[CANCER RESEARCH 53, 3908–3913, September 1, 1993]

Chemoprevention of Colon Carcinogenesis by the Natural Product of a Simple Phenolic Compound Protocatechuic Acid: Suppressing Effects on Tumor Development and Biomarkers Expression of Colon Tumorigenesis[1]

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ABSTRACT

Our previous study has shown that dietary administration of protocatechuic acid (PCA) acts as potential chemopreventive agent in inhibiting diethylnitrosamine-induced liver carcinogenesis in male F344 rats. The present study was designed to determine the modifying effect of PCA on azoxymethane (AOM)-induced colon carcinogenesis in male F344 rats and the effect on intermediate biomarkers, *i.e.*, colonic mucosal ornithine decarboxylase activity and colonic epithelial proliferation, which can be used as effective predictors of colon cancer. Staring at 6 weeks of age, groups of animals were fed the basal diet and experimental diet containing PCA at dose levels of 250, 500, and 1000 ppm. At 7 weeks of age, all animals except the PCA alone group (1000 ppm) and untreated controls were given s.c. injections of AOM at a dose level of 15 mg/kg body weight/week for 3 weeks. PCA at 3 doses was fed during the initiation phase (before 1 week, during, and after 1 week of AOM exposure) or postinitiation phase (for 28 weeks starting 1 week after the last injection of AOM). All animals were then killed at 32 weeks after the start and colonic tumor incidence and multiplicity were determined. Animals intended for cell proliferation study were given injections of bromodeoxyuridine/5-fluoro-2′-deoxyuridine (1 ml/100 g body weight) 1 h prior to be killing. The rate of colonic cell proliferation in the distal portion was assessed by immunohistochemistry using antibromodeoxyuridine and by counting silver-stained nucleolar organizer regions protein. The colonic mucosal ornithine decarboxylase activity was also measured at the termination. The results indicate that dietary PCA administration at 500 and 1000 ppm during the initiation or postinitiation phase significantly inhibited intestinal carcinogenesis induced by AOM as revealed by the reduction of tumor incidence and multiplicity. The data also demonstrate that PCA at 500 ppm and 1000 ppm significantly inhibited bromodeoxyuridine labeling index and also silver-stained nucleolar organizer regions protein number at three doses when animals were fed PCA at the initiation or postinitiation stage. Also, feeding of PCA at 1000 ppm during the initiation and postinitiation phase exerted a pronounced inhibitory effect on the colonic ornithine decarboxylase levels. PCA feeding did not cause any toxicity. These results demonstrate that PCA is a possible new chemopreventive agent for colon carcinogenesis through the suppression of manifestation of intermediate biomarkers induced by AOM, although the precise mechanisms of PCA-induced inhibition during the initiation and postinitiation phases remain to be elucidated.

INTRODUCTION

The best evidence of a major role for nutritional customs, and especially fat intake (1), is the appreciable increase in cancer of the breast and colon in Japan (2). This may be associate with the progressive introduction of Western dietary habits, especially an increasing fat and decreasing carbohydrate intake (3). Epidemiological investigations indicate an increased incidence of colorectal cancer in humans in geographic regions (4). In Japan, the predictive study of cancer incidence by Tsukuma *et al.* (5) indicated that while the cumulative risk was 19.8% in 1985, it was expected to reach 26.2% in 2015 and the incidence of the patients with colon cancer will be the third most common form of cancer in all organs. Thus, diets are known to play an important role in human health and in the development of certain diseases, especially cancer (1, 6, 7). However, some dietary factors may also be protective against cancer development (3, 8–13). The frequent consumption of fresh fruits and vegetables is associated with a low canecr incidence (14). This may be partly due to the presence of several vitamins in these foodstuffs (8). These also contain certain naturally occurring phenolic compounds that have antioxidative property (15). Scientific interest in phenolic antioxidants in food recently has been extremely active since they have antimutagenic and anticarcinogenic potency (16). Because of widespread occurrence of phenolic compounds, human ingests a large amounts of these substances (17). Most naturally occurring phenolic compounds in food are plant flavonoids, but others include chlorogenic acid, caffeic acid, ferulic acid, α-tocopherol, polyphenolic catechins, and curcumin (16). Besides antioxidative activity, these have been reported to be antimutagenic and/or anticarcinogenic and to possess several other biological properties. These include the ability to scavenge active oxygen species, the ability to scavenge electrophiles, the ability to inhibit nitrosation, the ability to chelate metals, the potential for autoxidation producing hydrogen peroxide under the presence for certain metals, and the capability to modulate certain cellular enzyme activities. Although phenolic antioxidants generally have antimutagenic and/or anticarcinogenic potential, some of them exert carcinogenic activity in rodents when given at a high dose (18–20). A simple phenolic acid, PCA,[3] is one of the constituents of edible plants, fruits, and vegetables and belongs to benzoics (21). A recent study has indicated that PCA in the extracts from the rind of *Citrus reticulata* BLANCO shaddock has a strong antioxidative effect, being 10 times higher than that of DL-α-tocopherol (22).

