RESEARCH COMMUNICATION

Dietary Protocatechuic Acid During the Progression Phase Exerts Chemopreventive Effects on Chemically Induced Rat Tongue Carcinogenesis

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Abstract

The modifying effects of dietary administration of protocatechuic acid (PCA) during the progression phase of tongue carcinogenesis initiated with 4-nitroquinoline 1-oxide (4-NQO) were investigated in male F344 rats. For tumor progression we developed a new animal model, where rats initiated by 4-week treatment of 20 ppm 4-NQO in drinking water, received four cycles of 20 ppm 4-NQO to induce advanced tongue cancer (one cycle: 2 weeks of 4-NQO followed by 2 weeks of tap water), starting at 14 weeks after the initiation. In this model, metastasis of tongue cancer occurred in lungs. Starting two weeks before the cycle treatment with 4-NQO, animals were fed the 2000 ppm PCA containing diet and continued on this diet until the end of the study. At the termination of the experiment (week 32), the incidences of tongue neoplasms and preneoplastic lesions, polyamine levels in the tongue tissue, and cell proliferation activity estimated by morphometric analysis of silver-stained nucleolar organizer regions' protein were compared among the groups. Feeding with PCA containing diet during the progression phase significantly decreased the occurrence of advanced tongue squamous cell carcinoma with metastasis ($P<0.05$) and preneoplasia (hyperplasia and dysplasia) ($P<0.001$). In addition, PCA exposure decreased polyamine levels in the tongue tissue ($P<0.001$) during progression phase. Our results suggest that dietary PCA inhibits progression of 4-NQO-induced oral carcinogenesis, and such inhibition might be related to suppression of cell proliferation by PCA.

Key Words: protocatechuic acid - 4-NQO - tongue carcinogenesis - inhibition - progression - rats

Abbreviations: PCA, protocatechuic acid; 4-NQO, 4-nitroquinoline 1-oxide; AgNORs, silver-stained nucleolar organizer regions; NO, nitric oxide; iNOS, inducible nitric oxide synthase.

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Introduction

The incidence of oral cancer, the major site being the tongue, exhibits marked geographic variation, with the highest morbidity and mortality rates appearing in southern Asia where people have the habit of chewing betel quid and tobacco, but recently it has been increasing worldwide, particularly in young adults (Atula et al., 1996; Johnson, 1997; Moore et al., 2000). Oral cancer development is associated with lifestyle, viruses, dietary, and genetic changes (Binnie, 1991; Daftary et al., 1991). Epidemiological data provide strong support for exogenous factors such as tobacco and alcohol use as being major causative agents (Macfarlane et al., 1996). In spite of surgical advances of diagnosis and therapy, oral carcinoma still remains difficult to cure: 30–40% of patients with oral carcinoma have 5 years' survival rate (Swango, 1996). Therefore, it is necessary to embrace bright new ideas that prevent oral carcinogenesis. Chemoprevention is one of such promising approaches and a rapidly expanding field, and promising one in the management of cancer (Cohen and F.R., 2002; Gupta and Mukhtar, 2002; Gustin and Brenner, 2002; Pappas and Jordan, 2002; Tanaka, 1995).

The frequent consumption of edible plants is associated with a low incidence of a variety of cancer (Shibata et al., 1992). This may be partly due to the presence of several

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antioxidants as like phenolic compounds in foodstuffs. Protocatechuic acid (PCA, Fig. 1), a simple phenolic acid, is a constituent of many edible plants. Recently, PCA from the leaves of Smallanthus sonchifolius and Mesona procumbens Hemsl was reported to have a strong antioxidant property (Hung and Yen, 2002; Valentova et al., 2003). The roles of dietary antioxidants have received much attention and appear to have a wide range of anticancer properties (Ito and Hirose, 1989; Tanaka et al., 2001). Furthermore, some phenolic compounds contained in plants or vegetables have anti-inflammatory, antimutagenic and/or anticarcinogenic properties (Huang and Ferrano, 1992). Indeed, our previous studies demonstrated a strong chemopreventive effect of dietary PCA on chemically induced carcinogenesis in liver (Tanaka et al., 1993), colon (Tanaka et al., 1993), urinary bladder (Hirose et al., 1995) and oral cavity (Ohnishi et al., 1997; Tanaka et al., 1994). However, there are a few studies on the chemopreventive ability of certain compounds in progression of carcinogenesis (Kawamori et al., 1999; Reddy et al., 1999).

