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Inhibitory Effects of Dietary Protocatechuic Acid and Costunolide on 7,12-Dimethylbenz[a]anthracene-induced Hamster Cheek Pouch Carcinogenesis

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The modifying effects of dietary exposure to two natural products, protocatechuic acid (PCA) and costunolide during the development of neoplasms in oral carcinogenesis initiated with 7,12-dimethylbenz[a]anthracene (DMBA) were investigated in male Syrian golden hamsters. All hamsters except those in the test chemical alone and control groups received DMBA (0.5%) in mineral oil to the right buccal pouch 3 times per week for 4 or 6 weeks. At 13 weeks of age, the groups exposed to DMBA were fed diet containing PCA or costunolide at a dose of 0.2 g/kg diet (200 ppm) for 17 weeks. The other groups consisted of hamsters given mineral oil alone for 6 weeks, or given 200 ppm PCA or costunolide alone, or untreated. All animals were necropsied at the termination of the experiment (week 24). PCA or costunolide significantly decreased the tumor burden ($P<0.001$–$P<0.05$) and the extent of dysplastic areas (%) ($P<0.001$–$P<0.05$). PCA significantly decreased the mean number of AgNORs/nucleus ($P<0.05$). The BrdUrd-labeling index was reduced by dietary administration of test compounds, though not significantly. These results suggest that PCA and costunolide inhibited hamster buccal pouch carcinogenesis and such inhibition may be related to suppression of cell proliferation in the buccal mucosa. It was also found that telomerase activity expressed in neoplastic and preneoplastic lesions of hamster buccal pouch epithelium after DMBA treatment correlated with the histopathological degree of malignancy.

Key words: 7,12-Dimethylbenz[a]anthracene — Hamster cheek pouch carcinogenesis — Protocatechuic acid — Costunolide — Telomerase activity

Several studies have revealed that neoplasms in the head and neck, including oral cavity, arise through multistage and multifocal carcinogenesis.[1–3] Patients with oral cancer also have an increased incidence of second primary tumors in the oral cavity.[4,5] The increased risk of cancer development in the entire upper aerodigestive tract after prolonged exposure to carcinogens is defined as "field cancerization."[1] Many oral or pharyngeal cancers are difficult to treat surgically because of the importance of maintaining organ function and form. Treatment of premalignant diseases, including dysplasia, which often occurs over wide areas of the oral mucosa, can also be difficult for similar reasons.

The term "cancer chemoprevention" refers to the prevention of cancer by intervention using nontoxic synthetic or natural chemicals before malignancy.[6] We have found several natural and synthetic compounds possessing cancer chemopreventive action against oral cancers.[7,8] Protocatechuic acid (PCA), a simple phenolic acid, is a constituent of many edible plants, fruits, and vegetables. Recently, PCA from the rind of *Citrus reticulata* Blanco has been reported to have a strong antioxidative property.[9] Therefore, PCA might modify hamster buccal pouch carcinogenesis. Our recent studies demonstrated a remarkable chemopreventive effect of dietary PCA on diethylnitrosamine-induced rat liver carcinogenesis, azoxymethane-induced colon carcinogenesis, and 4-nitroquinoline 1-oxide-induced tongue carcinogenesis in rats.[10–12] Costunolide, a sesquiterpene, is a constituent of Saussureae Radix ('Mokko' in Japanese), the dried root of *Saussurea lappa* Clarke (Compositae), and has been proven to have cholagogic or anti-ulcer effects and antioxidative properties.[13–15] We have also found a remarkable chemopreventive effect of dietary costunolide on azoxymethane-induced colon carcinogenesis in rats.[16]

The buccal pouch carcinogenesis model in Syrian golden hamsters is a well known animal model of premalignant and malignant lesions in human oral cancers. Therefore experimental studies on chemoprevention in the oral cavity have mainly been conducted using the hamster buccal pouch carcinogenesis model with a carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) and a variety of natural and pharmacological agents.[17] Most studies have employed painting of test chemicals on, or injection into, the buccal pouch epithelium.[18–21] In the present study, PCA or costunolide was given in the diet during DMBA-induced male Syrian golden hamsters buccal pouch carcinogenesis. The modifying effects of test compounds were investigated by measuring the 5-

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bromodeoxyuridine (BrdU)-labeling index and the number of silver-stained nucleolar organizer regions' proteins (AgNORs) as parameters of the alteration of proliferative potential of the buccal mucosa to clarify the underlying mechanism(s) of modification.