Increase in the polyamine levels is commonly associated with normal, abnormal, and induced proliferative states (23–25). Evidence that polyamine biosynthetic activity and ODC, a rate-limiting enzyme of the polyamine biosynthesis pathway, play an important role in normal and neoplastic cell proliferation has been accumulated (25–28). A number of studies have demonstrated that ODC is induced during colon carcinogenesis (26–28) and that α-difluoromethylornithine, an irreversible inhibitor of ODC activity, inhibited colon carcinogenesis (27, 29, 30), suggesting a relationship between tumor development and the induction of ODC activity in the colon. Data on cell kinetic analysis in the colonic mucosa of individuals at increased risk for colon cancer have revealed an anomalous expansion of epithelial cells within the colonic crypts (31, 32). Such expansion of proliferative region of actively renewing epithelium has been reported in other organs of both humans and carcinogen-treated rodents with preneoplastic lesions (33–35). Thus, expansion of the proliferative compartment appears to represent a phenotypic marker associated with the development of a precancerous state.

Received 3/16/93; accepted 6/28/93.

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[1] Supported by Grant-in Aids for Cancer Research from the Ministry of Health and Welfare of Japan.

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[3] The abbreviations used are: PCA, protocatechuic acid; ODC, ornithine decarboxylase; AOM, azoxymethane; BrdUrd, bromodeoxyuridine; AgNORs, silver-stained nucleolar organizer regions protein.

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Our recent study demonstrated a remarkable chemopreventive effect of dietary PCA on diethylnitrosamine-induced rat liver tumorigenesis, and PCA feeding during the initiation or postinitiation phase reduced liver ODC activity as well as liver tumor incidence and multiplicity (36). In the present study, possible modifying effects of dietary exposure of PCA during the initiation or postinitiation phase on AOM-induced colon carcinogenesis were investigated in male F344 rats. Also, the effects of PCA on cell proliferation in the colonic epithelium were evaluated by measuring BrdUrd labeling index, number of AgNORs, and ODC activity of colonic mucosa.

MATERIALS AND METHODS

Animals and Diet. Male F344 rats, 4 weeks of age, obtained from Shizuoka Laboratory Animal Center (Hamamatsu City, Japan), were quarantined for 14 days and randomized into experimental and control groups. All animals were housed, with three or four rats per wire cage. The holding room was maintained on controlled conditions at 23 ± 2°C, 50 ± 10% humidity, and a 12-h light/dark cycle. Powdered CE-2 (Clea Japan, Inc., Tokyo, Japan) was used as basal diet throughout the experiment.

Chemicals. AOM was purchased from Sigma Chemical Co., St. Louis, MO. PCA (97% purity) was obtained from Aldrich Chemical Co., Milwaukee, WI.

