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Suppression of Azoxymethane-induced Rat Colon Aberrant Crypt Foci by Dietary Protocatechuic Acid

Toshihiko Kawamori, Takuji Tanaka,[1] Toshihiro Kojima, Masumi Suzui, Masami Ohnishi and Hideki Mori

First Department of Pathology, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu 500

The modifying effect of dietary exposure to protocatechuic acid (PCA) on the development of azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF) was investigated in male F344 rats. The effects of PCA feeding on the silver-stained nucleolar organizer regions protein (AgNORs) count in the colonic epithelial cells and on the ornithine decarboxylase (ODC) activity in the colonic mucosa were also estimated. Animals were given weekly s.c. injections of AOM (15 mg/kg body weight) for 3 weeks to induce ACF. These rats were fed diet containing 1000 or 2000 ppm PCA for 5 weeks, starting one week before the first dosing of AOM. All rats were killed 2 weeks after the last AOM injection, to measure the number of ACF, ODC activity, and AgNORs count per nucleus in the colon. In rats given AOM and PCA, the frequency of ACF/colon was significantly decreased compared with that in rats given AOM alone ($P<0.005$ at 1000 and $P<0.05$ at 2000 ppm). ODC activity in the colon of rats given AOM and PCA at both doses was also significantly lower than that of rats treated with AOM alone ($P<0.05$). Similarly, the mean AgNORs count in rats fed PCA was significantly smaller than that of rats treated with AOM alone ($P<0.0001$). Treatment with PCA alone did not affect these three biomarkers. These results provide further evidence that PCA could be a chemopreventive agent against rat colon carcinogenesis.

Key words: Protocatechuic acid — Chemoprevention — Aberrant crypt focus — Azoxymethane

Colorectal cancer is a leading form of cancer in the United States and in countries where Western-style diets are generally consumed. In Japan, the progressive introduction of Western dietary habits, especially an increasing fat intake and decreasing carbohydrate intake, has been paralleled by an increase in colon and breast cancers.[1)] It has been suggested that chemopreventive agents might have considerable impact on the mortality rate in the United States from common cancers such as those of colon, breast and prostate.[2)]

A simple phenolic acid, PCA,[2] is one of the constituents of edible plants, fruits, and vegetables.[3)] A high level of PCA, which is a 10 times more potent antioxidant than D,L-α-tocopherol, was found in the extracts from the rind of *Citrus reticulata* Blanco.[4)] Our recent studies demonstrated a remarkable chemopreventive effect of dietary PCA on diethylnitrosamine-induced rat liver tumorigenesis,[5)] and AOM-induced colon carcinogenesis in rats.[6)] PCA feeding also lowered carcinogen-induced ODC activities in the liver[5)] and colon,[6)] and the bromodeoxyuridine (BrdU)-labeling index and AgNORs count in the colonic epithelium.[6)] The authors of the latter study suggested that PCA may act at the initiation and postinitiation stages of colon carcinogenesis. However, the results were obtained at the end of the study, whereas PCA was administered during carcinogen exposure.

Since the first description of ACF in carcinogen-treated rodent colon,[7)] these crypts have been shown to be dysplastic in nature[8)]; dysplasia is a precancerous state that leads to malignancy through the dysplasia-carcinoma sequence.[9, 10)] The number, size and multiplicity of ACF are useful as indicators of tumorigenicity.[11)] In addition, Pretlow *et al.*[12)] demonstrated the presence of these putative preneoplastic lesions in colonic mucosa of patients with colon carcinoma. Therefore, ACF are a useful biomarker to evaluate the chemopreventive effects of candidate compounds on colon carcinogenesis.

ODC is the first enzyme in the polyamine biosynthesis pathway.[13)] Polyamines play essential roles in cell proliferation and differentiation, and participate in macromolecular synthesis. Inhibitors of ODC block aspects of tumor promotion and induce cellular differentiation in several animal carcinogenesis models.[14)] Thus, the induction of ODC may be important for carcinogenesis,[15, 16)] and ODC activity[17–19)] may be useful as an intermediate biomarker of cell proliferation in chemopreventive studies. Similarly, AgNORs count/nucleus in the target organ is suggested to be a useful biomarker, since AgNORs count correlated well with BrdU-labeling index, which is an accurate measure of S-phase cells.[20)]

In the present study, using a short-term experiment, the modifying effect of dietary PCA during carcinogen

[1] To whom correspondence should be addressed.

