

Chemoprevention of Colorectal Cancer by Grape Seed Proanthocyanidin Is Accompanied by a Decrease in Proliferation and Increase in Apoptosis

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Abstract: Effects of proanthocyanidin (PA), procyanidin B-2 (B-2), and epigallocatechin gallate (EGCG) on azoxymethane (AOM)-induced colonic preneoplastic aberrant crypt foci (ACF) formation were investigated using F344 rats. The numbers of total ACF in rats treated with 0.002% PA and 0.05% B-2 were significantly decreased compared with the AOM alone group (control). Cell proliferation in the colon, as shown by proliferating cells nuclear antigen (PCNA), was also reduced in those treatments. The single-stranded DNA (ssDNA) labeling index, a marker for apoptosis, was significantly increased in 0.002% PA and 0.05% B-2 groups compared with control. Moreover, the numbers of CD11b/c⁺ cells (macrophages) and NKR-P1A⁺ cells (NK cells) in the all groups were significantly increased compared with control. In an *in vitro* study using rat colon cancer cell line RCN-9, PA, especially 5–10mer of PA (PA5/10), strong growth inhibition was shown. PA5/10 caused the most remarkable apoptosis as cleared by FACS analysis. These cells showed significantly increased caspase-3 activity. The results would suggest that the PA, especially PA5/10, might strongly enhance caspase-3 activity and cause apoptosis in cancer cells. PA at fairly low doses in the long term might serve as an effective means for preventing colon carcinogenesis.

Introduction

Colorectal cancer is one of the most prevalent cancers in developed societies, which may be associated with dietary factors. A large number of epidemiological studies and animal experiments have indicated that vegetables, fruits, and their constituents may exert a protective influence (1), and the beneficial effects may be partly attributable to polyphenolic compounds, which have antioxidant and free radical scavenging properties (2,3). Epidemiological studies

have also shown a close link between low incidence of coronary heart disease (the French paradox) or breast cancer (4–6) and moderate consumption of red wine that contains large amounts of such natural antioxidants.

Proanthocyanidin (PA), one of the polyphenolic compounds found in grape seeds and red wine, differs from all other natural polyphenols in that it is polymeric, made of flavan-3',4'-diol units. Generally, polyphenolic compounds display biochemical activities that would be expected to influence processes that are important for cancer development. Thus, they have antioxidant (7) and scavenging effects on activated carcinogens and mutagens (8,9) and also affect proteins that control cell cycle progression (10) and gene expression (11). Because biochemical activities differ from the individual polyphenolic structure, each compound needs to have animal studies to assess its individual biological potential. Recently, PA was reported as an effective inhibitor of 7,12-dimethyl-benz[*a*]anthracene (DMBA)-induced and 12-*O*-tetradecanoyl-phorbol 13-acetate-promoted skin tumorigenesis in mice (12). In the present study, we investigated the effect of purified PA, procyanidin B-2 (B-2) and (-)-epigallocatechin gallate (EGCG) on colon carcinogenesis by azoxymethane (AOM) in F344 male rats. AOM induces colonic aberrant crypt foci (ACF), which is reported to be preneoplastic lesions that have the potential to form colon tumors in the rat (13–16). In addition, the effects of PA on proliferative cells and apoptotic cells in the colon and immune cells in the lamina propria of small intestine were investigated to clarify possible mechanisms of action.

Because many polyphenols have selective antiproliferative activity on cancer cell lines (17–21), we also investigated the potential of various PAs (monomer, 2–4mer, 5–10mer) to inhibit proliferation and induce apoptosis of the rat colon carcinoma cell line RCN-9 *in vitro*. Enhancement of apoptosis of cancer cells may inhibit carcinogenesis (22).

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Materials and Methods

Animals

F344 rats, five-week-old males, were purchased from Charles River Japan (Atsugi, Japan). They were allowed free access to a basal diet (Oriental MF, Oriental Yeast, Tokyo, Japan) and tap water, and were maintained in plastic cages on wood-chip bedding under specific pathogen-free conditions in our animal facility with a controlled temperature ($24 \pm 2^\circ\text{C}$), humidity ($60\% \pm 10\%$), and a 12-h light-dark cycle. All experiments were initiated when the rats became six weeks old. The experiments were conducted according to the guidelines for the care and use of laboratory animals of the Animal Study Committee of our National Cancer Center Research Institute.

