Effects of vitamin A and β-carotene on respiratory tract carcinogenesis in hamsters - *in vivo* and *in vitro* studies

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Effects of vitamin A and β-carotene on respiratory tract carcinogenesis in hamsters - *in vivo* and *in vitro* studies

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Proefschrift

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Stellingen

1. De beschermende werking van vitamine A en β-caroteen tegen de vorming van benzo[a]pyreen-DNA adducten moet naast het effect van deze verbindingen op het metabolisme van benzo[a]pyreen ook worden toegeschreven aan een effect van deze verbindingen op het herstel van DNA schade.
(dit proefschrift)

2. Het effect van benzo[a]pyreen en vitamine A op proliferatie van hamstertrachea-epitheelcellen is sterk afhankelijk van het gebruikte weefselkweekmedium, waarbij de concentratie van Ca^{2+} in het medium een belangrijke rol speelt.
(dit proefschrift)

3. De longtumorrespons van hamsters na intratracheale toediening van benzo[a]pyreen-deeltjes is afhankelijk van de 'contact-tijd' tussen de benzo[a]pyreendeeltjes en het ademhalingswegepitheel, die wordt bepaald door de dosis benzo[a]pyreen en de grootte van de benzo[a]pyreendeeltjes.
(dit proefschrift)

4. Hamstertrachea-orgaancultures vormen een beter model voor het bestuderen van benzo[a]pyreen-DNA adductvorming en DNA-repair processen dan rattetrachea-orgaancultures.
(dit proefschrift)

5. Alhoewel veel onderzoekers steeds minder belang hechten aan de initiatiefase van het kankerproces, richt men zich bij onderzoek van biomarkers met een voorspellende waarde voor het ontstaan van kanker juist op parameters die betrokken zijn bij de initiatiefase.

6. De verschuiving van de incidentie van adenocarcinomen in de long ten opzichte van de incidentie van plaveiselcelcarcinomen is het gevolg van het toegenomen gebruik van filtersigaretten.
(Wynder and Hoffman, 1994, Cancer Research 54, 5284-5295)

7. De immunocytochemische bepaling van celproliferatie met behulp van antilichamen gericht tegen het "proliferating cell nuclear antigen" leidt tot een overschatting van de celproliferatie.

8. Het aannemen van gewoontes die een nadelige invloed hebben op de gezondheid is een negatief aspect van emancipatie.

9. Door de grote nadruk die bij solliciteren wordt gelegd op "netwerken" wordt de aandacht ten onrechte verschoven van kennis naar kennissen.

10. "Zwervende wetenschappers" leveren geen bijdrage aan de continuïteit van onderzoek binnen laboratoria.

11. Objectieve wetenschap bestaat niet; wetenschap is juist geworteld in maatschappelijke belangen, waardoor feiten gewoonlijk doorspekt zijn met waarden en normen. (wetenschapsfilosoof Bruno Latour, Volkskrant 1 oktober 1994)

Stellingen behorend bij het proefschrift:
'Effects of vitamin A and β-carotene on respiratory tract carcinogenesis in hamsters - in vivo and in vitro studies'.
André Wolterbeek, Wageningen 10 mei 1995.

Voor Hetty
Voor mijn ouders

Contents

Chapter 1

GENERAL INTRODUCTION

1.1 Scope of this thesis

Cancer is a major cause of morbidity and mortality throughout the world. Evidence has accumulated from epidemiological studies that relate life-style factors to the aetiology of some of the most common cancer types. Doll and Peto (1) showed that diet, alcohol and tobacco may account for about 70% of all cancer deaths in the USA. Although it has frequently been shown that the diet is an important factor influencing cancer (2-5), the association between the intake of specific nutrients and cancer risk is less clear. Various dietary components influencing cancer are summarized in Table I. By modifying the process of carcinogenesis, these components are able to increase or decrease the risk of cancer.

Lung cancer is the major cause of death by cancer in Western countries (6-9) and therefore, considerable effort has been directed towards identifying factors that induce lung cancer or protect against lung cancer. It has been shown that cigarette smoking is the main cause of lung cancer; about 90% of lung cancers are attributable to smoking (10-14). For protection against lung cancer, one of the promising dietary components is (pro)vitamin A (vitamin A and β-carotene) (15-17). The protective effects of vitamin A and β-carotene against respiratory tract cancer has been shown in a large number of epidemiological and experimental studies (18-23). However, the results are not always consistent; some studies have failed to show such a protective effect (24,25), or even showed an enhancing effect of vitamin A and β-carotene on the incidence of respiratory tract cancer (26,27). In spite of the sometimes promising results, the molecular mechanisms by which (pro)vitamin A influences the development of respiratory tract cancer are still not fully understood. Elucidation of these mechanisms might enhance the use of these compounds as anti-cancer agents. The studies described in this thesis are an attempt to better understand the mechanism by which vitamin A and β-carotene affect chemical-induced (pre)neoplastic changes in the respiratory tract.

1.2 Respiratory tract cancer

1.2.1 Introduction

Lung cancer is the leading form of cancer in nearly all parts of the world, particularly in the Western countries (6,7,10). The incidence of lung cancer and the mortality due to

Table I: Effect of various nutritional components on carcinogenesis[1,2].

Components	Effect on cancer	Organ
Vitamins		
Vitamin A and β-carotene	protection	Bladder, skin, mammary gland, respiratory tract, colon
Vitamin C	protection	Oesophagus, stomach, cervix
Vitamin D	protection	Colon, blood
Vitamin E	protection	Mammary gland, respiratory tract, intestine
Amino acids		
Methionine	protection	Liver, mammary gland, pancreas, colon
Minerals		
Calcium	protection	Colon
Iron	protection	Oesophagus, stomach,
Selenium	protection	Colon, liver, skin, stomach, mammary gland, ovary, rectum, respiratory tract.
Zinc	protection	Oesophagus
Other		
Proteins	aggravation	Mammary gland, prostate, colon, kidney, pancreas
Carbohydrates	aggravation	Pancreas, liver, mammary gland
Lipids	aggravation	Mammary gland, colon, pancreas, skin
Alcohol	aggravation	Head, neck, oral cavity, pharynx, oesophagus, larynx, stomach
Fibre	protection	Colon, mammary gland, pancreas, prostate

1) Data from references 2-5

2) The protective or aggravating effect of the components on the development of cancer in the target organs listed is not equally clear for each organ. Furthermore, the intake of each component is frequently accompanied by the consumption of other components, making it difficult to ascribe an effect to one particular component.

Table II: Incidence and mortality of (lung) cancer in the Netherlands in the period of 1980 - 1992

	1980		1985		1989		1990		1991		1992	
	M[2]	F	M	F	M	F	M	F	M	F	M	F
incidence of cancer[1,3]	-	-	-	-	449	313	444	318	445	320	-	-
incidence of lung cancer[1,3]	-	-	-	-	109	17	109	17	109	19	-	-
lung cancer as % of all cancer	-	-	-	-	24	5	25	5	25	6	-	-
mortality[1,4]	900	720	880	690	850	680	830	670	820	670	800	660
mortality due to cancer[1,4]	260	175	268	192	274	204	269	202	270	204	272	203
mortality due to lung cancer[1,4]	99	9	103	13	100	16	95	16	96	17	95	18
mortality due to lung cancer as % of total mortality	11	1	12	2	12	2	12	2	12	3	12	3
mortality due to lung cancer as % of mortality due to cancer	38	5	38	7	37	8	35	8	36	8	35	9

[1)] data of (lung) cancer incidence and mortality are expressed per 100,000

[2)] M; male; F; female

[3)] data from Vereniging van Integrale Kankercentra (28,29).

[4)] data from Centraal Bureau voor de Statistiek (30,31)

lung cancer in the Netherlands from 1985 to 1991 is shown in Table II. Both the incidence and the mortality in the Netherlands appeared to be one of the highest in the world (28,29,32). Epidemiological studies show a clear correlation between smoking and lung cancer (6,7,8,10). Smoking appears to be the main cause of human lung cancer; more than 90% of deaths from lung cancer and about 40% of deaths from all cancers can be attributed to smoking (10-14). Furthermore, albeit to a much lesser extent, environmental exposure to heavily polluted air (33-35) and occupational exposure to various carcinogenic agents (36-38) may contribute to the high incidence of lung cancer.

There are four major histological types of lung cancer arising in the epithelium of the respiratory tract: squamous cell carcinoma (representing 45% of all lung cancer cases in men and 22% in women), adenocarcinoma (men 15%; women 29%), small cell carcinoma (men 18%; women 25%), large cell carcinoma (men 14%; women 16%) and other types (men 8%; women 8%) (28). The difference in distribution of histological type between men and women, especially with regard to squamous cell carcinoma and adenocarcinoma, is generally explained by differences in smoking habits between the sexes. However, other factors such as hormones and differences in environmental exposure to carcinogenic compounds may also play a role (39-41).

At the molecular level it has been demonstrated that, depending on histological type, lung cancers are characterized by multiple genetic changes in growth regulatory genes, including activation or overexpression of several proto-oncogenes (erb, fod, jun, myc, raf, ras) as well as inactivation of several tumour suppressor genes (Rb, p53, RAR-β, PTP-γ) (42-47). Other aspects such as the development of drug resistance (43) and the over-production of growth factors and their receptors (46,48) are also considered to play a role in lung carcinogenesis. Frequently observed mutations in oncogenes and suppressor genes in respiratory tract cancer are G:C to T:A transversions (42,49,50). A relationship has been shown between this specific mutation and cigarette smoking (51,52).

Although 90% of all lung cancers are attributable to smoking, only 10% of all smokers develop lung cancers. Recently, it has been shown that genetic variability might determine the individual susceptibility to respiratory tract carcinogens. A high expression of the cytochrome P450 1A1 gene, responsible for the activation of some carcinogenic compounds in cigarette smoke, is associated with a higher risk for development of lung cancer. Furthermore, there is a negative correlation between the expression of GST-μ, involved in the detoxification of some carcinogenic compounds, and the incidence of lung cancer (53-58).

To study experimental respiratory tract cancer, several *in vivo* and *in vitro* models have been developed. The models used in this thesis are described in Chapter 2. In these models, polycyclic aromatic hydrocarbons, in particular benzo(a)pyrene, are frequently used to induce cancer.

1.2.2 Polycyclic aromatic hydrocarbons

In cigarette smoke, about 4000 chemicals have been identified of which about 40 are known carcinogenic compounds, including tobacco-specific nitrosamines, aromatic amines and polycyclic aromatic hydrocarbons (PAHs) (59,60).

According to the International Agency for Research on Cancer (61), there is evidence that some PAHs are carcinogenic to experimental animals. PAHs themselves are lipid-soluble, non-carcinogenic and chemically inactive compounds which tend to accumulate in the organism. To facilitate the excretion of PAHs, they are metabolized to more hydrophilic compounds, which are more water-soluble. The metabolism of various PAHs has extensively been investigated (62-66). The initial step in the metabolism of PAHs is the formation of an epoxide by cytochrome P450-dependent monooxygenases, as is presented schematically in Figure 1. Further metabolism of these epoxides results in hydration to dihydrodiols by epoxide hydrolase, isomerization to phenols or conjugation with glutathione by glutathione *S*-transferases. Glutathione conjugates can be easily excreted as mercapturic acid. Since, the dihydrodiols and phenols are not sufficiently hydrophilic to be excreted, these metabolites are conjugated with glucuronic acid by glucuronyl-transferases or with sulphuric acid by sulphotransferases to facilitate excretion. As a consequence of the metabolism of PAHs, highly reactive intermediate metabolites can be formed that react easily with cellular components, such as proteins, RNA and DNA.

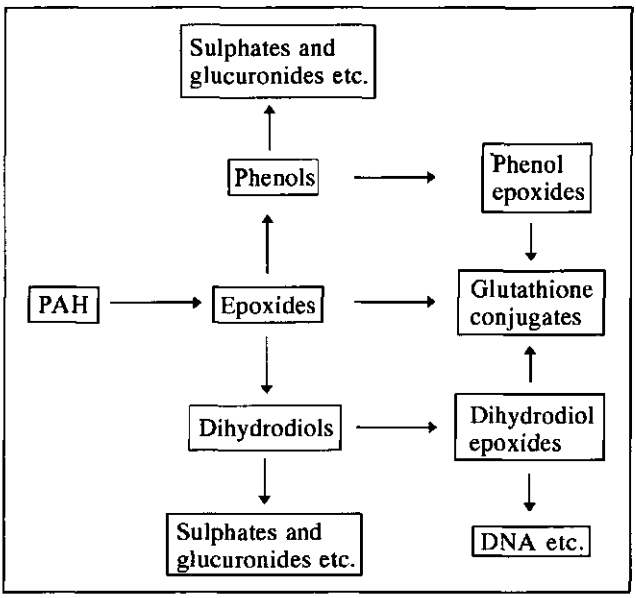

Figure 1: Schematic presentation of PAH metabolism (adapted from reference 64)

1.2.3 Benzo(a)pyrene

One of the most extensively studied PAHs is benzo(a)pyrene [B(a)P], which has been classified by the IARC (61) as carcinogenic in experimental animals and as suspect carcinogenic in humans. Human exposure to B(a)P largely derives from industrial activities and life-style factors. The most important routes of B(a)P exposure are inhalation, oral ingestion and dermal absorption (67-68). Respiratory tract exposure to B(a)P through inhalation of cigarette smoke, polluted indoor and outdoor air and in the occupational setting can be very substantial. In cigarette smoke, the concentration of B(a)P ranges from 20 to 40 ng per cigarette in mainstream smoke and from 40 to 80 ng per cigarette in sidestream smoke (69). In a study of Lioy *et al.* (68) it was shown that the indoor concentration of B(a)P ranges from 0.1 to 8.1 ng/m^3 depending on smoking habits, stove and heating system. The environmental outdoor concentration of B(a)P is <1 ng/m^3 in clean air and can be as high as 100 ng/m^3 in heavily polluted air (33). In the working environment, B(a)P can be released in the atmosphere by, among others, the heating of coal tar products in industrial processes such as aluminium melting and steel and asphalt production (70-73). Hemminki *et al.* (71) have shown workers in a Polish cokerie to be heavily exposed to B(a)P in the working environment (90 μg B(a)P/m^3).

Figure 2 shows the metabolism of B(a)P. B(a)P can be metabolized to the very reactive metabolite (+)-*anti*-B(a)P-7,8-dihydrodiol-9,10-epoxide (BPDE). This metabolite has been shown to bind covalently to DNA of target cells, preferentially to the guanine nucleotide (74,75). The major guanine adduct is formed by the binding of the C-10 of (+)-*anti*-BPDE with the 2-amino group of guanine (BPDE-dG adduct, see Figure 2) (62-65). The binding of carcinogenic compounds, such as B(a)P, with DNA is considered to be a critical step in tumour initiation (76-78). The presence of DNA adducts or improperly repaired DNA damage during cell proliferation may result in permanent changes in DNA (Figure 3). If these mutations occur in critical growth-regulating genes, such as proto-oncogenes and tumour suppressor genes, this alteration may lead to the development of cancer. The main mutation induced by BPDE are G:C to T:A transversions (80,81), as has been frequently observed in the growth-controlling genes in human lung cancers (42,49,50). This indicates that B(a)P may be an important component in cigarette smoke for the induction of human lung cancer. Several epidemiological studies demonstrate a negative correlation between the dietary intake of (pro)vitamin A and the incidence of respiratory tract cancer. Furthermore, in experimental studies it has been shown that the development of B(a)P-induced respiratory tract cancer is inhibited by (pro)vitamin A.

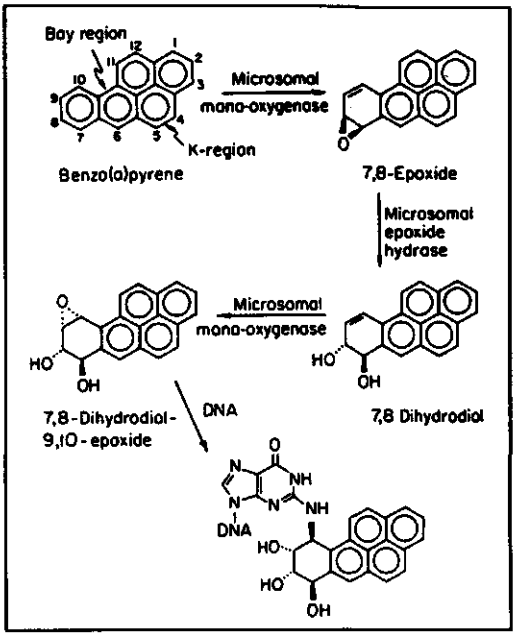

Figure 2: Metabolism of B(a)P and binding to DNA (source reference 74)

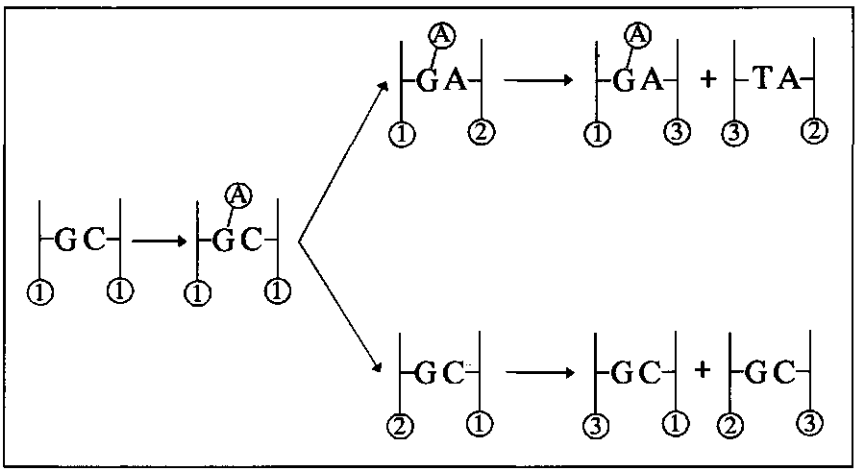

Figure 3: Fixation of a permanent change in the DNA. DNA synthesis in the presence of a damaged nucleotide results in a G:C to T:A transversion (adapted from reference 79).

1.3 (pro)Vitamin A and respiratory tract cancer

1.3.1 (pro)Vitamin A

Vitamin A belongs to the class of retinoids. Retinoids are a class of compounds consisting of four isoprenoid units joined in a head-to-tail manner (Figure 4). The term vitamin A should be used to describe all compounds that exhibit qualitatively the biological activity of retinol, whereas the term retinoids includes all natural forms of vitamin A as well as the many synthetic analogues of retinol, both biologically active or inactive forms. Some carotenoids are naturally occurring precursors of vitamin A, consisting of eight isoprenoid units. The most important carotenoid is β-carotene, exhibiting the highest vitamin A activity in man (Figure 4).

Animals and humans are not capable of *de novo* synthesis of vitamin A. The major dietary sources for vitamin A are retinyl esters from animal sources and β-carotene from green and yellow plants. Retinoids play an essential role in the control of cellular differentiation and proliferation of epithelial cells in various parts of the body. These cells show biochemical, morphological and functional changes in response to the absence or abundance of retinoids (83-86). Moreover, retinoids play a role in a variety of biological processes, including vision, reproduction, growth and development (87-89), and retinoids are required for a proper functioning of the immune system (90). Because of the serious effects caused by an excess or deficiency of vitamin A, the concentration of vitamin A in the plasma and at the cellular level is strictly regulated. In this process, the liver plays a major role as is schematically shown in Figure 5 which shows the major pathways for vitamin A uptake and transport in the body (91-93).

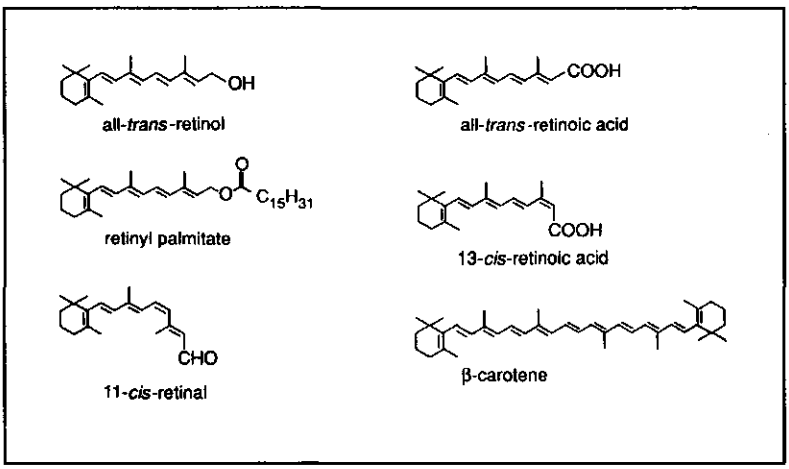

Figure 4: Structural formulas of β-carotene and some naturally occurring retinoids (source reference 82)

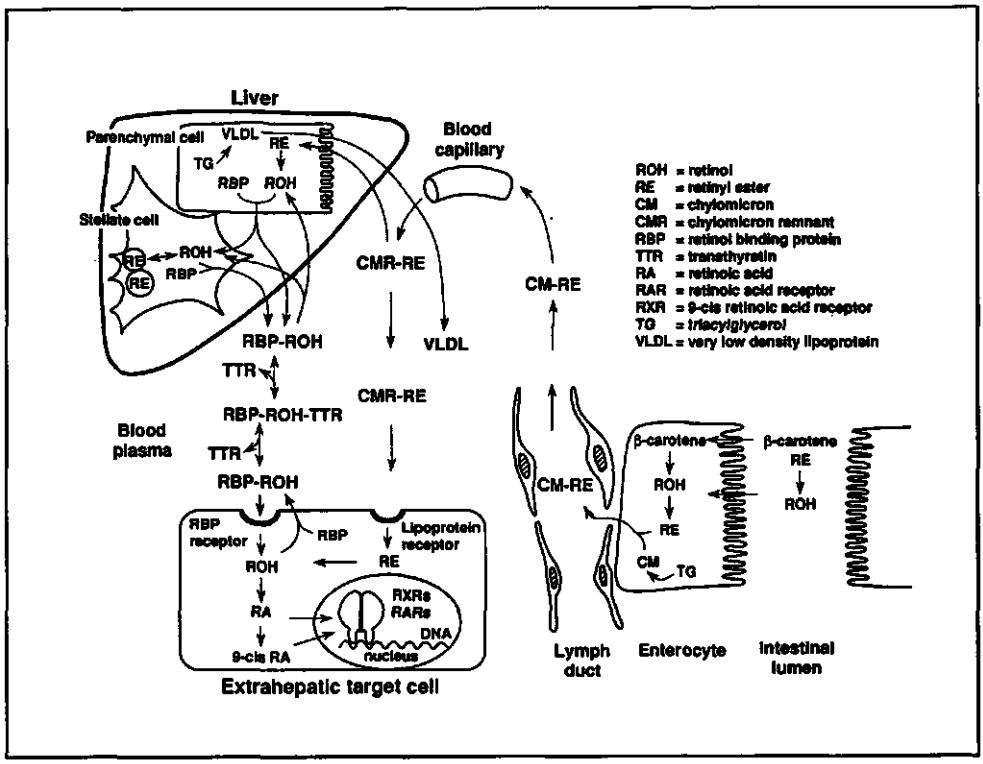

Figure 5: Major pathways for retinoid transport uptake and transport in the body. REs are hydrolysed to ROH before absorption. Carotenoids are partially converted to ROH in the enterocytes, where ROH is esterified and incorporated into CMs together with TGs. β-carotene can also be absorbed intact, and transported by CM in the lymph (not shown in figure). CMs reach the general circulation via the intestinal lymph. CMRs contain almost all the absorbed retinol, and are cleared mainly by the liver parenchymal cells. In the liver parenchymal cells, REs are hydrolysed to ROH, which binds to RBP. Most of the ROH in the liver parenchymal cell is transferred to stellate cells, which store REs in lipid droplets. Most of the RBP-ROH secreted from the liver is complexed with TTRs in plasma. The RBP-ROH is presumably taken up by cell surface receptors. In cells some ROH is metabolized to all-*trans* retinoic acid, and other retinoic acid isomers and derivates (9-*cis* retinoic acid and 3,4-didehydroretinoic acid), which are ligands for nuclear receptors like RARs and RXRs (from reference 93).

Although the pivotal role of retinoids in various vital processes has been known for a long time, only in the past 10 years the molecular mode of action of retinoids has become more clear. A major step forward in understanding the molecular mechanism mediated by retinoids was the discovery of two nuclear receptors for retinoids (see also Figure 5). Both are members of the superfamily of ligand-inducible transcriptional regulators, which activate the transcription of several genes by binding to specific DNA sequences (hormone

responsive elements). The first subfamily of receptors has a high affinity to all-*trans*-retinoic acid; these receptors are called retinoic acid receptors (RARs). The second structurally different subfamily of receptors has a high affinity to 9-*cis*-retinoic acid; they are called retinoic X receptors (RXRs) (84,94,95). A major mechanism by which retinoids influence the expression of proteins involves the direct (de)activation at the transcriptional level of various genes (84,96). The effects of retinoic acid on the process of cellular differentiation and proliferation result primarily from the ability of this molecule to regulate the expression of specific genes including several cytokeratines (97-98), proto-oncogenes, tumour suppressor genes (99) and growth factors and their receptors (100) at either the transcriptional or post-transcriptional level.

1.3.2 Modifying effect of (pro)vitamin A on chemically induced respiratory tract cancer

During the past decades, several epidemiological and experimental studies have shown an inverse relationship between vitamin A and β-carotene consumption and the risk of respiratory tract cancer.

Bjelke was one of the first to report epidemiological evidence showing a protective effect of vitamin A against lung cancer (18). Later, more epidemiological studies showed that vitamin A and, especially, β-carotene protects against respiratory tract cancer in man (17,23,101).

In experimental studies with animals, an important role for vitamin A in cancer was already suggested in 1925 by Wolbach and Howe (86) who showed histopathological resemblances between the epithelia of vitamin A-deficient organs and neoplastic tissues. Later, many reports demonstrate that consumption or application of vitamin A and β-carotene protects against the formation of chemically-induced respiratory tract cancer (19-22,102). However, the results of both epidemiological and experimental studies have been equivocal and in some cases even contradictory (24-26,103). The results of experimental studies appeared to be strongly dependent on experimental conditions, such as animal species, carcinogen and retinoid used, time, dose and route of administration of carcinogen and retinoid, dose regimen and animal housing conditions. Saffiotti *et al.* (22) were the first to show a protective effect of supplemental retinoid treatment against B(a)P-induced respiratory tract cancer in hamsters. The incidence of lung tumours induced by once-weekly intratracheal intubations of B(a)P adsorbed to ferric oxide for 10 weeks was reduced by 20% by twice weekly intragastric instillations of 5 mg retinyl palmitate, beginning 7 days after the last treatment and continuing during life-span. Other investigators have not been as succesful in using (pro)vitamin A to prevent the development of respiratory tract cancers induced by B(a)P. In studies of Beems (24,25), no effects of a vitamin A-deficient diet or a diet rich in vitamin A or β-carotene on B(a)P-induced lung cancers in hamsters were observed. Smith *et al.* (26) even showed an enhancing effect of

intragastrically instilled retinyl acetate (twice weekly 2400 μg) on B(a)P-induced respiratory tract tumours in hamsters.

In vitro, it has been shown that vitamin A strongly influences cellular proliferation and differentiation in tracheal epithelium in organ culture (83,85). Several studies showed a protective effect of (pro)vitamin A against the formation of chemically induced hyperplasia and squamous metaplasia, precursors of respiratory tract tumors *in vivo*, in tracheal epithelium (104-106).

Although many studies have shown more or less protective effects of (pro)vitamin A against respiratory tract cancer, the molecular mechanism of action of (pro)vitamin A remains largely unknown. To better understand the effects of (pro)vitamin A on respiratory tract cancer at the molecular level, it is necessary first to describe the process of carcinogenesis in general. The generally accepted theory today is that cancer is a multi-stage process, divided into 3 stages referred to as initiation, promotion and progression (Figure 6).

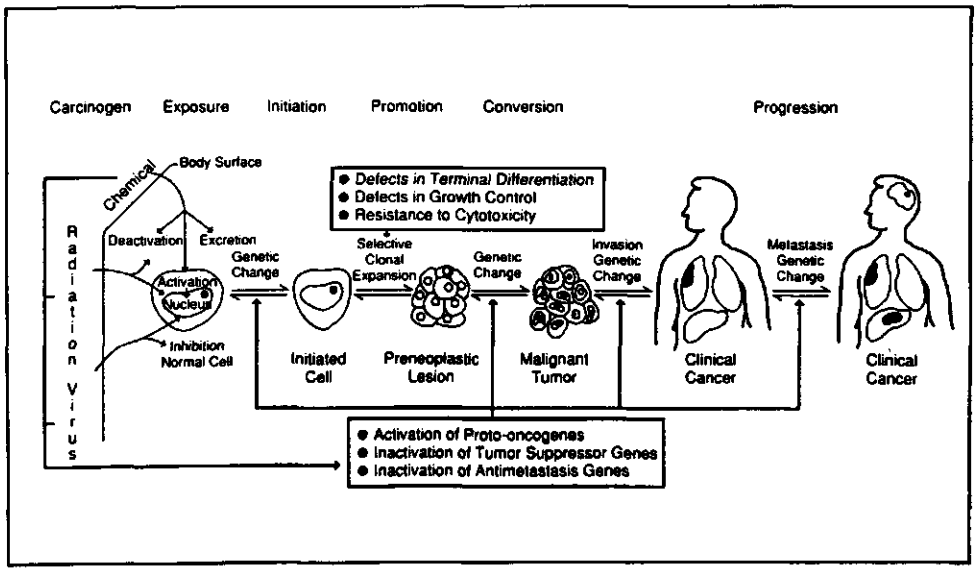

Figure 6: Schematic presentation of multistage lung carcinogenesis (from reference 107)

The initiation phase involves exposure of normal cells to agents that can cause genetic changes in critical target genes. As a consequence, the initiated cells have an altered responsiveness to their microenvironment and exert a selective clonal expansion advantage compared with the surrounding normal cells. Generally, initiated cells are less responsive

to growth regulating mechanisms, such as negative growth factors and inducers of terminal differentiation. In the promotion phase the proliferation and survival of initiated cells is enhanced, increasing the selective clonal expansion. Due to the enhanced cellular proliferation of initiated cells, the probability of accumulating additional genetic changes is increased. The progression phase is characterized by increased changes in growth-regulating genes associated with cellular proliferation, ultimately resulting in an invasive and malignant phenotype (107-108).

(pro)Vitamin A has been shown to modify each of the three stages of respiratory tract cancer. It influences the initiation phase by preventing or decreasing DNA damage caused by carcinogenic agents. In general, the inhibition of DNA damage by (pro)vitamin A is ascribed to a specific effect on the metabolism of carcinogenic compounds, resulting in a decreased amount of reactive metabolites (109-112). Moreover, (pro)vitamin A has also been shown to protect against cancer through scavenging of free radicals (113,114). The majority of research into the effect of (pro)vitamin A on lung cancer has been directed to the promotion phase, in particular to the effect of (pro)vitamin A on cellular proliferation and differentiation. *In vitro*, it has been shown that vitamin A inhibits the formation of squamous metaplasia and hyperplasia in tracheal epithelium in organ culture treated with chemical carcinogens such as B(a)P and cigarette smoke condensate (104-106,114,115). In addition, (pro)vitamin A has been shown to exert a variety of effects on glycoprotein and glycolipid synthesis in the cell membrane, resulting in an effect on cell adhesion properties (117,118) and cellular communication (119-121). Furthermore, it has been shown that (pro)vitamin A is able to counteract the development of cancer by increasing both the humoral and cell-mediated immune response (90,122,123)

1.4 Objectives of the studies described in this thesis

The studies described in this thesis were carried out in the TNO Nutrition and Food Research Institute. This institute has a long-standing history in research on chemically-induced respiratory tract cancer and the effects of nutritional components on its development (124-126). The studies described in this thesis mainly deal with the effect of vitamin A (all-*trans* retinol) and β-carotene on B(a)P-induced (pre)neoplastic changes in the respiratory tract of Syrian golden hamsters. Using both an *in vivo* and an *in vitro* approach, described in Chapter 2, the effects of vitamin A and β-carotene on various stages in the process of respiratory tract cancer were investigated. The emphasis in these studies was laid on the effects of vitamin A and β-carotene on B(a)P-induced DNA-adduct formation, DNA-repair activities, cell proliferation and histopathological changes in hamster tracheal epithelium. Furthermore, the relationships between DNA-adduct formation, DNA-repair activities, cell proliferation and the expression of the tumour suppressor gene p53 were investigated. Where possible, the role of specific tracheal epithelial cells in these processes was determined.

This thesis contains 11 Chapters. Chapter 1 is a general introduction to the scope of the thesis. An introduction to the technical approaches is given in Chapter 2. The *in vivo* model used was subjected to a critical analysis to display its strengths and limitations (see Chapters 7, 8 and 9).

In Chapter 3, the relation between the formation and repair of B(a)P-DNA adducts in hamster tracheal epithelial cells is investigated. Furthermore, DNA adducts formed *in vitro* and *in vivo* were qualitatively compared. In Chapter 4, both quantitative and qualitative comparisons are made between the formation and repair of B(a)P-DNA adducts in tracheal epithelium of hamster and rat.

Chapters 5 and 6 deal with the effects of vitamin A and β-carotene on the formation and repair of B(a)P-induced DNA damage and on cell proliferation in hamster tracheal epithelium. Furthermore, the studies described in Chapter 6 concern the influence of medium components and growth factors on cell proliferation.

Chapter 7 describes a study originally planned to investigate the effect of vitamin A and β-carotene on B(a)P-induced respiratory tract cancer in hamsters. Since no reasonable tumour response was obtained, an effect of vitamin A and β-carotene could not be studied. However, a remarkable finding in this study was a very low mortality of hamsters fed the high-β-carotene diet.

Chapters 8 and 9 describe a second *in vivo* experiment into the possible effect of a high dietary level of β-carotene on B(a)P-induced respiratory tract cancer in hamsters. In Chapter 8, the molecular aspects (DNA adducts, cell proliferation and p53 expression) are described, while Chapter 9 deals with the histopathological examinations, including cytokeratin and glutathione *S*-transferase isoenzyme Pi expression.

A summary and concluding remarks are presented in English in Chapter 10 and in Dutch in Chapter 11.

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Chapter 2

INTRODUCTION TO THE TECHNICAL APPROACH

2.1 *In vivo* hamster intratracheal instillation model[1]

Summary

Several experimental models have been developed to study respiratory tract carcinogenesis. The most widely applied *in vivo* model uses Syrian golden hamsters which receive intratracheal instillations of a suspension of benzo[a]pyrene (B[a]P) particles attached to ferric-oxide (Fe_2O_3) particles in saline; it was first described by Saffiotti and co-workers [1]. This model has several benefits compared with other experimental models, however, the large number of variables affecting the tumour response is a clear disadvantage because the tumour response is difficult to control. In this review, we describe a systematic analysis of various variables that may influence the tumour response of the respiratory tract with the aim to further standardize the method and increase, through that, its suitability and predictabilty. The most important variables influencing the tumour response, as shown by statistical analysis of 29 representative studies, turned out to be the administered dose and the particle size. Both variables influence the actual dose and the contact-time of the B[a]P particles with the target cells. The present analysis of literature data does not support the widespread opinion that ferric-oxide particles enhance the tumour response of the respiratory tract. Some recommendations for increasing the predictability of the model are made.

Introduction

The search for an adequate experimental animal model capable of reproducing the main characteristics of human lung cancer had been largely unsuccesful for several decades when in 1968 Saffiotti *et al.* [1] published their landmark paper on an effective method for the induction of tracheobronchial carcinomas in Syrian golden hamsters. They used repeated intratracheal instillations of a saline suspension of fine crystalline benzo[a]pyrene (B[a]P) particles attached to the carrier ferric oxide. Clear benefits of the Saffiotti method in comparison with other methods [2,3] for the induction of respiratory tract cancer are: a) extensive tissue damage could be avoided, b) no special (organic) solvents were required

[1] This section is based on : Wolterbeek A.P.M., Schoevers E.J., Rutten A.A.J.J.L. and Feron V.J. (1995) A critical appraisal of intratracheal instillation of benzo[a]pyrene to Syrian golden hamsters as a model in respiratory tract carcinogenesis. *Cancer Letters,* **89**, 107-116.

for administration of the carcinogen, c) the route of administration closely resembled that of human exposure to respiratory tract carcinogens and d) the morphology and spectrum of the induced lesions were close to those observed in humans. Syrian golden hamsters were selected because: a) histologically the respiratory tract of the hamster resembles quite well the human airway system, b) the morphology of the induced lesions in hamsters is close to that of human lesions, c) spontanous lung tumours are rarely observed in hamsters and d) hamsters have a relatively large resistance to chronic respiratory diseases.

In spite of the great benefits of the Saffiotti method and its broad applicability, a major disadvantage of the method is the large number of variables affecting the response of the respiratory tract rendering it thereby difficult to control this response with respect to type, degree, incidence and time-to-appearance of (pre)neoplastic changes. These variables can be divided into 3 groups; variables influencing 1) the retention of B[a]P particles, 2) the proliferation of respiratory tract epithelial cells, and 3) the metabolism and DNA-binding characteristics of B[a]P.

As a follow up to a recently published study into the effect of β-carotene on B[a]P-induced respiratory tract cancer in which a very low tumour response was observed [4], in the present paper we will evaluate variables regarding their role in the induction of (pre)neoplastic respiratory tract lesions in hamsters by intratracheal instillations of B[a]P. Major variables evaluated are dose of B[a]P, size of B[a]P particles, duration of exposure to B[a]P, and type of carrier for B[a]P particles. Minor variables considered are physiochemical properties of the suspension, tissue injury, respiratory tract infections, age, gender and nutritional factors.

Major variables

Dose of B[a]P

Table I shows that the total dose of B[a]P administered in 29 typical respiratory tract carcinogenicity studies in Syrian golden hamsters ranges from 3.25 to 111 mg per animal. Statistical analysis of the data in Table I revealed that the total dose is a dominant factor in determining tumour response. A significant positive correlation was found between total-B[a]P dose and tumour response (Table I and Fig 1; linear regression: $P=0.001$, $r=0.41$). This overall picture is strongly supported by the results of several individual studies showing a positive correlation between the dose of B[a]P and the tumour response [5-8]. Moreover, indirect evidence of the B[a]P dose being of crucial significance for the induction of respiratory tract tumours was obtained from a study by Henry *et al.* [9] who showed that the retention of B[a]P particles of similar size in the respiratory tract of hamsters increased with increasing B[a]P dose (Table II, upper part). Furthermore, statistical analysis of the data in Table I revealed a statistically significant negative correlation between total dose and latency time (time to appearance of first tumour; Table

I and Fig 1; linear regression: P=0.006, r=-0.41).

Particle size of B[a]P

The size of the B[a]P-particles instilled intratracheally has been shown to be an important variable in inducing respiratory tract tumours: the larger the particles the greater the tumour response. Statistical analysis of the data presented in Table III showed that the tumour response is largely determined by the size of the B[a]P particles (Table III; linear regression: P=0.003, r=0.74). This observation is indirectly confirmed by the data presented in the lower part of Table II, showing that larger B[a]P particles are cleared from the respiratory tract of hamsters much more slowly than smaller ones.

Duration of exposure to B[a]P

The period during which hamsters are exposed to B[a]P is a third factor that may affect the tumour response of the hamster respiratory tract (Table I). Statistical analysis did not show any effect of exposure time on tumour response (linear regression: P=0.71, r=-0.05). However, there was a significant correlation between exposure time and latency period (linear regression: P<0.001, r=0.52).

B[a]P particles attached to Fe_2O_3 particles

Fe_2O_3 particles were originally used to enhance penetration of B[a]P particles into the respiratory tract and to reduce their clearance from the respiratory tract [1]. However, Feron *et al.* [10] clearly showed that the clearance of B(a)P particles was not significantly influenced by Fe_2O_3 particles; Persson *et al.* [11] even showed that the B[a]P recovery was decreased by Fe_2O_3. Furthermore, statistical analysis of the data shown in Table I did not reveal a significant difference in tumour incidence between hamsters treated with a suspension of B[a]P attached to Fe_2O_3 as compared with animals treated with B[a]P alone (Student's t-test: P=0.69). However, latency time was clearly decreased in hamsters instilled with a suspension of B[a]P attached to Fe_2O_3 (Student's t-test: P<0.001).

