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β-Carotene and/or vitamin E as modulators of alkylating agents in SCC-25 human squamous carcinoma cells*

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Summary. Dietary levels of β-carotene and vitamin E have been associated with cancer prevention and to a lesser extent, with therapeutic enhancement of cancer treatment. We report on the cytotoxicity of β-carotene, vitamin E, and the combination of β-carotene and vitamin E in human SCC-25 squamous carcinoma cells under various environmental conditions found in solid tumor masses. β-Carotene was selectively cytotoxic toward normally oxygenated cells and was generally more cytotoxic at normal pH than at acidic pH (6.45). Vitamin E was selectively cytotoxic toward normally oxygenated cells following 6 h exposure at normal pH and was generally equally cytotoxic toward normally oxygenated and hypoxic cells under the other conditions tested. β-Carotene was an effective modulator of cisplatin (CDDP) cytotoxicity toward SCC-25 cells, whereas vitamin E was not. Both β-carotene and vitamin E were effective modulators of melphalan cytotoxicity toward SCC-25 cells. Treatment of SCC-25 cells with β-carotene (70 μM, 2 h) resulted in a reduction in superoxide dismutase activity, in glutathione-S-transferase activity, and in nonprotein sulfhydryl levels in the cells. Exposure to vitamin E or to a combination of β-carotene and vitamin E increased the glutathione-S-transferase activity in SCC-25 cells by 40%–45% over the control value. Treatment with β-carotene, vitamin E, or canthaxanthin reduced the incorporation of [^{3}H]-thymidine into SCC-25 cells but not that into normal human karotinocytes. The most marked reduction in [^{3}H]-thymidine incorporation into SCC-25 cells occurred following treatment with the combination of β-carotene and melphalan. We hope to continue to explore the mechanisms of this effect and to study these combinations in vivo.

Introduction

An association between levels of the fat-soluble vitamins A (retinol) and E (α-tocopherol) and β-carotene, a carotenoid that can be converted to vitamin A, and the susceptibility or resistance of cells to carcinogenesis and malignant transformation has been recognized for some time [20, 25, 44–45]. Vitamin A is involved in the control of the differentiation and proliferation of epithelial cells [17], and it has been demonstrated that the progression of premalignant cells to cells exhibiting invasive, malignant characteristics can be slowed, delayed, arrested, or even reversed in experimental animals by the administration or retinoids or carotenoids [13, 45]. This effect has been seen in cells of epithelial and mesenchymal origin in which malignancy has been induced either chemically or virally or via their transformation using radiation or growth factors [13]. Epedemiology studies have found an inverse relationship between dietary vitamin A, carotenoids, and tocopherols and the incidence of various human cancers [19]. However, as the toxicity of the retinoids has reduced their usefulness in the clinical treatment of cancers, recent studies have focused on the biologic and biochemical actions of the less toxic carotenoids, especially β-carotene and tocopherols [7, 22].

Over the past several years, our work has focused on characterizing the prevention, inhibition, and regression of chemically induced experimental oral cancers of the hamster buccal pouch by β-carotene, vitamin E, and other carotenoids [28–35, 40–43, 47, 48]. In one study on chemical carcinogenesis, a decrease in the activity of the transmembrane enzyme α-glutamyl transpeptidase (GGT) was observed following treatment with β-carotene. This decreasein activity was assumed to indicate a reduction in the intratumoral levels of reduced glutathione (GSH) because GGT functions to provide cysteine and glycine residues for the synthesis of reduced GSH in the oral mucosal cells [48]. Recent studies on the SCC-25 human head-and-neck carcinoma cell line showed that exposure of the cells to 70 μM β-carotene led to decreased proliferation of these tumor cells and to the concomitant expression of a

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70-kDa protein that was presumed to be a stress protein [34].

