Beta-carotene and lung cancer*

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Abstract: Does beta-carotene increase, rather than decrease, human lung cancer rates? A large body of observational epidemiologic study has demonstrated that individuals who eat more fruits and vegetables rich in carotenoids and/or who have higher levels of serum beta-carotene have a lower risk of cancer, particularly lung cancer. This inverse relationship has been particularly strong in lung cancer patients with a history of heavy smoking. However, there is contradictory evidence from recent human intervention studies using beta-carotene supplements (20–30 mg per day). An increase in risk of lung cancer among smokers who took beta-carotene supplements was reported in the Alpha Tocopherol, Beta-carotene Cancer Prevention (ATBC) Trial and among smokers and asbestos-exposed workers in the Beta-Carotene and Retinol Efficiency Trial (CARET), but not among male physicians in the United States in the Physicians Health Study (only 11% of whom were current smokers). Whether there is a true hazard associated with beta-carotene has been evaluated in control studies using the ferret. This animal mimics the human tissue metabolism of beta-carotene, and has been used for studies of tobacco smoking and inhalation toxicology. In the first study, ferrets were given a high-dose beta-carotene supplement equivalent to 30 mg per day in humans, and exposed cigarette smoke or both for six months. A strong proliferative response in lung tissue and squamous metaplasia were observed in all beta-carotene-supplemented animals, and this response was enhanced by exposure to tobacco smoke. When compared to the control group, beta-carotene-supplemented animals (with or without smoke exposure) had statistically significantly lower concentrations of retinoic acid in lung tissue, and they exhibited reductions in RAR-beta gene expression (a tumor suppressor gene). Further, ferrets given a high-dose beta-carotene supplement and exposed to tobacco smoke had fourfold elevated expressions of \textit{c-\textit{jun}} and \textit{c-\textit{fos}} genes. In a second study, ferrets were given either physiological- or pharmacologic-dose beta-carotene supplementations, which were equivalent to 6 mg vs. 30 mg per day in humans, respectively. The animals were exposed to cigarette smoke for six months. The retinoic acid concentration and RAR beta-gene expression were reduced in the lung tissues, whereas the expression of AP1, cyclin D1, and proliferative cell nuclear antigen were greater in the high-dose, beta-carotene-supplemented animals with or without smoke, as well as the smoke-exposed, low-dose, beta-carotene-supplemented animals—but not in the low-dose, beta-carotene-supplemented animals alone, as compared with the control group. Squamous metaplasia was only observed in the lung tissues of high-dose, beta-carotene exposed groups with or without smoke (but not the low-dose beta-carotene plus smoke group, the low-dose beta-carotene-supplemented group, or the control group). These data show that in contrast with the pharmacologic dose of beta-carotene, a physiologic dose of beta-carotene in smoke-exposed ferrets has no detrimental effect—and, in fact, may afford weak protection against lung damage induced by cigarette smoke.

Further studies from our laboratory have revealed an instability of the beta-carotene molecule in the lungs of cigarette smoke-exposed ferrets. Oxidized beta-carotene metabolites may play a role in lung carcinogenesis: by inducing carcinogen-bioactivating enzymes, facilitating the binding of metabolites of benz[a]pyrene to DNA, enhancing retinoic acid metabolism by P450 enzyme induction with subsequent down-regulation of RAR-beta, and acting as pro-oxidants, causing damage to DNA. Ferret studies under highly controlled experimental conditions using high- and low-dose beta-carotene in the presence of alpha tocopherol and ascorbic acid (thereby stabilizing the beta-carotene molecule) showed protective effects against smoke-induced lung squamous metaplasia in ferrets.

