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Beta-carotene promotes the development of NNK-induced small airway-derived lung adenocarcinoma

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Abstract

Aim—Beta-carotene has shown cancer preventive effects in preclinical studies while increasing lung cancer mortality in clinical trials. We have shown that β -carotene stimulates cAMP signaling in vitro. Here, we have tested the hypothesis that beta-carotene promotes the development of pulmonary adenocarcinoma (PAC) in vivo via cAMP signaling. **Methods:** PAC was induced in hamsters with the carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) followed by β -carotene for 1.5 years. Incidence, multiplicity and size of lung tumors were recorded and phosphorylated CREB and ERK1/2 in tumour cells determined by Western blots. Cyclic AMP in blood cells was analysed by immunoassays, retinoids in serum and lungs by HPLC. **Results:** beta-carotene increased lung tumor multiplicity, lung tumour size, blood cell cAMP, serum and lung levels of retinoids and induced p-CREB and p-ERK1/2 in lung tumours. **Conclusions:** Our data suggest that beta-carotene promotes the development of PAC via increased cAMP signaling.

Keywords

beta-carotene; lung adenocarcinoma; tumor promotion; cAMP-signaling

Introduction

Lung cancer is the leading cause of cancer death in industrialized countries and smoking is a major risk factor estimated to cause about 80% of all lung cancers (1). Pulmonary adenocarcinoma (PAC) is the predominating histological type of lung cancer today. The prognosis of PAC is poor, with 5-year survivals below 20%. Novel strategies for the prevention of these cancers in populations at risk are therefore urgently needed.

The pro-vitamin A, beta-carotene, vitamin A (retinol), and its metabolites all-trans-retinoic acid (ATRA), 9-cis-retinoic acid (9-cis-RA) and 13-cis-retinoic acid (13-cis-RA) have shown cancer preventive effects in preclinical studies (2). However, the alpha-tocopherol beta-carotene supplementation trial (ATBC) and the beta-carotene and retinoid efficacy trial (CARET) in smokers and former smokers had to be stopped due to a significant increase in

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mortality from lung cancer (3,4). In order to arrive at the identification of better cancer preventive agents it is important to understand the reasons for these failed trials.

Most PACs in people are thought to arise from the epithelial cells of small airways in the lung periphery (5). This epithelium consists mostly of conciliated Clara cells (6). PAC induced in Syrian golden hamsters by the nicotine-derived nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a model for human small airway-derived PAC (7). Similar to human PAC (8), these tumors in hamsters express activating point mutations in K-ras (9) while over-expressing cyclooxygenase-2 (COX-2) and the epidermal growth factor receptor (EGFR) (10). We have shown that human PAC cell lines that express the Clara cell-specific CC10 protein, immortalized human small airway epithelial cell line HPL1D (CC10 positive) and the NNK-induced PACs in hamsters (CC10 positive) are under growth control by G_{α_s} -coupled beta-1 and beta-2-adrenoreceptors that increase intracellular cAMP via activation of adenylyl cyclase. Classic agonists for beta-1 and beta-2-ARs as well as NNK initiated mitogenic signaling in these cells, via cAMP-induced activation of PKA, CREB and the PKA-dependent transactivation of the EGFR (11,12). Specific inhibitors of adenylyl cyclase or PKA blocked all of these downstream responses (12). These findings are consistent with the "classic" concept of beta-AR signaling via G_{α_s} adenylyl cyclase cAMP PKA CREB and transactivation of the EGFR (13). The beta-AR agonist epinephrine or the phosphodiesterase inhibitor theophylline each promoted the development of NNK-induced PAC whereas the beta-blocker propranolol significantly inhibited tumor development (14,15). P-CREB and p-ERK1/2 were overexpressed in the NNK-induced PACs and in hyperplastic lesions (10,16). These data identify cAMP-mediated signaling as an important regulator of small airway derived PAC.

Vitamin A deficiency causes squamous cell metaplasia in large airways of hamsters, an effect reversed by treatment with retinol (17). Numerous preclinical studies have shown genomic effects of high concentrations of retinol via nuclear retinoid receptors that suggested this agent and its metabolites as cancer preventive agents (2). Based on these preclinical results, the alpha-tocopherol, beta-carotene supplementation trial (ATBC) and the beta-carotene and retinoid efficacy trial (CARET) in smokers and former smokers were conducted. Both trials had to be stopped because of significant increases in lung cancer mortality in the beta-carotene or retinoid treated groups (3,4).