The telomere structure at the ends of eukaryotic chromosomes consists of protein-bound, randomly repeated, simple DNA sequences.[22,23] The telomere has important functions in protection and replication of chromosomes. However, chromosomes lose nucleotides of the terminal sequences at each cell division owing to incomplete replication of linear DNA molecules by undirectional RNA-primed DNA polymerase.[24,25] Telomerase is a ribonucleoprotein that synthesizes telomeric DNA on chromosome ends and is a kind of reverse transcriptase that uses a segment of its RNA component as a template.[26,27] Telomere length and telomerase activity have recently been implicated in the control of the proliferative capacity of normal and malignant cells.[28] Accordingly, we examined telomerase expression and activity in hamster buccal pouch neoplasms induced by DMBA, and the modifying effect of PCA or costunolide on them.

MATERIALS AND METHODS

Animals and diet Male Syrian golden hamsters, 5 weeks old, were purchased from Japan Shizuoka Laboratory Animal Center (Hamamatsu). After acclimation for 1 week, 184 hamsters (6 weeks of age) were transferred to the holding room under controlled conditions at 23±2°C, 50±10% humidity, and a 12-h light/dark cycle and randomized into experimental and control groups. They were housed three or four to a plastic cage, with wood chips for bedding. Powdered CE-2 (CLEA Japan, Inc., Tokyo) was used as basal diet during the experiment. It contained 50.4% crude carbohydrate, 24.8% crude protein, 4.6% crude fat, 7.2% crude ash, 4.2% crude cellulose, and 8.8% water, but no plant components.

Chemicals DMBA was purchased from Sigma Chemical Co., St. Louis, MO. PCA (97% purity) was obtained from Aldrich Chemical Co., Milwaukee, WI. Costunolide was extracted from Saussureae Radix, the dried root of *Saussureae lappa* CLARKE (Compositae) and purified by Yamahara, one of the present authors. The purity of this chemical was more than 99% (Fig. 1).

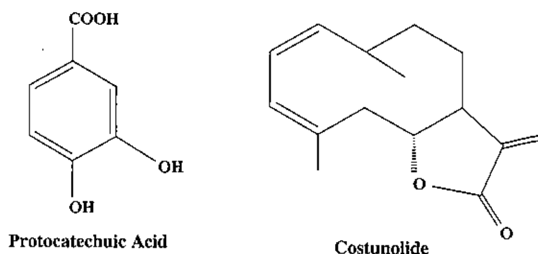

Fig. 1. Chemical structures of test compounds.