BETA-CAROTENE AND LUNG CANCER

Up until the mid-1990s, there was great hope that beta-carotene would be an effective agent for the prevention of lung cancer, particularly among heavy smokers. A large body of observational epidemiologic studies had consistently demonstrated that individuals eating more fruits and vegetables, and individuals having higher serum beta-carotene levels had a lower risk of developing lung cancer, especially among smokers. But in 1994, two studies were published that showed that supplementation with beta-carotene resulted in more—rather than fewer—lung cancers in high risk, smokers [1]. In the ATBC trial conducted in Finland, almost 30 000 male smokers smoking at least one pack of cigarettes per day were exposed to either a 20-mg beta-carotene supplement, a 50-mg supplement of vitamin E, the combination of both, or a placebo, and were followed for a mean of 6.1 years. Vitamin E had no effect on lung cancer incidence over this period of time, whereas high-dose beta-carotene intervention increased the incidence of lung cancers, as compared to the groups not exposed to beta-carotene. A second trial from the United States used daily 30 mg of beta-carotene combined with 25 000 units of retinol in smokers or asbestos-exposed workers who were at high risk for the development of lung cancer. Once again, it was found that beta-carotene supplementation gave rise to an increased number of lung cancers with a 20 % increase in the incidence over a four-year period [2]. A third study conducted among physicians in the United States (only 11 % of whom were smokers) showed neither a detrimental nor beneficial effect of beta-carotene supplementation at a dose of 50 mg given every other day [3]. It is interesting to note in these studies that because of different beta-carotene preparations that were used, average serum levels of beta-carotene reached 200 to 300 µg/dl in the ATBC and CARET studies, whereas in the Physicians Health Study, the average serum beta-carotene level was only 120 µg/dl [1–3]. These levels can be contrasted with those representative of the American population as found in the National Health and Nutrition Examination Survey (NHANES) study, ranging between 5 and 50 µg/dl [4].

Several questions arose due to these intervention studies. First of all, how does one explain the paradox of an apparent beneficial effect of beta-carotene against lung cancer in the observational epidemiologic studies, vs. either a detrimental or no effect in the high-dose intervention studies; and secondly, is there a true hazard associated with high doses of beta-carotene? The ferret model has been used to answer these questions, since this animal mimics human beta-carotene absorption and metabolism in tissues [5]. Moreover, this animal has been used for studies of tobacco smoking and inhalation toxicology [6,7]. Lung beta-carotene and retinol levels are similar in humans and ferrets not exposed to smoke or supplemental beta-carotene, as well as in humans and ferrets exposed to smoke and beta-carotene together. These observations set the stage for an intervention study to be conducted in ferrets.

In the first intervention study, ferrets were either exposed to supplemental beta-carotene, or not exposed to beta-carotene, at levels that were calculated to be equivalent to 30 mg per day in humans [8]. In each group of animals, beta-carotene- and nonbeta-carotene-supplemented, the ferrets were further divided into smoking and nonsmoking groups. Ferrets were exposed to cigarette smoke twice in the morning and twice in the afternoon, 10 cigarettes over a 30-min period each time, in a chamber connected to a smoking device. Animals that were not exposed to smoke underwent the same procedures...
as the animals exposed to smoke, except they did not receive exposure to the smoke. At the end of a six-month period, the animals were killed, and their blood and tissues were harvested for analysis.

Marked changes were seen in the animals exposed to smoke and high-dose beta-carotene together; localized proliferation of alveolar cells and alveolar macrophages, as well as keratinized squamous epithelium (confirmed by antikeratin antibody) were seen in all six ferrets of this group given high-dose beta-carotene with smoke exposure. Surprisingly, in the beta-carotene-exposed alone group (that is, not exposed to smoke, but only exposed to high-dose beta-carotene), some of these same detrimental changes were seen, although the changes were not as marked as in the animals exposed to beta-carotene and smoke together. To quantify cell proliferation activities in the lungs of the ferrets, lung tissue was analyzed for proliferative cell nuclear antigen (PCNA) expression via Western blot analyses. The highest PCNA activity was found in the animals exposed to both beta-carotene and smoke together.

