in rats so that DAG itself is not absorbed in the lower gastro-intestinal tract (Osaki et al., 2005). DAG oil is also reported not mutagenic (Kasamatsu et al., 2005). Furthermore, in DMBA-induced mammary carcinogenesis in rats, DAG oil did not enhance mammary tumor development more than edible TAG (Sugano et al., 2002). Accordingly, safety of the DAG oil has been generally recognized.

1,2-DAG has been shown to stimulate protein kinase C (PKC) an enzyme that plays a key role in growth control and signal transduction (Reddy et al., 1996; Sozeri et al., 1992). These functions are similar to a potent tumor promoting agent, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which contains a 1,2-DAG-related structure in its molecule and is reported to directly activate PKC (Castagna et al., 1982). PKC is normally activated by endogenous 1,2-DAG released from membrane phospholipids (Hurley et al., 1997; Nishizuka, 1984) and exogenous 1,2-DAG can also stimulate intracellular signaling pathways (Friedman et al., 1989), resulting in mitosis in a certain carcinoma cells *in vitro* (Liscovitch and Cantley, 1994). 1,2-DAG can also mediate cellular responses through a binding site unrelated to PKC that possesses a C1 domain like Ras guanyl nucleotide-releasing protein (RasGRP) (Kazanietz, 2002) and activated RasGRP has been implicated in activation of the Ras effector Raf-1 and thereby activation of the MAP kinase cascade (Lorenzo et al., 2000). Thus, increased levels of exogenous 1,2-DAG may be considered a tumor-promoting risk factor in many organs.

Several studies have shown that a high-fat diet results in increased fecal concentrations of 1,2-DAG (Choe et al., 1992; Pickering et al., 1995). Moreover, fecal bacteria in the colon can produce 1,2-DAG (Vulevic et al., 2004) which could impact directly on colon carcinogenesis (Friedman et al., 1989). Furthermore, direct absorbance by the tongue and other tissues in the oral cavity is conceivable so that carcinogenesis in these sites could be affected. To determine whether DAG oil enhances tongue carcinogenesis induced by 4-nitroquinoline 1-oxide (4NQO) (Suzui et al., 1995; Tang et al., 2004), the present study was therefore performed with Tg rats which over-express *ras* and are highly susceptible to induction of mammary, urinary bladder, esophagus, skin and tongue (Suzuki et al., 2006) tumors by chemical carcinogens (Asamoto et al., 2000; Park et al., 2004; Tsuda et al., 2005).

2. Materials and methods

2.1. Animals and experimental protocol

A total of 372 Tg and wild type rats of both sexes derived from Sprague–Dawley strain bred by CLEA (CLEA Japan Inc., Tokyo, Japan) at 7 weeks of age were maintained in plastic cages in an air conditioned room at 22 ± 2 °C and 55 ± 10% humidity with free access to standard AIN-93G basal diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water. The experimental protocol is shown in Fig. 1. After 1 week of acclimatization, they were randomly allocated to six groups (each with male and female Tg and wild-type sub-groups). The rats in Groups 1–4 received deionized water containing 10 ppm 4NQO (Wako Pure Chemical

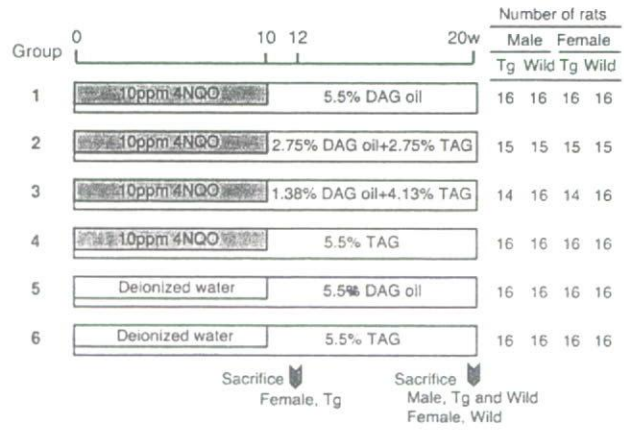

Fig. 1. Experimental protocol. Group 1–4 rats were treated with 4NQO (10 ppm) and Groups 5 and 6 were given the vehicle for the initial 10 weeks. Group 1–3 rats were fed DAG oil added to the AIG-93G basal diet at 5.5%, 2.75% and 1.38% (1:2 serial dilution), respectively. Fat content was adjusted to 5.5% in all groups by adding TAG as appropriate. Group 4 was treated with TAG alone for comparison of modification effects on tumor induction (control group). The experiment was terminated at week 12 for female Tg rats and at week 20 for female wild type and all the male rats. The effective number of animals in each group is shown on the left. M, male; F, female.

Ind., Osaka, Japan) for 10 weeks (see legend for Fig. 1), as well as basal diets containing DAG and/or TAG (total fat, 5.5%) (Kao Corporation, Tokyo, Japan) throughout the initiation and post-initiation periods. Groups 5 and 6 were fed DAG oil and TAG without 4NQO treatment. Body weights were monitored every other week throughout the experiment, and food and water intakes were measured every other week for the initial 10 weeks then once a month. Gross observation and palpation were regularly performed to monitor the development of tongue and mammary tumors throughout the experimental period. The surviving animals were sacrificed by exsanguination under deep anesthesia with diethyl ether, 12 weeks (Tg females) and 20 weeks (Tg males, wild males and females) after the start of the experiment. The experiments were conducted according to the "Guidelines for Animal Experiments in National Cancer Center" of the Committee for Ethics of Animal Experimentation of the National Cancer Center, Japan. At necropsy, all major organs except the brain were removed and assessed for any macroscopic lesion development. The oral cavity and tongue were carefully inspected and the lumen of the esophagus, stomach and intestines were inflamed with in 10% buffered formalin for fixation. The tongue, lung, liver, and kidney issues were also fixed in 10% buffered formalin, embedded in paraffin blocks, and sectioned (2.5 μm) for hematoxylin and eosin staining.

2.2. Diets

DAG oil and TAG were supplied and added into powdered diet by Kao Corp., (Tokyo, Japan). The rats were fed dry powdered AIN93-based purified diets containing 5.5% fat (Groups 1 and 5, 5.5% DAG oil; Group 2, 2.75% DAG oil + 2.75% TAG; Group 3, 1.38% DAG oil + 4.18% TAG; Groups 4 and 6, 5.5% TAG) as shown in Fig. 1. Fatty acid compositions in DAG oil and TAG were almost the same (linoleic acid 46.6% and oleic acid 38.9% of total fatty acids). The diet was prepared 2 weeks before use and stored in the cold room at 4 °C. Chemical structures of 1,3-DAG, 1,2-DAG and TAG, and fatty acid composition are shown in Fig. 2.

2.3. Analysis of biochemical parameters of the blood

For biochemical analysis, coagulated blood was centrifuged and serum was collected and stored in a deep freezer at −80 °C until measurement of the following parameters: triglycerides (TG), free fatty acids (FFA), total

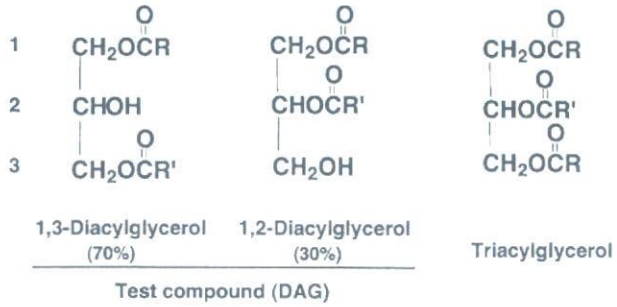

Fig. 2. Structure and composition of the test compound DAG oil and control oil TAG. Both have oleic acid (R) at 38.9% and linoleic acid (R') at 46.6%. DAG oil is a mixture of 1,3-DAG and 1,2-DAG at 70:30 ratio with R and R' at random position and TAG has R' in mostly "2" position.

cholesterol (T-Cho), lipoprotein (LP), glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT).