The current study was designed to determine whether β-carotene and/or vitamin E could be significantly cytotoxic toward SCC-25 human squamous carcinoma cells under various conditions that model the physiologic environments in solid tumors and whether these substances could modulate the cytotoxic actions of antitumor alkylating agents.

Materials and methods

Drugs. β-Carotene, d,l-α-tocopherol succinate (vitamin E), canthaxanthin, and melphalan (L-PAM) were purchased from Sigma Chemical Company (St. Louis, Mo.). *cis*-Diamminedichloroplatinum(II) (CDDP) was a gift from Dr. A. Crosswell, Bristol-Myers-Squibb, Inc. (Wallingford, Conn.). Melphalan was dissolved in HCl-acidified ethanol and diluted with phosphate-buffered saline (PBS) just prior to its use. The other drugs were prepared in PBS and stored at −20° C. Stock solutions of β-carotene, canthaxanthin, and vitamin E were checked by reverse-phase high-pressure liquid chromatography to determine the exact concentration of these agents [34].

Cell line. SCC-25 human squamous head-and-neck carcinoma cells grow as monolayers in Dulbecco-Vogt modified Eagle's minimum essential medium (DMEM) supplemented with antibiotics and 5% fetal bovine serum (FBS) [9]. This cell line exhibits a plating efficiency of 10%–30% and a doubling time of 48–50 h in vitro [23]. For experiments, SCC-25 cells were grown in plastic culture flasks and harvested when they had reached the exponential growth phase [12].

Human Epidermal keratinocytes. Epidermal keratinocytes (10^6 cells/60-mm tissue-culture plate) derived from foreskin were grown on a feeder layer of 3T3 fibroblasts (10^5 cells/plate) that had been pretreated with 60 G X-irradiation in medium that contained Ham F12 and DMEM (1 : 1, v/v) as well as 20% FBS and was supplemented with epidermal growth factor, cholera toxin, adenine, thyroxine, and hydrocortisone, according to the method of Rheinwald and Green [24]. In other experiments, keratinocytes derived from surgical mammoplasties (Clonectics; EpiPak, San Diego, Calif.) were grown in the absence of feeder layer in MCDB 153 medium supplemented with pituitary extract, epidermal growth factor, adenine, and hydrocortisone.

Production of Hypoxia. Flasks containing exponentially growing monolayers in complete medium were fitted with sterile rubber septa and exposed to a continuously flowing humidified atmosphere comprising 95% N_2 and 5% CO_2 for 4 h at 37° C as previously described [24]. Parallel flasks were maintained in an atmosphere consiting of 95% air and 5% CO_2. At the end of 4 h, the drug or vehicle was added to the flasks by injection through the rubber septum without disturbing the hypoxia, which was verified at various points during the experimental procedure using a platinum electrode (Diamond General Corp., Ann Arbor, Mich.). The pO_2 value was found to be 2.2 ± 1.1 mmHg across these measurements.

Alterations in pH. The pH of the medium was adjusted using a sodium bicarbonate ($NaHCO_3$)/5% CO_2 buffer system [11, 12]. For altered-pH experiments, the original bicarbonate-buffered medium (pH 7.4) was replaced by media that contained no $NaHCO_3$; the deletion of bicarbonate resulted in media displaying a pH of 6.45. Flasks were then purged for 4 h with a mixture of either 95% air and 5% CO_2 for normally oxygenated conditions or 95% N_2 and 5% CO_2 for hypoxic experiments as described above [11, 12]. Representative flasks were monitored throughout the experimental procedures, and the pH of the media did not vary by more than 0.05 pH units.

Drug-combination survival studies. SCC-25 cells in exponential growth were exposed to β-carotene (50 μM), vitamin E (50 μM), or β-carotene (50 μM) plus vitamin E (50 μM) for 6 h [4]. The solutions of β-carotene and vitamin E were prepared in 0.9% PBS containing 0.1% dimethylsulfoxide (DMSO). During the 4th h of exposure to the nutrient molecules, some experimental groups were also exposed to CDDP (5–100 μM) or L-PAM (5–100 μM).

