Original

Preventive Effects of Powdered Broccoli Sprout on Azoxymethane-induced Rat Colonic Aberrant Crypt Foci

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Abstract: The modifying effects of dietary feeding of powdered broccoli sprout (PBS), which contained a high concentration of sulforaphane, on the development of azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF) were investigated in male F344 rats. We also determined the influence of PBS on proliferating cell nuclear antigen (PCNA) index in “normal-appearing” crypts from rats treated with AOM and/or PBS, and activities of detoxifying enzymes of cytochrome P450 (CYP) and glutathione *S*-transferase (GST) in the liver. Rats were given two weekly subcutaneous injections of AOM (20 mg/kg body wt) to produce ACF. They also received experimental diets containing PBS at a dose of 20 or 100 ppm for 4 weeks, starting 1 week before the first dose of AOM. AOM exposure induced a substantial number of ACF (106 ± 10) at the end of the study (week 4). Dietary administration of PBS caused significant reduction in the frequency of ACF: 20 ppm PBS, 56 ± 11 (47% reduction, $P < 0.001$) and 100 ppm PBS, 64 ± 23 (40% reduction, $P < 0.001$). Immunohistochemically, PBS administration at both doses significantly lowered the PCNA index in “normal-appearing” crypts. In addition, feeding with PBS significantly reduced the activities of CYP 2B and CYP 3A, and slightly increased GST activity in the liver. These findings suggest a possible chemopreventive ability of PBS through alterations to the activities of cell proliferation in cryptal cells in the colon and activities of drug metabolizing enzymes in the liver. (*J Toxicol Pathol* 2004; 17: 119–126)

Key words: broccoli sprout, aberrant crypt foci, inhibition, rat colon

Introduction

Cancer death is an important public health concern around the world. Despite advances in early detection, and basic and clinical trials of promising new therapies, morbidity and mortality of cancer remain major factors in public health¹. Among the cancers, colorectal cancer is a leading cause of cancer deaths in the United States and is increasing in Asia^{2,3}. The dietary intake of agents preventing cancer or dietary recommendations is probably of great importance in cancer prevention⁴. A large number of epidemiologic and animal studies imply that consumption of higher levels of vegetables and fruits reduces the risks of several types of cancer, including colon cancer^{5–8}. Many potentially anticarcinogenic agents are found in food sources⁶, and particularly cruciferous vegetables are associated with a reduced incidence of cancer^{9–12}.

Broccoli is a cruciferous vegetable which contains some potentially anticarcinogenic phytochemicals such as carotenoids, phenols, indoles, and isothiocyanates. Sulforaphane is one of the isothiocyanates and is the main isothiocyanate found in broccoli sprout. There are several studies showing its chemopreventive potential for carcinogenesis in the prostate¹³, mammary¹⁴, stomach¹⁵, and colon^{16,17}. Broccoli also has other active constituents, namely indole-3-carbinol, chlorogenic acid, protocatechuic acid, and β -carotene. Broccoli sprout is an especially good source of the chemoprotective, glucosinolate, which is a precursor to isothiocyanate¹⁸, and is found in quantities 20–50 times greater than in mature broccoli¹⁹. Although the exact role of each compound that contributes to the protective effect needs to be identified, it is of interest to investigate the beneficial effects of broccoli, especially these of broccoli sprout on carcinogenesis.

Cytochrome P450 (CYP) isoenzymes are one major kind of phase I enzymes and play an important role in the oxidation of chemical compounds, often resulting in the formation of highly reactive compounds that are ultimately carcinogens²⁰. Phase II enzymes, including glutathione *S*-transferase (GST), are responsible for catalyzing the

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biotransformation of a variety of electrophiles, and have a central role in the detoxification of activated metabolites of procarcinogens produced by phase I reactions. Thus, phase II detoxifying enzyme inducers are considered to be promising chemopreventive agents against cancer²¹.

