Effects of physiological versus pharmacological β-carotene supplementation on cell proliferation and histopathological changes in the lungs of cigarette smoke-exposed ferrets

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There remains a remarkable discordance between the results of observational epidemiological studies and intervention trials using β-carotene as a potential chemopreventive agent. One question that needs to be examined is whether the adverse outcomes of human β-carotene trials are related to the large doses of β-carotene that were administered. In the present study, ferrets were given a physiological (low) dose or a pharmacological (high) dose of β-carotene supplementation (0.43 mg versus 2.4 mg/kg body wt/day, which is equivalent to 6 mg versus 30 mg/day in humans) and exposed to cigarette smoke for 6 months. We investigated the effects of these doses of β-carotene on retinoid concentrations, expression of retinoic acid receptors (RARs), activator protein 1 (AP-1; c-Jun and c-Fos), cyclin D1, proliferating cellular nuclear antigen (PCNA), and histopathological changes in the lungs of both normal and cigarette smoke-exposed ferrets. Thirty-six male ferrets were treated in six groups—control, smoke-exposed (SM), low-dose β-carotene (LBC), high-dose β-carotene (HBC), low-dose β-carotene plus smoke exposure (LBC+SM) or high-dose β-carotene plus smoke exposure (HBC+SM)—for 6 months. Retinoic acid concentration and RARβ gene expression, but not expression of RARα and RARγ, was reduced in the lung tissue of HBC+SM, HBC, SM and LBC+SM ferrets, but not in that of LBC ferrets, as compared with the control group. Expression of AP-1 and PCNA was greater in HBC+SM, HBC, SM and LBC+SM ferrets, but not in the LBC ferrets, as compared with the control group. Increased amounts of cyclin D1 and keratinized squamous metaplasia were observed in the lung tissue of HBC+SM, HBC and SM groups but not in that of the LBC+SM, LBC or control groups. These data suggest that, in contrast with a pharmacological dose of β-carotene, a physiological dose of β-carotene in smoke-exposed ferrets has no potentially detrimental effects and may afford weak protection against lung damage induced by cigarette smoke.

Introduction

Observational epidemiological studies have consistently demonstrated that people who eat more fruits and vegetables (which are rich in antioxidants) and those with higher serum β-carotene levels have a lower risk of cancer (1,2). The consistency of the results from observational studies is particularly strong for lung cancer (2). Animal and laboratory studies have shown that β-carotene can block the carcinogenic process and inhibit growth of tumor cells (3,4). In contrast to these observations, two human intervention studies using β-carotene supplementation at a pharmacological dose revealed an increased risk of lung cancer among smokers and asbestos workers (5–8). Two other randomized trials showed no overall benefit or risk in lung cancer among male physicians in the USA (11% current smokers) (9) or among female health professionals in the USA (13% current smokers) (10).

Understanding the mechanism(s) of the carcinogenic response to high-dose β-carotene supplementation reported in human intervention trials is important, due to a continuing interest in the potential of β-carotene as a chemopreventive agent. Recent reports (11–16), including ours (11), from both in vitro and in vivo studies have provided useful information on the controversy regarding the chemopreventive activity of β-carotene: the carcinogenic response to high-dose β-carotene supplementation reported in the human intervention trials may be related to the instability of the β-carotene molecule in the free radical-rich yet antioxidant-poor environment of the lungs of cigarette smokers (17). The presentation of high doses of β-carotene to the highly oxidative environment of lungs exposed to cigarette smoke results in increased levels of oxidative metabolites of β-carotene (11). The increased β-carotene oxidative metabolites could promote carcinogenesis by (i) induction of carcinogen-bioactivating enzymes that activate tobacco-smoke procarcinogens (12); (ii) facilitation of binding of metabolites of benzo[α]pyrene to DNA (15); and (iii) down-regulation of RARβ, which may function as a tumor suppressor (11). Nevertheless, it remains to be clarified whether the adverse outcomes of human β-carotene trials of smokers are related to the pharmacological doses of β-carotene that were administered. More specifically, would a physiological dose of β-carotene provide antioxidant protection, while not giving rise to undesirable metabolic by-products and thus afford protection against carcinogenesis?