[2] Abbreviations: PCA, protocatechuic acid; ACF, aberrant crypt foci; AOM, azoxymethane; ODC, ornithine decarboxylase; AgNORs, silver-stained nucleolar organizer regions protein.

exposure on the development of AOM-induced colonic ACF was investigated in male F344 rats. Also, the effects of PCA on two cell proliferation intermediate biomarkers, AgNORs in the colonic epithelium and ODC activity in the colonic mucosa, were examined.

MATERIALS AND METHODS

Animals and diet Male F344 rats, 4 weeks of age, purchased from Shizuoka Laboratory Animal Center (Hamamatsu), were quarantined for one week and then randomized into experimental and control groups. All animals were housed three to four to a wire cage. The holding room was controlled at 23±2°C, 50±10% humidity, and a 12-h light/dark cycle. Powdered CE-2 (Clea Japan, Inc., Tokyo) was used as the basal diet during 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 50 male rats was divided into five groups of 10 rats (Fig. 1). Starting at 6 weeks of age, rats in groups 1–3 were given s.c. injections of AOM (15 mg/kg body weight) once a week for 3 weeks. Animals in groups 2 and 3 were fed the diets containing 1000 and 2000 ppm PCA, respectively, for 5 weeks, starting at 5 weeks of age. Rats in group 4 were fed the diet containing 2000 ppm PCA alone during the experiment. Group 5 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* and weighed weekly. All animals were killed by decapitation at 4 weeks after the first administration of AOM, and complete necropsies were performed on all animals.

Identification of ACF At the termination of the study, the colons of five animals randomly selected from each group were removed, flushed with saline, slit open longitudinally from cecum to anus, placed between 2 pieces of filter paper and fixed in buffered 10% formalin for 24 h. Then they were stained with 0.2% methylene blue in saline according to the procedure of Bird[7] to observe ACF. The number of ACF/colon and the number of aberrant crypts in each focus were determined by microscopy at a magnification of ×40. The criteria used to identify an aberrant crypt focus topographically were as follows: (1) increased size, (2) thicker epithelial cell lining and (3) increased pericryptal zone relative to normal crypts.[7]

ODC activity At necropsy of the remaining five animals, their colons were rapidly removed. Each colon was rinsed in saline, slit open longitudinally, and freed from all 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). Colonic mucosa from each of 5 rats were pooled and homogenized in 1.5 ml of homogenizing buffer (250 m*M* sucrose, 50 m*M* Tris-HCl, pH 7.4, containing 1 m*M* dithiothreitol, 1 m*M* EDTA, and 0.4 m*M* pyridoxal 5′-phosphate) using a Polytron. The homogenates were centrifuged at 15,000 rpm, 4°C, for 30 min. The resulting cytosol fraction was used for determination of ODC activity and protein. ODC activity in the colonic mucosa was determined by a modification of the methods described previously.[21,22] The incubation mixture, in a final volume of 40 μl [50 m*M* N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid, pH 7.4, 1 m*M* EDTA, 0.25 m*M* pyridoxal phosphate, 1 m*M* dithiothreitol, and 0.25 μCi of DL-[1-^{14}C]ornithine (specific activity, 42.47 mCi/mmol; NEN, Boston, MA)], was incubated at 37°C for 1 h. The reaction was stopped by adding 50 μl of 6 *N* HCl, and the $^{14}CO_2$ released was collected on 10% KOH-saturated discs for another 15 min. ^{14}C in the form of $K_2{}^{14}CO_3$ was counted in a scintillation counter. The results were expressed as pmol $^{14}CO_2$/h/mg protein.