2.4. Statistical analysis

Statistical analyses of the incidences of lesions were performed using the Fisher's exact-test and the Cochran–Armitage trend test. Multiplicity, number of tumor per rat, of the lesion was analyzed by coefficients for linear contrast trend test. The data for body and liver weights, and biochemical parameters were analyzed by the Dunnet's test. Statistical significance was concluded with P-values less than 0.05.

3. Results

There were no significant compound-related effects noted on food consumption, body and organ weights. Data for tongue tumor incidences and multiplicities in the different groups for Tg and wild males are summarized in Tables 1–4. The macroscopic appearances of tongue tumors in Groups 1 and 4 are illustrated in Fig. 3. Representative histological features of SCC are shown in Fig. 4. In male Tg rats, incidences of tongue SCC were 43.8, 20, 14.3 and 12.3% in groups treated with 5.5, 2.75, 1.38 and 0% DAG oil, respectively (Table 1). Similarly, quantitative values (number/rat) of tongue SCC were 0.44, 0.20, 0.14 and 0.13, and combined values of squamous cell papilloma (papilloma) with SCC were 0.69, 0.73, 0.21 and 0.31 (Table 2). The values were significantly increased with linear trend test by coefficient for linear contrast ($P < 0.05$). In female Tg and wild type rats (not shown), incidences of tongue papilloma and SCC (Tables 3 and 4) were not correlated with the treatment.

Table 1
Incidence of tongue tumors (Tg, male)

Group	Treatment	No. of rats	Incidence (%)		
			Papilloma	SCC	Papilloma + SCC
1	4NQO + 5.5%DAG	16	4 (25.0)	7 (43.8)*	9 (56.3)
2	4NQO + 2.75%DAG + 2.75%TAG	15	6 (40.0)	3 (20.0)*	8 (53.3)
3	4NQO + 1.38%DAG + 4.13%TAG	14	1 (7.1)	2 (14.3)*	3 (21.4)
4	4NQO + 5.5%TAG	16	3 (18.8)	2 (12.3)*	5 (31.3)
5	5.5%DAG	16	0	0	0
6	5.5%TAG	16	0	0	0

Papilloma, squamous cell papilloma; SCC, squamous cell carcinoma.
* $P < 0.05$, linear trend by Cochran-Armitage trend-test.

Table 2
Multiplicity of tongue tumors (Tg, male)

Group	Treatment	No. of rats	No./rat[a]		
			Papilloma	SCC	Papilloma + SCC
1	4NQO + 5.5%DAG	16	0.25 ± 0.45	0.44 ± 0.51*	0.69 ± 0.70*
2	4NQO + 2.75%DAG + 2.75%TAG	15	0.53 ± 0.74	0.20 ± 0.41*	0.73 ± 0.80*
3	4NQO + 1.38%DAG + 4.13%TAG	14	0.07 ± 0.27	0.14 ± 0.36*	0.21 ± 0.43*
4	4NQO + 5.5%TAG	16	0.19 ± 0.40	0.13 ± 0.34*	0.31 ± 0.48*
5	5.5%DAG	16	0	0	0
6	5.5%TAG	16	0	0	0

Papilloma, squamous cell papilloma; SCC, squamous cell carcinoma.
* $P < 0.05$, linear trend by a test using coefficients for linear contrast.
[a] Mean ± SD.

Table 3
Incidence of tongue tumors (wild, male)

Group	Treatment	No. of rats	Incidence (%)		
			Papilloma	SCC	Papilloma + SCC
1	4NQO + 5.5%DAG	16	1 (6.3)	1 (6.3)	2 (12.5)
2	4NQO + 2.75%DAG + 2.75%TAG	15	0	2 (13.3)	2 (13.3)
3	4NQO + 1.38%DAG + 4.13%TAG	16	0	0	0
4	4NQO + 5.5%TAG	16	0	0	0
5	5.5%DAG	16	0	0	0
6	5.5%TAG	16	0	0	0

Papilloma, squamous cell papilloma; SCC, squamous cell carcinoma.

Table 4
Multiplicity of tongue tumors (wild, male)

Group	Treatment	No. of rats	No./rat[a]		
			Papilloma	SCC	Papilloma + SCC
1	4NQO + 5.5%DAG	16	0.06 ± 0.25	0.06 ± 0.25	0.13 ± 0.34
2	4NQO + 2.75%DAG + 2.75%TAG	15	0	0.13 ± 0.35	0.13 ± 0.35
3	4NQO + 1.38%DAG + 4.13%TAG	16	0	0	0
4	4NQO + 5.5%TAG	16	0	0	0
5	5.5%DAG	16	0	0	0
6	5.5%TAG	16	0	0	0

Papilloma, squamous cell papilloma; SCC, squamous cell carcinoma.
[a] Mean ± SD.

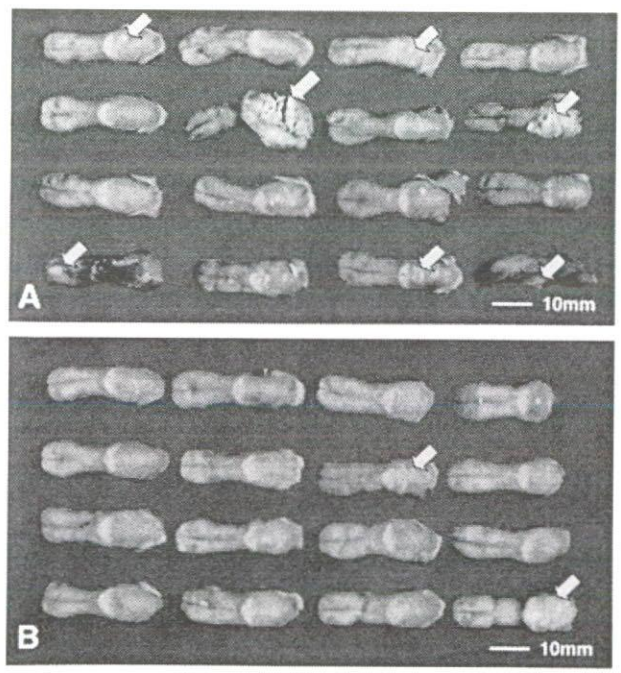

Fig. 3. Macroscopic appearance of tongue carcinomas; (A) 4NQO and 5.5%DAG (Group 1) and (B) 4NQO alone (Group 4). Arrows indicate carcinoma lesions.

In the forestomach of male Tg rats, incidences of the papilloma were 12.5%, 26.7%, 21.4%, and 6.3% in the groups receiving diets containing 5.5%, 2.75%, 1.38% and 0% DAG oil, respectively. These differences were not significant. Incidence and multiplicity of papilloma in male wild type rats and female Tg and wild type rats did not vary with the treatment.

In the mammary glands of male Tg rats, incidences of adenocarcinoma were 37.5%, 26.7%, 28.6% and 31.3% in the groups treated with 5.5%, 2.75%, 1.38% and 0% DAG oil, respectively. The female Tg rat group was sacrificed at week 12 because of early palpable tumor development, the incidence in the all group reaching almost 70%. These differences were not significant. Also in other organs, there was no significant variation in quantitative data for neoplastic lesion development.

Data for serum parameters are summarized in Tables 5 and 6. The levels of TG and GOT were significantly lowered with 5.5% DAG oil in male Tg and wild rats ($P < 0.05$). However, differences were not consistent across the carcinogen initiated and non-initiated groups and there was no clear dose dependence.