It is highly unlikely that another human intervention study will be conducted in smokers receiving β-carotene supplements to address key mechanistic questions of the promoting versus inhibitory actions of β-carotene in lung carcinogenesis. However, an appropriate animal model could be used to resolve the mechanistic issues. The ferret offers an excellent model for use in mimicking the conditions of human β-carotene intervention studies, considering the similarities between ferret and human in terms of (i) absorption of intact β-carotene (18); (ii) accumulation of β-carotene in lung tissue (11); (iii) appearance of oxidative metabolites of β-carotene in the lung (11,19); (iv) lung architecture (20); and (v) lung pathology induced by cigarette smoke (11,21,22).

In the present study, ferrets were given either a physiological...
Materials and methods

Animals and study groups

Male adult ferrets (1.0–1.2 kg) from Marshall Farms (North Rose, NY, USA) were housed in an American Association of Accreditation of Laboratory Animal Care (AAALAC)-accredited animal facility at the Human Nutrition Research Center on Aging at Tufts University (HNRCa). Animal maintenance and husbandry and experimental procedures, including the method of smoke exposure, have been described in a previous paper (11). Thirty-six male ferrets were randomly assigned to six groups of six animals for 6 months as follows: (i) control; (ii) smoke-exposed; (iii) low-dose β-carotene (0.43 mg/kg body wt/day); (iv) high-dose β-carotene (2.4 mg/kg body wt/day); (v) smoke-exposed plus low-dose β-carotene; (vi) smoke-exposed plus high-dose β-carotene. During the 6 month experimental period, ferrets' body weights were recorded weekly. After the experimental period, all ferrets were killed by puncturing the heart under deep isoflurane anesthesia.

Exposure to cigarette smoke

Ferrets were exposed to cigarette smoke as previously described (11). In brief, the method was as follows. Ferrets were placed in a closed transparent plastic chamber connected to a smoking device. Cigarette smoke was drawn out of the cigarettes (Standard Research Cigarettes, type 1R4F; Tobacco and Health Research Institute, University of Kentucky, Lexington, KY, USA) by vacuum and then exhausted into the chamber. During the first 2 weeks of study, the number of cigarettes smoked gradually increased to a rate of 10 cigarettes over a 30 min period twice in the morning and twice in the afternoon and then maintained for the rest of the 6 month experimental period. In our previous study (11), we showed that this amount of smoke exposure in the ferret is similar to that found in humans smoking one and a half packs of cigarettes per day in terms of the concentration of urinary cotinine equivalents (~12 µg/ml urine in the ferret). The ferrets had free access to food and water. Ferrets not exposed to smoke were housed in a separate room and treated in exactly the same way as the smoke-exposed animals, except that they received no smoke exposure.

β-Carotene supplementation

All-trans-β-carotene (type IV; Sigma, St Louis, MO, USA) was dissolved in 1 ml corn oil and fed orally (not by gavage) to the ferrets every morning for 6 months. Ferrets like to eat corn oil and lick it spontaneously. Ferrets in the control group were fed the basal diet plus 1 ml corn oil without β-carotene. The concentrations of β-carotene and vitamin A in the ferret basal diet were controlled by using a single batch of ferret diet. The calculation of the ferret equivalents of β-carotene doses used in humans was based on total absorption of intact β-carotene (18). The average intake of β-carotene from the basal diet during the experimental period was 0.16 mg/kg body wt/day. Since the total absorption of β-carotene by ferrets is about five times less than that in human (18), β-carotene intake from the diet was ~0.03 mg/kg body wt/day, which is equivalent to intake of 2.1 mg β-carotene/day in a 70 kg person. The low-dose β-carotene group received 0.43 mg β-carotene/kg body wt/day, including the β-carotene in the basal diet. This dose of β-carotene in the ferret is three times higher than that in their basal diet and equivalent to an intake of 6 mg β-carotene/day in humans. The group supplemented with high-dose β-carotene received 2.4 mg β-carotene/kg body wt/day including the β-carotene in the basal diet. This high dose of β-carotene in the ferret is equivalent to an intake of 30 mg β-carotene/day in a 70 kg person, a dose used in smokers in human intervention trials (7,8).

