Inhibitory Effects of the Extract of Soy Protein Fermented with Lactic Acid Bacteria and Yeasts on 1,2-Dimethylhydrazine-Induced Colon Cancer and Aberrant Crypt Foci of Mice

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We investigated the influence of the extract of soy protein fermented with lactic acid bacteria and yeast (ESFL) on 1,2-dimethylhydrazine (DMH)-induced colon cancer and aberrant crypt foci (ACF) in ICR female mice. In all the experiments, 8-week old mice were given subcutaneous DMH injections at 35 mg/kg once a week. In the experiment for colon cancer, mice were divided into groups A, B and C, with 20 mice each, and groups A and B were given DMH-injections for 10 weeks. Group C were injected with only PBS. Group A were fed a 5%-ESFL diet and groups B and C were fed normal mouse diets throughout the experiment for 35 weeks. Group A showed a 28.5% inhibition in incidence and a 59.1% inhibition in average number of tumors compared to group B, but the results were not statistically significant.

In ACF experiments, mice were divided into 4 groups, 10%-ESFL group, 1%-ESFL group, 0.1%-ESFL group, and a control with 10 mice each. All the mice were given DMH injections for 4 weeks. ESFL-supplemented diets were given throughout the experiment for 8 weeks. ACF formation decreased in all the ESFL-fed groups in a dose-dependent manner and the results were statistically significant in the 10%-ESFL group (69% inhibition, p < 0.001) and 1%-ESFL group (40% inhibition, p < 0.05) compared to the control. The present study suggests that ESFL could contain an effective anticarcinogenic substance(s).

Key words: ESFL; DMH; colon cancer; ACF

INTRODUCTION

Colon cancer, which was rare in Japan, has been increasing recently among Japanese people, and the increase is attributed to the growing consumption of animal fat in the modern Japanese diet (19, 34). A traditional Japanese diet contains not only little animal saturated fat but employs many soy foods, such as miso (fermented soybean paste), natto (fermented soybean), soy sauce and tofu (soy curd).

There are many reports that fermented soy foods have a preventive effect on cancer. An epidemiological study showed a strong association between consumption of miso soup and a reduction of gastric cancer mortality (12). Miso prevented N-nitroso-N-methylurea-induced mammary cancer in rats (8) and soy sauce inhibited benzo[a]pyrene-induced mouse forestomach neoplasia (4, 13, 21). With regard to colon cancer, reports on the preventive effect of miso on azoxymethane-induced aberrant crypt foci (ACF) in rats have recently been published (17, 22). Therefore, the lower amount of animal fat and the higher amount of fermented soy foods in a traditional Japanese diet may partly explain the reason why colon cancer was seen less in Japan than in Western countries.

Miso and soy sauce are mainly made of soybeans which are fermented with fungi, yeasts and lactic acid bacteria (LAB). The preventive effects of both soybeans and LAB on colon cancer have been also demonstrated in many reports. In epidemiological studies, soy protein was associated with a low risk of colon cancer in Asian populations (1). In animal models, soy protein protected against azoxymethane-induced colon cancer (3, 9). LAB, including Bifidobacteria, also have preventive effects on colon cancer, which were mostly examined in animal models (7, 28, 30).

In Japan, there is a soy protein product designated “the extract of soy protein fermented with LAB and yeasts (ESFL).” The product is categorized and sold as a health food, and is widely consumed among health-conscious people in Japan. The major component of the product is a soy protein medium cultured with LAB and yeasts. Because ESFL is made of soy protein fermented with LAB and yeasts, it is naturally expected
Table 1. Composition of the extract of soy protein fermented with lactic acid bacteria and yeasts.a

<table>
<thead>
<tr>
<th>General analysisb,c</th>
<th>Free amino acidsd,e</th>
<th>Organic acidd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>92.7</td>
<td>2-Oxoglutaric acid</td>
</tr>
<tr>
<td>Protein</td>
<td>1.5</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Fat</td>
<td>0.1</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>4.0</td>
<td>Alanine</td>
</tr>
<tr>
<td>Fiber</td>
<td>0.4</td>
<td>Total amino acids</td>
</tr>
<tr>
<td>Minerals</td>
<td>1.7</td>
<td>Total organic acids</td>
</tr>
</tbody>
</table>

a The sample is a supernatant obtained from ESFL by centrifugation at 3000 rpm for 20 min, and was analyzed in routine methods.

b mg/100 ml

c pH: 4.5

d mg/100 ml

e Major amino acids are presented.

to have a preventive effect on colon cancer.

We report in the present paper the inhibitory effects of ESFL on 1,2-dimethylhydrazine (DMH)-induced colon cancer and ACF in mice.

