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Carotenoids suppress proliferating cell nuclear antigen and cyclin D_1 expression in oral carcinogenic models☆

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Abstract

The purpose of this study was to investigate the chemopreventive effect of carotenoids on proliferating cell nuclear antigen (PCNA) and cyclin D_1 expression in betel (*Areca catechu*) quid extract (BQE)-induced hamster oral cancer and human KB cell models, respectively. In the in vivo animal study, 41 hamsters were divided into six groups and treated with 0.3 ml of 0.5% 9,10-dimethyl-1,2-benz[a]-anthracene, BQE, α-tocopherol, β-carotene, lycopene, lutein and mixed carotenoids for 12 weeks. After treatment, the pouches were excised and graded using an immunohistochemical assay of PCNA. In the in vitro cell experiment, KB cells were cultured, and the inhibitory effect of carotenoids (β-carotene, lycopene and lutein) on cell proliferation was evaluated. Cyclin D_1 and PCNA were evaluated in terms of cell differentiation. In the results, most of the animal lesions showed no overexpression of PCNA. However, in dysplastic lesions, PCNA expressions by the β-carotene, lutein, lycopene, mixed and vitamin E groups were less than that of the control group. In papilloma lesions, PCNA expressions by the β-carotene, mixed and vitamin E groups were less severe than that of the control group. PCNA expression by the vitamin E-treated group was less severe than that of the control group. No carcinoma was found in the lycopene or mixed groups. In the cell study, all carotenoids exerted a significant inhibitory effect on KB cell proliferation. Although lycopene suppressed KB cell proliferation at the G_0/G_1 phase with a significant decrease in PCNA expression, β-carotene and lutein possessed less of an inhibitory effect and even exhibited elevated cell proliferation at the G_2/M phase. These results indicate that different carotenoids present various suppressive abilities against PCNA and cyclin D_1 expressions in cell proliferation. In conclusion, carotenoids suppressed the carcinogenesis of induced hamster oral cancer and a cancer cell line by acting as a suppressor which inhibited the expressions of PCNA and cyclin D_1.

Keywords: Carotenoids; Betel quid extract; Oral cancer; Proliferating cell nuclear antigen; Cyclin D_1

1. Introduction

Betel quid (BQ) chewing is thought to contribute to leukoplakia, submucosal fibrosis and oral cancer in Taiwan, India and many Southeast Asian countries [1]. The traditional formula of the Taiwanese BQ contains the tender areca nut (*Areca catechu*), slaked lime, catechu (an astringent), the inflorescence of the piper betel or piper betel leaves. Some studies have reported that the extract of the areca quid is associated with the onset of early malignant changes in the hamster cheek pouch [2,3]. The components of the BQ accelerate the proliferation and differentiation of oral mucous cells, especially in combination with slaked lime and the nitrosative products of areca nut — arecoline (i.e., guvacoline, guvacine and arecaidine) [4]. One of the possible mechanisms leading to submucosal fibrosis is an elevation of reactive oxygen species (ROS) and then induction of DNA damage in vitro. However, the mechanism of BQ chewing-induced oral cancer still remains unknown.

The immunohistochemical assay of proliferating cell nuclear antigen (PCNA) has been utilized by many

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investigators as a useful marker of cell proliferation and differentiation. PCNA is one of the helper proteins of DNA polymerase σ, which is a key protein in cell cycle regulation [5,6] and a trigger of cell proliferation and differentiation [7,8]. Some studies have indicated that PCNA shows different expression extents in normal, precancerous and malignant tissues: the greater the degree of metaplastic tissue, the greater the expression of PCNA is. In cancer progression, e.g., from normal mucosal tissues to metaplasia, then dysplasia to squamous cell carcinoma in neck and oral tissues, PCNA expression is elevated [9], and the assay can be used to diagnose throat cancer progression [10]. Cyclin D_1 is an important nuclear protein in the G_1/S phase of the cell cycle. Bova et al. [11] revealed that cyclin D_1 overexpression and cyclin D_1 gene amplification were found in patients with oral squamous cell carcinoma. This implied that cyclin D_1 expression may be as an early indicator of oral carcinogenesis.

In our previous studies, BQ was shown to play a role as a promoter during the carcinogenesis of hamster buccal pouch carcinoma, and carotenoids presented an inhibitory effect in induced hamster oral carcinogenesis [12,13]. Carotenoids have been indicated as possessing a strong ability to protect cells against active oxygen species. Carotenoids are common lipotrophic phytochemicals in nature, which prevent cancers by interfering with protein expression in the cell cycle. Dietary carotenoids may serve to lower the risk of cancer in humans, based on their reported antioxidant capabilities to quench singlet oxygen and other oxidizing species. If such a free radical mechanism is involved in the initiation and promotion of carcinogenesis, carotenoids may undergo oxidation in order to protect against cellular oxidative damage. Thus, In this study, we investigated the chemopreventive effect of carotenoids on PCNA and cyclin D_1 expression in BQ extract (BQE)-induced hamster oral cancer and KB cell (a human oral epidermoid carcinoma cell line) models, respectively.

2. Materials and methods

2.1. Animal protocols

During the experiment, 41 8-week-old adult male hamsters (National Science Council, Taipei, Taiwan), weighing 110–120 g, were housed under a 12-h light-dark cycle, in a temperature- (22–24°C) and humidity-controlled (60%) room. Rodent chow (Labdiet #5001) and water were available ad libitum.