Chemicals and Antibodies

The carcinogen AOM was purchased from Sigma Chemical Co. (St. Louis, MO). PA (mean PA, 5.5mer; purity, more than 90%; Kikkoman Corp., Noda, Japan), B-2 (purity, more than 90%), and EGCG (purity, more than 95%; Tokyo Food Techno Co. Ltd., Tokyo, Japan) were purchased. Various procyanidins were kindly supplied by Kikkoman Corp. They were the monomer (PA1), 2–4mer (PA2/4), and 5–10mer (PA5/10), purified by HPLC (purity, more than 95%). These fractions contained limited amounts of EGCG.

Purified mouse anti-rat CD8a mAb (clone OX-8), mouse anti-rat CD11b/c mAb (OX-42), and mouse anti-rat NKR-P1A mAb (10/78) were purchased from PharMingen (San Diego, CA), and mouse antiproliferating cell nuclear antigen (PCNA) mAb and polyclonal rabbit anti-single stranded DNA (ssDNA) Ab were purchased from DAKO (Kyoto, Japan).

Experimental Protocols

Experiment 1: The experimental design is illustrated in Fig. 1. In groups 1–4, rats received subcutaneous injections of 15-mg/kg AOM three times for three weeks. Then, groups 1–3 received drinking water containing 0.002, 0.01, or 0.05% PA (15 rats each); 0.002, 0.01, or 0.05% B-2 (15 rats each); or 0.002, 0.01, or 0.05% EGCG (15 rats each), respectively, for five weeks, starting at the first dosing of AOM. Group 4 served as the carcinogen control and was without chemicals (20 rats). Group 5 was untreated (5 rats). All animals were sacrificed under ether anesthesia at the end of week 5 for quantitative analysis of ACF and aberrant crypts (AC). Body, liver, and kidney weights were also determined.

Experiment 2: The experimental design was as in Experiment 1. All animals were sacrificed under ether anesthesia at the end of week 5. The liver, kidneys, and colon of each rat were removed, fixed in 10% buffered formalin, embedded in paraffin blocks, and processed for routine histological observation with hematoxylin and eosin staining. Small intestine

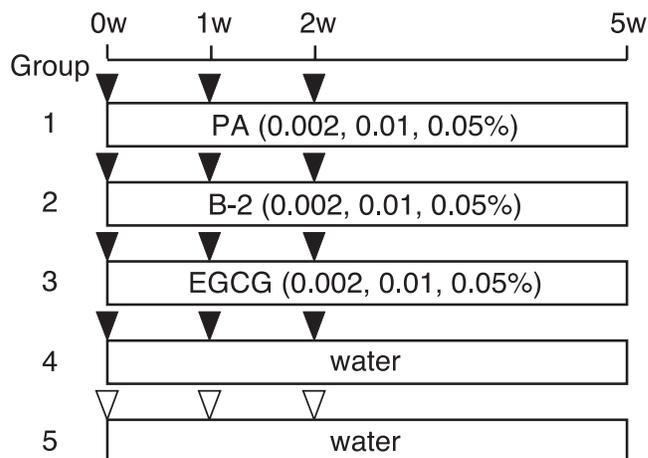


Figure 1. Experimental protocol for the assay of modification of azoxymethane (AOM)-induced aberrant crypt foci in F344 rats. ▼, AOM 15 mg/kg body weight, subcutaneous injection; ▽, saline subcutaneous injection. Abbreviations: PA, proanthocyanidin; B-2, procyanidin B-2; EGCG, (-)-epigallocatechin gallate; W, water.

tine was removed, fixed in acetone, embedded in paraffin blocks, and processed for routine immunohistological observation.

ACF Analysis

For quantitation of ACF, the colons were removed, inflated with 10% buffered formalin, cut longitudinally from cecum to anus, spread on filter paper, fixed in 10% buffered formalin and then stained with 0.2% methylene blue dissolved in saline for 2 min, according to the procedures described by Sutherland and Bird (23). The numbers of ACF and AC per entire colon (about 15 cm) were counted under a microscope at a magnification of $\times 40$. Because it has been indicated that large ACF (composed of four or more crypts) are more closely related to the occurrence of colon tumors, the lesions were divided into four size-based categories, one to three and four or more crypts/foci.

Immunohistochemical Staining Methods

After taking blood from each rat anesthetized with ethyl ether, the small intestine (jejunum, about 10 cm) and the large intestine were excised and washed with physiological saline for immunostaining. The jejunum was cut perpendicularly with scissors, opened onto a sheet, and fixed in acetone at 4°C for embedding in paraffin. Serial sections were cut at $4 \mu\text{m}$ and stained with mouse anti-rat NKR-P1A mAb or mouse anti-rat CD11b/c mAb. Immunoreactions were detected using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Results were expressed as mean numbers of positive cells in 10 fields per rat.