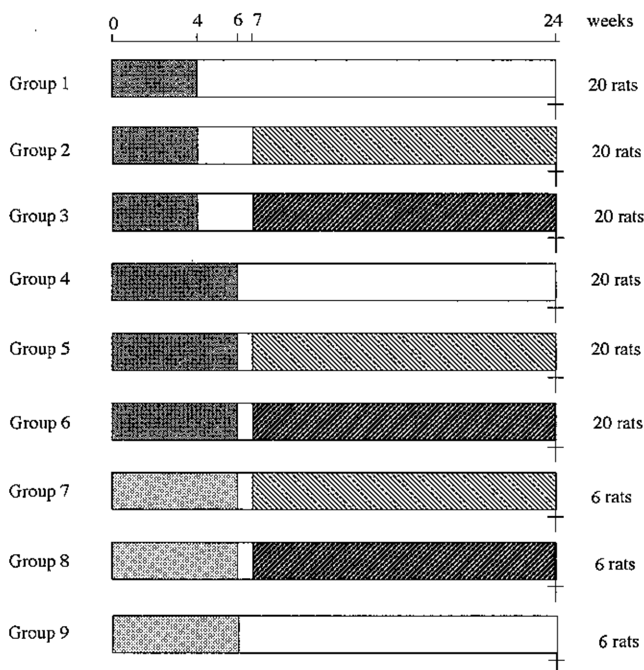

Fig. 2. Experimental protocol. ▦, treated with DMBA 3 times/wk. DMBA was administered as a 0.5% solution in mineral oil, applied to the entire mucosal surface of the right buccal pouch using a No. 3 sable brush at a dose of 0.25 mg on each buccal pouch per treatment (i.e., 0.75 mg/wk). ▒, treated with mineral oil alone; ▧, 200 ppm PCA; ▨, 200 ppm costunolide; □, basal diet.

Experimental procedure The experiment was designed to examine the modifying effects of PCA, and costunolide on the development of neoplasms in DMBA-induced oral carcinogenesis in male Syrian golden hamsters (Fig. 2).

Animals in groups 1, 2 and 3 were painted on the right buccal pouch, 3 times/week for 4 weeks, with a 0.5% solution of DMBA in mineral oil (Sigma Chemical Co.) using a No. 3 sable brush at a dose of 0.25 mg on each buccal pouch per treatment (i.e., 0.75 mg/week). Groups 4, 5 and 6 were painted 3 times/week for 6 weeks as in groups 1, 2 and 3. Each location was painted with approximately 0.6 mg of DMBA in oil upon the epithelial surface of the pouch. Groups 2 and 3 were given the diets containing 200 ppm PCA and costunolide, respectively, starting at 3 weeks after the cessation of DMBA treatment and continued on these diets for 17 weeks. Groups 5 and 6 were fed the diets mixed with PCA, and costunolide, respectively, at a concentration of 200 ppm,

starting 1 week after the cessation of DMBA treatment and continued on these diets for 17 weeks. Groups 7, 8 and 9 were painted 3 times/week for 6 weeks with mineral oil alone as a control, and groups 7 and 8 were given the diets containing PCA alone and costunolide alone, respectively, for the last 17 weeks of the experiment. Group 9 was given the basal diet throughout the study and served as an untreated control. All animals were provided with the diet and tap water *ad libitum*. Animals were weighed weekly until they reached 14 weeks of age, and then they were weighed every 4 weeks. All animals were killed by decapitation at 24 weeks after the start of the experiment, and complete necropsies were performed on all animals. After gross examination, all organs except for the buccal pouch were fixed in 10% buffered formalin. The right buccal pouches were carefully examined, and the number of tumors and the area of leukoplakial lesions were recorded. A part of each tumor and leukoplakial lesion was fixed in 10% buffered formalin for histopathological examination and cell counting, and the remaining portions were stored at −80°C until used for telomerase assay. Epithelial lesions (dysplasia and neoplasm) in the oral cavity were diagnosed according to the criteria described by Bánóczy and Csiba[29] and the WHO.[30] The tumor burden (number of tumors per group multiplied by mean volume of tumors per group in mm^3)[18] and extent of leukoplakial lesions (dysplastic areas per total area except neoplastic lesions of right buccal pouch epithelium ×100) were also measured.

Determination of proliferative activity in the tongue epithelium by AgNORs enumeration and BrdUrd-labeling index To assess the proliferative activity of buccal pouch squamous epithelium, the number of AgNORs per nucleus and the BrdUrd-labeling index of five randomly selected animals from each experimental group were quantified according to the methods described previously.[31] For measurement of BrdUrd-incorporated nuclei, the animals were given an i.p.-injection of 50 mg/kg body weight BrdUrd (Sigma Chemical Co.) 1 h prior to killing. The tumors and leukoplakial lesions were then cut into two. One half was used for molecular biology and the other was fixed in 10% buffered formalin for histopathology, AgNORs counting, and determination of BrdUrd-labeling index. Three serial sections (3 μm thick) were made after embedding in paraffin. On one section, a one-step silver colloid method for AgNORs staining was carried out and computer-assisted image analysis quantification using an image analysis system SPICCA II (Japan Avionics Co., Tokyo) with an Olympus BH-2 microscope (Olympus Optical Ind. Co., Ltd., Tokyo) and a color-charged coupled device camera (Hamamatsu Photonics Co., Hamamatsu) was performed on 100 nuclei of interphase cells from nonlesional areas. Another section was used for immunohistochemical detection of BrdUrd incorporation using a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). The labeling indices of BrdUrd (percentage) were calculated by counting labeled nuclei of 100 cells at ×400. The remaining section was used for histological diagnosis.