Plasma and lung sample extraction and HPLC analysis

β-Carotene, retinol, retinyl esters and retinoic acid in blood and lung tissue homogenates were assayed by HPLC as previously described (11). Briefly, 100 µl of an ethanolic solution of 0.5 N KOH and 0.5 ml H2O2 were added to 2.0 ml of ferret plasma and then the internal standards, echinenone and retinyl acetate each in 50 µl of ethanol, were added. The mixture was extracted by adding 2 ml hexane, rotary shaking and then centrifuging for 5 min at 800 × g at 4°C. The hexane layer was removed and the residue was acidified by adding 50 µl of 6 N HCl. A second extraction was performed with 2 ml hexane. The two extractions were pooled, dried under nitrogen and resuspended in 50 µl ethanol for injection into the HPLC system. Lung tissue was homogenized with ice-cold HEPES buffer and methanol (2:1 v/v). After homogenization, samples of lung were extracted twice without saponification using 6.0 ml of CHCl3/CH3OH (2:1 v/v). The internal standards (echinenone and retinyl acetate) were added to the samples before the extraction. The two extracts were collected and evaporated under nitrogen. A 50 µl aliquot of the extract reconstituted with ethanol was injected into the HPLC system. A gradient reversed phase HPLC system was used for the analysis of retinol, retinyl palmitate and plasma retinoic acid. The gradient procedure, at a flow rate of 1 ml/min, was as follows: 100% solvent A (acetone/tetrahydrofuran:water:50:20:30 by volume, with 0.35% acetic acid and 1% ammonium acetate in water) for 3 min, followed by a 6 min linear gradient to 40% solvent A and 60% solvent B (acetone/tetrahydrofuran:water:50:44:6 by volume, with 0.35% acetic acid and 1% ammonium acetate in water), a 12 min hold at 40% solvent A/60% solvent B, then a 7 min gradient back to 100% solvent A. In this HPLC system, retinolic acid, retinol, retinyl palmitate and β-carotene eluted at 6.8, 6.6, 18.6 and 20.1 min, respectively. A Waters 490E multielectric spectrophotometer detector was set at 340 nm for retinoids and 380, 400 and 450 nm for carotenoids. An additional Waters 994 programmable photodiode array detector was set to measure absorption spectra. Individual carotenoids and retinoids were identified by co-elution with standards and quantified relative to the internal standard (echinenone for carotenoids and retinyl acetate for retinoids), by determining peak areas calibrated against known amounts of standards. In all experiments, all preparations were carried out under red light to prevent photodamage to the compounds.

Histopathology and immunohistochemistry

The right upper lobe of each lung was inflated and fixed by intratracheal instillation of 10% formalin. The samples were embedded in paraffin. Five micrometer sections were made using an AO microtome and stained with hematoxylin and eosin for histopathological examination. For immunohistochemistry analysis, 5 µm thick paraffin sections were deparaffinized, rehydrated and incubated with 0.3% (v/v) H2O2 in absolute methanol at 37°C to inhibit endogenous peroxidase. The sections were incubated for 60 min at 37°C with a monoclonal anti-pan cytokeratin antibody (mixture; Sigma, St Louis, MO, USA) that reacts with simple, cornified squamous epithelium. The sections were then rinsed with phosphate-buffered saline (PBS) and incubated with a peroxidase-labeled goat anti-mouse immunoglobulin antibody (Bio-Rad) at a dilution of 1:1000 in PBS, in which sections were incubated with a peroxidase-labeled goat anti-mouse immunoglobulin antibody (Bio-Rad) at a dilution of 1:1000 in PBS, in which sections were incubated with HistoMark Black substrate solutions and counter-stained with contrast green solution (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA). Sections were air-dried, mounted in xylene-based mountant and examined by two independent investigators by light microscopy. In this animal study, we examined the presence or absence of lung squamous metaplasia. Since squamous metaplasia lesions are spotty and localized, we recorded an animal as positive if any keratinized squamous metaplasia lesion was observed (histological examination plus confirmations by immunohistochemistry with anti-keratin antibody) in the right upper lobe of lung. Otherwise the animal was considered negative.