A water-soluble quinoline derivative, 4-nitroquinoline 1-oxide (4-NQO) can produce a spectrum of preneoplastic and neoplastic lesions in the oral cavity, especially tongue, of rats following applications appear to have significant advantages. In our searching powerful cancer chemopreventive agents against oral cancer, we have used a 4-NQO-induced rat tongue carcinogenesis model (Tanaka et al., 1994; Tanaka et al., 2002; Tanaka et al., 1997). To produce the distinct progression stage, we have modified this model by cycle exposure of 4-NQO after initiation. The present study was therefore designed to specifically investigate whether dietary PCA is effective when administered during the progression stage in this modified tongue carcinogenesis model. In addition, the effects of PCA on expression of the proliferation biomarkers such as polyamine levels (Martinez et al., 2003; Tanaka et al., 1995) and silver-stained nucleolar organizer regions (AgNORs) number per nucleus (Tanaka et al., 1991) to assess clarify the underlying mechanism(s) of modification.

Materials and Methods

Animals, Chemicals and Diets.

Male F344 rats aged 4 weeks obtained from Japan SLC Inc. (Hamamatsu City, Japan) were used. They were housed in plastic cages with hardwood chips for bedding under controlled conditions of humidity (50±10%), lightning (12-hour light/dark cycle) and temperature (23±2Åé). All rats were free access to drinking water and basal diet, powdered CE-2 (CLEA Japan Inc., Tokyo, Japan). All animals were quarantined for 14 days and randomized by body weight into experimental and control groups. 4-NQO for tongue tumor induction was purchased from Tokyo Kasei Organic Chemicals Co., Ltd. (Tokyo, JAPAN) and PCA was obtained from Sigma Aldrich Japan K.K. (Tokyo, Japan). 4-NQO was dissolved in tap water to a final concentration of 20 ppm and stored in a dark and cold room (4 °C). PCA was blended into a powdered basal diet at a dose of 2000 ppm and stored in a cold room (4 °C).

Experimental Procedure.

A total of 88 male F344 rats were divided into five groups as shown in Fig. 2. At 6 weeks of age, rats in groups 1 through 3 were given 20 ppm 4-NQO in drinking water for 4 weeks. At weeks 18, groups 2 and 3 were exposed to four cycles of 20 ppm 4-NQO (one cycle: 2 weeks of 4-NQO followed by 2 weeks of tap water) to accelerate of progression: the 4th cycle did not include the treatment with 2 weeks of tap water. Group 3 was given the diet containing 2000 ppm PCA at weeks 16 and continues on this diet until the end of the study. Group 4 did not received 4-NQO treatment and was given PCA-containing diet, as did in group 3. Group 5 was given the basal diet and tap water throughout the experiment, and served as an untreated control. All rats were carefully observed daily and consumption of the drinking water containing 4-NQO or the diets mixed with PCA was recorded to estimate intake of chemicals. To monitor the occurrence of preneoplastic and neoplastic tongue lesions, rats in groups 1 and 5 were at 16, 24, and 32 weeks, those in groups 2 through 4 at 24 and 32 weeks were sacrificed. At necropsy, all organs, especially the tongue, were inspected grossly to evaluate the preneoplastic and neoplastic lesions. Tongues were cut approximately into two halves; one portion was used for polyamine assay and the other was used for histopathology and counts of cell proliferation biomarkers. For histopathological confirmation, tissue and gross lesions were fixed in 10% buffered formalin solution, embedded in paraffin blocks, and processed by the conventional histological methods using hematoxylin and eosin stain. Epithelial lesions in the oral cavity were diagnosed according to the criteria by Banoczy and Csiba (Banoczy and Csiba, 1976) and WHO (WHO, 1978).

Polyamine Level of Tongue Tissue.

The polyamines in the oral cavity tissues were measured by means of a new enzymatic method developed by Koide et al. (Koide et al., 1990). The results obtained by this method correlated well with those obtained by high performance liquid chromatography. At sacrifice, one-half of the tongues of all rats were collected, and the amounts of diamine, spermine, and spermidine and the sum of these were determined by an enzymatic differential assay.