Colony formation. Following treatment, cells were washed three times with PBS and suspended by treatment with 0.25% trypsin/0.1% ethylenediaminetetraacetic acid (EDTA), and known numbers of cells were then plated in duplicate at three dilutions for colony formation. After 2 weeks, the colonies were visualized by staining with crystal violet, and colonies of ≥ 50 cells were counted. The results were expressed as the surviving fraction of treated cells as compared with vehicle-treated control.

Nonprotein sulfhydryl determinations. SCC-25 cells in exponential growth (4×10^6) that either had been left untreated or had been treated with β-carotene (70 μM), d,l-α-tocopherol succinate (70 μM), canthaxanthin (70 μM), reduced glutathione (70 μM), or β-carotene (70 μM) plus d,l-α-tocopherol succinate (70 μM) for 2 h were lysed in 2 ml 5% perchloric acid. The protein was removed with 5 M potassium phosphate by centrifugation at 1,000 *g* for 3 min at 5° C. The supernatant was neutralized and then passed through a 0.2-μM filter prior to derivatization. Glutathione was assayed at four dilutions of the supernatants. The sample (0.1 ml) was added to 3 ml 0.1 M potassium phosphate buffer containing 5 mM EDTA (pH 8), and then 0.15 ml of an *ortho*-phthaldialdehyde (OPT; Aldrich Chemical Co., Milwaukee, Wis) solution (1 mg/ml OPT in methanol) was added [15]. The derivatization was allowed to continue for 15 min at room temperature in a dark environment. Fluorescence was measured using an excitation wavelength of 350 nm and an emission wavelength of 420 nm. The calibration curve was linear from 0.05 to 50 nmol glutathione/ml [6, 16]. The results represented the mean values for five determinations.

Glutathione-S-transferase activity measurements. SCC-25 cells in exponential growth that either had been left untreated or had been treated with β-carotene (70 μM), d,l-α-tocopherol succinate (70 μM), canthaxanthin (70 μM), reduced glutathione (70 μM), or β-carotene (70 μM) plus d,l-α-tocopherol succinate (70 μM) were harvested by trypsinization. The cells were washed three times with PBS and suspended in 50 mM sodium phosphate buffer (pH 6.5). The cell suspensions were kept at 4° C, disrupted by sonication (Sonifier 200; Bronson, Inc.), and centrifuged at 16,000 *g* for 30 min. Glutathione-S-transferase (GST) activity in the supernatant was measured according to the method of Habig et al. [10] using 1 mM 1-chloro-2,4-dinitrobenzene as the electrophilic substrate. GST activity was expressed as the nanomoles of GSH-1-chloro-2,4-dinitrobenzene conjugate formed per minute per milligram of protein. The results were obtained using cells that had been harvested on three separate occasions.

Superoxide dismutase measurements. SCC-25 cells in exponential growth that either had been left untreated or had been treated with β-carotene (70 μM), d,l-α-tocopherol succinate (70 μM), canthaxanthin (70 μM), reduced glutathione (70 μM), or β-carotene (70 μM) plus d,l-α-tocopherol succinate (70 μM) were lysed in a buffer containing 10 mM TRIS, 150 mM NaCl, 1% Triton X-100, 100 Kallikrein units aprotinin, 1 mM phenylmethylsulfonylfluoride, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate. Aliquots of the cell lysate were added to a reaction mixture containing 0.216 M phosphate buffer (monobasic) at pH 7.8, 10.7 mM EDTA, 1.1 mM ferricytochrome C, and 16.6 units xanthine oxidase/ml [2, 17]. Controls included only the reaction mixture, the reaction mixture plus superoxide dismutase (SOD, 5,000 units/ml), and, finally, the reaction mixture, cell lysate, and SOD (5,000 units/ml). Quantitation of the SOD level in the cell lysate was determined as:

$$\% \text{ Inhibition} = A - B \times 100,$$

where A = water A_{550}/min in cuvette A and B = SOD or experimental value A_{550}/min in cuvette B. Absorbance was read at least ten times at 1-min intervals. The amount of protein (in milligrams) present in a 100-μl aliquot of cell lysate was determined using a modified Lowry assay [8].