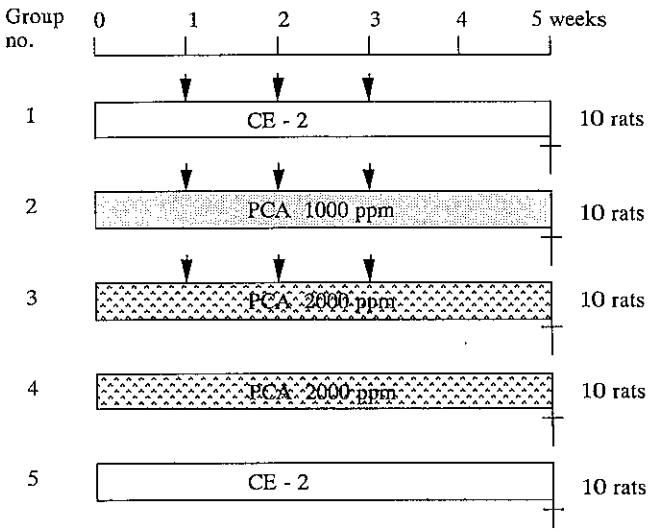

Fig. 1. Experimental protocol. ▼, AOM 15 mg/kg body weight s.c.; CE-2, basal diet; PCA, protocatechuic acid; †, termination (animals killed).

AgNORs count The AgNORs count of the mucosal epithelium of colon in each group was determined. The colon was removed and divided into three equal portions, named the upper, middle, and lower colon, and fixed in 10% buffered formalin. All portions of colon were embedded in paraffin and two serial sections (3 μm thickness) were made. One section was used for staining AgNORs and the other was stained with hematoxylin and eosin for histological examination. AgNORs staining was carried out according to the method described previ-

ously.[23] For determination of AgNORs count on the cell nuclei, 20 well oriented crypts, in which the base, lumen, and top of the crypts could be seen, were used. AgNORs were counted on AgNORs-stained sections under a microscope at a magnification of ×400. Data were expressed as AgNORs count per nucleus.

Statistical analysis The data in this experiment were analyzed by the use of Student's *t* test.

RESULTS

The body weight gains during the experiment and liver weights at death are presented in Table I. No significant effect of AOM and/or PCA treatment on body and liver weights was observed. Food consumption for PCA treatment groups was approximately 12.9–13.2 g/day/animal. No neoplasms were found on macroscopic and microscopic examinations of all organs of rats in each group.

Table II summarizes the mean number of ACF/colon, total number of aberrant crypts/colon and the mean number of aberrant crypts/focus. The rats treated with AOM (groups 1–3) showed a 100% incidence of ACF. No ACF were seen in the colons of rats without AOM treatment (groups 4 and 5). The number of ACF/colon was significantly decreased by PCA administration at either 2000 ppm ($P<0.01$) or 1000 ppm ($P<0.005$). The effect was more marked in rats fed 1000 ppm PCA. PCA administration at both doses significantly decreased the total number of aberrant crypts/colon ($P<0.05$). The number of aberrant crypts/focus was slightly increased by PCA administration at both doses. As for the size distribution of ACF, the number of foci consisting of one crypt was significantly decreased by PCA administration at 1000 ppm ($P<0.01$) and 2000 ppm ($P<0.05$) (Fig. 2). PCA administration did not affect the numbers of foci consisting of 2–4 crypts, although foci of 5–8 crypts were not found in groups 2 and 3 (AOM+PCA).

The ODC activity in colonic mucosa is indicated in Table III. The mean ODC activity of colonic mucosa in group 1 (AOM alone) was greater than those of the other groups. In the groups of rats treated with AOM and PCA (groups 2 and 3), mean colonic ODC activities were significantly lower than that of group 1 ($P<0.05$). The mean ODC activities in groups 2 and 3 were smaller than that in group 4 (PCA alone), but the difference lacked statistical significance.

The data on AgNORs count are also shown in Table III. The mean number of cells/crypt column in group 5 (no treatment) was the greatest among all the groups. The mean numbers of cells/crypt column in groups 2 and 3 were significantly smaller than that of group 1 ($P<0.0005$ and $P<0.005$, respectively). The value in

Table I. Mean Body Weights of Male F344 Rats Treated with Azoxymethane and Fed a Control Diet or a Diet Supplemented with Protocatechuic Acid

Group no.	Treatment	Mean body weight (g) of rats at:						Mean liver weight (g)
		Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	
1	AOM alone	86.8±1.7[a]	112.6±1.9	140.2±4.3	164.4±10.2	152.5±15.1	160.7±11.7	5.7±0.5
2	AOM+PCA 1000 ppm	80.8±3.7	107.3±5.0	127.5±8.7	140.7±9.3	153.4±10.8	159.3±10.3	5.2±0.5
3	AOM+PCA 2000 ppm	85.1±2.0	109.3±3.1	135.4±5.6	154.0±6.9	150.9±14.6	154.7±12.0	5.1±0.6
4	PCA 2000 ppm alone	86.5±3.6	112.4±4.4	137.4±7.0	155.7±8.2	152.6±13.7	156.7±12.4	4.8±0.9
5	No treatment	83.6±4.7	115.2±3.8	147.1±6.0	169.9±8.2	159.6±17.6	156.6±18.7	4.8±0.8

a) Mean±SD.