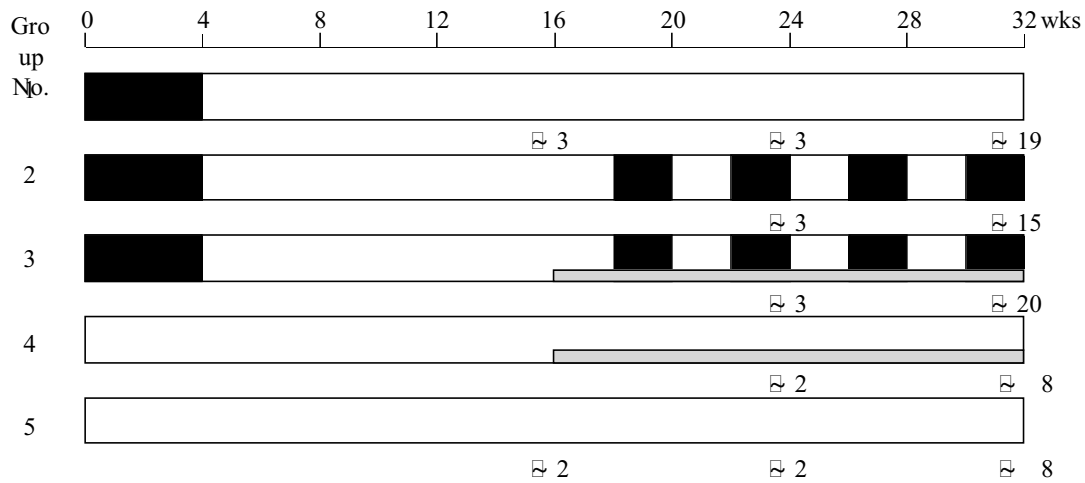**Figure 1. Protocatechuic Acid Chemical Structure**

Figure 2. Experimental Protocol

■ :4-NQO (20 ppm) in the drinking water

▨ :protocatechuic acid (2000 ppm) in the diet

□ :basal diet and tap water

X :sacrifice

Determination of Proliferative Activity in the Tongue Epithelium by AgNORs Enumeration.

To assess the proliferative activity, the numbers of AgNORs molecules per nucleus of squamous epithelium of the tongue in all rats were quantified. One-step silver colloid method for AgNORs staining was carried out, and computer-assisted image analysis quantification was based on the use of the image.

Statistical evaluation

Where applicable, data were analyzed using Student's t-test or Fisher's exact probability test. The results were considered statistically significant if P was 0.05 or less.

Results

General Observations.

The rats in groups 1-4 tolerated the oral administration of 4-NQO and/or PCA. Food consumption (g/day/rat) did not differ significantly among the group (data not shown). There were no significant differences on total intake of 4-NQO/rat between groups 2 and 3. The mean body weights

and mean and relative liver weights (g/100 g body weight) at the end of the study are indicated in Table 1. The mean body weight of group 4 given PCA alone was significantly greater than those of group 5 (untreated) (P<0.001). The mean liver weights of groups 3 and 4 were greater than those of group 1 (4-NQO alone) (P < 0.05) and group 5 (untreated) (P<0.01), respectively. The relative liver weight was insignificant among all groups.

Incidence of Tumors and Preneoplastic Lesions.

At weeks 16 and 24, no tongue neoplasms developed in rats of groups 1-3. The incidence of tongue tumors (squamous cell papilloma and carcinoma) in each group at the end of the study (week 32) is shown in Table 2. In group 1 (4-NQO alone), the incidences of tongue squamous cell carcinoma and papilloma were 0 and 16%, respectively. On the other hand, rats given 4-weeks of 4-NQO treatment followed by four cycles of 4-NQO exposure (group 2) had 47% incidence of tongue squamous cell carcinoma and 32% incidence of papilloma. The incidence of squamous cell carcinoma of group 2 was significantly higher than group 1 (P<0.005). Two rats of group 2 have tongue squamous cell