The large intestine was also cut perpendicularly with scissors, opened onto a sheet, and fixed in buffered formalin at 4°C for embedding in paraffin. One representative paraffin block from each rat, in which many ACF were present, was

used for this study. The tissue segments were sectioned at 4 μm and applied to poly-L-lysine-coated glass slides (Matsunami Glass Ind., LTD., Japan), dewaxed in xylene, dehydrated in ethanol, and then incubated with 3% hydrogen peroxide for 5 min. After having been washed with PBS, the sections were incubated with 1% skim-milk solution for 1 h at room temperature to block nonspecific binding of the primary mAb. The sections were then incubated with appropriately diluted rabbit polyclonal antibody (pAb) against ssDNA or mouse monoclonal antibody against PCNA overnight at 4°C and washed three times with PBS, then incubated with biotin-conjugated goat anti-rabbit IgG or biotin-conjugated goat anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA). Subsequently, the sections were washed three times with PBS and incubated with avidin-biotin peroxidase complexes (Vectastain ABC kit; Vector Laboratories, Inc.). Histochemical color development was achieved with 3,3-diaminobenzidine (DAB) (WAKO, Osaka, Japan). The sections were counterstained with hematoxylin for counts.

Cell Line and Culture Conditions

The rat colon cancer cell line (RCN-9) was cultured and passaged in RPMI 1640 tissue culture medium (Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (Sigma) and 1% penicillin-streptomycin (Invitrogen Corp., Grand Island, NY). Cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were passaged at preconfluent density using Trypsin-EDTA (Invitrogen).

Cell Proliferation

For determination of proliferation, RCN-9 cells were seeded at a density of 3×10^3 per well onto 96-well cell culture plates (Falcon, Franklin Lakes, NJ) and allowed to adhere for 24 h. Thereafter, medium was replaced with fresh culture medium containing test compound, and cells were allowed to grow for another 72 h. Total cell counts were determined with a Cell Counting Kit-8 (Dojindo, Masushiro, Japan), which allows very convenient assays with water-soluble tetrazolium salt, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye upon bioreduction in the presence of an electron carrier, 1-methoxy PMS (1-methoxy-5-methylphenazinium methylsulfate). The amount of the formazan produced is directly proportional to the number of living cells. Absorption of the corresponding cell numbers was measured at 450 nm with a XFLUOR4 (TECAN, Austria).

Nuclear Fragmentation Detection

Nuclear fragmentation as a late marker of apoptosis was determined by staining of DNA with Hoechst 33342. RCN-9 cells (3×10^4) were incubated with test compound for 24 or

72 h. Thereafter, cells were washed in PBS, allowed to air-dry for 30 min, and fixed in 3.7% formaldehyde before staining with 1 $\mu\text{g}/\text{ml}$ Hoechst 33342, and visualized under an inverted fluorescence microscope.

Cell Cycle Analysis

RCN-9 cells were seeded at a density of 1×10^6 onto 60-mm dishes and incubated from 24–72 h in the presence or absence of test compound. The cells were trypsinized, pelleted by centrifugation at 500 g for 5 min, washed twice with PBS, adjusted to 1×10^6 cells/ml PBS, and fixed in 70% ethanol at -20°C for 12 h. Cells were washed with PBS and treated with 100kU RNase and stained by adding propidium iodide solution for 30 min at room temperature. The stained cells were analyzed by fluorescent activated cell sorter (FACS) on a FACSCalibur (Becton Dickinson, NJ, USA) flow cytometer for relative DNA content based on red fluorescence.

Caspase-3 Activity

RCN-9 cells were seeded at a density of 5×10^5 onto 60 mm dishes and incubated for 24 h in the presence or the absence of the test compounds. The cells were trypsinized and pelleted by centrifugation at 500 g for 5 min. Cytosolic extracts were prepared by adding 100 μl cell lysis buffer (0.067M phosphate buffer, pH7.2, 1% Nonidet P-40, 0.5% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, 1mM phenylmethylsulfonyl fluoride, 2.5 $\mu\text{g}/\text{ml}$ leupeptin, and 20 U/ml aprotinin) to each pellet, homogenizing with 10 strokes and centrifugating at 3,500 g for 10 min. The supernatant was incubated with the chromophore caspase-3 substrate Ac-DEVD-pNA (BioVision Research Products, Mountain View, CA) at a final concentration of 200 μM . Cleavage of the caspase-3 substrate was measured at 405 nm with a XFLUOR4.