MATERIALS AND METHODS

Animals. Eight-week-old ICR female mice were purchased from Charles River Japan (Yokohama, Japan) and were kept under controlled conditions, 25 ± 2°C room temperature and 50 ± 10% humidity with a 12 hr light-dark cycle throughout the experiments. Mice were freely fed the prepared diets and water was given ad libitum.

Preparation of experimental diets. A crude ESFL was provided by Kigen Bio-Institute (Nagano, Japan). The crude ESFL was a culture medium mainly composed of 2% (w/v) of defatted soy protein powder which was fermented with Lactobacillus casei subsp. rhamnosus IFO 12521, Lactobacillus fermentum IFO 3071, Leuconostoc mesenteroides IFO 3426, Saccharomyces cerevisiae AHU 3035, IFO 0304 and IFO 0877 for 5 days. Then, the culture was processed in Techno-Sakaki Laboratory of Kigen (Nagano, Japan) for the present study. Namely, the culture was autoclaved at 115°C for 20 min to stop further fermentation, and was centrifuged at 1000 rpm for 5 min in order to remove any remnants of crude materials and heat-coagulated protein from the culture. The supernatant was used as an ESFL in this study which inevitably contained heat-killed LAB and yeasts. ESFL requires several additional procedures to make a final product for sale, i.e. addition of excipient, but it is essentially the same as the culture supernatant mentioned above. The composition of ESFL is shown in Table 1. ESFL was added to commercial mouse diets (CMF, Oriental Yeast, Tokyo, Japan) at concentrations of 10, 5, 1 and 0.1% (v/w).

Experimental design. 1,2-Dimethylhydrazine (DMH, Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) was dissolved in 0.5 ml of sterilized PBS at 35 mg/kg body weight, and then the solution was subcutaneously (sc) injected once a week.

For the colon cancer experiment, 60 ICR mice were divided into 3 groups, groups A, B and C, with 20 mice in each group. From groups A and B, 40 mice were injected with the DMH solution for 10 weeks. Group C were injected with only PBS. A 5%-ESFL supplemented diet was given group A from the day of the first injection, day 0, until the day of sacrifice. Groups B and C were given a normal mouse diet, CMF. Body weights of mice were checked weekly and they were sacrificed 35 weeks after day 0. The colon was dissected, cut lengthwise, and the contents were removed. The number of tumors were counted, and the width and length of each tumor was measured with a micrometer. All the mice which had died during the experimental period were excluded from analysis. Lesions of tumors were stained with hematoxylin and eosin for histology.

For the experiment of aberrant crypt (AC) foci (ACF), 40 ICR mice were divided into 4 groups: 10% group, 1% group, 0.1% group and a control one. All the mice were given DMH injections sc once a week for four weeks. The 10%, 1% and 0.1% groups were respectively given 10%, 1%, and 0.1% ESFL-supplemented diets. The control group was given only CMF. The diets were fed to the mice from the start to the end of the experiment, and mice were sacrificed 8 weeks after day 0, based on our preceding experiments. The colon was dissected, cut open lengthwise, pinned on a cork board to be fixed with 10% formalin/PBS overnight, and stained with 0.2% methylene blue/PBS for 5 min. AC
Fig. 1. Experimental design.
(Top; experiment for colon cancer) ICR mice were injected sc with DMH (35 mg/kg) or PBS once a week for 10 weeks and sacrificed 35 weeks after day 0. An ESFL-supplemented diet at 5% or CMF were given the mice throughout the experiment. (Bottom; experiment for ACF) ICR mice were injected sc with DMH (35 mg/kg) once a week for 4 weeks and sacrificed 8 weeks after day 0. Diets supplemented with ESFL at 10, 1, 0.1% or CMF were given the mice throughout the experiment.

Fig. 2. Time courses of average body weights of mice in the experiment for colon cancer.
The average body weights in Group A were bigger than those in Group B from the 20th week to the end but there was no significant difference among the 3 groups throughout the experiment.

and ACF were counted through a microscope at a magnification of 100 x.

Time schedules for the experiments are shown in Figure 1.

Statistics. All the data were statistically analyzed. We used Tukey’s HSD test following ANOVA, Student’s t-test and χ² test. A p < 0.05 was considered as being significant.

RESULTS

1. Inhibitory Effect of ESFL on Colon Cancer

Figure 2 shows changes in body weights of the mice
Table 2. Weights of body and main organs of mice in the experiment on DMH-induced colon cancer.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>n</th>
<th>Body (g)</th>
<th>Liver (g)</th>
<th>Spleen (g)</th>
<th>Kidneys (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>ESFL + DMH</td>
<td>14</td>
<td>40.8 ± 6.1</td>
<td>1.77 ± 0.25</td>
<td>0.195 ± 0.083</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>B</td>
<td>CMF + DMH</td>
<td>15</td>
<td>38.5 ± 6.1</td>
<td>1.77 ± 0.28</td>
<td>0.253 ± 0.150*</td>
<td>0.48 ± 0.05</td>
</tr>
<tr>
<td>C</td>
<td>CMF + PBS</td>
<td>20</td>
<td>41.6 ± 5.8</td>
<td>1.87 ± 0.26</td>
<td>0.145 ± 0.040</td>
<td>0.50 ± 0.05</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD.
Data were statistically evaluated by ANOVA and Tukey's HSD test.
*p < 0.05 to group C.