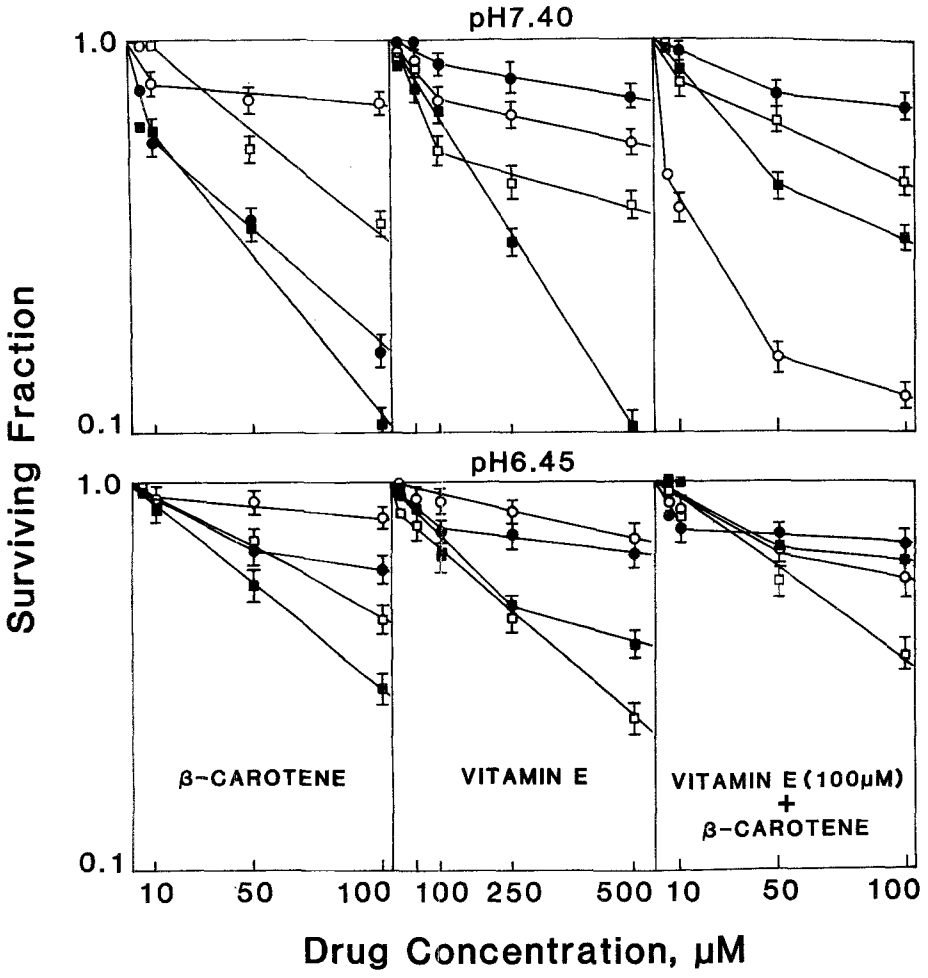

Fig. 1. Survival curve for exponentially growing, normally oxygenated (●, ■), and hypoxic (○, □) SCC-25 cells exposed to various concentrations of β-carotene, vitamin E, or vitamin E (100 μM)/β-carotene for 1 (●, ○) or 6 h (■, □) at pH 7.40 (*upper panels*) or pH 6.45 (*lower panels*). Points represent the mean values for 3 indpendent determinations; bars indicate the SEM