4. Discussion

Tg rats carrying three copies of the human c-Ha-ras proto-oncogene are highly susceptible to various carcino-

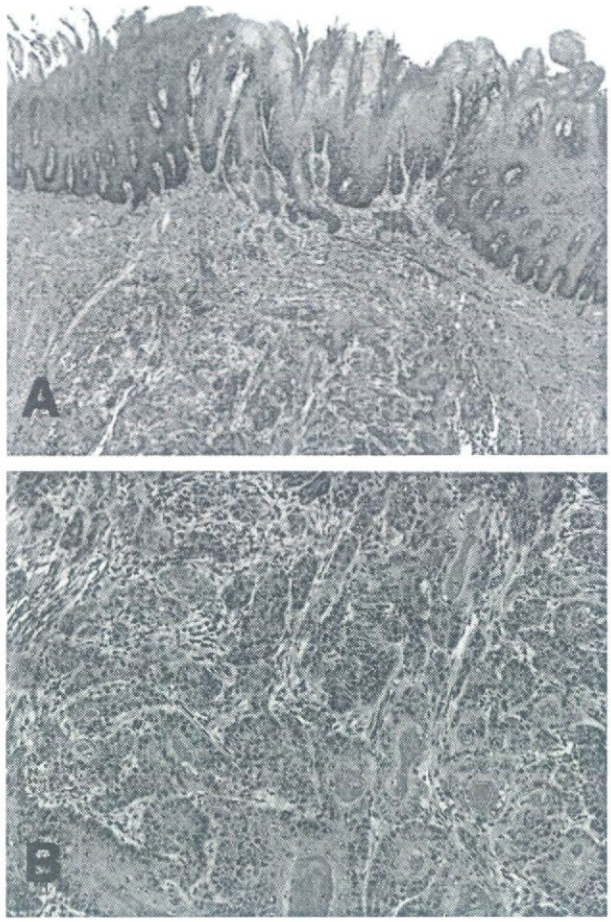

Fig. 4. Squamous cell carcinoma induced in a rat in Group 1; (A) lower magnification and (B) a higher magnification showing obvious invasion of submucosal and muscular tissue.

gens, developing mammary, tongue, skin and esophagus tumors (Tsuda et al., 2005). The present study provided evidence that DAG oil may indeed exert enhancing potential on SCC development in the tongue of male Tg rats after treatment with 4NQO. Both tumor incidence and multiplicity were significantly increased in trend analysis in the treatment groups, without obvious effects on food consumption or organ or body weights, pointing to a direct effect on the tongue epithelium.

The enhancing effect of DAG on 4-NQO-induced tongue carcinogenesis was observed only in male Tg rats. The susceptibility of the Tg rats to 4NQO-induced tongue (Suzuki et al., 2006) and DMBA-induced skin (Park et al., 2004) carcinogenesis is higher in males than in females. As estradiol inhibited development of skin tumors in male Tg rats (Han et al., 2002), the sex hormone may affect carcinogenesis of skin and maybe tongue. The mechanism remains to be clarified, but sex-dependence of tongue, mammary and skin susceptibility might be involved.

As shown in Tables 1 and 2, the formation of papillomas was not significantly affected by low doses of DAG, however, higher doses of DAG promoted progression from papilloma to SCC. The incidence of SCC and the multiplicity of SCC and papilloma + SCC were significantly increased (linear trend test, $P < 0.05$). These statistical analysis show that DAG oil administration enhances dose dependent 4NQO-induced tongue carcinogenesis in male Tg rats.

Earlier studies of 2-year feeding of 2.65% DAG oil plus 2.65% edible oil or 5.3% DAG oil to Sprague–Dawley rats demonstrated no effects, with reference to clinical signs, body and organ weights, food consumption, hematology, blood chemistry, or microscopic non-neoplastic changes as compared to controls. In our study, these physical findings and biochemical parameters in the blood also did not show a clear difference between DAG and TAG oil feeding groups.

No significant increase in mammary carcinoma development was observed between DAG and high edible oil, linoleic and oleic safflower oil, fed groups, although a significant increase was noted when the DAG group was compared to a rapeseed and soybean oil fed group (Soni et al., 2001). Recently, negative results for carcinogenic potential were reported with chronic feeding tests of DAG oil in rats, mice and dogs (Chengelis et al., 2006a,b; Chengelis et al., 2006c). At the dietary level of 7% DAG oil, irrespective of the source of DAG (composition of 1,2-DAG and 1,3-DAG not given), effects were

Table 5
Blood chemical analysis of male Tg rats

Group	Treatment	TG	FFA	T-Cho	LP	GOT	GPT	BUN	CR
1	4NQO − 5.5%DAG	130 ± 80*	1084 ± 461	105 ± 17	6.0 ± 0.7	126 ± 22*	25 ± 18	52 ± 77	0.48 ± 0.27
2	4NQO − 2.75%DAG + 2.75%TAG	157 ± 48	570 ± 170	105 ± 10	5.8 ± 0.4	77 ± 32	18 ± 6	14 ± 2	0.34 ± 0.03
3	4NQO − 1.38%DAG + 4.13%TAG	239 ± 100	1119 ± 470	110 ± 9	7.0 ± 0.8	134 ± 31	35 ± 25	20 ± 4	0.35 ± 0.07
4	4NQO − 5.5%TAG	302 ± 143	666 ± 68	108 ± 17	6.6 ± 1.1	75 ± 14	18 ± 4	16 ± 1	0.31 ± 0.04
5	5.5%DAG	160 ± 66#	691 ± 154	97 ± 3	6.8 ± 0.8	104 ± 27#	22 ± 8	18 ± 3	0.35 ± 0.04
6	5.5%TAG	139 ± 35	836 ± 177	121 ± 17	6.8 ± 0.4	126 ± 43	28 ± 11	17 ± 3	0.31 ± 0.02

* $P < 0.05$ as compared to 4NQO + 5.5%TAG group (Group 4).
\# $P < 0.05$ as compared to 5.5%TAG group (Group 6).

Table 6
Blood chemical analysis of male Wild-type rats

Group	Treatment	TG	FFA	T-Cho	LP	GOT	GPT	BUN	CR
1	4NQO + 5.5%DAG	147 ± 55	571 ± 78	83 ± 19	6.6 ± 0.9	81 ± 29	16 ± 8	17 ± 3	0.36 ± 0.05
2	4NQO + 2.75%DAG + 2.75%TAG	191 ± 57	624 ± 151	102 ± 15	6.4 ± 0.5	94 ± 45	42 ± 71	16 ± 1	0.32 ± 0.02
3	4NQO + 1.38%DAG + 4.13%TAG	207 ± 61	758 ± 112	112 ± 20	6.8 ± 0.8	105 ± 40	20 ± 8	17 ± 3	0.34 ± 0.06
4	4NQO + 5.5%TAG	136 ± 52	573 ± 117	88 ± 6	6.4 ± 0.5	97 ± 33	21 ± 17	19 ± 2	0.38 ± 0.04
5	5.5%DAG	165 ± 58[#]	742 ± 155	109 ± 8	7.0 ± 1.2	122 ± 43	28 ± 13	23 ± 2	0.33 ± 0.05
6	5.5%TAG	234 ± 104	1266 ± 508	109 ± 24	8.8 ± 5.7	133 ± 63	25 ± 12	20 ± 2	0.40 ± 0.12

[#] $P < 0.05$ as compared to 5.5%TAG group (Group 6).

similar to those of TAG on mammary carcinogenesis. Thus the tumor incidence and multiplicity did not demonstrate inter-group variation during a 90-day feeding period (Sugano et al., 2002).

