidences of large intestinal tumors in groups 3–5 were lower than in group 1 (\( P < 0.01 \) and \( P < 0.05 \), respectively).

As to the multiplicity of colonic carcinomas (number of carcinomas/rat), a significant reduction was found in rats fed the rice-germ preparations (groups 4 and 5) when compared with group 1 (\( P < 0.01–0.05 \)). The values for the large intestines of groups 2–5 were smaller than of group 1.

**PCNA-positive index**

The results on PCNA-positive index in the colonic mucosal epithelium are shown in Figure 4. PCNA-positive indices with the rice-germ preparations (groups 3 and 4) during or after AOM injection were significantly smaller than that of rats exposed to AOM alone (\( P < 0.05 \)). Supplementation with the rice-germ preparations produced a downward shift in the proliferative region of the crypt compared with crypts exposed to AOM alone (\( P < 0.02 \)).

**Discussion**

Feeding of rice-germ and GABA-enriched defatted rice-germ significantly decreased the development of ACF (number of ACF/colon), as revealed by quantification of ACF in the pilot study. These results suggest that rice-germ and GABA-enriched defatted rice-germ could inhibit the growth of colonic ACF and suppress progression of preneoplasia to malignant neoplasms but that defatted rice-germ had no inhibitory effects. These observations suggest that rice-germ and GABA-enriched defatted rice-germ may be an important class of dietary cancer preventives and that further investigations of effects of dietary rice-germ on carcinogenesis would be worthwhile. Our data suggest that rice-germ and GABA-enriched defatted rice-germ are new dietary preventive agents against colon cancer development.

The effects of dietary rice-germ and GABA-enriched defatted rice-germ on AOM-induced colonic ACF indicate that this short-term marker of colon carcinogenesis may be useful in screening chemopreventive agents (25) of colon tumorigenesis. This lesion has been suggested to be the premalignant lesion of chemically induced colon cancer. However, it would probably be prudent to use tumor incidence as the end-point for definitive investigations because there are many sites at which chemopreventive compounds may affect tumorigenesis (14). Though rice-germ and GABA-enriched defatted rice-germ had an inhibitory effect on AOM-induced ACF, rice-germ may have blocking and suppressing effects on AOM-induced colon carcinogenesis when fed in the diet during the initiation and post-initiation phases. GABA-enriched defatted rice-germ may have solely suppressing effects on AOM-induced colon carcinogenesis compared with rice-germ.
Inhibition of colon cancer by rice-germ fig. 4. PCNA-positive cell ratio in the colonic mucosa.

Several dietary factors are known to modulate carcinogenesis in humans (26) and rodents (27). The results in the present study confirmed the epidemiological data suggesting that the consumption of dietary fiber and, particularly, mixtures of soluble and insoluble fibers are inversely related to cancer risk, including colon cancer (28,29). In this experiment it may be that water-soluble fibers were important as the active principle from the two types of rice-germ agents. It has been established that short chain fatty acids can stimulate growth in the alimentary canal (18). Phytic acid (inositol hexaphosphate) is an abundant plant constituent, constituting 1–5% by weight of edible legumes, cereals, oil seeds and nuts. The modifying effects of phytic acid on carcinogenesis have been investigated in several experiments (15,30,31). Although most of these experiments have been directed to the effectiveness of phytic acid in wheat bran, we used rice-germ. A diet high in wheat bran, which inhibits colorectal carcinogenesis (32), is known to lower the increased proliferation of crypt cells caused by AOM treatment. Similar results have been reported for other possible dietary cancer preventive agents and other natural materials. 

Table III. Multiplicity of intestinal neoplasms of rats in each group (a long-term study)

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment</th>
<th>No. of animals</th>
<th>Entire intestine</th>
<th>Small intestine</th>
<th>Large intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total</td>
<td>AD</td>
<td>ADC</td>
</tr>
<tr>
<td>1</td>
<td>AOM alone</td>
<td>20</td>
<td>1.10 ± 0.79a</td>
<td>0</td>
<td>1.10 ± 0.79</td>
</tr>
<tr>
<td>2</td>
<td>AOM + GABA-enriched defatted rice-germ</td>
<td>14</td>
<td>1.00 ± 1.04</td>
<td>0</td>
<td>1.00 ± 1.04</td>
</tr>
<tr>
<td>3</td>
<td>AOM + rice-germ</td>
<td>14</td>
<td>0.71 ± 0.99</td>
<td>0</td>
<td>0.71 ± 0.99</td>
</tr>
<tr>
<td>4</td>
<td>AOM → GABA-enriched defatted rice-germ</td>
<td>15</td>
<td>0.40 ± 0.51b</td>
<td>0</td>
<td>0.40 ± 0.51b</td>
</tr>
<tr>
<td>5</td>
<td>AOM → rice-germ</td>
<td>15</td>
<td>0.80 ± 0.77</td>
<td>0.20 ± 0.40</td>
<td>0.60 ± 0.80</td>
</tr>
<tr>
<td>6</td>
<td>GABA-enriched defatted rice-germ</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>Rice-germ</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>No treatment</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

AD, adenoma; ADC, adenocarcinoma.

a Mean ± SD.
b Significantly different from group 1 (P < 0.01, cP < 0.05).
substances (37). Increased cell proliferation is suggested to play an important role in multistage carcinogenesis (38), including colon tumorigenesis (36). Zheng et al. (39) have also reported a better correlation of ACF with ability to reduce PCNA labeling index in ACF than with reduction in the size of the proliferative component in ACF in rats. As for the effects of rice-germ preparations, several other mechanisms may also operate. The type of fiber may influence the profile, the shift from propionate to butyrate observed in animals fed on hydrolyzed guar being suggested to be of importance to carcinogenesis. (40). These rice-germ preparations may have produced a decrease in pH in both the cecum and colon, as increased acidity may be a cause of proliferation (41) and effects on pH have been demonstrated with both soluble and non-soluble types of fiber, correlated with fermentability but only loosely linked to proliferation (42).

Thus, in the present study inhibitory effects of rice-germ and GABA-enriched defatted rice-germ on AOM-induced colon tumorigenesis paralleled suppression of cell proliferation in colonic crypts. Therefore, it is possible that a significant anticancer property of these rice-germ preparations may be partly due to their antiproliferative effects on carcinogen-exposed crypts. Although the exact mechanism(s) of the chemopreventive effects and constituents of rice-germ need to be determined, the evidence described here warrants further research on the modifying effects of the constituents of rice-germ on colon cancer. The modifying action of ferulic acid in a long-term bioassay is now on-going in our laboratory.

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References


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