Lung levels of beta-carotene and retinyl palmitate were highest in the beta-carotene-supplemented group that was not exposed to smoke (Table 1). Smoke exposure in the beta-carotene-supplemented animals caused a marked decrease in lung tissue levels of beta-carotene and a decrease in lung retinyl palmitate levels, as well. As compared to controls, the smoke-exposed animals that were not supplemented with beta-carotene showed only trace levels of beta-carotene in the lung, whereas retinyl palmitate levels were not affected. It was also noteworthy in this experiment, that lung retinoic acid levels were highest in the control animals, but became markedly reduced with beta-carotene supplementation alone, and were nondetectable in the smoke-exposed groups with or without beta-carotene supplementation. It was hypothesized that under the highly oxidative conditions of the lung due to cigarette smoke, a great number of oxidized metabolites of beta-carotene would be formed, and that these metabolites could subsequently somehow reduce the formation or result in the excess destruction of retinoic acid. Decreased tissue levels of retinoic acid could be one explanation of the enhanced cell proliferation seen in the ferrets and the enhanced lung cancer formation seen in the human beta-carotene intervention trials.

In order to demonstrate the appearance of oxidative products of beta-carotene in lung, postnuclear fractions of lung tissue were incubated with 10 µmol of beta-carotene. An increased formation of excentric cleavage products of beta-carotene was demonstrated if the tissue was derived from ferrets that were exposed to smoke, as compared to when lung postnuclear fractions were from ferrets not exposed to smoke. Thus, the dramatic decrease in beta-carotene that was seen in the animals exposed

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to smoke and beta-carotene supplementation together, as compared to the beta-carotene-supplemented animals alone, appears to be the result of enhanced destruction of beta-carotene and the formation of oxidative metabolites. These metabolites include beta apo 8′, 10′, 12′, and 14′ carotenals, which are structurally similar to retinoids and could interfere with the metabolism of retinoic acid and subsequent retinoid signaling.

In seeking an explanation for the lower levels of retinoic acid that were seen in all treatment groups, it was hypothesized that cytochrome P450 enzymes might be induced and result in the destruction of retinoic acid in the lung. It had been reported by Gradelet and colleagues that cytochrome P450/1A1 could be induced in rat tissue by beta apo 8′ carotenal [9]; and Paolini et al., in 1999 [10] had shown a significant increase in cytochrome P450/1A1 in the lungs of rats that were supplemented with very high doses of beta-carotene (500 mg/kg/day), possibly resulting in high levels of eccentric cleavage products.

To help prove that retinoic acid can be destroyed by P450 enzymes, we incubated retinoic acid with microsomal fractions of ferret lung derived from the various groups in the presence or absence of liarazole, which is a cytochrome P450 inhibitor [11]. We then assayed for two oxidative polar metabolites of retinoic acid, 4 oxo-retinoic acid and 18 hydroxy-retinoic acid. We found that in all three treatment groups (that is, the smoke-exposed, beta-carotene-supplemented alone, or smoke plus beta-carotene-treated groups) in the presence of the cytochrome P450 inhibitor, liarazole, the formation of 4 oxo-retinoic acid was markedly inhibited, as was the formation of 18 hydroxy-retinoic acid (unpublished results). Thus, cytochrome P450 induction appeared to be the explanation for why retinoic acid levels are lower in the lungs of the smoke-exposed and beta-carotene-supplemented animals.

The induction of cytochrome P450 enzymes either by beta-carotene oxidative cleavage products or by cigarette smoke has two possible detrimental actions in the lungs: (1) bioactivation of carcinogens, and (2) destruction of retinoic acid, thereby enhancing lung carcinogenesis. Since lung tissue levels of retinoic acid were found to be lower in smoke-exposed ferrets with or without beta-carotene supplementation, interference in retinoid signaling would be expected in these animals’ tissues. Lung carcinogenesis is associated with an alteration in retinoid signaling involving the AP1 complex which mediates signals from inflammatory peptides, growth factors, oncoproteins, and tumor promoters usually resulting in cell proliferation [12]. Activator Protein 1 (AP1) is formed by the c-Fos, c-Jun complex, which can bind to a DNA sequence motif not recognized by retinoid receptors, and which is referred to as the AP1 binding site. Retinoid receptors and the transcription factor, AP1, can inhibit each other’s activities. When retinoic acid is present, and becomes liganded to RXR-RAR, it allows these receptors to react with AP1—thereby preventing it from binding to the AP1 binding site and thus inhibiting a sequence of events that would have resulted in proliferation. Conversely, when retinoic acid levels are diminished, there is less binding of the RAR-RXR complex to AP1, and the AP1 complex is thus able to bind to the DNA sequence motif resulting in cell proliferation. Cigarette smoke itself can activate AP1 by increased oxidative stress. However, smoke can also indirectly activate AP1, as a result of retinoic acid destruction due to P450 induction by the oxidative metabolites of beta-carotene. c-Fos and c-Jun expression in nuclear protein extracts from the lungs of ferrets were increased in the beta-carotene plus smoke-exposed group. In addition, RAR-beta levels were examined by Western blotting. Several lines of evidence have suggested that RAR-beta plays an important role in lung carcinogenesis [13–15]. Primary lung tumors lack RAR-beta expression, and loss of tumor suppressor function by mutation or transcriptional repression leads to enhanced cell proliferation [13,16–18]. In our experiments, RAR-beta gene expression was down-regulated in the three treatment groups (smoke-exposed, high-dose beta-carotene, or both), as compared to the control group in a step-wise fashion—with beta-carotene plus smoke exposure together resulting in the most profound depression of gene expression. RAR-alpha and RAR-gamma expression were not similarly affected.