Nuclear protein preparations and western blotting analysis

Nuclear protein extracts from the lungs of ferrets in each group were prepared as described (11). Briefly, the lung tissues were homogenized gently in a Brinkmann (Westbury, NY, USA) Polytron homogenizer with ice-cold buffer A [10 mM Tris–HCl (pH 7.5), 10% glycerol, 10 mM potassium glycolycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 µg/ml leupeptin and 0.5 µg/ml aprotonin] and nuclei were collected by centrifugation for 30 min at 3200 × g. Nuclear pellets were solubilized in buffer B [10 mM Tris–HCl (pH 7.5), 10% glycerol, 600 mM KCl, 1 mM dithiothreitol (DTT), 10 mM monothioglycolycerol, 1 mM PMSF, 0.5 µg/ml leupeptin and 0.5 µg/ml aprotonin] for 60 min. The extracts were centrifuged for 30 min at 100 000 × g; the resulting supernatants are referred to as the nuclear extract. Proteins were quantified using a bicinchoninic acid protein assay kit (Pierce Co., Rockford, IL, USA). Western blotting analyses were carried out using monoclonal or polyclonal antibodies against proliferating cell nuclear antigen (PCNA), RARα, RARβ, RARγ, c-Jun, c-Fos and cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); signals on the blots were quantified by densitometry, as described (17).

Statistical analysis

Results are expressed as means ± standard deviations. Significant differences were compared using analysis of variance, followed by Tukey’s test among the groups at P < 0.05.

Results

There were no differences in body weight among the six groups of ferrets at the start of the study, during treatment or
after 6 months of treatment (data not shown). Assessment of smoke-exposure efficiency showed that there were no differences in the concentration of urinary cotinine equivalents in ferrets exposed to cigarette smoke alone, those exposed to cigarette smoke plus low-dose β-carotene and those exposed to cigarette smoke plus high-dose β-carotene (13.5 ± 1.3, 13.7 ± 0.9 and 14.1 ± 1.1 µg/ml urine, respectively).

**Concentrations of β-carotene and retinoids in plasma and lung tissue of ferrets after 6 months of treatment**

Supplementation with low- or high-dose β-carotene increased the concentration of β-carotene in both plasma and lung tissue of ferrets (Table I). Compared with controls, plasma concentrations of β-carotene were four-fold higher in the low-dose β-carotene group and 17-fold higher in the high-dose β-carotene group. Lung tissue concentrations of β-carotene were 25-fold higher in the low-dose β-carotene group and 202-fold higher in the high-dose β-carotene group. Smoke exposure decreased plasma concentrations of β-carotene in both the low-dose β-carotene group (by 76%) and the high-dose β-carotene group (by 64%) (Table I). Lung tissue concentrations of β-carotene were reduced by 93% by smoke exposure in both the low-dose β-carotene group and the high dose β-carotene group (Table I). Plasma concentrations of retinoids (retinoic acid, retinol and retinyl palmitate) did not differ among the six groups of ferrets (Table I). However, retinoic acid concentrations in lung tissue were significantly lower in the three smoke-exposed groups and in the high-dose β-carotene group, as compared with the control group and the low-dose β-carotene group. We observed no significant differences in the concentrations of retinol or retinyl palmitate in the lung tissue among the six groups (Table I).