Results

In an effort to model the various environmental conditions that occur in solid tumors, we investigated the cytotoxicity of β-carotene and vitamin E under normally oxygenated and hypoxic conditions at normal and acidic pH and over short (1 h) and long (24 h) exposure periods. Following exposure for 1 h, β-carotene was more cytotoxic toward SCC-25 cells under normally oxygenated conditions than under hypoxic conditions at pH 7.4 (Fig. 1). Extension of the exposure period to 6 h produced little increase in the cytotoxicity of β-carotene toward normally oxygenated SCC-25 cells. At higher concentrations, we observed a larger increase in the cytotoxicity of β-carotene toward hypoxic cells as the exposure period increased to 6 h. The cytotoxicity of β-carotene toward normally oxygenated SCC-25 cells after 1 or 6 h exposure at pH 6.45 was lower than that observed following treatment at pH 7.4. There was no pH-dependent difference in the cytotoxicity of β-carotene toward hypoxic SCC-25 cells.

Vitamin E was much less cytotoxic than β-carotene toward SCC-25 cells (Fig. 1). When SCC-25 cells were exposed to 500 μM vitamin E for 1 h at normal pH (7.4), only 30% of the normally oxygenated cells and 45% of the hypoxic cells were killed. When the exposure period was extended to 6 h, about 90% of the normally oxygenated cells and ca. 63% of the hypoxic cells were killed. The cytotoxicity of vitamin E toward normally oxygenated SCC-25 cells after 6 h exposure at pH 6.45 was significantly lower than that observed following treatment for the same period at pH 7.4. No pH-dependent difference was found in the cytotoxicity of vitamin E toward hypoxic cells.

The combination of β-carotene and vitamin E was assessed with the concentration of vitamin E being held at 100 μM and that of β-carotene being varied (Fig. 1). At normal pH (pH 7.4) and following 1 h exposure, the combination was markedly cytotoxic toward hypoxic SCC-25 cells, even at the lower concentrations (5 and 10 μM) of β-carotene tested. In contrast, the cytotoxicity of the combination of β-carotene and vitamin E was comparable with that of vitamin E alone in normally oxygenated SCC-25 cells. Extension of the duration of exposure to the combination to 6 h produced much lower cytotoxicity toward normally oxygenated cells than that exhibited by β-carotene alone. The cytotoxicity of the combination toward hypoxic cells was about the same as that of β-carotene alone following 6 h exposure. Under acidic (pH 6.45) conditions, the combination was not very cytotoxic but was slightly more so toward hypoxic cells than toward normally oxygenated cells after both periods of exposure.

CDDP kills SCC-25 cells in a log-linear manner with increasing drug concentration (Fig. 2). Exposure of the

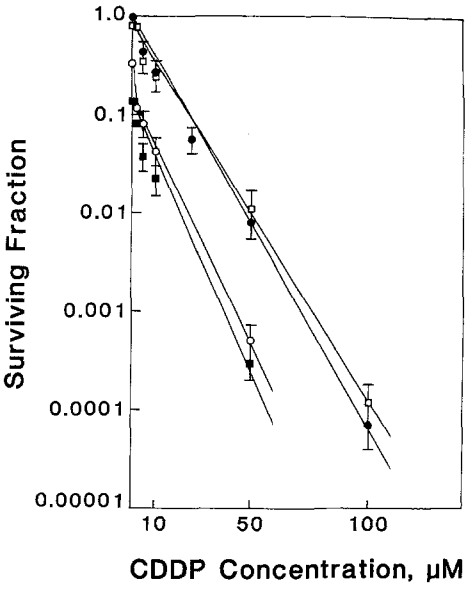

Fig. 2. Survival of SCC-25 cells exposed for 6 h to 50 μM β-carotene (○), 50 μM vitamin E (□), or 50 μM vitamin E plus 50 μM β-carotene (■) either alone or in combination with various concentrations of CDDP for 1 h during the 4th h of exposure to the nutrient molecule. ●, CDDP alone. Points represent the mean values for 3 independent experiments; bars indicate the SEM