With our wild-type rats, no consistent trend was noted across the sexes but yields of tumors were relatively low. Thus, the over expression of *ras* appeared to be of importance. DAG has been reported to act as an effector of the clonal expansion of mutated *ras* containing cells and a mechanism whereby an increase in endogenous DAG could contribute to clonal expansion of cells containing *ras* has been proposed (Mills et al., 1993). In another study, a TPA-independent irreversible decrease and alteration of the subcellular distribution of PKC-alpha and beta 2 in DMBA-initiated/TPA-promoted skin papillomas was been found (Mills et al., 1992). This effect occurred in the absence of any elevation in the total pool of endogenous DAG. Thus, alterations of PKC isozymes could also be important early events in multistage carcinogenesis.

DAG can cause conversion of GDP to GTP by two Ras exchange factors, PKC and RasGRP1, leading to mitogen-activated protein (MAP) kinase phosphorylation and cellular proliferation (Marais et al., 1998; Roose et al., 2005). Moreover, over expression of PKC species is known to be important for induction of squamous cell tumors in mice (Reddig et al., 2000). It is possible that the administered DAG was directly absorbed and activated pathways involved in carcinogenesis. Further studies to examine this possibility are obviously required. Regarding the possible role of *ras* mutations, it is of interest to note that exposure to DAG in populations carrying genetic alterations, such as smokers, may enhance the risk of oral cancer development (Das et al., 2000; Mishima et al., 1998; Scully et al., 2000; Xu et al., 1998).

While dietary 1,3-DAG has been found to reduce fat deposition in the viscera and body of rats (Meng et al., 2004), such alteration was not evident in the present study. Furthermore, no anti-obesity or lipid-lowering effects of DAG oil were reported (Sugimoto et al., 2003a,b). Thus, it appears that the metabolic effects of DAG oil were not essential for tumor development in the present study, in line with the lack of consistent influence on serum parameters.

In conclusion, the present study indicates that DAG oil, a mixture of 1,3-DAG and 1,2-DAG, is capable of enhancing tongue carcinogenesis by 4NQO in male Tg rats over expressing *ras*. Although DAG oil alone has not been demonstrated to be carcinogenic to the rat, the risk that DAG oil in combination with carcinogens in foodstuffs may act on the tongue and possibly pharyngeus, and esophagus in man indicates that further studies with an increased number of animals, higher dose and longer duration are warranted.

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Cancer prevention and anti-metastatic effects by oral administration of procyanidins

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Abstract

Procyanidins (PAs) are naturally occurring compounds, particularly rich in grape seed extract and red wine, which have been shown to exert carcinopreventive effects in a variety of model systems. PAs exist in a variety of forms, for example, monomers, dimmers, and trimmers of catechin and epicatechin. The polymeric nature of the PAs in the extracts used in most studies has rendered the properties of individual PA isoforms opaque.

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Using HPLC we have isolated PA fractions containing PA monomers (PA1), dimmers (PA2), trimmers (PA3), 2mer to 4mers (PA2/4), 5mer to 10mers (PA5/10), and more than 11mer (>PA11). Among these fractions PA5/10 and >PA11 showed significantly stronger cytotoxicity against the human colon cancer cell line HT-29 and the rat colon cancer cell line RCN-9 compared to the other isolates. Importantly, PA5/10 influenced apoptotic events in both cell lines: it markedly enhanced nuclear condensation, sub-G1-phase DNA content, and caspase-3 activity. In addition to this cytotoxic effect, PA5/10 also inhibited angiogenesis in the chick embryo chorioallantoic membrane (CAM) assay, possibly due to a block of endothelial cell function.

Oral administration of PAs has been shown to significantly inhibit lung metastasis in mice bearing subcutaneous (s.c.) implants of the highly metastatic colon carcinoma 26 (Co26Lu) cell line and to inhibit colon carcinogenesis in rats. The IC_{50} of a three day exposure of PA1, PA2/4, and PA5/10 against Co26Lu cells in vitro was about 2, 50 and 10 μM, respectively. However, while daily treatment with PA2/4 at 300 mg/kg or two times a day at 150 mg/kg significantly inhibited tumor growth and metastasis to the lung from s.c. implanted tumors, PA1 and PA5/10 had no effect. Another difference found in vivo was that significantly higher levels of PAs were found in the mucosa of the small intestine at 30 min after oral administration of PA2/4 compared to PA5/10. (The serum concentrations, however, were below the level of detection by Vanillin's method.) The proportion of $DX5^+$ (NK) cells in white blood cells was significantly increased following oral administration of PA2/4, and these cells showed strong cytotoxicity against Co26Lu cells in vitro. NK cells were also significantly increased in the lamina propria of the small intestine after oral treatment with PA2/4. Moreover, both PA2/4 and PA5/10 induced significant increases in IFN-γ levels in the mucosa of the small intestine, but only PA2/4 induced significant increases in IL-12 levels; PA2/4 also produced increases (although not statistically significant) in IL-1β and IL-18 levels. These results suggest orally administered PA2/4 is absorbed into mucosa of the small intestine where is induces expression of cytokines and consequent activation of NK cells. These activated NK cells could be an important tumor inhibitory agent.

In sum, these results suggest that PA mixtures containing PA5/10 and PA2/4 may be able to exert anti-carcinogenesis and anti-metastasis activities through PA5/10 mediated apoptosis of abnormal cells and inhibition of angiogenesis and PA2/4 mediated enhancement of host immune function.

Introduction

Epidemiological studies have provided evidence that the consumption of food products containing polyphenolic compounds with antioxidant and free

radical scavenging activities may be chemopreventive for various cancers (1,2). In particular, a high dietary intake of flavonoids may be associated with a low colon cancer incidence in humans (3, 4). The flavonoids comprise broadly distributed plant pigments and are responsible for much of the coloring of plants in nature. Animal studies and investigations using different cellular models suggest that certain flavonoids inhibit tumor initiation as well as tumor progression (5). A well studied flavonoid is (-)-epigallocatechin-3-gallate (EGCG), the major polyphenolic constituent of green tea. EGCG has anti-carcinogenic effects in *in vitro* and *in vivo* models (6-9), and this effect is mediated in part by its ability to induce apoptosis in cancer cells without affecting normal cells (10-14).

The major polyphenolic constituents of grape seed extract (GSE) and red wine are mainly catechin and the procyanidins (PAs). It has been reported that PAs have potent antioxidant activity (15-17). Recent studies show that PAs extracted from various natural products are effective inhibitors of carcinogenesis in several animal models (18, 19). PAs also inhibit the growth of various human cancer cell lines (20, 21) due to induction of cell cycle arrest and apoptotic cell death (22-24). In our previous report, oral feeding of 0.002% PAs in the diet was shown to significantly decrease azoxymethane-induced colonic preneoplastic aberrant crypt foci (ACF) formation (25). These PAs were a mixture of several different PA isoforms, mostly dimmers (PA2), trimmers (PA3) and higher order oligomers of catechin and epicatechin and their

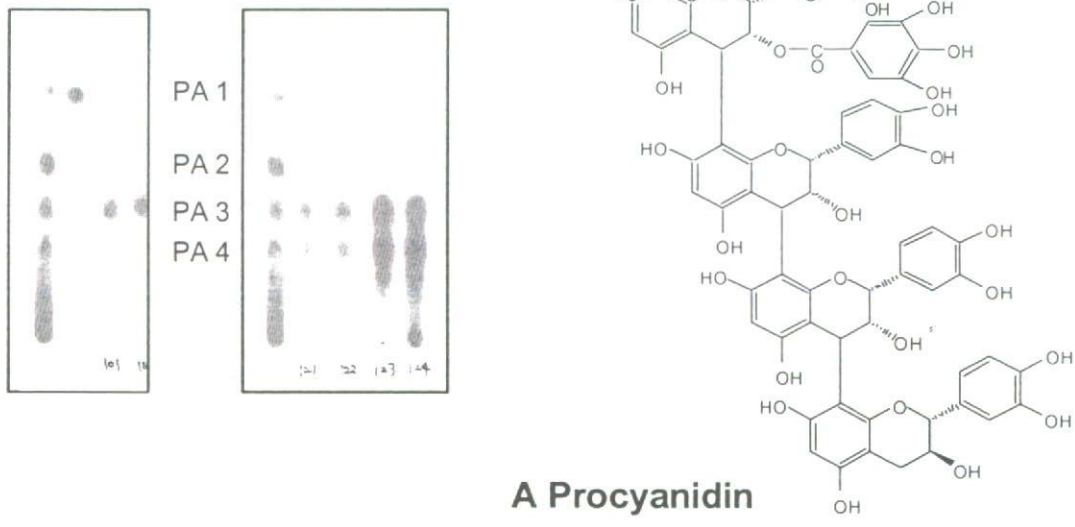

Figure 1. Various procyanidins purified from grape seed extract and the structure of one of the PA4 isoforms. The extracts were spotted on a silica gel thin-layer plate. Developing solvent: acetone-toluene-folic acid (6:3:0.8).