Table 3. Effects of ESFL on DMH-induced colon cancer.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>n</th>
<th>Number of tumor-bearing animals</th>
<th>Incidence (%)</th>
<th>Total number of tumors</th>
<th>Tumors/total animals</th>
<th>Tumors/tumor-bearing animals</th>
<th>Average length of tumors (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>ESFL + DMH</td>
<td>14</td>
<td>6</td>
<td>42.9</td>
<td>11</td>
<td>0.79 ± 1.05</td>
<td>1.83 ± 0.75</td>
<td>4.78 ± 1.31</td>
</tr>
<tr>
<td>B</td>
<td>CMF + DMH</td>
<td>15</td>
<td>9</td>
<td>60.0</td>
<td>29</td>
<td>1.93 ± 2.34</td>
<td>3.22 ± 2.22</td>
<td>4.60 ± 2.07</td>
</tr>
<tr>
<td>C</td>
<td>CMF + PBS</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.80 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD.
There was no statistically significant difference between A and B groups by Student’s t-test or χ² test.

Fig. 3. Histology of colonic tumors.
Well-differentiated tubular adenocarcinoma (A) from group A (ESFL + DMH), well-differentiated tubular adenocarcinoma (B) from group B (CMF + DMH) and normal colon mucosa (C) from group C (CMF + PBS). Hematoxylin-eosin. Original magnification × 10 (A) (B) and × 25 (C).

during the experiment. The body weights showed no apparent difference between groups A and B until the 19th week but group A showed higher weight gain compared to group B from the 20th week to the last week, with no statistical significance. According to our previous data, feeding of ESFL at this percentage did not affect the body weight of mice (data not shown). Table 2 expresses general observations of the experiment. The average spleen weight of group B was significantly higher than that of group C (p < 0.05). Table 3 expresses the suppressive effects of ESFL on colon cancer. The ratio of incidence of groups A and B were 42.9% (6/
2. Dose-Dependent Inhibitory Effect of ESFL on ACF

Figure 4 shows dose-dependent inhibition of ESFL on DMH-induced ACF and AC in ICR mice. Those inhibitory effects were statistically significant. Average numbers of total ACF and total AC per colon in the 10% group were 16.8 ± 8.5 (p < 0.001 to control) and 37.1 ± 19.7 (p < 0.001 to control), respectively. The results from the 1% group were 32.3 ± 13.1 (p < 0.05 to control) and 64.8 ± 22.4 (p < 0.05 to control), 39.1 ± 13.0 and 80.8 ± 28.3 in the 0.1% group, and 53.5 ± 23.0 and 122.2 ± 54.5 in the control, for ACF and AC, respectively. The inhibitory rates using the average ACF and AC numbers from the 10% group and the control were 69% ((1-16.8/53.5) x 100) and 70% ((1-37.1/122.2) x 100), respectively, and those of the 1% group compared to the control were 40% ((1-32.3/53.5) x 100) and 47% ((1-64.8/122.2) x 100), respectively. The number of ACF with 1–3 ACs per focus and with ≥4 ACs per focus showed the same trend as the results of the total ACF and AC. Average numbers of ACF with more than 4 ACs per focus were 1.80 ± 1.02 in 10% group, 1.88 ± 1.39 in 1% group, 2.0 ± 1.78 in 0.1% group, and 5.58 ± 3.41 in control group, respectively (p < 0.05 between 10% group and control).

DISCUSSION

ESFL had inhibitory effects on both colon cancer and ACF induced with DMH in mice, and the effect in the latter was dose-dependent and statistically significant. ESFL was a soy protein culture fermented with LAB and yeasts, and it contained heat-killed LAB and yeast. Thus, the function of ESFL could be a combination of the functions of soy protein, other substances extracted from soybeans such as isoflavones and saponin, the components of heat-killed LAB and yeast, and the products produced through the process of fermentation.