Minor variables affecting tumor response

Physicochemical properties of B[a]P-Fe_2O_3 suspension

The physical properties of B[a]P-Fe_2O_3 suspensions may also influence the retention time of B[a]P particles and thereby the induction of respiratory tract cancer. In a comparative study, Henry *et al.* [12] showed that in hamsters intratracheally instilled with suspensions of Fe_2O_3 particles ground, coated or mixed with B[a]P in 0.5% gelatin solution, tumor incidences were 84%, 73% and 12% respectively. Retention time of B[a]P from the coated and grounded particles was longer than that of B[a]P from the mixed

Table I: Experimental characteristics of 29 respiratory tract carcinogenicity studies in hamsters, tumour latency time and percentage of animals bearing respiratory tract tumours.

Total dose (mg B[a]P)	Numer of instillations	Exposure time (days)	Ferric[b] oxide	Vehicle[c]	Latency time[d] (weeks)	Tumour response (%)	Reference
30.0	10	70	+	S	33	32.0	Saffiotti *et al.* 1967 [22]
45.0	15	105	+	G	10	64.0	Saffiotti *et al.* 1968 [1]
36.0	36	252	-	S	40	66.0	Feron 1972 [31]
5.0	1	1[a]	+	S	52	3.7	Saffiotti *et al.* 1972 [47]
15.0	5	35	+	S	77	14.0	
15.0	5	125	+	S	30	14.5	
30.0	10	70	+	S	20	28.5	
30.0	10	70	+	S	32	41.5	
37.5	1	1[a]	+	S	28	15.0	
45.0	15	105	+	S	10	62.0	
45.0	15	105	+	S	15	65.0	
7.5	30	210	+	S	22	11.4	Saffiotti *et al.* 1972 [6]
15.0	30	210	+	S	42	30.0	
30.0	30	210	+	S	22	66.0	
60.0	30	210	+	S	12	60.0	
3.25	52	364	-	S	78	10.0	Feron *et al.* 1973 [5]
6.5	52	364	-	S	78	13.0	
13.0	52	364	-	S	70	30.0	
26.0	52	364	-	S	57	86.0	
52.0	52	364	-	S	50	93.0	
1110	8	56	-	G	18	40.0	Henry *et al.* 1973 [9]
30.0	10	140	+	S	50	39.0	Sellakumar *et al.* 1973 [29]
30.0	10	140	+	S	40	40.0	
30.0	10	140	+	S	10	44.0	
100.0	25	175	+	G	25	45.8	Farrell *et al.* 1974 [30]
100.0	25	175	+	G	25	50.0	
100.0	25	175	+	G	25	51.0	
100.0	25	175	+	G	25	51.0	
45.0	15	105	+	S	28	68.5	Feron 1975 [48]
26.1	30	210	+	G	50	7.3	Henry *et al.* 1975 [12]
26.3	30	210	+	G	15	12.0	
26.4	30	210	-	G	50	17.0	
27.4	30	210	+	G	15	84.0	

Table I: continued

Total Dose (mg B[a]P)	Number of Instillations	Exposure time (days)	Ferric[a] oxide	Vehicle[c]	Latency time[d] (weeks)	Tumour response (%)	Reference
30.0	30	210	-	S	24	50.0	Kobayashi 1975 [49]
36.0	12	84	+	S	12	58.0	Smith *et al.* 1975 [44]
36.0	12	84	+	S	22	60.0	Smith *et al.* 1975 [45]
45.0	15	105	+	S	24	70.0	Stenbäck *et al.* 1975 [50]
30.0	10	70	-	S	-	15.0	Sellakumar *et al.* 1976 [40]
30.0	10	70	+	S	-	71.0	
11.2	8	56	-	G	-	2.0	Little *et al.* 1978 [51]
75.0	15	105	-	G	-	71.0	Nettesheim *et al.* 1978 [33]
75.0	15	105	-	G	-	81.0	
75.0	15	105	-	G	-	84.0	
54.0	18	126	-	S	-	11.0	Stenbäck *et al.* 1978 [52]
54.0	18	126	-	S	-	68.0	
60.0	20	140	-	S	-	11.0	Stenbäck *et al.* 1979 [53]
60.0	20	140	-	G	-	13.0	
26.0	52	364	-	G	-	13.5	
26.0	52	364	-	G	-	35.0	
52.0	52	364	-	G	60	17.5	
52.0	52	364	-	G	48	52.5	
52.0	52	364	-	G	44	51.0	
18.2	52	364	-	S	-	12.5	Feron *et al.* 1982 [8]
36.4	52	364	-	S	65	46.0	
50.0	5	70	+	S	20	36.0	Beems 1984 [42]
60.0	15	105	+	S	20	35.0	Beems *et al.* 1984 [40]
75.0	15	105	-	G	15	32.0	Godleski *et al.* 1984 [54]
26.0	26	364	-	G	-	11.0	Feron *et al.* 1985 [55]
48.0	6	42	+	S	-	42.0	Reijnders *et al.* 1985 [32]
64.0	8	112	+	S	-	68.0	Beems 1986 [46]
64.0	8	112	+	S	-	63.0	Beems 1987 [43]
45.0	15	105	+	S	66	44.0	Keenan *et al.* 1989 [14]

[a] single dose; [b] + = present, - = absent; [c] S = saline, G = gelatin in saline; [d] - = not given in reference

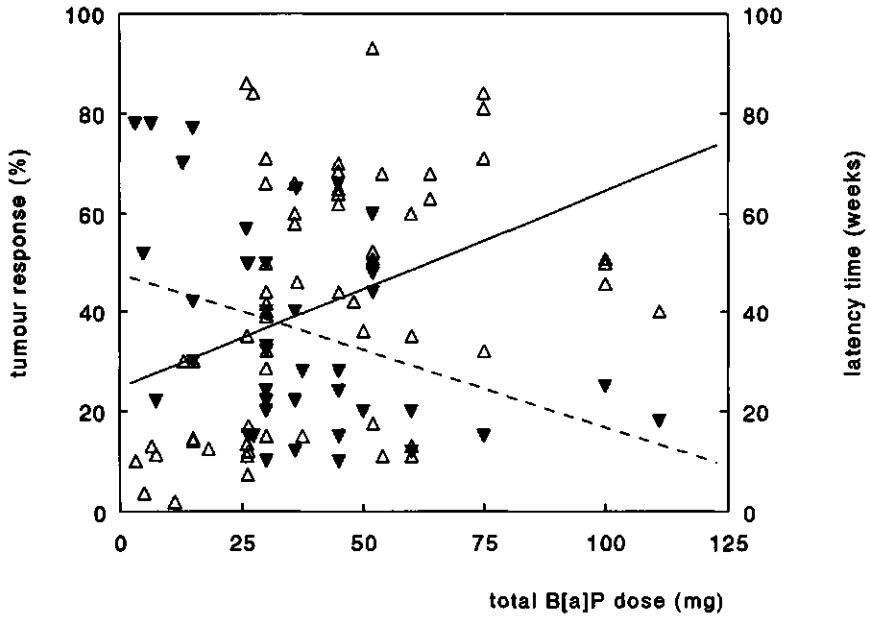

Figure 1: Relationship between total B[a]P-dose administered and the tumour response (left Y-axis, open triangles, line) and latency time (right Y-axis, closed triangles, dotted line). Data are taken from Table I.

particles. However, the differences in retention time and tumour incidence in this study could also be due to the different sizes of the B[a]P-Fe_2O_3 particles (see also Table II and III). Furthermore, the tumour response may have been affected by the vehicle used for administration. However, analysis of all data presented in Table I with respect to type of vehicle (0.9% saline versus 0.02% - 0.5% gelatin in 0.9% saline) as a potential variable did not show any effect of vehicle on tumour response (Student's t-test: P=0.9).

Tissue injury

Since cell proliferation is essential in the formation and progression of (pre)neoplastic changes, the role of epithelial cell injury and the subsequent regenerative hyperplasia in tumour induction have been the subject of many experiments. Keenan et al [13,14] showed that intratracheal intubation as such induced hyperplasia and metaplasia of the tracheal epithelium, and submucosal fibroplasia whereas intralaryngeal intubation did not. In an accompanying carcinogenesis study, intratracheal instillation of N-methyl-N-nitrosourea (MNU) or B[a]P induced tracheal tumours, whereas intralaryngeal instillation caused proliferative lesions and cancer of the larynx. The authors concluded that the effect

Table II: Recovery of B[a]P in the respiratory tract of hamsters following a **single** intratracheal instillation of B[a]P using various dose or different particle size[a].

Dose (mg)	Particle size (μm) Range (median)	Recovery of B(a)P (% of administered dose)		Reference
		16 - 24 h	7 days	
Effect of dose				
2.96	1.0 - 5.0 (2.0)	>1.0		Henry *et al.* 1973 [9]
15.52	1.0 - 5.0 (2.0)	40.0	2.0	
18.16	1.0 - 5.0 (2.0)	58.0	2.0	
Effect of particle size				
2.0	1.0 - 17.5 (17.5)	40.0	7.0	Saffiotti 1970 [56]
2.0	1.0 - 12.0 (9.0)		3.0	
2.0	< 8.5 (5.0)		0.7	
0.87[b]	3.0 - > 15.0 (>15.0)	30.0	5.0	Henry *et al.* 1975 [12][c]
0.91[b]	4.5 - > 15.0 (>15.0)	40.0	4.0	
0.88[b]	2.0 - 10.0 (7.0)	13.0	0.0	
0.88[b]	< 5.0 (2.0)	>1.0	0.0	
3.0	3.0 - 26.0 (13.0)	25.0		Stenbäck *et al.* 1978 [52]
3.0	1.0 - 9.0 (3.5)	1.0		
1.0[b]	1.0 - 5.0 (3.5)	11.5	1.0	Feron *et al.* 1980 [7]
1.0[b]	16.0 - 48.0 (31.0)	51.5	20.5	
1.0[b]	5.0 - 48.0 (19.0)	30.0	1.5	

a) in the study in the upper part of the table the dose varies with similar particle size, in the studies in the lower part of the table the particle size varies with similar dose.

b) B[a]P suspended in saline with gelatine.

c) the B[a]P particles in the various groups did not only differ in size but differed also in physiochemical properties.

Table III: Tumour response of hamsters intratracheally treated with various dose levels of B[a]P particles of different size.

Total dose (mg B[a]P)	Particle size (μm) Range (median)	Response (%)	Reference
26.1	3.0 - >15.0 (>15.0)	73.0	Henry *et al.* 1975 [12][a]
27.4	4.5 - >15.0 (>15.0)	84.0	
26.3	2.0 - 10.0 (7.0)	12.0	
26.4	< 5.0 (2.0)	17.0	
54.0	1.0 - 9.0 (3.5)	11.0	Stenbäck *et al.* 1978 [51]
54.0	3.0 - 26.0 (13.0)	68.0	
60.0	<20.0 (3.4)	11.0	Stenbäck *et al.* 1979 [52]
60.0	<20.0 (3.4)	13.0	
52.0	1.0 - 5.0 (3.5)	17.5	Feron *et al.* 1980 [7]
26.0	1.0 - 5.0 (3.5)	13.5	
52.0	16.0 - 48.0 (31.0)	52.5	
26.0	16.0 - 48.0 (31.0)	35.0	
52.0	5.0 - 80.0 (19.1)	51.0	

[a] The B[a]P particles in the various groups did not only differ in size but differed also in physiochemical properties.

of respiratory tract carcinogens was greatly enhanced by (mechanically induced) changes in epithelial target cell proliferation.

Respiratory tract infections

Respiratory tract infections have been shown to influence chemically-induced respiratory tract cancer. Various mechanisms have been suggested, e.g. local immune competence [15], changes in pulmonary carcinogen metabolism [16], and disturbance of pulmonary clearance [17]. However, findings are rather inconsistent. Although most data suggest enhancement of respiratory tract cancer by infectious agents [15-17], other studies show a decrease in tumour response due to infections [18]. Loss of target cells and changes in the metabolism of carcinogens were considered aspects involved [18].

Age and gender

It has been shown that the respiratory tract of young animals is more sensitive to carcinogens than that of adult animals [19]. The metabolism of carcinogenic compounds is age dependent [20], and young animals have a higher mitotic rate in the respiratory tract epithelium than older animals [21]. However, statistical analysis did not show differences in tumour incidence or latency time between animals younger or older than 10 weeks at the first instillation of B[a]P (data not shown).

Although some studies showed an effect of gender on the incidence of respiratory tract tumours induced by B[a]P in hamsters [7], statistical analysis of all appropriate data did not reveal such an effect of gender (data not shown).

Nutritional factors

Nutritional factors such as vitamin A, fat, fibers and selenium are known to influence chemically-induced respiratory tract carcinogenesis. The possible protective effect of (pro)vitamin A has been studied extensively. Saffiotti et al [22] were the first to report a protective effect of vitamin A against B(a)P-induced respiratory tract cancer in hamsters and several other groups have confirmed this finding [23,24]. Generally, (pro)vitamin A is believed to play an important role in the regulation of epithelial cell proliferation and differentiation [25]. Furthermore, effects have been found on the metabolism of B[a]P [26,27] and, recently, on B[a]P-induced DNA-repair activities [28] resulting in decreased B[a]P-induced DNA-adduct levels.

Discussion

In experimental lung cancer research a number of animal models has been developed based on various ways of tumour induction such as intratracheal instillation of carcinogens, implantation of carcinogen-containing threads or pellets, irradiation, administration of systemically acting carcinogens that induce cancer in the respiratory tract, and transplantation experiments [2,3]. One of these models described by Saffiotti *et al.* [1] has proved to be one of the most convenient and relatively simple *in vivo* methods for studying respiratory tract carcinogenesis. The method has frequently been used for examining the role of carrier particles [29,30], for studying interaction of various respiratory tract carcinogens [8,14,31], for exploring the role of irritants [32], and for studying nutritional factors in respiratory tract carcinogenesis [22-28]. It is in these types of studies that the limitations of the model become most obvious. The data summarized in Table I clearly showed that it is rather difficult to control and predict the tumour incidence. This disadvantage of the model has been clearly demonstrated by Nettesheim *et al.* [2]. Three groups of hamsters treated with the same amount of B[a]P under identical experimental conditions showed significant differences between the groups, particularly with respect to the tumour incidence in various parts of the respiratory tract [2]. Furthermore, using the

Saffiotti model it is difficult to predict the latency time and the type and target site of the tumours induced.

In this review paper, we described a systematic analysis of various experimental variables that may influence the tumour response of the respiratory tract with the aim to further standardize the method and to increase, through that, its suitability, controlability and predictability. Important variables influencing tumour response and latency time, as shown by statistical analysis of variables from 29 representative studies were the administered dose and the particle size. Both administered dose and the particle size largely determine the actual dose and 'contact-time' (retention time) to which epithelial target cells are exposed to B[a]P. In this respect it may be emphasized that Dontenwill *et al.* [33] and Pott *et al.* [34] have demonstrated that B[a]P administered intratracheally to hamsters, as a solution in Sesam oil or polyethyleneoxide, disappeared very quickly form the lungs (24 to 48 hours after instillation of B[a]P the B[a]P content was very low). According to the authors the rapid elimination of the carcinogen from the lungs has to be attributed to the fact that dissolved B[a]P reaches the pulmonary tissue in an absorbable form, whereas suspended B[a]P particles have to dissolve in the lung tissue before absorption can take place. Continuing this line of thoughts it is obvious to assume that small B[a]P particles will disappear more rapidly from the lungs than an equal amount (by weight) of larger B[a]P particles. Suspensions of B[a]P in saline of the type used in the studies by Feron *et al.* [10] contained relatively large B[a]P particles, and their repeated intratracheal instillation has been shown to result in a high incidence of respiratory tract tumours in hamsters [5,7,8]. This strong tumour response was very probably due to the use of relatively large B[a]P crystals which were cleared relatively slowly. In addition it is reasonable to assume that after instillation of a large number of relatively small B[a]P particles, a large area of the respiratory tract is exposed to a relatively low (sub-effective) dose of the carcinogen, whereas following administration of a small number of relatively large B[a]P particles only a small area of the airway system is exposed to a relatively high (effective) dose of the carcinogen. This hypothesis is confirmed by an experiment of Benfield and Hammond [35] who showed that B[a]P-containing sustained release implants (with a prolonged contact-time) induced a high respiratory tract tumour response in hamsters.

Exposure time did not appear to influence the tumour response, but a positive correlation was observed between exposure time and latency period.

Mechanical tissue damage caused by the intratracheal intubation itself appeared to play an important role in the enhancement of tumor response. Tissue damage may also increase the susceptibility of the hamsters to respiratory tract infections, which has also been shown to significantly influence the tumour response [15-18].

In accordance with findings of several investigators [10,29], but fully in contrast to the observations of others [36-39], the present analysis did not provide evidence of an enhancing effect of Fe_2O_3 particles on the tumour response of the respiratory tract, leading to the recommendation to use no Fe_2O_3 or only a small amount of Fe_2O_3 particles relative

to the amount of B[a]P. In this way overloading of the lungs with the insoluble Fe_2O_3 dust is prevented.

The effect of nutritional factors in B[a]P-induced respiratory tract carcinogenesis have been studied extensively showing that retinoids [22-28], fat [40] and selenium [41] influence tumour response; however, the results are not always consistent [42-46].

This review confirms the usefulness of the Saffiotti model in research on respiratory tract carcinogenesis. The multitude of (modifying) factors involved in this respiratory tract cancer model is, on the one hand, one of its strenghts because the model offers the possibility to study each of these factors in detail. On the other hand, the involvement of so many factors indicates the vulnerability of the model to the effect that all factors should be carefully controlled to allow prediction of a certain desired response of the respiratory tract in terms of type, degree, incidence and time to tumour appearance. Special care has to be given to dose and size of the B[a]P particles because these factors appeared to be dominant factors influencing the induction of respiratory tract tumours. In accordance with our own experience with the Saffiotti model over the years the present analysis of the model allows the following recommendations: depending on the desired tumour response use a relatively high dose administered in a relatively short period of time, use relatively large B[a]P particles suspended in saline solution containing a low concentration of gelatin, do not use Fe_2O_3 particles or use, in comparison with the amount of B[a]P, a (very) small amount of Fe_2O_3 mixed and shortly ground with B[a]P particles.

2.2 *In vitro* hamster tracheal organ culture model

The trachea consists of three main structures: pseudostratified epithelium, fibroelastic lamina propria and several C-shaped cartilage rings [57]. The pseudostratified tracheal epithelium lining is composed of a heterogeneous, phenotypically different population of four major cell types: basal cells, ciliated cells, small mucous granule cells and mucous goblet cells (Figure 2). Less frequently observed cells are intermediate cells, brush cells, serous cells and neuroendocrine cells (58-60). Ciliated cells, small mucous granule cells and mucous goblet cells rest on the basal lamina and reach the tracheal lumen, whereas basal cells also rest on the basal lamina but do not reach the lumen, resulting in an epithelial lining that looks stratified. The main function of the secretory cells is to produce a viscous glycoprotein mixture which is secreted onto the luminal surface of the epithelial lining. Ciliated cells move the secreted mucous layer together with trapped particles towards the upper parts of the respiratory tract where it is secreted. The lamina propria contains blood vesels, mast cells, fibroblasts, muscle cells and serous glands with excretory ducts (Figure 2), the main function of the lamina propria is providing nutrition for the epithelium. The main function of the cartilage ring is to support the tissue.

In tracheal organ cultures, the tissue organization, morphology and the control of cel-

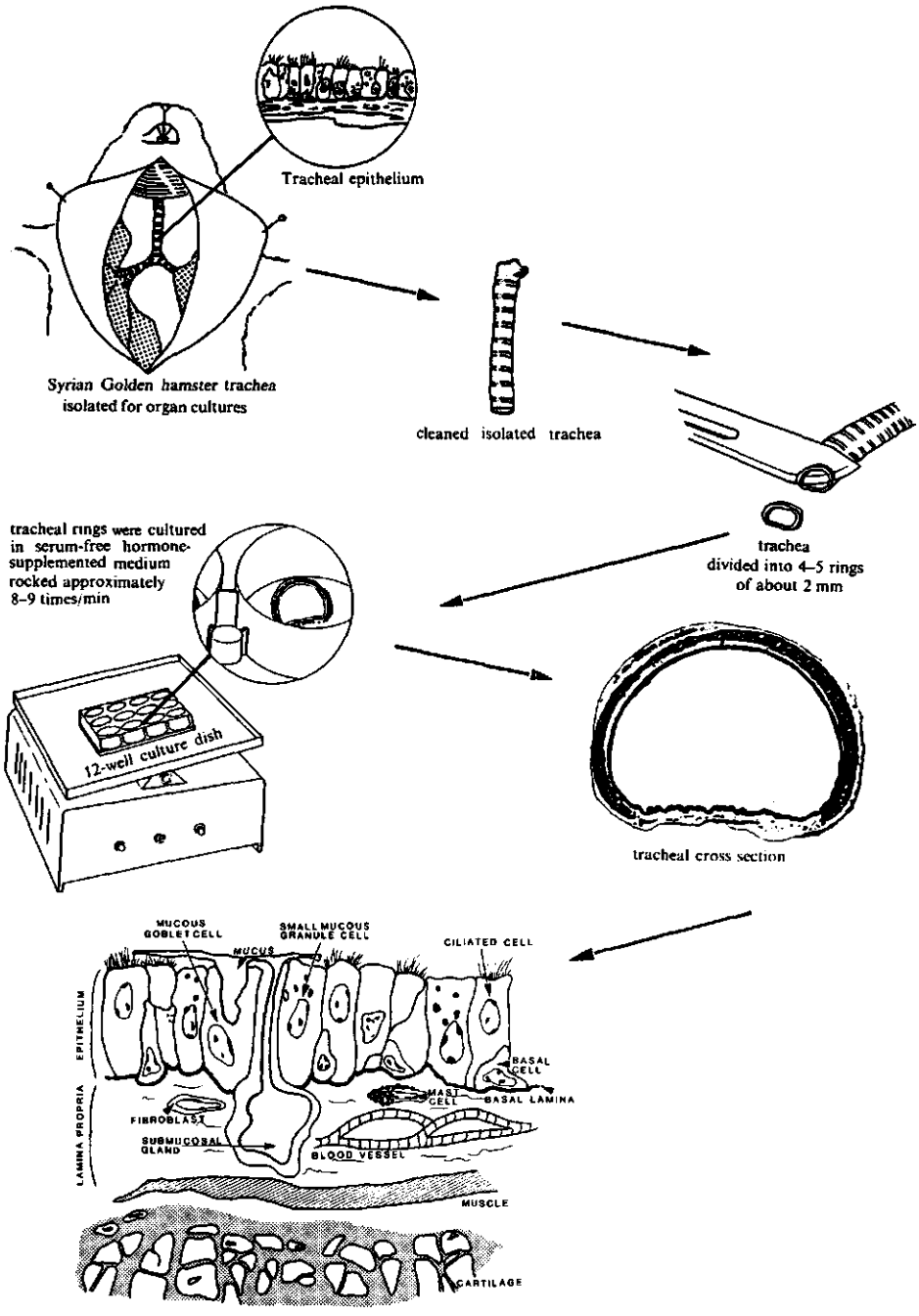

Figure 2: Isolation procedure of tracheal rings for organ culture and a schematical presentation of the basic organization of the tracheal epithelium (adapted from 57 and 72).

lular differentiation and proliferation are almost similar to the in vivo situation, although tissue innervation, blood supply and immune responses are absent.

Hamster tracheas, cultured in serum-free hormone supplemented medium, have been frequently used to study the pathogenic effects of chemical carcinogens on respiratory tract epithelium and to study the effects of compounds inhibiting the development of respiratory tract cancer. Tracheas exposed to B(a)P or cigarette smoke develop hyperplasia and squamous metaplasia [61-63], which are generally considered to be precursors of respiratory tract tumors *in vivo* (see previous section). Retinoids have been shown to prevent the development of these effects [64-66], as have also been shown *in vivo* [22].

To culture hamster tracheas, the tracheas were isolated aseptically and the external surface was cleaned to remove adherent tissue (the isolation procedure of tracheas is illustrated in Figure 2). Thereafter, tracheas were divided in 2 to 6 rings and cultured in serum-free hormone-supplemented Ham's F12 medium containing 2 mM L-glutamine, 1 μM hydrocortisone, 25 ng/ml epidermal growth factor and 50 μg/ml gentamycin. The tracheas were cultured in 12 or 24-well culture dishes for up to 15 days. The medium was replaced every day by changing the medium or by transferring the tracheal rings to wells of new culture dishes. The cultures were gassed with 40% CO_2, 55% N_2 and 5% CO_2 in a humidified incubator at 37^0C. The culture dishes were rocked 8 to 9 times/min to allowcontact of the tracheal rings with both gas and culture medium.

2.3 ^{32}P-postlabeling analysis of B(a)P-DNA adducts

^{32}P-postlabeling analysis, developed by Randerath *et al* [67], is currently the most sensitive technique for the detection of DNA-adducts, especially of polycyclic aromatic hydrocarbons-DNA adducts. Additional procedures have been developed to increase the sensitivity of the technique up to 1 adducts per 10^8 to 10^{10} nucleotides [68-71]. Another advantage of the postlabeling assay is that unidentified adducts can be detected and quantified, although the qualification of unknown adducts is difficult. Drawbacks of the method are the requirement of large quantities of radiolabeled ^{32}P and that the method is very laborious.

In the present study, the ^{32}P-postlabeling method was used to detect the formation of B(a)P-DNA adducts in hamster tracheal epithelial cells and to investigate the effect of vitamin A and β-carotene on the formation and repair of B(a)P-DNA adducts. Therefore, hamster tracheas were exposed to B(a)P with or without (pro)vitamin A, whereafter epithelial cells were scraped-off and the DNA was isolated by phenol extraction and ethanol precipitation. The general principles of the ^{32}P-postlabeling procedure is shown in Figure 3. The method involves the enzymatic digestion of the DNA to deoxyribonucleoside 3'-monophosphates by micrococcal nuclease and spleen phosphodiesterase. Hereafter, the modified nucleoside fraction was enriched by dephosphorylation of unmodified

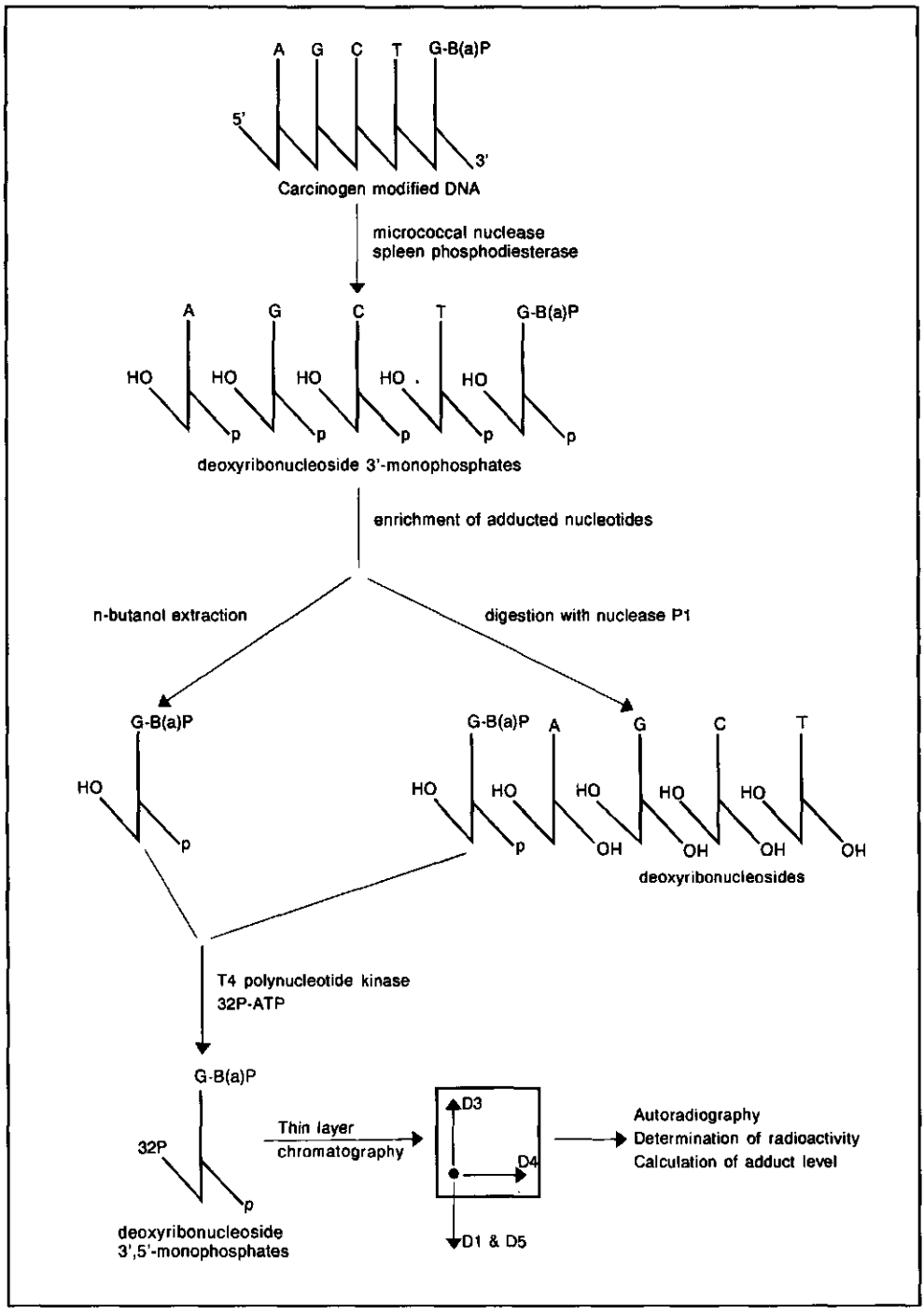

Figure 3: Schematic presentation of the ^{32}P-postlabeling method.

nucleosides by Nuclease P1 or by extraction of the adducted nucleosides by n-butanol. In the present studies, the reproductability of the experiments was enhanced using the n-butanol method. The digest is then labeled with [γ-^{32}P]ATP by incubation with T4 polynucleotide kinase to deoxyribonucleoside 3',5'-biphosphates. Subsequently, the ^{32}P-labeled nucleoside 3',5'-biphosphates were separated by anion exchange poly(ethyl-enimine)(PEI)-cellulose thin-layer chromatography in 3 directions by 4 different specific solvents. In the first elution step (D1), the excess of ^{32}P-ATP and labeled nucleosides without adducts migrate away from the origin onto a paper wick which attached to the PEI sheet. The paper wick is removed prior to subsequent elutions. During the second (D3) and third (D4) elution, the modified nucleoside 3',5'-biphosphates are migrated from the origin to specific locations on the PEI sheet. The fourth (D5) elution is employed to reduce the background radioactivity on the plates. The adducts were then qualified by their locations on the PEI thin-layer sheets by autoradiography and the different adducts were quantified by determination of radioactivity of cut-out spots of the thin-layer chromatogram. The amount of B(a)P-DNA adducts was calculated on the basis of the analysis of standard samples with known amounts of B(a)P-DNA adducts. The exact amount of input DNA was determined by chromatography of an aliquot of the DNA digest on FPLC, which also served to verify the absence of RNA.

Another, indirect, way to detect DNA damage and DNA repair is the measurement of unscheduled DNA synthesis (UDS), which is described in the next section.

2.4 Autoradiography

Autoradiography is a method to visualize radioactive compounds in cellular components. The method is based on the activation of silver bromide crystals in a photographic emulsion by radioactive radiation, resulting in black grains of metallic silver after treatment of the emulsion with a developing agent. In the present studies, the method was principally used to detect the incorporation of [methyl-^{3}H]thymidine in the DNA of tracheal epithelial cells during UDS and cell proliferation. For this purpose, tracheas were cultured for 18h in the presence of radiolabeled [methyl-^{3}H]thymidine. Thereafter, the tracheas were fixed and embedded in plastic. Semi-thin plastic cross-sections (1 or 2 μm thickness) of the tracheas on microscope slides were dipped in a photo-graphic emulsion and exposed. Then the autoradiograms were developed and stained. UDS is visible as black grains, and expressed as net grains over the nucleus which was calculated as the number of grains over the nucleus minus a nucleur-sized area in the cytoplasm. The labeling was interpreted as UDS when less than 20 grains were over the nucleus, else, the cell was scored as dividing. Generally, cell proliferation was observed as a big black dot over the nucleus (see Figure 1, Chapter 6) and quantified by counting the number of labeled basal cells and non-basal cells. Cell proliferation was expressed as a labeling index

(LI), which was defined as the precentage of labeled cells amongst the total number of epithelial cells counted. Another method for the quantification of cell proliferation is the immunocytochemical detection of the thymidine analogue bromodeoxyuridine (BrdU). This method is described in the next section.

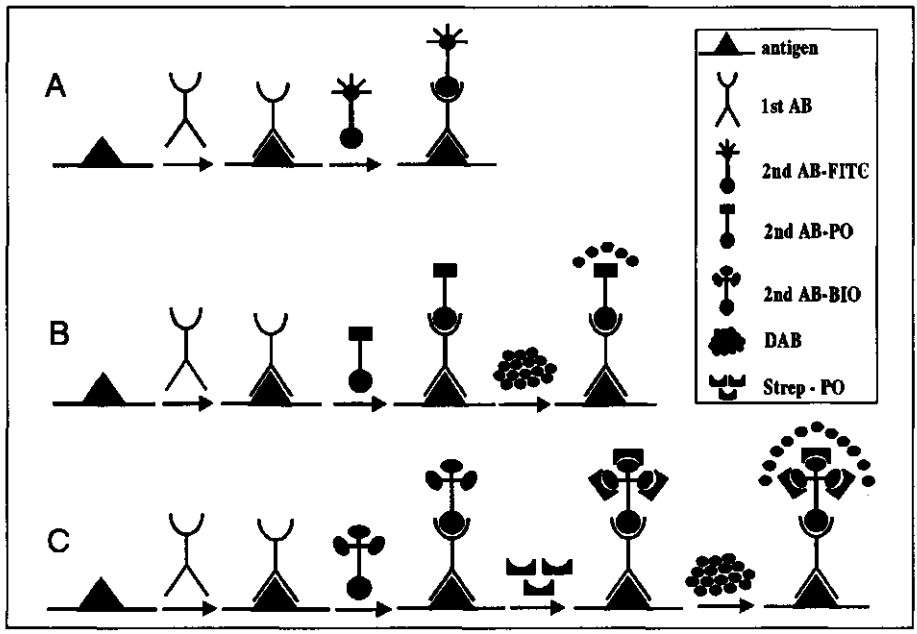

Figure 4: Schematic presentation of 3 immunocytochemical methods. For description see text.

2.5 Immunocytochemistry

The immunocytochemical method is based on the interaction between antigens (e.g. proteins) and specific antibodies raised against these antigens. Using antibodies labeled with a fluorochrome or with enzymes which catalyse a precipitation reaction of a coloured substrate, these antigens can be visualized. Several immunocytochemical methods are available. In the studies described in this thesis, 3 methods have been used which are shown in Figure 4. Common to all the methods used is that each antigen is detected by a specific antibody raised in mouse (monoclonal antibodies) or in rabbit (polyclonal antibodies). Subsequently, this first antibody is detected by a second, raised against the first. To detect B(a)P-DNA adducts (see Chapter 5), a second antibody was used labeled with a fluorochrome (fluorescein isothiocyanate, FITC), which is detectable by a fluores-

cence- or laser-scan microscope (Figure 4a). To detect cytokeratines (see Chapter 9), we used a second antibody labeled with peroxidase (PO). This enzyme catalyses, in the presence of H_2O_2, the precipitation reaction of diaminobenzidine (DAB), which is visible as a brownish precipitate (Figure 4b). To increase the sensitivity of this method, biotine labeled second antibodies were used, which react strongly with PO-labeled streptavidine (Figure 4c). Using this method, BrdU (Chapter 8), p53 (Chapter 8) and GST-isoenzyme pi (Chapter 9) were detected in hamster-tracheal sections.

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Chapter 3

Formation and repair of benzo(a)pyrene-DNA adducts in cultured hamster tracheal epithelium determined by ^{32}P-postlabeling analysis and unscheduled DNA synthesis.

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Summary

Hamster tracheal organ cultures were used to investigate the relationship between DNA adduct formation measured directly by the ^{32}P-postlabeling assay, and the DNA damage measured indirectly by the unscheduled DNA synthesis (UDS) assay. The hamster tracheas were treated with three concentrations of benzo[a]pyrene (B[a]P) for two days. Postlabeling and UDS assays were also carried out a few days after removal of the B[a]P. Furthermore, the types of B[a]P-DNA adducts formed in the *in vitro* organ culture were qualitatively compared with those formed *in vivo* after intratracheal intubation of B[a]P attached to Fe_2O_3 particles. *In vivo* only one adduct was detected by ^{32}P-postlabeling. This adduct co-chromatographed with the *trans*-addition product of dG and (+)-*anti*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE). *In vitro*, also a clear B[a]P-DNA adduct formation was found with the ^{32}P-postlabeling assay. Four different adducts were found. The main adduct spot migrated to the same position on the thin-layer chromatogram as the *in vivo* adduct. B[a]P-DNA adduct formation was both time- and dose-dependent. During the first day after removal of the B[a]P the adduct levels still increased, thereafter they decreased at all B[a]P concentrations. A time- and dose-dependent increase in UDS was observed in the tracheal epithelial cells treated with B[a]P *in vitro*. After removal of the B[a]P, UDS decreased immediately, in contrast to the formation of DNA-adducts. The results of the present study show that B[a]P induces time- and dose- dependently both DNA adducts and UDS in hamster tracheal organ culture. Moreover, the main DNA adduct formed *in vitro*, dG-(+)-*anti*-BPDE, was the same as that found *in vivo*.

Introduction

Genotoxic agents may cause a variety of lesions in DNA of exposed cells. When these lesions are improperly repaired, they may be converted during DNA replication to permanent changes in the genome, such as point mutations, deletions, gene amplifications or DNA rearrangements. These changes are considered to be initiating events in the neoplastic transformation of cells (1,2).

Benzo[a]pyrene (B[a]P), one of the most extensively studied carcinogens of the class of polycyclic aromatic hydrocarbons (PAH), is a constituent of burned products such as cigarette smoke. It is readily converted into reactive intermediates by the cytochrome P450-dependent mixed-function oxidase system (3-5). Covalent binding of specific reactive B[a]P metabolites to DNA of target cells is considered to be a critical step in tumour initiation (6,7).

Carcinogenic effects of B[a]P on the respiratory tract epithelium of experimental animals have been studied extensively, *in vivo* as well as *in vitro*. To induce respiratory tract tumours in rodents, B[a]P is usually intratracheally administered in combination with Fe_2O_3 (8). In this way, a broad spectrum of respiratory tract tumours can be induced in hamsters and rats, including papillomas, adenomas, carcinomas and adenocarcinomas (8,9). Morphologically, these types of tumours resemble those frequently observed in humans (10,11).

In addition to the *in vivo* models, a variety of *in vitro* methods have been developed to study pathogenic processes in tracheal epithelium (12-14). Both cigarette smoke and B[a]P induce hyperplasia and squamous metaplasia in cultured hamster trachea (15), which are generally considered to be precursors of respiratory tract tumours (11,16).

To assess the initial DNA damage after exposure to B[a]P, sensitive biochemical methods, e.g. ^{32}P-postlabelling and immuno(histo)chemical techniques, were used. These methods allow detection of very small amounts of B[a]P-DNA adducts (17,18). Another, indirect way to assess early effects of chemical carcinogens on DNA is to determine the induced unscheduled DNA synthesis (UDS) as an indicator of DNA damage. B[a]P or its metabolites are known to induce a clear increase in UDS in respiratory tract epithelial cells of rodents as well as humans (19,20,21).