In the current study, we investigated the possible inhibitory action of powdered broccoli sprout (PBS), which contained a high concentration of sulforaphane, in azoxymethane (AOM)-induced rat colonic aberrant crypt foci (ACF), putative precursor lesions of colon adenocarcinoma²². We also evaluated the proliferating cell nuclear antigen (PCNA) index to assess whether PBS modifies cell proliferation activity in the cryptal cells. In addition, we assayed the effect of PBS on CYP and GST activities in the liver.

Materials and Methods

Animals, chemicals, and diets

Male F344 rats (Charles River Japan, Inc., Tokyo, Japan) aged 5 weeks were used. The animals were maintained at Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. They were housed in plastic cages (4 rats/cage) with free access to tap water and diet, under controlled conditions of humidity ($50 \pm 10\%$), lighting (12-h light/dark cycle), and temperature ($23 \pm 2^\circ\text{C}$). They were quarantined for 7 days and randomized by body weight into experimental and control groups. AOM for ACF induction was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Powdered MF diet (Oriental Yeast, Co., Ltd., Tokyo Japan) was used as basal diet throughout the study. Powdered broccoli sprout (*Oryza Oil & Fat Chemical Co., Ltd., Ichinomiya, Japan*) is a powder in which sulforaphane is concentrated in high content by machining broccoli (*Brassica oleracea var. botrytis*) sprouts. The composition of PBS is listed in Table 1. The experimental diet was made by mixing PBS (20 or 100 ppm) with the powdered basal MF diet using a rocking mixer (RM-10-2, Aichi Electric Co., Ltd., Kasugai City, Japan).

Experimental procedure for ACF assay

A total of 32 male F344 rats were divided into four experimental groups and a control group (Fig. 1). Animals in groups 1 through 3 were initiated with AOM by two weekly subcutaneous injections (20 mg/kg body weight). Rats in groups 2 and 3 were fed diets containing 20 and 100 ppm PBS for 4 weeks, respectively, starting one week before the first dosing with AOM. Group 4 did not receive AOM and were given the diet containing 100 ppm PBS. Group 5 served as an untreated control. Rats were sacrificed under ether anesthesia at week 4 to assess the occurrence of colonic ACF. They underwent careful necropsy, with emphasis on the colon, liver, kidney, lung, and heart. All grossly abnormal lesions in any tissue, and the organs such as the liver (caudate lobe), kidney, lung and heart were fixed in 10% buffered formalin.

Table 1. Composition of PBS (100 g)

Moisture	2.2 g
Protein	45.2 g
Fat	12.5 g
Fiber	21.2 g
Ash	5.4 g
Sulforaphane	1.2 g
Indole-3-carbinol	0.08 g
Vitamin A	0.0005 g
Others	12.2 g

Others include sugar, minerals, and unknown compounds.

Fig. 1. Experimental protocol.

□ : Basal diet, □ : 20 ppm PBS, ■ : 100 ppm PBS,
 ↑ : AOM, 20 mg/kg bw s.c., × : Sacrifice.

Determination of ACF

The frequency of ACF was determined according to the method described in our previous report²³. At necropsy, the colons were flushed with saline, excised, cut open longitudinally along the main axis, and then washed with saline. They were cut and fixed in 10% buffered formalin for at least 24 h. Fixed colons were dipped in a 0.5% solution of methylene blue in distilled water for 30 sec, briefly washed with the distilled water, and placed on a microscope slide for counting ACF.