Table 1. Body, Liver, and Relative Liver Weights of Rats at the End of the Study

Group no.	Treatment	No. of rats	Body weight (g)	Liver weight (g)	Relative liver weight (g/100g body weight)
1	4-NQO	19	333 ± 32[a]	13.2 ± 1.9	3.95 ± 0.29
2	4-NQO → 4-NQO	15	345 ± 17	12.9 ± 0.8	3.75 ± 0.29
3	4-NQO → 4-NQO + PCA	20	350 ± 21	14.6 ± 2.0[b]	4.14 ± 0.52
4	PCA	8	364 ± 9[c]	13.7 ± 1.1[d]	3.76 ± 0.32
5	No treatment	8	335 ± 17	11.8 ± 1.3	3.49 ± 0.22

[a] Mean ± SD.
[b] Significantly different from group 1 by Student's t-test (P<0.05).
[c,d] Significantly different from 5 by Student's t-test ([c]P<0.001 and [d]P<0.01).

Table 2. Incidence of Tongue Lesions in Each Group

Group no.	Treatment	No. of rats examined	No. of rats with :	
			Squamous cell papilloma	Squamous cell carcinoma
1	4-NQO	19	3 (16%)	0 (0%)
2	4-NQO → 4-NQO	15	5 (32%)	7[a] (47%)
3	4-NQO → 4-NQO + PCA	20	2 (10%)	2[b] (10%)
4	PCA	8	0	0
5	No treatment	8	0	0

[a] Significantly different from group 1 by Fisher's exact probability test (P<0.005).
[b] Significantly different from group 2 by Fisher's exact probability test (P<0.05).

malignancy with invasion to esophagus and metastasis to lungs (Fig. 3). Meanwhile, only a few rats (10%) fed PCA during cycle administration of 4-NQO (group 3) possessed tongue squamous cell carcinoma without metastasis or invasion: the incidence was statistically significant lower than group 2 (P<0.05). Animals in groups 4 and 5 did not have any preneoplastic or neoplastic lesions.

Besides these neoplasms, a number of hyperplasia or dysplasia, which are considered to be preneoplastic lesions for tongue cancer, were also present in the tongue of rats in groups 1-3 at weeks 16, 24, and 32 (Table 3 and Fig. 4). The incidence of total hyperplasia and total dysplasia in group 2 was higher than that in group 1. Also in group 2, enhancement in the frequency of simple and papillary hyperplasia by cycle treatment with 4-NQO was prominent (P<0.05, P<0.001), when compared with group 1. Among various degrees of dysplasia, an increase in the frequency of severe dysplasia in group 2 was remarkable (P<0.001) compared to group 1. In group 3, reduction in the incidence of various degrees of these preneoplastic lesions by feeding of PCA was found: 71% reduction in papillary hyperplasia (P<0.001) and 75% reduction in severe dysplasia (P<0.001) when compared to group 2.

As shown in Fig.4, the incidence of tongue dysplasia of group 1 was 33% at week 16, and increased gradually with time (67% at week 24 and 58% at week 32) (Fig. 4 A). In group 2, the value reached 100% at weeks 24 and 32. The incidence of tongue dysplasia of group 3 was 67% at week 24 and 90% at week 32. The incidences of tongue dysplasia

with various degrees of stypia and those lesions per cm are also shown in Fig. 4 B-E. The incidence of rats with severe dysplasia of group 2 (100%) was significantly higher than that of group 1 (21%) (P<0.05) at week 32 (Fig.4 D). Number of dysplasia per cm in group 2 (0.98±0.17) is lower than group 1 (0.39±0.23) (P<0.05) at week 32 (Fig.4 E). At week 32, PCA administration effectively lowered the incidence of severe dysplasia (25%) compared to group 2 (100%) (P<0.001) (Fig.4 D) and number of dysplasia per cm (0.25±0.10) is lower than group 2 (0.98±0.17) (P<0.01) (Fig.4 E).

Expression of Cell Proliferation Biomarkers.

The data on expression of cell proliferation biomarkers of the tongue epithelium are shown in Figs. 5 and 6. As for polyamine levels (nmol/mg, mean±SD), the significant differences were present among groups 1, 2, 3, and 5 at the end of the experiment (week 32): 3.41±0.21 in group 1 (P<0.05 vs. group 5); 3.80±0.44 in group 2 (P<0.01 vs. group 1); 3.23±0.34 in group 3 (P<0.001 vs. group 2); 3.28±0.53 in group 4; and 3.16±0.22 in group 5.

