Nutrition and Cancer

Lycopene Inhibits Proliferation and Enhances Gap-Junction Communication of KB-1 Human Oral Tumor Cells

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ABSTRACT Cell-cell interaction via gap junctions is considered to be a key factor in tissue homeostasis, and its alteration is associated with the neoplastic phenotype. Experimental and epidemiologic data suggest that carotenoids, particularly lycopene and β-carotene, can reduce the risk of certain cancers. The aim of this study was to assess whether lycopene and β-carotene interfere at some stage with the carcinogenic processes in human cancer cells derived from the oral cavity. KB-1 cells, originating from a human oral cavity tumor, were incubated with different concentrations of lycopene or β-carotene delivered via the cell culture media from stock solutions in tetrahydrofuran. Lycopene strongly and dose dependently inhibited proliferation of KB-1 human oral tumor cells. β-Carotene was a far less effective growth inhibitor. Lycopene (3 and 7 μmol/L) significantly upregulated both the transcription ($P < 0.005$) and the expression ($P < 0.05$) of connexin 43, a key protein in the formation of gap-junctional communication. β-Carotene (3 μmol/L) tended to upregulate connexin 43 expression ($P = 0.07$) and significantly affected transcription of connexin 43 at 7 μmol/L ($P < 0.05$). Gap-junctional communication measured by scrape-loading dye transfer and electron microscopy showed that lycopene enhanced gap-junctional communication between the cancer cells, whereas β-carotene was less effective in this regard. The pattern of cellular uptake and incorporation into cancer KB-1 cells differed significantly between the carotenoids. β-Carotene was avidly and rapidly incorporated into KB-1 cells, whereas lycopene uptake into the cells took place after longer incubation periods and only at the highest concentrations. The results of the present study further support the hypothesis that carotenoids in general, and lycopene in particular, may be effective anticarcinogenic agents in oral carcinogenesis. J. Nutr. 132: 3754–3759, 2002.

KEY WORDS: • *lycopene* • *oral cancer cells* • *gap-junctional communication* • *connexin 43*

Oral cavity cancer is the sixth most frequent cancer in the world. Some of the highest rates are in developing countries where up to 25% of all malignancies are found in the oral cavity (1). Although this neoplasia is treatable with surgery or radiotherapy in its early stages, most patients are diagnosed only at advanced stages of the disease. At these late stages, therapy outcomes have not dramatically improved in recent years. Reduced incidence of this disease may be attainable through preventive measures (2,3). Preventive strategies are designed to suppress, reverse or prevent the formation of premalignant lesions and their subsequent development through the multistep process of initiation, promotion and progression into squamous cell carcinoma (4,5).

Epidemiologic studies suggest that a diet rich in lycopene is related to decreased risk of certain diseases, particularly cancers of the digestive tract, prostate and pancreas, as well as cardiovascular disease (6). Most of the reports concerning the anticarcinogenic activity of carotenoids emphasize their ability to be converted into vitamin A, which has been associated with differentiation and cancer regression (7,8). All-*trans* retinoic acid has been shown to be effective as a therapeutic agent that induces differentiation of promyelocytic leukemia cells (9), and 13-*cis* retinoic acid has shown great promise in inhibiting second primaries in head and neck cancer (10). However, the use of retinoic acid is limited in chemoprevention because of its high toxicity. Carotenoids may provide a nontoxic alternative treatment, but with far less potent activity. β-Carotene has shown activity against oral leukoplakia, a premalignant lesion in the oral cavity (11).

Experimental data have demonstrated lycopene's notable anticarcinogenic effect in vitro (8,12). The carotenoid lycopene differs from β-carotene mainly in the latter's ability to be converted into vitamin A. The reported biological activities of lycopene include potent antioxidant activity involving singlet-oxygen quenching, peroxyl-radical scavenging and in addition, induction of cell-cell communication and growth control (13,14).

The present study was conducted to investigate whether the inhibitory effect exerted by lycopene and β-carotene on

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oral cancer cell proliferation is associated with upregulation of connexin 43 mRNA and protein expression, concomitant with enhanced gap-junctional communication.

MATERIALS AND METHODS

Materials. Synthetic β-carotene was generously provided by Hoffman-La Roche (Basel, Switzerland). Natural lycopene extracted from tomatoes was prepared by Makhteshim (Beer Sheva, Israel). Tetrahydrofuran (THF)[2] was purchased from Sigma Chemical (St. Louis, MO). Rabbit polyclonal anti-connexin 43 was purchased from Zymed Laboratories (San Francisco, CA).