Table II. Effect of PCA on AOM-induced Aberrant Crypt Foci in Rat Colon

Group no.	Treatment	Incidence[a]	No. of foci/colon	Total no. of aberrant crypts/colon	No. of aberrant crypts/focus
1	AOM alone	5/5	149.4±26.2[b]	208.6±45.8	1.40±0.24
2	AOM+1000 ppm PCA	5/5	68.6±21.1[c]	116.4±41.9[d]	1.66±0.15
3	AOM+2000 ppm PCA	5/5	91.0±13.6[e]	128.0±25.1[d]	1.41±0.18

a) No. of rats with aberrant crypt foci/total rats.
b) Mean±SD.
Significantly different from group 1 by Student's *t* test: *c*, $P<0.005$; *d*, $P<0.05$; *e*, $P<0.01$.

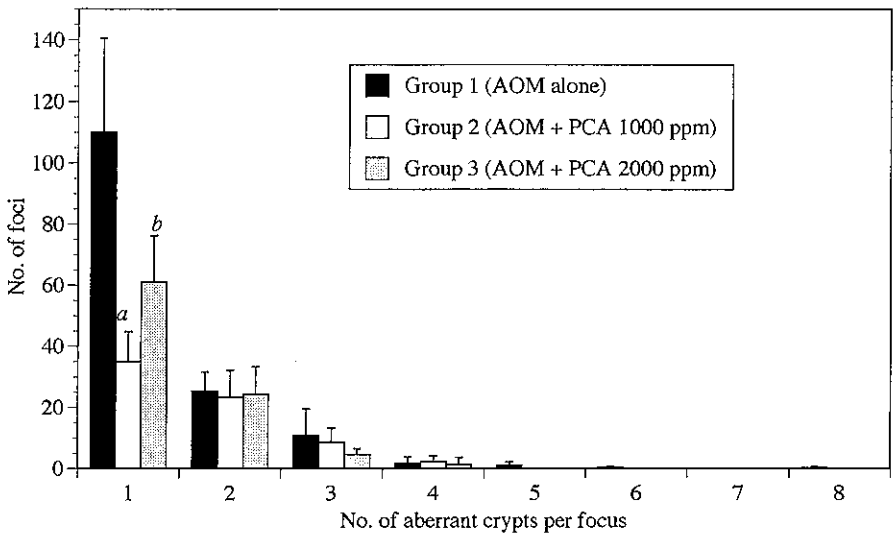

Fig. 2. Size distribution of AOM-induced aberrant crypt foci in the colon of rats in each group. Columns, average; bars, SD. *a, b)* Significantly different from group 1 at $P<0.01$ and $P<0.05$, respectively.

Table III. ODC Activity, Number of Cells per Crypt Column and AgNORs Count of the Colonic Epithelium in Each Group

Group no.	Treatment	Colonic ODC activity (pmol $^{14}CO_2$/h/mg protein)	No. of cells/crypt column	AgNORs count/nucleus
1	AOM alone	10.5±7.15[a)]	34.4±5.2	1.47±0.28
2	AOM+PCA 1000 ppm	0.44±0.44[b)]	31.1±4.2[c)]	1.13±0.33[d)]
3	AOM+PCA 2000 ppm	0.42±0.72[b)]	31.5±4.3[e)]	0.96±0.29[d)]
4	PCA 2000 ppm alone	7.58±7.01	29.4±4.1	0.85±0.27
5	No treatment	4.89±3.46	36.7±4.9	1.01±0.28

a) Mean±SD.
Significantly different from group 1 by Student's *t* test: *b*, $P<0.05$; *c*, $P<0.0005$; *d*, $P<0.0001$; *e*, $P<0.005$.

group 4 was also significantly smaller than that in group 5 ($P<0.0001$). The mean AgNORs count/nucleus in groups 2 and 3 was significantly lower than that of group 1 ($P<0.0001$).

DISCUSSION

In the present study, dietary administration of a simple phenolic acid, PCA, clearly inhibited the development of AOM-induced ACF in rats colon. PCA feeding also reduced ODC activity, AgNORs count and the height of the colonic mucosa.