gallate derivatives (Figure 1). We also reported on the effect which individual purified PA isolates had on HT-29 human colon carcinoma cells and RCN-9 rat colon carcinoma cells *in vitro*. Here we expand on our previous findings and report on the *in vivo* effects of individual purified PA isolates.

1. Inhibitory effect of procyanidins (PAs) against colon carcinoma cells *in vitro* and enhancement of apoptosis

In our previous report, PAs significantly inhibited the number of aberrant cript foci (ACF) in rat colon following treatment with azoxymethane (AOM) (25). To elucidate the mechanisms of anti-carcinogenesis, we investigated the effects of HPLC purified PA isolates on apoptosis using the rat colon carcinoma RCN-9 and human colon carcinoma HT-29 cell lines.

Materials and methods

Chemicals and antibodies

EGCG (purity: more than 95%, MW 458.4) was purchased from Tokyo Food Techno Co. Ltd., Tokyo, Japan. HPLC purified isolates (purity is more than 95%) of PA monomers (PA1, MW 290.3), dimmers (PA2, MW 578.5), trimmers (PA3, MW 866.8), 2-4mers (PA2/4, 866.8 mean molecular weight), 5-10mers (PA5/10, 2019.8 mean molecular weight), and more than 11mer (more than MW 3172.6) were obtained from Kikkoman Corp., Noda, Japan.

Cell lines

The rat colon carcinoma RCN-9 and human colon carcinoma HT29 cell lines were 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 densities using Trypsin-EDTA (Invitrogen).

Cell proliferation

For determination of proliferation, HT-29 and RCN-9 cells were seeded at a density of 3×10^3 cells per well into 96-well cell culture plates (Falcon) and allowed to adhere for 24 hr. Thereafter, medium was replaced with fresh culture medium containing the test compound, and cells were allowed to grow for another 72 hr. Total cell counts were determined with a Cell Counting Kit-8 (WAKO). Absorption of the formazan product was measured at 450 nm with a XFLUOR4 (TECAN, Austria).

Nuclear condensation detection in HT-29 cells

Nuclear condensation as a late marker of apoptosis was determined by staining DNA with Hoechst 33342. HT-29 cells (3×10^4) were incubated with test compounds for 24 and 72 hr. Thereafter, cells were washed in PBS, allowed to air-dry for 30 min and fixed in 3.7% formaldehyde before staining with 1 μg/ml Hoechst 33342 and visualized under an inverted fluorescence microscope.

Cell cycle analysis

HT-29 cells were seeded at a density of 1×10^6 cells per dish into 60 mm dishes and incubated from 24 to 72 hr in the presence or absence of test compounds. The cells were trypsinized, pelleted by centrifugation at 500 x 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 hr. Cells were washed with PBS and treated with 100 kU 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 FACSCallibur (Becton Dickinson, NJ, USA) flow cytometer for relative DNA content based on red fluorescence.

Caspase-3 activity

HT-29 cells were seeded at a density of 5×10^5 cells per dish into 60 mm dishes and incubated for 24 hr in the presence or the absence of test compounds. The cells were trypsinized, and pelleted by centrifugation at 500 x 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, 1 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin and 20 U/ml aprotinin) to each pellet, homogenizing with 10 strokes of a homogenizer and centrifuging at 3,500 rpm for 10 min. The supernatant was incubated with the chromophore caspase-3 substrate Ac-DEVD-pNA (Kamiya Biomedical Co., Seattle, USA) at a final concentration of 200 μM. Cleavage of the caspase-3 substrate was measured at 405 nm with a XFLUOR4.

Results

Cell proliferation

Various PAs inhibited proliferation of human (HT-29) or rat (RCN-9) colon carcinoma cell lines dose-dependently. PA1, PA2, PA3, PA2/4, PA5/10, >PA11 and EGCG led to 50% growth inhibition at concentrations (IC_{50}) of 55.6 μM, 222.2 μM, 94.5 μM, 103.1 μM, 15.2 μM, 7.7 μM and 35.8 μM against HT-29, respectively (Table 1). IC_{50}s against RCN-9 were similar to values against HT-29. More than 5mer of PA (PA5/10 and >PA11) showed much stronger inhibition of cell growth than the other PA isolates.

Table 1. IC_{50} values of HT-29 and RCN-9 cells treated with various PAs.

HT-29 cells			
PA 1	55.6 μM	PA 2/4	103.1 μM
PA 2	222.2	PA5/10	15.2
PA 3	94.5	>PA11	7.7
		EGCG	35.8
RCN-9 cells			
PA 1	54.5 μM	>PA11	20.1 μM
PA 2/4	150.6	EGCG	42.0
PA 5/10	19.5		

Detection of nuclear condensation of HT-29 cells

After 24 hr exposure of HT-29 cells to 30 μM PA5/10 nuclear condensation was readily detectable and was prominent at 72 hr (Figure 2); results similar to nuclear condensation in RCN-9 cells after exposure to PA5/10 (data not shown).

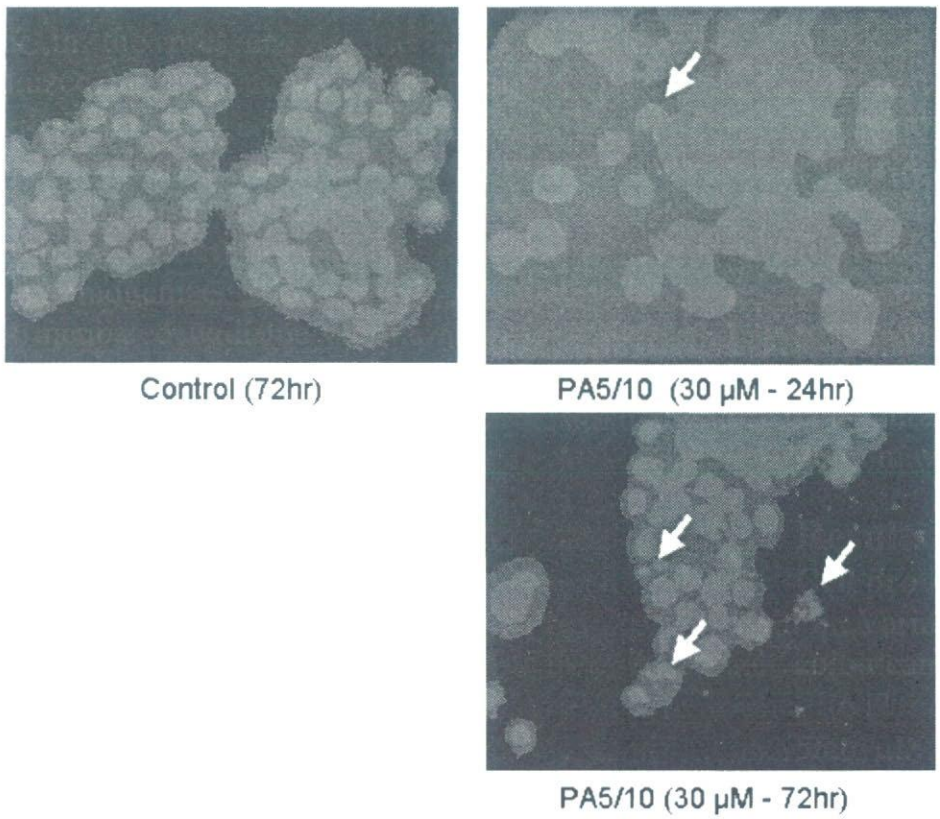

Figure 2. Apoptotic nuclear condensation in HT-29 cells treated with 30 μM PA5/10.