Experimental Procedure. A total of 169 rats were divided into nine groups as shown in the tables. Starting at 7 weeks of age, rats in groups 1–7 groups were given s.c. injections of AOM (15 mg/kg body weight) once a week for 3 weeks. Animals in groups 2–4 were fed the diets containing 250, 500, and 1000 ppm PCA, respectively, for 4 weeks, starting at 6 weeks of age. Groups 5–7 were fed the diets mixed with PCA at concentrations of 250, 500, and 1000 ppm for 28 weeks, starting 1 week after the final AOM injection. Rats in group 8 were fed the diet containing 1000 ppm PCA during the experiment. Group 9 was given the basal diet throughout the study and served as an untreated control. All rats were provided with the diet and tap water *ad libitum*.

Animals were weighed weekly until they reached 14 weeks of age, and then they were weighed every 4 weeks. The experiment was terminated 32 weeks after the start. Complete necropsies were performed on all animals that were dying or moribund and on all remaining rats at the termination of the experiment. All organs, especially the intestine, were carefully inspected grossly and all abnormal lesions were examined histologically. All tissues, after fixed in 10% neutral buffered formalin, were processed for histological examination by conventional methods and stained with hematoxylin and eosin. Intestinal neoplasms were diagnosed according to the criteria described by Ward (37). Renal tumors were diagnosed according to the criteria described by Hard and Butler (38).

Ornithine Decarboxylase Activity. At the termination of the study, five animals from each group were randomly selected and sacrificed by CO_2 euthanasia. The colon was rapidly removed, rinsed in ice-cold saline, slit open longitudinally, and freed from all the contents. It was laid flat on a glass plate, and the mucosa was scraped with a stainless steel disposable microtome bladed knife, S35 (Feather Safety Razor Co., Ltd., Osaka, Japan). Colon mucosa from each of 5 rats was pooled and homogenized in 1.5 ml of homogenizing buffer (50 mM sodium phosphate buffer, pH 7.2, containing 5 mM dithiothreitol, 0.1 mM EDTA, and 0.1 mM pyridoxal 5′-phosphate) using a polytron. The homogenates were centrifuged at 100,000 *g*, 4°C, for 1 h. The resulting cytosol fraction was used for determination of ODC activity and protein. ODC activity in the colonic mucosa was determined by the modification of the methods described previously (39, 40). The incubation mixture in a final volume of 250 μl [50 mM sodium phosphate, 2 mM pyridoxal phosphate, 5 mM dithiothreitol, 20 mM L-ornithine, and 0.25 μCi of DL-[1-^{14}C]ornithine (specific activity, 58 mCi/mmol; Amersham)] was incubated at 37°C for 1 h. The reaction was stopped by adding 300 μl of 2 N sulfuric acid, and the $^{14}CO_2$ released was collected on barium hydroxide-saturated discs for another 30 min. ^{14}C in the form of $Ba^{14}CO_3$ was counted in a scintillation counter. The results were expressed as pmol $^{14}CO_2$/h/mg protein.

BrdUrd Labeling Index and AgNORs Count. At the end of the study, BrdUrd labeling index and the number of AgNORs of the mucosal epithelium of colon from five rats randomly selected from each group were determined. For the measurement of BrdUrd, animals were given an i.p. injection of BrdUrd/5-fluoro-2′-deoxyuridine (1 ml/100 g body weight; Cell Proliferation Kit, RPN.20; Amersham) 1 h prior to being killed. The colon was removed, fixed in 10% buffered formalin, and the distal colon (3 cm from the anal orifice) was cut. The distal colon was embedded in paraffin and two serial sections (3 μm in thickness) were made. The one section was used for immunohistochemical detection of BrdUrd incorporation using an immunohistochemical analysis kit (Amersham) and the other for staining AgNORs. AgNORs staining was carried out according to the method described previously (41, 42). For determination of the labeling index, 15 well oriented crypts in which the base, lumen, and top of the crypts could be seen were observed for each animal in the distal portion of the colon. The number and the position of the labeled cells in each crypt column were recorded in terms of serial position counting upwards from position 1, at the base of crypt up to the mouth of the crypt. The percentage of labeled cells (labeling index) was determined for the whole crypt by calculating the ratio of labeled cells to total number of cells × 100. For each group the number of cells/crypt column of each animal was also determined. AgNORs enumeration on the cell nuclei in the proliferative zone was carried out using a computer-assisted image analysis system SPICCA II (Japan Avionics Co., Tokyo, Japan) with an Olympus BH-2 microcope and a color CCD camera (Hamamatsu Photonics Co., Hamamatsu City, Japan) (41). Data were expressed as number of AgNORs/nucleus.