ACF are one of the earliest hallmarks of colon carcinogenesis.[19)] They are readily utilizable as intermediate endpoints in colon cancer prevention studies since they are easily and rapidly quantified topographically in a methylene-blue-stained whole colon from rodents fed test chemicals. In the present study, PCA reduced the total number of ACF and aberrant crypts/colon. Although PCA reduced the number of foci consisting of one aberrant crypt and those of more than 5 crypts, PCA slightly increased the number of aberrant crypts (2–4 crypts)/focus. These results may suggest that PCA suppresses the induction of ACF, which show increased cell proliferation activity and some genetic alterations,[24–26)] but does not alter the proliferative activity in ACF themselves. Therefore, it might be that the blocking effect of PCA is stronger than its suppressing ability on colon carcinogenesis. Further studies on alterations of proliferative activity in ACF by dietary PCA are needed.

Cell proliferation might play an important role in the carcinogenic process.[27)] Cell kinetic analysis in the colonic mucosa of individuals at increased risk for colon cancer has revealed an anomalous expansion of epithelial cells within the colonic crypts. We have demonstrated that AgNORs count is a useful index for cell proliferation

and we have recently used it as a biomarker in chemoprevention studies of liver, tongue, and colon carcinogenesis.[6, 28–31] ODC induction precedes cell proliferation in many cells.[27] Several initiators and promoters of carcinogenesis elevate ODC activities in the target organs.[18, 32–34] Our previous study[6] demonstrated that PCA inhibited AOM-induced rat colon carcinogenesis, and decreased ODC activities in the colonic mucosa and liver tissue, while the AgNORs count in the colonic epithelium paralleled the tumor reduction. In that study, PCA was fed during or after the AOM treatment, namely in the initiation or postinitiation phase. However, ODC activities and AgNORs count were determined at the termination of the study (32 weeks after the start). In the current study, PCA treatment together with AOM significantly reduced ODC activity of colonic mucosa as well as number of ACF/colon at 5 weeks after the start. Colonic mucosa of rats treated with AOM showed a decreased number of total cells/crypt column and increased AgNORs count/nucleus. PCA treatment decreased such indices. These results suggested that PCA decreased the number of S-phase cells, and so protected DNA in the colonic epithelial cells from AOM injury. Thus, PCA may be an effective chemopreventive agent with a blocking effect against colon carcinogenesis.

Cancer chemoprevention is defined as intervention with agents at a pre-malignant lesion to reverse or suppress progression to malignancy. Many biomarkers have been proposed for use as intermediate endpoints in chemoprevention studies. Kelloff *et al.*[35] classified these biomarkers into genomic biomarkers, markers of cell proliferation, markers of cell differentiation and premalignant lesions. However, there are rather few reliable biomarkers. Searching for potential inhibitors of carcinogenesis using routine *in vivo* long-term assay is expensive and time-consuming. Therefore, short-term *in vivo* assay using a combination of several biomarkers is needed, as described here and by Rao *et al.*[19] Similar short-term experiments have been reported,[36, 37] but only the incidence of ACF was used as a biomarker.

In summary, PCA inhibited the formation of AOM-induced rat colon ACF, reducing ODC activity of colonic mucosa, and decreasing the AgNORs count in the colonic epithelium. The methodology of this short-term model may be developed into a very useful prescreening tool for chemopreventive agents, especially blocking agents that are effective against colon cancer.