Carotenoid solutions. Carotenoids were dissolved in THF to a final concentration of 2 mmol/L and stored at −70°C. Immediately before the experiment, aliquots from these stock solutions were added to the cell culture medium under a N_2 environment, and stirred vigorously. The final concentration of the carotenoids in the medium was measured by spectrophotometry after extraction in 2-propanol and *n*-hexane-dichloromethane (12). The exact carotenoid concentration, which varied within narrow limits in each medium preparation, was recorded. The final THF concentration in each experiment was 0.5%, a concentration that does not affect cell growth compared with control media. All procedures, including incubation with the carotenoids, were performed under dim light.

Cells. KB-1 human oral tumor cells (kindly supplied by Prof. Ruth Arnon, Weizmann Institute, Rehovot, Israel) were cultured on RPMI medium (Biological Industries, Bet Haemek, Israel) containing penicillin, streptomycin, fungyzone, 5% fetal calf serum (FCS) and 1% L-glutamine.

Assessment of cell growth. Cells were seeded onto six-well plates (5×10^5 cells/well) in a medium containing 5% FCS. After 1 d, the medium was replaced with medium containing the solubilized carotenoid or THF alone. At different times, cells were harvested by trypsinization and vital cells, excluding trypan blue, were counted in a Bright Line Neubauer counting chamber (Sigma-Aldrich Chemie, Steinheim, Germany).

Protein isolation. Cell cultures were grown and treated in six-well dishes for different periods of time. The cells were scraped and lysed in 250 μL of lysis buffer (20 mmol/L Tris, pH 7.8, 1% Nonidet P-40, 100 mmol/L NaCl, 50 mmol/L NaF, 10% glycerol, 1 mmol/L sodium orthovanadate). Expression of solubilized connexin 43 was assessed in the supernatant (15). Protein concentration was determined by the Lowry method (16) using bovine serum albumin as the standard protein.

Western blot determination of connexin 43. Protein (30 μg) from the lysates' supernatants was electrophoresed on a 12% SDS-polyacrylamide gel. Proteins were electroblotted onto a nylon-transfer membrane (Amersham, Buckinghamshire, UK) and subsequently incubated with a polyclonal antibody against the C-terminal region of connexin 43 (rabbit anti-connexin 43, Zymed Laboratories). The bound antibody was visualized using a chemiluminescent detection system (17).

Immunocytochemistry. Cells were grown and treated with the different carotenoid solutions for 3 d on chamber slides. Fixation and abrogation of endogenous peroxidase activity were performed in a solution of methanol/hydrogen peroxide/H_2O (95:3:2). The fixed cells were incubated with polyclonal connexin 43 antibody (rabbit anti-connexin 43). Detection was performed with a Histostain kit (Histostain-SP, Zymed Laboratories). Counterstaining was performed with hematoxylin (18).

Connexin 43 reverse transcription-polymerase chain reaction (RT-PCR). cDNA was synthesized from 1 μg of total RNA [extracted with Tri Reagent (MRC, Cincinnati, OH) according to manufacturer protocol] from control, β-carotene or lycopene-treated cells, using RT-PCR beads (Amersham, Piscataway, NJ) essentially according to manufacturer's instructions. RT-PCR was performed using the primers of the connexin 43 gene 5′-ACA TCA GGT CGA CTG TTT CCT-3′, bases 579–600; and 5′-ACG ACT GCT GGC TCT GCT T-3′, bases 1110–1091. The PCR products were analyzed on a 1% agarose gel in Tris-acetate/EDTA buffer, and molecular weight was confirmed with the 100-bp DNA Ladder (Promega, Madison, WI). Gels were stained with ethidium bromide and photographed (19,20).

Transmission electron microscopy (EM). Cells grown in flasks and incubated with different β-carotene and lycopene concentrations (3 d) were fixed in 1% glutaraldehyde in 0.1 mol/L PBS, pH 7.4, for 1 h at room temperature. Fixed cells were then removed with a rubber policeman, washed in PBS and postfixed in 1% OsO_4 for 1 h. Samples were dehydrated in graded ethanol solutions and embedded in Epon. Ultrathin sections were stained on grids with uranyl acetate and lead citrate and examined with a Jeol 100 CX or 1200 EX transmission electron microscope (Jeol, Tokyo, Japan) (21).