The quantitative relationship between B[a]P treatment dose, DNA-adduct levels and UDS in tracheal epithelial cells in organ culture is not known. The objective of the present study was to investigate the formation and the repair of B[a]P-DNA adducts in cultured hamster tracheal epithelium of Syrian golden hamsters to compare the DNA-adduct level measured directly by ^{32}P-postlabeling and the DNA damage measured indirectly by the UDS asay. Furthermore, the types of B[a]P-DNA adducts formed *in vitro* was compared qualitatively to the B[a]P-DNA adducts formed after *in vivo* treatment of hamsters with B[a]P.

Materials and methods

In vivo exposure

Ten-weeks old Syrian golden hamsters were obtained from the Central Institute for the Breeding of Laboratory Animals, Zeist, The Netherlands. Five hamsters were intratracheally instilled with a mixture of 18 mg B[a]P (Sigma Chemicals, St. Louis, MO) and 18 mg Fe_2O_3 (Fisher Scientific Company, New Jersey) suspended in 0.2 ml saline on 2 consecutive days. The suspension was made as described by Saffiotti *et al* (8). Just before each intubation, 3700 kBq ^{3}H-B(a)P (specific activity 3 TBq/mmol; Amersham, Houten, The Netherlands), dissolved in acetone, was added to the suspension. The concentration of acetone was 0.5%. After 2 days of exposure, the animals were killed by an overdose of Nembutal (Ceva, Paris, France), and the tracheas were isolated. From each trachea a small piece (ca. 3 mm thickness) was cut off, washed thoroughly with acetone for 1 h to remove all unbound B[a]P and processed for autoradiography for the localization of the ^{3}H-label. The remaining part of the epithelium was scraped off and prepared for ^{32}P-postlabeling analysis.

In vitro exposure

Hamster tracheas were isolated aseptically and the external surface was cleaned to remove the adherent tissue. Thereafter, tracheas were divided into two pieces (~3-4 mm thickness) and cultured in 24-well culture dishes in serum-free, hormone-supplemented Ham's F12 medium [L-glutamine 2 mM (Flow Laboratories, Herts, UK), hydrocortisone 1 μM, bovine pancreatic insulin 5 μg/ml (Sigma Chemicals, St. Louis, MO), human transferrin 5 μg/ml (Sigma), epidermal growth factor 25 ng/ml (Sigma) and gentamycin 50 μg/ml (Flow)]. Cultures were gassed with 40% O_2, 55% N_2 and 5% CO_2 in a humidified incubator at 37^{0}C. The culture dishes were rocked 8-9 times/min to allow contact of the tracheal rings with both gas and culture medium. On the first day after isolation, the tracheas were exposed for two days to B[a]P (5, 10 or 15 μg/ml). The B[a]P was added from a stock solution in DMSO, and the final concentration of DMSO in the culture medium was 0.1% (v/v). The tracheas were then incubated with fresh culture medium on the rocker for 3 h to remove the B[a]P. Thereafter, the tracheas were cultured for another 3 days in complete culture medium without B[a]P. On each day of the study, the epithelial cells of four tracheas per concentration of B[a]P were scraped off and frozen (-80 ^{0}C). In DNA from these cells the level of B[a]P-DNA adducts was quantified by ^{32}P-postlabeling assay (see below). In addition, a smaller ring (~2 mm thickness) was cut off and cultured for 18 h in the presence of 370 kBq/ml [methyl-^{3}H]thymidine (sp. act. 1.81 TBq/mmol; Amersham) to assess UDS (see below).

^{32}P-postlabeling method

DNA was isolated from the tracheal epithelial cells by means of phenol extractions and ethanol precipitation. The concentration of DNA in the samples was determined spectrophotometrically (1 mg/ml of DNA was equivalent to 20 absorbance units at 260 nm). The DNA adducts were determined by the ^{32}P-postlabeling assay according to the nuclease P1-modified procedure as described by Reddy and Randerath (22). Four micrograms of DNA/sample were digested with micrococcal nuclease (Sigma), spleen phosphodiesterase (Sigma) and with nuclease P1 (Boehringer Mannheim, Almere, The Netherlands). Therafter, the modified nucleotides were labelled with 2.3 MBq [γ^{32}P]ATP/sample (sp. act. > 185 TBq/mmol, Amersham) by incubation with T4 polynucleotide kinase (Biolabs, Beverly, MA) for 30 min at 37^{0}C. The postlabeled mixtures were spotted on 20 * 20 cm poly(ethyleneimine)(PEI)-cellulose sheets (JT Baker) with a paper wick attached to the top of each TLC plate. The plates were developed for two days in sodium phosphate buffer [1M, pH 6.0 (D1)]. The plates were washed in water and developed in a solution containing 8.5 M urea and 3 M lithium formate [pH 3.5 (D3)] for 6.5 h, washed again and then developed in 8.5 M urea, 0.8 M LiCl and 0.5 M Tris [pH 8.0 (D4)] also for 6.5 h. To remove the remaining impurities, the plates were finally developed overnight in sodium phosphate buffer [1.7 M, pH 6.0 (D5)], in the direction of D1.

The DNA adducts were visualized by autoradiography on Kodak XAR-5 films using an intensifying screen. The spots were localized on the chromatograms and cut out. The radioactivity was determined by liquid scintillation counting.

The amount of DNA-adducts was calculated on the basis of the analysis of standard samples with known amounts of modifications. The exact amount of input DNA was determined by chromatography on FPLC, which also served to verify the absence of RNA (23).

Unscheduled DNA synthesis

For the DNA-repair assay tracheal rings were cultured for 18 h in the presence of 370 kBq/ml [methyl-^{3}H]thymidine (sp. act. 1.81 TBq/mmol, Amersham). Then the tracheas were washed three times with phosphate-buffered saline (PBS), fixed in 4% aqueous phosphate-buffered formaldehyde solution (pH 7.0), dehydrated and embedded in Technovite 7100 plastic (Kulzer, Wehrheim, F.R.G.).

Semi-thin sections (1 μm thickness) of the tracheal rings on microscope slides were dipped in Kodak NTB-2 emulsion (Eastman Kodak, New York), diluted 1:1 with a 2% aqueous glycerol solution. Autoradiograms were exposed in dry, light-tight boxes for 4-6 weeks at -30^{0}C, developed in Kodak D19, stained with 0.01% Toluidine Blue solution (Gurr, Chadwell Heath, UK) and embedded in DePex.

DNA-repair was expressed as net grains per nucleus which was calculated as the number of silver grains over the nucleus minus a nucleur-sized area in the cytoplasm. For each experimental point, two cross sections were counted (50 nuclei per section).

Statistical analysis

The mean coefficient of variation (CV) for the ^{32}P-postlabeling method was 24.5 ± 18.6 whereas the CV for the UDS was 34.9 ± 44.8. Data obtained with the ^{32}P-postlabeling method and UDS were tested for significant differences using the Tukey studentized range method (BMDP statistical software manual, 1988).

Results

DNA-adducts in vivo

^{32}P-Postlabeling analysis of DNA isolated from tracheal epithelial cells from hamsters treated with B[a]P *in vivo* showed only one spot (Figure 1a). The single adduct co-chromatographed with the standard reference B[a]P-DNA adduct. This reference sample was prepared by incubation of (+)-*anti*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo-[a]pyrene (BPDE) with deoxyguanosine-3'-monophosphate and resulted in the *trans*-addition product of dG and (+)-*anti*-BPDE (Figure 1b).

In the autoradiogram of tracheal epithelium of hamsters treated with radioactive B[a]P *in vivo* most of the silver grains were observed in exfoliated cells and hyperplastic regions (Figure 2).

DNA-adducts in vitro

In vitro incubations of hamster tracheas with B[a]P resulted in one main spot and three minor adduct spots as detected by ^{32}P-postlabeling analysis (Figure 1c). The main adduct (~95%) co-chromatographed with the standard DNA adduct (Figure 1d), the *trans*-addition product of dG and (+)-*anti*-BPDE (Figure 1b). The minor adducts were 3.5 ± 0.6% (adduct 2), 1.6 ± 0.6% (adduct 3) and 0.7 ± 0.3% (adduct 4) of total adduct formation. In hamster tracheal organ cultures, a marked time- and dose-dependent B[a]P-DNA adduct formation was found by ^{32}P-postlabeling analysis (Figure 3). B[a]P-DNA adduct levels increased up to day 3 (5 and 15 μg/ml) or day 4 (10 μg/ml). B[a]P-DNA adduct formation still increased for 1 or 2 days after washing out the B[a]P on day 2. Ultimately, the DNA-adduct levels decreased at all three B[a]P concentrations indicating the removal of DNA adducts. Increasing concentrations of B[a]P in the culture medium resulted in an increase in the B[a]P-DNA adduct levels in the tracheal epithelial cells (Figure 3).

Relatively high DNA-adduct levels were observed on days 3 and 4 of the experiment, i.e. 1 and 2 days after removal of the B[a]P. On day 5, the adduct levels were comparable to levels obtained on the first day (1 day of treatment with B[a]P). No difference in B[a]P-DNA adduct levels were observed between hamster tracheal epithelial cells treated with 10 or 15 μg/ml B[a]P on day 4 and 5 (Figure 3).

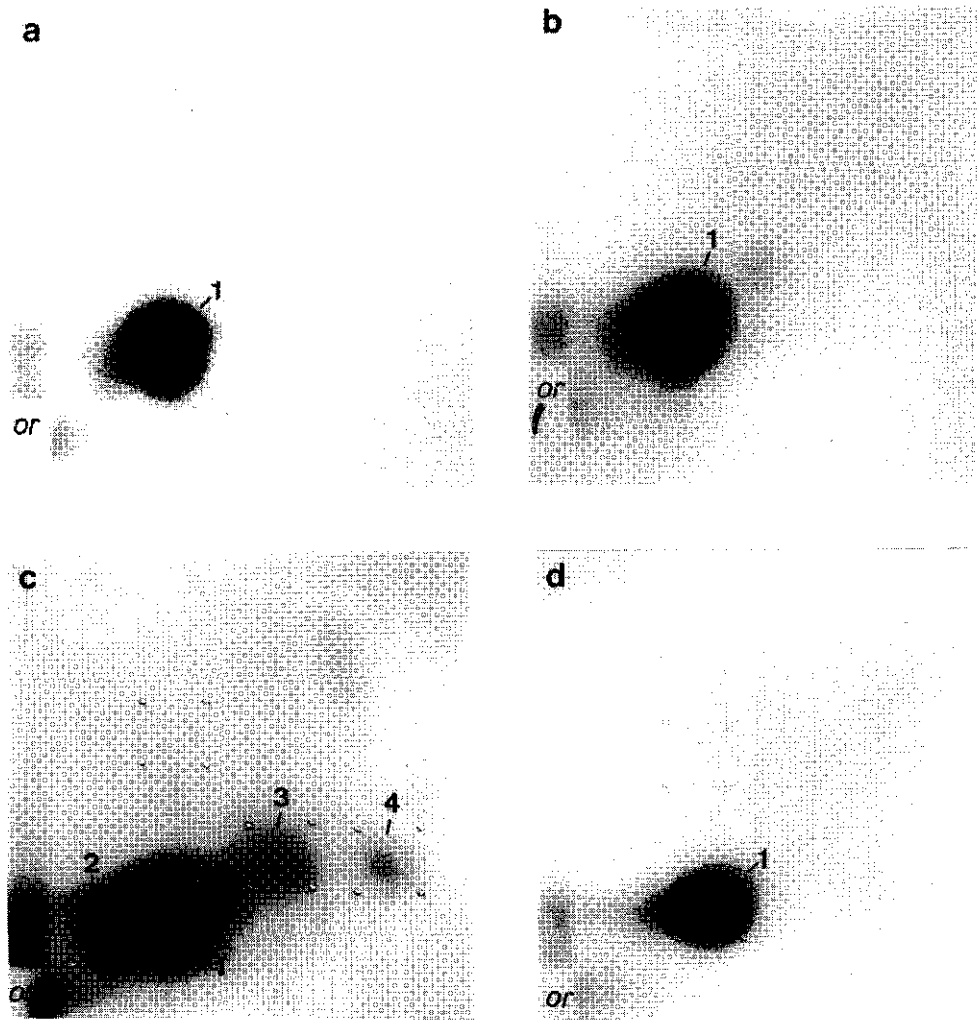

Figure 1: Autoradiograms of ^{32}P-postlabelled DNA. (**a**) ^{32}P-postlabeling analysis of DNA isolated from tracheal epithelial cells obtained from a hamster that had been treated intratracheally with 18 mg B[a]P + 18 mg Fe_2O_3 + 37 MBq [^{3}H]B[a]P suspended in 0.2 ml saline on each of 2 consecutive days. (**b**) ^{32}P-postlabeling analysis of standard DNA (*trans*-addition product of dG and (+)-*anti*-BPDE). (**c**) ^{32}P-postlabeling analysis of DNA isolated fron hamster tracheal epithelial cells treated with 15 μg/ml B[a]P for 4 days in organ culture. Four different DNA adducts were detected. The main adduct (**1**) correspond with ± 95% of the radioactivity, the minor adduct with 3.5 ± 0.6% (**2**), 1.6 ± 0.6% (**3**) and 0.7 ± 0.3% (**4**) of total adduct formation. (**d**) ^{32}P-Postlabeling analysis of DNA isolated from hamster tracheal epithelial cells treated with 10 μg/ml B[a]P for 3 days in organ culture co-chromatographed with standard DNA (see b). The main adduct co-chromatographed with the standard DNA adduct.

Figure 2: Semi-thin cross-section of a trachea from a hamster intratracheally treated with 18 mg B[a]P + 18 mg Fe_2O_3 + 100 μCi ^{3}H-B[a]P suspended in 0.2 ml saline on each of two consecutive days. Most silver grains are observed in the hyperplastic region and in exfoliated cells. Toluidine Blue Staining. x 640

DNA-repair in vitro

Treatment of hamster tracheal epithelium in organ culture with B[a]P resulted in a dose- and time-dependent increase in UDS as measured by the incorporation of [methyl-^{3}H]-thymidine (Figure 4). The maximal level of UDS was observed in the tracheas treated with 15 μg/ml B[a]P for two days. Before removal of the B[a]P, a clear increase in UDS was observed in tracheal epithelial cells treated with 5 μg/ml B[a]P compared to the untreated controls (Figure 4). After washing out the B[a]P, UDS was clearly higher in the tracheas treated with 10 and 15 μg/ml B[a]P compared to the tracheas treated with 5 μg/ml B[a]P. Figure 4 shows the time-dependent increase in UDS levels before the B[a]P was washed out of the culture medium on day 2. After removal of the B[a]P, UDS decreased at all three B[a]P concentrations.

Discussion

Tracheal organ cultures are widely used to study the formation of B[a]P-DNA adducts, mainly measured by scintillation counting of radiolabeled B[a]P (24-27). Autrup *et al* (26) and Mass and Kaufman (24) showed that the binding of B[a]P to the DNA of epithelial cells in tracheal organ culture was higher than that in tracheal epithelium of other species such as rat, mice and humans. By HPLC analysis four different DNA adducts were detected in cultured hamster tracheas treated with [^{3}H]B[a]P (27). In the present study, the

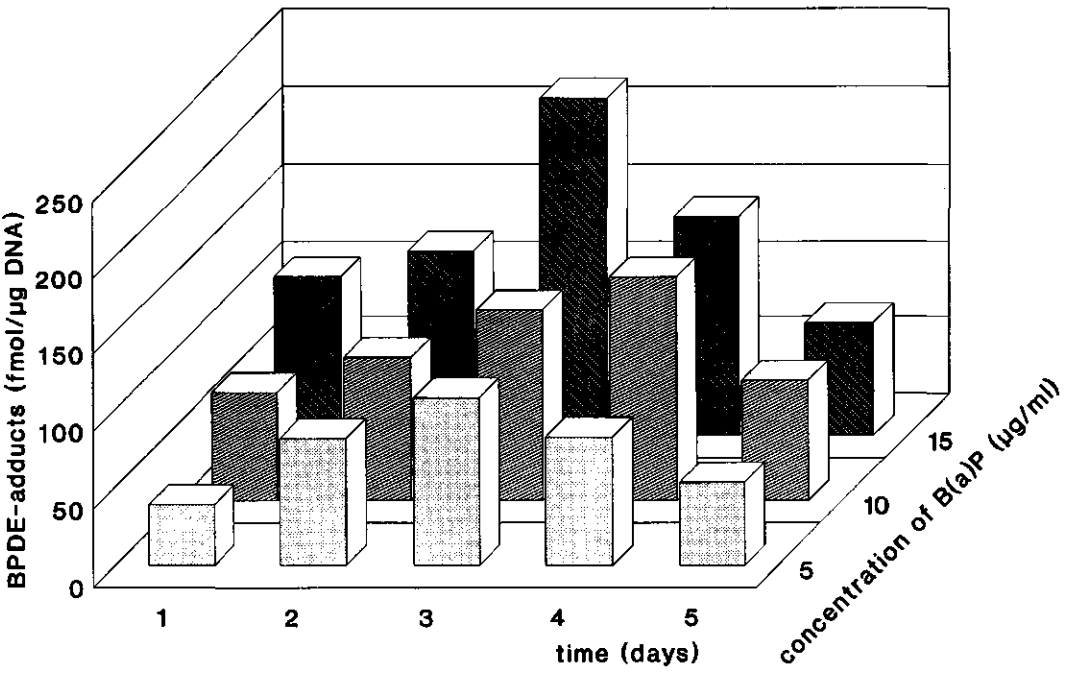

Figure 3: Formation and removal of B[a]P-DNA adducts in hamster tracheal epithelial cells. Isolated hamster tracheas were treated with three concentrations of B(a)P *in vitro*. The B[a]P-DNA adduct (BPDE-adducts) levels were measured by the ^{32}P-postlabeling assay. After two days the B[a]P was removed. B[a]P-DNA adduct levels increased both time- and dose-dependently. After removal of the B[a]P, adduct levels still increased for one or two days. BPDE-adduct levels in the tracheas treated with 5 µg/ml B[a]P are significantly different from the BPDE-adduct levels in the tracheas treated with 15 µg/ml B[a]P ($p<0.05$). BPDE-adduct levels on day 3 are significantly different from the BPDE-adduct levels on day 1 and day 5 ($p<0.05$).

adduct pattern of DNA isolated from hamster tracheal epithelial cells treated with B[a]P in organ culture also consisted of four spots. The main DNA adduct (~95%) co-chromatographed with the dG-(+)-*anti*-BPDE adduct. The other adducts are only a small percentage (~5%) of total adduct formation. However, *in vivo* intratracheal intubation with B[a]P + Fe_2O_3 resulted in only one B[a]P-DNA adduct in tracheal epithelial cells. This single adduct co-chromatographed with the reference adduct, and is the same as the main DNA-adduct formed in organ culture.

In the *in vitro* experiment relatively high concentrations of B[a]P were used to induce BPDE adducts and UDS. However, these concentrations are in the same order of magnitude as those used by Chopra and Cooney (28) to induce squamous metaplasia in hamster tracheas (5 µg/ml) and by Harris *et al* (26) to induce BPDE-adducts in cultured human bronchus (16.25 µg/ml). Furthermore, histopathological examination of the tracheas treated

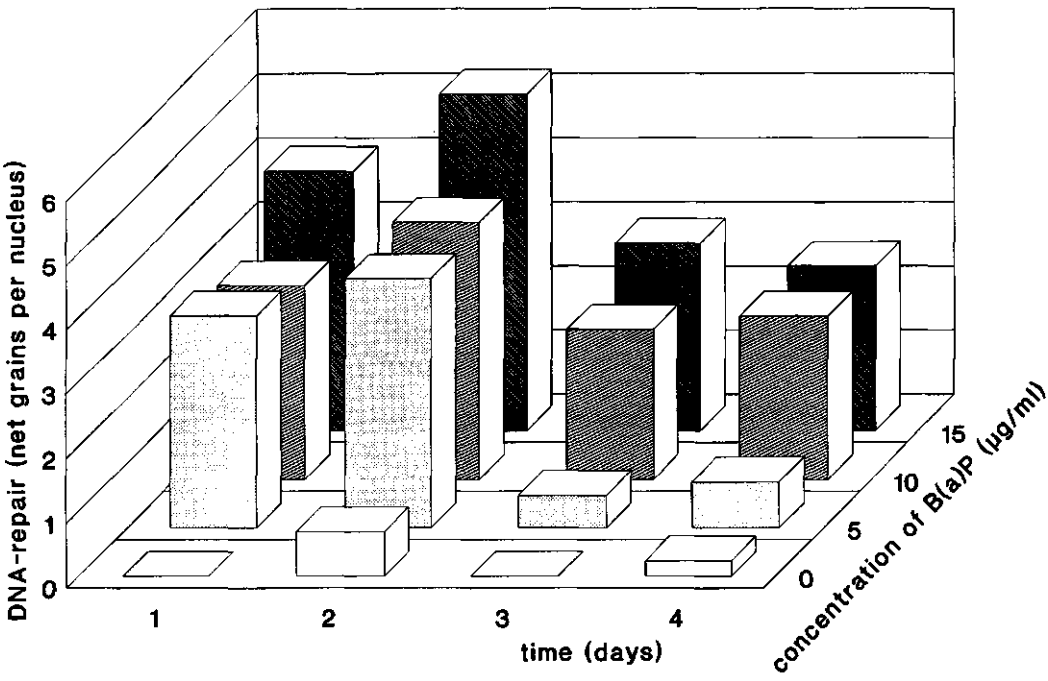

Figure 4: Time- and dose-dependent effect on unscheduled DNA synthesis in hamster tracheas epithelial cells *in vitro* treated with different concentrations of B[a]P. After two days the B[a]P was removed from the culture medium. Before removal of the B[a]P, UDS-levels increased, after removal the UDS-levels decreased immediately. UDS levels in the tracheas treated with B[a]P are significantly different from the UDS levels in the control tracheas (B[a]P 5 μg/ml $p<0.05$, B[a]P 10 and 15 μg/ml $p<0.01$). For treated tracheas UDS levels on day 2 are significantly different from the UDS levels on day 3 and 4 ($p<0.01$).

with B[a]P did not reveal signs of cytotoxicity.

In the *in vitro* experiment with hamster tracheal epithelial cells, the formation of B[a]P-DNA adducts and the subsequent DNA-repair was both time- and dose-dependent. This is in agreement with experiments in human bronchus organ culture (26).

Following removal of the B[a]P from the culture medium, adduct levels still increased for 1 or 2 days. This increase is most likely due to residual B[a]P inside the cells or to B[a]P that remained bound to the external surface of the tracheas.

The levels of B[a]P-DNA adducts found in previously published studies using radiolabeled B[a]P are of the same order of magnitude as those determined in the present work with the ^{32}P-postlabeling assay, i.e. in the femtomole range per μg DNA. In hamster tracheas exposed to 1.5 (27) or 20 μM (24) [^{3}H]B[a]P for 24 hours, ~2.5 fmol/μg DNA were detected by scintillation counting of the isolated DNA. In the present study an adduct level of ~40 fmol/μg DNA was detected by the ^{32}P-postlabeling assay in hamster tracheas

exposed to 5 μg/ml (20 μM) B[a]P for 24 h. This adduct level was higher than that in the study of Mass and Kaufman (24).

B[a]P induces a marked UDS in rat tracheal epithelium (19) as well in human bronchial epithelial cells (20). In the present study, B[a]P induces UDS in hamster tracheal epithelium in organ culture in a time- and dose-dependent manner. After the B[a]P had been removed UDS decreased. Gill *et al* (29) showed a dose relationship between DNA adduct formation and UDS in cultured mouse epidermal keratinocytes, in which UDS increased in parallel with increases in adduct levels. In the experiments with hamster tracheal organ culture described in this paper, in the first 2 days increasing doses of B[a]P lead to increased UDS and B[a]P-DNA adduct levels concomitantly. During the first days following removal of B[a]P, there was an inverse relationship in time between DNA-adduct formation and UDS. DNA adduct formation continues to increase for another 1 (5 and 15 μg/ml B[a]P) or 2 (10 μg/ml B[a]P) days whereas UDS decreased immediately at all three B[a]P concentrations. In the experiments of Gill *et al* (29), keratinocytes were treated with dimethylbenz[a]anthracene or B[a]P (or metabolites) for only 24 h, a correlation was found between carcinogen concentration, DNA-adduct levels and UDS. In our experiment, the hamster tracheas were treated with B[a]P for several days. Every experimental day there was a clear relation between B[a]P concentration, adduct level and UDS (Figure 3 and 4). However, after removal of the B[a]P, DNA-adduct levels increased for 1 or 2 days while UDS levels decreased immediately. This could be explained by assuming that the UDS decreases and plateaus by depletion of the enzymes for UDS in the tracheal epithelial cells due to longer treatment time. Further investigations into the formation and repair of DNA-adducts and into their relationship in tracheal epithelial cells are in progress.

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Chapter 4

DNA-Adduct formation and repair in hamster and rat tracheas exposed to benzo[a]pyrene in organ culture

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Summary

Syrian golden hamsters are much more susceptible than Wistar rats to the induction of tracheal tumours by benzo[a]pyrene (B[a]P). To investigate whether this difference is reflected in the pattern of DNA-adduct induction and removal, tracheas from either species were isolated and exposed to B[a]P (5 μg/ml) in organ culture. At various time points B[a]P-DNA adducts were quantified by ^{32}P-postlabeling; unscheduled DNA synthesis (UDS) and cell proliferation were determined by [^{3}H]thymidine incorporation during 18 h before sampling. In an induction-repair experiment tracheas were exposed to B[a]P for two days, and cultured for another 4 days without B[a]P. After 2 days of exposure total B[a]P-DNA adduct levels were 10 times higher in hamster compared to rat tracheas. In hamster tracheas one major adduct was formed (95%), *viz.* the adduct between (+)-*anti*-B[a]P-diolepoxide and deoxyguanosine (BPDE-N^{2}dG). In rat tracheas BPDE-N^{2}dG comprised about 60% of the total B[a]P-DNA adduct level. The other major adduct found in rat tracheas is probably derived from interaction of *syn*-BPDE and deoxyadenosine. During exposure to B[a]P in hamsters the adduct level increased to 36 ± 19 adducts/10^{6} nucleotides (add/10^{6}n) on day 2. Two days after removal of B[a]P the B[a]P-DNA adduct level had decreased to 60% of that on day 2; there was no further decrease in the B[a]P-DNA adduct level, despite considerable cell proliferation at the end of the six-day culture period. UDS increased during exposure to B[a]P and decreased after removal of B[a]P. In rats removal of B[a]P did not lead to a decrease in the B[a]P-DNA adduct level, which agreed with the observed absence of UDS. In a second experiment tracheas were exposed to B[a]P continuously for 15 days. In hamster tracheas the total B[a]P-DNA adduct level increased from 11 ± 0.7 add/10^{6}n after 1 day of exposure to 105 ± 2 add/10^{6}n after 15 days; also UDS increased with increasing exposure until day 11. Cell proliferation was low at the end of the culture period. In rat tracheas no progressive increase in the B[a]P-DNA adduct level was observed, UDS was not increased, and cell proliferation had

increased significantly at the end of the exposure period. The extent of adduct induction in the tracheas of the two species corresponded with the different susceptibilities to B[a]P-induced tumour formation.

Introduction

The lung is a major site of tumour formation in humans. Epidemiological studies show a higher relative risk of lung cancer for the smoking population compared to non-smoking controls (1). With the ^{32}P-postlabeling method tobacco-smoke specific DNA adducts could be detected in lung tissue from cigarette smokers (2-5). Morphologically, the type of tumours observed in the lungs of humans shows a close similarity to benzo[a]pyrene (B[a]P)-induced respiratory tract tumours in hamsters (6-8).

In vitro exposure of hamster tracheas to B[a]P or cigarette-smoke condensate (9) resulted in hyperplasia and squamous metaplasia, which are generally considered to be precursors of respiratory-tract tumours (7,10). The Syrian golden hamster is susceptible to tumour formation in the trachea after exposure to B[a]P (11). After multiple intratracheal instillations of B[a]P epithelial hyperplasia and squamous metaplasia with various degrees of atypia, and tumours were observed in the trachea of hamsters. In contrast, similarly-treated rats did not show any epithelial alterations including tumours in the trachea, but developed lung tumours. This difference in B[a]P-susceptibility of the trachea between hamsters and rats was attributed to differences in the mucosal barrier of the trachea, preventing B[a]P to penetrate the tracheal epithelial cells of rats. However, the results of a study by Autrup *et al.* (12) indicated that B[a]P-DNA adducts could be detected in rat trachea epithelial cells after *in vitro* exposure to B[a]P.

Several groups (12-14) already reported a difference in total B[a]P-DNA adduct levels between hamster and rat tracheas, exposed to B[a]P *in vitro* during 24 h. The objective of the experiments described in the present paper was to study differences in DNA-adduct formation and DNA-repair between B[a]P-exposed hamster and rat tracheas in organ culture. In an induction-repair experiment B[a]P (5 μg/ml) was present for two consecutive days, whereafter B[a]P was removed while the tracheas were kept in culture for another four days. In an induction-accumulation experiment B[a]P was present continuously during 15 days. At various time-points in both experiments tracheas were prepared for analysis of UDS and cell proliferation by incorporation of [^{3}H]thymidine. DNA-adducts were analysed by ^{32}P-postlabeling. In the latter assay, the reaction product of deoxyguanosine with the reactive *anti*-B[a]P-diolepoxide (*anti*-BPDE) was used as a calibration standard. In an attempt to identify the unknown adducts observed in the postlabeling chromatograms, control experiments were carried out with the *syn*-isomer of the reactive B[a]P-diol-epoxide.

Materials and methods

Chemicals and enzymes

Racemic *anti*-BPDE was obtained from Chemsyn, Lenexa, KS. Racemic *syn*-BPDE was a kind gift of Dr A. Dipple. [γ^{32}P]-ATP was purchased from Amersham, Buckinghamshire, UK. B[a]P, micrococcal endonuclease, spleen phosphodiesterase, and RNAse A were obtained from Sigma Chemicals, St. Louis, MO. Nuclease P1 and RNAse T1 were obtained from Boehringer Mannheim, Mannheim, Germany. RNAse T1 was heated at 80°C for 5 min to remove DNAse activity. T4 polynucleotide kinase was purchased from New England Biolabs, Beverly, MA and proteinase K was purchased from Merck, Darmstadt, Germany.

Culturing of tracheal rings

Ten-week old male Syrian golden hamsters and Wistar rats (obtained from Harlan/CPB, Zeist, The Netherlands) were sacrificed by an *i.p.* overdose of Nembutal (Ceva, Paris, France). Tracheas were isolated aseptically, and adherent connective tissue was removed. Thereafter, hamster and rat tracheas were cut into three to four and eight to nine rings, respectively. Tracheal rings were randomly allocated to the wells of 24-well plates (Costar, Cambridge, MA) and cultured in serum-free, hormone-supplemented Ham's F12 medium containing 2 mM L-glutamine (Flow Laboratories, Herts, UK), 1 μM hydrocortisone (Sigma), 5 μg/ml bovine pancreatic insulin (Sigma), 5 μg/ml human transferrin (Sigma), 25 ng/ml epidermal growth factor (Sigma), and 50 μg/ml gentamycin (Flow). Each well contained four tracheal rings derived from four different hamsters or rats. Plates were placed on a rocker (8 to 9 cycles per minute) in an incubator in a humidified atmosphere of 40% O_2, 55% N_2, and 5% CO_2 at 37°C.

Induction-repair experiment

On the day before treatment (day −1) hamster (n=40) and rat (n=16) tracheas were isolated, cleaned and placed in culture. Starting on day 0, the tracheal rings were exposed to B[a]P (5 μg/ml) for 48 h. B[a]P was dissolved in DMSO; the final concentration of DMSO in the medium was 0.1% (v/v). Samples were taken at day 0 (control), day 1 and day 2. At the end of day 2, just after sampling, all remaining tracheal rings were washed, transfered to a medium without B[a]P, and cultured for another four days during which samples were taken at 24-h intervals (see Figure 1). Sampling comprised scraping off epithelial cells for DNA isolation and adduct analysis (^{32}P-postlabeling), and preparation of sections for the assays of UDS and cell proliferation (see below).

Induction-accumulation experiment

Hamster (n=30) and rat (n=12) tracheas were isolated, cleaned and placed in culture on day −1. On day 0 trachea rings were exposed to B[a]P (5 μg/ml) which continued for 15

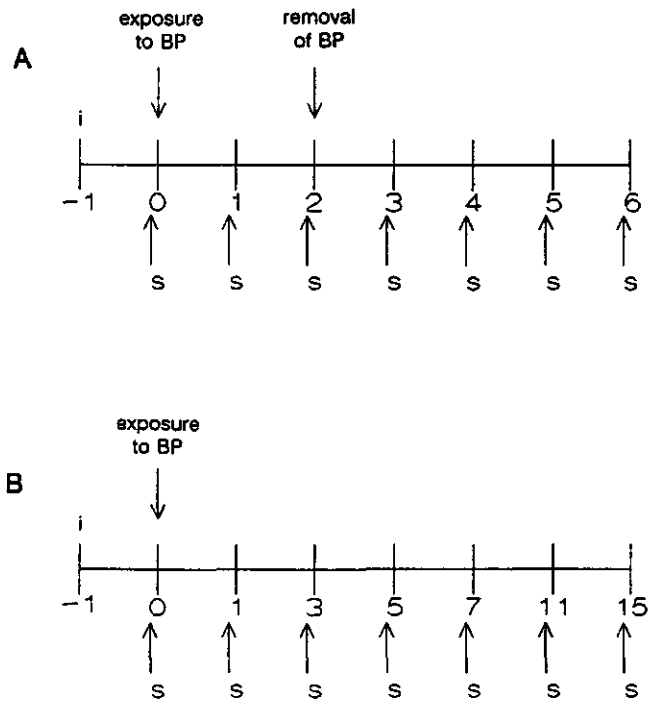

Figure 1: Diagram of the induction-repair (A) and the induction-accumulation (B) experiment. The "i" indicates the isolation of the tracheas. The "s" indicates the sampling-points on the respective days.

days. Every day medium was removed and replaced with fresh medium containing freshly dissolved B[a]P. Samples were taken on days 1, 3, 5, 7, 11, and 15 (after 24, 72, 120, 168, 264, and 360 h of exposure, respectively).

DNA isolation

At each selected time-point during both the induction-repair and the induction-accumulation experiment the epithelial cells were scraped off the sampled tracheal rings (12 per time-point). Cells were collected in PBS and centrifuged at 125 g. The cell pellet was suspended in 250 μl TEN buffer (20 mM Tris-HCl pH 7.6, 1 mM EDTA.Na_2, and 100 mM NaCl). Thereafter, 250 μl of TEN containing 1% SDS and proteinase K (100 μg/ml) was added and the mixture was incubated for 18h at 37°C. DNA was isolated by phenolic extractions with equal volumes of phenol, phenol-chloroform-isoamylalcohol (25:24:1, v/v/v), and chloroform-isoamylalcohol (24:1, v/v). The DNA was precipitated by addition of 0.1 volume of 3 M sodium acetate, pH 6.0 and 3 volumes of ethanol at -20°C. DNA

was washed with 70 % ethanol in water at -20°C and dried *in vacuo*. Subsequently, DNA was dissolved in 250 μl TE buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA.Na_2) and treated serially with a mixture of RNAses A (50 μg/ml) and T1 (50 U/ml) for 90 min and proteinase K (100 μg/ml) for 60 min at 37°C. Phenolic extractions were repeated and finally DNA was precipitated, washed, and dried as described above. DNA was dissolved in Millipore-filtered (MQ) water and the concentration was determined spectrophotometrically (1 mg of DNA per ml = 20 absorbance units at 260 nm).

^{32}P-postlabeling analysis of B[a]P-DNA adducts

The ^{32}P-postlabeling assay was performed as described by Reddy and Randerath (15) with some modifications. DNA (2-3 μg in 18 μl) was digested with micrococcal nuclease (0.4 U) and spleen phosphodiesterase (0.008 U) for 3 h at 37°C, and subsequently treated with nuclease P1 (2 units) for 40 min at 37°C. The modified nucleotides were labelled with 37 MBq γ-[^{32}P]-ATP (sp.act. > 185 TBq/mmol) by incubation with 50 U T4-polynucleotide kinase for 30 min at 37°C.

The postlabeled mixtures were applied to 20-cm x 20-cm poly(ethylenimine)(PEI)-cellulose sheets (JT Baker, Phillipsburg, NJ, USA). A paper wick was attached to the top of each TLC sheet; the sheets were developed overnight in 1 M sodium phosphate, pH 6.0 (D1). After two washes with water the plates were developed in 8.5 M urea, 3 M lithium formate, pH 3.5 (D3) for 6.5 h and in 8.5 M urea, 0.8 M lithium chloride, 0.5 M Tris, pH 8.0 (D4), also during 6.5 h. In order to remove any remaining impurities, the plates were developed overnight in 1.7 M sodium phosphate, pH 6.0 (D5, in the direction of D1). The adducts were localized by autoradiography at -70°C on Kodak XAR-5 film with intensifying screens.

The spots on the PEI-cellulose sheets detected by autoradiography were cut out and the radioactivity was determined by liquid scintillation counting. DNA-adduct levels were calculated on the basis of the results of concurrent postlabeling analysis of standard DNA samples carrying known amounts of B[a]P-adducts prepared by *in vitro* reaction with *anti*-BPDE (16). The exact amount of input DNA and the absence of RNA were determined by chromatography of the DNA digest on FPLC, as described previously (16).

In the induction-repair experiment the B[a]P-DNA-adduct levels in the first postlabeling experiment were twice as high as the levels in the second postlabeling experiment; this was due to differences in labeling of the standard samples. However, kinetics of adduct-formation and removal were comparable between the two experiments.

Reaction of (±)-syn-BPDE with DNA, dAMP and dGMP

In an attempt to identify the unknown adducts observed after postlabeling of B[a]P-modified DNA from rat and hamster, various reference adducts were prepared, by incubation of DNA or nucleotides with the *syn*-isomer of the reactive BPDE. One volume of purified rat-liver DNA (1 mg/ml in 0.1 M Tris-HCl, pH 7) was incubated with 0.1

volume of (±)-*syn*-BPDE (1 mg/ml in $MgSO_4$-dried acetone) for 12 h at 37°C. The reaction mixture was extracted three times with an equal volume of water-saturated 1-butanol, and twice with an equal volume of water-saturated ether. The DNA was then precipitated from the aqueous phase with alcohol as described above (see DNA isolation).

Solutions of dAMP (40 μM in MQ water) and of dGMP (75 μM in MQ water) were each mixed with an equal volume (±)-*syn*-BPDE (1 mg/ml in dry acetone) for 12 h at 37°C. The B[a]P-modified dNMPs were purified by Sephadex-LH20 chromatography. Both the (±)-*syn*-BPDE modified DNA and the (±)-*syn*-BPDE modified dNMPs were subjected to ^{32}P-postlabeling analysis, run in parallel with treated DNA samples.

UDS and cell proliferation

Slides were prepared for UDS as described by Wolterbeek *et al.* (17). Briefly, tracheal rings were cultured for 18 h in the presence of 370 kBq/ml [methyl-^{3}H]thymidine (Amersham, sp. act. 1.81 TBq/mmol). Semi-thin sections (1 μm thickness) of the tracheal rings were dipped in Kodak NTB-2 emulsion (Eastman Kodak, New York). Autoradiograms were exposed for 4-6 weeks at -30°C. UDS was expressed as net grains per nucleus which was calculated as the number of silver grains over the nucleus minus the number of silver grains over a nuclear-sized area in the cytoplasm. At each time-point a total of four sections (on two different slides) were scored. Per section 50 nuclei were scored.

Cell proliferation was determined in the sections that were also used for UDS (18). In the epithelium the total number of labeled cells (basal and non-basal cells) was counted. Furthermore, the total number of epithelial cells per section was counted. At each time-point cells were counted around the whole circumference (600-1200 cells) of eight different sections (on four slides). The labeling index (LI) was defined as the percentage of labeled cells amongst the total number of cells counted.