Fig. 6 indicates the number of AgNORs/nucleus in the non-lesional area of tongue. Although the value of group 2 was higher than group 1 at weeks 24 and 32, there was no significantly differences. The values of groups 1 and 3 were comparable at week 24 and 32.

Discussion

In the present study, dietary administration of PCA during the progression phase inhibited the development of tongue carcinogenesis initiated with 4-NQO. Our previous studies provided evidence that PCA inhibits carcinogenesis during the initiation or post-initiation stage (Hirose et al., 1995; Ohnishi et al., 1997; Tanaka et al., 1994; Tanaka et al., 1993; Tanaka et al., 1993). In the present study, we develop a progression model of 4-NQO-induced rat tongue carcinogenesis, and PCA could inhibit progression of tongue carcinogenesis. Our results also showed that the experiment model of the present study can be used as a beneficial model of chemoprevention of tongue carcinogenesis during progression with the potential for detailed molecular studies of neoplastic progression to 4-NQO.

Although the mechanisms by which PCA exerts its

Figure 3. Metastasis from Tongue Tumor to Lung.

Table 3. Incidence of Preneoplastic Tongue Lesions in Each Group

Group no.	Treatment	No. of rats examined	Hyperplasia (HP[a])			Dysplasia (DYS[a])			
			Total	Simple	Papillary	Total	Mild	Moderate	Severe
1	4-NQO	19	13 (68%)	13 (68%)	5 (26%)	11 (58%)	5 (26%)	5 (26%)	4 (21%)
2	4-NQO→4-NQO	15	15[b] (100%)	15[b] (87%)	13[c] (100%)	15[d] (27%)	4 (33%)	5 (100%)	15[c] (87%)
3	4-NQO→4-NQO+PCA	20	20 (100%)	20 (100%)	5[e] (25%)	18 (90%)	6 (30%)	7 (35%)	5[e] (25%)
4	PCA	8	0	0	0	0	0	0	0
5	No treatment	8	0	0	0	0	0	0	0

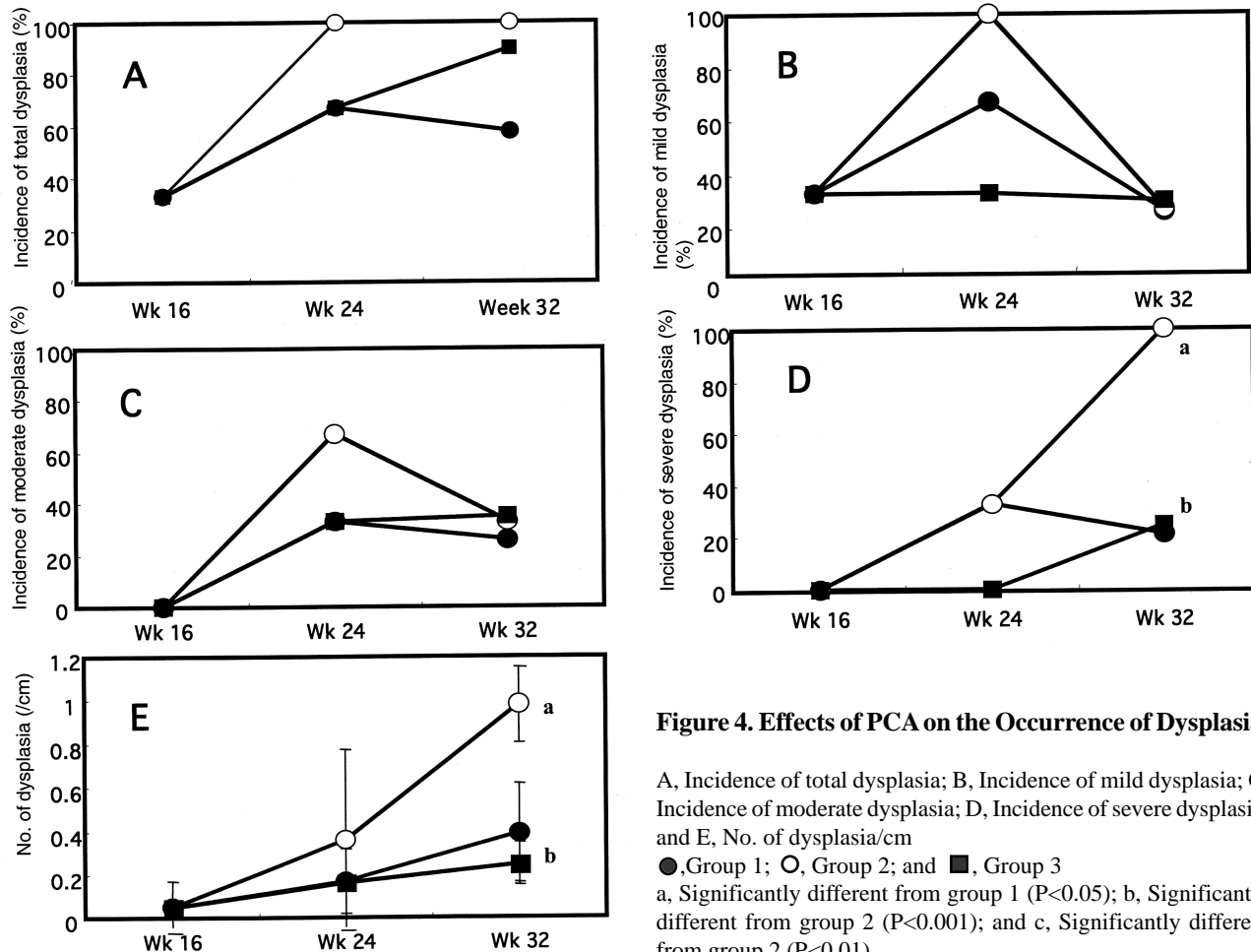[a] HP, hyperplasia; DYS, dysplasia.
[b-d] Significantly different from group 1 by Fisher's exact probability test ([b]P<0.05, [c]P<0.001, [d]P<0.005).
[e] Significantly different from group 2 by Fisher's exact probability test ([e]P<0.001).