Cell cycle analysis

To determine the mechanism of inhibition of cell proliferation of HT-29 cells by PA5/10, we examined whether PA5/10 could induce programed cell death. FACS analysis of HT-29 cells exposed to 30 μM PA5/10 showed that

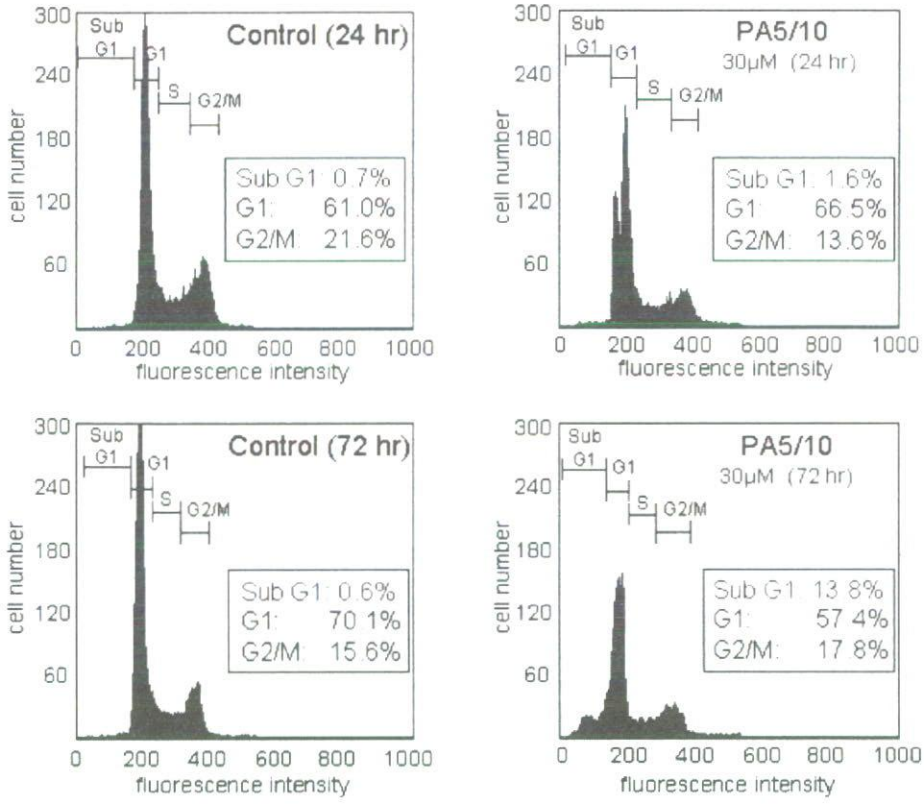

Figure 3. Flow cytometric analysis of HT-29 cells treated with 30 μM PA5/10.

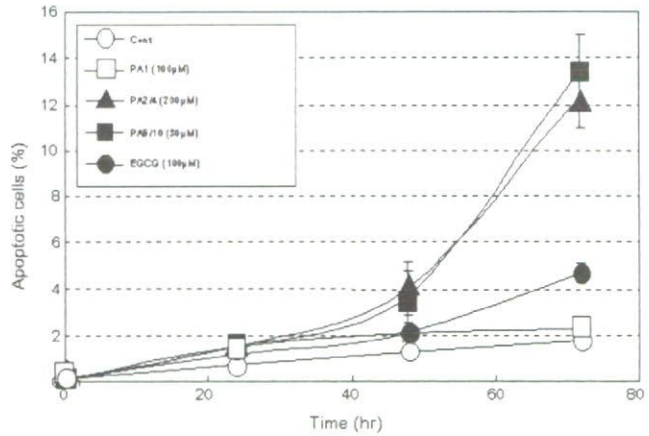

Figure 4. Ratio of apoptotic cells following treatment with various PAs. The concentrations of PA1, PA2/4, PA5/10, and EGCG were 100, 200, 30 and 100 μM, respectively.

PA5/10 arrested the cells in the sub-G_1-phase (Figure 3), indicative of apoptosis. PA5/10 (30 μM), PA2/4 (200 μM), and EGCG (100 μM), but not PA1 (100μM), treated cells showed significant increases in apoptosis (Figure 4).

Caspase-3 activity

Caspase-3 is a key protease associated with apoptosis. Effects of PA5/10 on the activity of caspase-3 were examined in HT-29 cells and its activity was markedly increased (17.0-fold) (Figure 5). The presence of the active fragment of caspase-3 was confirmed by Western blot analysis (Figure 6).

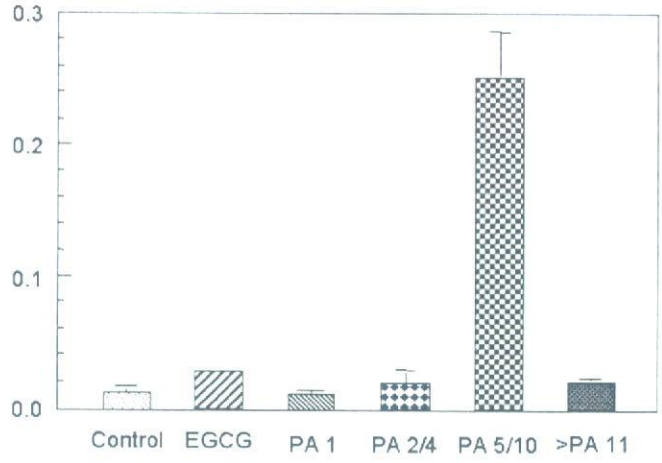

Figure 5. Caspase-3 activity enhanced by PA5/10. The concentrations of PA1, PA2/4, PA5/10, and EGCG were 100, 200, 30 and 100 μM, respectively.

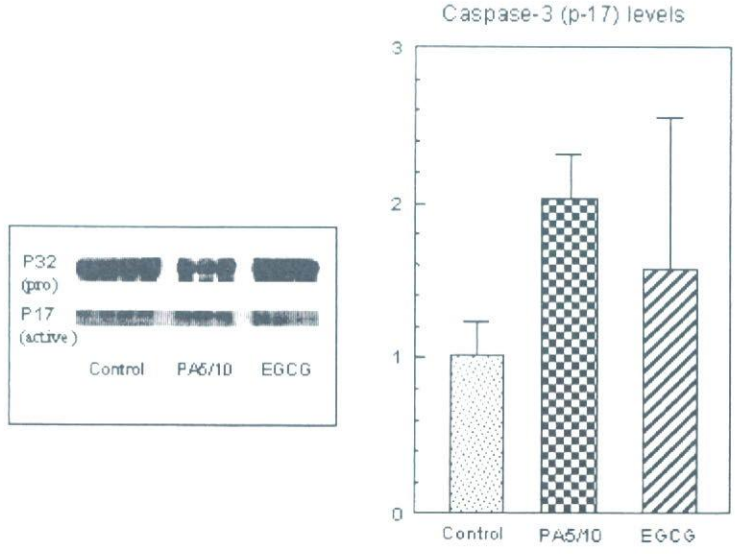

Figure 6. Pro- and active caspase-3 by Western blot analysis. The concentrations of PA5/10 and EGCG were 30 and 100 μM, respectively.