Results

Induction-repair experiment

Hamster and rat tracheas were exposed to B[a]P (5 μg/ml, ~20 μM) for two consecutive days, whereafter B[a]P was removed and tracheas were kept in culture for another four days. In DNA isolated from hamster trachea epithelial cells an increase in the total B[a]P-DNA adduct level, as measured with the ^{32}P-postlabeling method, from day 0 (start of exposure to B[a]P) to day 2 (end of exposure to B[a]P) was observed (Figure 2). The adduct level on day 2 corresponded to 36 ± 19 adducts per 10^6 nucleotides (add/10^6n). One day after washing out B[a]P the adduct level had decreased to 60% ± 2% of the level on day 2. There was no further decrease in adduct level during culture. A typical adduct pattern obtained with DNA from the hamster trachea is depicted in Figure 3. At all time-points the TLC results showed one predominant adduct in the hamster trachea (adduct 1),

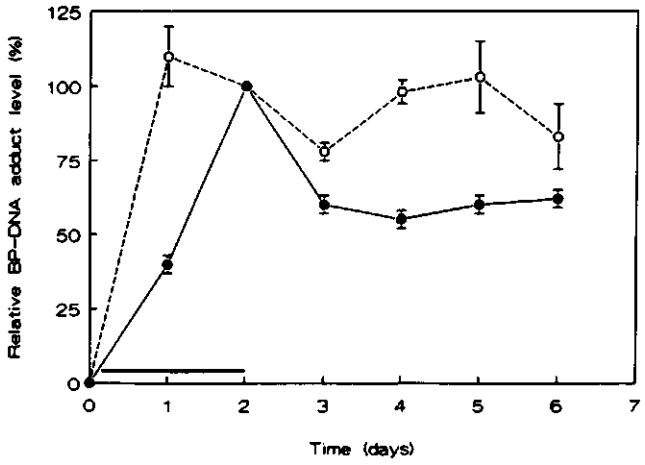

Figure 2: Relative B[a]P-DNA adduct level in hamster (-•-) and rat (-o-) tracheas exposed to B[a]P (5 μg/ml) for two days. The 100% level at 48 h corresponds to 36 ± 19 add/10^6n in case of hamster tracheas and 3.5 ± 1.7 add/10^6n in case of rat tracheas. The horizontal bar indicates the period of B[a]P-exposure. For each of two postlabeling experiments, the results obtained with the sample collected on day 2 was set at 100%. Error bars represent range of values.

which co-migrated with the adduct formed between (+)-*anti*-BPDE and deoxyguanosine. It accounted for 95% of the total adduct level.

In the DNA of rat trachea epithelial cells the B[a]P-DNA adduct level was 3.7 ± 1.5 add/10^6n after one day exposure to B[a]P. However, contrary to the hamster, there was no further increase in the B[a]P-DNA adduct level after two days of exposure. Also in the rat trachea B[a]P-DNA adducts could still be detected four days after removal of B[a]P (Figure 2). Actually, this was almost 90% of the level found at day 2. After 48 h of exposure to B[a]P the total B[a]P-DNA adduct level in the rat trachea was 10-fold lower than in the hamster trachea. As depicted in Figure 3, the adduct pattern on TLC of rat trachea epithelial cells differed considerably from that of hamster; while adduct 1 was almost the only adduct in the hamster, the relative amount in rat trachea was about 60 %. The other substantial adduct in the rat trachea, *viz.* adduct 3, comprised about 30 % of total adducts. The chromatographic behaviour of adduct 3 closely resembles that of one of the two adducts derived from incubation of (±)-*syn*-BPDE with dAMP (see Figure 4). This suggests that adduct 3 is derived from interaction of (±)-*syn*-BPDE and deoxyadenosine. Adduct 2 was a minor adduct (10% of the total adduct level).

In order to determine DNA-repair activity, the unscheduled DNA synthesis (UDS) was scored in trachea sections. In hamster trachea epithelial cells the exposure to B[a]P led to a well measurable induction of UDS (Figure 5). The control level on day 0 of 0.22 ± 0.09

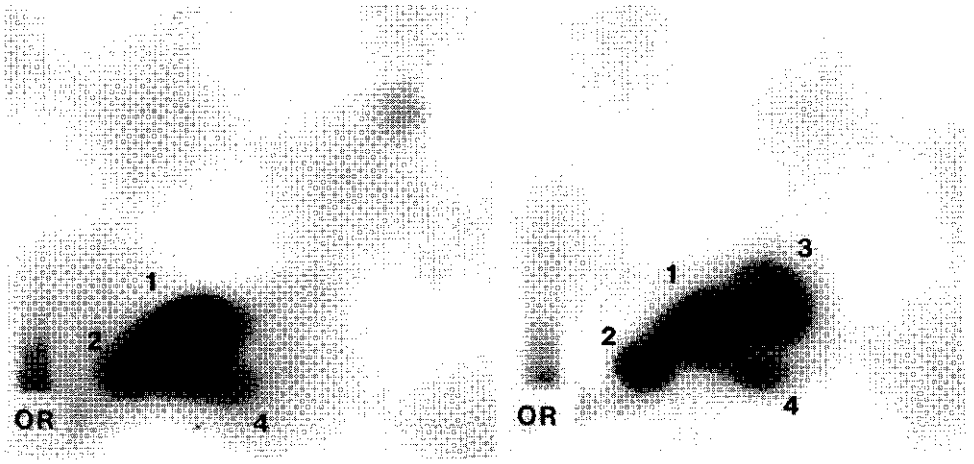

Figure 3: Typical adduct pattern on TLC of postlabeled DNA from hamster (left) and rat (right) tracheas. The films were exposed for 6 h (hamster) and 26 h (rat) at -70°C. Adduct 1 was identified by co-chromatography as the interaction product of (+)-*anti*-BPDE and dG.

increased to 0.54 ± 0.03 on day 1 (statistically significant; Student's t-test; p=0.0056). On day 2 (48 h exposure to B[a]P) and day 3 (one day after removal of B[a]P) the UDS was also significantly higher than on day 0 (Student's t-test; p=0.0003 and p=0.0017, respectively). UDS at days 4, 5, and 6 was not increased compared to that on day 0. In contrast, in rat trachea epithelial cells UDS was not increased after exposure to B[a]P at any timepoint (see Figure 5).

During exposure to B[a]P, cell proliferation was low in hamster tracheas (LI < 2.5%). On days 4, 5, and 6 cell proliferation had recovered (LI varied from 15 to 20%). Despite this rather extensive cell proliferation there was no further decrease in the total B[a]P-DNA adduct level on days 4, 5, and 6 compared to day 2. In general, cell proliferation in hamster tracheas was higher in non-basal cells than in basal cells. In rat tracheas the LI varied from 6 to 12% during exposure to B[a]P. On days 4, 5, and 6 the LI decreased from 12% to <1%. No clear difference was found between non-basal and basal cells with respect to cell proliferation in rat tracheas.

Induction-accumulation experiment

Figure 6 shows the postlabeling data of DNA from tracheas that had been exposed to B[a]P continuously during 15 days. In hamster trachea epithelial cells, a steady increase in the total B[a]P-DNA adduct level from 11 ± 0.7 add/10^6n on day 1 to 105 ± 2 add/10^6n on day 15 was observed. Adduct 1 comprised about 95% of the total adducts, comparable

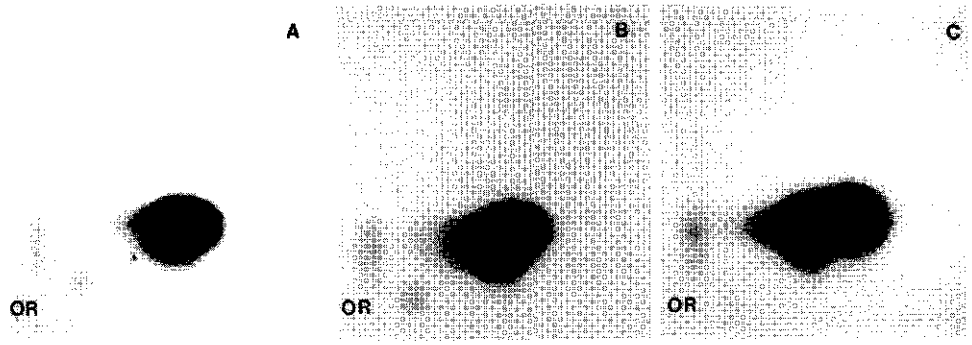

Figure 4: Adduct pattern on TLC of postlabeled (±)-*syn*-BPDE-modified DNA (A), dGMP reacted with (±)-*syn*-BPDE (B), and dAMP with (±)-*syn*-BPDE (C). Film (A) was exposed for 30 min and films (B) and (C) were exposed for 4 h at -70°C.

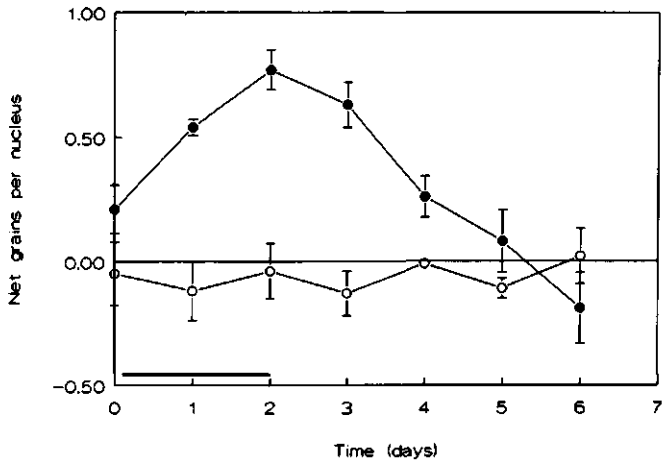

Figure 5: UDS in hamster (-•-) and rat (-o-) tracheas exposed to B[a]P (5 μg/ml) for two days. The horizontal bar indicates the period of B[a]P-exposure. Error bars represent SD (n=8).

with the results of the induction-repair experiment.

In rat trachea epithelial cells the total adduct level on day 1 was 6.1 ± 1.5 add/10^6n. Contrary to the hamster, in the rat trachea no steady increase in adduct level was found; on day 15 the total adduct level was 8.1 ± 0.3 add/10^6n. The adduct patterns of both the induction-repair and the induction-accumulation experiment were comparable, with the exception of adduct 4, a minor adduct (<10%), which was not observed in the induction-repair experiment. The difference in total adduct level between the hamster and rat trachea

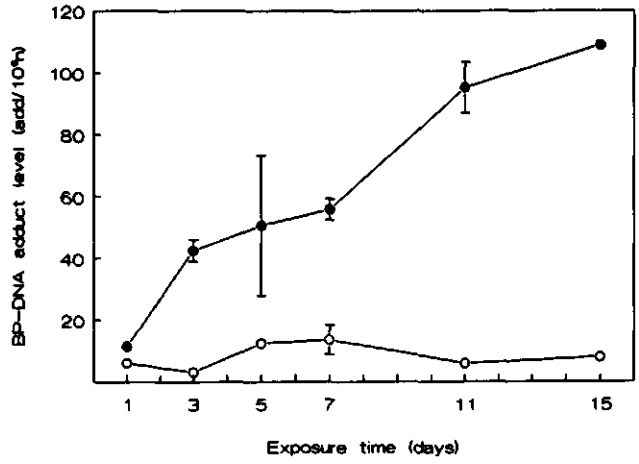

Figure 6: B[a]P-DNA adduct level in hamster (-•-) and rat (-o-) tracheas exposed to B[a]P (5 μg/ml) continuously. Error bars represent range of values of two postlabeling assays (error bars sometimes within the size of the symbol).

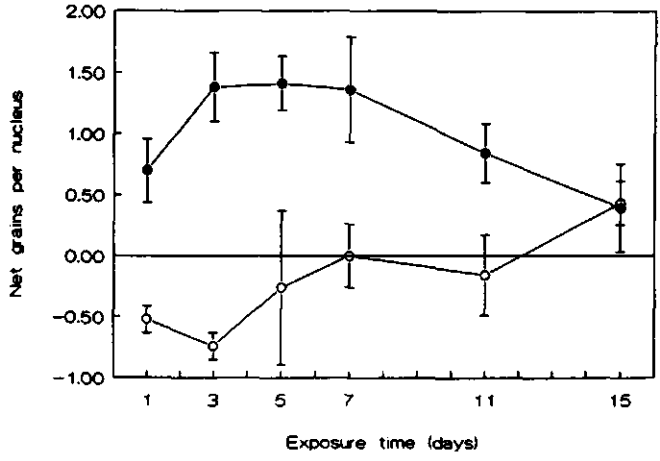

Figure 7: UDS in hamster (-•-) and rat (-o-) tracheas exposed to B[a]P (5 μg/ml) continuously. Error bars represent SD (n=8).

increased from about 2-fold at day 1 to about 13-fold on day 15.

In the hamster trachea epithelial cells UDS (Figure 7) was significantly increased on days 1, 3, 5, 7, and 11 (Student's t-test; $p<0.025$ at all time-points) compared to the unexposed control in the induction-repair experiment (Figure 5, day 0). In rat trachea

epithelial cells UDS did not increase with exposure time. Cell proliferation activity in hamster tracheas decreased during culture to less than 1% at day 15. In rat tracheas, however, cell proliferation increased from a LI of 5% at day 1 to 20% at day 15.

Discussion

In view of the remarkable difference between the hamster and the rat with respect to the susceptibility to B[a]P-induced tumour formation in the trachea, it is of interest to establish whether a similar difference can be observed during the early stages of tumorigenesis, *viz.* with respect to DNA-adduct formation. In this paper the results are presented of experiments in which tracheas from Syrian golden hamsters and Wistar rats were exposed to B[a]P *in vitro* during various periods of time. This *in vitro* organ culture has proven to be an attractive model to study the initial stages of tumour development in the respiratory tract (9). The amount of DNA adducts in the epithelial cells of the tracheas was determined at different time points by use of the ^{32}P-postlabeling assay, which also yielded the adduct pattern as revealed by TLC. Furthermore, DNA-repair synthesis (UDS) and the extent of cell proliferation were determined in preparations sampled at the same time points.

In an induction-repair experiment tracheas from the two species were exposed to B[a]P for two days, followed by post-treatment incubation for four days to allow repair to proceed. The kinetics of adduct formation in epithelial cells during B[a]P exposure and adduct removal during the post-treatment incubation were different: in the hamster trachea the amount of DNA adducts increased during B[a]P treatment, reached a maximum on day 2, then dropped to a level at ~60% of the maximum value, which remained constant on days 4, 5 and 6. In the rat trachea the adduct level reached a plateau value already on day 1 and remained more or less the same during days 2-6. There was a striking difference between the two species as to the total B[a]P-DNA adduct level, which was about 8 times as high in hamster than in rat trachea. In an induction-accumulation experiment tracheas were exposed continuously to B[a]P for 15 days. A steady increase in the DNA-adduct level was observed in the hamster, and a constant, low level in the rat trachea epithelial cells. At the end of the incubation period there was a 13-fold difference between the adduct levels in hamster and rat. A few other studies have been published in which B[a]P-DNA adduct formation in rat and hamster tracheas was compared, after exposure for 24 h to 1 or 1.5 μM [^{3}H]B[a]P (12-14); the differences in total adduct levels varied from 2- to 17-fold between the species, which is similar to our observations. This difference in B[a]P-DNA adduct induction in hamster and rat trachea is in line with the difference in susceptibility of the two species to B[a]P-induced tracheal tumour formation.

Besides quantitative data on adduct levels, the ^{32}P-postlabeling assay also provides information on adduct patterns. The TLC chromatogram of postlabeled hamster tracheal DNA showed that one adduct (adduct 1) was formed almost exclusively (95% of the total

amount), whereas another adduct (adduct 2) comprised only about 2%. Adduct 1 was tentatively identified by co-migration as the reaction product of (+)-*anti*-BPDE and deoxyguanosine (BPDE-N^2dG). Adduct 2 was not identified; it might originate from the reaction of 9-hydroxy-B[a]P-4,5-epoxide with DNA (19). In rat tracheal DNA, adducts 1, 2 and a third adduct (adduct 3) were observed in a 60:10:30 ratio. The chromatographic behavior of adduct 3, when compared with that of appropriate standards, suggested that this adduct results from interaction of the *syn*-isomer of the B[a]P-diolepoxide ((±)-*syn*-BPDE) and deoxyadenosine. Autrup *et al* (12) reported that in rat tracheas BPDE-deoxyadenosine adducts accounted for about 20% of the total modification. Adduct 4 was not identified. However, its low recovery in the butanol extraction procedure (results not shown) suggests that the adduct is more polar than adduct 1, possibly as a consequence of more than two hydroxyl groups on the B[a]P-moiety.

From the qualitative assessment of the adduct patterns on TLC it appears that epithelial cells in both hamster and rat trachea are capable of metabolic conversion of B[a]P into reactive metabolites, under the experimental conditions used. In tracheal DNA from both species the *anti*-BPDE-N^2dG adduct (adduct 1) was detected, albeit in different proportions. This adduct is considered to play a role in B[a]P-induced carcinogenesis. In addition, in rat DNA the *syn*-BPDE-dA adduct was detected. Although there are qualitative differences in the adduct patterns of hamster and rat tracheal DNA, it can be concluded that the different susceptibilities of these species to B[a]P-induced tracheal tumour formation cannot be attributed to a specific adduct that is present in the hamster and absent in the rat. This conclusion appears to be a valid one since there are indications that B[a]P-metabolism and B[a]P-DNA adduct formation during *in vitro* organ culture are not too different from those *in vivo*. This follows from the observation that adduct patterns of postlabeled DNA from the trachea of *in vivo* B[a]P-treated hamsters (intratracheal intubation, ref. 17) were qualitatively comparable with those from the present *in vitro* study.

Bulky lesions, such as those induced by B[a]P, are repaired through the excision-repair pathway, which can be visualized by incorporation of [^{3}H]thymidine during resynthesis of DNA (UDS). In parallel to adduct analysis by ^{32}P-postlabeling, the extent of UDS was determined in samples collected at the same time points. In the induction-repair experiment a significant increase in UDS was detected in hamster trachea cells. The time interval of maximum UDS coincided with the time point at which the highest B[a]P-adduct level was observed. After removal of B[a]P, UDS gradually decreased to control levels on days 5 and 6, despite the fact that ~60% of the adducts were still present. One explanation could be that part of the adducts is not susceptible to repair, and appears persistent. However, this is less likely because the adduct patterns observed in the TLC chromatograms at the various time points were similar. Other explanations could be the absence of active DNA repair in certain cell types, *e.g.*, non-dividing cells (see below), or gradual loss of overall repair activity under the conditions of the *in vitro* organ culture. A

similar UDS pattern in the hamster trachea was observed in the induction-accumulation experiment, albeit at a somewhat higher level. In this case, an increased UDS level was observed up to day 7, and a gradual decrease to control values on day 15, despite the fact that a steady accumulation of DNA adducts was seen. This may be explained by a deficit of the repair system due to the large amount of DNA lesions by that time. In both experiments, rat tracheal cells did not show significant levels of UDS. Apparently, the low level of DNA damage does not evoke a measurable UDS response.

Reduction of DNA-adduct levels, which are expressed per unit amount of DNA, may be the result not only of active repair but also of ongoing cell proliferation. Along with the analysis of repair, the incorporation of [^{3}H]thymidine was also used to determine the relative number of cells in S-phase (expressed as the labeling index), as an indicator of cell proliferation. In the epithelial cells of the hamster trachea, a relatively high labeling index was observed on days 4, 5 and 6 of the induction-repair experiment, while the total amount of DNA isolated was approximately the same at all time points. Furthermore, tissue sections of the treachea did not show an increase of the thickness of the epithelial wall during the course of the experiment. This may indicate that cell proliferation in the epithelium is counterbalanced by cell loss at the epithelial surface. Remarkably, the total amount of B[a]P-DNA adducts per μg DNA did not change during that time period, despite the rather high labeling index. This may be explained by a preferential occurrence of proliferation of those cells which contained only few adducts or which had their adducts removed before entering into S-phase owing to rapid DNA-repair, and/or by an uneven distribution of B[a]P-DNA adducts over the various cell types. Also, the time needed to proceed through the S-phase in B[a]P-exposed tracheal epithelial cells may be important in this respect; since indications point out that the cell-cycle in this *in vitro* system amounts to 3-4 days, the S-phase may very well take a few days, in which case we may not be able to detect any substantial change in the B[a]P-DNA level per μg DNA over such a period. The use of adduct-specific antibodies will allow the study of adduct formation and repair *in situ*, in different epithelial cell types within the trachea. Experiments along this line are ongoing.

Acknowledgements

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Chapter 5

Vitamin A and β-carotene influence the level of benzo[a]pyrene-induced DNA adducts and DNA-repair activities in hamster tracheal epithelium in organ culture.

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Summary

Although most studies concerning the effect of vitamin A and β-carotene on chemical carcinogenesis are focused on tumour promotion and progression, these compounds may affect initiation as well. In this study the influence of vitamin A and β-carotene on unscheduled DNA synthesis (UDS) was investigated in hamster tracheal epithelium in organ culture exposed to benzo[a]pyrene (B[a]P). DNA-repair activities were compared with the level of B[a]P-DNA adducts as measured both by ^{32}P-postlabeling and by immunocytochemical detection. In hamster tracheal epithelial cells, both vitamin A and β-carotene significantly increased B[a]P-induced UDS, with 40% and 45%, respectively. At the same time, vitamin A and β-carotene decreased the level of B[a]P-DNA adducts in these cells with 18% and 40%, respectively as measured by ^{32}P-postlabeling and with 12% and 35%, respectively as measured by immunocytochemistry. The effect of vitamin A on B[a]P-induced UDS and DNA-adduct levels in hamster tracheal epithelium appeared to depend on the dose of B[a]P *vis-à-vis* the concentration of vitamin A. The results of the present study show that both vitamin A and β-carotene cause a decrease in B[a]P-DNA adduct levels by enhancing DNA-repair activities. Because the formation of B[a]P-DNA adducts is considered to be an early step in respiratory tract carcinogenesis, it is suggested that enhancement of DNA-repair activities by vitamin A and the subsequent removal of DNA adducts may be one of the mechanisms involved in vitamin A-mediated protection against cancer.

Introduction

Several epidemiological and experimental studies have shown an inverse relationship between the intake of retinoids or their precursor molecule β-carotene and the incidence of

respiratory tract cancer (1-6). Saffiotti *et al.* (3) were the first to report on the modifying effect of vitamin A on respiratory tract cancer in hamsters after intratracheal instillation of benzo[a]pyrene (B[a]P) attached to ferric oxide particles. Later on, more studies demonstrated the protective effect of vitamin A and β-carotene against the formation of (pre)neoplastic lesions of the respiratory tract in experimental animals (4-6). However, other studies failed to show a protective effect of β-carotene (7), or even showed an enhancing effect of vitamin A on respiratory tract cancer [8]. The mechanism by which vitamin A influences respiratory tract cancer at the molecular level is still largely unknown. Most experiments concerning the role of vitamin A in carcinogenesis have focused on tumour promotion and progression (4,9-12). However, vitamin A has also been shown to modulate initiation (4,13-15).

One of the critical events in chemical carcinogenesis is the formation of DNA adducts. Improper DNA repair or lack of repair may result in permanent changes in the DNA during the subsequent DNA replication. Vitamin A and β-carotene have been shown to prevent or to decrease DNA damage caused by chemical carcinogens, as detected directly by measuring the level of DNA adducts (16,17) or indirectly by measuring sister-chromatid exchanges (18), chromosome aberrations (18,19) or DNA repair (20,21). In general, the inhibition of DNA-damage induction by vitamin A is ascribed to an effect on the metabolism of carcinogenic compounds, resulting in a decreased amount of ultimate carcinogen. However, it has also been shown that vitamin A inhibited the formation of DNA damage by agents that do not require metabolic activation such as UV light (22). Furthermore, Decoudu *et al.* (23) showed that vitamin A deficiency decreased the activities of enzymes involved in the activation of aflatoxin B1 but increased the level of DNA damage. It was suggested that the protection by chromosomal constituents to DNA damage was decreased by vitamin A deficiency.

In previous studies (24,25), we showed a positive relation between the formation of B[a]P-DNA adducts and B[a]P-induced DNA repair in hamster tracheal epithelial cells. The objective of the present study is to investigate the effect of vitamin A and β-carotene on the repair of B[a]P-DNA adducts in hamster tracheal epithelial cells in organ culture. DNA-repair activities were assessed by unscheduled DNA synthesis (UDS) and related to the level of B[a]P-DNA adducts as measured by ^{32}P-postlabeling or by immunocytochemical analysis.

Materials and methods

Culturing of hamster tracheas

Tracheas from Syrian golden hamsters (obtained from Harlan/CPB, Zeist, The Netherlands) were cultured as described previously (24). Briefly, hamster tracheas were isolated aseptically and the external surface was cleaned to remove the adherent tissue. Thereafter,

tracheas were divided into 4-5 rings of approx. 2-3 mm, which were randomly allocated to the wells of 24-well culture dishes (Costar, Cambridge, MA). The tracheas were cultured in serum-free, hormone-supplemented Ham's F12 medium [L-glutamine 2mM (Flow Laboratories, Herts, England), hydrocortisone 1 μM, bovine pancreatic insulin 5 μg/ml, human transferrin 5 μg/ml, epidermal growth factor 25 ng/ml (Sigma Chemicals, St. Louis, MO) and gentamycin 50 μg/ml (Flow)]. Cultures were gassed with 40% O_2, 55% N_2 and 5% CO_2 in a humidified incubator at 37^0C. The culture dishes were rocked 8-9 times/min to allow contact of the tracheal rings with both gas and culture medium.

Experimental protocol

In a first experiment (Figure 1a) tracheas were exposed for 2 consecutive days to B[a]P (0, 20, 40 and 60 μM, Sigma). Thereafter, tracheas were cultured in B[a]P-free medium, supplemented with vitamin A (all-*trans*-retinol, 1 μM, Sigma) or dimethyl sulfoxide (DMSO, 0.1% v/v, Sigma) for another 3 days. On each day, the epithelial cells of 4 tracheas per treatment were gently scraped off, collected in phosphate-buffered saline (PBS, pH 7.4, Flow) and stored at -80^0C for quantification of the level of B[a]P-DNA adducts by ^{32}P-postlabeling (see below). Furthermore, tracheas were cultured in the presence of 370 kBq/ml [methyl-^{3}H]thymidine (Amersham, sp. act. 1.81 Tbq/mmol) for 18 h before sampling to assess UDS (see below).

In a second experiment (Figure 1b), tracheas were exposed for up to 12 days to 10 μM B[a]P in combination with equimolar concentrations of vitamin A or β-carotene (Merck, Darmstadt, Germany). As a control, tracheas were cultured in medium containing DMSO (0.1% v/v). After 2 days, the epithelial cells of 2 tracheas per treatment were collected as described above for quantification of B[a]P-DNA adducts by ^{32}P-postlabeling (see below). Another trachea per treatment was incubated overnight in 0.05% trypsin in PBS with 0.025% EDTA at 4^0C. Thereafter, the epithelial cells were flushed out with and suspended in foetal calf serum (Flow) and subsequently cytospin preparations were made on 3-amino-propyltriethoxysilane (Sigma)-precoated slides. Next, cells were fixed with methanol for 10 min at room temperature and the level of B[a]P-DNA adducts was quantified by immunocytochemical analysis (see below). UDS was assessed every other day as described below.

In both experiments, medium was refreshed every day. B[a]P and vitamin A were added from a stock solution in DMSO, the β-carotene solution in DMSO was made fresh every day. The final concentration of DMSO in the culture medium was 0.1%.

^{32}P-postlabeling analysis of B[a]P-DNA adducts

DNA was isolated from tracheal epithelial cells by means of phenolic extractions and ethanol precipitation as described previously (26). In the first experiment, the ^{32}P-postlabeling assay was performed according to the nuclease P1-enrichment procedure as described by Reddy and Randerath (27), with some modifications as described previously

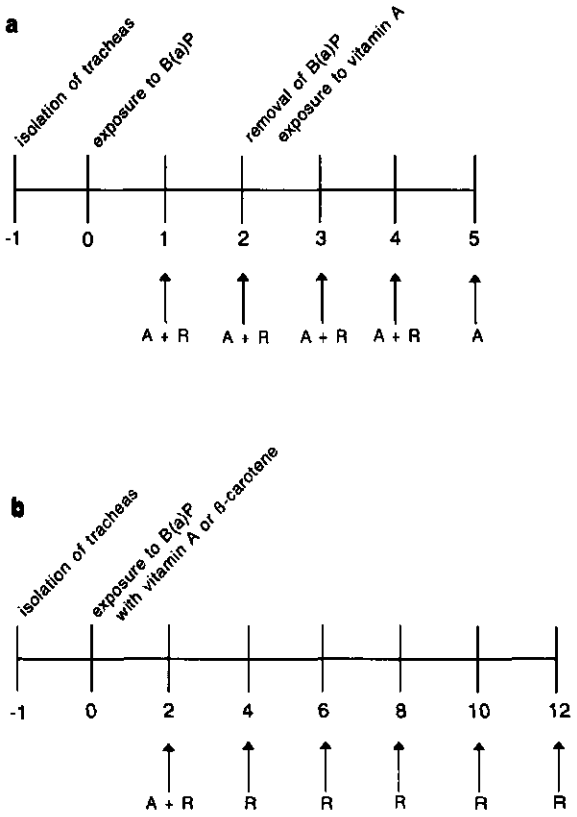

Figure 1: Diagram of experiment 1 (a) and 2 (b). The "A" and "R" indicate sampling points (days) for adduct quantification (A) and for assessment of DNA-repair activities (R).

(26). In the second experiment, B[a]P-DNA adducts were isolated by n-butanol extraction (28). In both cases, adducts were labeled with 2.3 MBq [γ^{32}P]ATP/sample (Amersham, sp.act. > 185 TBq/mmol) by incubation with T4 polynucleotide kinase (Biolabs, Beverly, MA) as described by Gupta (27). Multidirectional thin-layer chromatography of labeled nucleotides and quantification of B[a]P-DNA adducts were performed as described recently (26,29).

Immunocytochemical analysis of B[a]P-DNA adducts

Methanol-fixed tracheal epithelial cells were rehydrated in 0.1 N HCl at 4^0C for 3 min, treated with RNAse A (0.1 mg/ml) at 37^0C for 1 h and dehydrated in ethanol-series. Thereafter, DNA in the cells was denaturated with 0.15 N NaOH in 70% ethanol for 2 min and fixed in 3.5% formaldehyde (Riedel-de Haën, Seelze, Germany) in 70% ethanol

for 30 sec. Cells were pretreated with proteinase K buffer (2 mM Tris/HCl, pH 7.4 and 0.2 mM $CaCl_2$) at 37^0C for 1 min and treated with 2 μg/ml proteinase K (Sigma) in this buffer for 10 min at 37^0C. Next, cells were preincubated with 5% normal hamster serum in Tris-buffered saline (TBS, 20 mM Tris/HCl, pH 7.4 and 150 mM NaCl) for 1 h. Cells were then incubated with a rabbit antiserum directed against B[a]P-DNA adducts [30] in 5% normal hamster serum in TBS for 18 h at 4^0C and subsequently incubated with an fluorescein isothiocyanate (FITC)-conjugated goat-anti-rabbit antibody in 5% normal hamster serum in TBS for 1 h at 37^0C. Finally, DNA was counterstained with propidium iodide (50 ng/ml in TBS) for 10 min and cells were mounted with TBS/glycerol (1:9). FITC fluorescence, as a measure of DNA-adduct levels, was quantified by use of laser-scanning microscopy and image-processing software as described by Roggeband *et al.* (30). These authors also presented comparative analysis of adduct detection by ^{32}P-postlabeling and immunocytochemistry, which showed a fair agreement between the two methods (30).

Unscheduled DNA synthesis

Slides were prepared for UDS as described previously (24). Briefly, semi-thin (1 μm) plastic sections of tracheal rings were dipped in Kodak NTB-2 emulsion (Eastman, Kodak, New York). Autoradiograms were exposed for 4 weeks at -30^0C, developed in Kodak D19 and stained with 0.01% toluidine blue solution (Gurr, Chadwell Heath, UK). UDS was expressed as net grains per nucleus, which was calculated as the number of silver grains over the nucleus minus the number of silver grains over a nuclear-sized area in the cytoplasm. Per section 50 nuclei were scored.

Statistical analysis

The mean coefficient of variation (CV) for the UDS analysis was 25.5, the CV for the ^{32}P-post- labeling was 36.3 and the CV for the immunocytochemical analysis was 18.1. Data were tested for significant differences by analysis of variance (ANOVA) followed by the Student's t-test (BMDP statistical software manual, 1988).

Results

UDS in tracheal epithelial cells

In the first experiment (see outline in Fig. 1a), B[a]P induced a time- and dose-dependent increase in UDS (Fig. 2 and 3). Replacement of the medium with 20 μM B[a]P by medium supplemented with DMSO or vitamin A on day 2 resulted both in a decrease in UDS with 87% and 52%, respectively, on day 3, and 82% and 45%, respectively, on day 4 (Fig. 2). UDS in tracheas treated with vitamin A was increased compared with UDS in control tracheas, the increase being about 4-fold on day 3 and 3-fold on day 4 (Fig. 2).

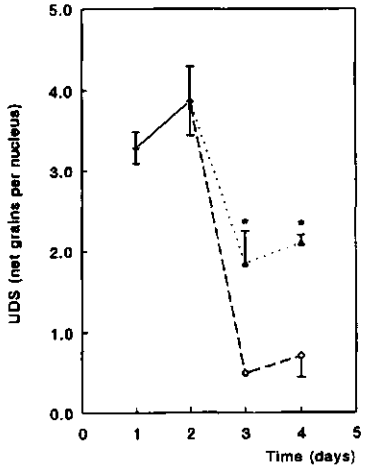

Figure 2: Effect of vitamin A on UDS in hamster tracheal epithelial cells exposed to B[a]P (20 μM). Tracheas were exposed to B[a]P for 2 days (+-+). Thereafter, the tracheas were cultured in B[a]P-free medium supplemented with 1 μM vitamin A (▲-▲) or 0.1% DMSO (○-○) for 2 days. UDS levels in tracheas treated with vitamin A were significantly increased compared with the UDS levels in the tracheas treated with DMSO (*: $P<0.05$). Statistics; unpaired t-test, error bars represent SEM (n=2). (Error bars sometimes within the size of the symbol).

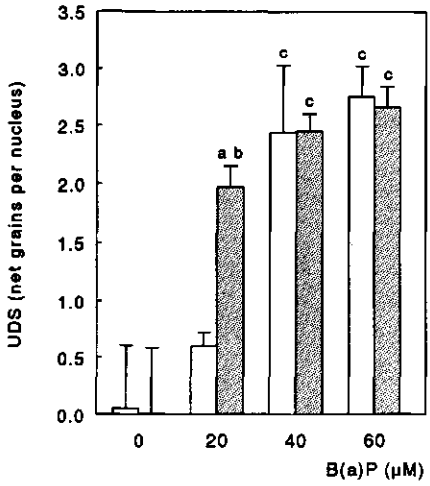

Figure 3: Effect of vitamin A on UDS in hamster tracheal epithelial cells pre-treated with B[a]P (0, 20, 40 or 60 μM) for 2 days. After this period, the tracheas were cultured in B[a]P-free medium supplemented with 1 μM vitamin A (hatched bars) or 0.1% DMSO (open bars) for another 2 days. Data are presented as mean of UDS values of day 3 and 4. In tracheas pre-treated with 20 μM B[a]P, vitamin A increased UDS compared to the tracheas treated with DMSO (a: $P<0.05$). UDS in tracheas pre-treated with B[a]P was significantly increased compared with control tracheas *i.e.* B[a]P 0 μM (b: $P<0.01$, c: $P<0.001$). At high concentrations of B[a]P (40 and 60 μM), vitamin A did not influence UDS. Error bars represent SEM (n=4).

UDS was significantly increased in the tracheal epithelial cells pre-exposed to B[a]P compared with UDS in the tracheas pre-exposed to DMSO (B[a]P 0 μM, Fig. 3). In the tracheas pre-treated with the high concentrations of 40 and 60 μM B[a]P for 2 days, vitamin A did not affect UDS (Fig. 3).

In the second experiment (see outline in Fig. 1b), analysis of variance did not show a significant time-dependent effect of the various treatments on UDS. Analysis of the data grouped by treatment showed a clear statistically significant increase in UDS in tracheal epithelial cells exposed to 10 μM B[a]P compared with the untreated controls (Fig. 4). In the tracheal epithelial cells treated with the combination of B[a]P and vitamin A or β-carotene, UDS was significantly increased compared with tracheal epithelial cells treated with B[a]P alone viz. with 40% and 45%, respectively (Fig. 4).

B[a]P-DNA adducts in tracheal epithelial cells

In the first experiment (see outline in Fig. 1a), a time-dependent formation of B[a]P-DNA adducts was found in tracheas exposed to 20 μM B[a]P as detected by ^{32}P-postlabeling (Fig. 5). After replacement of the B[a]P-containing medium by medium with DMSO, the level of B[a]P-DNA adducts still increased for 1 more day, whereas in tracheal epithelial cells post-treated with vitamin A, the level of B[a]P-DNA adducts decreased immediately (Fig. 5). In the tracheal epithelial cells treated with vitamin A the level of B[a]P-DNA adducts, avaraged over days 3 to 5, slightly decreased with 25% and 10% in the tracheas treated with 20 and 40 μM B[a]P, respectively (Fig. 6). Vitamin A increased the level of B[a]P-DNA adducts in hamster tracheal epithelium pre-exposed to the high concentration of 60 μM B[a]P with 23% compared with the tracheas post-treated with DMSO.

In the second experiment (see outline in Fig. 1b), the level of B[a]P-DNA adducts in hamster tracheal epithelial cells exposed for 2 days to 10 μM B[a]P was 17.3 ± 6.7 B[a]P-DNA adducts/10^6 nucleotides (add/10^6n). Vitamin A and β-carotene decreased the levels of B[a]P-DNA adducts to 14.4 ± 3.5 and 10.6 ± 3.5 add/10^6n, respectively (a decrease of 17% and 39%, respectively) (Fig. 7). Figure 8 shows the effect of vitamin A and β-carotene on B[a]P-DNA adduct formation, the immunocytochemical assay being used to quantify the level of B[a]P-DNA adducts. In the tracheal epithelial cells exposed to B[a]P the fluorescence signal was clearly higher than in the control epithelial cells. Tracheal epithelial cells treated with the combination of B[a]P and vitamin A or β-carotene showed fluorescence signals that were decreased by 23% and 50% respectively, compared with the fluorescence signal in the tracheal epithelial cells treated with B[a]P alone (Fig. 8). This indicates that vitamin A and β-carotene decreased the level of B[a]P-DNA adducts.

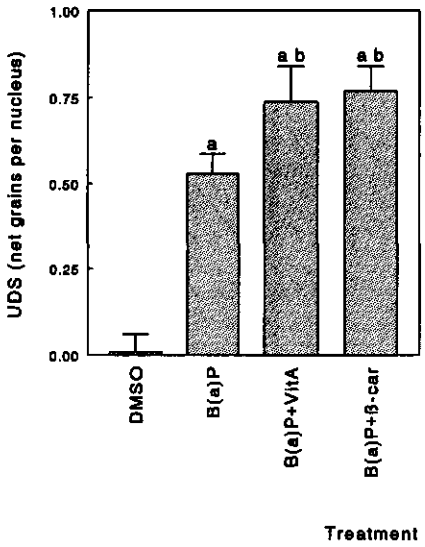

Figure 4: Effect of vitamin A and β-carotene on UDS in hamster tracheal epithelial cells exposed to 10 μM B[a]P in combination with equimolar concentrations of vitamin A or β-carotene up to 12 days. Data are presented as mean of UDS values of each experimental day. UDS in tracheas treated with B[a]P or with a combination of B[a]P and vitamin A or β-carotene was increased compared to UDS in the tracheas treated with DMSO (a: $P<0.001$). Vitamin A and β-carotene significantly increased UDS compared to B[a]P alone (b: $P<0.05$). Error bars represent SEM (n=12).