Telomerase activity assay Telomerase activity assay was basically performed according to the method of Kim *et al.*[32] with modifications.[33] The stored tissues were washed once in ice-cold washing buffer [10 m*M* HEPES-KOH pH 7.5; 1.5 m*M* $MgCl_2$; 10 m*M* KCl; and 1 m*M* dithiothreitol] and centrifuged. Then, tissues were homogenized in ice-cold lysis buffer [10 m*M* Tris-HCl, pH 7.5; 1 m*M* $MgCl_2$; 1 m*M* EGTA; 0.1 m*M* phenylmethylsulfonyl fluoride; 5 m*M* β-mercaptoethanol; 0.5% CHAPS (Pierce Chemical Co., Rockford, IL); and 10% glycerol], incubated for 30 min on ice, and then centrifuged for 45 min at 15,000*g* at 4°C. The supernatant was removed and its protein concentration was measured with a BCA protein assay kit (Pierce Chemical Co., Rockford, IL). The samples were diluted to 1–5 μg/μl with lysis buffer and stored at −80°C until the telomerase activity assay was conducted.

The assay was performed in two steps, instead of as in the original protocol. Each microtube contained 40 μl of reaction solution containing 20 m*M* Tris-HCl, pH 8.3; 1.5 m*M* $MgCl_2$; 63 m*M* KCl; 0.005% Tween-20; 1 m*M* EGTA; 50 μ*M* dNTPs; 1 μg of T4gene32 protein (Amersham Intl. plc, Amersham, Bucks., U.K.); 2.5 μg of bovine serum albumin; 0.1 μg of nontelomeric oligonucleotide; TS upstream primer; and 1 μl of the stored extract. The mixture was incubated at 23°C for 30 min to allow the telomerase in the extracted proteins to extend the TS primer and then at 99°C for 5 min to stop the enzyme activity. For radiolabeling of PCR products, we then added 10 μl of PCR mix (1× PCR buffer, 0.2 μl of [α-^{32}P]dCTP (10 μCi/μl, 3000 Ci/mmol, Amersham Intl. plc), 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer Cetus, Emeryville, CA), and 0.1 μg of CX downstream primer to each microtube and transferred the tubes to a thermal cycler for 30 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min. Fifteen microliters of the reaction mixture was analyzed by electrophoresis in 1× Tris-glycine-EDTA buffer on 15% polyacrylamide nondenaturing gels (Joey Gel Casting System, Owl Scientific Inc., Wobarn, MA). The gels were exposed to Kodak XAR film (Eastman Kodak Co., Rochester, NK) overnight after being dried. The DNA size marker used was pUC19DNA/Msp I (MBI Fermetas, Vilnius, Lithuania) labeled with [γ-^{32}P]ATP (10 μCi/μl, >5000 Ci/mmol, Amersham Intl. plc).

Thirteen hamster buccal pouch epithelia (identified by histopathological examination as five squamous cell carcinomas, three dysplasias, two treated normal epithelia

and three untreated normal epithelia) were examined at a protein concentration of 2 μg/μl.

Quantification of telomerase activity To remove non-reacted [α-^{32}P]dCTP from labeled telomeres, 10 μl of PCR products was transferred to a Microcon 100 (Amicon, Inc., Beverly, MA) and centrifuged. Amplified DNA fragments were collected by washing the membrane with 20 μl of distilled water and added to a scintillation cocktail solution. The radioactivity was then measured with a liquid scintillation counter (Packard Instrument Co., Inc., Meriden, CT). The background was subtracted from the observed values.