**RARs gene expression in lung tissue of ferrets**

Expression of the RARβ gene, but not RARα or RARγ, was reduced in smoke-exposed ferrets (by 27%) and in those given high-dose β-carotene alone (by 61%) or smoke-exposed plus high-dose β-carotene (by 70%) (Figure 1). No difference in RARβ gene expression was observed between the group given low-dose β-carotene alone and the control group (Figure 1). However, RARβ gene expression in the smoke-exposed plus low-dose β-carotene group was 20% lower than that in the control group (Figure 1).

**AP-1 gene expression in lung tissue of ferrets**

c-Jun gene expression was greatly increased in the groups subjected to smoke exposure and given high-dose β-carotene after 6 months of treatment (data not shown). Assessment of smoke-exposure efficiency showed that there were no differences in the concentration of urinary cotinine equivalents in ferrets exposed to cigarette smoke alone, those exposed to cigarette smoke plus low-dose β-carotene and those exposed to cigarette smoke plus high-dose β-carotene (13.5 ± 1.3, 13.7 ± 0.9 and 14.1 ± 1.1 µg/ml urine, respectively).

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Fig. 2. Effect of β-carotene supplementation (low versus high dose), smoke exposure or the combination of smoke exposure with β-carotene supplementation (low versus high dose) on AP-1 [c-Jun (upper panel) and c-Fos (lower panel)] expression in the lung tissue of ferrets. The figure shows the intensity of the protein signal determined by densitometry (six samples in each group) and expressed as relative values (means ± SD). Different letters for given bars indicate that those values are significantly different from each other (P < 0.05). The relative values in the panel were defined as the intensity of signal of each sample of five treatment groups divided by the intensity of signal of each control sample in each run (a total of six runs). The insets show representative western blot analyses of the groups in the same order as in the graph. The size of the detected c-Jun and c-Fos were 39 and 62 kDa, respectively.

(by 308%), those given high-dose β-carotene alone (by 101%), and those exposed to smoke (by 43%), as compared with the control group (Figure 2, upper). No difference in c-Jun gene expression was observed between the low-dose β-carotene alone group and the control group (Figure 2). However, there was no difference in the expression of c-Jun or c-Fos between ferrets exposed to smoke alone and those also given low-dose β-carotene (Figure 2).

Cyclin D1 and PCNA gene expression in lung tissue of ferrets
Cyclin D1 gene expression was markedly greater in the smoke-exposed plus high-dose β-carotene group (by 283%) and high-dose β-carotene alone group (by 104%) than in the control group (Figure 3, upper). We observed no difference in

Fig. 3. Effect of β-carotene supplementation (low versus high dose), smoke exposure or the combination of the two on expression of cyclin D1 (upper panel) and PCNA (lower panel) in lung tissue of ferrets. The figure shows the intensity of the protein signal determined by densitometry (six samples per group) and expressed as relative values (means ± SD). Different letters for given bars indicate that those values are significantly different from each other (P < 0.05). The relative values in the panel were defined as the intensity of signal of each sample of five treatment groups divided by the intensity of signal of each control sample in each run (total of six runs). The insets show representative western blot analyses in the same order as in the graph. The size of the detected cyclin D1 and PCNA proteins were 39 and 34 kDa, respectively.
cyclin D1 gene expression among smoke-exposed ferrets with or without low-dose β-carotene or low-dose β-carotene, compared with controls (Figure 3, upper).

To evaluate the potential increase in cell proliferation in the lung tissue, we analyzed PCNA expression in all groups of ferrets. Compared with the control group, PCNA expression was increased significantly by 32% in the smoke-exposed group, 123% in the high-dose β-carotene alone group and 244% in the smoke-exposed plus high-dose β-carotene group (Figure 3, lower). PCNA overexpression was correlated with pathological changes in the lung tissue of all groups. We observed no difference in PCNA expression between the control group and the low-dose β-carotene alone group, or between the smoke-exposed and the smoke-exposed plus low-dose β-carotene group (Figure 3, lower).