Statistics

The data for body and organ weights; food consumption; numbers of ACF, PCNA, and ssDNA; and immunohistochemical labeling indices were analyzed using the JMP software package (Version 3.1, SAS Institute Japan) on a Macintosh computer.

Results

General Observations

There were no significant differences in daily intake of water per body weight (Table 1) and body weights among the groups. However, relative liver and kidney weights in group 1 given 0.05% PA were significantly decreased compared with the group 4 values ($P < 0.05$).

Table 1. Water Intake, Proanthocyanidin (PA), Procyanidin B-2 (B-2), and (-)-Epigallocatechin Gallate (EGCG) Intake^a

Group	Treatment	Concentration	Intake	
			Water (g/kg bw/day)	Estimated Intake (mg/kg bw/day)
1	AOM + PA	0.002%	107.4 ± 7.8	2.2 ± 0.2
		0.01%	101.7 ± 3.6	10.2 ± 0.4
		0.05%	99.1 ± 7.1	49.6 ± 3.5
2	AOM + B-2	0.002%	95.4 ± 3.4	1.9 ± 0.1
		0.01%	98.4 ± 7.5	9.8 ± 0.8
		0.05%	100.2 ± 2.2	50.1 ± 1.1
3	AOM + EGCG	0.002%	97.8 ± 1.8	2.0 ± 0.0
		0.01%	99.2 ± 2.4	9.9 ± 0.2
		0.05%	94.0 ± 2.0	47.0 ± 1.0
4	AOM alone		97.9 ± 3.3	
5	No treatment		92.7 ± 0.0	

a: Abbreviation is as follows: bw, body weight.

Table 2. Effects of Proanthocyanidin (PA), Procyanidin B-2 (B-2), and (-)-Epigallocatechin Gallate (EGCG) on Aberrant Crypt Foci (ACF) in the Colon^a

Group	Treatment	Concentration	No. of Rats	Number of ACF/Colon Containing		
				1–3 Crypts	≥ 4 Crypts	Total
1	AOM + PA	0.002%	15	101.4 ± 45.0	10.2 ± 6.7**	111.6 ± 50.3*
		0.01%	15	115.1 ± 47.8	12.7 ± 10.8	127.8 ± 56.7
		0.05%	15	139.1 ± 10.4	16.5 ± 8.2	155.6 ± 50.02
2	AOM + B-2	0.002%	15	123.6 ± 54.1	15.6 ± 8.3	139.2 ± 58.1
		0.01%	15	116.5 ± 50.0	12.7 ± 10.8	129.2 ± 54.7
		0.05%	15	94.8 ± 37.4*	12.9 ± 5.2	107.7 ± 40.7*
3	AOM + EGCG	0.002%	15	107.2 ± 36.3	14.5 ± 6.6	121.7 ± 40.4
		0.01%	15	128.8 ± 27.9	19.1 ± 7.4	147.9 ± 33.1
		0.05%	15	109.3 ± 29.9	14.2 ± 4.9	123.5 ± 33.1
4	AOM alone		20	143.5 ± 54.4	20.2 ± 7.8	163.7 ± 57.9
5	No treatment		5	0	0	0

a: * $P < 0.05$, ** $P < 0.01$ compared with azoxymethane (AOM) alone group.

ACF

The data for total ACF and AC counts are summarized in Table 2. The numbers of total ACF in group 1 given 0.002% PA and group 2 given 0.05% B-2 were significantly decreased compared with the AOM alone (group 4) values ($P < 0.05$). Similarly, values for ACF of larger than four crypts in group 1 given 0.002% PA were significantly decreased compared with group 4 ($P < 0.01$). Values for one to three crypts in group 2 given 0.05% B-2 were significantly decreased compared with group 4 ($P < 0.05$). EGCG (group 3) did not show significant inhibition effects on ACF.

PCNA Labeling Indices

PCNAs were examined to the level of cell proliferation. Values for PCNA-positive cells are summarized in Fig. 2. Significant decreases compared with the AOM alone (group 4) values were observed for 0.002% and 0.01% PA, for 0.002% and 0.05% B-2, and for 0.002% and 0.01% EGCG.