Thus, from these studies it appears that the harmful effect of high-dose beta-carotene supplementation in smokers is due to the free-radical atmosphere in the lungs of cigarette smokers enhancing beta-carotene oxidation with the formation of oxidative metabolites via eccentric cleavage. These
metabolites in turn diminish retinoic acid levels and, thus, retinoid signaling by down-regulating RAR-beta expression and up-regulating AP1. Further investigations into the molecular mechanisms whereby beta-carotene metabolites could act to promote carcinogenesis (in addition to up-regulation of AP1 and down-regulation of RAR-beta), are warranted, such as effects on P53 expression.

As to how excentric cleavage products of beta-carotene are formed in excess amounts in the smoke-exposed lung, it is probable that oxidation can come about by several mechanisms including enzymatic oxidation, co-oxidation, and simple oxidation. Wang et al. demonstrated considerable excentric cleavage activity in the postnuclear fractions of lungs with no additional antioxidant added to the system [19]. However, Yeum et al., using postmitochondrial fractions of tissue, showed that in the presence of alpha-tocopherol, beta-carotene is converted in the intestine almost exclusively to retinal, whereas, in the absence of alpha-tocopherol both retinal and apocarotenoids are formed [20]. In the free-radical rich environment of the lungs, it is highly conceivable that beta-carotene can be cleaved randomly by either enzyme-related radicals (that is, co-oxidation) or by direct oxidation to produce these apocarotenoids.

Further, Kiefer and colleagues recently reported the molecular identification of a cDNA from BALB/c mice encoding an oxygenase that catalyzes excentric oxidative cleavage of beta-carotene at the 9′, 10′ double bond in humans [21]. Since the formation of apo 10′ carotenal from beta-carotene was observed in the lungs of smoke-exposed ferrets, we examined whether there was an mRNA encoding this excentric carotene oxygenase in the lungs of ferrets. Using primer sets from BALB/c mice, we showed that beta-carotene 9′, 10′ oxygenase is expressed in the lungs of the ferret (i.e., a specific RT-PCR product of a size similar to that in the mouse was detected in ferret lung tissue). The effects of the beta-carotene metabolites on the expression and activity of this enzyme are as yet unexplored.

As already discussed, excentric oxidative products of beta-carotene can induce P450 enzymes, resulting in a local deficiency of retinoic acid. Further, and building on the work of Salgo and Perocco, [22,23], it has been shown by Prakash in our laboratory, that oxidative metabolites of beta-carotene, beta-apo 10′ and 14′ apocarotenals, facilitate the binding of metabolites of benzo[a]pyrene (a smoke-derived carcinogen) to DNA (unpublished results). This binding could thus induce DNA damage, giving rise to another mechanism for lung carcinogenesis in conditions of high beta-carotene concentrations and smoke exposure.