Pathological evaluation

No pathological changes were observed in the lung tissue of ferrets in the control group or in the low-dose β-carotene group after 6 months of intervention (Figure 4A). Mild aggregation and proliferation of macrophages and squamous metaplasia was observed in lung tissue of two of the six ferrets exposed to smoke alone (Figure 4B). The proliferation of macrophages caused by smoke exposure were somewhat reduced when smoke exposure was combined with low-dose β-carotene (Figure 4B). No squamous metaplasia was observed in the smoke-exposed plus low-dose β-carotene group (Figure 4B). Localized proliferation of alveolar macrophages and keratinized squamous epithelium was observed in the lung tissue of ferrets in the high-dose β-carotene group (Figure 4A). Severe focal proliferation of alveolar cells, squamous metaplasia and destruction of alveolar walls were observed in the smoke-exposed plus high-dose β-carotene group (Figure 4B). Keratinized squamous metaplasia was confirmed by immunohistochemistry with anti-keratin antibody (Figure 4A and B).

Discussion

The presentation of high doses of β-carotene to the oxidative lung environment results in increased concentrations in lung tissue of oxidative metabolites of β-carotene, which could promote carcinogenesis by several mechanisms (17). However, an important question remains, namely, is the harmful effect of β-carotene dependent on the dosage used? More specifically, what would be the impact on lung cancer risk of a physiological dose, such as 6–7 mg of β-carotene/day, as would be contained in approximately five to nine servings of fruits and vegetables, rather than the pharmacological dose used in the human intervention studies? In the present study, we demonstrate that, in contrast to high-dose β-carotene supplementation, low-dose β-carotene (equivalent to 6 mg of β-carotene in a human) supplementation in smoke-exposed ferrets has no harmful effects, and may, in fact, afford weak protection against the development of squamous metaplasia.

Retinoic acid is required for normal epithelial cell growth and regulation through its nuclear receptors (RARs). RARβ, which can be induced by retinoic acid, has been proposed to function as a tumor suppressor gene (23). Primary lung tumors and lung cancer cell lines lack RARβ expression, and loss of expression may be an early event in lung carcinogenesis (24). Most abnormalities in RARβ expression involve reduced or absent expression of the RARβ2 isoform, which is the most abundant isoform in normal human lung tissue (25). Further, restoration of RARβ2 in a RARβ-negative lung cancer cell line has been reported to inhibit tumorigenicity in nude mice (26). In our study, concentrations of retinoic acid in lung tissue were significantly decreased in ferrets after either smoke exposure or high-dose β-carotene supplementation (with or without smoke exposure) (Table 1). This decreased concentration of retinoic acid could be due to increased catabolism of retinoic acid into more polar metabolites by a cytochrome P450-dependent process. Cigarette smoke is a strong inducer of cytochrome P450 enzymes in lung tissue (27). It has been reported that cytochrome P450 enzymes can be induced by high-dose β-carotene (12) and β-apo-8'-carotenal, an excrent clevage product of β-carotene, in rats (28). Although little is known about the specific enzymes that could be responsible for increased retinoic acid catabolism, we have recently shown that the formation of polar metabolites of retinoic acid increased significantly in a lung microsomal fraction after either smoke exposure or high-dose β-carotene supplementation, as compared with the control (C. Liu et al., unpublished data). Similar to the reduction in retinoic acid concentration, RARβ gene expression, but not expression of RARGα or RARγ, was reduced in the lungs of ferrets given high-dose β-carotene with or without cigarette smoke exposure, as compared with the control group (Figure 1). Furthermore, the decrease in RARβ expression in these three groups of animals was associated with overexpression of PCNA (Figure 3) and squamous metaplasia (Figure 4A and B) in the ferret lung.