PCNA immunohistochemistry

Immunohistochemical staining for PCNA was performed by the avidin-biotin complex method (Vecstain Elite ABC Kit, Vector, Burlingame, CA). Tissues were fixed in 10% buffered formalin for at least 24 h, embedded in paraffin blocks, and 3 μm -thick paraffin-embedded sections were prepared. After that, they were deparaffinized with xylene, hydrated through a graded ethanol series, immersed in 0.3% hydrogen peroxide in absolute methanol for 30 min at room temperature to block endogenous peroxidase activity, and then washed in phosphate-buffered saline (pH 7.2). Following incubation with normal rabbit serum at room temperature for 10 min to block background staining, the sections were incubated with an anti-PCNA antibody (mouse monoclonal PC10, 1:100 dilution: Dako Co., Kyoto) for 12 h in a humidified chamber at room temperature. They

Table 2. Body, Liver, and Relative Liver Weights and Food Consumption

Group no.	Treatment (no. of rats examined)	Terminal			Mean intake of food/day/rat (g)
		Body wt (g)	Liver wt (g)	Relative liver wt (g/100 g body wt)	
1	AOM alone (8)	212 ± 7 ^a	10.7 ± 1.0	5.07 ± 0.32	12.0 ± 1.2
2	AOM + 20 ppm PBS (8)	211 ± 7	10.7 ± 1.2	5.06 ± 0.46	12.3 ± 1.9
3	AOM + 100 ppm PBS (8)	218 ± 6	11.3 ± 0.7	5.21 ± 0.26	12.4 ± 1.4
4	100 ppm PBS (4)	223 ± 13	10.5 ± 0.6	4.69 ± 0.16	14.5 ± 3.4
5	No treatment (4)	214 ± 7	10.7 ± 0.6	4.97 ± 0.20	12.9 ± 1.9

a: Mean ± SD.

were then reacted with 3,3'-diaminobenzidine and lightly counterstained with Harris' hematoxylin. For determination of the PCNA-positive index, 10 full-length crypts of each colon were examined. The number and position of PCNA positively stained nuclei in each crypt column were recorded and expressed as the PCNA-positive index (number of positive stained nuclei × 100/total number of nuclei counted). The observer was unaware of the group to which the specimens belonged.

Measurement of drug metabolizing enzymes in rat liver

The liver without caudate lobe was rinsed in cold 0.9% NaCl, homogenized in ice-cold 50 mM Tris buffer (pH 7.4) containing 0.15 M KCl to yield a 10% (wt/vol) homogenate, and then centrifuged at 10,000 × g for 60 min at 4°C. The supernatant (cytosol fraction), after discarding any floating lipid layer and appropriate dilution, was used for enzyme assays.

The concentration of total amount of CYP and cytochrome *b*₅ were quantified by the method of Omura and Sato²⁴. NADPH-cytochrome P450 reductase activity was measured by the method of Phillips *et al.*²⁵. For measurement of activities of CYP 1A1, CYP 1A2, and CYP 2B levels using ethoxyresorufin *O*-deethylase, methoxyresorufin *O*-demethylase, and pentoxyresorufin *O*-depentylase as substrate, respectively^{26,27}. *P*-Nitrophenol hydroxylase or testosterone 6β-transferase activities were used to determine CYP 2E1²⁸ or CYP 3A²⁹, respectively.

The cytosolic GST activity was determined spectrophotometrically at 340 nm with 1-chloro-2,4-dinitrobenzene as a substrate according to the procedure of Habig *et al.*³⁰

Statistical evaluation

Where applicable, data were analyzed using Student's *t*-test or Welch's *t*-test with *P*<0.05 as the criterion for significance.

Results

General observation

Body, liver, relative liver weights (g/100 g body weight), and food consumption (g/day/rat) in all groups are shown in Table 2. All animals remained healthy throughout the experimental period. Food consumption (g/day/rat) did not differ significantly among the groups. At the end of the study, there were no significant differences in the mean weights of body and liver, and the relative liver weights among the groups.