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REFERENCES

1) Tajima, K., Hirose, K., Nakagawa, N., Kuroishi, T. and Tominaga, S. Urban-rural difference in the trend of colorectal cancer mortality with special reference to the subsites of colon cancer in Japan. *Jpn. J. Cancer Res.*, **76**, 717–728 (1985).
2) Weinstein, I. B. Cancer prevention: recent progress and future opportunities. *Cancer Res.*, **51**, 5080s–5085s (1991).
3) Spanos, G. A. and Wrolstad, R. E. Phenolics of apple, pear, and white grape juices and their changes with processing and storage: a review. *J. Agric. Food Chem.*, **40**, 1478–1487 (1992).
4) Ohkawa, Y., Tanizawa, H., Takino, Y., Miyase, T., Ueno, A., Kageyama, T. and Shikano, E. Studies on the antioxidative activity in citrus fruit. II. *Proc. Jpn. Pharmacol. Soc., 111th Annu. Meet.*, 174 (1991).
5) Tanaka, T., Kojima, T., Kawamori, T., Yoshimi, N. and Mori, H. Chemoprevention of diethylnitrosamine-induced hepatocarcinogenesis by a simple phenolic acid protocatechuic acid in rats. *Cancer Res.*, **53**, 2775–2779 (1993).
6) Tanaka, T., Kojima, T., Suzui, M. and Mori, H. 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. *Cancer Res.*, **53**, 3908–3913 (1993).
7) Bird, R. P. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer Lett.*, **37**, 147–151 (1987).
8) Chang, W. W. L. Histogenesis of colon cancer in experimental animals. *Scand. J. Gastroenterol.*, **19** (Suppl. 104), 27–44 (1984).
9) Day, D. W. and Morson, B. C. The adenoma-carcinoma sequence. *In* "The Pathogenesis of Colorectal Cancer," ed. B. C. Morson, pp. 58–71 (1987). W.B. Saunders, Philadelphia.
10) Bird, R. P., McLellan, E. A. and Bruce, W. R. Aberrant crypts, putative precancerous lesions, in the study of the role of diet in the aetiology of colon cancer. *Cancer Surv.*, **8**, 189–200 (1989).
11) McLellan, E. A. and Bird, R. P. Sequential analyses of the growth and morphological characteristics of aberrant crypt foci: putative preneoplastic lesions. *Cancer Res.*, **51**, 5270–5274 (1991).
12) Pretlow, T. P., Barrow, B. J., Ashton, W. S., O'Riordan, M. A., Pretlow, T. G., Jurcisek, J. A. and Stellato, T. A. Aberrant crypts: putative preneoplastic foci in human