2.1.1. Experimental design

Animals were divided into six experimental groups and were treated with 0.3 ml of 0.5% of the experimental agents (approximately 2.7 mg for each treatment), which included 9,10-dimethyl-1,2-benz[a]-anthracene (DMBA), BQE, α-tocopherol, β-carotene, lycopene, lutein and a mixture of equal amounts of these carotenoids, respectively. In the 16-week experimental period, the buccal pouches of animals in the test groups were initially daubed with DMBA for 4 weeks. In the subsequent 12 weeks, groups were daubed with either BQE or a mineral oil solution containing carotenoids three times per week on alternate days. DMBA+BQE daubed treatments were set as a control for tissue carcinogenic process. The process of manufacturing BQE was modified from the description by Lin et al. [14]. Vitamin E and carotenoids were purchased from Roche (Nutley, NJ, USA). All chemicals used in this study were obtained from Sigma (St. Louis, MO, USA). The components for the BQE were purchased from a local market.

2.1.2. Sample preparation

At the end of the experiment, all animals were anesthetized under a CO_2 atmosphere. Hamster buccal pouch carcinomas and esophageal tissues were removed, extended and fixed on a 25-cm^2 paperboard under a 10% formaldehyde solution for at least 24 h. After the paraffin fixation process, the tissue lesions were sliced into 5-μm sections under poly-L-lysine treatment for further immunohistochemical staining and were evaluated by a senior pathologist.

2.1.3. Immunohistochemical staining of PCNA

For the immunohistochemical staining analyses, tissue sections cut from the paraffin blocks were first deparaffinized and rehydrated in graded alcoholic solutions and phosphate-buffered saline (PBS). Specific antibody staining for various antigens (LSAB2 System, HRP, DAKO, Carpentaria, CA, USA) was used. Primary antibody incubations of PCNA (DAKO-PCNA PC10 monoclonal antibody) were carried out at room temperature for 45 min with nonimmune mouse IgG; after careful washing, sections were incubated in biotinylated secondary immunoglobulin of appropriate species specificity (Sigma; diluted to 1:200 with PBS) for 30 min at room temperature. After incubation with the secondary antibody, sections were washed in PBS three times for 10 min each and then incubated with an avidin–biotin complex for 30 min at room temperature. Histochemical localization was accomplished using an avidin–biotin horseradish peroxidase (HRP) complex (DAKO Liquid DAB+Substrate–Chromogen System) with 3-3′ diaminobenzidine tetrahydrochloride as the chromogen. Normal goat serum was applied to the sections for 30 min to bind nonspecific sites. After immunohistochemical staining, tissue sections were counterstained with hematoxylin. Control sections were processed in parallel with mouse nonimmune IgG at the same concentration as the primary antibodies. After sectioning and staining, multiple degrees (score, 1–5: mild, moderate, marked, massive and extreme massive) were examined to determine the percentage of epithelial cells showing positive immunoreactivity against PCNA. For each individual degree, the percentage of expressing cells was counted in five separate low-magnification microscopic fields by a qualified oral pathologist.

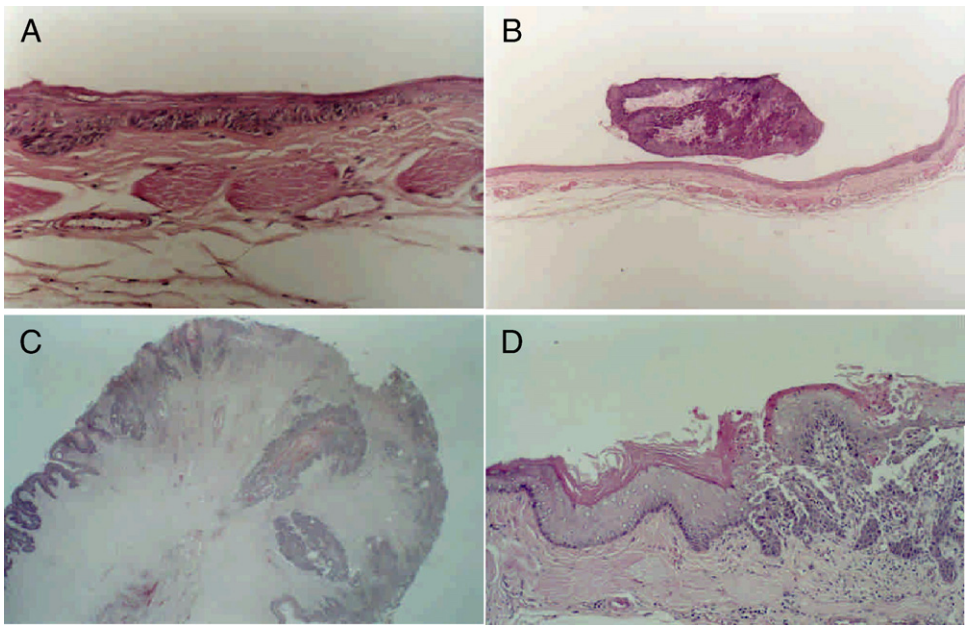

Fig. 1. Lesion characteristics of the control and experimental groups with hematoxylin and eosin stain. In the control group, some lesions presented with a disordered cell arrangement, condensed chromosomes of the basal layer and dysplasia (A) (original magnification ×100); some lesions developed papilloma with hyperplasia (B) (original magnification ×20) and some lesions exhibited squamous cell carcinoma with keratin overexpression (C) (original magnification ×20). In the mixed carotenoid group, lesions showed less hyperplasia and more-normal tissue morphology (D) (original magnification ×40).