Statistical analysis The data in this experiment were analyzed by the use of Fisher's exact probability test, the χ^2 test, Student's t test or Welch's t test.

RESULTS

General observations The mean body and liver weights at the end of the study are listed in Table I. The mean body weights of hamsters in all groups given test compounds were comparable with that of a control group (group 9). The mean liver weight of hamsters in group 4 was significantly larger than that of group 9 ($P<0.001$). However, this group (group 4) was not significantly different from group 9 in mean relative liver weight (g/100 g body weight).

Tumor burden and extent of preneoplastic lesions (%) of buccal pouch In this study, exophytic tumors occurred only in the oral cavity. They were located at the right buccal pouch treated with DMBA and were squamous cell papillomas, or well or moderately differentiated squamous cell carcinomas, histologically. The incidence of buccal pouch neoplasms (squamous cell papillomas and carcinomas) showed no significant differences among groups (Table II). However, statistical analysis revealed a significant decrease in the tumor burden (number of tumors per group multiplied by mean volume of tumors per group in mm^3) in group 3 when compared to that of group 1 ($P<0.001$) and the values of groups 5 and 6 were significantly smaller than that of group 4 ($P<0.001$ and $P<0.05$) (Table III). Tumor volume was obtained by use of the formula $4/3\pi r^3$ where r is the mean radius of the tumor.

The extent of dysplastic areas (%) (dysplastic areas per total areas except neoplastic lesions of right buccal

Table I. Body, Liver and Relative Liver Weights in Each Group

Group no.	Treatment	No. of hamsters examined	Body wt. (g)	Liver wt. (g)	Relative liver wt. (g/100g b.w.)
1	DMBA 4 wks alone	20	166±15[a]	11.8±1.7[b]	7.09±0.65
2	DMBA 4 wks+PCA	20	166±11	11.5±1.1	6.90±0.68
3	DMBA 4 wks+Costunolide	20	163±14	11.6±1.8	7.10±0.82
4	DMBA 6 wks alone	20	166±10	11.9±1.1[c]	7.16±0.55
5	DMBA 6 wks+PCA	20	162±20	11.5±1.9	7.06±0.71
6	DMBA 6 wks+Costunolide	20	168±12	11.7±1.1	6.96±0.62
7	PCA alone	6	165±9	10.1±0.4	6.13±0.30
8	Costunolide alone	6	155±17	9.7±1.4	6.25±0.51
9	No treatment	6	154±11	9.7±1.4	6.71±0.60

a) Mean±SD.
b, *c*) Significantly different from group 9 by Student's t test (*b*) $P<0.05$ and *c*) $P<0.001$).

Table II. Incidence of Buccal Pouch Neoplasms in Hamsters of Each Group

Group no.	Treatment	No. of hamsters examined	No. of hamsters with buccal pouch neoplasms		
			Total	Papilloma	Carcinoma
1	DMBA 4 wks alone	20	13	7	8
2	DMBA 4 wks+PCA	20	11	3	8
3	DMBA 4 wks+Costunolide	20	14	3	12
4	DMBA 6 wks alone	20	19	6	19
5	DMBA 6 wks+PCA	20	16	2	16
6	DMBA 6 wks+Costunolide	20	20	4	17
7	PCA alone	6	0	0	0
8	Costunolide alone	6	0	0	0
9	No treatment	6	0	0	0

Table III. Tumor Burden—Total Number of Tumors×Mean Volume ($4/3\pi r^3$) per Group

Group no.	Treatment	No. of hamsters examined	No. of tumors	Mean diameter of tumors (mm)	Tumor burden (mm^3)
1	DMBA 4 wks alone	20	16	1.88±1.34[a]	55.67
2	DMBA 4 wks+PCA	20	11	2.22±1.30	63.02
3	DMBA 4 wks+Costunolide	20	13	1.66±1.08	31.14[b]
4	DMBA 6 wks alone	20	57	2.80±2.30	655.16
5	DMBA 6 wks+PCA	20	32	2.40±1.80	231.62[c]
6	DMBA 6 wks+Costunolide	20	37	2.80±2.12	425.28[d]
7	PCA alone	6	0	0	0
8	Costunolide alone	6	0	0	0
9	No treatment	6	0	0	0

a) Mean±SD.
b) Significantly different from group 1 by Welch's *t* test ($P<0.001$).
c, d) Significantly different from group 4 by Welch's *t* test (*c*) $P<0.001$ and *d*) $P<0.05$).