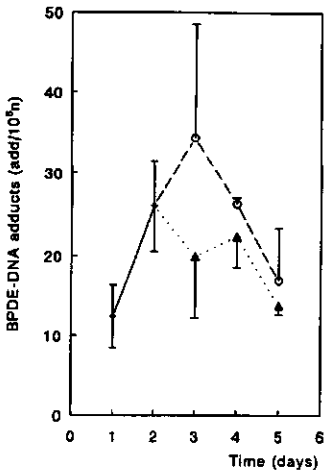

Figure 5: Effect of vitamin A on the removal of B[a]P-DNA adducts in hamster tracheal epithelial cells as detected by ^{32}P-postlabeling. Tracheas were exposed to B[a]P (20 μM) for 2 days (+-+). Thereafter, the tracheas were cultured in B[a]P-free medium, supplemented with 1 μM vitamin A (▲-▲) or 0.1% DMSO (○-○) for 3 days. B[a]P-DNA adduct levels were lower in tracheas treated with vitamin A than in tracheas treated with DMSO. Error bars represent SEM (n=2).

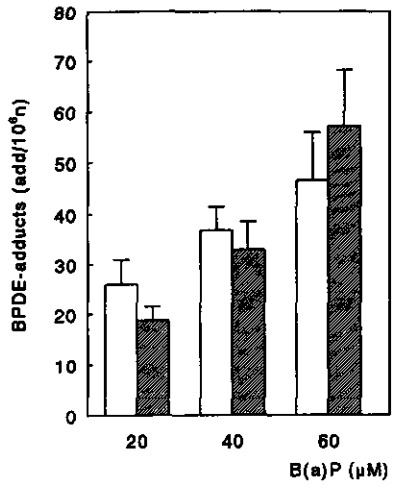

Figure 6: Effect of vitamin A on B[a]P-DNA adduct levels in hamster tracheal epithelial cells. Tracheas were exposed to B[a]P for 2 days. Thereafter, the tracheas were cultured for 3 days in B[a]P-free medium, supplemented with 1 μM vitamin A (hatched bars) or 0.1% DMSO (open bars). The B[a]P-DNA adduct levels were measured by ^{32}P-postlabeling. Data are presented as the mean of B[a]P-DNA adduct levels of day 3, 4 and 5. The B[a]P-DNA adduct levels in tracheas exposed to 20 and 40 μM B[a]P were decreased by vitamin A compared with B[a]P-DNA adduct levels in tracheas treated with DMSO only. B[a]P-DNA adduct levels in the tracheas exposed to 60 μM B[a]P were increased by vitamin A. Error bars represent SEM (n=6).

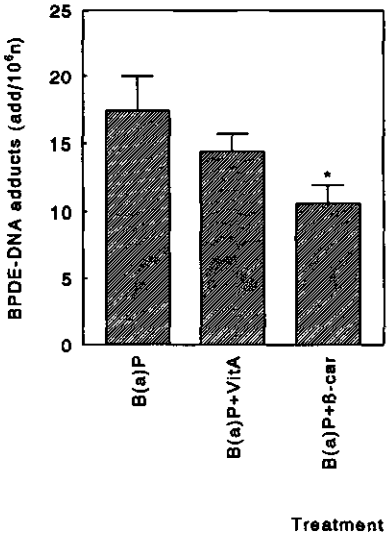

Figure 7: Effect of vitamin A and β-carotene on the formation of B[a]P-DNA adducts in hamster tracheal epithelial cells. Hamster tracheas were exposed to 10 μM B[a]P in combination with equimolar concentrations of vitamin A or β-carotene for 2 consecutive days. B[a]P-DNA adduct levels were measured by ^{32}P-postlabeling. Both vitamin A and β-carotene inhibited the formation of B[a]P-DNA adducts (*: $P<0.05$). Error bars represent SEM (n=8).

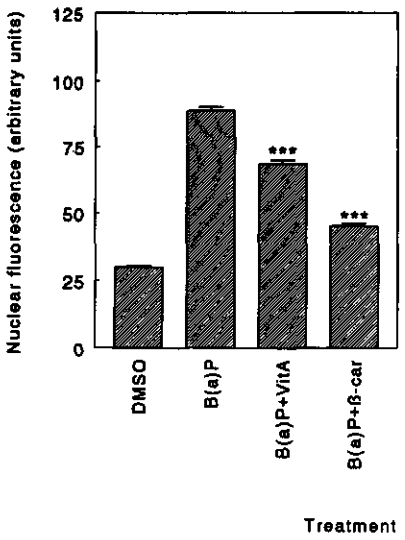

Figure 8: Effect of vitamin A and β-carotene on the formation of B[a]P-DNA adducts in hamster tracheal epithelium as detected by immunocytochemical analysis. Hamster tracheas were exposed to 10 μM B[a]P in combination with an equimolar concentration of vitamin A or β-carotene for 2 consecutive days. Both vitamin A and β-carotene inhibited the formation of B[a]P-DNA adducts (***: $P<0.001$). Bars represent the mean fluoresence signal of 150 - 250 epithelial cells, error bars represent SEM.

Discussion

In previous studies, we have shown that there was a positive correlation between the formation of the (+)-*anti*-benzo[a]pyrenediolepoxide (BPDE) guanosine adduct, the major B[a]P-DNA adduct formed in hamster tracheal epithelial cells, and DNA-repair activities (24, 25). Although the data in the present study show considerable variation (the CV for UDS analysis was 25.5, the CV for ^{32}P-postlabeling was 36.3 and the CV for the immunocytochemical analysis was 18.1), the results of UDS and B[a]P-DNA adduct levels obtained in the various experiments carried out independently and obtained with different experimental techniques showed similar effects. Vitamin A and β-carotene increased B[a]P-induced DNA-repair activities in hamster tracheal epithelial cells. This increase in DNA-repair activities was not due to enhancement of the formation of B[a]P-DNA adducts (adverse effect) by vitamin A or β-carotene but, on the contrary, coincided with decreased levels of B[a]P-DNA adducts (beneficial effect).

In the first experiment of this study, vitamin A was added to the culture medium after removal of B[a]P to preclude an effect of vitamin A on the metabolism of B[a]P. At the low concentration of B[a]P, vitamin A decreased the level of B[a]P-DNA adducts and increased UDS. So far, a direct effect of vitamin A on DNA-repair activities induced by chemical carcinogens has not been reported. Effects of vitamin A on DNA-repair activities

as described in other studies had been explained as an indirect effect by changes in the metabolism of carcinogenic compounds (20). The mechanism underlying the direct effect of vitamin A on UDS is still unknown. Sharma *et al.* (31) recently showed that vitamin A influenced the activity of the poly(ADP-ribose)polymerase enzym in human neonatal foreskin fibroblasts, an enzym which is involved in DNA-repair. Furthermore, vitamin A strongly influences cell proliferation in hamster tracheal epithelial cells (32,33). Part of the enzymes involved in cell proliferation are also involved in UDS (34). Probably, vitamin A increased UDS by enhancing DNA synthesis.

At higher concentrations of B[a]P, vitamin A was unable to increase UDS and thereby decrease B[a]P-DNA adduct levels. Apparently, the effect of vitamin A on the level of B[a]P-DNA adducts depends on the dose of B[a]P *vis-à-vis* the concentration of vitamin A. Similar results were described by Rutten *et al.* (9) for the effect of vitamin A on intercellular communication in tracheal epithelial cells.

In the second experiment, the concentration of B[a]P used was decreased compared with the B[a]P-concentrations used in the first experiment, because the effect of vitamin A on the formation and removal of B[a]P-DNA adducts was observed more clearly at lower concentrations of B[a]P. In this experiment we were unable to show a statistically significant time-dependent effect of B[a]P on UDS (ANOVA), so UDS-data obtained at the different experimental days were combined for further statistical analysis. The results of this experiment showed that vitamin A also increased B[a]P-induced UDS and decreased the levels of B[a]P-DNA adducts when administered in combination with B[a]P. Furthermore, the results showed that β-carotene was even more efficient in decreasing B[a]P-DNA adduct levels than vitamin A. However, the stronger effect of β-carotene on B[a]P-DNA adduct levels was not reflected by a stronger effect on UDS, which suggests that for β-carotene besides the effect on UDS other mechanisms may be involved to influence the level of B[a]P-DNA adducts (35).

As has been shown in the second experiment, the effect of vitamin A and β-carotene on the formation of B[a]P-DNA adducts as measured by immunocytochemistry was of the same order as the effect measured by ^{32}P-postlabeling. This is in agreement with a previous study of Roggeband *et al.* (30). They showed a fairly good correlation between the level of B[a]P-DNA adducts in hamster tracheal epithelial cells detected by ^{32}P-postlabeling or immunocytochemistry. Therefore, we can conclude that the immunocytochemical method to detect B[a]P-DNA adducts in tracheal epithelial cells isolated by incubation with trypsin appears to be a suitable alternative for the laborious ^{32}P-postlabeling assay to quantify B[a]P-DNA adducts in hamster tracheal epithelial cells exposed to B[a]P. Furthermore, this method gives the opportunity to detect B[a]P-DNA adducts in specific cells as has been shown previously (30).

In conclusion, the results of the present study show that both vitamin A and β-carotene increase DNA-repair activities and decrease the formation of B[a]P-DNA adducts in hamster tracheal epithelial cells. Because the formation of B[a]P-DNA adducts is involved

in respiratory tract carcinogenesis, it is suggested that enhancement of DNA-repair activities by vitamin A and the subsequent removal of DNA-adducts may be one of the mechanisms involved in the protection against cancer by vitamin A.

Acknowledgements

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Chapter 6

Influence of growth factors and medium composition on benzo(a)pyrene and vitamin A-induced cell proliferation and differentiation in hamster tracheal epithelium in organ culture.

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Summary

Tracheal organ cultures and isolated tracheal epithelial cells are frequently used to study effects of carcinogens and retinoids on both proliferation and differentiation of respiratory tract epithelial cells. For each of these *in vitro* models, optimal culture conditions have been established, varying in type of culture medium and composition of growth factor and hormone supplementation, which by themselves may influence cellular proliferation and differentiation. In this study, we investigated the influence of medium composition and growth factor supplementation on the effect of benzo(a)pyrene (B[a]P) and vitamin A on cellular proliferation and differentiation in hamster tracheal epithelium in organ culture. In tracheas cultured in Ham's F12 medium, cell proliferation was decreased by B[a]P relative to untreated controls, whereas vitamin A in combination with B[a]P increased cell proliferation compared with that in tracheas treated with B[a]P alone. The effects in tracheas cultured in CMRL-1066 medium were just the opposite: B[a]P increased cell proliferation and vitamin A decreased B[a]P-induced proliferation. To explain this difference in cell proliferation, the effects of various growth factors (epidermal growth factor and transferrin) and medium components (nucleotides, NAD^+/NADP and $CaCl_2.2H_2O$) on B[a]P and vitamin A-induced cell proliferation were investigated. The main factor responsible for the different effects on cell proliferation appeared to be the concentration of Ca^{2+} in the culture medium; addition of $CaCl_2.2H_2O$ to Ham's F12 medium resulted in effects of B[a]P and vitamin A on cell proliferation comparable to those observed in tracheas cultured in CMRL-1066 medium.

These results clearly show that the composition of the culture medium, and particularly the concentration of Ca^{2+}, strongly influences the effect of vitamin A and B[a]P on cell proliferation in hamster tracheal epithelium in organ culture.

Introduction

Various *in vitro* systems for culturing isolated tracheal epithelial cells, tracheal explants and tracheal organ cultures have been developed and are used to study factors that influence both aetiology and pathogenesis of respiratory tract cancer.

Tracheas can be cultured in serum-free hormone-supplemented CMRL-1066 medium for several weeks, in the course of which the normal pseudostratified columnar mucociliary differentiation is preserved. This renders this model appropriate to study effects of compounds that influence the delicate balance between cellular proliferation and differentiation of the epithelium. In this regard, the effects of retinoids on proliferation and differentiation of epithelial cells have been extensively investigated. Retinoids are essential for maintenance of the normal tracheobronchial epithelium. Both *in vivo* and *in vitro* experiments have shown that vitamin A deficiency resulted in hyperplasia and squamous metaplasia of the tracheal epithelium (1-5), which can be reversed by the addition of vitamin A (6,7). Furthermore, vitamin A inhibited the formation of squamous metaplasia and hyperplasia in tracheal epithelium treated with chemical carcinogens such as benzo[a]-pyrene (B[a]P) or cigarette-smoke condensate (8-11).

In addition to tracheal organ cultures, isolated tracheal epithelial cells have also been used to investigate effects of retinoids and chemical carcinogens on cellular proliferation and differentiation (12-14). For each of the above-mentioned *in vitro* models, optimal culture conditions have been established, which vary in type of culture medium and composition of growth factor or hormone supplementation. Among these components, several have been shown to influence cellular proliferation and differentiation to a large extent (12,15-20), and some of them may influence, directly or indirectly, the effect of vitamin A and B[a]P on differentiation and proliferation of tracheal epithelial cells. Furthermore, in addition to growth factors, the concentration of calcium in the culture medium has a strong effect on proliferation and differentiation of epithelial and epidermal cells (21,22).

The objective of the present study was to investigate the influence of culture medium composition and growth factor supplementation on B[a]P- and vitamin A-induced tracheal epithelial cell proliferation and differentiation in organ culture.

Materials and methods

Culturing of hamster tracheas

Ten-week old male Syrian golden hamsters, obtained from Harlan/CPB, Zeist, The Netherlands, were killed by an *i.p.* overdose of Nembutal (Ceva, Paris, France). Tracheas were isolated aseptically and adherent connective tissue was removed. Thereafter, tracheas were cut into 4 or 5 rings (ca. 2 mm thickness). Tracheal rings were placed in the wells of 24-well plates (Costar, Cambridge, MA) and cultured in serum-free, hormone-supple-

mented Ham's F12 (Flow Laboratories, Herts, UK) or CMRL-1066 medium (Flow; see Experimental protocol). Each well contained 2 tracheal rings derived from different hamsters. Cultures were gassed with 40% O_2, 55% N_2 and 5% CO_2 in a humidified incubator at 37^0C. The culture dishes were rocked 8 or 9 times/min to allow contact of the tracheal rings with both gas and culture medium. One day after isolation, the tracheas were treated as described in Experimental protocol.

Experimental protocol

Three independent experiments were carried out. In the first experiment, tracheas were cultured in Ham's F12 medium or CMRL-1066 medium supplemented with cofactor mix 1 (Ham's $F12^{mix1}$ and CMRL-1066^{mix1}; see Table I for the composition of the cofactor mix) and exposed to B[a]P (10 μM, Sigma Chemicals, St Louis, MO) with or without vitamin A (all-*trans* retinol, 10^{-5} M, Sigma) for 4 days. Control tracheas were cultured in medium containing the solvent control DMSO (0.1% v/v, Sigma).

Table I: Composition of the cofactor mixtures.

	Mix 1	Mix 2
L-glutamine[a]	2 mM	2 mM
hydrocortisone[b]	0.36 μg/ml[c]	0.1 μg/ml
bovine pancreatic insulin[b]	5 μg/ml	1.0 μg/ml
human transferrin[b] (Tf)	5 μg/ml	-
epidermal growth factor[b] (EGF)	25 ng/ml	-
gentamycin[a]	50 μg/ml	50 μg/ml

[a] Flow Laboratories, Herts, UK; [b] Sigma Chemicals, St. Louis, MO; [c] equal to 1 μM.

In a second experiment, tracheas were cultured in Ham's $F12^{mix1}$ and treated with B[a]P (5, 10, 20 and 40 μM) for 4, 6, 8 and 10 days. Furthermore, tracheas were exposed to B[a]P (10 μM) in combination with vitamin A (10^{-9}, 10^{-7} and 10^{-5} M) for 4, 6, 8 and 10 days. As controls, tracheas were cultured in Ham's $F12^{mix1}$, supplemented with either DMSO (0.1% v/v) or vitamin A (10^{-9}, 10^{-7} and 10^{-5} M). To study the role of growth factor and culture medium components, in a third experiment, tracheas were cultured for 4 days in Ham's $F12^{mix1}$ without epidermal growth factor (EGF, Sigma) or transferrin (Tf,

Sigma), or in Ham's F12 medium or CMRL-1066 medium supplemented with cofactor mix 2 (Ham's $F12^{mix2}$ and CMRL-1066^{mix2}; see Table I). Furthermore, tracheas were cultured in Ham's $F12^{mix1}$ or Ham's $F12^{mix2}$ medium supplemented with $CaCl_2.2H_2O$ (Sigma), nucleotides (Sigma) and NAD^+/NADP (Sigma) for 4 days. The final concentrations of these supplements in Ham's F12 medium were identical to those in the CMRL-1066 medium ($CaCl_2.2H_2O$ 1.2 mM, nucleotides 10 mg/l, NAD 7 mg/l, NADP 1.03 mg/l). In this experiment, tracheas were treated with either DMSO (0.1%, v/v), B[a]P (10 μM), B[a]P (10 μM) plus vitamin A (10^{-5} M) or with vitamin A (10^{-5} M) alone for 4 days.

Cell proliferation

For quantification of cell proliferation, tracheal rings were cultured for 18 h in the presence of 370 kBq/ml [methyl-^{3}H]thymidine (sp. act. 1.81 TBq/mmol; Amersham, Houten, The Netherlands). Then the rings were washed three times with phosphate-buffered saline (PBS) and fixed in 4% phosphate-buffered formaldehyde solution (pH 7.0). Thereafter, tracheal rings were dehydrated and embedded in Technovite 7100 plastic (Kulzer, Wehrheim, Germany). Semi-thin sections (1 - 2 μm thickness) of the tracheal rings were placed on microscope slides and dipped in Kodak NTB-2 emulsion (Eastman Kodak, New York), diluted with an equal volume of 2% aqueous glycerol solution. Autoradiograms were exposed in dry, light-tight boxes for 3-6 weeks at -30^{0}C, developed in Kodak D19, stained with 0.05% toluidine blue solution (Gurr, Chadwell Health, UK) and embedded in DePex (BDH Laboratory Supplies, Poole, UK).

Quantitative measurement of cell proliferation

Cell proliferation was assessed by counting the number of labelled basal cells (cells in contact with the basal lamina and with cytoplasm not reaching the tracheal lumen), and labelled non-basal cells (cells in contact with the basal lamina and with cytoplasm reaching the tracheal lumen or epithelial cells that were not in contact with the basal lamina). A cell showing more than 10 silver grains over the nucleus was considered to be labelled. The mean values for cell proliferation are expressed as the labelling index (LI) which is defined as the percentage of labelled cells amongst the total number of epithelial cells counted. For each tracheal ring, the LI was determined around the whole circumference of 2 to 4 different cross-sections, separated by several cell layers.

Determination of squamous metaplastic changes

Metaplastic changes in the tracheal epithelium were assessed in the same (coded) sections that were used for quantification of cell proliferation. Epithelium was scored as metaplastic when the normal epithelial columnar structure was replaced by flattened or enlarged cells with rotated or irregularly shaped nuclei.

Statistical analysis

All data were analysed for statistically significant differences by use of analysis of variance (ANOVA) followed by Dunnett's or Tukey's t-test (Instat Biostatistics, V2.05a, 1994, GraphPad Software, San Diego, CA).

Results

In the first experiment, a relatively high cell proliferation (LI ca. 14%, Fig. 1a and Fig. 2) was observed in control tracheas cultured in Ham's $F12^{mix1}$ medium. Tracheas exposed to 10 μM B[a]P for 4 days showed a slightly lower cell proliferation than control tracheas (Fig. 2), whereas cell proliferation was significantly increased in tracheas treated with the combination of B[a]P (10 μM) and vitamin A (10 μM) compared to tracheas treated with 10 μM B[a]P alone (Fig. 2). For the CMRL-1066^{mix1} culture medium, the direction of the effects of the various treatments was reversed compared to that observed in tracheas cultured in Ham's $F12^{mix1}$. For all treatments, values determined for cell proliferation in tracheas cultured in Ham's $F12^{mix1}$ medium were significantly different from those in tracheas cultured in CMRL-1066^{mix1} medium (Fig. 2). Proliferation of the control tracheal epithelial cells cultured in CMRL-1066^{mix1} medium and treated with DMSO was relatively low (LI ca. 4.5%, Fig. 1b and Fig. 2). Cell proliferation was significantly enhanced by B[a]P compared to that observed in tracheas treated with DMSO alone, whereas vitamin A significantly decreased B[a]P-induced cell proliferation (Fig. 2). In all treatment groups, the proliferation of the non-basal cells was higher than that of the epithelial basal cells.

In the second experiment, a slightly lower cell proliferation was observed in tracheas treated with either DMSO (0.1%) or vitamin A (10^{-9} M) compared to tracheas cultured in Ham's $F12^{mix1}$ medium alone. However, no significant effects on cell proliferation were observed of the various control treatments (Fig. 3). B[a]P treatment for 4 and 6 days resulted in a significant dose-dependent decrease in cell proliferation compared to that in corresponding control tracheas (Fig. 4). The effect of B[a]P treatment for 8 and 10 days was less clear (Fig. 4). Furthermore, the overall level of cell proliferation on days 6, 8 and 10 was significantly lower than on day 4 (Fig. 4). Figure 5 shows the time- and dose-dependent effect of a combined treatment of vitamin A (10^{-9} M, 10^{-7} M and 10^{-5} M) and B[a]P (10 μM) on cell proliferation in hamster tracheal epithelial cells. Vitamin A, particularly the highest concentration used (10^{-5} M), in combination with B[a]P increased cell proliferation compared to that in tracheas treated with B[a]P alone. No significant time-dependent effect on cell proliferation was observed. In this second experiment, proliferation of the non-basal cells was also higher than that of the basal cells.

To study the factors responsible for the observed differences in cell proliferation between tracheas cultured in the two different media (Ham's F12 *vs.* CMRL-1066), a third experiment was set up in which the possible effect was investigated of growth factors (EGF and Tf) and medium components ($CaCl_2.2H_2O$, nucleotides and NAD^+/NADP) on

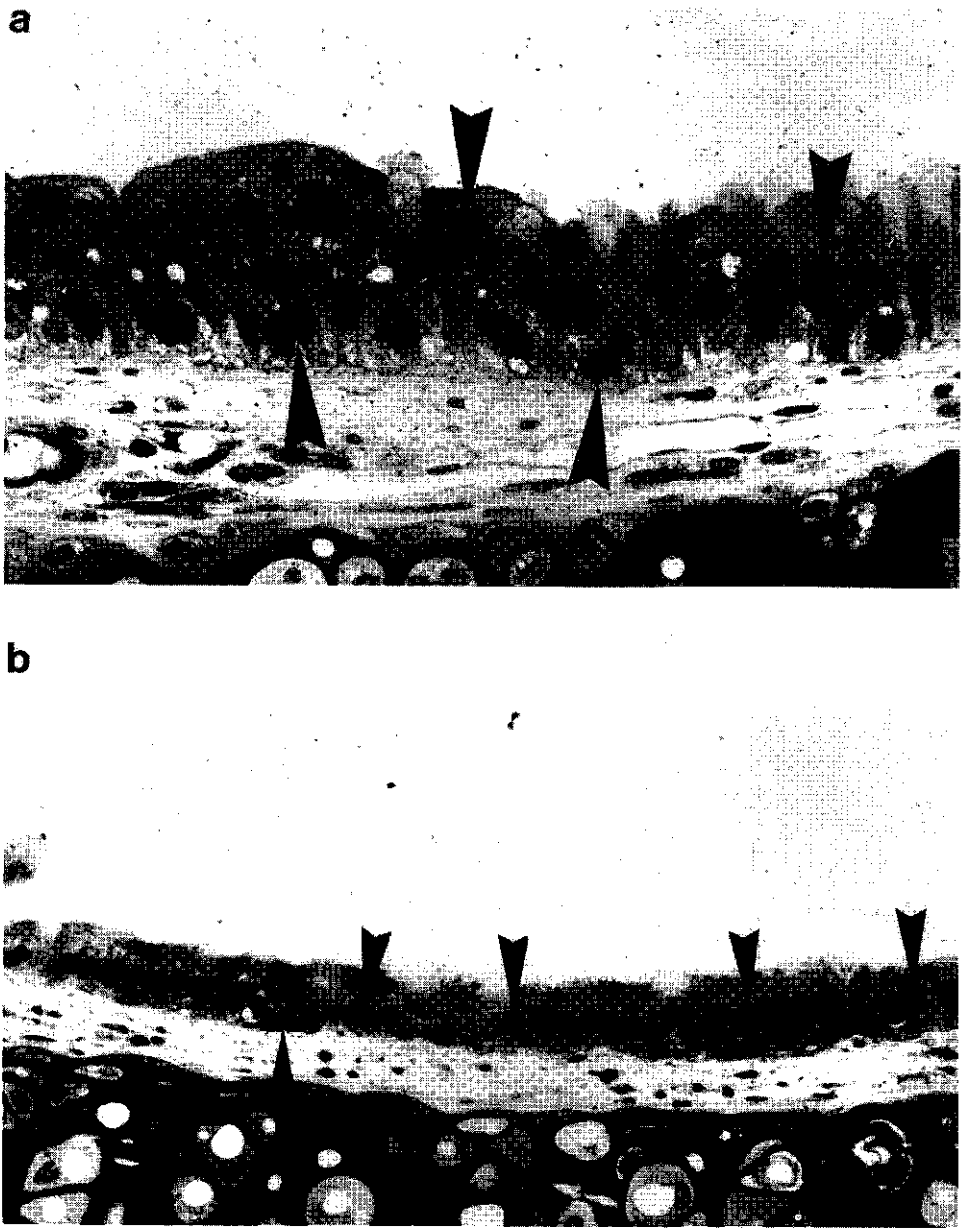

Figure 1: Autoradiographs of DMSO (0.1% v/v)-treated control tracheas cultured in Ham's $F12^{mix1}$ medium (**A**) and $CMRL\text{-}1066^{mix1}$ medium (**B**). Cell proliferation is recognizable by silver grains over the nucleus (arrowheads). In the tracheas cultured in Ham's F12 medium the cell proliferation is higher than in the tracheas cultured in CMRL-1066 medium.

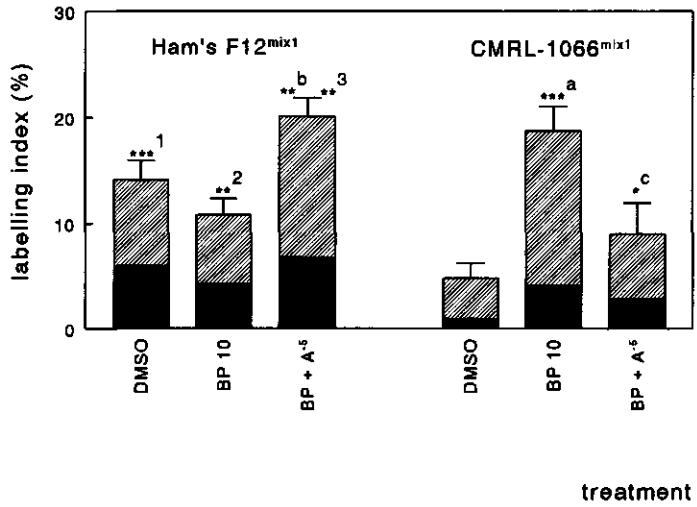

Figure 2: Effect of medium on B[a]P- and vitamin A-induced proliferation of basal (closed bars) and non-basal (hatched bars) hamster tracheal epithelial cells cultured in Ham's $F12^{mix1}$ medium or $CMRL\text{-}1066^{mix1}$ medium for 4 days. DMSO: DMSO 0.1% (v/v); BP 10: B(a)P 10 μM; BP + A^{-5}: B(a)P 10 μM + vitamin A 10^{-5} M. Error bars represent SEM of total (basal+non-basal) cell proliferation (n = 14 to 20; ^{***1}: P<0.001, ^{**2}, ^{**3}: P<0.01 compared to correspondingly treated tracheas cultured in $CMRL\text{-}1066^{mix1}$. ^{***a}: P<0.001 compared to the corresponding DMSO control; ^{**b}: P<0.01, ^{*c}: P<0.05 compared to correspondingly B[a]P-treated tracheas).

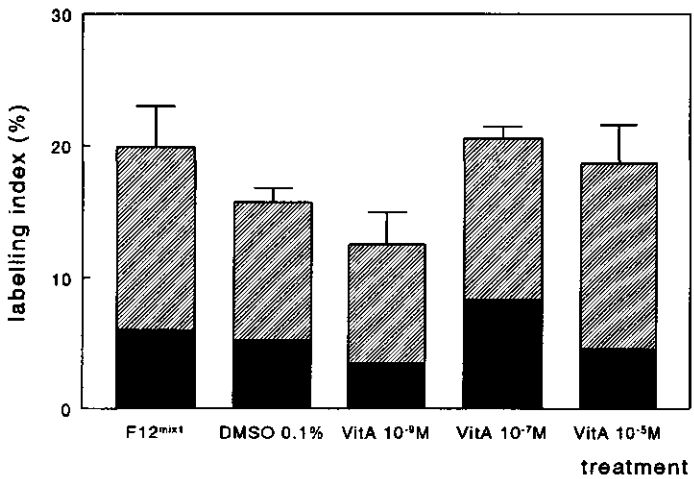

Figure 3: Labelling indices in hamster tracheal basal (closed bars) and non-basal (hatched bars) epithelial cells cultured in Ham's $F12^{mix1}$ medium for 4 days. Tracheas were not treated ($F12^{mix1}$), treated with DMSO (0.1% v/v), or treated with vitamin A (10^{-9} M, 10^{-7} M and 10^{-5} M). No significant differences were observed between the different treatments. Error bars represent SEM of total (basal+non-basal) cell proliferation (n = 7 to 12).

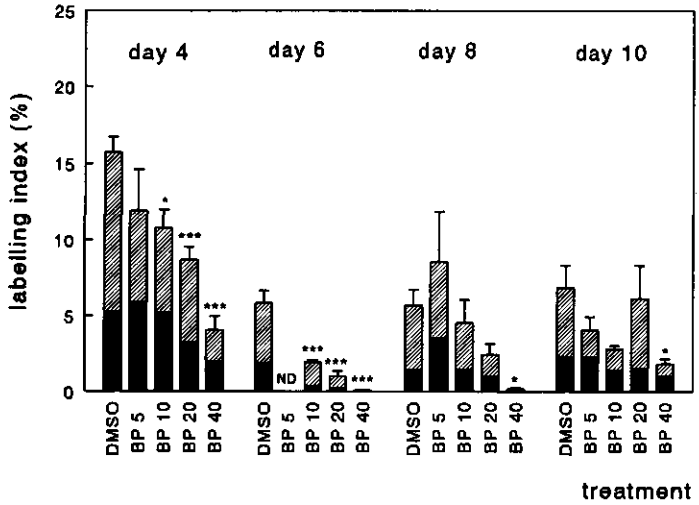

Figure 4: Time- and dose-dependent effect of B[a]P on basal (closed bars) and non-basal (hatched bars) cell proliferation in hamster tracheal epithelium cultured in Ham's $F12^{mix1}$ medium. DMSO: DMSO 0.1% (v/v); BP 5: B[a]P 5 μM; BP 10: B[a]P 10 μM; BP 20: B[a]P 20 μM; BP 40: B[a]P 40 μM. Error bars represent SEM of total (basal+non-basal) cell proliferation (n = 4 to 12; *: P<0.05, ***: P<0.001 compared to correspondingly DMSO-treated control tracheas). Furthermore, cell proliferation on day 4 was significantly higher than on the other days.

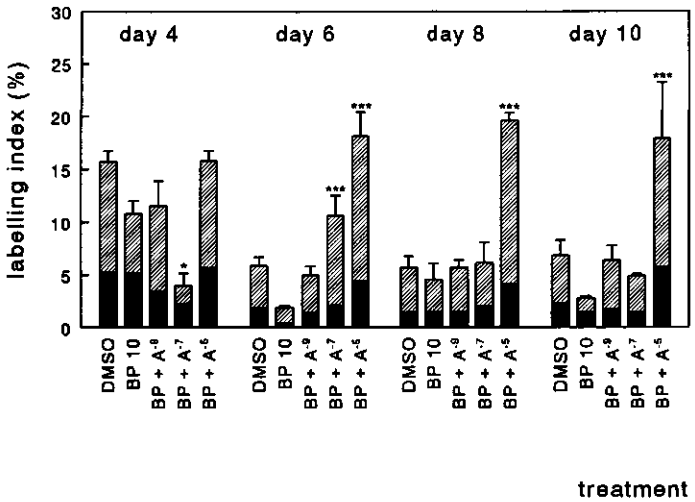

Figure 5: Time- and dose-dependent effect of vitamin A on basal (closed bars) and non-basal (hatched bars) cell proliferation of B[a]P-treated hamster tracheal epithelium cultured in Ham's $F12^{mix1}$ medium for several days. BP 10: B[a]P 10 μM; BP+A^{-9}: B[a]P 10 μM + vitamin A 10^{-9} M;
BP+A^{-7}: B[a]P 10 μM + vitamin A 10^{-7} M; BP+A^{-5}: B[a]P 10 μM + vitamin A 10^{-5} M. Error bars represent SEM of total (basal+non-basal) cell proliferation (n = 4 to 12; *: P<0.05, ***: P<0.001 compared to correspondingly B(a)P-treated tracheas).

B[a]P/vitamin A-induced cell proliferation. Depletion of EGF from Ham's $F12^{mix1}$ medium resulted in a 3-fold lower overall cell proliferation (Fig. 6A) compared to that in tracheas cultured in Ham's $F12^{mix1}$ medium (Fig. 2). Compared to the cell proliferation in control tracheas, B[a]P slightly decreased cell proliferation but vitamin A had no significant effect, in contrast to the effect of vitamin A in tracheas cultured in complete Ham's $F12^{mix1}$ medium (Fig. 6A and Fig. 2). The effect of depletion of Tf from Ham's $F12^{mix1}$ medium was less clear (Fig. 6B). For all treatments, cell proliferation was lower than in tracheas cultured in Ham's $F12^{mix1}$ (Fig. 2), but the effect of B[a]P and vitamin A was similar. In the tracheas cultured in Ham's $F12^{mix2}$ medium (Fig. 6C), cell proliferation was 4- to 10-fold lower than in tracheas cultured in Ham's $F12^{mix1}$ (Fig. 2). Comparable with the effect in Ham's $F12^{mix1}$, B[a]P slightly decreased cell proliferation in Ham's $F12^{mix2}$ compared to that in the control tracheas. However, in contrast to the effect in Ham's $F12^{mix1}$ (Fig. 2), vitamin A in combination with B[a]P slightly decreased cell proliferation in tracheas cultured in Ham's $F12^{mix2}$. In tracheas cultured in CMRL-1066^{mix2} (Fig. 6D) the effects of the various treatments on cell proliferation were similar to those observed in tracheas cultured in CMRL-1066^{mix1} (Fig. 2). B[a]P significantly increased cell proliferation compared to that in control tracheas. Vitamin A in combination with B[a]P significantly decreased cell proliferation compared to tracheas treated with B[a]P alone (Fig. 6D).

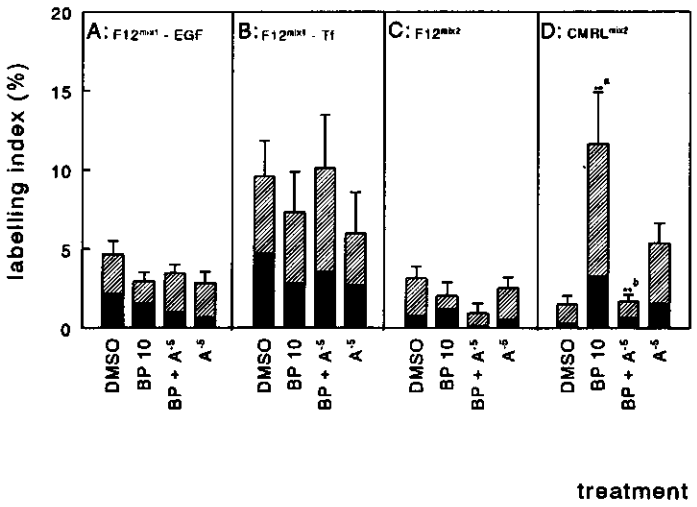

Figure 6: Effect of medium and cofactor mix composition on basal (closed bars) and non-basal (hatched bars) cell proliferation in hamster tracheal epithelium treated with DMSO: DMSO (0.1% v/v), BP 10: B[a]P (10 μM), BP+A^{-5}: B[a]P (10 μM) + vitamin A (10^{-5} M) and A^{-5}: vitamin A 10^{-5} M for 4 days. Error bars represent SEM of total (basal+non-basal) cell proliferation (n = 4 to 10; ^{**a}: P<0.01 compared to corresponding DMSO-treated control tracheas, ^{**b}: P<0.01 compared to corresponding B[a]P-treated tracheas).

Figure 7 shows the effect of $CaCl_2.2H_2O$ (Ca^{2+}) addition to Ham's F12 medium on cell proliferation. In the tracheas cultured in Ham's $F12^{mix1}$ medium supplemented with Ca^{2+} (Fig. 7A), B[a]P increased cell proliferation compared to that in control tracheas, whereas vitamin A decreased B[a]P-induced cell proliferation. These effects are thus similar to those observed in tracheas cultured in CMRL-1066^{mix1} medium (Fig. 2). No effect on cell proliferation was observed of the addition of Ca^{2+} to Ham's $F12^{mix2}$ (Fig. 7B) compared to the tracheas cultured in Ham's $F12^{mix2}$ without Ca^{2+} (Fig. 6C). Addition of nucleotides or NAD^+/NADP to Ham's $F12^{mix1}$ or Ham's $F12^{mix2}$ culture media did not influence the effect of the various treatments on cell proliferation (data not shown).

Histological examination of the tracheal epithelium revealed B[a]P-induced metaplasia independent of the type of medium (Table II). However, the degree of squamous metaplasia was found to strongly depend on the culture medium used. Furthermore, vitamin A lowered the formation of B[a]P-induced squamous metaplastic changes. Overall, the degree of metaplasia was lowest in the tracheas treated with 10^{-5} M vitamin A alone (Table II).

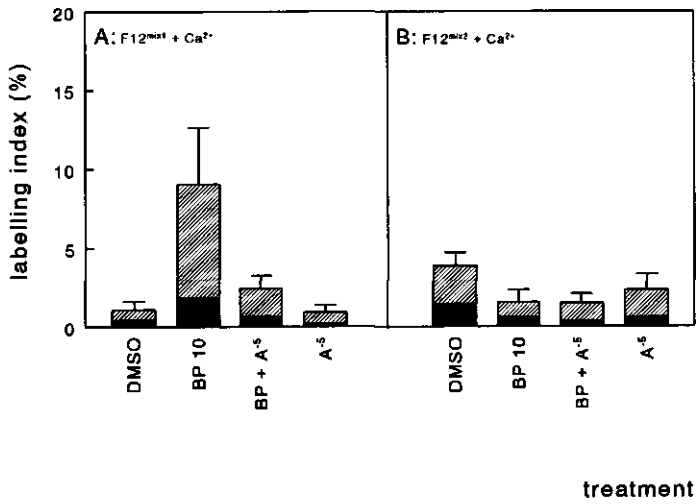

Figure 7: Effect of $CaCl_2.2H_2O$ (Ca^{2+}) supplementation on basal (closed bars) and non-basal (hatched bars) cell proliferation in hamster tracheal epithelium cultured in Ham's $F12^{mix1}$ or Ham's $F12^{mix2}$ medium and treated with DMSO: DMSO (0.1% v/v), BP 10: B[a]P (10 μM), BP+A^{-5}: B[a]P (10 μM) + vitamin A (10^{-5} M) and A^{-5}: vitamin A (10^{-5} M) for 4 days. Error bars represent SEM of total (basal+non-basal) cell proliferation (n = 6 to 8). **Note** that as a consequence of supplementation of Ca^{2+} to Ham's $F12^{mix1}$ medium, the effects of the various B[a]P and vitamin A treatments on cell proliferation are similar to those in CMRL-1066 medium (see Fig. 2 and Fig. 6).