Scrape-loading and dye transfer. Cells were treated with β-carotene (7 μmol/L) lycopene (7 μmol/L) or solvent (THF) devoid of carotenoids for 3 d. At the end of the treatment procedure, the cells were rinsed with PBS before addition of the fluorescent dye mixture; 2 mL of 0.05% lucifer yellow (purchased from Molecular Probe, Eugene, OR) dissolved in PBS were added to the cells and scrape-loaded at room temperature using a rubber policeman or wooden probe. The dye solution was left on the cells for 2 min then discarded, and the plates were rinsed with PBS to remove detached cells and background fluorescence. Media (2 mL) were replaced and cells were examined under a Nikon (Tokyo, Japan) epifluorescence phase microscope illuminated with an Osram HBO 200 W lamp (22).

Incorporation of lycopene and β-carotene into KB-1 cells. KB-1 cells were incubated with the carotenoids for various periods. On d 1, cells were harvested every 2 h until 12 h of incubation, and then at 24, 36 and 72 h. Harvested cells were exposed to different concentrations of lycopene (3–7 μmol/L) or β-carotene (3–7 μmol/L). SDS (0.5 mL, 10 mmol/L) and ethanol were added to the harvested cell samples (0.5 mL), and the carotenoids were extracted with hexane and separated by HPLC. The intracellular concentrations of lycopene and β-carotene in the cancer cells after the different incubation periods were analyzed with a multiwavelength detector. The peak identity of each carotenoid was confirmed by comparison with chromatographic (retention time) and spectral characteristics of authentic standard carotenoids (23).

Statistical analysis. All values are expressed as mean ± SEM. Data were analyzed by one- or two-way ANOVA, and then differences among means were analyzed using Tukey-Kramer multiple comparison tests. Differences were considered significant at $P < 0.05$.

RESULTS

The inhibitory effects of lycopene and β-carotene on the growth of KB-1 human oral tumor cells were detected as early

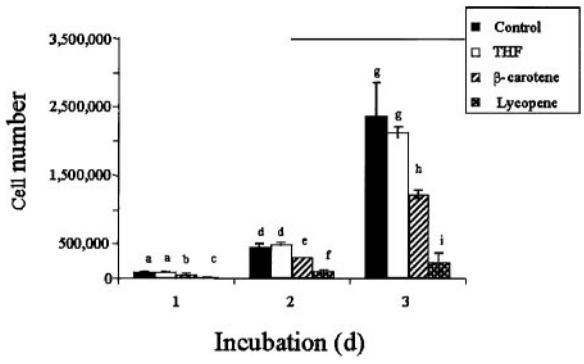

FIGURE 1 Effect of carotenoid treatments for various incubation periods on KB-1 cell proliferation. KB-1 cells were incubated in medium (control), in the presence of the carotenoid solvent tetrahydrofuran (THF) at a final concentration of 0.5%, or in the presence of 7 μmol/L lycopene or β-carotene, replaced daily. Results are means ± SEM, $n = 4$. Data were evaluated by ANOVA and the Tukey-Kramer multiple comparison tests. Means at a time without a common letter differ, $P < 0.003$.

[2] Abbreviations used: EM: electron microscopy, FCS: fetal calf serum; RT-PCR: reverse transcription-polymerase chain reaction; THF, tetrahydrofuran.