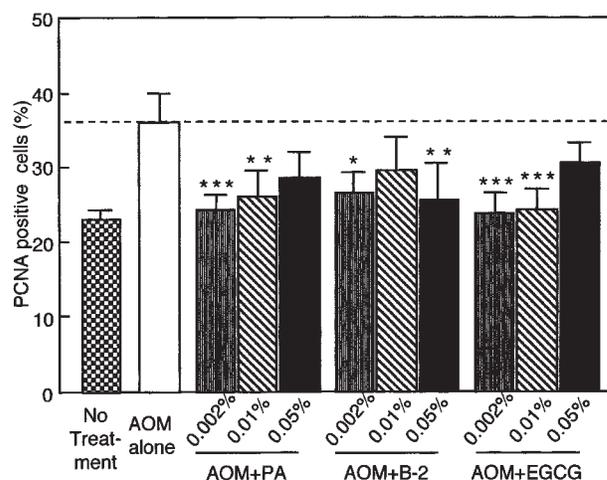


Figure 2. Effects of proanthocyanidin (PA), procyanidin B-2 (B-2), and (-)-epigallocatechin gallate (EGCG) on proliferating cells nuclear antigen (PCNA) labeling in rat colonic epithelium. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with azoxymethane (AOM) alone group.

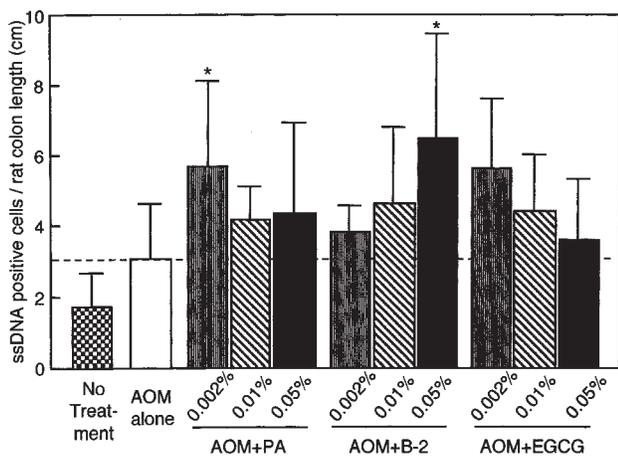


Figure 3. Effects of proanthocyanidin (PA), procyanidin B-2 (B-2), and (-)-epigallocatechin gallate (EGCG) on apoptosis as detected by single-stranded DNA (ssDNA) labeling in rat colonic epithelium. * $P < 0.05$ compared with azoxymethane (AOM) alone group.

ssDNA Labeling Indices

Apoptosis was detected in epithelial cells in the colon treated with PA by the ssDNA method. The incidence of apoptosis in groups 1–4 were significantly increased compared with no-treatment group (group 5). In group 4, treated with AOM alone, the incidence of apoptosis was increased compared with the no-treatment group. The finding suggests that apoptosis was induced to exclude the cells damaged by the AOM treatment. In group 1 given 0.002% PA and group 2

given 0.05% B-2, the apoptosis induced was higher than that for AOM alone (group 4), suggesting that 0.002% PA and 0.005% B-2 enhanced induction or apoptosis, presumably to eliminate damaged cells (Fig. 3).

Intestinal Mucosal Immunity

We investigated whether PA has immune-modulating functions in lamina propria of the small intestine. The number of CD11b/c⁺ macrophages in group 1 given 0.002% and 0.01% PA, group 2 with all doses of B-2, and in group 3 given 0.002% EGCG were significantly increased compared with the AOM alone group (group 4) values (Fig. 4A). Furthermore, the number of NKR-P1A⁺ NK cells in group 1 given 0.002% and 0.01% PA, group 2 given 0.01% and 0.05% B-2, and group 3 with all doses of EGCG were significantly higher than the AOM alone group (group 4) values (Fig. 4B). There were no significant differences in CD8a⁺ cells among the groups (data not shown).

Cell Proliferation

PAs reduced proliferation of the rat colon carcinoma cell line RCN-9 dose-dependently. PA1, PA2/4, and PA5/10 led to one-half maximal growth inhibition at concentrations of 54.5 μ M, 150.6 μ M, and 19.5 μ M, respectively (Fig. 5). Under the same experimental conditions, equivalent values for PA (molecular weight > 3000) and EGCG were 20.1 μ M and 42.0 μ M, respectively (data not shown).