Table II: Effect of B[a]P and vitamin A on the degree of squamous metaplasia[a] in hamster tracheal epithelium. Tracheas were cultured in two different culture media with various supplements and exposed to B[a]P (10 μM), B[a]P (10 μM) in combination with vitamin A (10^{-5} M) or vitamin A alone (10^{-5} M) for 4 days. Control tracheas were cultured in medium containing DMSO (0.1% v/v).

	DMSO	B[a]P	B[a]P + vit A	vit A
$F12^{mix1,c}$	1.1 (27)[b]	1.7 (27)	1.3 (15)	1.7 (16)
$F12^{mix2}$	1.5 (10)	1.7 (10)	1.1 (8)	0.4 (8)
$CMRL^{mix1}$	1.8 (11)	2.3 (11)	1.4 (11)	0.6 (6)
$CMRL^{mix2}$	0.3 (6)	1.0 (6)	0.3 (6)	0.3 (6)
$F12^{mix1} + Ca^{2+}$	2.0 (4)	2.5 (4)	2.3 (4)	1.8 (4)
$F12^{mix2} + Ca^{2+}$	0.6 (4)	1.6 (4)	1.5 (4)	0.6 (4)
$F12^{mix1}$ - EGF	1.0 (10)	1.2 (10)	1.0 (10)	0.8 (10)
$F12^{mix1}$ - Tf	1.5 (4)	2.0 (3)	1.5 (4)	1.5 (4)

[a] Metaplasia is expressed as the mean of the scores of each individual tracheal ring examined; metaplasia is graded 3 if more than 40% of the epithelium is affected, 2 if between 10 and 40% is affected, 1 if less than 10% is affected and 0 if the tracheal epithelium is not affected (adapted from Clamon *et al.* 1974).

[b] value in parenthesis is the number of tracheal rings examined.

[c] medium supplemented with cofactor mix 1 $^{(mix1)}$ or cofactor mix 2 $^{(mix2)}$(see Table I for composition of the cofactor mixtures).

Discussion

Tracheal organ cultures have been frequently used to study the effect of vitamin A on chemically induced respiratory tract carcinogenesis (8-11). In tracheas cultured in the relatively nutrient-rich CMRL-1066 culture medium, supplemented with a limited amount of growth factors and hormones, the effects of vitamin A on B[a]P-induced cell proliferation and differentiation were similar to those observed *in vivo* (23). In addition to the tracheal organ culture model, isolated tracheal cells have also been used to study effects of respiratory tract carcinogens and vitamin A on cell proliferation and differentiation (13,14). Frequently, these cells are cultured in the relatively nutrient-poor Ham's F12 culture medium supplemented with an ample amount of growth factors (13,19,24). Although both models are used to study the process of chemically induced respiratory tract

cancer and the role of vitamin A in this process, there are differences between the two media with respect to overall composition and the content of growth factors that may influence the effect of vitamin A and carcinogens on cellular proliferation and differentiation. This may lead to differences in results and in the interpretation of the effects observed.

In the first experiment it was clearly shown that cell proliferation in control tracheas cultured in Ham's $F12^{mix1}$ medium was significantly higher than in control tracheas cultured in CMRL-1066^{mix1} medium (Student t-test: $P<0.001$). This finding indicates that the composition of the medium is critical with regard to tracheal epithelial cell proliferation in organ culture. This is in agreement with the results of Mossman and Craighead (18), who showed profound effects of 6 different media, with or without serum, on cell proliferation in hamster tracheal epithelium in organ culture. Remarkably, the effect of B[a]P alone or of B[a]P in combination with vitamin A on cell proliferation in the tracheas cultured in the two media was completely different. The results observed in the tracheas cultured in CMRL-1066^{mix1} are in agreement with data of other *in vitro* studies and with results obtained *in vivo* (9,23). However, in the experiments of Rutten *et al.* (9), the CMRL-1066 medium used was supplemented with a poorer cofactor mix than that used in the first experiment described here, *i.e.*, similar to cofactor mix 2. When cell proliferation in tracheas cultured in CMRL-1066 medium of the first experiment, using cofactor mix 1 (Fig. 2), is compared to that in tracheas cultured with the poorer cofactor mix 2 (Fig. 6), it is clear that the composition of the cofactor mixtures as used in these experiments did not influence the effect of vitamin A and B[a]P on cell proliferation in CMRL-1066 medium. However, the overall level of cell proliferation was decreased by depletion of growth factors. This is in accordance with results in the literature showing that EGF and insulin strongly influence cell proliferation in tracheal epithelial cells (15,19,20).

The inhibition of cell proliferation by B[a]P and the stimulation of cell proliferation by vitamin A in hamster tracheas cultured in Ham's $F12^{mix1}$ is in contrast with the results observed *in vivo* (23) and *in vitro* (9). In an other study (25) we showed that the level of cell proliferation inhibition by B[a]P in tracheas cultured in Ham's $F12^{mix1}$ medium was inversely correlated with the level of B[a]P-DNA adducts in hamster tracheal epithelial cells, which suggests that the lower cell proliferation was a consequence of the formation of B[a]P-DNA adducts. The presence of B[a]P adducts in the DNA has been shown to inhibit DNA polymerase (26,27) and DNA damage may also block the G1 phase of cell cycle, possibly mediated by the p53 tumour-suppressor gene (28). However, in tracheas cultured in Ham's $F12^{mix1}$ or CMRL-1066^{mix1}, no differences were observed in the formation of B[a]P-DNA adducts, and the expression of the p53 tumour-suppressor protein, as detected by immunocytochemical analysis, was very low and could not explain the differences in cell proliferation between the two media (data not shown). These results suggest that the level of B[a]P-DNA adducts is a factor of less concern than medium

composition in determining cell proliferation in hamster tracheal epithelium.

In tracheas cultured in Ham's F12 medium, the effect of the different cofactor mixtures on the overall level of cell proliferation was much more pronounced than in the tracheas cultured in CMRL-1066 medium. Furthermore, the composition of the cofactor mix strongly influenced the effect of vitamin A and B[a]P on cell proliferation. Cell proliferation in the tracheas cultured in Ham's $F12^{mix2}$ medium and treated with the combination of vitamin A and B[a]P was not increased compared to tracheas treated with B[a]P only, whereas such an increase was observed in tracheas cultured in Ham's $F12^{mix1}$ medium. This indicates that EGF and, possibly, Tf and insulin influence the effect of vitamin A on B[a]P-induced cell proliferation in tracheas cultured in Ham's F12 culture medium, but not in CMRL-1066 medium. These results show that the composition of the culture medium strongly influenced the effect of growth factors and of vitamin A and B[a]P on cell proliferation in hamster tracheal epithelium, which indicates that the effect of cell proliferation-regulating factors can be modulated by nutrient components in the medium.

In the present study supplementation of NAD^+ and NADP did not influence cell proliferation in tracheal epithelial cells (data not shown). However, Stierum *et al.* (29) showed that a correlation exists between inhibition of cell proliferation by B[a]P and a decrease in intercellular NAD^+ level in PHA-stimulated human lymphocytes. Probably, in this study, the epithelial cells are unable to take up the exogenously administered NAD^+ and NADP (30). Furthermore, supplementation of nucleotides to Ham's F12 medium did not influence proliferation of hamster tracheal epithelial cells (data not shown), which suggests that the *de novo* biosynthesis of nucleotides in tracheal epithelial cells is sufficient to support the relatively high cell proliferation.

The calcium concentration has been shown to be an important regulator of cellular proliferation and differentiation in various epidermal and epithelial cell types (12,19,22, 31). In the present study, a relatively low Ca^{2+} concentration (0.2 mM) in Ham's $F12^{mix1}$ culture medium results in a high cell proliferation in control tracheas, whereas cell proliferation was decreased when Ca^{2+} was supplemented. This is in contrast to the results of Thomassen *et al.* (19) who showed that the clonal proliferation of isolated rat tracheal epithelial cells was maximal at 0.8 mM Ca^{2+}. However, the results are in agreement with those of Lechner *et al.* (17,31) showing that clonal growth of human bronchial epithelial cells is maximal at a low Ca^{2+} concentration (0.1 mM), whereas high concentrations (0.45 - 0.6 mM) induced terminal differentiation. Moreover, Hennings *et al.* (22) and Boyce *et al.* (32) showed that the colony-forming efficiency of keratinocytes is maximal at 0.3 mM Ca^{2+} and that squamous metaplasia was induced at 1.0 mM Ca^{2+}. Addition of Ca^{2+} to Ham's $F12^{mix2}$ did not influence cell proliferation compared to tracheas cultured in Ham's $F12^{mix2}$ without Ca^{2+}, which indicates that the effect of Ca^{2+} on cell proliferation depends on the presence of other growth-regulating factors.

Histomorphological examination of the tracheas revealed a relatively high degree of squamous metaplasia in control tracheas, probably due to the absence of vitamin A in the

culture medium. The results of the different treatments are generally in agreement with previous studies (10); B[a]P increases the degree of squamous metaplasia, whereas vitamin A decreases it. The high level of squamous metaplasia in the tracheas cultured in Ham's $F12^{mix1}$ supplemented with Ca^{2+} is in agreement with the results described by Jetten (12) which show that high calcium concentrations stimulate the expression of the squamous differentiated phenotype in human tracheobronchial epithelial cells at confluence. In tracheas cultured in CMRL-1066 medium or in Ham's F12 medium supplemented with Ca^{2+}, both of which have a relatively high concentration of Ca^{2+}, the degree of squamous metaplasia was higher in the media supplemented with the rich cofactor mix (mix 1) than in the media supplemented with the relatively poor cofactor mix (mix 2). This result is comparable with that of Lechner *et al.* (31) who showed that the degree of squamous metaplasia induced in human bronchial epithelial cells by a high concentration of Ca^{2+} was potentiated by serum. In the present study, the effect of Ca^{2+} on squamous metaplasia was potentiated by growth factors.

The results presented here show that non-basal cell proliferation was, independent of the treatment protocol, higher than proliferation of basal cells. Although the basal cell is the cell from which other cell types originate (12,15), it has also been shown that non-basal cells are able to divide (15,33). The cause of this discrepancy is not very clear. In addition to the vitamin A status of the hamsters, an important difference between the treatment protocols of tracheas in previous studies of Rutten *et al.* (9) and of tracheas cultured in $CMRL\text{-}1066^{mix2}$ in this study, is the concentration of [methyl-^{3}H]thymidine (74 kBq/ml in the studies of Rutten *vs.* 370 kBq/ml in the present study). Probably, a higher concentration of ^{3}H-thymidine in the culture medium results in an increased uptake of ^{3}H-thymidine by non-basal cells, resulting not only in a higher number of labelled non-basal cells but also in increased total cell proliferation compared to the results obtained in the studies of Rutten *et al.* (9).

In conclusion, the results of this study clearly show that the composition of the culture medium, in particular the concentration of Ca^{2+}, strongly influences the effects of vitamin A and B[a]P on cell proliferation of hamster tracheal epithelium in organ culture. Furthermore, the effect of various growth factors on proliferation of tracheal epithelial cells is influenced by medium components.

Acknowledgements

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Chapter 7

Short communication

High survival rate of hamsters given intratracheal instillations of benzo(a)pyrene and ferric oxide and kept on a high β-carotene diet.

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Carcinogenesis 15, 133-136, 1994

Summary

The study described in this paper was primarily conducted to identify the cell types involved in the formation, progression and regression of metaplastic changes in the respiratory tract epithelium of hamsters after intratracheal intubations with benzo(a)pyrene. Furthermore, the role of vitamin A and β-carotene in these processes was studied. In the course of the study a remarkable effect of dietary β-carotene on survival of hamsters became a subject of investigation. Hamsters were fed diets with various levels of vitamin A or β-carotene and were treated intratracheally with a suspension of benzo(a)pyrene with ferric oxide in saline. The tumour response of the respiratory tract was very low (2.8%) and hyper- and metaplasia of respiratory epithelium were virtually absent. However, an interesting observation was an exceptionally low mortality of only 2% after 69 weeks in the group of hamsters fed a high β-carotene diet (1% w/w), whereas in the other groups mortality after 69 weeks amounted to 25%. Although the exact cause of death of most of the hamsters could not be established, a 40% reduction of lipid peroxidation in the livers was found in the high β-carotene group. Moreover, in this group degree and incidence of nephrosis and of focal mineralisation of kidneys and heart were lower than in the other groups. These favourable effects of the high β-carotene diet may have contributed to the unusually high survival in hamsters fed this diet. Further studies are planned to verify and study this observation.

Several epidemiological and experimental studies have shown an inverse relationship between the intake of vitamin A or provitamin A (carotenoids) and the incidence of (pre)neoplastic changes in the respiratory tract (1-4). Furthermore, a positive correlation has been observed between dietary levels of vitamin A or β-carotene and mortality (5-7), probably related to an effect of these vitamins on the immune system. For instance, the number of T- and B-cells, macrophages and natural killer cells increases after supplementation of (pro)vitamin A (8,9). Another way (pro)vitamin A could influence health is by quenching of singlet oxygen and other free radicals, resulting in decreased lipid peroxidation (10,11). A further important health determining factor is the serum level of various lipoproteins. It has been shown by Costantino *et al.* that these levels are influenced by β-carotene (12).

The study described in this paper was primarily conducted to identify cell types involved in the formation, progression and regression of metaplastic changes in the respiratory tract epithelium of hamsters after intratracheal intubations with benzo(a)pyrene [B(a)P]. Furthermore, the role of vitamin A or β-carotene in these processes was studied. In the course of the study a remarkable effect of dietary β-carotene on survival of hamsters became subject of investigation.

The experiment comprised four groups of weanling male Syrian golden hamsters (Central Institute for the Breeding of Laboratory Animals, Zeist, The Netherlands) which were fed a pelleted purified diet (Table 1) containing various levels of vitamin A (Table 2). Diets were provided ad libitum. Since a large number of animals had to be treated intratracheally, the experiment was split-started, the second part being started three months later then the first part (in fact, this resulted in two very similar but independent assays).

Table I: Composition of the diet (adapted from 13)

Ingredients	(%)
Casein[1]	20.0
dl-Methionine	0.3
Wheat starch[2]	53.0
Corn oil[3]	5.0
Cellulose[4]	5.0
Choline bitartrate	0.2
Mineral mixture (based on AIN-76A)[5]	3.5
Vitamin mixture (based on AIN-76A, vitamin A deficient)[6]	1.0
$CaHPO_4$	1.5
Vitamin A or β-carotene mixture[7]	10.5

1) Acid-precipitated, containing protein 89.10% (N x 6.38); moisture 8.9%; ash 4.67%; pH of a 10% aqueous suspension 4.5%.

2) 12.2% of native wheat starch was replaced by pregelatinized wheat starch to improve the quality of the pellets.

3) No antioxidants were added

4) Dicacel, highly purified and bleached fibrous filter powder, consisting of: 87-90% pure α-cellulose; average length of fibres about 44 μm; water 4%; ash 0.12-0.15%; and lignin 0.04%.

5) Mineral mixture (g/kg mixture) NaCl (110). $K_3C_6H_5O_7.H_2O$ (394). K_2SO_4 (51.8), MgO (28.4), $MnCO_3.xH_2O$ (3.5), $FeC_6H_5O_7.5H_2O$ (24), $5ZnO.2CO_3.4H_2O$ (1.6), $CuCO_3(OH)_2.H_2O$ (0.3), KIO_3 (0.08), $Na_2SeO_3.5H_2O$ (0.01), $CrK(SO_4).12H_2O$ (0.55), NaF (0.063), $CoCl_2.6H_2O$ (0.127) and finely powdered sucrose to make up 1.0 kg.

6) Vitamin mixture (g or IU/kg mixture): thiamin-HCl (2.0 g), riboflavin (1.5 g), pyridoxine-HCL (0.7 g), nicotinic acid (9.0 g), Ca-D(+)-pantothenate (4.0 g), folic acid (0.2 g), D(+)biotin (0.06 g), vitamin B12 (0.005 g), inositol (10 g), cholecalciferol (248,400 IU), dl-α-tocopheryl acetate (5000 IU), menadione Na bisulphite (0.4 g), and finely powdered sucrose to make up 1.0 kg.

7) Vitamin A or β-carotene mixture. Vitamin A premix contained retinyl palmitate (1.0 m.I.U./g) 0.08% and pregelatinized wheat starch 99.92%. Mixtures (g/kg mixture); Group A and B: vitamin A premix (47.62) and wheat starch (952.38); group C: vitamin A premix (4.76) and wheat starch (995.24); group D: vitamin A premix (47.62) and 10% β-carotene preparation (952.38).

Table II: Number of animals per group, dietary vitamin A and β-carotene levels, and liver and serum retinol and β-carotene levels.

group	n	vitamin A in diet IU/kg	treatment	liver[1]		serum[2]	
				retinol IU/g mean±sd	β-carotene μg/g mean±sd	retinol μmol/l mean±sd	β-carotene μmol/l mean±sd
A	64	4000	Fe_2O_3	280±196	0.30±0.54	1.32±0.28	<0.01
B	106	4000	B(a)P + Fe_2O_3	240± 98	0.15±0.06	--	--
C	106	400	B(a)P + Fe_2O_3	113± 67	0.12±0.06	--	--
D	106	4000 + 1% β-carotene	B(a)P + Fe_2O_3	1183±836***	7.32±4.44***	1.10±0.12	0.05±0.01

n: initial number of animals per group; --: not analysed; IU: international units
1) determined in 4 (group A) or 11 animals per group
2) determined in 5 animals per group
Statistics: pairwise Student's t-test with Bonferroni adjusted P-values (29); ***P<0.001 when compared to group B.

After a three month adaptation period to the diets, hamsters were instilled intratracheally with saline suspensions of B(a)P particles (Sigma Chemicals, St. Louis, MO) attached to Fe_2O_3 particles (Fisher Scientific Company, NJ), once every 2 weeks for 3 months according to the method of Saffiotti *et al.* (14). This method has been proven very successful to induce lung cancer (14-17). A broad spectrum of respiratory tract tumors can be induced in hamsters and rats, which morphologically resemble those frequently observed in humans (18,19). Controls were given intratracheal instillations of a saline suspension of Fe_2O_3 particles alone (Table 2). At five interim kills and one final kill 10 months after the last instillation, lungs, tracheas, larynx, heart, kidneys, livers and macroscopically aberrant organs were collected and processed for further investigations, including histopathology. In addition, blood and part of the liver were sampled for determination of vitamin A and β-carotene levels according to the method of van Vliet *et al.* (20).

No statistically significant differences in body weights (Figure 1) and food consumption (Figure 2) were observed between the various groups. The tumour response of the respiratory tract in hamsters treated with B(a)P + Fe_2O_3 was dramatically low (2.8%, mostly papillomas of trachea and larynx), and hyperplasia and metaplasia of respiratory epithelium were virtually absent. The possible reasons for this extremely low response will be discussed in a separate paper dealing with the strengths and limitations of this hamster model.

However, a striking and most interesting observation was the exceptionally low mortality in the group of hamsters fed the high β-carotene diet (Figure 3). In the groups fed the normal or the vitamin A-deficient diet about 25% of the hamsters died from effects most likely unrelated to B(a)P treatment. Most hamsters that died showed similar clinical signs. Some weeks before they died, animals began to eat and drink less and soon did not eat and drink anymore, resulting in emaciation and death. Gross autopsy findings were essentially negative, and as a consequence no or only a few organs and tissues were

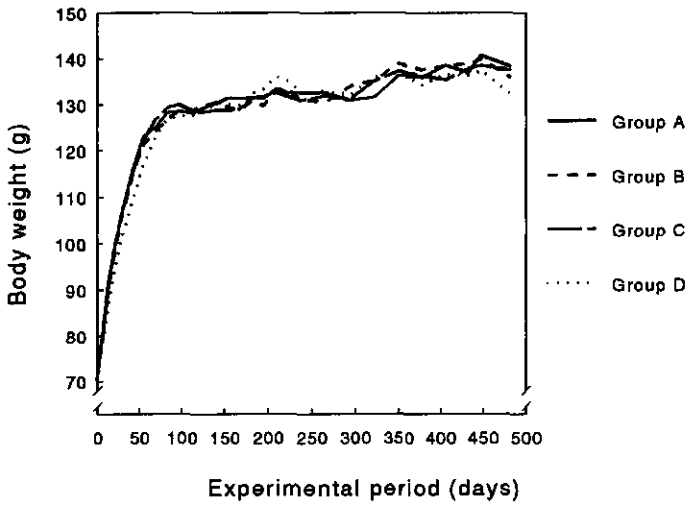

Figure 1: Body weights of the hamsters of the various groups. Group A: 4000 IU vitamin A/kg diet, Fe_2O_3; group B: 4000 IU vitamin A/kg diet, B(a)P + Fe_2O_3; group C: 400 IU vitamin A/kg diet, B(a)P + Fe_2O_3; group D: 4000 IU vitamin A/kg diet + 1% β-carotene, B(a)P + Fe_2O_3.

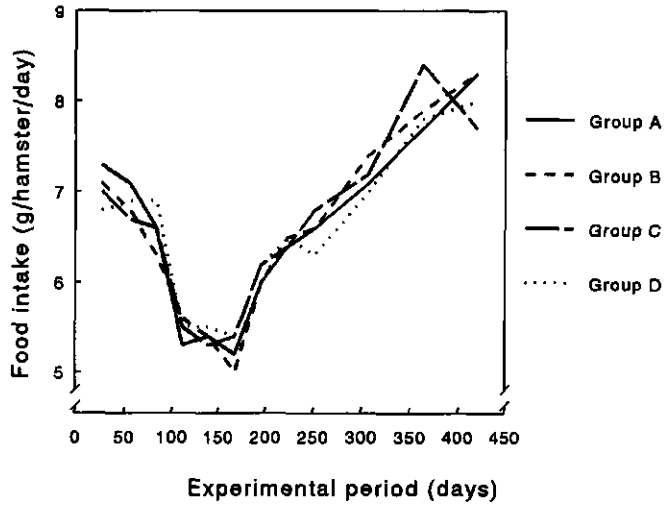

Figure 2: Food intake of the hamsters of the various groups. Group A: 4000 IU vitamin A/kg diet, Fe_2O_3; group B: 4000 IU vitamin A/kg diet, B(a)P + Fe_2O_3; group C: 400 IU vitamin A/kg diet, B(a)P + Fe_2O_3; group D: 4000 IU vitamin A/kg diet + 1% β-carotene, B(a)P + Fe_2O_3.

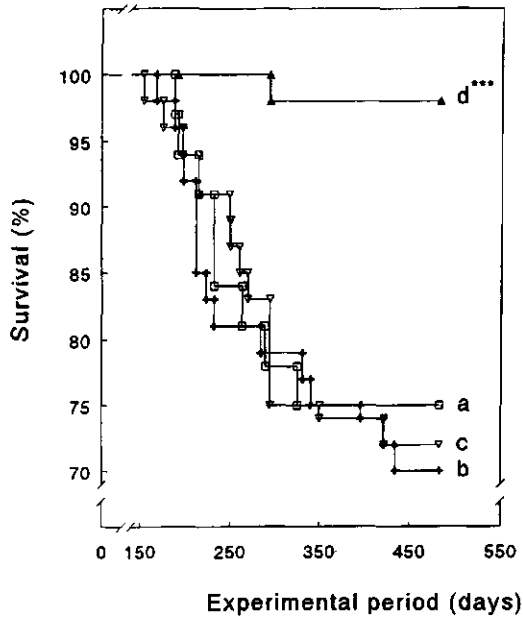

Figure 3: Survival of the hamsters in the different groups. Note the exceptionally low mortality in the β-carotene group. Group A: 4000 IU vitamin A/kg diet, Fe_2O_3; group B: 4000 IU vitamin A/kg diet, B(a)P + Fe_2O_3; group C: 400 IU vitamin A/kg diet, B(a)P + Fe_2O_3; group D: 4000 IU vitamin A/kg diet + 1% β-carotene, B(a)P + Fe_2O_3. Group D is significantly different from group A, B and C (statistics: generalised savage (mantel-cox) test (29) , $^{***}P<0.001$)

collected for histopathological examinations, particularly in the early phase of the study. In the group fed the diet containing 1% β-carotene only 2% of the animals died. Also in comparison with the survival in control groups of male hamsters from other studies, ranging from 40 to 80% after about 18 months (21), the survival of hamsters on the high β-carotene diet (98%) is remarkably high. This strikingly high survival occurred in both separately started parts of this study, strongly reducing the probability of this high survival being a chance effect. In a recent long-term study with SENCAR mice, high dietary levels of β-carotene (up to 600 μg/kg diet) did not increase survival (22), whereas in short-term studies, both β-carotene and vitamin A increased the survival of mice (7,23). High dietary levels of vitamin A were found to decrease the survival rate of hamsters in a long-term carcinogenicity study with B(a)P (24).

The levels of vitamin A and β-carotene in serum and liver varied strongly among the different groups (Table 2). There was no evidence of the immune system mediating the protective effect of β-carotene. No difference was observed in T- and B-cell activation in the spleen between hamsters fed the different diets (data not shown). However, lipid peroxidation in livers, determined according to the method of Ohkawa *et al.* (25), was statistically significantly decreased in the hamsters on the high β-carotene diet compared to hamsters fed the control diet (Figure 4). Serum levels of lipoproteins (triglycerides, high and low density lipid proteins, phospholipids and triglycerides) were not influenced by dietary β-carotene or vitamin A (data not shown).

In elderly hamsters, the most common fatal disease is amyloidosis of liver and kidneys, and heart failure due to thrombosis (21,26,27). Surprisingly, degree and incidence of

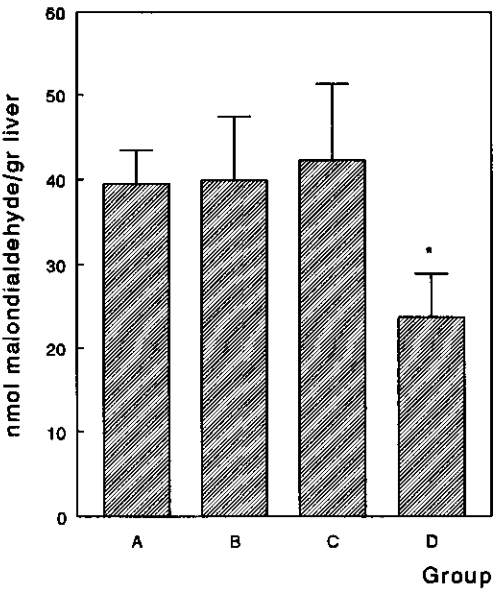

Figure 4: Lipid peroxidation in liver homogenate of 5 scheduled killed hamsters per group killed at the end of the study. Group A: 4000 IU vitamin A/kg diet, Fe_2O_3; group B: 4000 IU vitamin A/kg diet, B(a)P + Fe_2O_3; group C: 400 IU vitamin A/kg diet, B(a)P + Fe_2O_3; group D: 4000 IU vitamin A/kg diet + 1% β-carotene, B(a)P + Fe_2O_3. (statistics: pairwise Student's t-test with Bonferroni adjusted P-values (29), $^*P<0.05$ when compared to group B).

amyloidosis were exceptionally low in all groups (Table 3), probably related to the purified diet used in the present study (Table 1). Degree of and incidence of nephrosis and of focal mineralization in kidneys and heart were lowest in animals fed the high β-carotene diet.

In most animals that died or were killed in extremis, we could not unequivocally establish the cause of death. The statistically significant 40% reduction in lipid peroxi-dation in livers of animals of the high β-carotene group is considered the most important lead in searching for the mechanism underlying the positive effect of β-carotene on survival of hamsters. Although the protective effect of β-carotene on the development of nephrosis and mineralization in kidneys and heart was not very strong, the reduced occurrence of these renal and cardiac lesions in the high β-carotene group might partly explain the high survival in this group (28). The widely accepted view that β-carotene stimulates the immune response could not be confirmed in the present study.
Further studies are planned to elucidate the mechanism responsible for the effect of β-carotene on the survival of hamsters.

Table III. Histopathology of kidneys and heart of hamsters in the different groups[1].

	Number and percentage (in brackets) of animals showing lesions in group:			
Type of lesion	A [26][2]	B [44]	C [46]	D [68]
Kidneys				
- nephrosis, minimal to slight	15 (58)	34 (77)	33 (72)	37 (55)*
moderate	3 (12)	1 (2)	8 (17)*	0
severe	0	0	1 (2)	0
total	18 (69)	35 (80)	42 (91)	37 (55)**
- focal mineralization	8 (31)	12 (27)	18 (39)	6 (9)*
- mononuclear-cell infiltrate	0	0	1 (2)	0
- pyelitis	0	0	1 (2)	0
- hyperplasia of pelvic epithelium	1 (4)	0	4 (9)	0
- amyloid deposits	1 (4)	0	1 (2)	0
- small adenoma	0	0	0	1 (2)
- transitional-cell carcinoma	0	0	0	1 (2)
Heart				
- focal mineralisation	4 (15)	2 (5)	7 (15)	0

1) Group A: 4000 IU vitamin A/kg diet, Fe_2O_3; group B: 4000 IU vitamin A/kg diet, B(a)P+Fe_2O_3; group C: 4000 IU vitamin A/kg diet, B(a)P+Fe_2O_3; group D: 4000 IU vitamin A/kg diet + 1% β-carotene, B(a)P+Fe_2O_3

2) number of animals examined.

Statistics: Fisher Exact Probability Test (29); *P<0.05, **P<0.01 when compared to group B.

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Chapter 8

Relation between benzo[a]pyrene-DNA adducts, cell proliferation and p53 expression in tracheal epithelium of hamsters fed a high β-carotene diet.

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Carcinogenesis, in press

Summary

Vitamin A and β-carotene protect against respiratory tract cancer by inhibiting the formation of DNA-damage and controling cellular proliferation and differentiation. Recently, it has been shown that the p53 tumor-suppressor gene plays a crucial role in the etiology of respiratory tract cancer. In the present study, we investigated the relationship between benzo[a]pyrene (B[a]P)-DNA adducts, cell proliferation and p53 expression and the possible effect of β-carotene on such a relationship in tracheal epithelium of hamsters given intratracheal instillations of B[a]P-Fe_2O_3 particles suspended in saline. DNA-adducts were quantified by the ^{32}P-postlabeling assay, cell proliferation was quantified by immunocytochemical detection of incorporated BrdU during S-phase, and p53 protein was detected by immunohistochemistry with an antibody that recognized both the wild-type and the mutated protein (BioGenex, Clone BP53-12-1). A clear relationship appeared to exist between the extent of B[a]P-DNA adduct formation, the induction of cell proliferation and the expression of p53 protein in hamster tracheal epithelium. These results suggest that B[a]P induces cell proliferation in hamster tracheal epithelial cells most likely by the induction of mutations in the p53 gene. Furthermore, β-carotene was not found to influence the formation of B[a]P-DNA adducts, which is probably due to the high B[a]P dose. Moreover, β-carotene did not statistically significantly affect cell proliferation and p53-protein expression in hamster tracheal epithelial cells.

Introduction

Recently, overwhelming evidence has been presented that the tumour-suppressor gene p53 plays a key role in the etiology of respiratory tract cancer. In all histological types of lung cancer, genetic abnormalities have been detected in the p53 gene (1-5). The wild-type p53 protein has been shown to down-regulate cell proliferation following treatment with

DNA-damaging agents (6-8).

G to T transversions which have been detected frequently in the mutated p53 tumour-suppressor gene, may be the consequence of DNA damage induced by, *e.g.*, benzo[a]pyrene (B[a]P, 9-11), a well-known respiratory tract carcinogen.

The various stages of B[a]P-induced respiratory tract cancer have been extensively investigated, both *in vivo* and *in vitro* (12-16). To induce respiratory tract cancer in experimental animals, B[a]P is usually administered intratracheally in combination with Fe_2O_3 according to the method of Saffiotti *et al.* (12). In this way a broad spectrum of (pre)neoplastic changes are induced in the respiratory tract epithelium of hamsters.

Both vitamin A and the provitamin β-carotene have been shown to protect against the development of chemically induced respiratory tract cancer (19,20). The molecular mechanism, however, is not yet fully elucidated. Vitamin A plays a very important role in regulation of cellular proliferation and differentiation of respiratory tract epithelium (21,22). It has been demonstrated that vitamin A decreased chemically induced cell proliferation *in vivo* and *in vitro* (23-25). Moreover, (pro)vitamin A has been shown to decrease the formation of DNA-damage, which is an important early step in the initiation of cancer (26,27).

Although (pro)vitamin A and p53 both play an important role in the formation of respiratory tract cancer, little is known about the effect of (pro)vitamin A on the expression of the p53 protein. The goal of this study was first to study the relationship between B[a]P-DNA adducts, cell proliferation and p53 expression in hamster tracheal epithelium, and second, to find out whether β-carotene affects such a relationship.

Materials and Methods

Experimental Design

Weanling male Syrian golden hamsters (Charles River Wiga GmbH, Sulzfeld, Germany) were kept on sterile saw dust in macrolon cages, 2 animals per cage, under standard conventional laboratory conditions. Hamsters were fed a pelleted purified diet as has been described recently (28) containing a normal level of vitamin A (4000 IU retinyl palmitate/kg food, Hoffman-La Roche, Basle, Switzerland) or 4000 IU vitamin A/kg food and 1% β-carotene (w/w, Hoffman-La Roche) (Table I). Diets and drinking water were provided *ad libitum*. After a 1-month adaptation period to the diets, hamsters were given saline suspensions (0.2 ml) of 8 mg B[a]P particles (Sigma Chemicals, St. Louis, MO) attached to 8 mg Fe_2O_3 particles (Fisher Scientific Company, NJ) by intratracheal instillation. The animals were treated 10 times over a period of 12 weeks according to the method described by Saffiotti *et al.* (12). Control animals were given intratracheal instillations of a suspension in saline of Fe_2O_3 particles alone (Table I). One week (subgroup 1) and 6 weeks (subgroup 2) after the last instillation, animals were sacrificed and the respiratory

tract was collected and processed for quantification of B[a]P-DNA adducts, cell proliferation and p53 expression (see below).

Table I. Experimental design; number of animals per group, dietary vitamin A and β-carotene levels and treatments.

Group	n (1)	n (2)	Vitamin A in diet (IU/kg)	Treatment
A	4	9	4000	Fe_2O_3
B	4	9	4000	B(a)P + Fe_2O_3
C	4	9	4000 + 1% β-carotene	Fe_2O_3
D	4	9	4000 + 1% β-carotene	B(a)P + Fe_2O_3

n: number of animals per subgroup; (1): animals in subgroup 1 were sacrificed one week after the last intubation; (2): animals in subgroup 2 were sacrificed 6 weeks after the last intubation; IU: international units.

^{32}P-postlabeling analysis of B[a]P-DNA adducts

For quantification of B[a]P-DNA adducts by ^{32}P-postlabeling, 1/3 of the trachea and part of the lungs of 5 animals per group were collected. The tracheal rings were cut open and the epithelial cells were gently scraped off and pooled. Lungs were homogenised in phosphate-buffered saline (PBS) and pooled. DNA was isolated by means of phenolic extractions and ethanol precipitation as described previously (29). Following enzymic digestion of the DNA, B[a]P-DNA adducts were enriched by n-butanol extraction (30) and labeled with 2.3 MBq γ-[^{32}P]-ATP/sample (Amersham, Buckinghamshire, UK, sp.act. > 185 TBq/mmol) by incubation with T4 polynucleotide kinase (Biolabs, Beverly, MA) as described previously (30,31). Subsequently, the postlabeling mixtures were applied to poly(ethylenimine)(PEI)-cellulose sheets (JT Baker, Phillipsburg, NJ, USA), with a paper wick attached to the top of each sheet for multidirectional thin-layer chromatography. The sheets were developed in 1 M sodium phosphate, pH 6.0 overnight (D1), and subsequently in 8.5 M urea and 3 M lithium formate, pH 3.5 for 6.5 h (D3) and in 8.5 M urea, 0.8 M lithium chloride, 0.5 M Tris, pH 8.0 for 6.5 h (D4). To remove any remaining impurities, the plates were developed overnight in 1.7 M sodium phosphate, pH 6.0 (D5, in the same direction as D1). The B[a]P-DNA adducts were visualized by autoradiography on Kodak XAR-5 films using an intensifying screen. The spots on the PEI-cellulose sheets detected by autorafiography were cut out and the radioactivity was determined by liquid scintil-

lation counting.

The amount of DNA-adducts was calculated on the basis of the results of concurrent postlabeling analysis of standard DNA samples carrying known amounts of B[a]P-adducts prepared by *in vitro* modification of DNA with *anti*-BPDE as described recently (31). The exact amount of input DNA and the absence of RNA were determined by chromatography of the DNA digest on FPLC, as described by Steenwinkel *et al.* (31).

Immunocytochemical detection of BrdU incorporation

Three days before each sacrifice, osmotic mini-pumps (model 2001, release rate of 1 μl/h, Alza Corporation, Palo Alto, CA) filled with 200 μl bromodeoxyuridine (BrdU) solution (25 mg/ml in 0.01 N NaOH in PBS, Sigma) were implanted subcutaneously under ether anaesthesia. At necropsy, tracheas were fixed in phosphate-buffered formalin for 24 h and subsequently stored in 70% ethanol for 3 days at 4^0C. Tracheas were embedded in paraffin and cross sections (5 μm thickness) were put on 3-aminopropyltriethoxysilane (Sigma)-precoated slides. Sections were dewaxed in xylene and hydrated in a series of ethanol. Endogenous peroxidase was blocked using 0.3% H_2O_2 in methanol. DNA was denatured by 1 N HCl for 1 h at 37^0C. Other steps were performed at room temperature. Tissue sections were treated with 0.05% w/v pronase E (Sigma) in PBS, pH 7.8 for 10 min. Tissue sections were preincubated with 25% normal goat serum (Dakopatts a/s, Glostrup, Denmark) for 20 min, and incubated with mouse anti-BrdU monoclonal antibody (1:60 in PBS, Becton Dickinson Immunocytochemistry Systems, San José, CA) for 60 min, biotinylated rabbit anti-mouse antibody (1:400 in PBS, Dakopatts a/s) for 30 min and peroxidase-labelled streptavidin (1:400 in PBS, Dakopatts a/s) for 30 min, respectively. Between the incubations tissue sections were rinsed in PBS. Thereafter, tissue sections were stained with diaminobenzidine (DAB) solution (Sigma, 5 mg/ml aquous solution containing 0.015% H_2O_2) for 10 min and slightly counterstained with hematoxylin for 20 sec. Finally, sections were dehydrated and mounted in Tissue-tek coverslipping resin (Miles Inc, Elkhart, IN, USA).

Cell proliferation was assessed by counting the number of DAB-stained nuclei of basal cells (cells in contact with the basal lamina and not reaching the tracheal lumen) and DAB-stained nuclei of non-basal cells (cells in contact with the basal lamina and reaching the tracheal lumen or cells that have lost contact with the basal lamina). Cell proliferation is expressed by a labeling index (LI) which is defined as the percentage of BrdU-positive cells amongst the total number of epithelial cells counted per cross-section. Per animal 1 or 2 tracheal cross-sections were counted.

Immunocytochemical detection of p53 expression

Immunocytochemical detection of the expression of the p53 protein was performed on serial cross sections. The immunocytochemical procedure to detect the p53 protein was the same as described for BrdU staining except for DNA denaturation and pronase E treatment

which were left out. The p53 protein was detected by a mouse anti-human-p53 monoclonal antibody (Clone BP53-12-1, Biogenex, San Ramon, CA). This antibody recognizes a fixation-resistant epitope of both wild-type and mutant p53 protein located on the amino terminus between residues 1 and 45. As a negative control for the immunocytochemical procedure, the p53 monoclonal antibody was replaced by PBS. The level of p53 expression in tracheal epithelium is given as the number of p53-positive basal and non-basal cells divided by the total number of cells counted per tracheal cross section. Per animal, 1 or 2 tracheal cross sections were scored. Only clearly dark-brown stained nuclei were scored as positive, slightly pale-brown and grey-coloured nuclei were scored as negative.