In vitro studies in human small airway-derived PAC cells and small airway epithelia showed that concentrations of 1 pM – 2 μ M of beta-carotene, retinol, ATRA, 9-cis-RA or 13-cis-RA increased intracellular cAMP, activating PKA CREB and ERK1/2 (18,19). These findings suggest that beta-carotene may promote small airway-derived PAC in vivo. To test this hypothesis, we have investigated the effects of beta-carotene on the development of small airway-derived NNK-induced PAC in hamsters.

Materials and Methods

Bioassay experiment in hamsters

The main dependent variables of this study were tumour number per animal (lung tumour multiplicity) and tumour size. Survival was not used as endpoint, because animals showing any respiratory problems were euthanized prior to the end of the study. Under these conditions, the survival of NNK treated versus NNK+ beta-carotene treated animals was not significantly different. The animal experiment was conducted in compliance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee. Male, outbred Syrian golden hamsters (6 weeks old, Charles River) were randomly assigned to three treatment groups (20 hamsters per group) and housed under standard laboratory conditions with free access to tap water and food (Purina lab chow). The development of PAC derived from small airway epithelial cells was induced

in two treatment groups as previously described (7,10,16) by subcutaneous injections with NNK (2.5 mg/100g bodyweight in 0.2 ml sterile water 3X per week for 10 weeks). One week after the last NNK injection, injections with beta-carotene (Sigma) started in one group (5 μ M beta-carotene in 0.2 ml sesame oil subcutaneously 2X/week until end of study). Sesame oil by itself neither modulates NNK-induced small airway epithelial cell proliferation nor NNK-induced PAC development (20). The control group received subcutaneous injections with sterile water. The animals were observed for 1.5 years unless humane euthanasia by anaesthetic overdose (telazol, 50 mg/Kg by intraperitoneal injection) was indicated at an earlier time in animals showing symptoms of disease. Blood samples were harvested from each animal followed by full necropsies. All major organs were fixed in 70% ethanol v/v and processed for histopathology evaluation from paraffin embedded tissue sections stained by haematoxylin/eosin. The histopathology diagnosis of lung adenoma versus lung adenocarcinoma was in accordance with the histopathology classification of neoplastic and preneoplastic lung lesions in the mouse (21). Prior to tissue fixation, each lobe of the lungs was dissected into two halves by a longitudinal cut along its main lobar bronchus to allow for counting of lung tumors originating from airways. Each half of a lung lobe was embedded and resulting stained sections were scanned onto a Macintosh computer for the assessment of tumour sizes by image analysis (area measurements of the entire tumor surfaces by NIH Scion image analysis software). Statistical analysis of data from lung tumour multiplicity (number of lung tumors per animal) and tumor size (number of pixels per measured tumor area) was conducted by one way analysis of variance (ANOVA), unpaired t-test and Mann-Whitney test. The cellular fraction of the blood samples were used for determination of systemic cAMP levels. Snap frozen samples of serum and lung tissue from 4 randomly chosen control hamsters and hamsters treated with NNK+ β -carotene were sent to a commercial laboratory (Craft Technologies) for analysis of retinoids by HPLC.

Determination of systemic cAMP levels

The cellular fraction of blood samples comprised of erythrocytes, lymphocytes, granulocytes and thrombocytes was used. These cells express beta-adrenergic receptors, and lymphocytes are routinely used in human patients for the assessment of systemic cAMP-dependent signaling activity (22). The levels of cAMP were determined with an enzyme immunoassay kit according to the manufacturer instructions (Assay Designs Inc, Ann Arbor, Mi). Data are expressed as mean values and standard errors of triplicate samples per treatment group. Statistical analysis of data was by ANOVA, Tukey-Kramer multiple comparisons test and two-tailed unpaired t-test.