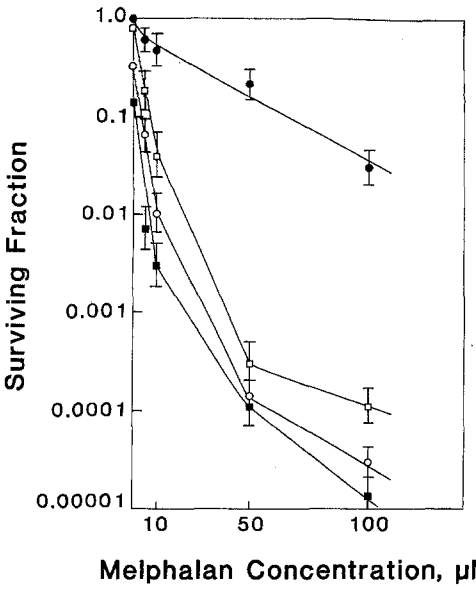

Fig. 3. Survival of SCC-25 cells exposed for 6 h to 50 μM β-carotene (○), 50 μM vitamin E (□) or 50 μM vitamin E plus 50 μM β-carotene (■) either alone or in combination with various concentrations of melphalan for 1 h during the 4th h of exposure to the nutrient. ●, melphalan alone. Points represent the mean values for 3 independent experiments; bars indicate the SEM

cells to β-carotene (50 μM) for 3 h prior to, during (1 h), and for 2 h after treatment with CDDP produced an increase of about 1–1.5 log in the cytotoxicity of CDDP toward SCC-25 cells. Vitamin E (50 μM) treatment on the same schedule did not alter the cytotoxicity of CDDP toward these cells. β-Carotene (50 μM) and vitamin E (50 μM) applied in combination for the same 6-h exposure period did not change the enhancement in CDDP cytotoxicity toward SCC-25 cells from that obtained using β-carotene alone.

Melphalan killed increasing numbers of SCC-25 cells with increasing concentration of the drug (Fig. 3). The addition of β-carotene (50 μM) for 3 hours prior to, during (1 h), and for 2 h after exposure to melphalan resulted in a marked enhancement in cytotoxicity. There was about a 3-log increase in cell killing at concentrations of 50 or 100 μM melphalan when β-carotene (50 μM) was added to treatment with the alkylating agent. On the same treatment regimen vitamin E (50 μM) produced about the same level of enhancement in the cytotoxicity of melphalan toward SCC-25 cells as did β-carotene. The combination of β-carotene (50 μM) and vitamin E (50 μM) produced the same enhancement in the cytotoxicity of melphalan at higher drug concentrations (50 and 100 μM melphalan) as did β-carotene (50 μM), or vitamin E (50 μM) alone. However, at the lowest melphalan concentration (5μM, the combination of β-carotene (50 μM) and vitamin E (50 μM) resulted in a 1-log increase in cell killing as compared with β-carotene and melphalan.

Exposure to 70 μM β-carotene for 2 h resulted in a 48% reduction in the SOD activity and a 43% reduction in the nonprotein sulfhydryl content in treated SCC-25 cells as compared with untreated control cultures (Table 1). Exposure to vitamin E under the same conditions resulted in a 15% increase in the SOD levels and a 26% increase in the nonprotein sulfhydryl content in the cells. The combination produced a 13% decrease in the SOD levels and a 30% decrease in the nonprotein sulfhydryl content in SCC-25 cells. CAN, a carotenoid that cannot be converted by cells to retinoid species, and GSH increased the nonprotein sulfhydryl content in SCC-25 cells by 63% and 25%, respectively, whereas CAN treatment produced a 17% decrease and GSH produced a 14.5% increase in the SOD activity in these cells. The same treatment with β-carotene slightly lowered the GST activity in SCC-25 cells. In contrast, exposure to vitamin E or to a combination of β-carotene and vitamin E increased the GST activity in SCC-25 cells by 40%–45% over the control value. Neither CAN nor GSH significantly altered the GST activity from that of controls.