Statistical analysis

All data were analysed for statistically significant differences by analysis of variance (ANOVA) followed by Tukey's t-test (Instat Biostatistics, GraphPad Software, San Diego, CA)

Results

B[a]P-DNA adducts

Treatment of hamsters with B[a]P-Fe_2O_3 suspension by intratracheal instillation resulted in the formation of only one major DNA adduct in lung and trachea as detected by ^{32}P-postlabeling, the *trans*-addition product of deoxyguanosine (dG) with (+)-*anti*-7,8,dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE) (Fig 1). One week after the last instillation, about 6 BPDE-DNA adducts/10^6nucleotides (add/10^6n) were found by ^{32}P-postlabeling analysis of trachea and lung DNA of B[a]P-treated animals (Fig 2, subgroup 1). In the tracheas and lungs of control animals (groups A and C) no BPDE-DNA adducts were detected (Fig 2). No differences were observed between BPDE-DNA adduct levels in either tracheas or lungs of hamsters fed a normal diet and hamsters that received the β-carotene diet (Fig 2). ^{32}P-postlabeling analysis of the tracheal and pulmonary DNA in subgroup 2, sacrificed 6 weeks after the last instillation, showed a decrease in the level of pulmonary BPDE-DNA adducts as compared to subgroup 1, and a statistically non-significant increase in tracheal BPDE-DNA adduct level. Although the level of B[a]P-DNA adducts in tracheas of subgroup 2 was increased by β-carotene, no statistically significant effect of β-carotene on the level of BPDE-DNA adducts in either tracheas or lungs of subgroup 1 and 2 was observed.

Cell proliferation

Immunocytochemical analysis of BrdU incorporation during S-phase in hamster tracheal epithelial cells showed clear nuclear staining (Fig 3). B[a]P induced a significant increase in cell proliferation in tracheal epithelium collected 1 and 6 weeks after the last intubation compared with that observed in control hamsters (Fig 4). In both subgroups, B[a]P-

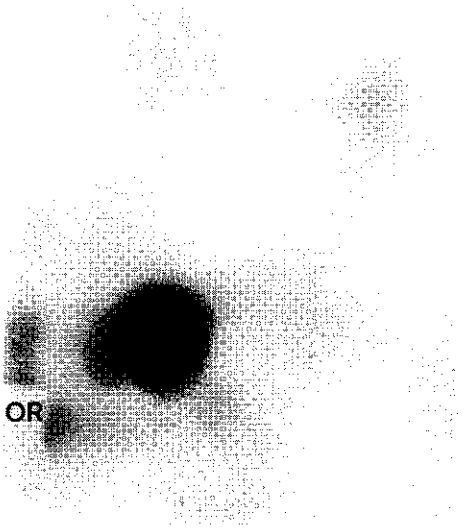

Figure 1. Autoradiogram of ^{32}P-postlabelled DNA isolated from tracheal epithelial cells obtained from a hamster that had been treated intratracheally with B[a]P + Fe_2O_3 showing only one adduct which has been previously identified as the interaction product of (+)-*anti*-BPDE and dG (15,16).

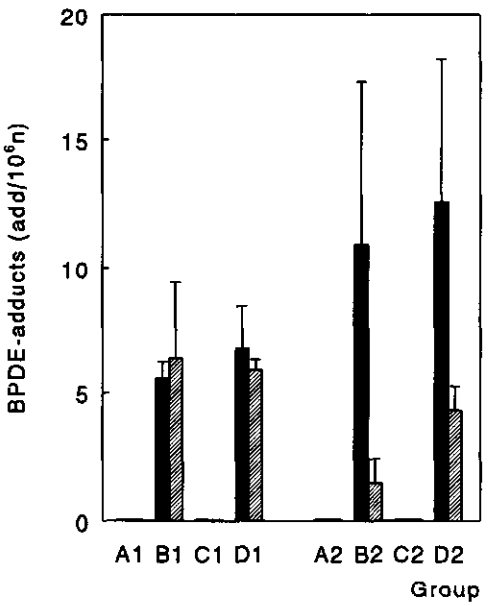

Figure 2. Effect of β-carotene on the level of BPDE-DNA adducts in hamster tracheas (closed bars) and lungs (hatched bars); 1 (subgroup 1) and 6 (subgroup 2) weeks after 10 intratracheal intu- bations. Group A: 4000 IU vitamin A/kg diet, Fe_2O_3; Group B: 4000 IU vitamin A/kg diet, B[a]P+Fe_2O_3; group C: 4000 IU vitamin A/kg diet + 1% β-carotene, Fe_2O_3; group D: 4000 IU vitamin A/kg diet + 1% β-carotene, B[a]P+Fe_2O_3. Error bars represent range of values of 2 indepedent postlabeling assays.

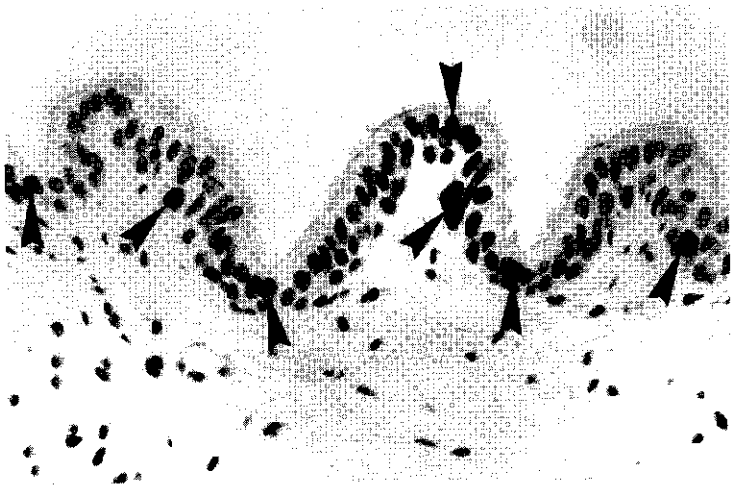

Figure 3: Tracheal epithelium of a hamster of group C (4000 IU vitamin A/kg diet + 1% β-carotene, Fe_2O_3) showing BrdU-positive nuclei (arrowheads). Haematoxylin counter-staining, x400.

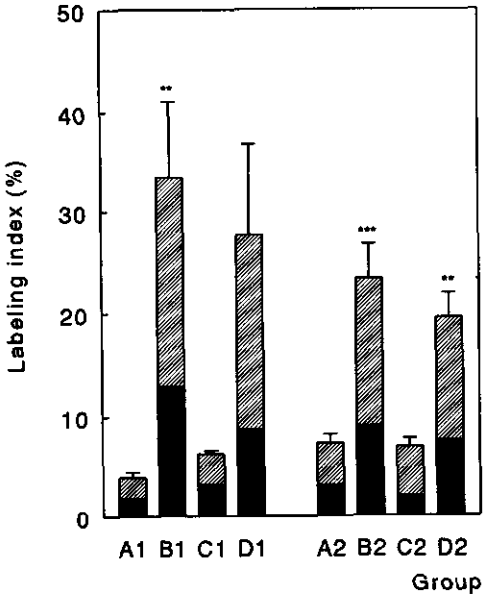

Figure 4: Effect of β-carotene and B[a]P on proliferation of basal (closed bar) and non-basal (hatched bar) hamster tracheal epithelial cells; 1 (subgroup 1) and 6 (subgroup 2) weeks after 10 intratracheal intubations. Group A: 4000 IU vitamin A/kg diet, Fe_2O_3; group B: 4000 IU vitamin A/kg diet, B[a]P+Fe_2O_3; group C: 4000 IU vitamin A/kg diet + 1% β-carotene, Fe_2O_3; group D: 4000 IU vitamin A/kg diet + 1% β-carotene, B[a]P+Fe_2O_3. Error bars represent SEM of total (basal+non-basal) cell proliferation of 4 (subgroup 1) or 9 (subgroup 2) hamsters per group ($^{**}P<0.01$, $^{***}P<0.001$ compared with corresponding control group, *e.g.* B with A and D with C).

induced cell proliferation was slightly decreased only in hamsters fed the β-carotene diet compared with hamsters receiving the control diet, but this difference was not statistically significant. Furthermore, cell proliferation in the tracheal epithelium was slightly decreased in hamsters of subgroup 2 (at 6 weeks) compared with hamsters of subgroup 1 (at 1 week). In tracheal epithelium, proliferation of non-basal cells was higher than that of basal-cells (Fig 4).

Expression of p53

Immunocytochemical staining of the p53 protein in hamster tracheal epithelium showed a broad range of staining intensities of the nuclei, which varied from pale-grey to dark-brown (Fig 5). Only the dark-brown stained nuclei were scored. The presence or absence of p53-positive cells was not related with any histopathological changes. In tracheal epithelial cells of hamsters treated with B[a]P, the number of epithelial cells expressing p53 protein was statistically significantly increased compared with epithelial cells of control hamsters (Fig. 6). The expression of p53 protein in tracheal epithelial cells of hamsters fed the β-carotene diet (group D) was slightly increased compared with that in tracheal epithelial cells of hamsters receiving the control diet (group B), but the difference was not statistically significant. Furthermore, the expression of p53 protein in non-basal cells was higher than that in basal cells. In epithelial cells of control hamsters (groups A and C), no nuclear expression of p53 protein could be detected at all. However, granular staining was observed in the apical cytoplasm of some tracheal epithelial cells of hamsters of all groups (Fig. 7).

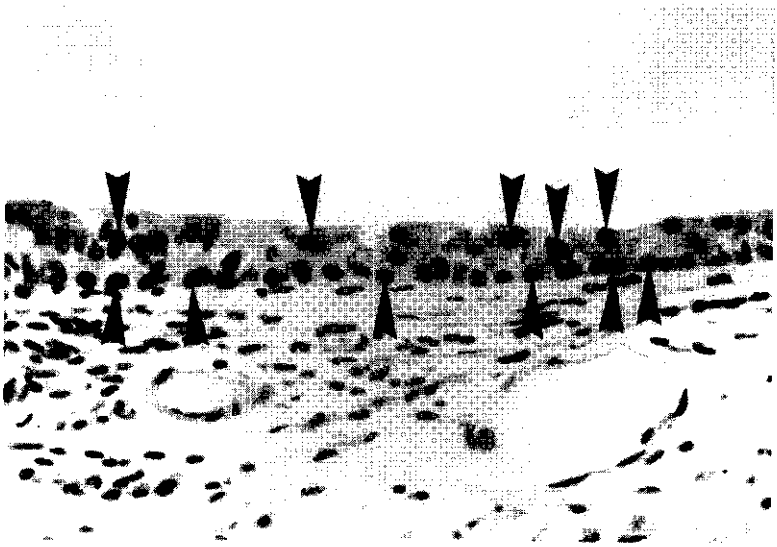

Figure 5: Tracheal epithelium of a hamster of group D (4000 IU vitamin A/kg diet + 1% β-carotene, B[a]P-Fe_2O_3) showing P53-positive nuclei (arrowheads). Haematoxylin counter-staining, x400.

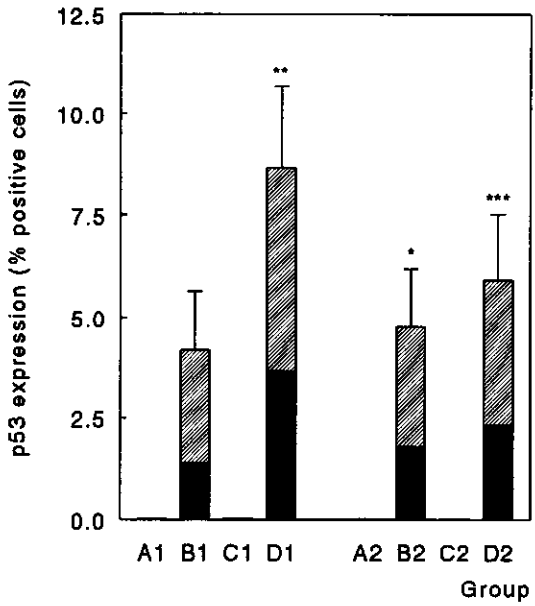

Figure 6: Effect of β-carotene and B[a]P on P53 expression in basal (closed bar) and non-basal (hatched bar) hamster tracheal epithelial cells; 1 (subgroup 1) and 6 (subgroup 2) weeks after 10 intratracheal intubations. Group A: 4000 IU vitamin A/kg diet, Fe_2O_3; group B: 4000 IU vitamin A /kg diet, B[a]P+Fe_2O_3; group C: 4000 IU vitamin A/kg diet + 1% β-carotene, Fe_2O_3; group D: 4000 IU vitamin A/kg diet + 1% β-carotene, B[a]P+Fe_2O_3. Error bars represent SEM of total (basal+non-basal) LI of P53 expression of 4 (subgroup 1) or 9 (subgroup 2) hamsters per group ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with corresponding control group, *e.g.* B with A and D with C).

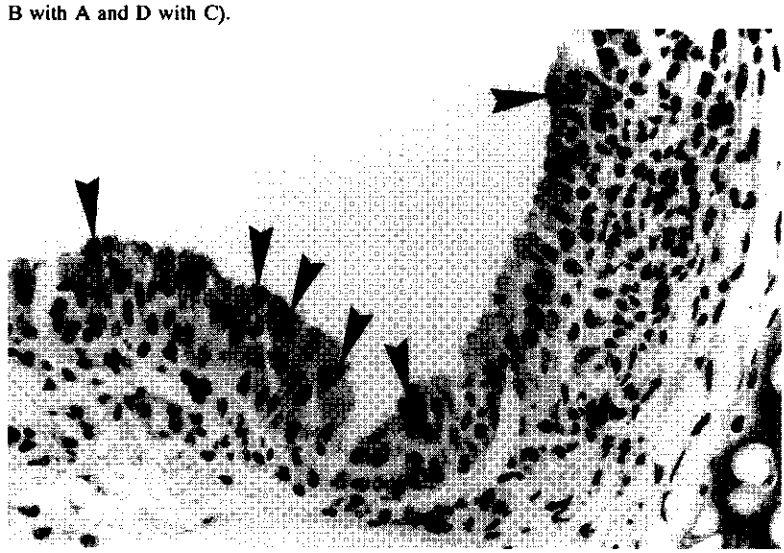

Figure 7: Tracheal epithelium of a hamster of group B (4000 IU vitamin A/kg diet, B[a]P-Fe_2O_3) showing apical cytoplasmic p53 staining (arrowheads). Haematoxylin counter-staining, x400.

Discussion

To study experimental respiratory tract carcinogenesis, the most widely applied *in vivo* model is the model developed by Saffiotti *et al.* (12) based on intratracheal instillation of B[a]P-Fe_2O_3 particles in suspension. We used this experimental model to investigate the relation between the formation of B[a]P-DNA adducts, induction of cell proliferation and the expression of p53 protein in hamster tracheal epithelial cells and the possible effect of a high dietary level of β-carotene on these parameters. The amount of DNA adducts was determined by the ^{32}P-postlabeling assay, cell proliferation was determined by use of immunohistochemical detection of BrdU incorporation during S-phase and p53 expression was determined by immunohistochemistry with an antibody recognizing the wild-type as well as the mutated p53 protein. The half-life of the native wild-type p53 protein however, has been shown being too short to allow immunohistochemical detection (1). Several mechanisms has been described stabilizing the p53 protein in cells resulting in an increased immunohistochemical staining. Mutation of the p53 gene has frequently been shown to result in an overexpression of the p53 protein in cells. Furthermore, binding of viral or cellular proteins to the p53 protein stabilizes the nonmutant p53 protein (32,33), so it is difficult to discriminate between wild-type and mutated p53 protein as detected by immunohistochemistry.

In this study, only nuclei of tracheal epithelial cells of B[a]P-treated hamsters stained clearly positive for p53 protein, whereas in tracheas of control hamsters no nuclear staining was observed (even no pale-brown or grey coloured nuclei were present). Furthermore, in this study a clear correlation exists between the extent of B[a]P-induced DNA damage, induction of cell proliferation and expression of p53 protein in tracheal epithelium of hamsters intratracheally instilled with B[a]P-Fe_2O_3. Since B[a]P treatment frequently has been shown to induce G to T transversions and because this mutation is the most encountered mutation in the p53 gen which results in lose of cell proliferation control, these results suggest that B[a]P increases cell proliferation in hamster tracheal epithelial cells by inducing alterations in the p53 gene, the guardian of cell cycle control. These results are in agreement with data reported by several authors showing an accumulation of the p53 protein in cells of respiratory tract cancers (1-5). Furthermore, recently Bjelogrlic *et al.* (34) reported a positive relation between p53 protein expression and DNA damage in mouse skin treated with B[a]P.

In tracheas of B[a]P-treated hamsters and in control hamsters cytoplasmic p53 protein was detected. The meaning of cytoplasmatic p53-protein staining is still under discussion. Possibly, cytoplasmatic p53 protein staining is an artefact due to treatment protocol of the tissues. Brambilla *et al.* (5) suggested that cytoplasmic staining may reflect accumulation of an abnormal p53 protein in a conformational state that prevents its transport through the nuclear membrane. Furthermore, Moll *et al.* (35) suggested that the level of cytoplasmatic p53 protein in breast tissue cells increases caused by a physiological process to permit transient cell proliferation. Sun *et al* (36) showed that cytoplasmatic labeling of p53 in

colorectal adenocarcinoma cells of humans was associated with a poor survival. However, since we did not observed any differences in cytoplasmatic staining between control and B[a]P-treated cells, it is suggested that cytoplasmic p53 staining may be of minor importance in respiratory tract cancer in hamsters.

Under the experimental conditions applied here, β-carotene did not significantly influence the expression of p53 protein in tracheal epithelial cells of hamsters treated with B[a]P. However, both one week and six weeks after B[a]P treatment, a slight but not significant increase ($P > 0.10$) in the expression of p53 protein by β-carotene corresponds to a small decrease in cell proliferation. This result is in agreement with those of Joiakim and Chopra (37) who showed that retinoic acid down-regulates the growth of human tracheal gland epithelial cells by increasing the expression of p53 protein.

In a previous *in vitro* study, we showed that vitamin A and β-carotene protect against the formation of B[a]P-DNA adducts in hamster tracheal epithelium in organ culture. However, the effect was strongly dependent on the concentration of vitamin A *vis-a-vis* the concentration of B[a]P. Using a low concentration of B[a]P, vitamin A decreased the level of B[a]P-DNA adducts, whereas vitamin A increased the level of B[a]P-DNA adducts in hamster tracheal epithelial cells treated with a high concentration of B[a]P (publication submitted). Although the concentration of β-carotene in the diet in the present study was relatively high (1%), no significant effect of
β-carotene on B[a]P-DNA adduct formation was observed both in tracheas and lungs. Apparently, the dose of B[a]P administered in this study was too high to observe a protective effect of
β-carotene against the formation of B[a]P-DNA adducts. Six weeks after the last instillation, the level of B[a]P-DNA adducts in the lungs was decreased compared with that 1 week after the last instillation. In tracheas, however, the level of B[a]P-DNA adducts was increased after 6 weeks. This may be explained by an increased concentration of B[a]P in the trachea after clearance of the lung via the mucociliary escalator. However, the relatively short retention time of B[a]P particles in the lungs after intratracheal instillation (13,38) fails to make a reasonable case for this explanation.

Generally, the modulating effect of (pro)vitamin A on respiratory tract cancer is ascribed to its role in cellular differentiation and proliferation (21-25). In this study, β-carotene did not sig-nificantly influence the B[a]P-induced cell proliferation in hamster tracheal epithelium, although, both 1 and 6 weeks after the last instillation, β-carotene tended to inhibit cell proliferation. These results are in accordance with the results of Beems *et al.* (39) who showed that β-carotene did not influence the B[a]P-induced tumor response in the hamster respiratory tract. In the present study, cell proliferation in the tracheas of the hamsters treated with Fe_2O_3 alone was relative high (4-8%), probably because of mechanically-induced tissue damage caused by instillation (40). Non-basal cell proliferation was higher than basal cell proliferation, probably due to the relatively prolonged BrdU-treatment period of 3 days. Labeled basal cells could have divided and differentiated to

mucous or ciliated cells during this period. Subsequently, they are scored as BrdU-positive non-basal cells.

In conclusion, in this study a clear quantitative correlation between B[a]P-induced DNA damage, induction of cell proliferation and expression of p53 protein has been shown in tracheal epithelium of hamsters intratracheally instilled with a B[a]P-Fe_2O_3, suggesting that B[a]P increases cell proliferation by inducing alterations in the p53 gene. Furthermore, we were unable to show a statistically significant effect of β-carotene on the formation of B[a]P-DNA adducts, cell proliferation and p53-protein expression, probably because of the dose of B[a]P was too high.

Acknowledgements

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Chapter 9

Benzo[a]pyrene-induced respiratory tract cancer in hamsters fed a high β-carotene diet. A histomorphological study.

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Submitted for publication

Summary

The effect of a high dietary level of β-carotene on the formation of preneoplastic and neoplastic respiratory tract lesions was studied in hamsters intratracheally treated with benzo[a]pyrene (B[a]P) attached to ferric oxide (Fe_2O_3) and suspended in saline. In addition to conventional histopathological examinations, the expression of cytokeratins and the glutathione *S*-transferase isoenzyme Pi (GST-Pi) was determined in tracheal epithelium using immunocytochemical techniques. B[a]P treatment increased the expression of cytokeratins in tracheal mucous and ciliated epithelial cells as detected by antibody RCK102 (cytokeratins 5 and 8) which normally recognizes basal cells only. The expression of cytokeratins in mucous and ciliated cells as detected by antibody RGE53 (cytokeratin 18) was decreased by B[a]P treatment. Furthermore, the expression of the cytokeratin detected by antibody RKSE60 (cytokeratin 10), characteristic of metaplastic squamous cells, and the expression of the GST-Pi, characteristic of metaplastic changes, was increased in tracheal epithelium of hamsters treated with B[a]P.

There was no evidence for dietary β-carotene affecting the expression of cytokeratins or GST-Pi. The incidence of preneoplastic changes and tumours of the respiratory tract was not reduced by dietary β-carotene. On the contrary, the tumour response of the respiratory epithelium was almost twice as high in hamsters fed the high-β-carotene diet than in hamsters on the low-β-carotene diet. However, this difference was not statistically significant ($P = 0.15$); hence, the present study did not produce evidence for a clear effect of β-carotene on B[a]P-induced respiratory tract cancer in hamsters.

Introduction

Lung cancer is the leading form of cancer in nearly all parts of the world, particularly in Western countries (1,2). Because elimination of all factors responsible for the induction of lung cancer seems to be an almost impossible task, considerable effort has been

directed towards identifying naturally occurring or synthetic compounds which may prevent or suppress the formation of respiratory tract cancer. In this regard, (pro)vitamin A (vitamin A and β-carotene) have been shown to protect against lung cancer in a large number of epidemiological and experimental studies (3,4), although the results are not always consistent (5,6).

To study the effect of (pro)vitamin A on chemically induced respiratory tract cancer of experimental animals, the experimental hamster-trachea model introduced by Saffiotti and co-workers (7) has been shown to be very useful. In this model, hamsters receive repeated intratracheal instillations of a saline suspension of benzo[a]pyrene (B[a]P) particles attached to ferric oxide (Fe_2O_3) particles. In this way, a broad spectrum of (pre)neoplastic changes can be induced in the lining epithelium of the respiratory tract of hamsters, including hyperplasia, squamous metaplasia, papillomas, adenomas, squamous cell carcinomas and adenocarcinomas (3,7,8), which can be prevented by (pro)vitamin A (3).

Epithelial cells are well characterized by a specific combination of cytokeratins (9). Both *in vivo* and *in vitro* studies have shown that changes in the expression of various cytokeratins coincide with changes in tracheal epithelial cell differentiation, rendering the cytokeratin expression pattern in tracheal epithelial cells a good marker for differentiation (10,11). Furthermore, the expression of glutathione *S*-transferase Pi-class isoenzymes (GST-Pi) has frequently been reported to be a marker for (pre)neoplastic changes including those developing in the respiratory tract (12-14).

The aim of this experiment was to investigate possible effects of a high dietary level of β-carotene on the development of B[a]P-induced (pre)neoplastic lesions in the respiratory tract of hamsters. Various cytokeratins and GST-Pi were determined immunohistochemically as markers for changes in the differentiation stage of hamster tracheal epithelial cells.

Materials and Methods

Experimental Design

Weanling male Syrian golden hamsters (Charles River Wiga GmbH, Sulzfeld, Germany) were kept on sterile saw dust in Macrolon cages, 2 animals per cage, under standard conventional laboratory conditions. Hamsters were fed a pelleted diet as described (15) containing a normal level of vitamin A (4000 IU retinyl palmitate/kg diet, Hoffmann-La Roche, Basle, Switzerland) or 4000 IU vitamin A/kg diet and 1% w/w β-carotene (Hoffmann-La Roche). Food and drinking water were provided *ad libitum*. After a 1-month period of adaptation to the diets, hamsters were given saline suspensions (0.2 ml) of 8 mg B[a]P particles (Sigma, St. Louis, MO) attached to 8 mg Fe_2O_3 particles (Fisher, NJ) by intratracheal instillation according to the method described by Saffiotti and co-workers (7). Control hamsters were given intratracheal instillations of a suspension in saline of Fe_2O_3 particles alone (Table I). The animals were treated 10 times over a period

Table I: Experimental design: number of animals per group, dietary vitamin A and β-carotene levels and treatments.

Group	n^1	Vitamin A[2] in diet (IU/kg)	β-carotene in the diet (w/w)	Treatment[3]
A	20	4000		Fe_2O_3
B	50	4000		B[a]P + Fe_3O_3
C	20	4000	1 %	Fe_2O_3
D	50	4000	1 %	B[a]P + Fe_2O_3

[1]: *n* is the number of animals per group; [2]: retinyl palmitate; [3]: 10 intratracheal intubations of a saline suspension of 8 mg Fe_2O_3 and 8 mg B[a]P or 8 mg Fe_2O_3 alone over a period of 12 weeks.

of 12 weeks. One week (subgroup 1) and 6 weeks (subgroup 2) after the last instillation, animals were sacrificed and the respiratory tract was collected and fixed in phosphate-buffered formalin for 24 h and subsequently stored in 70% ethanol for 3 days at 4^0C and embedded in paraffin wax. Cross sections (5 μm thick) were stained with haematoxylin and eosin for conventional histopathological examination. Furthermore, serial tracheal cross-sections were collected on 3-aminopropyltriethoxysilane (APTS)-precoated slides for immunocytochemical detection of GST-Pi (see below). At autopsy, a small part of the trachea of each hamster was embedded in Tissue-Tek cryomatrix (Miles, Elkhart, IN), frozen in solid-CO_2-cooled isopentane and stored at -80^0C for the immunocytochemical detection of various cytokeratins (see below). The respiratory tract of animals that died or were killed *in extremis* during the experiment were only used for conventional histopathological examination.

Immunocytochemical detection of cytokeratins

Cryostat cross sections (7 μm thick) were put on APTS-precoated slides, air-dried and fixed in acetone for 10 min. Subsequently, tissue sections were rinsed in phosphate-buffered saline (PBS, pH 7.4) and preincubated with 25% normal goat serum (Dakopatts, Glostrup, Denmark) in PBS for 20 min, and incubated with primary antibodies directed against various cytokeratins [RCK102 directed against human cytokeratin (HC) 5 and 8; RGE53 directed against HC 18 and RKSE60 directed against HC 10; the antibodies were kindly provided by Dr. G. Schaart and Professor G.P. Vooijs, Nijmegen University Hospital, Department of Pathology, The Netherlands] for 60 min at room temperature. Then sections were rinsed with PBS and incubated with a peroxidase-labelled rabbit anti-

mouse antibody (1:200, Dakopatts) in PBS with 4% normal rat serum (Dakopatts) for 30 min. Thereafter, tissue sections were stained with diaminobenzidine (DAB) solution (Sigma, 5 mg/ml solution containing 0.015% H_2O_2) for 10 min and slightly counterstained with haematoxylin for 20 sec. Finally, sections were dehydrated and mounted in Tissue-Tek coverslipping resin (Miles).

Immunocytochemical detection of GST-Pi

Sections (5 μm thick) of paraffin-embedded tracheas on APTS-precoated slides were dewaxed in xylene and hydrated in a series of ethanol solutions. Tissue sections were preincubated with 25% normal goat serum (Dakopatts) for 20 min and incubated with a mouse anti-rat GST-7.7 monoclonal antibody (1:800 dilution, kindly provided by Professor P.J. van Bladeren) or a rabbit anti-rat GST-7.7 polyclonal antibody (1:8000, kindly provided by Dr. K. Satoh, Second Department of Biochemistry, Hirosaki University School of Medicine, Hirosaki, Japan). Subsequently, cross-sections were rinsed with PBS and incubated with a biotinylated rabbit anti-mouse antibody (1:400 in PBS, Dakopatts) or a biotinylated swine anti-rabbit antibody (1:400 in PBS, Dakopatts) for 30 min. Thereafter, sections were rinsed and incubated with peroxidase-labelled streptavidin (1:400 in PBS, Dakopatts) for 30 min, rinsed with PBS and stained with DAB, counterstained with haematoxylin and embedded as described above.

Both antibodies directed against rat GST-7.7 were tested for cross-reactivity with other classes of purified hamster GST isoenzymes (described by Bogaards *et al.* [16]) by immuno dot-blotting on nitrocellulose paper. At the antibody concentrations used in this study, both antibodies only reacted with hamster GST isoenzyme P_1 (hamster GST isoenzyme of Pi-class [16]). No cross-reactivity was observed with hamster GST isoenzymes from other classes.

Quantification of labelling

Expression of various cytokeratines and GST-7.7 was assessed in serial cross-sections of tracheas of hamsters killed according to the schedule. Immunocytochemical labelling was graded 4 if more than 90% of the tracheal epithelium was positive, 3 if 40 - 90% was positive, 2 if 10 - 40% was positive, 1 if between 1 - 10% was positive and 0 if less than 1% was positive. Values are given as the mean value of 5 to 10 tracheas (adapted from Rutten *et al.* [11]).

Results

Histopathology

The results of the histopathological examinations are presented in Table II. The tumour response in the 1% β-carotene group (15/41) appeared to be higher than in the group

Table II: Site, type and incidence of non-neoplastic and neoplastic changes of respiratory tract epithelium of hamsters treated intratracheally with benzo[a]pyrene and kept on a diet with or without 1% β-carotene for a period of at most 22 weeks.

Site and type of changes	Incidence of changes					
	Interim kill		Final kill		Intercurrent deaths	
	Group B[1]	Group D	Group B	Group D	Group B	Group D
LARYNX	(8)[2]	(6)	(10)	(9)	(16)	(13)
Hyperplasia/squamous metaplasia:						
slight	0	0	6	4	7	5
moderate	4	4	2	3	5	3
severe	3	2	1	2	3	2
Squamous cell carcinoma	0	0	0	0	1	0
TRACHEA	(8)	(8)	(11)	(11)	(21)	(21)
Hyperplasia/squamous metaplasia:						
slight	0	0	2	3	4	3
moderate	2	2	4	4	9	5
severe	6	5	5	4	7	9
Carcinoma *in situ*	0	0	0	0	0	2
Squamous cell carcinoma	0	0	0	0	2	3
BRONCHI	(8)	(8)	(11)	(12)	(20)	(21)
Hyperplasia/squamous metaplasia:						
slight	0	0	1	3	4	3
moderate	0	2	6	3	6	5
severe	6	3	3	5	5	7
Papilloma	0	0	0	1	0	0
Squamous cell carcinoma	1	1	0	1	2	4
Adeno-squamous carcinoma	0	0	0	0	1	0
Adenocarcinoma	0	0	1	2	0	0
Carcinosarcoma	0	0	0	0	0	1
Total number of tumours	1	1	1	4	6	10

[1] For group code see Table I
[2] Number of organs examined are given in brackets.

without additional dietary β-carotene (8/39). However, this difference was not statistically significant (P = 0.15; two-sided Fisher Exact Probability Test). The average "time to tumour appearance" was 19 weeks in the high-β-carotene group and 18 weeks in the group without β-carotene after the beginning of the experiment. Several hamsters of both groups showed varying degrees of acute laryngitis, tracheitis and/or bronchopneumonia. Moreover, highly malignant fibrosarcomas of the respiratory tract were encountered in a total of 17 B[a]P-treated hamsters, *viz* in 6 of 41 hamsters of the high-β-carotene group and in 11 of 39 hamsters of the group without β-carotene. In hamsters not treated with B[a]P no respiratory tract tumours were found and the non-neoplastic respiratory tract epithelial changes were confined to very slight or slight focal hyperplasia of the larynx, trachea and/or bronchi.

Cytokeratin expression

The effects of the different treatments on cytokeratin expression in hamster tracheal epithelium are shown in Table III. No marked differences were observed in cytokeratin expression between hamsters of subgroup 1 and those of subgroup 2 (data per subgroup are not shown). The expression of cytokeratins, as detected by RCK102 (HC 5 and 8), in basal cells was not influenced by the various treatments. All basal cells were positively stained by antibody RCK102. The number of RCK102-positive mucous and ciliated tracheal cells of hamsters of Group B and D was clearly elevated compared with the corresponding control groups, A and C, respectively (Table III and Figure 1a), whereas β-carotene did not influence the expression patterns of cytokeratins detected by RCK102.

All mucous and ciliated cells in control groups A and C were positively stained by the RGE53 antibody (detecting HC 18). The number of RGE53-positive non-basal cells was lower in tracheas of hamsters treated with B[a]P (groups B and D) than in those of corresponding controls. Furthermore, some RGE53-negative non-basal cells were positively stained by the RCK102 antibody (Figure 1a and 1b). No differences were observed in immunocytochemical staining of HC 18 as detected by the RGE53 antibody between group A and group C or between group B and group D, showing that β-carotene did not affect the expression of HC 18.

Table III: Expression of various cytokeratins in hamster tracheal epithelium.

Antibodies[1]	Group A[2]		Group B		Group C		Group D	
	B[3]	M-C[4]	B	M-C	B	M-C	B	M-C
RCK102	3.8[5]	0.7	4.0	2.2	3.7	0.4	4.0	2.2
RGE53	0.8	4.0	0.3	2.7	0.6	3.9	0.0	3.0
RKSE60	0.0	0.0	0.0	1.3	0.0	0.0	0.0	1.5

[1] More detailed information about the antibodies has been published by Rutten *et al.*[10,11]; [2] For group code see Table I; [3] B: basal cells; [4] M-C: mucus producing and ciliated cells; [5] Labeling was graded 4 if more than 90% of the tracheal epithelium was positive, 3 if between 40% and 90% was positive, 2 if between 10% and 40% was positive, 1 if between 1% and 10% was positive and 0 if less than 1% was positive. Values are given as the mean value of 10 tracheas (adapted from Rutten *et al.* [10,11]).

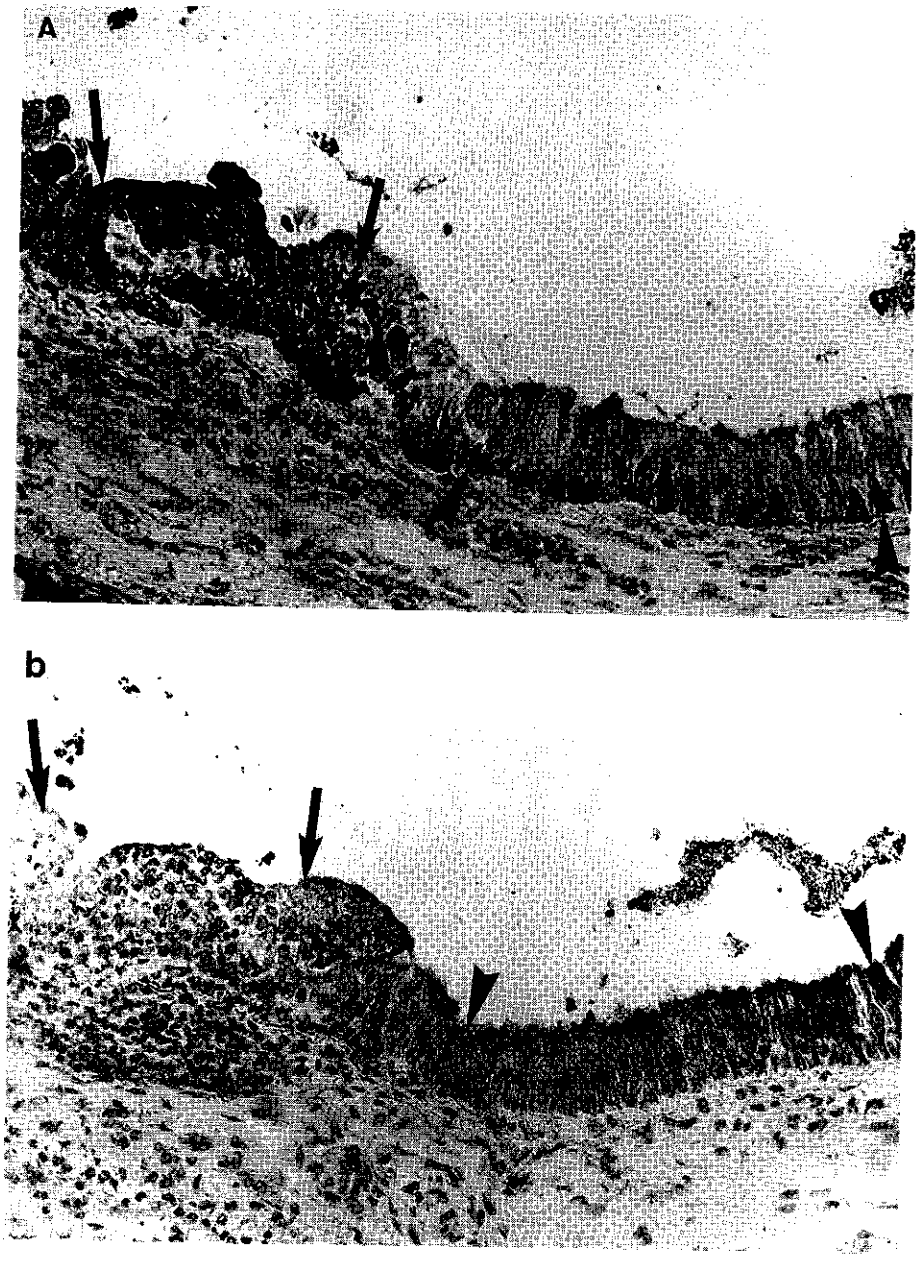

Figure 1: Serial cryostat cross-sections of a trachea of a hamster of group B. **A**: RCK102-positive basal cells (arrowheads) and a region of mucus-producing and ciliated RCK102-positive cells (arrows). **B**: Serial section with RGE53-positive (arrowheads) and RGE53-negative (arrows) non-basal cells. The RGE53 negative cells in **B** are RCK102-positive in **A**. Haematoxylin counter-staining, x 200.

Positive staining by the RKSE60 antibody, detecting HC 10 in squamous metaplastic lesions, was observed in tracheal epithelial cells of hamsters treated with B[a]P (groups B and D), showing early or mild squamous metaplastic lesions, and in some single intermediate cells (Figure 2). Severe or more mature squamous metaplastic lesions were not stained by the RKSE60 antibody. Tracheal epithelial cells of control hamsters were not stained by RKSE60. No effect of β-carotene was observed on the expression of HC 10 as detected by the RKSE60 antibody.

GST-P1 expression

The expression of hamster GST-P1 class isoenzyme P_1, immunocytochemically detected by two different antibodies directed against rat GST isoenzyme 7.7, is shown in Table IV. No clear differences were observed in GST-P_1 isoenzyme staining between the monoclonal and polyclonal antibodies used. Some tracheas of control hamsters (Group A and C) were positively, but very weakly, stained by both antibodies. In tracheas of B[a]P-treated hamsters, staining of GST-P_1 isoenzyme was more pronounced. Although both hyperplastic and metaplastic changes were observed in the tracheal epithelium of B[a]P-treated hamsters, no correlation was observed between any of these lesions and the expression of GST-P_1 isoenzyme (Figure 3). No effect of β-carotene treatment on GST-P_1 isoenzyme expression was observed either (Table IV).

Table IV: Expression of hamster glutathione S-transferase P_1 in tracheal epithelium.