2.2. KB cell culture

2.2.1. Chemicals

Bovine serum albumin, tetramethylene diamine, dimethyl sulfoxide, β-carotene and lutein were commercially obtained from Sigma Chemical, while the lutein required partial purification. Lycopene, propidium iodium and ribonuclease A (RNase A) were purchased from MP Biomedicals (Illkirch, France). Minimum essential medium (MEM), fetal bovine serum (FBS), a trypsin/EDTA solution and penicillin–streptomycin were purchased from GIBCO (Grand Island, NY, USA). Cyclin D_1 mouse monoclonal IgG_1 antibody, PCNA mouse monoclonal $IgG_2\alpha$antibody and goat antimouse IgG-HRP antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The β-actin mouse monoclonal IgG1k antibody was obtained from Chemicon International (Temecula, CA, USA). Absolute ethanol was purchased from Merck (Darmstadt, Germany). All other laboratory chemicals were of the highest quality available and were purchased from Sigma, TEDIA (Fairfield, OH, USA) and USB (Cleveland, OH, USA).

2.2.2. Cell culture conditions

The human KB cell line [CCL-17 human oral epidermoid carcinoma cell line, American Type Culture Collection number (ATCC, Rockville, MD, USA)] originated from the Food Industry Research and Development Institute, Hsinchu, Taiwan. Cells were grown as monolayers in MEM supplemented with 10% FBS and 100 IU/ml of penicillin–streptomycin in an atmosphere of 95% air and 5% CO_2 at 37°C. β-Carotene, lycopene and lutein were dissolved in absolute 9 ethanol, and the concentration of absolute ethanol added to the media never exceeded 0.2% (v/v). Because β-carotene, lycopene and lutein (5, 10, 20 and 30 μM) showed no significant cytotoxicity, these concentrations were used in this study.

2.2.3. MTT assay

The inhibition of KB cell proliferation by carotenoids was determined by the MTT assay, as described below [15], for which cells were plated onto 96-well tissue culture dishes at a density of 5×10^3 well^{-1} in 100 μL medium. After plating, cells were allowed to attach for 24 h. Cells were incubated with various concentrations of the agents for 24 h, at which time 20 μL of 2 mg/ml MTT was added, and the absorbance at 492 nm was determined by a microtiter plate reader. The extract was added using tetrahydrofuran (THF) as the vehicle, at a maximum concentration of 0.1%. Live cells convert 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium to a formazan dye that can be detected at 492 nm by a microplate reader. The absorbance at 690 nm was also measured as a reference. The same aliquot of carotenoid-free emulsion alone was added to the control cells. Experiments were conducted in six replicates.

2.2.4. Protein extraction and Western blotting

At termination of the cultures, treated KB cells were incubated at 4°C for 60 min in the presence of lysis buffer consisting of 150 mM/L NaCl, 2 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 1% sodium deoxycholate,

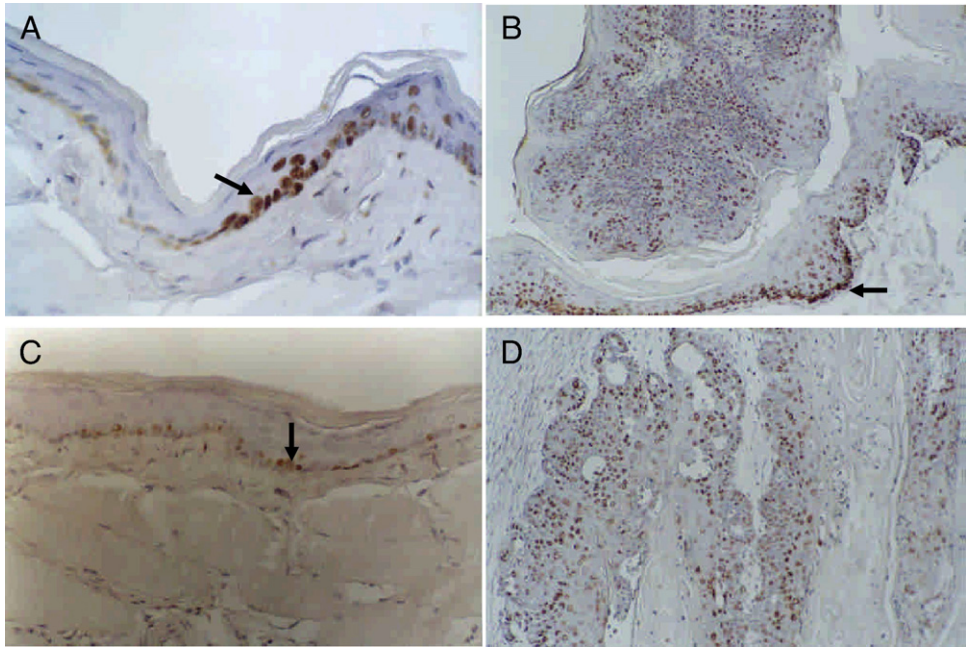

Fig. 2. PCNA expression of lesions. In the control group, the basal layer with dysplasia presented greater PCNA expression (A) (original magnification ×100); some lesions developed a papilloma with hyperplasia (B) (original magnification ×40); and some lesions with carcinoma also showed high PCNA expression (C) (original magnification ×100). In the mixed carotenoid group, PCNA was only evident in the basal layer (D) (original magnification ×40).