[^{3}H]-Thymidine incorporation was found to be significantly reduced after 2 h treatment of exponentially growing SCC-25 cells with 70 μM β-carotene, vitamin E, or canthaxanthin, whereas similar treatment with GSH resulted in no change in [^{3}H]-thymidine incorporation from that observedin controls (Fig. 4). in contrast, following the same treatment with β-carotene, vitamin E, or canthaxanthin, [^{3}H]-thymidine incorporation into normal human epidermal keratinocytes (NHK), was increased. Exposure for 2 h to 50 μM melphalan alone or in the presence of β-carotene or vitamin E produced no decrease in [^{3}H]-thymidine incorporation into NHK cells but reduced that into SCC-25 cells.

Table 1. Non-protein sulfhydryl levels, superoxide dismutase, and glutathione-S-transferase activity in SCC-25 cells after various treatments

Treatment group	SOD[a] (units ml^{-1} mg^{-1}	NPS[b] (nmol/10^7 cells)	GST activity[c] (nmol min^{-1} mg^{-1})
Controls	17.9±2.5	4.38±0.96	386±14
β-Carotene	9.2±1.5	2.51±0.71	328±20
Vitamin E	20.6±5.5	5.54±0.92	547± 6
β-Carotene/ vitamin E	15.5±4.3	3.03±0.63	554±13
Canthaxanthin	14.7±8.4	7.15±1.13	394±7
Glutathione	20.5±4.5	5.49±1	420±6

[a] SOD, Superoxide dismutase; units present to catalyze the reduction of ferricytochrome C per ml lysate per mg protein

[b] NPS, Nonprotein sulfhydryl (glutathione), as measured by fluorescence emission at 420 nm of an OPT derivative

[c] GST, Glutathione-S-transferase; activity determined by absorption at 340 nm of the product formed by the reaction of GSH with 1-chloro-2,4-dinitrobenzene

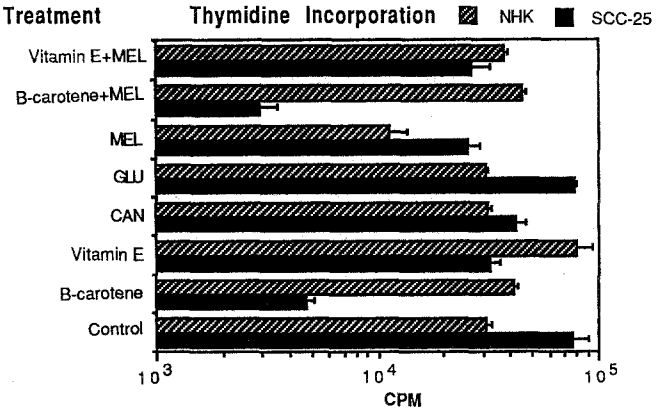

Fig. 4. Incorporation of [^{3}H]-thymidine over 24 h into SCC-25 tumor cells and normal human epidermal keratinocytes. (*NHK*) cells were exposed to β-carotene (70 μM, 2 h), vitamin E (70 μM, 2 h), canthaxanthin (70 μM, 2 h), reduced glutathione (70 μM, 2 h), melphalan (50 μM, 2 h), or β-carotene (70 μM) with vitamin E (70 μM) for 2 h and, after a washing, to melphalan (50 μM, 2 h). The data represent the mean counts per minute (*CPM*) derived from triplicate identical wells containing 1×10^5 cells/well; bars indicate the SEM