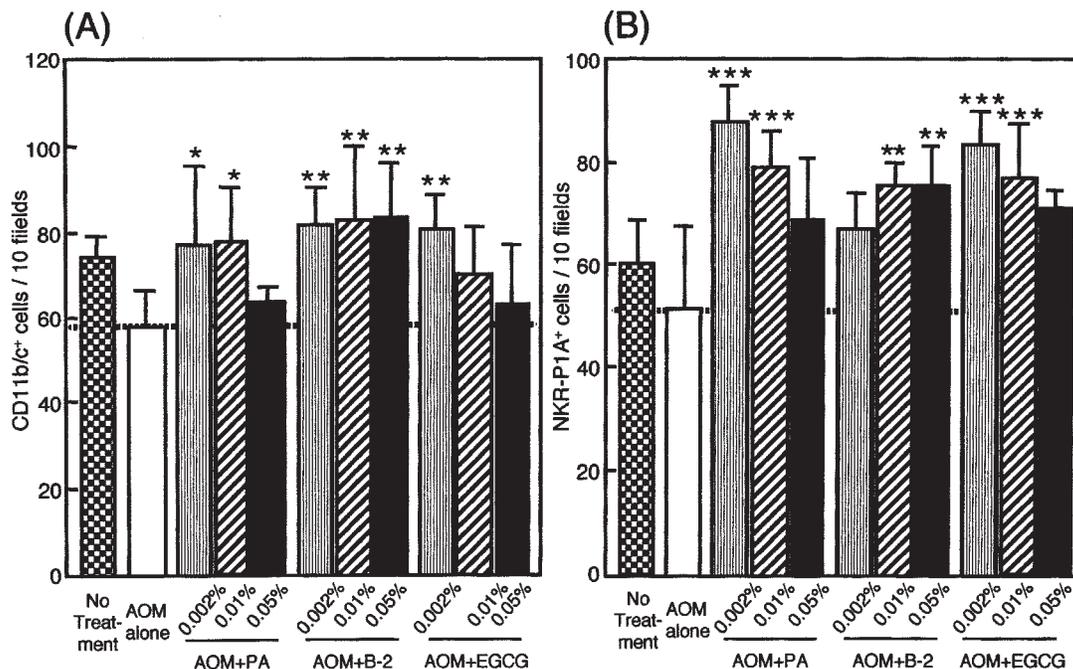


Figure 4. Effects of proanthocyanidin (PA), procyanidin B-2 (B-2), and (-)-epigallocatechin gallate (EGCG) on A: number of CD11b/c⁺ and B: NKR-P1A⁺ cells in the lamina propria of the small intestine. * $P < 0.05$ and ** $P < 0.01$, respectively, compared with AOM alone group.

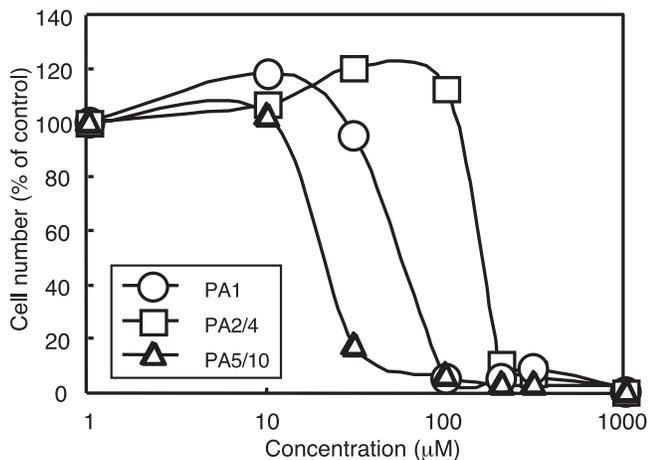


Figure 5. Growth inhibition of rat colon cancer RCN-9 cells by PA1, PA2/4, and PA5/10.

Detection of DNA Fragmentation

Nuclear fragmentation after 24 h of exposure of cells to PA was readily detectable, being prominent at 72 h (data not shown).

Cell Cycle Analysis

To determine the mechanism of inhibition of cell proliferation by PA, we examined whether PA can induce program

cell death in RCN-9 cells. FACS analysis of RCN-9 cells exposed to 25 µM PA5/10 from 24 to 72 h showed that PA5/10 arrested the cells in the sub-G1-phase. The hypodiploid cells in the sub-G1-phase were markedly increased, as shown in Fig. 6A, in a dose-dependent manner (Fig. 6B).

Caspase-3 Activity

Caspase-3 is a key protease associated with the apoptosis. Effects of PA5/10 on activity of caspase-3 were examined in RCN-9 cells. PA5/10-treated cells with 6.25, 12.5, 25, and 50 µM for 24 h showed significant increases (11.5%, 84.7%, 80.3%, and 112.1%, respectively; Fig. 7).