1 mg/L aprotinin, 0.1% sodium dodecyl sulfate (SDS) and 50 mM Tris–HCL (pH 7.4). Cells were subsequently scraped off the plates. The lysates were centrifuged at 4°C and 15,000*g* for 15 min, and the supernatants were collected. Protein contents in the supernatants were determined using a protein assay kit (Bio-Rad Dc Protein Assay Reagent; Bio-Rad Laboratories, Hercules, CA, USA). Proteins (50 μg) were subjected to 12.5% SDS–polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Bedford, MA, USA). Membranes were blocked with nonfat dried milk, and immunoblotting was done using an anti-cyclin D_1 mouse monoclonal IgG_1 antibody (1:500) (Santa Cruz Biotechnology), anti-PCNA mouse monoclonal $IgG_{2\alpha}$ antibody (1:2000) (Santa Cruz Biotechnology) and anti-β-actin monoclonal IgG_{1K} antibody (1: 1000) (Chemicon) in Tween 20 base saline buffer (PBS-T). Membranes were probed with a goat anti-mouse IgG-HRP antibody (Santa Cruz Biotechnology) as a second antibody. The antigen-antibody complexes were detected with the secondary antibodies using an ECL chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were visualized by exposure to X-Omat film (Eastman Kodak, Rochester, NY, USA). The experiments were repeated in triplicate analyses in different cultured specimens with similar results, and representative results are reported.

2.2.5. Cell cycle analysis

Cell samples for cytometric analysis were prepared according to a method reported elsewhere [16]. The cell cycle distribution was determined using a FACScan laser flow cytometer (Becton Dickinson, San Jose, CA, USA). Cells were trypsinized, collected and washed twice with PBS. The pellets were resuspended in 1 ml of PBS, fixed in 2 ml of an ethanol–PBS solution (75/25) and stored at

Table 1
PCNA expression of epithelial cells treated with different carotenoids

Treatment	Animal number	Normal epithelium	Hyperplasia	Dysplasia	Papilloma	Carcinoma
DMBA+BQE	7	1.6±0.3 (0–2)	2.6±0.7 (2–3)	4.4±0.8 (4–5)	3.1±0.5 (3–4)	4.6±0.6 (4–5)
DMBA+BQE+vitamin E	6	0.3±0.0 (0–1)*	1.6±0.2 (1–2)*	1.3±0.2 (1–2)*	2.3±0.3 (2–3)*	2.8±0.5 (2–4)*
DMBA+BQE+β-carotene	7	1.5±0.4 (0–2)	2.1±0.2 (2–3)	2.1±0.1 (2–3)*	2.1±0.4 (2–3)*	3.2±0.4 (2–4)*
DMBA+BQE+lycopene	7	1.1±0.2 (0–2)	2.3±0.1 (2–3)	2.4±0.6 (2–3)*	2.5±0.6 (2–3)	0*
DMBA+BQE+lutein	7	0.3±0.0 (0–1)*	2.2±0.1 (2–3)	2.6±0.4 (2–3)*	3.2±0.5 (3–4)	3.9±0.7 (2–4)
DMBA+BQE+mixture	7	1.1±0.0 (0–2)*	1.7±0.7 (1–3)	2.2±0.2 (2–3)*	2.1±0.5 (2–3)*	0*

The expression and distribution of PCNA were graded as five degrees (scores 1–5, mild, moderate, marked, massive and extreme massive, a higher number indicated greater severity). Values are presented as the mean ± S.D.

The mixture consisted of equal amounts of β-carotene, lycopene, lutein and α-tocopherol.

"0" indicates no carcinoma production in the group.

* $P<.05$ comparing to control group (DMBA+BQE).