Statistics. The data on tumor incidence were analyzed statistically by the Fisher's exact probability test; tumor multiplicity, body weights, liver weights, and relative body weights (liver weight/100 g body weight) were subjected to analysis of the Welch's test. Data of BrdUrd labeling index, AgNORs enumeration, and biochemical determinations for ODC activity were analyzed by Student's t test. All statements of significance are $P < 0.05$.

RESULTS

General Observations. One rat from group 5, which was moribund and necropsied on the 125th day, had ear duct tumor (squamous cell carcinoma). Therefore, the rats alive on that day were counted as effective animals.

The mean body weight, liver weight, and relative liver weight (g/100 g body weight) in each group are indicated in Table 1. The average body weights of rats receiving AOM with PCA (groups 2–5) were slightly higher than that of rats given AOM alone (group 1), but

Table 1 *Body weight, liver weight, and relative liver weight of rats in each group*

Group	Treatment	No. of effective rats[a]	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)
1	AOM	20	348 ± 22[b]	13.1 ± 1.8	3.78 ± 0.48
2	AOM + 250 ppm PCA	20	357 ± 31	15.0 ± 2.5[c]	4.24 ± 0.77[d]
3	AOM + 500 ppm PCA	20	354 ± 53	14.8 ± 3.5	4.12 ± 0.61
4	AOM + 1000 ppm PCA	23	356 ± 55	14.9 ± 2.9[e]	4.18 ± 0.47[c]
5	AOM → 250 ppm PCA	20	351 ± 45	14.5 ± 2.2	4.17 ± 0.67[f]
6	AOM → 500 ppm PCA	20	333 ± 37	12.9 ± 2.2	3.85 ± 0.41
7	AOM → 1000 ppm PCA	23	333 ± 69	12.3 ± 3.0	3.65 ± 0.36
8	1000 ppm PCA	11	332 ± 25	12.2 ± 1.2	3.67 ± 0.36
9	No treatment	12	334 ± 18	11.7 ± 0.9[g]	3.51 ± 0.36

[a] Rats that survived more than 125 days.
[b] Mean ± SD.
[c–g] Significantly different from group 1 by Welch's test: [c] $P < 0.02$; [d] $P < 0.04$; [e] $P < 0.03$; [f] $P < 0.05$; [g] $P < 0.09$.

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Table 2 *Incidence of neoplasms in the intestine of rats in each group*

Group	Treatment	No. of effective rats[a]	No. of rats with tumors (%) at:								
			Total intestine			Small intestine			Large intestine		
			Total	AD[b]	ADC	Total	AD	ADC	Total	AD	ADC
1	AOM	20	16 (80)	3 (15)	16 (80)	7 (35)	1 ((5)	6 (30)	15 (75)	2 (10)	15 (75)
2	AOM + 250 ppm PCA	20	12 (60)	1 (5)	11 (55)	7 (35)	0	7 (35)	9 (45)	1 (5)	8 (40)
3	AOM + 500 ppm PCA	20	13 (65)	1 (5)	13 (65)	6 (30)	0	6 (30)	11 (55)	1 (5)	11 (55)
4	AOM + 1000 ppm PCA	23	14 (61)	3 (13)	13 (57)	8 (35)	1 (4)	8 (35)	9 (39)[c]	2 (9)	7 (30)[d]
5	AOM → 250 ppm PCA	20	12 (60)	4 (20)	11 (55)	5 (25)	0	5 (25)	11 (55)	4 (20)	8 (40)
6	AOM → 500 ppm PCA	20	11 (55)	3 (15)	9 (45)[e]	1 (5)[e]	0	1 (5)	10 (50)	3 (15)	8 (40)
7	AOM → 1000 ppm PCA	23	8 (35)[f]	3 (13)	7 (30)[g]	3 (13)	1 (4)	2 (9)	6 (26)[g]	2 (9)	5 (22)[h]
8	1000 ppm PCA	11	0	0	0	0	0	0	0	0	0
9	No treatment	12	0	0	0	0	0	0	0	0	0

[a] Rats that survived for more than 125 days.
[b] AD, adenoma; ADC, adenocarcinoma.
[c–h] Significantly different from group 1 by Fisher's exact probability test: [c] $P < 0.04$; [d] $P < 0.006$; [e] $P < 0.05$; [f] $P < 0.03$; [g] $P < 0.003$; [h] $P < 0.008$.