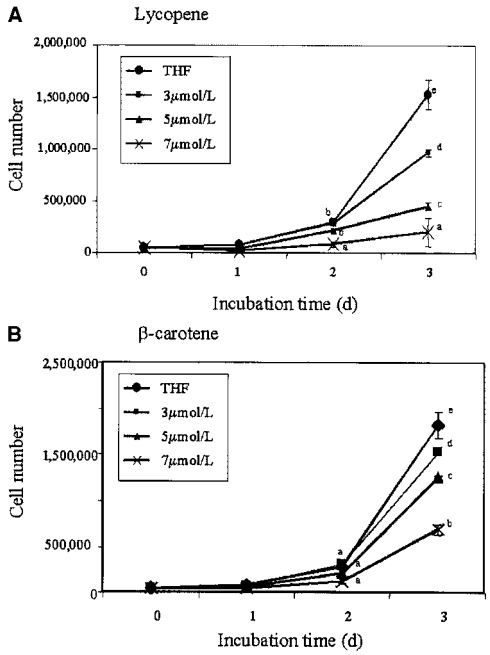

FIGURE 2 Effect of treatments with various carotenoid concentrations on KB-1 cell proliferation. KB-1 cells were incubated in medium (control), in the presence of tetrahydrofuran (THF; 0.5%), in 3, 5 or 7 μmol/L lycopene (*A*) or β-carotene (*B*). Media and treatments were replaced daily. Results are means ± SEM, $n = 5$. Data were evaluated by ANOVA and the Tukey-Kramer multiple comparison test for each carotenoid. Means for each carotenoid without a common letter differ, $P < 0.01$.

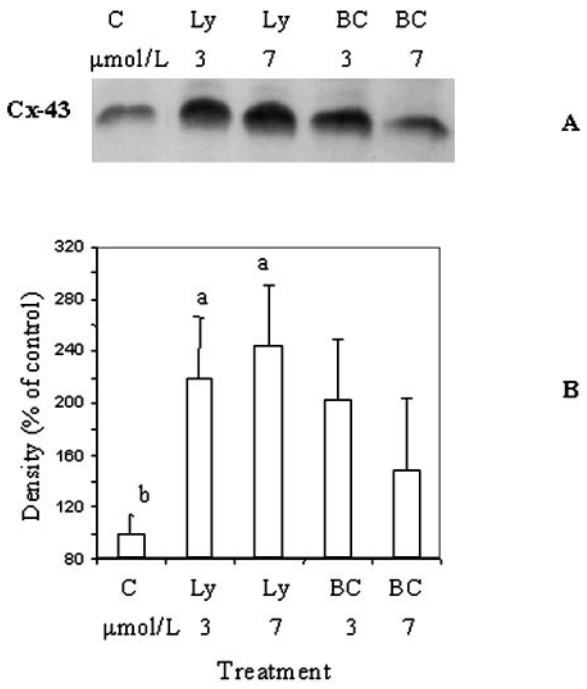

FIGURE 3 Effects of lycopene (Ly) and β-carotene (BC) on connexin-43 expression in KB-1 cells, as assessed by Western blot analyses. KB1 cells were treated with Ly, BC or medium (C) for 24 h. (*A*) Connexin-43 (Cx-43) expression in cell lysates of C; Ly: 3 and 7 μmol/L; BC: 3 and 7 μmol/L. (*B*) Relative density of the bands obtained after Western blot analysis was calculated with the program NIH image 1.51. Results are means ± SEM, $n = 4$. Data were evaluated by ANOVA and the Tukey-Kramer multiple comparison test. Means without a common letter differ, $P < 0.05$.