ACF Analysis

Table 3 summarizes the data on colonic ACF formation (Fig. 2a). All rats belonging to groups 1 through 3, which were given AOM, developed ACF. The mean number of ACF/colon in group 1 (AOM alone) was 106 ± 10. The dietary administration of 20 ppm (group 2) or 100 pm (group 3) PBS significantly reduced the number of ACF compared to group 1: 56 ± 11 (47% reduction, *P*<0.001) in group 2 and 64 ± 23 (40% reduction, *P*<0.001) in group 3. The percentages of ACF consisting of more than 4 crypts in group 2 (2.2 ± 1.4, *P*<0.001) and group 3 (2.2 ± 1.8, *P*<0.001) were significantly smaller than that of group 1 (9.2 ± 1.3). In groups 4 and 5, there was no microscopically observable change, including ACF, in colonic mucosa.

PCNA-labeling index in "normal-appearing" and normal colonic crypts

The PCNA-labeling indices in "normal-appearing" crypts from rats treated with AOM and/or PBS and normal colonic crypts from untreated rats are presented in Table 4 and Fig. 2 b–d. Dietary feeding with PBS significantly reduced the mean PCNA-labeling indices (%): group 2, 18.8 ± 3.2, *P*<0.01; and group 3, 14.4 ± 4.8, *P*<0.001 in "normal-appearing" crypts: vs. group 1, 24.8 ± 4.6. Feeding with 100 ppm PBS alone (group 4) did not affect the PCNA-labeling index in normal crypts of rats, when compared with an untreated control group (group 5).

Table 3. Effect of PBS on AOM-induced ACF Formation in Male F344 Rats

Group no.	Treatment (no. of rats examined)	Incidence (%)	Total no. of ACF/colon	% of ACF containing 4 or more ACs
1	AOM alone (8)	8/8 (100%)	106 ± 10 ^a (100%)	9.2 ± 1.3 (100%)
2	AOM + 20 ppm PBS (8)	8/8 (100%)	56 ± 11 ^b (53%)	2.2 ± 1.4 ^b (24%)
3	AOM + 100 ppm PBS (8)	8/8 (100%)	64 ± 23 ^b (60%)	2.2 ± 1.8 ^b (24%)
4	100 ppm PBS (4)	0/4 (0%)	0	0
5	No treatment (4)	0/4 (0%)	0	0

ACF, aberrant crypt foci.

a: Mean ± SD. b: Significantly different from group 1 by Welch's *t*-test or Student's *t*-test ($P < 0.001$). **Fig. 2.** Morphology of ACF and PCNA immunohistochemistry. a: ACF consisted of 2 and 9 aberrant crypts on methylene-blue-stained colonic mucosa of a rat in group 1; b: PCNA immunohistochemistry of "normal appearing" crypts of a rat from group 1; c: PCNA immunohistochemistry of "normal appearing" crypts of a rat from group 2; and d: PCNA immunohistochemistry of "normal appearing" crypts of a rat from group 3. Original magnifications, a × 4; and b–d × 20

Table 4. PCNA-labeling Index in Rectal Section of Colonic Mucosa

Group no.	Treatment (no. of rats)	“Normal-appearing” crypts	Normal crypts
1	AOM alone (8)	24.8 ± 4.6 ^a	—
2	AOM + 20 ppm PBS (8)	18.8 ± 3.2 ^b	—
3	AOM + 100 ppm PBS (8)	14.4 ± 4.8 ^c	—
4	100 ppm PBS (4)	—	11.5 ± 2.4
5	No treatment (4)	—	11.5 ± 1.3

a: Mean ± SD.

b, c: Significantly different from group 1 by Welch's *t*-test (b: *P*<0.01, and c: *P*<0.001).**Table 5.** Measurement of Drug Metabolizing Enzymes in Liver