In contrast to animals receiving high-dose β-carotene supplementation, neither the concentration of retinoic acid (Table 1) nor the expression of RARβ in the lung was affected in animals treated with low-dose β-carotene, as compared with the control group (Figure 1). In fact, compared with the smoke-exposed group, low-dose β-carotene supplementation in the smoke-exposed group resulted in a significantly smaller decrease in retinoic acid concentrations in the lung (Table 1). This supports the possibility, as shown previously (29), that β-carotene, when given at a low dose, could act to supply adequate retinoic acid to the lung tissue of smoke-exposed ferrets. However, when given at a high dose, oxidative by-products of β-carotene (e.g. β-apo-8'-carotenal (27)) could induce cytochrome P450 enzymes (28), thus destroying retinoic acid.

In our study, alveolar cell proliferation and keratinized squamous metaplasia were observed in the lung tissue of all ferrets supplemented with high-dose β-carotene with or without smoke exposure (Figure 4A and B), and the pathological changes were more profound in the high dose β-carotene-treated animals exposed to smoke (Figure 4B). In contrast, we did not observe any pathological lesions in ferrets given low-dose β-carotene alone (Figure 4A). Furthermore, low-dose β-carotene supplementation appeared to alleviate the keratinized squamous metaplasia caused by smoke exposure alone, since keratinized squamous metaplasia were found in none of the smoke-exposed animals receiving low-dose β-carotene exhibited, but in two of six animals exposed to smoke alone. These results indicate a dose relationship between β-carotene intake and the appearance of squamous metaplasia. That is, high-dose β-carotene supplementation with or without exposure to cigarette smoke results in a decrease in retinoic acid concentrations and reduced RARβ expression while low-dose β-carotene supplementation in the absence of smoke exposure does not. Furthermore, in contrast to high-dose β-carotene supplementation in smoke-exposed ferrets, low-dose β-carotene supplementation results in less down-regulation of RARβ.
induced by cigarette smoke (Figure 1), indicating that low-dose β-carotene supplementation in smoke-exposed ferrets may provide some protection against cigarette smoke-induced lesions. It has been shown in the SENCAR mouse skin carcinogenesis model (30) that excess β-carotene supplementation (600 µg/g of diet) causes more papilloma formation, whereas a lower dose of β-carotene (60 µg/g of diet) was associated with lower papilloma yield, when compared with groups that received very low β-carotene intake (0.6–6 µg/g of diet). Recently, Lowe et al. (31) have demonstrated that intact β-carotene can protect against xanthine/xanthine oxidase-induced oxidative DNA damage in HT29 cells at relatively low concentrations (1–3 µM), but rapidly loses this capacity at higher β-carotene doses (4–10 µM).

AP-1, the product of the c-jun and c-fos proto-oncogenes, binds to a DNA sequence motif, the AP-1 response element (AP-1 RE), and mediates the actions of signal transduction pathways, usually resulting in cell proliferation. Since recent evidence has accumulated supporting a role for reactive oxygen species in the regulation of AP-1 gene expression (32), we examined expression of AP-1 (c-Jun and c-Fos) and cell proliferation in the ferret lung after 6 months of treatment. In our study, cigarette smoke exposure for 6 months resulted in an increase in expression of c-Jun and c-Fos (Figure 2), PCNA expression (Figure 3) and squamous metaplasia (Figure 4B). However, both c-Jun and c-Fos expression were dramatically higher in the smoke-exposed, high-dose β-carotene supplemented ferrets than in the control animals (Figure 2). This overexpression of c-Jun and c-Fos was positively associated with increased PCNA expression (Figure 3) and with squamous metaplasia (Figure 4A and B) in the lungs of the high-dose β-carotene supplemented ferrets with or without smoke exposure. Recently, it has been reported that c-Jun is required for progression through the G1 phase of the cell cycle by a
Effects of β-carotene on lung cell proliferation