Taking into account the epidemiologic and intervention studies, it is highly likely that the dosage of beta-carotene is key to explaining its procarcinogenic effects; that is, that carcinogenesis is enhanced with high doses and high concentrations of beta-carotene, whereas no such enhancement is seen at lower levels. Possibly, a benefit could be seen at low levels of beta-carotene supplementation. Thus, we carried out a study to test whether a physiologic dose of beta-carotene would have the same effect as a pharmacologic dose [24]. Ferrets were once again divided into beta-carotene-supplemented and non-supplemented groups, and each of these groups was further divided into smoke-exposed or nonsmoke-exposed groups. The beta-carotene supplementation was either given at high dose, equivalent to 30 mg of beta-carotene per day in the human, or given at a physiologic dose, equivalent to 6 mg of beta-carotene per day in humans. The animals were exposed to these treatments for six months.

As seen previously, in animals exposed to smoke plus high-dose beta-carotene together, significant lung damage with squamous metaplasia resulted, whereas no such damage was seen in the smoke-exposed animals exposed to low-dose beta-carotene. No pathological lesions were seen in the ferrets given low-dose beta-carotene alone. Furthermore, low-dose beta-carotene supplementation appeared to alleviate squamous metaplasia caused by smoke exposure alone, since keratinized squamous metaplasia was found in none of the smoke-exposed animals receiving low-dose beta-carotene, whereas two of the six animals exposed to smoke alone exhibited squamous metaplasia.

In summary, decreased concentrations of retinoic acid and RAR-beta expression and increased AP1 and PCNA expression are most prominent in smoke- and high-dose beta-carotene-exposed ferrets, the same animals that show the most profound squamous metaplasia. The cell and molecular markers were only mildly affected in the smoke plus low-dose beta-carotene fed group, and there was no squa-
mous metaplasia seen in this group of animals. These data suggest that in contrast to a pharmacologic dose of beta-carotene, a physiologic dose of beta-carotene in smoke-exposed ferrets has no detrimental effects. Recently, it was reported that c-Jun was required for progression through the G-1 phase of the cell cycle via a mechanism involving the transcriptional control of the cyclin D-1 gene. In our latest experiments, this was supported by the demonstration of increased expression of cyclin D-1 in the lungs of ferrets supplemented with high-dose beta-carotene in either the absence or presence of smoke exposure [24].

It is interesting to reflect on the fact that in the observational epidemiologic studies, high dietary levels of beta-carotene (which are one-quarter or less of the doses received in the intervention trials), in conjunction with the other nutrients contained in fruits and vegetables, resulted in a lower prevalence of lung cancers in smokers. The failure of the beta-carotene intervention trials to show a benefit against lung carcinogenesis in smokers should now shift interest from the use of single micronutrients for chemoprevention (such as beta-carotene alone), to some of the micronutrient combinations found in food, particularly in fruits and vegetables. For example, moderate doses of beta-carotene, vitamin C, and vitamin E could act together in a network fashion to optimize antioxidant protection, and such a combination should now be studied for possible chemopreventive effects. Both vitamins E and C inhibit cytochrome P450-mediated lipid peroxidation as well as carcinogen activation [25]. In many observational epidemiologic studies, diets high in vitamin C are correlated with a lower risk for lung cancer. It is also known that smokers have significantly lower plasma levels of vitamin C as compared to non-smokers, and that passive smokers as well have reduced ascorbic acid concentrations in their plasma [26,27]. It has also been demonstrated that vitamin C can regenerate vitamin E from the vitamin E radical formed during lipid peroxidation [28] and that vitamin C can convert the beta-carotene radical back to beta-carotene, thus maintaining beta-carotene in its unoxidized form [29]. Moreover, it has recently been shown that oxidized vitamin E can also be recycled by beta-carotene [30]. Böhm and colleagues have shown that the combination of beta-carotene, alpha tocopherol, and vitamin C provides a synergistic protection against free-radical damage in in vitro cell systems [30]. Although the combination of beta-carotene and vitamin E was not found to be protective against smoke-related lung cancer in the ATBC study [1], vitamin C, which would facilitate both vitamin E recycling and beta-carotene stability, was not included in the ATBC study. It may be particularly important to have broad antioxidant protection when exposed to high doses of beta-carotene in order to prevent the production of carotene excentric cleavage products and the subsequent cascade of events that can result from them.

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