Group No.	1 (AOM)	2 (AOM + 20 ppm PBS)	3 (AOM + 100 ppm PBS)	4 (100 ppm PBS)	5 (no treatment)
Cytochrome P450 (nmol/mg)	0.39 ± 0.04 ^a	0.32 ± 0.02 ^b	0.40 ± 0.07	0.49 ± 0.04 ^c	0.35 ± 0.08
Cytochrome <i>b</i> ₅ (nmol/mg)	0.33 ± 0.03	0.34 ± 0.02	0.35 ± 0.03	0.4 ± 0.02	0.35 ± 0.04
NADPH- cytochrome P450 reductase (nmol/min/mg)	152 ± 8	143 ± 22	161 ± 9	170 ± 13	149 ± 17
EROD (pmol/min/mg)	90 ± 15	61 ± 3 ^b	86 ± 10	70 ± 12	75 ± 13
MROD (pmol/min/mg)	31.2 ± 4.6	22.7 ± 8.5	33.1 ± 4.0	24.4 ± 4.8	30.9 ± 4.7
PROD (pmol/min/mg)	7.4 ± 1.2 ^d	4.46 ± 0.56 ^e	4.59 ± 0.81 ^f	5.05 ± 0.93	3.71 ± 0.95
PNPH (nmol/min/mg)	0.66 ± 0.06	0.77 ± 0.16	0.83 ± 0.16	0.92 ± 0.20	0.77 ± 0.30
6β-OH (nmol/min/mg)	1.58 ± 0.10 ^g	0.63 ± 0.09 ^h	0.59 ± 0.03 ^h	0.58 ± 0.08	0.48 ± 0.07

EROD, Ethoxyresorufin *O*-deethylase, CYP1A1; MROD, Methoxyresorufin *O*-demethylase, CYP1A2; PROD, Pentoxyresorufin *O*-depentylase, CYP2B; PNPH, *p*-Nitrophenol 2-hydroxylase, CYP2E1; 6β-OH, Testosterone 6β-hydroxylase, CYP3A.

a: Mean ± SD.

b, e, f, h: Significantly different from group 1 by Welch's *t*-test or Student's *t*-test (b: *P*<0.05, e: *P*<0.005, f: *P*<0.01, and h: *P*<0.001).c, d, g: Significantly different from group 5 by Welch's *t*-test or Student's *t*-test (c: *P*<0.05, d: *P*<0.005, and g: *P*<0.001).

Alterations of the CYP and GST in the liver

Table 5 summarizes the content of CYP or cytochrome *b*₅ and the activity of NADPH-cytochrome P450 reductase in the liver. Dietary feeding with 20 ppm PBS significantly reduced the content of CYP (nmol/mg): group 2, 0.32 ± 0.02 vs. group 1, 0.39 ± 0.04 (*P*<0.05). A significant increase of CYP was detected in group 4 (0.49 ± 0.04, *P*<0.05) when compared to group 5 (0.35 ± 0.08). There were no significant differences among the groups for cytochrome *b*₅ and the activity of NADPH-cytochrome P450 reductase in the liver. Dietary feeding of PBS influenced the activities of CYP (Table 5). CYP 1A1 (pmol/min/mg) in group 2 (61 ± 3, *P*<0.05) was significantly lower than that of group 1 (90 ± 15). The value of CYP 2B (pmol/min/mg) in group 1 (7.4 ± 1.2, *P*<0.005) was significantly greater than that of group 5 (3.71 ± 0.95). The values in groups 2 (4.46 ± 0.56, *P*<0.005) and 3 (4.59 ± 0.81, *P*<0.01) were significantly smaller than that of group 1. AOM exposure significantly elevated CYP 3A activity (1.58 ± 0.10 nmol/min/mg, *P*<0.001) when compared to group 5 (0.48 ± 0.07). The treatment with PBS at both doses together with AOM significantly lowered this increase (group 2, 0.63 ± 0.09, *P*<0.001; and group 3, 0.59 ± 0.03, *P*<0.001) when compared with group 1. For activities

of CYP 1A2 and CYP 2E1, there were no significant differences among the groups.