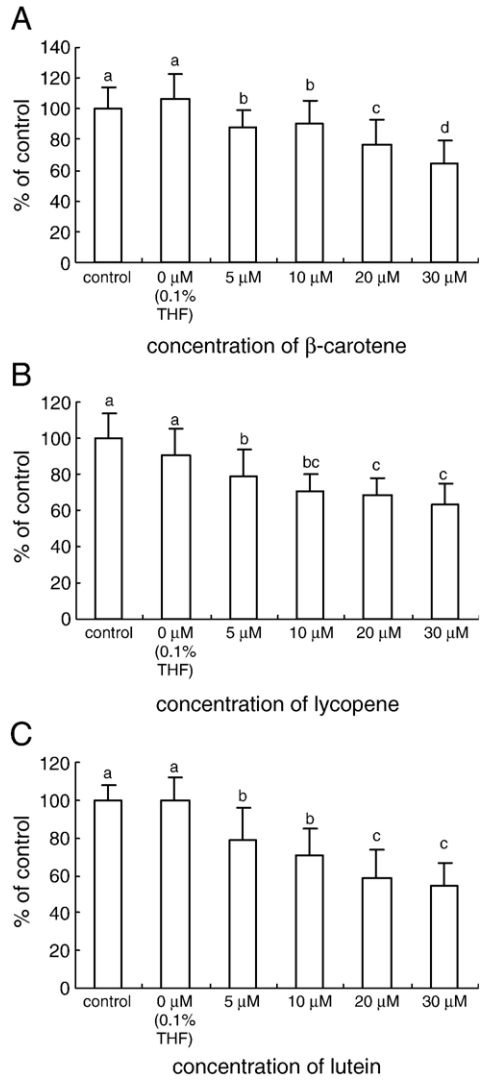

Fig. 3. Effect of carotenoids [β-carotene (A), lycopene (B) and lutein (C)] on KB cell proliferation after 24 h of incubation. Cells were incubated in medium (control), in the presence of the carotenoid solvent THF at a final concentration of 0.1% (0 μM), or in the presence of 5, 10, 20 and 30 μM of carotenoids. Results are presented as the mean±S.D. ($n=6$). Values were evaluated by one-way ANOVA and Fisher's Exact Test. Data with different superscripts significantly differ between groups ($P<.05$).

−20°C. Cells were washed twice with PBS then incubated for 30 min in 1 ml of PBS and 3 μl ribonuclease A (10 mg/ml) at room temperature. One milliliter of propidium iodide (40 μg/ml) was added, and the suspension was incubated in the dark at room temperature for 30 min and was then filtered through a 60-μm mesh filter. The percentages of cells in different phases of the cell cycle were determined by flow cytometry with a ModFit (Verity Software House) computer program.

2.3. Statistical analysis

Values are presented as the mean±S.D. All data were analyzed using the SAS system (Cary, NC, USA). Analysis of variance (ANOVA) with the least significant difference test was performed to analyze differences among different ethanol durations. The acceptable level of significance was established at $P<.05$, except where indicated otherwise.

3. Results

3.1. Effect of carotenoids on PCNA expression in hamster buccal pouch lesions

The results indicated that most of the normal tissues showed low expression of PCNA. PCNA expression merely appeared in the basal layer of the epithelium. In the basal layers, the expression of PCNA was significant, especially in peripheral cells of the basal layer. PCNA expression was obvious in dysplastic and hyperplastic lesions, carcinomas and papillomas in the control group. Cells along the inner border of the tumor and anterior tissue of invasive carcinomas also showed significant positive expression of PCNA. Precancerous hyperplastic and dysplastic lesions and keratocytes showed strong PCNA expression, especially papillomas and squamous cell carcinoma.

Fig. 1A–D shows morphologic changes in lesions treated with the mixed carotenoids. In the control, cells appeared disoriented during proliferation, and chromosomal aberrations, such as micronuclei and sister chromatid exchanges, were evident. Under a low microscopic field, lesions appeared dysplastic (Fig. 1A). Not surprisingly, lesions exhibiting PCNA expression showed inconsistencies (Fig. 2A–D). In hyperplastic lesions, PCNA expressions by the lutein, lycopene, mixture and vitamin E groups were less severe compared with that of the control group. In papillomas, PCNA expressions by the β-carotene, mixed and vitamin E groups were less severe compared with that of the control group. In dysplastic lesions, PCNA expressions by the carotenoid and vitamin E groups were less severe compared with that of the control group, but in carcinomas, only the PCNA expression of the β-carotene group was less severe than that of the control group. There was no carcinoma production in the lycopene and mixed groups thus no PCNA expression in those groups. For most of the lesions, vitamin E and β-carotene showed inhibitory effects on PCNA expression in buccal mucosal lesions. PCNA expression with carotenoid treatment is summarized in Table 1.

3.2. Carotenoids on PCNA and Cyclin D_1 Expression in KB cells

3.2.1. MTT assay

The inhibitory effects of carotenoids (β-carotene, lycopene and lutein) on the growth of KB human oral tumor cells were detected within 24 h (Fig. 3). The carotenoid solvent (THF) did not affect KB cell growth relative to growth in the control media. The cell-growth inhibition induced by carotenoids occurred in a dose-dependent manner. Surprisingly, cell-growth suppression induced by lutein (30 μM) was significantly more potent than that