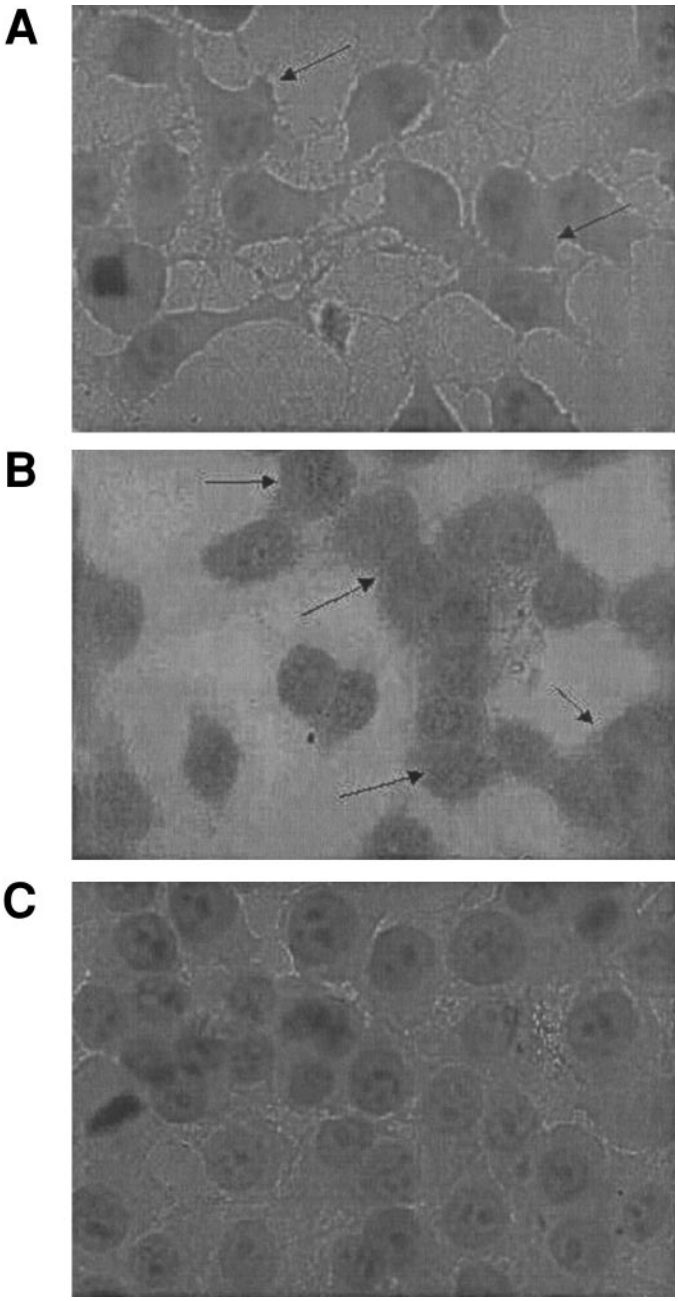

FIGURE 4 Effect of lycopene and β-carotene on connexin-43 expression in KB-1 cells, immunocytochemistry. KB-1 cells were incubated with medium containing 0.5% tetrahydrofuran (THF), 7 μmol/L lycopene or β-carotene for 24 h and the expression of connexin 43 in the cells was detected by immunocytochemistry. Original magnification, X100: (*A*) β-carotene: weak reaction, see arrow; (*B*) lycopene: positive reaction, see arrows; (*C*) negative control: no reaction.

as 24 h after incubation with the carotenoids and persisted throughout the 3-d experiment (**Fig. 1**). The treatments became much more effective after 3 d. However, at all time points, the cell-growth suppression induced by lycopene (7 μmol/L) was significantly more potent than that by β-carotene (7 μmol/L). The carotenoid solvent (THF) did not affect KB-1 cell growth relative to growth in the control media.

Lycopene inhibited KB-1 cell proliferation dose dependently at all times tested. A lycopene concentration of 3 μmol/L inhibited growth by 47 ± 5% after 3 d of incubation,

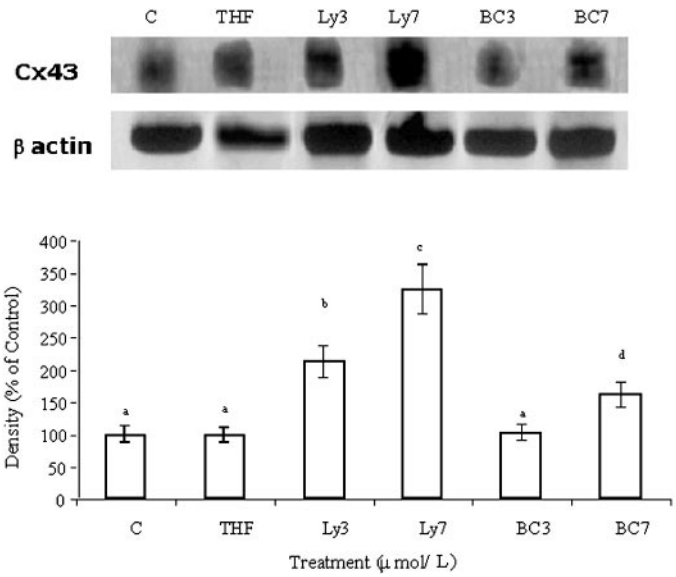

FIGURE 5 Effect of carotenoids on connexin 43 mRNA expression in KB-1 cells. (*A*) Reverse transcription-polymerase chain reaction (RT-PCR) assays were carried out to detect connexin 43 (Cx 43) transcript (531 bp) in KB-1 cells treated with different concentrations of the carotenoids. Lanes: C, control; Ly3, lycopene 3 μmol/L; Ly7, lycopene 7 μmol/L; BC3, β-carotene 3 μmol/L; BC7, β-carotene 7 μmol/L. Equal amounts of RNA were loaded (see β-actin expression; 602 bp). (*B*) Densitometry of mRNA transcripts relative to β-actin. Results are means ± SEM, $n = 5$. Data were evaluated by ANOVA and the Tukey-Kramer multiple comparison test. Means without a common letter differ, $P < 0.05$.

whereas the highest concentration (7 μmol/L) led to 89 ± 9% inhibition after this time (**Fig. 2***A*). β-Carotene had a similar concentration-dependent inhibitory effect on cell proliferation (Fig. 2*B*). However, although the highest concentration used (7 μmol/L) caused 62 ± 8% inhibition, the lowest concentration (3 μmol/L) resulted in a much smaller, although still significant inhibition (15 ± 5%) after 3 d.