Table 3 *Multiplicity of neoplasms in the intestine of rats in each group*

Group	Treatment	No. of effective rats[a]	Multiplicity (/rat) of neoplasms at:								
			Total intestine			Small intestine			Large intestine		
			Total	AD[b]	ADC	Total	AD	ADC	Total	AD	ADC
1	AOM	20	1.65 ± 1.27[c]	0.20 ± 0.52	1.45 ± 1.10	0.35 ± 0.49	0.05 ± 0.22	0.30 ± 0.47	1.30 ± 1.17	0.15 ± 0.49	1.15 ± 1.09
2	AOM + 250 ppm PCA	20	1.00 ± 1.03	0.05 ± 0.22	0.95 ± 1.05	0.40 ± 0.60	0	0.40 ± 0.60	0.60 ± 0.75[d]	0.05 ± 0.22	0.55 ± 0.76
3	AOM + 500 ppm PCA	20	1.00 ± 0.97	0.05 ± 0.22	0.95 ± 0.89	0.30 ± 0.47	0	0.30 ± 0.47	0.70 ± 0.80	0.05 ± 0.22	0.65 ± 0.75
4	AOM + 1000 ppm PCA	23	0.91 ± 0.85[d]	0.17 ± 0.49	0.74 ± 0.75[e]	0.39 ± 0.58	0.04 ± 0.21	0.35 ± 0.49	0.52 ± 0.73[f]	0.13 ± 0.46	0.39 ± 0.66[f]
5	AOM → 250 ppm PCA	20	1.05 ± 1.10	0.20 ± 0.41	0.85 ± 0.93	0.25 ± 0.44	0	0.25 ± 0.44	0.80 ± 1.01	0.20 ± 0.41	0.60 ± 0.88
6	AOM → 500 ppm PCA	20	0.65 ± 0.75[e]	0.20 ± 0.52	0.45 ± 0.51[g]	0.05 ± 0.22[h]	0	0.05 ± 0.22[i]	0.60 ± 0.75[d]	0.20 ± 0.52	0.40 ± 0.50[f]
7	AOM → 1000 ppm PCA	23	0.43 ± 0.66[j]	0.13 ± 0.34	0.30 ± 0.47[k]	0.13 ± 0.34	0.04 ± 0.21	0.09 ± 0.29	0.30 ± 0.56[l]	0.09 ± 0.29	0.22 ± 0.42[g]
8	1000 ppm PCA	11	0	0	0	0	0	0	0	0	0
9	No treatment	12	0	0	0	0	0	0	0	0	0

[a] Rats that survived more than 125 days.
[b] AD, adenoma; ADC, adenocarcinoma.
[c] Mean ± SD.
[d–l] Significantly different from group 1 by Welch's test: [d] $P < 0.04$; [e] $P < 0.007$; [f] $P < 0.02$; [g] $P < 0.002$; [h] $P < 0.03$; [i] $P < 0.05$; [j] $P < 0.0009$; [k] $P < 0.0004$; [l] $P < 0.003$.

no significant differences were present. The mean liver weights of rats in groups 2 and 4 and the average relative liver weights of animals in groups 2, 4, and 5 were significantly higher than that of group 1 ($P <$ 0.02–0.05). However, there was no clinical signs for toxicity, low survival, poor condition, or histopathological changes suggesting toxicity in the liver, kidney, and lung caused by dietary PCA administration.