Analysis of signaling proteins in lung cells

A PixCell Ile Laser Capture Microdissection system (Arcturus) was used to harvest lung tumour cells from hamsters treated with NNK or NNK+ β -carotene and small airway epithelial cells from control hamsters as previously described (16). Protein was determined using the BCA Protein Assay (Pierce, Rockford, IL). Protein samples were treated with loading buffer at 100°C for five minutes and electrophoresed in 12% w/v polyacrylamide gel, transferred onto nitrocellulose membranes in transfer buffer at 100 mV for one hour, treated with blocking buffer (5% v/v nonfat dry milk in tris buffered saline with Tween 20, TBST) for one hour, and incubated with primary antibody overnight at 4°C. The primary antibodies used were: anti-total CREB (Upstate Biotechnology, Lake Placid, N.Y., USA), anti-p-CREB, anti-p-ERK1/2 and anti-ERK1/2 (Cell Signaling). After incubation with horseradish peroxidase-labeled secondary antibody (goat anti-mouse, or goat anti-rabbit, Cell Signaling) for one hour, immunoreactive bands were detected using a chemiluminescent reaction (ECL, Amersham Biosciences, Piscataway, NJ) via autoradiography on Kodak Bio-Max film. Three separate Western blots were conducted for each antibody per sample and yielded similar data. Relative densities of the bands were determined by image analysis using NIH SCION image analysis

software. Mean values and standard errors from five densitometric readings per band were analyzed by ANOVA and Tukey-Kramer multiple comparison test.

Results

The absorption of dietary β -carotene by the gastrointestinal tract of laboratory rodents is very poor, necessitating the use of a high fat diet to yield significant levels of retinol and its metabolites formed from this provitamin in the lungs (20,23). However, a high fat diet can promote the development of PAC in people (24) and in laboratory rodents (25). We therefore used subcutaneous injections of β -carotene in a carrier (sesame oil) that facilitates β -carotene absorption while neither promoting NNK-induced proliferation of small airway epithelial cells nor the development of NNK-induced PAC (20). We found that β -carotene-induced increases of retinol, retinyl palmitate and retinoic acids in serum and lung tissue (Figure 1A). All trans-retinoic acid (ATRA) and 9-cis-RA were the predominating metabolites in serum (9-Cis-RA: 3.45-fold, $p<0.001$; ATRA: 3.5-fold, $p<0.001$) whereas in lung tissue retinyl palmitate (RP) and ATRA predominated (RP: 1.5-fold, $p<0.01$; ATRA: 4.4-fold, $p<0.001$). These findings suggest that the observed modulation of NNK-induced carcinogenesis was predominantly caused by ATRA, 9-cis-RA and RP.

Analysis of cAMP in the cellular fraction of blood samples by immunoassay showed a 1.8-fold increase ($p<0.001$) in the NNK-treated animals (Figure 1B). The levels of blood cell cAMP were further increased (2.3-fold, $p<0.001$) in the hamsters treated with β -carotene after discontinuation of NNK treatments (Figure 2). The observed increase of cAMP in the β -carotene treated hamsters was significantly ($p<0.001$) higher than in animals treated with NNK alone. These findings are suggestive of a generalized, systemic increase in cAMP-dependent signaling induced by NNK that was significantly enhanced further by β -carotene.

All of the control hamsters survived until the end of the experiment (1.5 years) and none of them developed tumours. By contrast, none of the animals treated with NNK alone or with NNK+ β -carotene survived beyond 12 months after start of the NNK treatment with no significant difference in survival times among these two groups. All hamsters treated with NNK or NNK + β -carotene developed multiple airway-derived adenomas and adenocarcinomas, yielding a lung tumour incidence of 100% in both treatment groups (Figure 2A). In addition, the hamsters treated with NNK alone developed a low incidence (three out of 20 hamsters) each of pancreatic ductal adenocarcinomas and olfactory adenocarcinomas of the nasal cavity, respectively (Figure 2a). The incidence of the pancreatic neoplasms was slightly increased by β -carotene treatment (from 3 to 4 out of 20 hamsters) whereas the incidence of olfactory adenocarcinomas almost quadrupled (from 3 to 11 out of 20 hamsters).