Connexin 43 expression was measured in control KB-1 cells or cells treated with different carotenoid concentrations for 3 d. **Figure 3***A* shows a representative gel; the densitometric quantitation of the protein relative to the control is depicted in Figure 3*B*. Lycopene significantly ($P < 0.05$) upregulated connexin 43 expression. β-Carotene tended to upregulate expression ($P = 0.07$) at 3 μmol/L but not at 7 μmol/L ($P = 0.42$) . Control and THF-treated cells did not differ; thus, the THF results are not shown. Expression of connexin 43 was also assessed by immunocytochemistry and the results were consistent with the Western blot results. Lycopene induced notable connexin 43 expression, localized to the cell membranes (**Fig. 4***A*), whereas cells treated with β-carotene did not show this pattern (Fig. 4*B*).

RT-PCR allowed us to determine whether the carotenoids affect connexin 43 mRNA levels by regulating transcription or transcript stability. A representative experiment is shown in **Figure 5***A*. Densitometric analysis of this gel and of four others is presented in Figure 5*B*. Lycopene treatment (3 and 7 μmol/L) significantly upregulated the expression of connexin 43 mRNA compared with the control untreated cells (212 ± 25 and 325 ± 39% of the control, respectively, $P < 0.005$). The effect of β-carotene (7 μmol/L) was smaller but significant (162 ± 19% of the control, $P < 0.05$). At both concentrations, the effects of lycopene were greater than those of β-carotene ($P < 0.05$).

EM of thin sections from KB-1 oral cancer cells grown in RPMI medium (control) showed highly undifferentiated cells lacking contact points and with no apparent intercellular junctions (**Fig. 6***A*). After the addition of carotenoids, morphological changes were evident, i.e., EM of thin sections from KB-1 oral cancer cells treated with β-carotene for 3 d revealed the presence of intermediate junctions and desmosomes (Fig. 6*B*) and the cultures contained cells that contacted one another. Interestingly, treatment with lycopene for the same period (3 d) resulted in cell cultures with tight contacts between the cells (desmosomes and intermediate junctions) and several intercellular junction-type structures resembling gap junctions (Fig. 6*C*).

To test whether carotenoids upregulate gap-junction intercellular communication, we probed the cells by the scrape-loading technique with lucifer yellow after treatment of KB-1 cells with the different carotenoid solutions. The greatest effect was seen in the cells treated with the highest concentration of lycopene (7 μmol/L) (**Fig. 7***F*). A weaker effect was seen in cells treated with 3 μmol/L lycopene (Fig. 7*E*). No effect was observed following treatment with 3 μmol/L β-carotene (Fig. 7*C*) and a very weak effect was detected with 7 μmol/L β-carotene (Fig. 7*D*); the untreated cells (control) did not show any positive reaction (Fig. 7*A*, *B*).

The rate of lycopene and β-carotene incorporation into KB-1 cells as a function of incubation time and carotenoid concentration in the media was determined. Intracellular lycopene concentration reached detectable levels only when