Discussion

Most research involving the role of fat-soluble vitamins, including retinoids, carotenoids, and d,l-α-tocopherol, in malignant disease has focused on their ability to prevent carcinogen-induced tumors [21, 32, 42, 45]. However, as early as in 1982, Seifter et al. [36] reported that feeding CBA/J mice bearing established Moloney sarcoma virus-induced tumors a β-carotene-enriched diet resulted in an increased rate of tumor regression. When CBA/J mice bearing established C3HBA tumors were treated with 30 G radiation plus diets that had been enriched in vitamin A or β-carotene, complete regression of all of the tumors was noted [37, 38]. A long-term follow-up of these animals indicated that maintenance on vitamin A- or β-carotene-enriched diets was beneficial. Quite similar results were obtained in the same tumor model system using the combination of cyclophosphamide and vitamin A or β-carotene [38, 39]. Regression of established experimental cancer has been demonstrated by the local injection of β-carotene or vitamin E directly into the tumor site [12, 32, 47]. Oral administration of either β-carotene or vitamin E has not been shown to be effective, although combined oval dosing with these two substances has produced a regression of established epidermoid carcinoma [41]. Evaluation of combinations of vitamin E and/or β-carotene with cytotoxic antitumor agents have not yet been carried out in the hamster cheek-pouch model system.

Burton and Ingold [3] hypothesized that in an oxic environment, β-carotene would act as a reactive oxygen molecule rather than as an antioxidant. These authors also predicted that vitamin E would act as a reactive oxygen molecule in a hypoxic environment. The data we obtained partially confirm this prediction in that β-carotene was clearly more cytotoxic toward normally oxygenated SCC-25 cells at pH 7.4. In addition, vitamin E was somewhat more cytoxic toward hypoxic cells, but only in an acidic environment. The presence of reactive oxygen molecules has been shown to cause damage to DNA and related structures [14]. SOD is one of the prime cellular constitutents controlling the superoxide anion; therefore, the depletion of this enzyme could result in an accumulation of free radicals [1]. Of the various treatments shown in Table 1, only β-carotene significantly reduced the SOD levels in SCC-25 cells.

The sensitivity of cells to the cytotoxicity of CDDP and melphalan may be affected by the glutathione levels in tumor cells [26, 49], and the development of resistance to these agents may also depende on maintenance of the intracellular levels of glutathione and glutathione-S-transferase [49]. As demonstrated in Fig. 2, whereas vitamin E in combination with CDDP did not alter the cytotoxicity of the drug, β-carotene and the combination of β-carotene and vitamin E with CDDP markedly decreased the surviving fraction of SCC-25 tumor cells as compared with CDDP alone. This difference in CDDP activity may be attributable in part to the effects of vitamin E and β-carotene on cellular glutathione concentration and glutathione-S-transferase activity (Table 1). The need for further analysis of the interaction of chemotherapeutic agents and β-carotene or vitamin E is illustrated by the results obtained following treatment with the combination of melphalan and either β-carotene or vitamin E, which resulted in significant reductions in the surviving fraction of tumor cells (Fig. 3). Further evidence for a relatively specific synergy between β-carotene and melphalan is provided by the data shown in Fig. 4, whereby only the combination of β-carotene and melphalan markedly reduced thymidine incorporation into SCC-25 cells. One possible mechanism for this effect may involve the increased uptake of melphalan by β-carotene-treated cells. Experiments testing this possibility are under way.

We have previously found that on treatment with β-carotene, a 70-kDa stress protein is expressed in SCC-25 cells [34]. Stress proteins (e.g. heat-shock proteins) have been shown to be expressed following the induction of an acute oxidative change [5, 18, 51]. The significant decrease in the surviving fraction of SCC-25 cells obtained using the combination of β-carotene and the alkylating agents CDDP or melphalan may be interpreted as an increase in the sensitivity of these cells to the drugs, and β-carotene treatment may therefore provide a clinically relevant means of overcoming resistance to alkylating agents. We hope to continue these studies at a mechanistic level and to examine the efficacy of these treatment combinations in vivo.

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