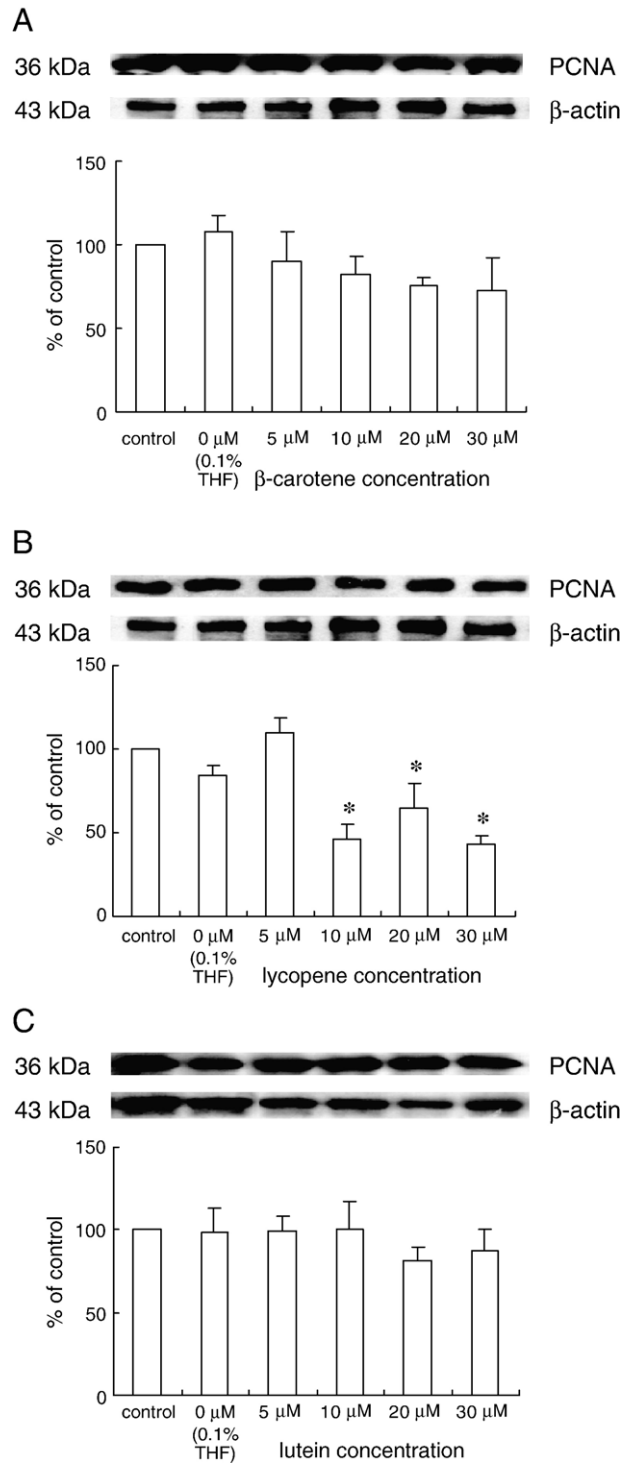

Fig. 4. Effects of carotenoids [β-carotene (A), lycopene (B) and lutein (C)] on PCNA (36 kDa) protein expression of KB cells after 24 h of incubation. β-Actin (43 kDa) served as the internal control. Results are presented as the mean±S.E.M. ($n=3$). Values were evaluated by Student's t test. Data with an asterisk significantly differ from the 0 μM group ($P<.05$).

by β-carotene and lycopene. β-Carotene and lycopene presented similar concentration-dependent inhibitory effects on cell proliferation. The lowest dose (5 μM) of β-carotene and lycopene caused 12%±7% and 21%±9% inhibition, respectively, while the highest dose (30 μM) resulted in a much higher and significant inhibition (35%±8% and 37%±10%) after 24 h. A lutein concentration of 5 μM inhibited growth by 21%±9% after 24 h of incubation, whereas the highest concentration (30 μM) led to 45%±8% inhibition after this time.

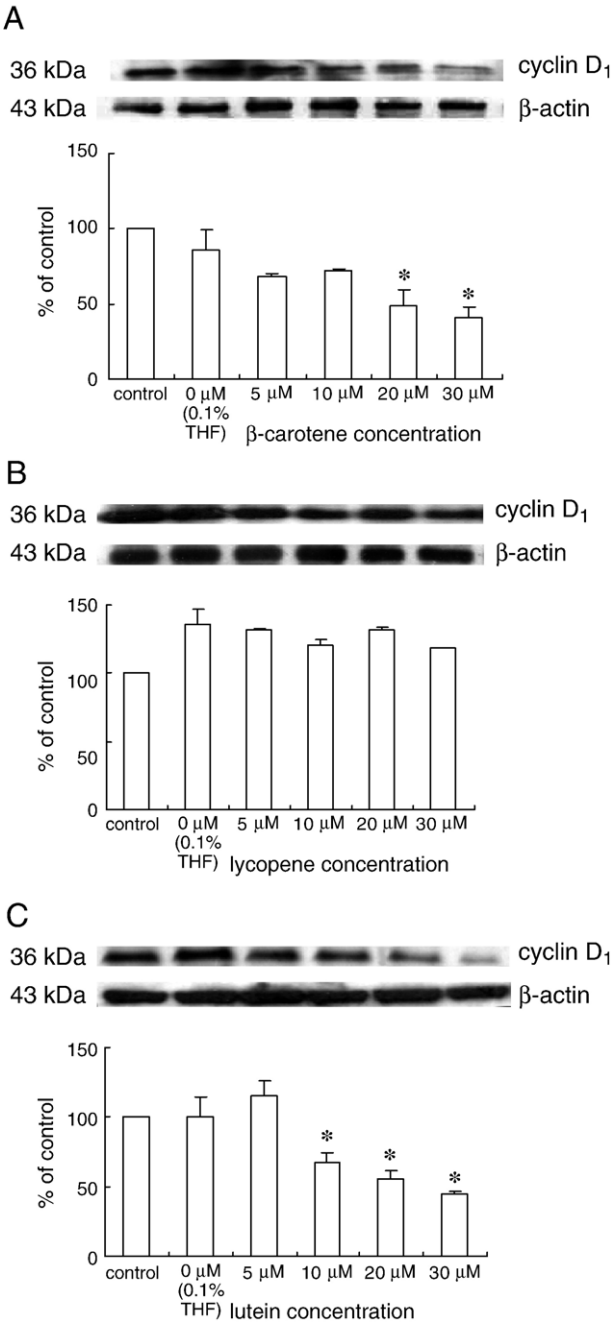

Fig. 5. Effects of carotenoids [β-carotene (A), lycopene (B) and lutein (C)] on cyclin D_1 (36 kDa) expression of KB cell after 24 h of incubation. β-Actin (43 kDa) served as the internal control. Data with an asterisk significantly differ from the 0 μM group ($P<.05$).