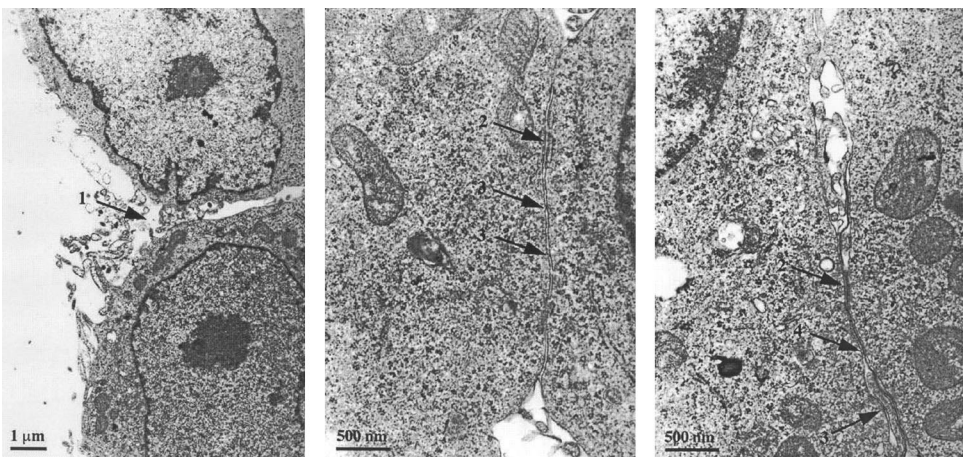

FIGURE 6 Electron microscopic photomicrographs of control-, lycopene- and β-carotene–treated KB-1 cells. (*A*) Micrograph of control KB-1 cells. Note the distance between the cells (arrow 1). (B) Micrograph of KB-1 cells treated with β-carotene (7 μmol/L) for 3 d. Note the desmosomes (arrow 2) and intermediate junctions (arrow 3) where the cells are in contact. (*C*) Micrograph of KB-1 cells treated with lycopene (7 μmol/L) for 3 d. Arrow 4 indicates gap junctions between cells contacting each other in addition to desmosomes (arrow 2) and an intermediate junction (arrow 3).

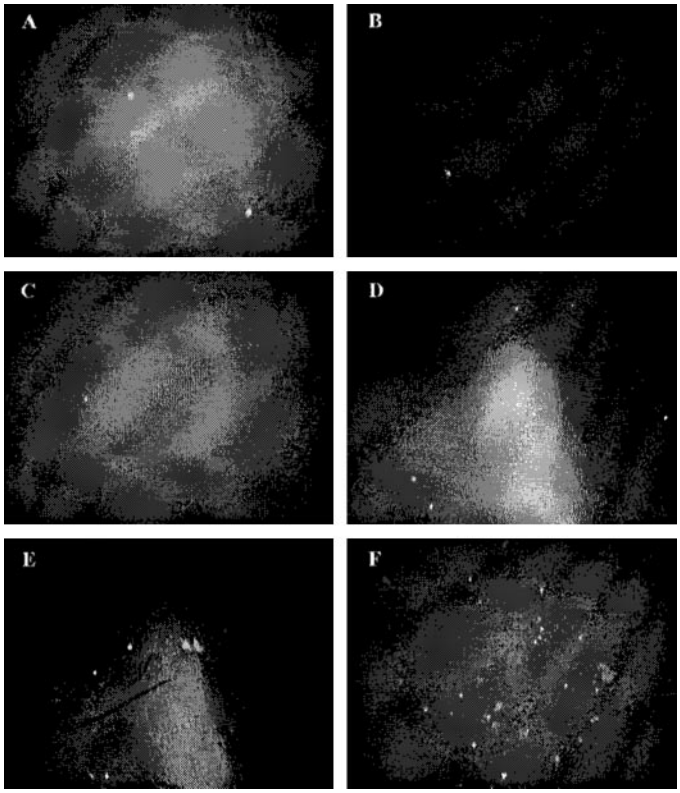

FIGURE 7 Scrape-loading of lucifer yellow in control-, lycopene- and β-carotene–treated KB-1 cells. (*A*) Negative reaction in the untreated cells (control). (*B*) Cells treated with tetrahydrofuran (THF; carotenoid vehicle): no reaction. (*C*) Cells treated with 3 μmol/L β-carotene: no reaction. (*D*) Cells treated with 7 μmol/L β-carotene: a few cells reacted. (*E*) Cells treated with 3 μmol/L lycopene: a few cells reacted. (*F*) Cells treated with 7 μmol/L lycopene: cells reacted strongly. The results are representative of three similar experiments.

lycopene concentrations in the incubation medium were 7 μmol/L for 48 h (**Table 1**). At 72 h, the intracellular lycopene concentration increased. At the lowest concentration of β-carotene tested (3 μmol/L) and as early as 24 h after treatment, intracellular β-carotene was detected (Table 1), an effect that was drastically different from any effect of dose or time for lycopene.