inhibitory effect on 4-NQO-induced rat tongue carcinogenesis remain to be elucidated, several mechanisms could be considered. Some plant phenolics have an antioxidant effect (Williamson et al., 1998), PCA also shows a strong antioxidative effect, 10-fold higher than that of α-tocopherol (Ueda et al., 1996) and even at a 100 ppm shows potent chemopreventive effects on oral carcinogenesis (Tanaka et al., 1994). Therefore, this antioxidant effect might contribute its inhibitory effect on progression of tongue carcinogenesis found in the current study.

Cell proliferation is considered to be an important factor

in tumorigenesis (Janne and Poso, 1978) including oral cavity (Reszec et al., 2002). Polyamines play an essential role in cell growth and differentiation and its contents are often elevated in rodent and human neoplastic cells/tissues, compared with relevant normal cells/tissues (Verma, 1990). The polyamine biosynthetic pathway may be an important target for the design of chemotherapeutic agents. In the current study, number of AgNORs/nucleus of the non-lesional tongue squamous epithelium in rats received cycle treatment with 4-NQO after the initiation (group 2) gradually increased with time when compared to that of animals treated
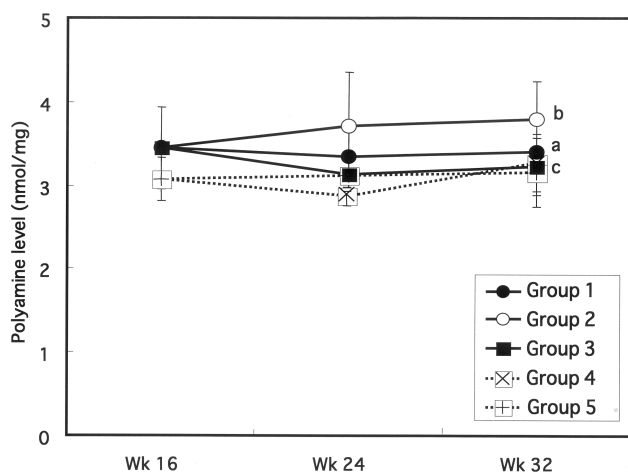

Figure 4. Effects of PCA on the Occurrence of Dysplasia.