Genistein, which is one of the aglycones of soy iso-
flavonoids, is a representative factor among soy isoflavones for preventing cancer (19), and the amount is said to be higher in fermented soy foods than in soybeans (6). Genistein acts as an inhibitor to some enzymes which are responsible for cell proliferation, such as tyrosine-specific protein kinase (33) and DNA topoisomerase II (16). Genistein exists as a β-glycoside conjugate, genistin, in soybeans and the genistin is extracted along with soy protein when the soy protein is extracted from soybeans. Genistin is usually turned into genistein by microbes having β-glucosidase in the intestines, and then it is absorbed into a body. This process appears during the fermentation of miso and soy sauce by fungi, which have strong β-glucosidase. During the preparation for the culture medium of ESFL, genistin is expected to be transferred into the medium but it is unknown whether genistin is turned into genistein in the process of the fermentation of ESFL because no fungi are used during the process. However, Matsuda et al. reported that some strains of LAB had ability to convert genistin to genistein and Lactobacillus casei subsp. rhamnosus IFO 12521, which is the one of the LABs used for ESFL, was one of them (18). Thus, there is a possibility that genistin extracted from soybeans may have been turned into genistein in ESFL and the genistein may have played a partial role in the cancer prevention seen in our study.

Bowman-Birk inhibitor (BBI) is also abundant in soybeans and it prevents DMH-induced colon tumors in mice (32, 36). However, BBI should be excluded as a candidate as a functional substance in ESFL because BBI is easily inactivated by heat and the ESFL used in this study was autoclaved at 115°C for 20 min.

Many reports using animal models have demonstrated that oral intake of LAB prevents colon cancer and several mechanisms for the effect have been proposed. Bifidobacterium longum prevents colon cancer by improving the environment of gastrointestinal tracts through interaction with the microflora (31). There has been no direct evidence that LAB intake reduces colon cancer by enhancing the gut-associated immune system. However, Pierre et al. showed that short chain fructo-oligosaccharides prevented colon tumors in C57BL-6-Apc (Min/+), mice (27), and that T cells in gut-associated lymphoid tissue were activated by the intake of fructo-oligosaccharides. The authors suggested that the activated T cells may have influenced the formation of colon cancer from surveying colon mucosa of mice. Although their study did not directly prove that the enhancement of the T cells resulted from an increase in LAB, it is highly possible that the intake of short chain fructo-oligosaccharides increased LAB in the intestines of mice, which would have resulted in the reduction of colon cancer.

As another mechanism for LAB to prevent colon cancer, LAB may excrete mutagens from food by physically binding them together (23). LAB in ESFL are dead. Thus, heat-killed bodies of LAB may have influenced colon cancer and ACF formation by activating the gut-associated immune system of mice and/or by excreting potential promoters in the intestines from their bodies.

Substances produced during fermentation have been drawing attention to other anticarcinogens in foods, as well as LAB and soy isoflavones. Melanoidins (MEL) are a brown pigmentary substance produced by Maillard reactions in the process of heating or aging foods. Fermented soy foods such as miso are rich in MEL and its anticarcinogenic activity has been reported (38). MEL scavenges active oxygen (10), and act as a trypsin inhibitor (11).

Soy peptides are another factor. Soy proteins are digested into peptides during fermentation and their antioxidative and immunostimulating effects have been reported (20, 39). Other functional substances, which are still unknown, are expected to exist in fermented foods.

The major purpose of the present study was to observe whether ESFL would have inhibitory effects on colon cancer in an animal model, and our aim was accomplished affirmatively. ESFL also significantly suppressed the formation of ACF in a dose-dependent manner.

ACF assay has been performed to assess functional substances for colon cancer prevention in many cases (5, 29, 37). Although ACF seems to be regarded as a useful biomarker for colon cancer, it is still controversial whether ACF really are preneoplastic lesions in the colon. However, a view that the large type of ACF with more than 4 crypts per focus represents the early events in colon carcinogenesis seems to be widely accepted (2, 15, 26) and several researchers have recently provided new evidence supporting the hypothesis that ACF represent preneoplastic lesions in the colon (14, 24, 25, 35). In our present study, we showed that ESFL was effective in the prevention of both colon cancer and ACF. Therefore, we regard ACF assay as a useful technique to evaluate the role of functional substances in foods toward cancer prevention, especially considering the fact that the assay is not time-consuming.

We have not yet proved whether ESFL contains the functional substances mentioned above. Thus, our next
aim is to identify the substance(s) responsible for the inhibitory effects on colon cancer and ACF and to understand the mechanism(s) for the inhibition.

Finally, several mice died within 24 hr after the first injection of DMH. The event was observed in both the cancer experiment and the ACF one. Although the dose was decided on the basis of our pre-experiments, the administration of DMH must have been slightly overdone. Several mice died during the experiment on colon cancer and we excluded the dead ones from the evaluation. Although all the mice which had died during the colon experiment underwent autopsy, we failed to prove that colon cancer was definitely the cause of the death because of the rapid putrefaction of the gut.

All of those problems will be addressed in our next study.

REFERENCES