DISCUSSION

We examined the putative inhibitory effect of lycopene on the proliferation of KB-1 oral epidermoid cancer cells and compared this activity with that due to β-carotene. In these cancer cells, both β-carotene and lycopene inhibited cell proliferation, but lycopene was much more potent (Fig. 1). The mode of action by which both carotenoids affect cell proliferation is unclear. Bertram et al. (24) demonstrated a significant correlation between the ability of diverse carotenoids to inhibit chemically induced neoplastic transformation (25) and their ability to stimulate junctional communication in 10T1/2 cells via increased levels of connexin 43 (26–28). It has previously been shown that increased levels of connexin 43 are associated with increased gap-junctional communication (29). Moreover, Zhang et al. (30) recently demonstrated that overexpression of connexin 43 is associated with suppressed proliferation of human osteosarcoma U2OS cells through inhibition of cell-cycle transition from the G_1 to the S phase.

In the present study, the expression of connexin 43 in KB-1 cells was upregulated mainly after treatment with lycopene, and to a much lesser extent, with β-carotene. These findings were confirmed by Western immunoblotting (Fig. 3) and immunocytochemistry (Fig. 4). EM micrographs of KB-1 cells treated with lycopene (Fig. 6C) showed many connections between neighboring cells and many points of close communication reminiscent of desmosomes, intermediate junctions and gap-junction communications. In contrast, in micrographs from cells treated with β-carotene (Fig. 6*B*), desmosomes and intermediate junctions were evident but no clear gap-junction-type structures were detected. EM micrographs of KB-1 cells incubated in medium without carotenoids (control) showed no clear communication, no signs of desmosomes, no

TABLE 1

Effect of various carotenoid concentrations and incubation times on lycopene and β-carotene uptake into KB-1 oral cancer cells[1]

Carotenoid	Lycopene (μmol/L)			β-carotene (μmol/L)		
	3	5	7	3	5	7
Incubation time (h)	Intracellular lycopene			Intracellular β-carotene		
				ng/mg protein		
2	UD	UD	UD	UD	UD	2.6 ± 0.1[c]
4	UD	UD	UD	UD	UD	7.2 ± 0.3[d]
6	UD	UD	UD	UD	UD	11.8 ± 1.1[e]
8	UD	UD	UD	UD	UD	16.4 ± 1.7[f]
10	UD	UD	UD	UD	UD	18.0 ± 1.8[g]
12	UD	UD	UD	UD	UD	18.9 ± 1.9[g]
24	UD	UD	UD	224 ± 17[h]	431.4 ± 41[i]	445 ± 51[i]
48	UD	UD	67 ± 7[a]	339 ± 31[j]	794.9 ± 82[k]	863 ± 91[l]
72	UD	UD	91 ± 10[b]	717 ± 81[m]	1226 ± 110[n]	1419 ± 125[o]

[1] The values are means ± SEM, $n = 5$. Each carotenoid was evaluated by two-way ANOVA (concentration × time) with Tukey-Kramer's post-hoc multiple comparison test. Means for a carotenoid without a common letter differ, $P < 0.05$. Data were not transformed before statistical analyses because the unequal variances did not affect the results.

[2] UD, undetectable.

intermediate junctions and no gap junctions between the cells (Fig. 6A). In KB-1 cells, lycopene was demonstrated to stimulate cell-to-cell communication, as a function of the concentration. β-Carotene was far less effective (Fig. 7A–7F). Similar data have been reported previously (31); however, the effect was detectable at lower lycopene concentrations (0.1 μmol/L) than the concentrations used in the present study (3, 5 and 7 μmol/L), which are close to the physiologic levels detectable in basal human serum (~1 μmol/L) (32). In supplemented people, serum lycopene can be doubled (33).

We addressed the question of whether differences in proliferation inhibition and modulation of gap-junctional communication induced by both carotenoids are due to differences in the uptake of those carotenoids by the cells. We found that significant differences exist between lycopene and β-carotene cell uptake. Very low concentrations of intracellular lycopene were detectable only after 48 h of incubation with the highest extracellular carotenoid concentration. In contrast, β-carotene was detectable in large amounts within 24 h of incubation and continued to increase on subsequent days (Table 1).

Carotenoid activity that takes place mainly within the extracellular domain appears to be more effective at inhibiting proliferation and regulation of cell-cell communication than that taking place mainly in the intracellular domain. The importance of these differences is being investigated in our laboratory. The present study adds to previous findings suggesting that lycopene is an efficient cancer preventive agent in oral carcinogenesis that deserves further investigation.

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