Tumor Incidence and Multiplicity. Macroscopic examination revealed that most tumors were present in the large intestine (mainly in the distal colon) and the small intestine. They were sessile or pedunculated polyps. They were histologically adenomas or adenocarcinomas with a higher incidence of adenocarcinomas. In group 1, a few rats had renal tumors diagnosed as mesenchymal tumors and liver cell foci, but these lesions were not present in other groups (groups 2–9). The incidences of intestinal tumors in all groups are shown in Table 2. The dietary exposure of PCA during the initiation phase of AOM-initiated carcinogenesis resulted in reduction of intestinal carcinogenesis as revealed by a low incidence of both adenomas and adenocarcinomas of the intestine. The incidence of adenocarcinomas and the combined incidence of tumors (adenomas and adenocarcinomas) of the large intestine of the rats in group 4 (AOM + 1000 ppm PCA) were significantly smaller than those of group 1 ($P < 0.006$ or $P <$ 0.04). Similarly, dietary administration of PCA during the postinitiation phase of AOM-induced intestinal carcinogenesis also inhibited intestinal carcinogenesis. The incidence of carcinomas and the combined incidence of neoplasms in the large intestine of rats of group 7 (AOM → 1000 ppm PCA) were significantly lower than those of group 1 ($P < 0.008$ or $P < 0.003$). In rats given PCA at a dose of 500 ppm for 28 weeks starting 1 week after AOM exposure (group 6), the combined incidence of small intestinal tumors was significantly smaller than that of group 1 ($P < 0.05$). In this group, the frequency of intestinal carcinomas was also smaller than that of group 1 ($P <$ 0.05). With regard to tumor multiplicity (number of tumors/rat), significant inhibition in the multiplicity of large intestinal adenocarcinomas, total large intestinal tumors (adenomas plus adenocarcinomas), total intestinal adenocarcinomas, and total intestinal tumors (tumors in the large intestine and small intestine) was observed in animals fed the diet containing 1000 ppm PCA during the initiation phase ($P < 0.04$ or $P < 0.02$) (Table 3). In the low PCA diet groups during the initiation phase, 250 and 500 ppm PCA showed a 43–67% inhibition of the multiplicity of colon adenomas and adenocarcinomas, as compared to the animals treated with AOM alone, but the differences did not reach statistical significance. PCA administration at doses of 500 and 1000 ppm during the postinitiation stage also significantly reduced the multiplicity of large intestinal adenocarcinomas and total intestinal tumors and adenocarcinomas ($P <$ 0.02–0.0009). Dietary administration of PCA at the lowest dose (250 ppm) during the postinitiation phase reduced the multiplicity of intestinal tumors (adenomas and adenocarcinomas), but the difference between the two groups was not significant.

Colonic Mucosal ODC Activity. The results of colonic mucosal ODC activity at the end of the study are indicated in Table 4. Mean

Table 4 *Colonic mucosal ODC activity in each group*

Group	Treatment	ODC activity (pmol $^{14}CO_2$/h/mg protein)	
		Mean ± SD	Range
1	AOM	169.7 ± 56.4[a]	122.6–278.1
2	AOM + 250 ppm PCA	129.6 ± 40.9	73.1–192.8
3	AOM + 500 ppm PCA	97.6 ± 43.6	68.9–180.8
4	AOM + 1000 ppm PCA	52.6 ± 5.4[b]	46.1–61.1
5	AOM → 250 ppm PCA	143.6 ± 64.5	79.0–205.8
6	AOM → 500 ppm PCA	98.6 ± 42.2	36.6–157.2
7	AOM → 1000 ppm PCA	92.1 ± 35.4[c]	48.7–145.8
8	1000 ppm PCA	84.8 ± 37.6	32.8–130.1
9	No treatment	86.0 ± 7.7	75.1–97.4

[a] Significantly different from group 9 by Student's t test ($P < 0.02$).
[b, c] Significantly different from group 1 by Student's t test: [b] $P < 0.01$; [c] $P < 0.05$.