For GST (μmol/min/mg), the activity of group 1 (0.90 ± 0.11) was significantly greater than that of group 5 (0.73 ± 0.04) (*P*<0.05, Fig. 3). Feeding with the diets containing PBS at both doses resulted in slight increases, when compared to the AOM alone group, and a significant difference was found between group 3 (0.95 ± 0.06) and group 5 (*P*<0.005).

Discussion

The results described here clearly indicate that dietary administration of PBS, which contains a high amount of sulforaphane, significantly reduced AOM-induced ACF formation in male rats. Also, rats fed the diets containing PBS showed no adverse effects on food consumption, growth rate, and no histological alterations in organs. These findings suggest that dietary PBS may suppress the early phase of chemically induced colon carcinogenesis.

Some explanations for the inhibitory effects of PBS on ACF-formation by AOM are considered. Cell proliferation has long been suspected to play a significant role in the

Fig. 3. Liver cytosolic GST activity.

initiation step as well as the promotion/progression of carcinogenesis^{31,32}. In the present study, the PCNA-labeling indices of “normal-appearing” crypts in the rectal section of colonic mucosa were significantly decreased by dietary administration of PBS. Thus, the inhibitory effect of PBS might be, in part, due to modification of cell proliferation activity in the cryptal cells. The apoptosis inducing effect of sulforaphane on HT-29 human colon cancer cell was recently reported¹⁷. This activity may also contribute to the inhibition of ACF formation, although we did not determine the apoptotic index in the colon.

CYP is known to play a prominent role in the modulation of xenobiotic metabolism including carcinogenic agent. CYP 2E1 is one of the important factors for converting AOM to methylazoxymethanol which can promote DNA adduct formation and follow the initiation event^{33,34}. Unexpectedly, feeding of PBS did not affect the activity of CYP 2E1 in the current study.

Dietary broccoli or isothiocyanate exposure modulates a variety of CYP activities in the liver, colon, and kidney of rats^{35,36}. Maheo *et al.*³⁷ reported that sulforaphane decreased the activities of CYP 1A1 and 2B1/2 in rat hepatocytes. In the current study, the enzyme activities of CYP 1A1, 2B and 3A were decreased by feeding with PBS. Thus, alterations in CYP 1A1 and 2B activities might be caused by highly concentrated sulforaphane in PBS. There are few reports describing the association between these types of CYP and AOM-induced colon carcinogenesis, to our knowledge. However, we recently found that the reduction effect of extract of *Ginkgo biloba* leaves on the activity of liver CYP 3A is related to its chemopreventive effect on AOM-induced

rat colonic ACF³⁸. Also, the amount of CYP 2B in FAO cells (rat hepatocyte cell line) was decreased by treatment with dexamethasone³⁹, which can inhibit the growth of colon carcinoma cell⁴⁰. These findings suggest that downregulation of CYP 2B and/or 3A might cause the reduction of AOM-induced ACF found in the current study.

There are some reports describing that dietary broccoli sprouts and their component glucosinolates and isothiocyanates induce phase II detoxication enzymes and this contributes to protection against chemical carcinogenesis in rodents^{41–43}. Unfortunately, in the current study, a slight increase of GST activity was observed after PBS feeding. We suggest that the GST system was not involved in the inhibitory effect of PBS on ACF development in this study.

In the current study, we investigated the modifying effects of PBS on early stage rat colon carcinogenesis at two doses, and our results on the inhibitory potency of PBS in ACF formation were not dose-dependent. Also, the influence of PBS at two doses on CYPs and GST activities and PCNA-labeling measured in this study were comparable. These results may indicate that feeding PBS at a dose of 20 ppm was enough to reduce colonic ACF development.

In conclusion, the findings described here demonstrate that dietary administration of PBS containing concentrated sulforaphane significantly inhibited the development of AOM-induced colonic ACF. Although the exact mechanisms by which PBS inhibits colonic ACF remain to be elucidated, it would be worthwhile to test the cancer chemoprevention ability of PBS in the colon with a long-term carcinogenesis model.

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