The multiplicity of NNK-induced lung tumours (Figure 2B) was significantly ($p<0.001$) increased by β -carotene (NNK alone mean number of tumors per animal: 6.9 ± 0.8 ; NNK+ β -carotene mean number of tumors per animal: 11.0 ± 0.7). Area measurements of tumour tissue by image analysis revealed that the lung tumors induced by NNK alone were significantly ($p<0.001$) smaller (106 ± 12.3 square pixels; Figures 3A, B and 4A.), and most of them were diagnosed as adenomas due to their good demarcation from surrounding normal tissue (Figure 4A). By contrast, each of the hamsters treated with NNK+ β -carotene had developed several very large adenocarcinomas that had replaced large portions of pulmonary lobes (Figures 3AB, 4B), yielding a 13.2-fold increase in mean lung tumor size (1401 ± 423 square pixels, $p<0.001$). In addition, these animals had multiple small adenomas similar in size and appearance to those induced by NNK alone (Figures 3A,B and 4B). Taken together, these data indicate that β -carotene not only promoted the development of NNK-induced lung tumors but additionally accelerated their progression.

We have shown that NNK activates cAMP-dependent signaling via p-CREB and p-ERK1/2 in human PAC cells in vitro (12) and in NNK-induced PACs in hamsters in vivo (10,16). We therefore investigated the expression levels of these two phosphorylated proteins in small airway epithelial cells from control hamsters in comparison to cells harvested from lung tumors of hamsters treated with NNK alone or with NNK+ β -carotene. Our data show that p-ERK1/2 and p-CREB were significantly ($p<0.001$) increased in lung tumor cells of hamsters treated with NNK alone (p-ERK1/2: 3.4-fold; p-CREB: 2.3-fold) over small airway epithelial cells of control hamsters (Figures 5AB). The expression levels of both these proteins were further enhanced significantly ($p<0.001$) in tumors from animals given NNK+ β -carotene (p-ERK1/2: 4.5-fold; p-CREB: 3.4-fold; Figures 5AB). These findings suggest that enhanced signaling via p-ERK and p-CREB contributed to the tumor promoting effects of β -carotene on NNK-induced lung carcinogenesis.

Discussion

The classic action of retinoids is thought to involve the activation of nuclear retinoid receptors (26). Our data show, for the first time, that β -carotene significantly promoted and accelerated the development of small airway-derived PAC induced by NNK in vivo by mechanisms that involved non-classical cellular signaling via p-ERK1/2 and p-CREB. The observed four-fold increase in the incidence of nasal cavity tumors may have been caused by similar mechanisms, even though regulatory signal transduction in this type of cancer has not been studied to date. Having harvested serum and lung tissues for HPLC analysis of retinoids two hours after the last β -carotene injection, we were able to identify ATRA, 9-cis-RA and RP as the predominating retinol metabolites in serum and lungs. These findings are in accord with reports that have described non-genomic signaling induced by β -carotene, retinol or its metabolites in human PAC cells of small airway epithelial phenotype, in immortalized normal human small airway epithelial cells and in human bronchial epithelial cells (18,19,27). While signaling via cAMP PKA CREB and PKA-dependent transactivation of ERK1/2 downstream of the EGFR stimulated the proliferation of small airway-derived PAC cells and its normal cells of origin, the same signaling cascade inhibited the proliferation of bronchial epithelial cells or small cell lung cancer cells (18,19). In conjunction with our current findings, these data suggest that the non classical signaling cascade stimulated by β -carotene and its metabolites modulates the regulation of lung cells with different outcomes for different types of cells. Small airway epithelia and the PACs derived from them are stimulated in their growth whereas large airway epithelia and SCLC derived from them are inhibited. Since PAC was the predominating lung cancer type in the CARET trial (28), the stimulation of non-genomic signaling in the current study may therefore justify the hypothesis that increased cAMP signaling may have contributed to the unfortunate outcome of this and similar clinical trials.

Immunoassays showed a significant increase in intracellular cAMP in blood cells from hamsters treated with NNK that was further increased by β -carotene treatment. These findings suggest that hyperactive cAMP signaling in response to NNK and β -carotene is a systemic event that can be detected by a simple blood test. This interpretation is in accord with the reported increase in lung cancer deaths in the CARET trial (4). In analogy to the assessment of cardiovascular function by analysis of cAMP in blood cells (22), it may hence be possible to identify individuals with elevated systemic cAMP who would respond with lung cancer promotion to β -carotene or retinoid treatment. On the other hand, individuals with below normal cAMP levels in blood cells may benefit from the selective cancer preventive effects of these agents.