Table IV. Extent of Preneoplastic Areas (%) in Buccal Pouch of Hamsters of Each Group

Group no.	Treatment	No. of hamsters examined	No. of hamsters with dysplastic lesions			
			Total	Mild	Moderate	Severe
1	DMBA 4 wks alone	20	1.28±0.81[a]	0.69±0.68	0.27±0.50	0.32±0.60
2	DMBA 4 wks+PCA	20	0.32±0.73[b]	0.18±0.65[c]	0.01±0.03[d]	0.13±0.34
3	DMBA 4 wks+Costunolide	20	0.32±0.58[b]	0.23±0.49[c]	0[d]	0.09±0.38
4	DMBA 6 wks alone	20	5.25±6.42	0.02±0.06	1.43±1.57	3.78±6.38
5	DMBA 6 wks+PCA	20	1.71±1.96[e]	0.25±0.34[f]	0.77±0.76	0.69±1.59
6	DMBA 6 wks+Costunolide	20	1.28±0.93[e]	0.08±0.14	0.76±0.79	0.44±0.62[e]
7	PCA alone	6	0	0	0	0
8	Costunolide alone	6	0	0	0	0
9	No treatment	6	0	0	0	0

a) Mean±SD.
b, c) Significantly different from group 1 by Student's *t* test (*b*) $P<0.001$ and *c*) $P<0.05$).
d) Significantly different from group 1 by Welch's *t* test ($P<0.05$).
e, f) Significantly different from group 4 by Welch's *t* test (*e*) $P<0.05$ and *f*) $P<0.01$).

pouch epithelium ×100) is shown in Table IV. Significant differences in the total areas of dysplastic lesions were obtained between group 1 and group 2 or 3 ($P<0.001$), and between group 4 and group 5 or 6 ($P<0.05$). Dysplasia was classified into three degrees (mild, moderate, and severe). The extents of mild and moderate dysplasia in groups 2 and 3 were lower than those of group 1 ($P<0.05$). The extent of mild dysplasia in group 5 was lower than that of group 4 ($P<0.01$). The value of severe dysplasia in group 6 was smaller than that of group 4 ($P<0.05$).

Enumeration of AgNORs and BrdUrd-labeling index The results of morphometric analysis of AgNORs and BrdUrd-labeling indices in the nonlesional squamous epithelium are shown in Table V. The mean number of AgNORs in the right buccal pouch epithelium exposed to DMBA alone (groups 1 and 4) was significantly larger than that of the untreated control (group 9) ($P<0.05$ and $P<0.001$). The mean number of AgNORs/nucleus in group 5 (dietary administration of PCA) was significantly smaller than in the group untreated with test compounds (group 4) ($P<0.05$). Those in groups 2 and 3 were smaller than that in group 1, and that in group 6 was smaller than that in group 4, though these differences were not significant. The BrdUrd-labeling index in the right buccal pouch epithelium exposed to DMBA alone (group 4) was significantly higher than that of the untreated control (group 9) ($P<0.001$). The BrdUrd-labeling index in groups given test compounds (groups 2 and 3, and groups 5 and 6) was lower than in groups not treated with test compounds (groups 1 and 4, respectively), though again the differences were not significant. The average number of AgNORs and BrdUrd-labeling indices in groups 7 and 8 (200 ppm test chemicals alone)

Table V. AgNORs Count and BrdUrd-labeling Index of Non-Lesional Areas of Buccal Pouch Squamous Epithelium