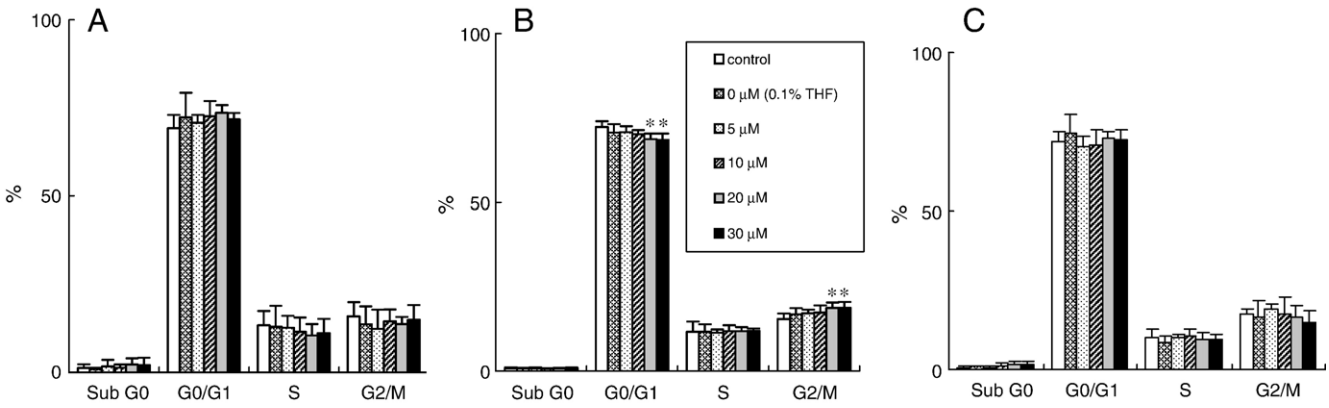

Fig. 6. Effect of carotenoids [β-carotene (A), lycopene (B) and lutein (C)] on cell cycle progression of KB cells after 24 h of incubation. Data with an asterisk significantly differ from the 0 μM group ($P<.05$).

3.2.2. Carotenoids on PCNA and cyclin D_1 expressions

The inhibitory effect of carotenoids on KB cells was additionally confirmed using the immunohistochemical marker PCNA. PCNA/cyclin regulates the initiation of cell proliferation by mediating DNA polymerase and is elevated in the G_1 and S phases of the cell cycle. Under culture conditions, lycopene remarkably suppressed the PCNA expression by KB cells at higher doses (10–30μM) with statistical significance. However, β-carotene and lutein presented lower inhibitory effects on PCNA expression beyond 20 μM. THF did not affect PCNA expression. Determination of PCNA expression in control and treated cells demonstrated that lycopene did affect the proliferation in KB cells in a dose-dependent manner (Fig. 4). Surprisingly, β-carotene and lutein suppressed cyclin D_1 expression in dose-dependent manners but not the lycopene group (Fig. 5). THF did not affect PCNA expression. Although THF affected cyclin D_1 expression in the lycopene group, the higher concentrations showed no inhibitory effectiveness.

3.2.3. Carotenoids on cell cycle progression

Cell cycle progression of KB cells under various carotenoid treatments is shown in Fig. 6. There were no significant differences in any concentration for treatment with β-carotene and lutein; however, lycopene (20 and 30μM) presented significant alterations in the G_0/G_1 and G_2/M phases.

4. Discussion

In this study, PCNA expression obviously showed dramatic alterations in each cancer stage. Due to the cancer development process, morphologic changes in lesions are progressive; nevertheless, PCNA expression presented more activity in later stages such as the transformation and translocation stages. The expression of PCNA was lower in the vitamin E and β-carotene groups than in the control, but PCNA was overexpressed in most of the developing cancerous lesions. The invasive and metastatic potential of carcinomas has been considered to be regulated by alterations in cell morphology and motility [17]. Compared to HE staining, there was no such difference between groups, in contrast to specific protein expression (such as PCNA and cytoskeletons), while the control showed much-higher protein expression, especially in the late stage of cancerous cell proliferation and displayed various extents of tissue disorientation.