Antibodies	Group A[1]	Group B	Group C	Group D
GST-7.7 (monoclonal)	1.3[2,3]	3.3	0.6[3]	4.0
GST-7.7 (polyclonal)	0.0	3.0	0.8[3]	3.0

[1] For group code see Table I; [2] Labeling was graded 4 if more than 90% of the tracheal epithelium was positive, 3 if between 40% and 90% was positive, 2 if between 10% and 40% was positive, 1 if between 1% and 10% was positive and 0 if less than 1% was positive. Values are given as the mean value of 5 tracheas; [3] staining in control tracheas is very weak, whereas staining in B[a]P-treated tracheas is very clear.

Discussion

The hamster intratracheal intubation model as developed by Saffiotti and co-workers (7) has been frequently used to investigate effects of (pro)vitamin A on B[a]P-induced respiratory tract cancer. Although it is generally accepted that (pro)vitamin A can protect against lung cancer (3,17), the results of various experimental studies have been equivocal and in

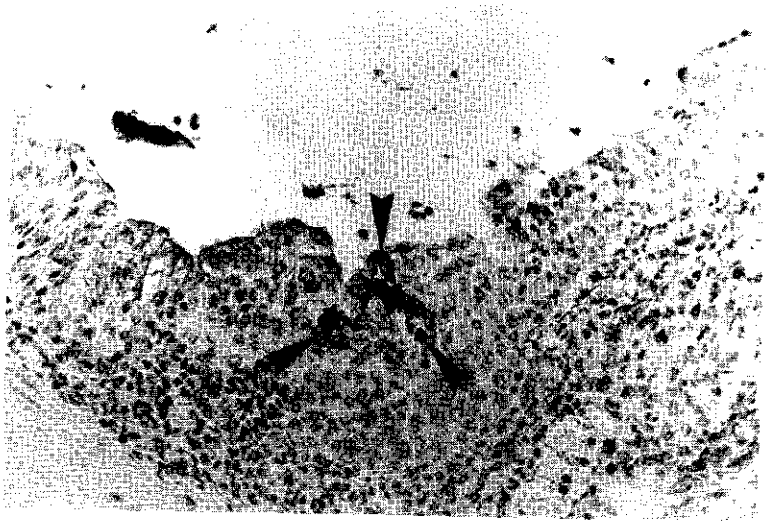

Figure 2: Cryostat cross-section of a trachea of a hamster of group B showing RKSE60-positive single cells (arrowheads). Haematoxylin counter-staining, x 400.

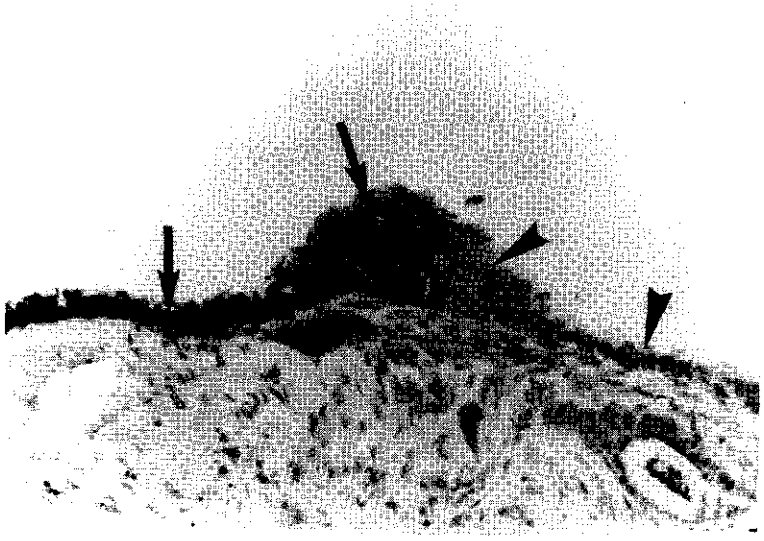

Figure 3: Tracheal cross-section of a trachea of a hamster of group D showing a hyperplastic region partly positively (arrow) and partly negatively (arrowhead) stained by the polyclonal antibody against rat GST-7.7. Haematoxylin counter-staining, x 400.

some cases even contradictory (5,18). The results appeared to be strongly dependent on experimental conditions, such as type of vitamin A used, dose of B[a]P, dose regimen and animal housing conditions. In the present study we were unable to show an inhibitory effect of a high dietary level of β-carotene on B[a]P-induced respiratory tract cancer in hamsters. On the contrary, although not significantly, the results were slightly suggestive of a potentiating effect of β-carotene on the tumour response of the respiratory tract epithelium. Furthermore, we were unable to show an effect of β-carotene on the expression of cytokeratins and GST-P_i, both in hamsters treated with B[a]P and in control hamsters. This absence of an effect is in accordance with the finding in a previous study showing that a high dietary level of β-carotene did not affect cell proliferation, DNA-adduct formation and p53 expression in tracheas of B[a]P-treated hamsters (19).

The results observed in the present study on the expression of cytokeratins in tracheal epithelium of hamsters intratracheally treated with B[a]P are largely in agreement with the findings of Rutten *et al.* (10,11) who studied the expression of various cytokeratins in tracheas exposed *in vitro* to cigarette smoke condensate and vitamin A. The expression of cytokeratins in basal cells was not influenced by B[a]P treatment. HC 5 and 8 as detected by the RCK102 antibody, normally occurring in basal cells only, were also expressed in mucous and ciliated cells of tracheas of B[a]P-treated hamsters. This confirms previous *in vitro* findings of Rutten *et al.* (10,11). Furthermore, the expression of HC 10 in early squamous metaplastic tracheal epithelial cells was increased by B[a]P treatment, whereas HC 10 was not detected in more mature metaplastic changes, suggesting that the expression of various cytokeratins changes during the development of squamous metaplastic lesions. The observation that β-carotene did not influence B[a]P-induced morphological changes was confirmed by the results for cytokeratin expression, showing no effect of β-carotene. Several authors have reported that the level of various GST isoenzymes are negatively correlated with the susceptibility of smokers to develop lung cancer (20). Furthermore, it has been shown that the expression of GST-Pi is increased in (pre)neoplastic changes of various organs, including lungs (12,21), rendering the expression of GST isoenzyme Pi a reliable marker for preneoplastic changes (14). In this study, we showed that the immunocytochemically detected expression of GST-P_1 in tracheal epithelium of hamsters intratracheally treated with B[a]P was increased compared with control hamsters. Yamamoto *et al.* (22) showed that the expression of GST-Pi was increased in lung squamous metaplasia of *N*-nitrosobis(2-hydroxypropyl)amine-treated rats but not in hyperplasia. No evidence was found in the present study that the increase in expression of GST-P_1 in hamster tracheal epithelium was associated with any specific lesion. Moore *et al.* (13) also showed an increased expression of GST-Pi in dihydroxy-di-*n*-propylnitrosamine-induced hyperplastic regions in the lungs of hamsters. In accordance with the pathological observations showing that β-carotene did not influence the formation of B[a]P-induced hyper- and metaplastic lesions in the tracheal epithelium, no effect of β-carotene was observed on the expression of GST-P_1.

In conclusion, in this study we were unable to show a significant effect of a high dietary level of β-carotene on B[a]P-induced respiratory tract cancer in hamsters. Furhermore, β-carotene did not influence the expression of various cytokeratins and hamster GST-P_1. The expression of various cytokeratins and GST-P_1 immunocytochemically detected in hamster tracheal epithelial cells was clearly changed by B[a]P treatment.

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Chapter 10

Summary and concluding remarks

Summary

Respiratory tract cancer is the leading cause of death by cancer in 'Western' countries. The greater part of lung cancers are caused by smoking. Furthermore, environmental air pollution and occupational exposure contribute to the high incidence of lung cancer. Because it seems to be an almost impossible task to eliminate exposure of man to all these factors, considerable effort has been focused on identifying naturally occurring or synthetic compounds which can prevent the formation of respiratory tract cancer. In this regard, (pro)vitamin A (vitamin A and β-carotene) have been shown very promising. In a large number of epidemiological and experimental studies it has been shown that (pro)-vitamin A inhibits the formation of respiratory tract cancer. However, the results of these studies are not always consistent and some studies even showed that (pro)vitamin A increases the incidence of lung cancer. Although the effect of (pro)vitamin A on the formation of respiratory tract cancer has been studied extensively, the mechanisms by which (pro)vitamin A influences the process of respiratory tract carcinogenesis are still not fully understood. In the studies described in this thesis, using both an *in vitro* and an *in vivo* approach, the effects of vitamin A and β-carotene on various stages of the process of chemically-induced respiratory tract carcinogenesis were investigated (Figure 1). The emphasis was on the effects of vitamin A and β-carotene on benzo[a]pyrene (B[a]P)-induced DNA-adduct formation, DNA-repair activities, cell proliferation and histomorphological changes in the hamster respiratory tract epithelium. Furthermore, the relationships between DNA-adduct formation, DNA-repair activities, cell proliferation and the expression of the tumour suppressor gene p53 were investigated.

In vitro studies

In the first *in vitro* experiments, the formation and repair of B[a]P-DNA adducts in hamster and rat tracheal epithelial cells was studied (Chapters 3 and 4). It was shown that *in vitro* the main DNA adduct formed in hamster tracheal epithelial cells was the *trans*-addition product of deoxyguanosine and (+)-*anti*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE-dG). This is the same DNA adduct as formed *in vivo* in tracheal epithelial cells of hamsters intratracheally treated with B[a]P. Furthermore, it is the same adduct as has been frequently observed in human respiratory tract cells. In rat

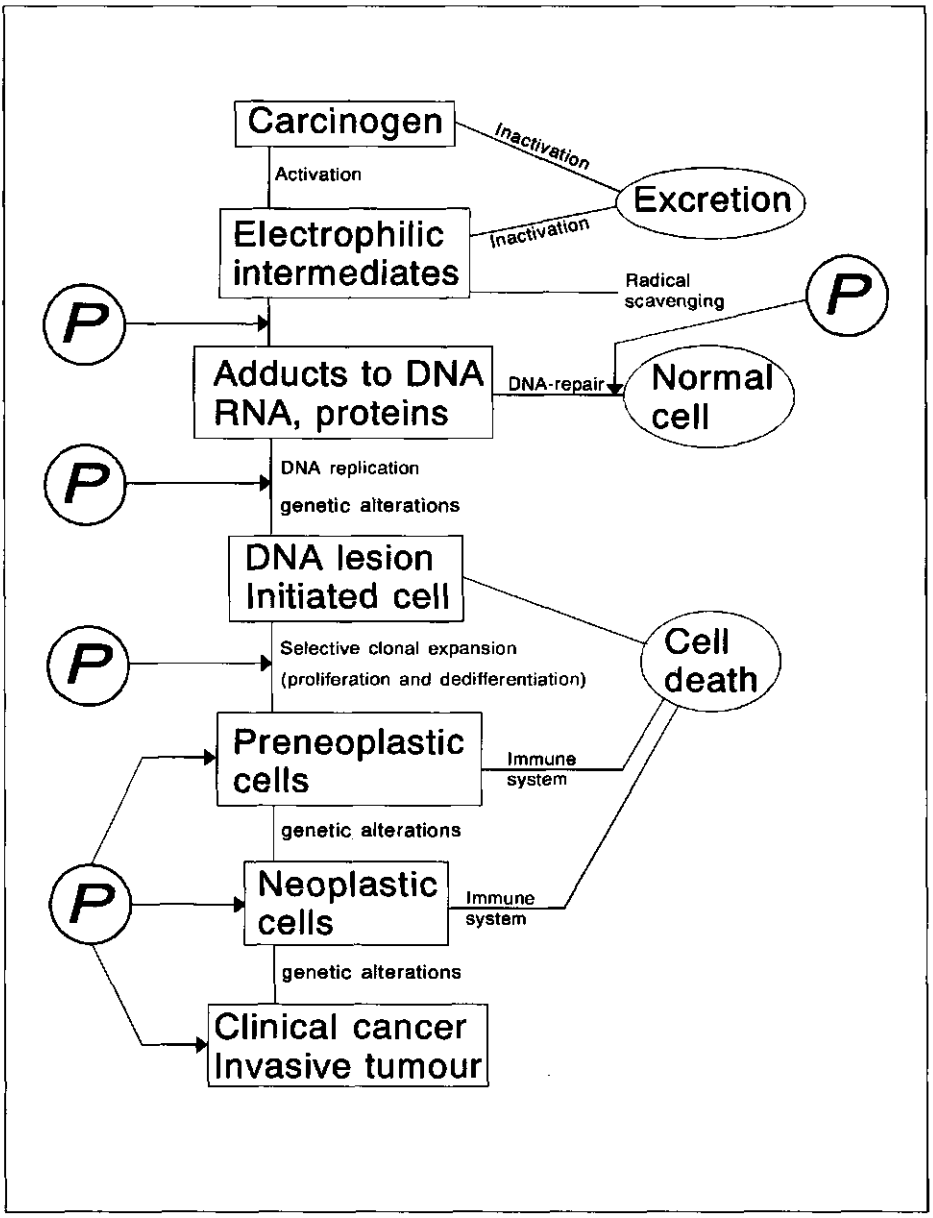


Figure 1: Schematic presentation of the process of chemically induced carcinogenesis including the possible sites of action of (pro)vitamin A (vitamin A and β-carotene) influencing this process. Ⓟ indicates the various action sites of (pro)vitamin A investigated in this thesis.

tracheal epithelial cells two major DNA adducts were found *in vitro*: the BPDE-dG adduct and an adduct that is probably derived from interaction of *syn*-BPDE and deoxyadeno- s-ine. Both the formation of B[a]P-DNA adducts and the B[a]P-induced DNA-repair activities in hamster tracheal epithelial cells were time- and concentration-dependent. In rat tracheal epithelial cells, the formation of B[a]P-DNA adducts was 10 times lower than in hamster tracheas. Furthermore, unlike in hamster tracheal epithelial cells, B[a]P did not induce DNA-repair activities in rat tracheal epithelial cells. In the studies described in Chapter 5, the effect of vitamin A and β-carotene on the formation and repair of B[a]P-DNA adducts in hamster tracheal epithelial cells was investigated. It was shown that both vitamin A and β-carotene slightly inhibited the formation of B[a]P-DNA adducts. In addition, vitamin A and β-carotene increased B[a]P-induced DNA-repair activities. This suggests that the observed decrease in B[a]P-DNA adducts is a positive effect of vitamin A and β-carotene, probably also partly caused by an increase in DNA-repair activities. The effect of vitamin A on DNA-adduct formation and DNA-repair activities depended on the concentration of B[a]P versus the concentration of vitamin A. At a low B[a]P concentration relative to the concentration of vitamin A the formation of B[a]P-DNA adducts was inhibited by vitamin A, whereas at a relatively high concentration of B[a]P the formation of DNA adducts was enhanced by vitamin A.

The role of B[a]P and vitamin A in cell proliferation in hamster tracheal epithelium in organ culture is described in Chapter 6. It was shown that the effects of B[a]P and vita-min A on cell proliferation strongly depended on the culture medium used; in tracheas cultured in Ham's F12 medium cell proliferation was decreased by B[a]P treatment compared to control tracheas, while cell proliferation in tracheas treated with vitamin A in combination with B[a]P was increased compared to tracheas treated with B[a]P alone. In tracheas cultured in CMRL-1066 medium, the effects of B[a]P and vitamin A on cell proliferation were opposite to those observed in tracheas cultured in Ham's F12 medium: cell proliferation in tracheas cultured in CMRL-1066 medium and treated with B[a]P was increased compared to control tracheas, while vitamin A decreased B[a]P-induced cell proliferation. To explain these opposite effects of B[a]P and vitamin A on cell prolifer-ation, various medium components and growth factors were investigated. The concen-tration of $CaCl_2.2H_2O$ revealed to be the most important factor: supplementation of $CaCl_2.2H_2O$ to the Ham's F12 culture medium mimicted the effects of B[a]P and vitamin A on cell proliferation in CMRL-1066 medium. These results clearly indicate that Ca^{2+} is an important regulator of proliferation of hamster tracheal epithelial cells. Furthermore, the results of these experiments showed that the level of B[a]P-DNA adducts was inversely related to cell proliferation in tracheas cultured in Ham's F12 medium. Although these results suggest that the tumour suppressor gene p53 might be involved by inhibiting cell proliferation as a consequence of DNA damage, we were unable to show a direct relationship between the level of B[a]P-DNA adducts, cell proliferation and expression of the p53 tumour suppressor protein in hamster tracheal epithelium in organ culture.

In vivo studies

The most widely applied *in vivo* model to study the aetiology and pathogenesis of respiratory tract cancer in experimental animals is based on repeated intratracheal instillations of a saline suspension of fine crystalline B[a]P particles attached to ferric oxide as a carrier. Various aspects of this method are discussed in Chapter 2, showing that the dose of B[a]P and the size of the B[a]P particles are the most important variables influencing the tumour response. In a first *in vivo* experiment into the effect of vitamin A and β-carotene on B[a]P-induced (pre)neoplastic changes in the respiratory tract of hamsters, the response of the respiratory tract epithelium was too low. This might be due to an insuficiently high B[a]P dose, possibly in combination with a relatively insensitive strain of hamsters used. The low response hampered studying potential effects of vitamin A or β-carotene on the (pre)neoplastic response (Chapter 7). An interesting observation in this experiment was an exceptionally low mortality of hamsters fed a high-β-carotene diet. Although we were unable to establish the exact cause of death of hamsters not receiving β-carotene, the most conspicuous difference between hamsters in the high-β-carotene group and hamsters in other groups was a decrease in lipid peroxidation in the livers of hamsters in the former group. Probably, this effect was not only due to the high concentration of β-carotene in the diet, but was also related to a high dietary level of α-tocopherol and ascorbyl palmitate (also present in the β-carotene beads and used to protect β-carotene from oxidation). To obtain a higher tumour response, hamsters were treated in a second experiment with a higher total dose of B[a]P (Chapter 8 and 9). In this study, a clear relationship appeared to exist between the extent of B[a]P-DNA adduct formation, the induction of cell proliferation and the immunocytochemically detected expression of the p53 protein in hamster tracheal epithelial cells. However, in this experiment the formation of B[a]P-DNA adducts was not found to be affected by a high dietary level of β-carotene, probably due to the high B[a]P dose. Furthermore, β-carotene did not affect B[a]P-induced cell proliferation and expression of the p53 protein in tracheal epithelial cells. Chapter 9 describes the histomorphological aspects of this hamster study, using conventional histopathology and immunohistochemical techniques for the detection of various cytokeratins and glutathione *S*-transferase (GST)-isoenzyme Pi. From this study, it appeared that B[a]P influenced both the expression of cytokeratins and the expression of the GST-isoenzym Pi. However, in accordance with the results described in Chapter 8, β-carotene did not inhibit B[a]P-induced lesions in the respiratory tract epithelium of hamsters.

Concluding remarks

Finally, the studies described in this thesis allow the following conclusions:

- *In vitro,* vitamin A and β-carotene decrease slightly but consistently the formation of B[a]P-DNA adducts, probably due to an increase in DNA-repair activities. The effect of

vitamin A on the formation of B[a]P-DNA adducts depends on the concentration of B[a]P versus the concentration of vitamin A.

- The effects of vitamin A and B[a]P on cell proliferation in hamster tracheal epithelial cells in organ culture strongly depend on the tissue-culture medium used, in particular on the concentration of Ca^{2+} in the medium. The effects of B[a]P and vitamin A on cell proliferation observed in tracheas cultured in CMRL-1066 medium are similar to the effects generally observed *in vivo*.
- The hamster tracheal organ culture model is very suitable to study the B[a]P-induced formation of DNA adducts and DNA-repair activities. Both the formation and repair of B[a]P-DNA adducts is dose and time dependent. Furthermore, the main adduct formed *in vitro* is similar to the adduct formed *in vivo* after intratracheal instillation of B[a]P, and moreover, this adduct is frequently observed in man.
- A high dietary dose of β-carotene, possibly in combination with a high level of α-tocopherol and ascorbyl palmitate, strongly increases the survival of hamsters.
- In tracheal epithelial cells of hamsters treated intratracheally with B[a]P, a relationship between the level of B[a]P-DNA adducts, cell proliferation and p53 expression is observed.
- The effect of vitamin A on B[a]P-induced DNA-adduct formation and cell proliferation, as observed in the *in vitro* experiments, was not found in *in vivo* experiments, probably due to the high B[a]P dose applied.
- β-carotene did not affect the formation of (pre)neoplastic changes in the respiratory tract epithelium of hamsters intratracheally treated with B[a]P as evaluated by conventional histopathology, cytokeratin expression, and glutathione *S*-transferase isoenzyme Pi expression.
- Although intratracheal instillation of B[a]P to Syrian golden hamsters is one of the most widely applied models to study respiratory tract cancer in experimental animals, the tumour response is difficult to control due to a large number of variables affecting the response. The most important variables influencing the tumour response are the dose of B[a]P and the size of the B[a]P particles.

In conclusion, although the *in vitro* experiments described in this thesis show that vitamin A and β-carotene may influence the process of respiratory tract carcinogenesis, *in vivo* it was not possible to show a modulating effect of vitamin A and β-carotene on B[a]P-induced respiratory tract cancer in hamsters. To explain the inconsistencies in the effect of vitamin A and β-carotene on respiratory tract cancer, further in-depth research should be focused on the molecular mechanisms underlying this effect. The concentration of vitamin A and β-carotene, in particular the concentration of the active metabolite retinoic acid, in target cells should be measured in relation to the action of these molecules on the genomic level.

Hoofdstuk 11

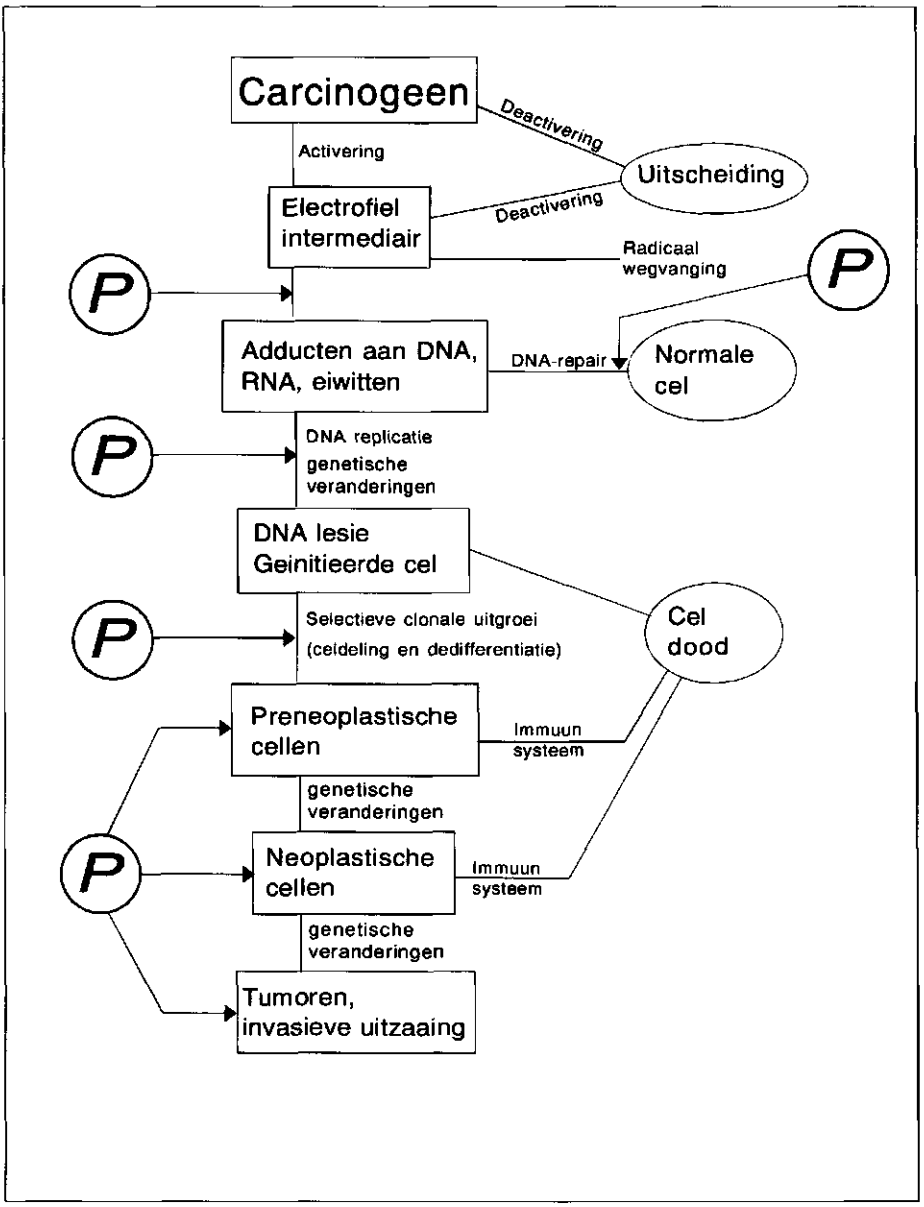
Samenvatting en slotbeschouwing

Samenvatting

Longkanker is de belangrijkste oorzaak van kankersterfte in westerse landen. De belangrijkste oorzaak van longkanker is roken. Verder dragen ook de luchtvervuiling en de bedrijfsmatige blootstelling aan kankerverwekkende stoffen bij aan de hoge incidentie van longkanker. Daar het bijna onmogelijk lijkt om blootstelling van mensen aan deze factoren te voorkomen, wordt veel onderzoek verricht naar het effect van natuurlijke en synthetische verbindingen die het ontstaan van longkanker kunnen tegengaan. Veelbelovende verbindingen wat dit betreft zijn vitamine A en β-caroteen [(pro)vitamine A]. In een groot aantal epidemiologische en dierexperimentele studies is namelijk aangetoond dat (pro)vitamine A het ontstaan van longkanker tegengaat. De resultaten van verschillende studies duiden echter niet altijd op een beschermend effect van (pro)vitamine A; in sommige studies is zelfs aangetoond dat (pro)vitamine A het ontstaan van longkanker bevordert. Hoewel er al veel onderzoek is verricht naar het effect van (pro)vitamine A op het ontstaan van longkanker, is het mechanisme waarop de werking van (pro)vitamine berust nog niet helemaal duidelijk. Bij het onderzoek dat in dit proefschrift wordt beschreven, werd, gebruik makend van zowel *in vivo* en *in vitro* onderzoeksmodellen, het effect van vitamine A en β-caroteen op de verschillende stadia van het longkankerproces onderzocht (Figuur 1). Hierbij lag de nadruk op het effect van vitamine A en β-caroteen op de binding van de kankerverwekkende stof benzo(a)pyreen (B[a]P) aan DNA (vorming van B[a]P-DNA adducten), het verwijderen van deze adducten (DNA-herstel), celdelingsactiviteit en histomorphologische veranderingen van het luchtwegepitheel van hamsters. Verder werd de relatie tussen de vorming van B[a]P-DNA adducten, DNA-herstel, celdeling en de expressie van het tumor-suppressor gen p53 onderzocht.

In vitro experimenten

Allereerst werd *in vitro* de vorming en de repair van B[a]P-DNA adducten in hamster- en rattetrachea-epitheelcellen bestudeerd (Hoofdstuk 3 en 4). Het belangrijkste B[a]P-DNA adduct dat *in vitro* werd gevormd, was het *trans*-additie produkt van deoxyguanosine (dG) en (+)-*anti*-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyreen (BPDE). Dit is hetzelfde adduct als hetgene dat *in vivo* wordt gevormd in trachea-epitheelcellen van hamsters na intratracheale toediening van B[a]P. Bovendien wordt dit adduct ook vaak gevonden in luchtweg-epitheelcellen van mensen. In rattetrachea-epitheelcellen werden



Figuur 1: Schematische weergave van het chemisch-carcinogenese proces, waarbij de mogelijke aangrijpingspunten van (pro)vitamine A zijn aangegeven. Ⓟ geeft de verschillende aangrijpingspunten van (pro)vitamine A aan die in dit proefschrift zijn onderzocht.

twee hoofdadducten gevonden; het BPDE-dG adduct en een adduct dat waarschijnlijk ontstond uit *syn*-BPDE en deoxyadenosine. In hamstertrachea-epitheelcellen bleek dat zowel de vorming als het herstel van B[a]P-DNA adducten afhankelijk was van de blootstellingstijd en de blootstellingsconcentratie. In hamstertrachea-epitheelcellen werden 10 keer meer adducten gevormd dan in rattetrachea-epitheelcellen. Bovendien werd er in rattetrachea-epitheelcellen door B[a]P behandeling anders dan in hamstertrachea-epitheelcellen geen DNA-repair geïnduceerd. In Hoofdstuk 5 worden studies beschreven waarin het effect van vitamine A en β-caroteen op de vorming en het herstel van B[a]P-DNA adducten werd onderzocht. Uit deze studies bleek dat vitamine A en β-caroteen de vorming van B[a]P-DNA aducten remden. Tegelijkertijd verhoogden vitamine A en β-caroteen het herstel van B[a]P-DNA adducten. Deze resultaten wijzen erop dat de waargenomen daling in de hoeveelheid B[a]P-DNA adducten een positief effect zou kunnen zijn van vitamine A en β-caroteen dat verklaard kan worden door een verhoging van de herstelcapaciteit van B[a]P-DNA adducten. Het effect van vitamine A op de vorming van B[a]P-DNA adducten was afhankelijk van de concentratie B[a]P ten opzichte van de concentratie vitamine A. Bij een relatief lage concentratie B[a]P werd een beschermend effect van vitamine A gevonden maar bij een hoge concentratie B[a]P ten opzichte van de concentratie vitamine A werd gevonden dat vitamine A de vorming van B[a]P-DNA adducten juist bevorderde.

In Hoofdstuk 6 wordt het effect van B[a]P en vitamine A op de delingsactiviteit van hamstertrachea-epitheelcellen beschreven. Het bleek dat het effect sterk afhankelijk was van het gebruikte weefselkweekmedium; in tracheas gekweekt in Ham's F12 medium en behandeld met B[a]P werd de celdeling geremd ten opzichte van de celdeling waargenomen bij controle tracheas; vitamine A verhoogde de door B[a]P geremde celdeling. In tracheas gekweekt in CMRL-1066 weefselkweekmedium bleken de effecten van B[a]P en vitamine A op celdelingsactiviteit precies tegenovergesteld te zijn aan die gevonden in tracheas gekweekt in Ham's F12 medium; B[a]P verhoogde de celdeling en vitamine A remde de door B[a]P-geïnduceerde celdeling. Om dit verschil in celdelingsactiviteit te kunnen verklaren werd het effect van verschillende verbindingen in het weefselkweekmedium en van groeifactoren op de door B[a]P en vitamine A geïnduceerde celdelingsactiviteit onderzocht. Hieruit bleek dat de concentratie van $CaCl_2.2H_2O$ in het weefselkweekmedium een belangrijke rol speelde; door verhoging van de concentratie $CaCl_2.2H_2O$ in het Ham's F12 medium werden dezelfde effecten van B[a]P en vitamine A op celdeling gevonden in tracheas gekweekt in het Ham's F12 medium als in tracheas gekweekt in het CMRL-1066 medium. Deze resultaten tonen aan dat de Ca^{2+} concentratie een belangrijke rol speelt bij de regulatie van celdeling in hamstertrachea-epitheel. Verder blijkt uit de resultaten van bovenbeschreven experimenten dat in hamstertrachea epitheel gekweekt in Ham's F12 medium de hoeveelheid B[a]P-DNA adducten omgekeerd evenredig is met de mate van celdeling. Hoewel deze resultaten er op wijzen dat het p53 tumor-suppressor gen hierbij betrokken zou kunnen zijn door de celdeling te remmen als gevolg van DNA-

schade, waren we niet in staat om een direct verband tussen de hoeveelheid B[a]P-DNA adducten, celdeling en de expressie van het p53 tumor-suppressor gen aan te tonen in hamstertrachea-epitheelcellen in orgaan-cultuur.

In vivo experimenten

Bij een van de meest toegepaste modellen voor het bestuderen van ademhalingsweg kanker worden hamsters een aantal maal intratracheaal blootgesteld aan een suspensie van B[a]P deeltjes en ijzeroxide deeltjes in fysiologisch zout. In Hoofdstuk 2 worden de voor- en nadelen van dit model uitvoerig beschreven. In dit hoofdstuk wordt aangetoond dat de tumorrespons sterk afhankelijk is van de B[a]P dosis en van de grootte van de B[a]P deeltjes. In een eerste *in vivo* experiment, met als doel bestudering van het effect van vitamine A en β-caroteen op door B[a]P-veroorzaakte (pre)neoplastische veranderingen van de ademhalingsweg bij hamsters, bleek de respons van het ademhalingswegepitheel erg laag te zijn. Hierdoor was het niet mogelijk een uitspraak te doen over het effect van vitamine A en β-caroteen op de deze respons (Hoofdstuk 7). De geringe respons zou veroorzaakt kunnen zijn door een te lage dosis B[a]P en mogelijk waren de hamsters ook ongevoeliger voor de behandeling. In dit experiment werd wel een opmerkelijke lage sterfte waargenomen bij hamsters die gevoerd werden met een dieet met een hoog gehalte aan β-caroteen. Hoewel het moeilijk was om de doods-oorzaak vast te stellen van de hamsters die geen β-caroteen hadden gekregen, bleek er een groot verschil te bestaan in lipid-peroxidatie in de levers van hamsters gevoerd met het β-caroteen dieet en hamsters in andere groepen. Waarschijnlijk is dit effect niet alleen toe te schrijven aan het hoge gehalte aan β-caroteen in het voer, maar ook aan een hoog gehalte aan α-tocopherol en ascorbyl palmitate (toegevoegd aan de β-caroteen-korrels om oxidatie van het β-caroteen tegen te gaan). Om verzekerd te zijn van een hogere tumor-respons, werden hamsters in een tweede experiment blootgesteld aan een hogere dosis B[a]P (Hoofdstuk 8 en 9). In deze studie werd een heel duidelijke relatie aangetoond tussen de hoeveelheid B[a]P-DNA adducten, celdeling en de immunocytochemisch waar- genomen expressie van het p53 gen in hamstertrachea-epitheelcellen. Er werd echter geen effect waargenomen van een hoog gehalte β-caroteen in het voer op de vorming van B[a]P-DNA adducten, mogelijk als gevolg van de hoge dosis B[a]P. Verder werd er ook geen effect waargenomen van β-caroteen op de door B[a]P-geïnduceerde celdelingsacti- viteit en op de expressie van het p53 tumor-suppressor gen in trachea-epitheelcellen. In Hoofdstuk 9 worden de histomorfologische aspecten van deze studie beschreven waarbij gebruik wordt gemaakt van gewone pathologie en van immunohistochemische technieken voor de detectie van verschillende cytokeratines en van het glutathion S-transferase isoenzym Pi. In deze studie werd een duidelijk effect van B[a]P gevonden op de expressie van zowel cytokeratines als glutathion S-transferase isoenzym Pi. Er werden echter, in overeenstemming met de resultaten beschreven in Hoofdstuk 8, geen opmerkelijke effecten van β-caroteen waargenomen op de door B[a]P-geïnduceerde lesies in het luchtwegepitheel van hamsters.

Slotbeschouwing

De experimenten beschreven in dit proefschrift leiden tot de volgende conclusies:

- *In vitro* wordt de vorming van B[a]P-DNA adducten in hamstertrachea-epitheelcellen zwak, maar reproduceerbaar, geremd door zowel vitamin A als β-caroteen. Waarschijnlijk wordt dit mede veroorzaakt door een verhoging in DNA-repair capaciteit door vitamine A en β-caroteen. Het effect van vitamine A en β-caroteen op de vorming en het herstel van B[a]P-DNA adducten is afhankelijk van de concentratie B[a]P ten opzichte van de concentratie vitamine A.
- Het effect van B[a]P en vitamine A op de delingsactiviteit van hamstertrachea-epitheelcellen is sterk afhankelijk van het gebruikte weefselkweekmedium; in het bijzonder van de Ca^{2+} concentratie in het medium. De effecten van B[a]P en vitamine A op de deling van hamstertrachea-epitheelcellen in tracheas gekweekt in CMRL-1066 medium zijn vergelijkbaar met de effecten zoals die in het algemeen *in vivo* worden gevonden.
- Het hamstertrachea-orgaankweekmodel is geschikt voor het bestuderen van de vorming en het herstel van B[a]P-DNA adducten. Zowel de vorming en het herstel van B[a]P-DNA adducten zijn afhankelijk van de B[a]P concentratie en van de blootstellingstijd aan B[a]P. Het belangrijkste adduct dat wordt gevormd is hetzelfde als het adduct dat *in vivo* wordt gevormd na intratracheale toediening van B[a]P. Bovendien wordt dit adduct vaak aangetoond in luchtwegepitheelcellen van de mens.
- Een hoog gehalte aan β-caroteen in het voer, waarschijnlijk in combinatie met een hoog gehalte aan α-tocopherol en ascorbyl palmitaat, verlengt de levensduur van hamsters.
- In trachea-epitheelcellen van hamsters die intratracheaal waren blootgesteld aan B[a]P, werd een duidelijk relatie gevonden tussen de hoeveelheid B[a]P-DNA adducten, celdeling en de expressie van het p53 eiwit.
- Het effect van vitamine A en β-caroteen op de vorming van B[a]P-DNA adducten en celdeling, zoals gevonden in de *in vitro* experimenten, werd niet gevonden in de *in vivo* experimenten. Mogelijk is dit het gevolg van de (te) hoge dosis B[a]P.
- De vorming van B[a]P-geïnduceerde (pre)neoplastische veranderingen, gekarakteriseerd door middel van histopathologie, cytokeratine-expressie en de expressie van het glutathion S-transferase isoenzym Pi, werd niet beïnvloed door β-caroteen.
- Hoewel het model waarbij hamsters intratracheaal worden blootgesteld aan B[a]P een van de meest gebruikte modellen is om ademhalingswegkanker in dieren te bestuderen is de tumorrespons moeilijk te controleren omdat er veel factoren zijn die de reposn beïnvloeden. De belangrijkste factoren die de tumorrespons bepalen zijn de B[a]P dosis en de grootte van de B[a]P deeltjes.

Hoewel de *in vitro* experimenten die in dit proefschrift zijn beschreven aantonen dat vitamine A en β-caroteen het chemisch-geïnduceerde carcinogenese proces in de ademhalingsweg kunnen beïnvloeden, was het *in vivo* niet mogelijk een effect van vitamine A en β-caroteen op de door B[a]P geïnduceerde tumorrespons aan te tonen. Om de verschil-

len in effect van vitamine A en β-caroteen op ademhalingswegkanker te kunnen verklaren zou toekomstig onderzoek gericht moeten zijn op de moleculaire mechnismen die hieraan ten grondslag liggen. Hierbij zou de concentratie van vitamine A en β-caroteen, en in het bijzonder de concentratie van de actieve metaboliet vitamine A-zuur in de doelcellen moeten worden bepaald in samenhang met het effect van deze moleculen op gen niveau.

Curriculum vitae

André Wolterbeek werd op 20 december 1961 in Hardinxveld-Giessendam geboren. In 1981 werd het VWO diploma behaald aan de Rijksscholengemeenschap te Gorinchem, en in datzelfde jaar werd begonnen met de studie Biologie aan de Landbouwuniversiteit Wageningen. Het doctoraalexamen van de oriëntatie "Cel", met als hoofdvakken Toxicologie (Prof. J.H. Koeman) en Celbiologie (Prof. W.B. van Muiswinkel), werd behaald in 1988. Tijdens de doctoraalfase werden na een afstudeervak Toxicologie (Dr. J.H.M. Temmink) stages gevolgd bij de afdeling Celbiologie van het Nederlands Kankerinstituut te Amsterdam (Dr. J.G. Collard) en bij de divisie Toxicologie van TNO Voeding te Zeist (Dr. A.A.J.J.L. Rutten).

Op 1 juli 1989 trad hij als assistent in opleiding in dienst van de Landbouwuniversiteit Wageningen. Gedurende het onderzoek was hij gedetacheerd bij de divisie Toxicologie van TNO Voeding te Zeist. Tijdens dit onderzoek heeft hij de post-doctorale opleiding toxicologie gevolgd.

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