Group no.	Treatment	No. of hamsters examined	No. of AgNORs/ Nucleus	BrdUrd-labeling indices (%)
1	DMBA 4 wks alone	20	2.35±0.25[a, b]	8.6±2.7
2	DMBA 4 wks+PCA	20	2.16±0.12	7.8±2.4
3	DMBA 4 wks+Costunolide	20	2.20±0.12	7.8±2.3
4	DMBA 6 wks alone	20	2.93±0.22[c]	12.0±1.9[c]
5	DMBA 6 wks+PCA	20	2.51±0.27[d]	10.5±1.8
6	DMBA 6 wks+Costunolide	20	2.68±0.30	10.7±2.0
7	PCA alone	6	1.83±0.22	6.0±1.6
8	Costunolide alone	6	1.91±0.13	6.4±1.3
9	No treatment	6	2.01±0.13	5.8±1.5

a) Mean±SD.
b, c) Significantly different from group 9 by Student's *t* test (*b*) $P<0.05$ and *c*) $P<0.001$).
d) Significantly different from group 4 by Student's *t* test ($P<0.05$).

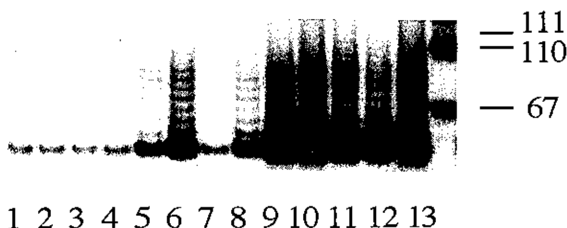

Fig. 3. Telomerase activity in hamster buccal pouch epithelium. Normal, DMBA-treated or untreated normal epithelium; DYS, dysplasia; CAR, squamous cell carcinoma induced by 7,12-dimethylbenz[*a*]anthracene. Telomerase activity was seen in samples histologically confirmed as squamous cell carcinoma and dysplasias, but not in normal epithelium.

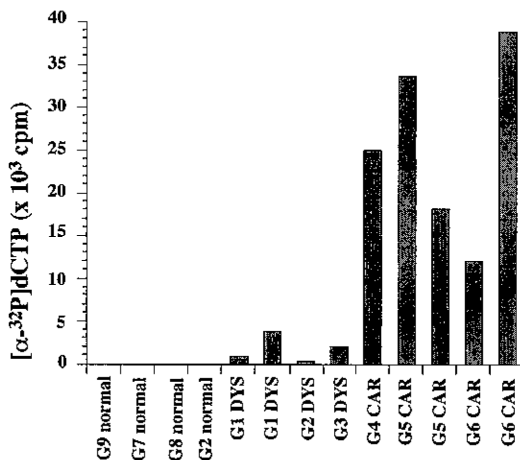

Fig. 4. Quantification of telomerase activity. Telomerase activity was generally greater in carcinoma than in dysplasia. ■, telomerase activity (cpm).

were similar to those of group 9 (untreated control).

Telomerase activity assay Telomerase activity was found in histologically confirmed squamous cell carcinomas and dysplasias, but not in normal epithelia (Fig. 3).

Quantification of telomerase activity The telomerase activity in carcinomas was generally greater than that in dysplasias (Fig. 4). There was no difference in telomerase activity between the neoplastic and preneoplastic lesions treated and untreated with the test compounds.

DISCUSSION

In the present study, dietary administration of PCA or costunolide during the postinitiation phase inhibited the development of oral carcinogenesis initiated with DMBA. PCA and costunolide also inhibited the development of DMBA-induced neoplastic lesions, AgNORs count and BrdUrd-labeling index in buccal pouch squamous epithelium.