mechanism that involves direct transcriptional control of the cyclin D1 gene (33). It is conceivable that the overexpression of c-Jun by chronic excess β-carotene intake may cause abnormal cell cycle regulation and drive cells into premature S phase, resulting in aberrant mitotic process. This hypothesis was supported by the increase of expression of cyclin D1 in the lungs of the ferrets supplemented with high-dose β-carotene in the absence or presence of smoke exposure (Figure 3). Thus, increased c-Jun and cyclin D1 expression may be an important mechanism for causing cell proliferation and promoting carcinogenesis by high-dose β-carotene. Retinoid receptors can inhibit AP-1’s activities, via a protein–protein interaction mechanism, thereby inhibiting cell proliferation (34). This inhibitory effect on AP-1 activity by retinoids contributes to the suppression of human bronchial epithelial squamous metaplasia (35). Kamei et al. (36) showed that the interaction between RAR and cAMP response element-binding protein (CBP) is responsible for some forms of repression of AP-1 activity (Figure 5). It is plausible that the decreased concentration of retinoic acid in the lung or the down-regulation in the absence or presence of smoke exposure (Figure 3). Thus, increased c-Jun and cyclin D1 expression may be of RAR β might reduce the inhibitory effect of retinoids on AP-1, so enhancing lung cell proliferation and potentially tumor formation (Figure 5). Squamous metaplasia, which often occurs in the lungs of cigarette smokers and is also present in

Fig. 4. Pathological changes in the lung tissue of ferrets after 6 months of treatment HE×5 and ×50, hematoxylin and eosin stained sections at original magnification of ×5 and ×50, respectively; IM, immunohistochemical staining for keratin (×50). Any keratinized squamous metaplasia lesion (histological examination with confirmation by immunohistochemistry with an anti-keratin antibody) appearing in the right upper lobe of the lung was defined as positive. (A) Controls, low-dose β-carotene (LBC) and high-dose β-carotene (HBC) groups. Squamous metaplasia were observed in all six HBC animals but in none of the control or LBC animals. (B) Smoke-exposed (SM), smoke-exposed plus low-dose β-carotene (SM+LBC) and smoke-exposed plus high-dose β-carotene (SM+HBC) groups of ferrets. Squamous metaplasia were observed in all six SM+HBC animals and in two of the six SM animals, but in none of the SM+LBC animals.

2251
the bronchial epithelium adjacent to lung tumors, is believed by many investigators to antedate the appearance of carcinoma of the lung. However, we did not observe lung tumors in the groups of ferrets exposed to cigarette smoke and/or excessive β-carotene supplementation in the present study. We are currently conducting a study to determine if cigarette smoke (with or without β-carotene) makes ferrets more susceptible to lung tumors induced by a chemical carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane.

In our study, we did not demonstrate significant protective effects of low-dose β-carotene supplementation in smoke-exposed ferrets, in terms of the expression of AP-1, PCNA to lung tumors induced by a chemical carcinogen, 4-(methyl-β-carotene) makes ferrets more susceptible to lung tumors induced by a chemical carcinogen, 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butane.

In our study, we did not demonstrate significant protective effects of low-dose β-carotene supplementation in smoke-exposed ferrets, in terms of the expression of AP-1, PCNA to lung tumors induced by a chemical carcinogen, 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butane.

In summary, our study provides evidence that high-dose β-carotene supplementation may diminish retinoid signaling and increase gene expression of AP-1 and cyclin D1, as well as cell proliferation; these responses are enhanced by smoke exposure. Low-dose β-carotene supplementation has no detrimental effects; rather, it has weakly protective effects against cigarette smoke-induced damage. The increased risk of lung cancer after β-carotene supplementation observed in the ATBC and CARET studies could be due to the pharmacological dose of β-carotene used in these studies. It will be interesting to see if low-dose β-carotene, used in combination with other dietary antioxidants, may have chemopreventive effects against lung cancer.

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2253