Discussion

Epidemiological studies have provided evidence that high dietary intake of flavonoids with fruits and vegetables could be associated with a low colon cancer incidence in humans (24,25). Animal studies and investigations using different cellular models have suggested that certain flavonoids can inhibit tumor initiation as well as tumor progression (26,27). The present study clearly demonstrated that dietary drinking of PA (0.002%) during the initiation stage significantly reduced the development of preneoplastic lesions of the colon induced by AOM. It should be noted that a 0.002% dose of

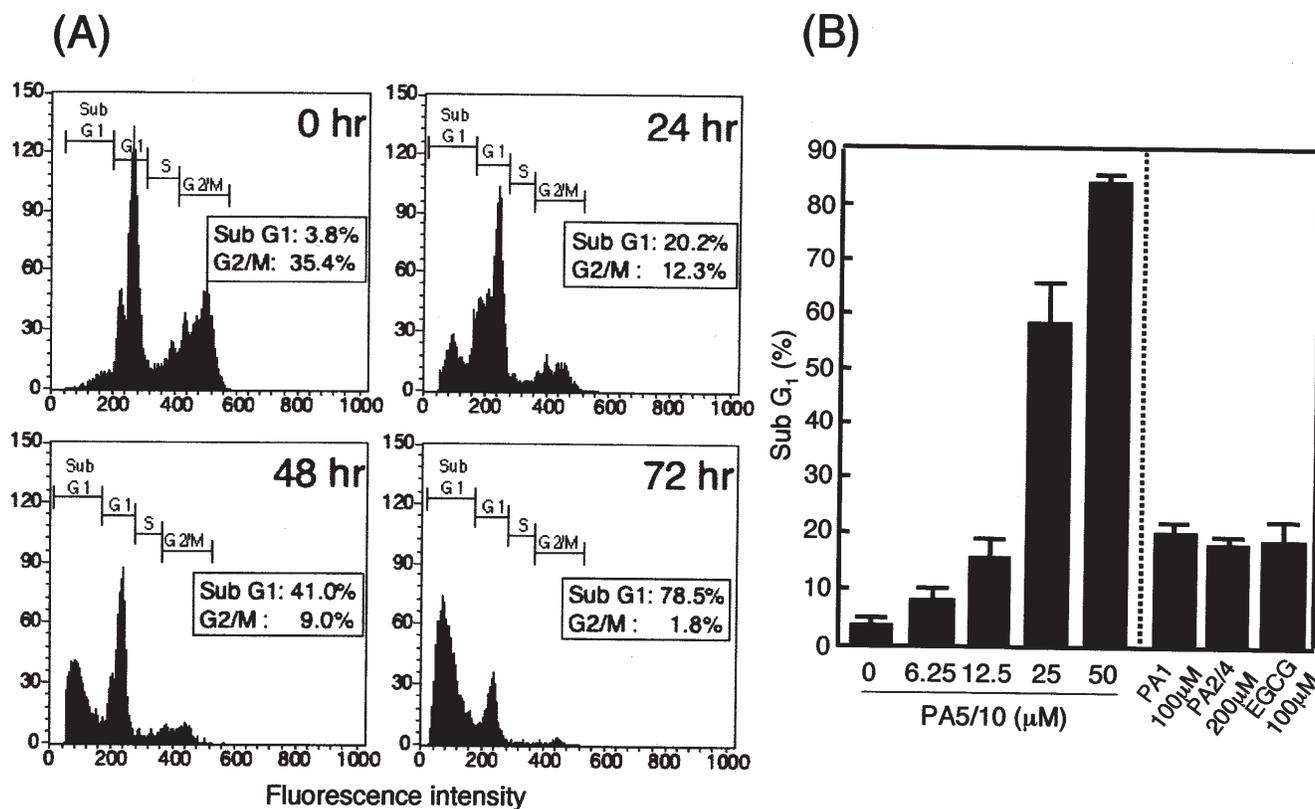


Figure 6. Flow cytometric analysis of rat colon cancer RCN-9 cells incubated with A: 25 µM PA5/10 for 0 to 72 h and B: 0–50 µM PA5/10, 100µM PAI, 200µM PA 2/4, and 100µM EGCG for 72 h.