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Table 5 *Cell number, BrdUrd labeling index and number of AgNORs of the colonic mucosal epithelium in each group*

Group	Treatment	No. of cells/crypt column	BrdUrd labeling index (%)	No. of AgNORs nucleus
1	AOM	34.4 ± 2.5[a, b]	13.4 ± 1.5[b]	4.0 ± 0.4[b]
2	AOM + 250 ppm PCA	32.5 ± 1.9	9.7 ± 2.3	3.5 ± 0.3[c]
3	AOM + 500 ppm PCA	30.0 ± 1.4[c]	9.0 ± 1.3[c]	3.5 ± 0.4[c]
4	AOM + 1000 ppm PCA	28.1 ± 0.5[d]	7.5 ± 1.4[e]	3.2 ± 0.4[c]
5	AOM → 250 ppm PCA	32.6 ± 1.9	10.0 ± 1.1	3.6 ± 0.4[c]
6	AOM → 500 ppm PCA	30.1 ± 0.7[c]	9.4 ± 1.8	3.4 ± 0.4[c]
7	AOM → 1000 ppm PCA	28.5 ± 0.2[d]	8.5 ± 1.1[c]	3.1 ± 0.4[c]
8	1000 ppm PCA	28.7 ± 1.0	4.9 ± 0.3	3.2 ± 0.3
9	No treatment	28.3 ± 0.9	5.0 ± 0.3	3.1 ± 0.4

[a] Mean ± SD.
[b] Significantly different from group 9 by Student's *t* test ($P < 0.02$).
[c–e] Significantly different from group 1 by Student's *t* test: [c] $P < 0.05$; [d] $P < 0.01$; [e] $P < 0.02$.

colonic mucosal ODC activity in group 1 (AOM alone) was significantly greater than that of group 9 (untreated control) ($P < 0.02$). In the groups of rats fed PCA during the AOM treatment (groups 2–4), mean ODC activities were low compared to that of group 1 and the value in group 4 (AOM + 1000 ppm PCA) was significantly smaller than that of group 1 ($P < 0.01$). Also, the mean values of ODC in the groups of rats fed PCA after AOM initiation were small when compared to group 1 and the mean ODC activity in group 7 (AOM → 1000 ppm PCA) was significantly lower than that of group 1 ($P < 0.05$). The mean value of colonic ODC activity in rats given PCA alone (group 8) was slightly lower than that of group 9 (untreated control), but no significant difference was present between the two groups.

BrdUrd Labeling Index and Number of AgNORs of Colonic Mucosal Epithelium. The data on BrdUrd labeling index and AgNORs enumeration are summarized in Table 5. The mean values of BrdUrd labeling indices in groups 3, 4, and 7 were significantly smaller than that of group 1 ($P < 0.05$ or $P < 0.02$). As for the AgNORs numbers per nucleus in groups 2–7 were significantly lower than that of group 1 ($P < 0.05$). The effect of PCA at 250 ppm (groups 2 and 5) on the number of cells per crypt column in the distal colon was minimal, but PCA at 500 and 1000 ppm (groups 3, 4, 6, and 7) exerted a significant inhibition of colonic cell number ($P < 0.05$ or $P < 0.01$).

DISCUSSION

In the present study, dietary administration of a simple phenolic acid, PCA, at three doses during the initiation or postinitiation phase inhibited the development of colon tumors induced by AOM. Such inhibition was prominent at a high dose of PCA.

Dietary factors are known to play a significant role in certain human diseases including cancer development (1, 6, 7). A number of naturally occurring substances have been intensively investigated previously for their chemopreventive potential on chemically induced colon cancer (43–45). On the other hand, many synthetic compounds showing inhibitory effects on colon carcinogenesis have been reported (43, 46–49). We have previously demonstrated the protective effect of a plant phenol chlorogenic acid, which is an ester of caffeic acid and quinic acid and major component of green coffee beans and dicotyledonous plants, on methylazoxymethanol acetate-induced colon carcinogenesis in hamsters (50). However, there have been no reports that simple phenolic compounds possessing anticarcinogenic action on colon tumor occurrence. More recently, in our studies on search for chemopreventive agents against colon cancer, inhibitory effects of a newly synthesized retinoid, 5-hydroxy-4-(2-phenyl-(*E*)-ethenyl)-2-(5H)-furanone, and a fungal metabolite (flavoglaucin) or herbs (shikonin and gingerol) on AOM-induced rat intestinal carcinogenesis has been demonstrated when they are fed during initiation phase (51, 52). Some of these potential chemopreventive agents might exert their inhibitory effects on colon carcinogenesis through suppression of cell proliferation induced by chemical carcinogens especially when they were applied during the postinitiation phase. For example, calcium, magnesium hydroxide, curcumin, and α-difluoromethylornithine inhibit cell proliferation in the target organs (45, 53–55).