colonic mucosa. *Cancer Res.*, **51,** 1564–1567 (1991).
13) Pegg, A. E. and McCann, P. P. Polyamine metabolism and function. *Am. J. Physiol.*, **243,** C212–C221 (1982).
14) Russell, D. H. Ornithine decarboxylase: a key regulatory enzyme in normal and neoplastic growth. *Drug Metab. Rev.*, **16,** 1–88 (1985).
15) O'Brien, T. G. The induction of ornithine decarboxylase as an early, possible obligatory, event in mouse skin carcinogenesis. *Cancer Res.*, **36,** 2644–2653 (1976).
16) Kingsnorth, A. N., King, W. W. K., Diekema, K. A., McCann, P. P., Ross, J. S. and Malt, R. A. Inhibition of ornithine decarboxylase with 2-difluoromethylornithine: reduced incidence of dimethylhydrazine-induced colon tumors in mice. *Cancer Res.*, **43,** 2545–2549 (1983).
17) Kojima, T., Tanaka, T., Kawamori, T., Hara, A. and Mori, H. Chemopreventive effects of dietary D,L-α-difluoromethylornithine, an ornithine decarboxylase inhibitor, on initiation and postinitiation stages of diethylnitrosamine-induced rat hepatocarcinogenesis. *Cancer Res.*, **53,** 3903–3907 (1993).
18) Rozhin, J., Wilson, P. S., Bull, A. W. and Nigro, N. D. Ornithine decarboxylase activity in the rat and human colon. *Cancer Res.*, **44,** 3226–3230 (1984).
19) Rao, C. V., Desai, D., Simi, B., Kulkarni, N., Amin, S. and Reddy, B. S. Inhibitory effect of caffeic acid esters on azoxymethane-induced biochemical changes and aberrant crypt foci formation in rat colon. *Cancer Res.*, **53,** 4182–4188 (1993).
20) Tanaka, T., Takeuchi, T., Nishikawa, A., Takami, T. and Mori, H. Nucleolar organizer regions in hepatocarcinogenesis induced by N-2-fluorenylacetamide in rats: comparison with bromodeoxyuridine immunohistochemistry. *Jpn. J. Cancer Res.*, **80,** 1047–1051 (1989).
21) Stanley, B. A. and Kazarinoff, M. M. Induction of ornithine decarboxylase in colon and liver by starvation and refeeding: a comparison of effects on total and holoenzyme. *Biochem. Biophys. Res. Commun.*, **105,** 773–777 (1982).
22) Lapointe, D. S. and Cohen, R. J. A rapid and efficient microassay of ornithine decarboxylase. *Anal. Biochem.*, **109,** 291–294 (1980).
23) Tanaka, T., Kojima, T., Okumura, A., Yoshimi, N. and Mori, H. Alterations of the nucleolar organizer regions during 4-nitroquinoline 1-oxide-induced tongue carcinogenesis in rats. *Carcinogenesis,* **12,** 329–333 (1991).
24) Stopera, S. A., Davie, J. R. and Bird, R. P. Colonic aberrant crypt foci are associated with increased expression of c-*fos*: the possible role of modified c-*fos* expression in preneoplastic lesions in colon cancer. *Carcinogenesis,* **13,** 573–578 (1992).
25) Stopera, S. A., Murphy, L. C. and Bird, R. P. Evidence for a *ras* gene mutation in azoxymethane-induced colonic aberrant crypts in Sprague-Dawley rats: earliest recognizable precursor lesions of experimental colon cancer. *Carcinogenesis,* **13,** 2081–2085 (1992).
26) Stopera, S. A. and Bird, R. P. Effects of all-*trans* retinoic acid as a potential chemopreventive agent on the formation of azoxymethane-induced aberrant crypt foci: differential expression of c-myc and c-fos mRNA and protein. *Int. J. Cancer,* **53,** 798–804 (1993).
27) Jänne, J., Pösö, H. and Raina, A. Polyamines in rapid growth and cancer. *Biochim. Biophys. Acta,* **473,** 241–293 (1978).
28) Tanaka, T., Yoshimi, N., Sugie, S. and Mori, H. Protective effects against liver, colon, and tongue carcinogenesis by plant phenols. *In* "Phenolic Compounds in Food and Their Effects on Health II: Antioxidants and Cancer Prevention, American Chemical Society Symposium Series 507," ed. M.-T. Huang, C.-T. Ho and C. Y. Lee, pp. 326–337 (1992). American Chemical Society, Washington, DC.
29) Tanaka, T., Kojima, T., Morishita, Y. and Mori, H. Inhibitory effects of the natural products indole-3-carbinol and sinigrin during initiation and promotion phases of 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis. *Jpn. J. Cancer Res.*, **83,** 835–842 (1992).
30) Tanaka, T., Kojima, T., Kawamori, T., Wang, A., Suzui, M., Okamoto, K. and Mori, H. Inhibition of 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis by the naturally occurring plant phenolics caffeic, ellagic, chlorogenic and ferulic acids. *Carcinogenesis,* **14,** 1321–1325 (1993).
31) Tanaka, T., Kojima, T., Hara, A., Sawada, H. and Mori, H. Chemoprevention of oral carcinogenesis by DL-α-difluoromethylornithine, an ornithine decarboxylase inhibitor: dose-dependent reduction in 4-nitroquinoline 1-oxide-induced tongue neoplasms in rats. *Cancer Res.*, **53,** 772–776 (1993).
32) Marks, F., Bertsch, S. and Furstenberger, G. Ornithine decarboxylase activity, cell proliferation, and tumor promotion in mouse epidermis *in vivo*. *Cancer Res.*, **39,** 4183–4188 (1979).
33) Matsushima, M., Takano, S., Erturk, E. and Bryan, G. Induction of ornithine decarboxylase activity in mouse urinary bladder by L-tryptophan and some of its metabolites. *Cancer Res.*, **42,** 3587–3591 (1982).
34) Kitchin, K. T., Brown, J. L. and Kulkarni, A. P. Ornithine decarboxylase induction and DNA damage as predictive assay for potential carcinogenicity. *Prog. Clin. Biol. Res.*, **369,** 137–144 (1991).
35) Kelloff, G. J., Malone, W. F., Boone, C. W., Steele, V. E. and Doody, L. A. Intermediate biomarkers of precancer and their application in chemoprevention. *J. Cell. Biochem. (Suppl.),* **16G,** 15–21 (1992).
36) Lam, L. K. T. and Zhang, J. Reduction of aberrant crypt formation in the colon of CF1 mice by potential chemopreventive agents. *Carcinogenesis,* **12,** 2311–2315 (1991).
37) Pereira, M. A. and Khoury, M. D. Prevention by chemopreventive agents of azoxymethane-induced foci of aberrant crypt in rat colon. *Cancer Lett.*, **61,** 27–33 (1991).