The results in the current study are in agreement with our previous findings showing a suppressing effect of PCA on chemically induced liver, colon and tongue carcinogenesis in rats.[10–12] The incidence of buccal pouch neoplasms (squamous cell papillomas and carcinoma) in the groups showed no significant differences (Table II). This may be related to the fact that a lower dose of PCA than that used in the previous study was used in this study to compare the effects of PCA and costunolide. In diethylnitrosamine-induced rat liver carcinogenesis, feeding of PCA at 500 and 1000 ppm during the initiation and postinitiation phases significantly reduced the occurrence of preneoplasia (liver cell foci) and hepatocellular neoplasms.[10] In azoxymethane-induced rat colon carci-

nogenesis, PCA significantly inhibited the development of intestinal tumors when fed at a dose of 1000 ppm during initiation and when fed at levels of 500 and 1000 ppm during postinitiation.[11] In 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis, PCA at 500, 1000, and 2000 ppm during the initiation and postinitiation phases significantly inhibited the occurrence of tongue neoplasms.[12] Chemopreventive agents that inhibit such processes have been categorized as "blocking agents" by Wattenberg.[6, 34] Other types of chemopreventives are "suppressing agents" that prevent the development of neoplasms after carcinogen exposure.[6, 34] The results in the previous studies suggest that PCA possesses both blocking and suppressing properties, although this should be confirmed by biochemical studies on the modifying effects of dietary PCA on the phase I and phase II enzymes involved in the metabolism, detoxification, and elimination of carcinogens.

The results of the current study are agreement with our earlier findings showing a suppressing effect of costunolide on chemically induced colon carcinogenesis in rats. Our previous study demonstrated that feeding of costunolide during the initiation phase inhibited azoxymethane(AOM)-induced intestinal tumorigenesis as well as the formation of aberrant crypt foci, ornithine decarboxylase activity, polyamine concentration, silver-stained nucleolar organizer region number, and bromodeoxyuridine-labeling index in the colon.[16, 35] Costunolide may have blocking and suppressing effects against AOM-induced intestinal carcinogenesis, and its effects seem to be related to the inhibition of cell proliferation.

Cell proliferation has an important role in carcinogenesis of rodents and humans.[36, 37] The BrdUrd-labeling index is a useful marker for cell proliferation and has been used as a biomarker in chemoprevention studies.[31] The tumor-suppressing effect of dietary PCA or costunolide administration may be due to suppressing effects on cell proliferation in the target organ. In the present study, PCA or costunolide exposure lowered the cell proliferation activity of the buccal pouch mucosal epithelium. These results were comparable to those of our earlier experiments testing the chemopreventive efficacy of several natural phenolic antioxidants[38, 39] and a synthetic compound, DL-α-difluoromethylornithine.[40] The results in the current study provide additional evidence that a natural product, PCA, could effectively inhibit tumor development without toxicity. PCA might be a possible cancer chemopreventive agent in the oral cavity, in addition to other organs.

Telomerase is probably essential for the survival of immortal cells,[28, 41] and seems to be related to the malignancy of neoplasms in humans.[42–45] Recently, a powerful PCR-based method was developed to detect telomerase activity.[32] However, there are few reports of telomerase activity in animal models.[33, 46] In the present study, we found that telomerase activity was elevated in neoplastic and preneoplastic lesions of hamster buccal pouch epithelia after DMBA treatment. In addition, telomerase activity was not detected in normal epithelia of DMBA-treated or untreated animals. This finding is the first report on telomerase activity in hamster carcinogenesis, though there is a report concerning mouse skin carcinogenesis.[46] Telomerase activity was expressed most prominently in the tissues of carcinomas, and the activity was detected in the tissues of even dysplasias. Accordingly, we hypothesize that telomerase activity could be a biomarker of the transition of normal lesions into preneoplastic lesions, and that the amount of telomerase activity is in proportion to the histopathological degree of malignancy. Bednarek *et al.*[46] reported that the telomerase activity in carcinomas was higher than that in papilloma in mouse skin carcinogenesis.

In conclusion, the results of the present study indicated that the natural products PCA and costunolide both have chemopreventive activities on DMBA-induced hamster buccal pouch carcinogenesis when given during the post-initiation phase of tumorigenesis. This protecting effect may be related to the control of carcinogen-induced hyper-proliferation. It was also found that telomerase activity was elevated more strongly in neoplastic and preneoplastic lesions of hamster buccal pouch epithelia after DMBA treatment. Telomerase activity may be proportional to the histopathological degree of malignancy.

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