A, Incidence of total dysplasia; B, Incidence of mild dysplasia; C, Incidence of moderate dysplasia; D, Incidence of severe dysplasia; and E, No. of dysplasia/cm
●,Group 1; ○, Group 2; and ■, Group 3
a, Significantly different from group 1 (P<0.05); b, Significantly different from group 2 (P<0.001); and c, Significantly different from group 2 (P<0.01)

Figure 5. Polyamine Levels of Tongue Tissue During the Experiment

a Significantly different from group 5 (P<0.01).
b Significantly different from group 1 (P<0.01).
c Significantly different from group 2 (P<0.001).

Figure 6. No. of AgNORs/nucleus in the Non-lesional Area

with the initiation procedure alone (group 1). PCA feeding (group 3) reduced this increase and the value reached to the level of group 1. Thus, the inhibitory effect of PCA on progression of tongue carcinogenesis found in the present study might be coincided with lowered cell proliferation activity in the non-lesional tongue tissue caused by administration of PCA.

Excess production of nitric oxide (NO) is involved in inflammatory and cancer (Alcaraz and Guilln, 2002) and also inducible nitric oxide synthase (iNOS) has been associated with the development of human and animal cancers including oral (Brennan et al., 2000; Chen et al., 2000). Recently, Lin et al. (Lin et al., 2003) show that PCA inhibited liver iNOS expression and serum NOx (NO2- and NO3-) induced by lipopolysaccharide in rat. PCA might inhibit iNOS expression in tongue lesions, as did other cancer chemopreventive agents (Tanaka et al., 2003; Yoshida et al., 2003). Investigation of such effect of PCA is underway in our laboratoty.

Various types of inhibitions of mutagenesis or carcinogenesis and their mechanisms have been postulated in available reviews (Cohen and F.R., 2002; Eckhardt, 2002; Greenwald, 2002; Gupta and Mukhtar, 2002; Pappas and Jordan, 2002; Wattenberg, 1997). Wattenberg (Wattenberg, 1985) broadly classified these into three categories based on the time period that chemopreventives exert their inhibitory activity in animal carcinogenesis models: inhibitors of carcinogenesis formation, "blocking" agents that are inhibitors of tumor initiation, and "suppressing" agent that are inhibitors of tumor promotion and/ or progression. The results of the present study suggest that PCA possesses suppressing property.

In the current study, the protective effect of PCA against progression of tongue carcinogenesis was investigated only 2000 ppm. Previously, we reported that PCA inhibited 4-NQO induced rat tongue carcinogenesis in both initiation
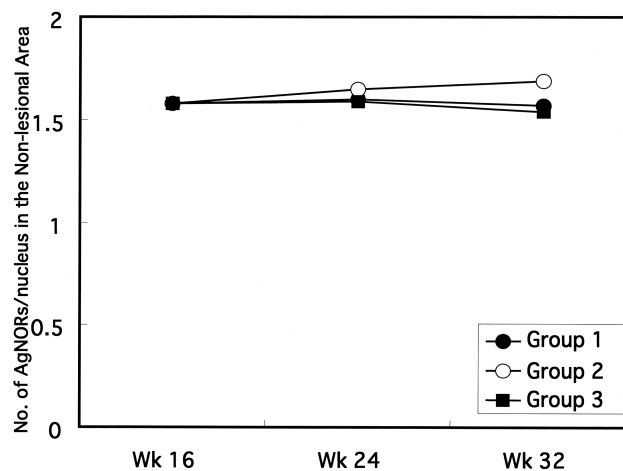
and postinitiation phases, when administered the diet containing PCA at 500 or 1000 ppm (Tanaka et al., 1994). Therefore, it is possible that less than 2000 ppm of PCA might inhibit all phases of tongue carcinogenesis.

Several studies have shown that the timing of administration of chemoprevention agents was important in modulating cancer risk (Ip et al., 1995; Song et al., 2000). Recently, Nakamura et al. demonstrated that topical application of PCA exerts contrasting effects on 12-O-tetradecanoylphorbol-13-acetate induced tumor promotion in mouse skin in a manner that is both dose and timing dependent (Nakamura et al., 2000). Therefore, much attention should be paid to the administration dose or time of PCA in chemoprevention studies.

In conclusion, the results of the present study indicate that a simple phenolic acid PCA has chemopreventive activities on 4-NQO-induced rat tongue carcinogenesis when given during the progression phase of tumorigenesis. Also, this study design allows us to elucidate the role of other chemoprevention agent in inhibiting the progression and growth of premalignant tongue tumors.

Acknowledgments

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