Meanwhile, PCNA expression was found not only in papillomas, dysplastic lesions and squamous cell carcinoma but also in normal basal cells and peripheral tissues. Thus, from the viewpoint of cell differentiation, some cells of the suprabasal layer had proceeded and even bypassed the normal cell-cycle regulation. Cell proliferation by normal cells and squamous cell carcinoma showed little PCNA expression; the reason for this situation could have been that cancer cells released some transforming growth factors or epithelium growth factors, which stimulated irregular cell differentiation or activated some DNA excision-repair actions [5,6]. Betel quid extract (BQE) has been demonstrated to promote oral carcinogenesis and to show strong genotoxicity, and DNA breakage may be the major cause of oral carcinoma [18,19]. Thus, it is suggested that the expression of PCNA is highly correlated with the genotoxicity to DNA and hyperproliferation of buccal mucosal tissues [20]. PCNA was expressed to a greater extent in the control group than in the experimental groups in our study. These results imply two aspects of clinical significance: (1) some components of BQE contribute to PCNA's overexpression via genotoxicity, and (2) carotenoids attenuated the DNA-breaking process and PCNA expression and, thus, may provide a chemopreventive effect against oral carcinogenesis.

The results indicated that buccal and esophageal histological symptoms (data not shown) in the mixed group were less severe, compared with those of the control group. With DMBA induction, the BQE intervention indeed promoted the development of oral cancer. Carotenoids effectively inhibited the development of oral mucosal malignant tumors, especially in the lycopene and mixed group.

Compared with the esophageal and buccal mucosal pathology, there were fewer buccal mucosal tumors than esophageal mucosal tumors. However, the volume of tumors in the esophagus was smaller than that of buccal tumors. Furthermore, the buccal mucosal tumor burden was much greater than that in the esophagus. From the aspect of the preventive ability of carotenoids, the tumor number and burden in the esophagus and the buccal mucosa were the most severe in the control group. The application of carotenoids significantly inhibited the development of both esophageal and buccal tumors, especially in the mixed group. Kozuki et al. [21] reported the anti-invasive effects of carotenoids on rat ascites hepatoma AH109A cells. Cancer cells cultured with hypoxanthine and xanthine oxidase showed highly invasive activity. β-Carotene and astaxanthin, at up to 5 μM, suppressed the ROS-potentiated invasive capacity. The results suggested that the antioxidative property of carotenoids may be involved in this anti-invasive action.

The impact of carotenoids on cell proliferation has been discussed [22–24]. Cellular micronuclei have been suggested to be involved in DNA damage and are replicated as end products. β-Carotene (10 μM) has been reported to indeed decrease the prevalence of micronuclei in KB cells [22]. Livny et al. [23] indicated that a lower lycopene concentration (7 μM) possessed the capability of suppressing KB cell proliferation. Another study showed alternative anticancer actions. β-Carotene (1 μM) and its derivatives (9-cis β-carotene isomers, 10 μM) had suppressive effects on proliferation and gene expression in murine 10T1/2 cells and human HaCaT keratinocytes [24]. The 9-cis isomer was less active than all-trans β-carotene in reducing proliferation and in up-regulating expression of connexin 43 in 10T1/2 cells and in suppressing the expression of keratin K1. Surprisingly, the 9-cis isomer of retinoic acid was approximately 10-fold more active in suppressing neoplastic transformation and inducing connexin 43 expression in both cell types than all-trans β-carotene. In our study, carotenoids caused a significant inhibitory effect on cell proliferation in a dose-dependent manner. β-Carotene showed suppressive action on the S phase and stayed on the G_1 phase of cell proliferation in fibroblasts/caused fibroblasts to remain in the G_1 phase of cell proliferation [25]. Nahum et al. [26] also reported that lycopene suppressed cyclin D_1 expression, cyclin-dependent kinase 4 activity and retinoblastoma protein phosphorylation of breast (MCF-7) and endometrial cancer cells.

It is noteworthy that lycopene showed greater chemopreventive effectiveness than other carotenoids in this study, especially toward PCNA expression. Cell–cell interactions via gap junctions have been considered to be a key factor in tissue homeostasis, and their alteration is associated with the neoplastic phenotype. Lycopene up-regulates both the transcription and expression of connexin 43 [27]. The report implied that the pattern of cellular uptake and incorporation into cancer KB-1 cells differed between the various carotenoids. β-Carotene was rapidly incorporated into KB-1 cells, whereas lycopene uptake into the cells took place after longer incubation periods and only at the highest concentrations. Regarding the change in PCNA expression correlated with the phase of the cell cycle that the agents acted upon, in our study, although lycopene suppressed KB cell proliferation at the G_0/G_1 phase with a significant decrease in PCNA expression, β-carotene and lutein possessed less inhibitory effects and even elevated cell proliferation at the G_2/M phase. These results indicate that different carotenoids present various suppressive abilities against PCNA and cyclin D_1 expression in cell proliferation. Furthermore, with the exception of its cellular regulatory property, lycopene also demonstrated strong free radical-quenching ability. In some in vitro studies, lycopene possessed the most powerful quenching capacity for singlet oxygen. Lycopene also showed an ability to deactivate some free radicals such as H_2O_2, NO_2, RS and RSO_2 [28]. Such potential anticancer mechanisms reveal that the molecular regulatory aspect of carotenoids may play a major role in these chemopreventive actions.

In conclusion, carotenoids suppressed the carcinogenesis of induced hamster oral cancer and a cancer cell line by acting as a suppressor inhibiting the expression of PCNA and cyclin D_1. The results suggest that diverse anticancer characteristics of carotenoids may be involved in the cell cycle progression.

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