2. Inhibitory effects of various PAs on angiogenesis in chick embryo chorioallantoic membranes (CAM)

Thus, induction of apoptosis could be induced by some PAs. Next, we investigated the anti-angiogenic effect exerted by these PAs using the chick embryo chorioallantoic membrane (CAM) assay.

Materials and methods

Reagents and eggs

HPLC purified isolates (purity is more than 95%) of PA monomers (PA1, MW 290.3), dimmers (PA2, MW 578.5), trimmers (PA3, MW 866.8), 2-4mers (PA2/4, 866.8 mean molecular weight), and 5-10mers (PA5/10, 2019.8 mean molecular weight) were obtained from Kikkoman Corp., Noda, Japan. Fertilized eggs were purchased from Omiya Kakin (Omiya, Japan).

Anti-angiogenesis assay using the chick embryo CAM assay

The anti-angiogenic activity of PAs was assayed using the chick embryo CAM assay. Various concentrations of PAs (10 μl) were applied to a ring (3 mm) on the surface of the CAM of 4-day-old chick embryos in the shell. After 48 hr exposure at 37 °C, a fat emulsion was injected into the CAM to clearly identify blood vessels. Each experimental group included 4 - 6 eggs. Angiogenic inhibition was indicated by the presence of a 3-mm-diameter avascular zone around the ring. The results are expressed as the percentage of embryos showing such inhibition.

Results

Saline-treated 6 day-old embryos completed formation of a blood vessel network in the CAM. This was strongly inhibited by PA5/10. The inhibitory activity of PA1, PA3, PA2/4 and PA5/10 was dose-dependent with an ED_{50} of 48, 25, 60, and 7 nmoles/CAM, respectively (Figure 7).

3. Anti-metastatic effects of PAs in mice bearing highly metastatic colon carcinoma 26 (Co26Lu) and PA mediated enhancement of NK activity

PAs show anti-carcinogenesis effects against rat colon cancer (25). Among the various PA isolates, PA2/4 and PA5/10 caused strong apoptosis against the HT-29 human colon carcinoma cell line (Figure 4), similar to their effect against the RCN-9 rat colon carcinoma cell line (25). Moreover, PA5/10 showed strong anti-angiogenesis in a CAM assay (Figure 7). Next, we investigated the effects which PA1, PA2/4, and PA5/10 isolates had on metastasis

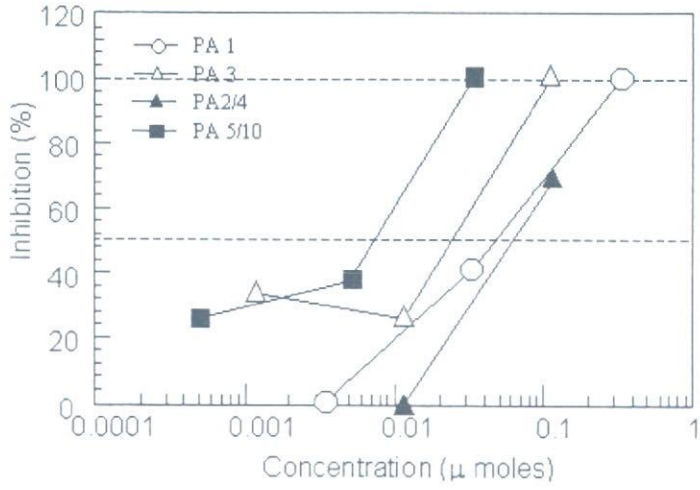

Figure 7. Inhibition of angiogenesis by various PAs.

in mice bearing highly metastatic Co26Lu cells, and the effects of PA administration on NK cell activity.

Materials and methods

Chemicals and antibodies

HPLC purified isolates (purity is more than 95%) of PA monomers (PA1, MW 290.3), 2-4mers (PA2/4, 866.8 mean molecular weight), and 5-10mers (PA5/10, 2019.8 mean molecular weight) were obtained from Kikkoman Corp., Noda, Japan. FITC anti-mouse DX5 (pan-NK), phycoerythrin (PE) anti-mouse CD3e (IgG, 145-2C11), and PE anti-mouse CD11b (M1/70) mAbs were purchased from eBioscience, USA. Purified rat anti-mouse DX5, rat anti-mouse CD4 (IgG, GK1.5), and rat anti-mouse CD11b mAbs were also purchased from eBioscience.

Animals and tumors

Inbred, 5-week-old male BALB/c mice weighing 18-20 g each were obtained from CLEA Japan Inc., Tokyo, and acclimatized for 7 days prior to starting experimentation. The animals were allowed free access to CE-2 pellet diets (CLEA Japan Inc., Tokyo, Japan) and water, and were maintained in plastic cages on woodchip bedding under specific-pathogen free conditions in our animal laboratory with a controlled temperature (24±2 °C) and a 12 hr light-dark cycle.

On day 0, aliquots of Co26Lu cells (3 x 10^5 cells/0.1 ml) were subcutaneously (s.c.) implanted into the right thighs of the BALB/c mice and the animals were then randomly allocated to control and treatment groups (8-12 mice per group). The experiments were repeated for confirmation of the results.

All experiments were conducted according to the "Guidelines for Animal Experiments in National Cancer Center" of the Committee for Ethics of Animal Experimentation of the National Cancer Center, Japan

Treatment

Various PAs (30, 100 and 300 mg/kg, daily, or 150 mg/kg, twice a day) were administered orally (p.o.) in physiological saline (0.1 ml/10g body weight) by stomach tube from day 3 for 3 weeks (5 days per week). Survivors were sacrificed on day 26. The s.c. implanted tumors were measured in two dimensions, longest (a) and shortest (b), and volumes were calculated using the formula: $ab^2/2$ (mm^3). At sacrifice the lungs were removed, rinsed in physiological saline and fixed for 5 days in acetone to allow determination of the number of macroscopic lung metastases.

Total PAs levels in the serum and mucosa in the small intestine

Serum samples and mucosa of the small intestines were collected at 15, 30 and 60 min after single administration of PAs at 300 mg/kg. Serum was obtained after centrifugation of whole blood at 3500 rpm for 10 min. Mucosa samples from the small intestine were obtained by scraping with a slide glass after the small intestines were washed with saline two times to remove contents, including administered PAs, and the residual saline removed. PAs in the mucosa were extracted in 500 μl of distilled water using a Polytron homogenizer (Kinematica, Swizerland) and centrifuged at 3500 rpm for 10 min. Extracted solutions were transferred to new tubes and perchloric acid added to a final concentration of 0.4M. The extracts were then neutralized by addition of 1M KOH and clarified by centrifugation at 15,000 rpm for 10 min at 4°C. Total PA levels in the serum and the mucosal extracts were assayed by Vanillin's method (26).

Cell mediated cytotoxicity

Calcein (DiC_{18}, Sigma) was dissolved in DMSO (5.5 μM) and Co26Lu cells were incubated for 1 hr with 5.5 μM DiC_{18}/well. Calcein-labeled Co26Lu cells (1×10^4) were washed twice with saline and incubated with white blood cells or spleen cells from mice (five mice/group) which had been treated 5 times with oral administration of PA2/4 at 300 mg/kg/day. Effector cells were mixed in 96-well plates (Falcon) with target Co26Lu cells at effector/target ratios ranging from 3:1 to 30:1. The mixed cells were incubated at 37°C for 4 hr. The supernatant was then removed from the wells and the adherent cells were washed twice with saline. Cells were extracted with lysis buffer (0.5 ml) and the extractions were measured by XFLUOR4 (Ex 480 nm, Em 530 nm) (TECAN, Austria).