It has been shown that vitamin A deficiency alone or in combination with benzo (a)-pyrene caused squamous metaplasia, a precursor of squamous cell carcinoma, in the epithelium of the trachea and stem bronchi in hamsters in vivo and in organ culture (17,29,30). Treatments with

β -carotene, retinol or other retinoids reversed or reduced this response (30,31). The hamster trachea and stem bronchi are coated by a pseudostratified respiratory epithelium found in large airways (trachea, stem bronchi, lobar bronchi, segmental bronchi) of humans (32) and comprised of basal cells, mucous cells and ciliated cells. In accord with the cited data in hamsters, recent studies with immortalized human large airway epithelial cells have shown a significant retinoid-induced inhibition of cell proliferation involving a cAMP-dependent inhibition of ERK1/2 phosphorylation (19). Dietary β -carotene administered to A/J mice enhanced bronchial epithelial cell proliferation in NNK-treated animals but did not promote the NNK-induced PACs (20). The mouse lung is a model for the human lung periphery, with all intrapulmonary airways being coated by the simple respiratory epithelium comprised of nonciliated Clara cells and sparse ciliated cells that is restricted to small airways (bronchioles) in humans (32). The stimulation of bronchial epithelial cell proliferation in mice is thus equivalent to the stimulation of human small airway epithelial cells in vitro by β -carotene and retinoids (18,19). However, spontaneous and NNK-induced lung tumors in the mouse are derived from alveolar type II cells (33) and not from small airway epithelia as most human PACs (5) or the PACs induced in the hamster by NNK (7). In turn, human PAC cell lines with features of alveolar type II cells are not stimulated in their growth by agents that increase intracellular cAMP (34). The observed β -carotene induced proliferation of bronchial epithelial cells in mice did therefore not promote the development of PAC.

Collectively, our data in NNK-induced small airway-derived PAC and the in vitro responses of human PAC cells of this phenotype (18,19) suggest that β -carotene and retinoids promote the development of this PAC type.

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Figure 1A

Figure 1B

Figure 1.

Figure 1A. Modulation of retinoid levels in serum and lung tissue. Retinyl palmitate and ATRA were the predominating metabolites and their increase over controls was highly significant ($p < 0.001$). Mean values and standard errors of samples from 5 hamsters per group expressed as fold increase over controls.

Figure 1B. Modulation of systemic cAMP levels in blood cells. NNK significantly ($p < 0.001$) increased systemic cAMP levels over controls. Animals treated with NNK+β-carotene showed an additional significant ($p < 0.001$) increase in systemic cAMP. Mean values and standard errors from 5 hamsters/group expressed as fold increase over controls.

Figure 2A

Figure 2B

Figure 2. Tumor incidences in lungs, pancreas and nasal cavity (A) and lung tumor multiplicity (B). Beta-carotene significantly ($p < 0.001$) increased lung tumor multiplicity. Data in both graphs are from 20 animals per group.

Figure 3A

Figure 3B

Figure 3. Modulation of NNK-induced lung tumor sizes by β -carotene. The graph in B shows the results of lung tumor area measurements. The lung tumors in animals treated with NNK+ β -carotene were significantly ($p < 0.001$) larger than in the hamsters treated with NNK alone.

Figure 4A

Figure 4B

Figure 4. Histopathology of lung tumor induced in a hamster by NNK alone (A) or by NNK+ β -carotene (B). Hematoxylin/eosin stain; X 40.

Figure 5A

Figure 5B

Figure 5.

Western blots (A) and densitometry values (B) showing levels of p-ERK1/2 and p-CREB in small airway epithelial cells and lung tumor cells. Protein induction by NNK ($p < 0.001$) was significantly ($p < 0.001$) increased by β -carotene. Bars in the graph are mean values and standard errors of five densitometric measurements per band expressed as ratio of p-ERK1/2 or p-CREB over ERK1/2.