Cell proliferation might play an important role in the carcinogenic process (23, 25, 56). In fact, in the gastrointestinal tracts, increased epithelial cell proliferation is reported to increase the susceptibility to tumor development (57). Tumor promoters are also known to enhance cell proliferation in the target organs (58). The BrdUrd labeling index is used as a useful marker for cell proliferation and defined as one of the biomarkers in chemopreventive studies (13). We have demonstrated that AgNORs number is also a useful index for cell proliferation and have recently used this marker as a biomarker in chemoprevention studies of liver and tongue carcinogenesis (50, 59–61). The results in the current study provide additional evidence that AgNORs enumeration is useful as a biomarker in chemoprevention studies in colon carcinogenesis. As for another biomarker of ODC, ODC induction precedes cell proliferation in many cells (23, 25). Chemical potential to cause either promotion or initiation induces ODC in the target organs (27, 62–64). ODC inhibitors like α-difluoromethylornithine have an inhibitory effect on tumor development in several organs including colon (27, 29, 30). Luk *et al.* (28) reported that biphasic increase of colonic mucosal ODC activity during AOM-induced rat colon carcinogenesis and an early peak might be associated with initiation phase and a subsequent second increase might be associated with postinitiation phase. In the present study, PCA given during or after the AOM initiation clearly inhibited colon tumor development and also decreased mucosal ODC activity, BrdUrd labeling index, and AgNORs number in the distal portion of the colon. Our previous study on the modifying effect of PCA on diethylnitrosamine-induced rat liver carcinogenesis also indicated the suppressing effect of dietary PCA during the initiation or postinitiation phase and such PCA-induced inhibition clearly paralleled the reduction of liver ODC activity (36). Thus, the inhibition of colonic tumor development by PCA at the postinitiation stage might be due to lowering cell proliferation and ODC activity of colonic mucosa by PCA. When PCA was given during the initiation phase of AOM-induced carcinogenesis, similar results were obtained. This might be caused by blocking the fixation of DNA injury by inhibition of cell proliferation as revealed by the reduction of ODC activity, BrdUrd labeling index, and AgNORs number. However, the inhibition of activation enzymes, activation of detoxifying enzymes, and reduction of DNA adduct by PCA exposure might be also considered. Further studies should be performed to elucidate the exact mechanism(s), since biological and pharmacological properties of PCA have not been examined well. Our recent investigations on chemopreventive effects of PCA on diethylnitro-

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samine-induced liver carcinogenesis (36) and 4-nitroquinoline 1-oxide-induced tongue carcinogenesis in rats[4] indicated a clear inhibitory effect of dietary PCA on tumor occurrence in the liver and tongue in dose-dependent manner. Also, in such studies and the present study, PCA did not cause any toxic effects in all organs and weight gain retardation. Thus, PCA might be a promising new chemopreventive agent in carcinogenesis of various organs including colon. Additional studies to examine the efficacy of PCA as chemopreventive agent for carcinogenesis in other organs are ongoing in our laboratory.

ACKNOWLEDGMENTS

We wish to thank Suzuyo Satoh, Kyoko Takahashi, and Hiroko Mori for excellent technical assistance and Man-ichi Ito for his animal care.

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Chemoprevention of Colon Carcinogenesis by the Natural Product of a Simple Phenolic Compound Protocatechuic Acid: Suppressing Effects on Tumor Development and Biomarkers Expression of Colon Tumorigenesis

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Cancer Res 1993;53:3908-3913.

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