Flow cytometry of white blood cells (WBC) treated with PA2/4

At 3 hr after the last of the treatments with PA2/4 at 300 mg/kg for 5 days, mice (5 mice/group) were anesthetized with diethyl ether and blood samples (0.6-1.0 ml) were collected from the descending vena cava into tubes (NIPRO, Osaka, Japan). WBC was collected by centrifugation after treatment with ammonium chloride lysing solution (BD PharMingen, San Diego, CA).

To investigate changes in subsets of white blood cells after PA2/4 treatment, lymphocytes were differentiated by two-color immunofluorescent staining with FITC anti-DX5 and PE anti-CD3e or PE anti-CD11b mAb. These experiments were repeated two times.

Tissue sections and staining

After taking blood from the mice for analysis of WBC, the small intestine (jejunum and ileum) was excised and then washed with physiological saline. Half of the jejunum was cut perpendicularly with scissors, opened onto a sheet, fixed in acetone at 4°C, embedded in paraffin, sectioned at 4 μm serial sections, and stained with anti-CD4 mAb (rat anti-mouse L3T4, GK1.5, Southern Biotechnology Association, Inc., Birmingham, AL), anti-mouse DX5 mAb (pan-NK) or anti-mouse CD11b mAb. Immunoreactions were detected using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA).

Results are expressed as the mean number of positive cells in 20 fields per mouse.

Enzyme-linked immunosorbent assay (ELISA) for IL-1β, IL-12, IL-13, IL-18 and IFN-γ

The remainder of the small intestine, after removal of half of the jejunum for sectioning, was cut perpendicularly with scissors and opened onto a plate, so that the whole surface (epithelium, lamina propria, submucosa) could be obtained by scraping with a slide glass. Samples were placed on an aluminum plate cooled with acetone-dry ice and frozen under strong pressure using a second plate. The samples were stored at –80 °C until extraction for ELISA. Frozen samples were extracted by homogenization with a Sonifier 450 (Branson Ultrasonics Corp., Daburg, CT) in lysis buffer (0.067 M phosphate buffer, pH7.2, 1% Nonidet P-40, 0.5% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, 1mM phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin and 20 U/ml aprotinin) and clarified by centrifugation at 15,000 rpm for 10 min. The supernatants were collected and stored at –80 °C until testing. The IL-1β, IL -13, and IFN-γ present in the samples were measured with specific ELISA kits from TECHNE, Corp., (Minneapolis, MN). The IL-12 levels were determined using a mouse IL-12 (p70) ELISA kit (Endogen, Inc., Woburn, MA). Active IL-18 levels were determined using a mouse IL-18 ELISA kit (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan).

Protein assay

Total protein content of the extracted samples was assayed using Coomassie Dry Protein Assay Plates (Pierce, Rockford, IL) with bovine serum albumin as the standard (Fraction V, Sigma Chemical Co. St. Louis, Mo).

Data analysis

Tumor volumes and cell numbers were statistically evaluated using Dunnett's modification of Student's *t* test. Data for numbers of lung metastases were analyzed with the Mann-Whitney U test.

Results

Anti-tumor and anti-metastatic effects of PAs in BALB/c mice bearing Co26Lu

Daily treatment with PA2/4 at 300 mg/kg or twice a day at 150 mg/kg significantly inhibited growth of s.c. implanted Co 26Lu cells (44 and 50% inhibition compared to Control, respectively: Table 2). Moreover, significant

Table 2. Effects of procyanidins on Co26Lu tumor growth and its lung metastases.

Dose of procyanidins (po) (mg/kg/day)		Tumor volume on day 20 (mm^3)	No. of lung metastatic colonies Median (range)	
I. Control		1784 ±155 (s.e.)	142.5	(76 - 218)
PA2/4	(30)	1849 ± 222	143.0	(115-206)
	(100)	1753 ± 203	136.5	(25 - 185)
	(300)	999 ± 177	76.0*	(19 - 154)
	(150 x 2)	890 ± 118*	72.0**	(32 -115)
PA5/10	(30)	1423 ± 266	104.5	(31 - 194)
	(100)	1193 ± 192	116.5	(16 - 180)
	(300)	1403 ± 180	109.5	(35 - 222)
	(150 x 2)	1580 ± 244	147.0	(29 - 235)
>PA11	(30)	1545 ± 284	130.5	(59 - 213)
	(100)	1921 ± 202	152.5	(35 - 200)
	(300)	1473 ± 240	117.5	(90 - 236)
	(150 x 2)	1575 ± 262	145.0	(58 - 215)
II. Control		1949 ± 108	124.0	(68 - 233)
PA1	(30)	2072 ± 278	185.0	(93 - 287)
	(100)	2038 ± 232	132.0	(41 - 216)
	(300)	1682 ± 161	142.5	(66 - 266)

* $P<0.05$, ** $P<0.01$ vs control.

suppression of spontaneous lung metastasis was observed with PA2/4 treatments (47 and 49% inhibition compared to Control, respectively: Table 2). PA1, PA5/10 and >PA11 did not significantly inhibit tumor growth or the number of the lung metastases.

Total PA levels in the serum and mucosa of the small intestine after oral administration of PA1, PA2/4 or PA5/10

PA levels in the serum and the mucosa of the small intestine were assayed by Vanillin's method. PA levels could not be detected in any of the serum samples (less than10 μg/ml). On the other hand, high levels of PAs in the mucosa of the small intestine were detected after oral treatment with PAs (Figure 8).

Inhibitory effects of PAs on the growth of the mouse colon carcinoma cell line Co26Lu

Co26Lu cells were incubated with various concentrations of PA1, PA2/4 and PA5/10 for 24, 48 and 72 hr. PA2/4 had much weaker cytotoxicity than PA1 or PA5/10: IC_{50} of PA1, PA2/4 and PA5/10 were about 2, 50, and 10 μM for 72 hr exposure, respectively (Figure 9).

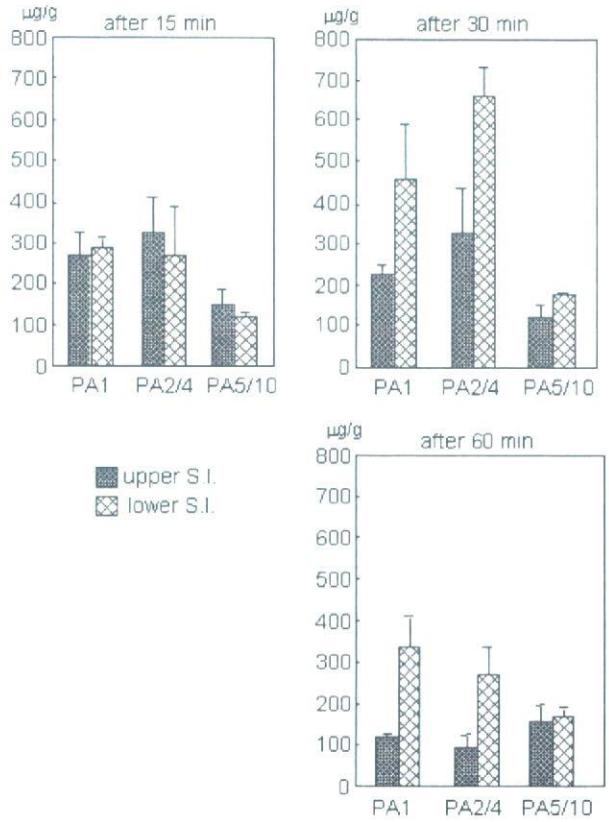

Figure 8. PA levels in the mucosa of the small intestine (upper and lower parts) after a single oral treatment with PA1, PA2/4, or PA5/10 at 300 mg/kg.