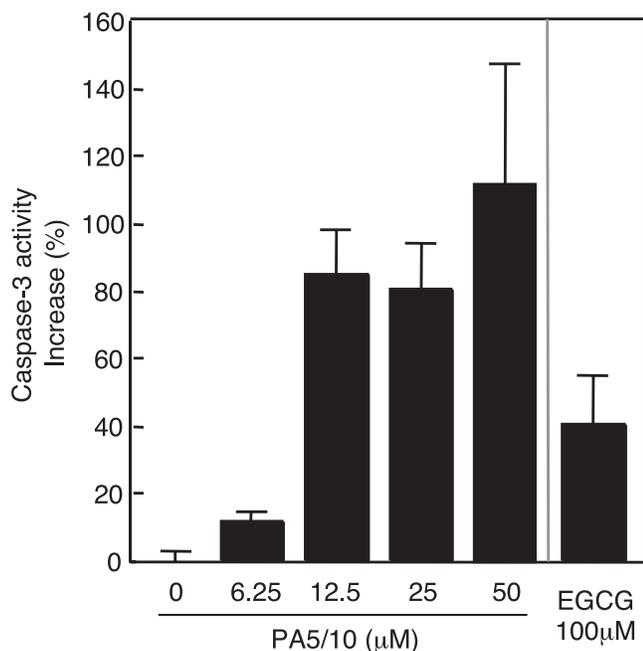


Figure 7. Effect of proanthocyanidin (PA) on caspase-3 activity in rat colon cancer RCN-9 cells treated with 0–50 μM for 24 h.

PA is equal to one or two glasses of wine per day for the average person. However, high doses of PA (0.01% and 0.05%) were without significant effects, and the possibility of toxicity must be taken into account. Indeed, relative liver and kidney weights of 0.05% PA were significantly decreased compared with the AOM alone group values. On the other hand, B-2 showed inhibitory effect only at a high dose, and EGCG did not show significant inhibition. PA exerted efficiency to inhibit carcinogenesis of the colon.

It has been reported that development of ACF correlates well with tumor yield in experimental colon carcinogenesis in the rat (28–31). ACF composed of multiple aberrant crypts, especially, have been shown to have a high potential for developing into colon carcinomas (32). Thus, from our results for ACF comprising four or more crypts, it is suggested that PA (0.002%) may be a candidate as a chemopreventive agent for colon carcinogenesis. It has been reported that the AOM treatment increases the number of proliferating cells as observed by detection of PCNA labeling (33). In the present study, the treatment with PA reduced the number of PCNA-positive cells to the level of the no-treatment group without AOM, suggesting that this treatment inhibited the cell proliferation induced by the carcinogen, which correlates with a decrease in the ACF number. However, in the case of EGCG treatment, there was no significant correlation with ACF inhibition. PA, B-2, and EGCG may exert different effects on ACF and normal cells.

Many anticancer drugs are known to induce apoptosis in cancer cells (34–37), and some polyphenols also have this potential (17,38–40). Cytotoxic effects of proanthocyanidin have been reported on MCF-7 breast cancer, A-427 lung cancer, and gastric adenocarcinoma cell lines, although it en-

hances the growth and viability of the normal human gastric mucosal cells and murine macrophage cells. These results suggest that proanthocyanidin exhibits cytotoxicity toward some cancer cells (41).

PA induced apoptosis in the colon treated with AOM *in vivo*, and also in the rat colon cancer RCN-9 cell line, as other polyphenols induce apoptosis. Especially, PA5/10 reduced stronger proliferation and induced more apoptosis of RCN-9 cells than PA1 and PA2/4, in a time and a dose-dependent manner. Moreover, PA5/10 activated the apoptosis-related protease caspase-3. These results suggest that PA causes apoptosis through a caspase-dependent mechanism. EGCG is reported to the FAS death receptor and initiates the caspase activation and apoptosis (42). Further studies need to analyze interaction of PA to apoptosis-related protein of cell membranes. The apoptosis induced by PA may relate to a reduced number of ACF in the colon *in vivo*. Thus, apoptosis induction in one of the cancer cells is of interest as one preventive agent.

We also could show that PA significantly increases CD11b/c⁺ and NKR-P1A⁺ cells in the small intestine. The gut-associated lymphoid tissue is a well-developed immune system that is involved in protection of the host against pathogens, including cancer cells. The activation of the immune system by PA might be an important mechanism of inhibition of colorectal cancer development. Clearly, further studies on detailed mechanisms are warranted.

In conclusion, a low dose of PA effectively induced apoptosis in one colon cancer cell line and large intestine treated with AOM. It also markedly reduced the number of ACF in the rat colon. These results suggest that PA can apply as a chemopreventive agent against colorectal cancer.

Acknowledgments and Notes

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