CYTOKINETICS AND HISTOGENESIS OF CULTURED HAMSTER TRACHEAL EPITHELIUM

Effects of vitamin A and cigarette smoke condensate

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ISBN 90-9002495-6

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen
op gezag van de rector magnificus,
dr. H.C. van der Plas,
in het openbaar te verdedigen
op vrijdag 16 december 1988
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

STELLINGEN

1. Het bifasisch effect van all-trans-retinol op de communicatie tussen cellen verklaart veel van de tegenstrijdige resultaten van experimentele studies met vitamine A; de nodige voorzichtigheid is daarom geboden bij het gebruik van vitamine A in de kankerpreventie.
   [Dit proefschrift; de Vries en van Zandwijk (1988), Tijdschrift Kanker 12, 176-177]

2. De bewering dat alleen slijmhoudende cellen in de trachea een rol spelen bij het onstaan van metaplasie naar plaveiselepitheel is onjuist.
   [Dit proefschrift; McDowell et al. (1984), Virchows Archiv B Cell Pathology 45, 197-219; Sigler et al. (1988), Virchows Archiv B Cell Pathology 55, 47-55]

3. De opvatting dat trilhaarcellen van de trachea zich niet delen is onjuist.
   [Dit proefschrift; Bindreiter et al. (1968), Experimental Cell Research 50, 377-382]

4. Karakteristieke eigenschappen van vitamine A laten zich uitstekend bestuderen in tracheakweken van vitamine A-deficiënte hamsters.
   [Dit proefschrift]

5. Orgaankweken lenen zich beter dan celkweken voor het bestuderen van biologische processen zoals celdifferentiatie, celdeling en het metabolisme van chemische verbindingen.
   [Dit proefschrift]

6. Gezien de voortdurende controverse in de literatuur over de beïnvloeding van diverse typen kanker door β-caroteen dan wel vitamine A verdient het ontwikkelen van een adequaat diermodel hoge prioriteit.
   [Beems (1988), Tijdschrift Kanker 12, 170-173]

7. In het toxicologisch onderzoek verdient optimalisering van bepalingen en testmethoden en standaardisatie tussen laboratoria meer aandacht.
   [Rutten et al. (1987), Archives of Toxicology 61, 27-33]

8. Bij onderzoek naar remming van intercellulaire communicatie als parameter voor tumorpromotie dient de vraag of deze remming de oorzaak dan wel een gevolg is van tumorpromotie voorrang te krijgen.

9. Bij de beoordeling van de toxicologische eigenschappen van ionogene verbindingen wordt onvoldoende rekening gehouden met de invloed van het tegenion op de zuur-base-balans.
   [De Groot et al. (1988), Food and Chemical Toxicology 26, 425-434]

10. Voedseladditieven zijn veiliger dan nutriënten.

11. Celdeling is niet altijd de gelukkigste oplossing voor het cellentekort, celvermeerdering wel.

Stellingen behorend bij het proefschrift "Cytokinetics and histogenesis of cultured hamster tracheal epithelium. Effects of vitamin A and cigarette smoke condensate".

Fons Rutten, 16 december 1988

Voorwoord

Het in dit proefschrift beschreven onderzoek is tot stand gekomen door de inspanning van zeer velen. Mijn dank gaat dan ook uit naar hen die hebben meegeholpen aan de voltooiing van dit proefschrift, en daarmee deze fase aanzienlijk hebben verlicht.

Mijn promotor, prof. dr. J.H. Koeman, wil ik bedanken voor de prettige samenwerking gedurende mijn verblijf bij de vakgroep Toxicologie in Wageningen en voor de hulp bij het tot stand komen van dit proefschrift.

Dr. J.W.G.M. Wilmer, mijn co-promotor dank ik voor de goede begeleiding, de steun bij het schrijven en het in mij gestelde vertrouwen. Beste Jan, als jij me niet zo enthousiast had gemaakt voor onderzoek was ik waarschijnlijk iets heel anders gaan doen. Zonder jouw inzet voor dit onderzoek zou dit proefschrift waarschijnlijk op een veel later tijdstip zijn voltooid.

Dr. V.J. Feron, beste Vic, mijn dank voor de mogelijkheid die je me gaf om het in dit proefschrift beschreven onderzoek binnen de afdeling Biologische Toxicologie uit te voeren. Bovendien dank ik je voor de stimulerende discussies die we met betrekking tot het trachea-epitheel met elkaar mochten hebben.

Dr. R.B. Beems, beste Dolf, je hebt me veel geleerd over de histomorfologie van de ademhalingsweg en over de effecten van vitamine A op trachea-epitheel.

De leden van de Wetenschappelijke Advies Raad Roken en Gezondheid (WARG), en met name dr. T. Vossenaar, ben ik zeer erkentelijk voor de ondervonden steun, de stimulerende gesprekken en voor de waardering voor het in dit proefschrift beschreven onderzoek.

Ing. B.J. Spit, beste Ben, het is verrassend wat jou allemaal opviel tijdens het bekijken van coupes, vooral die bijzonderheden waarvoor ik minder aandacht had. Jouw ideeën over vitamine A en vetbollen hebben mede ten grondslag gelegen aan het onderzoek.

J.P. Bruyntjes, E.G.J. Hendriksen, W.R. Leeman en W.G. Roverts, beste Joost, Ferry, Winfried en Gerard, coupes bekijken is een inspannende bezigheid, maar evenzeer plezierig als de coupes van zeer goede kwaliteit zijn.

Dr. W.M.F. Jongen en L.H.J. de Haan, beste Wim en Laura, veel dank voor alles wat ik geleerd heb over microinjectie, en voor de genoten gastvrijheid.

Dr. ir. S.A.G.F. Angelino en P.S. Peereboom, beste Steven en Piet, het isoleren en opzuiveren van hamster retinol-bindend eiwit lijkt een bijna onmogelijke opgave. Mijn dank voor jullie inzet.

A. van Welie, C. Weymans en M. van de Vaart, beste Fred, Carola en Martin, mijn dank voor alle dia's, foto's, figuren en posters.

J.M. Blom, beste Jasper, je hebt op zeer kundige wijze de vitamine A-deficiënte voeren gemaakt; de resten van de eerste semi-synthetische voeren in de korrelmal zul je waarschijnlijk nog niet vergeten zijn.

D.G. van der Hey, beste Dirk, mijn dank voor de correcties die je aanbracht in de manuscripten en hoofdstukken.

Dr. F.C.S. Ramaekers, beste Frans, de hoofstukken 5 en 6 waren zonder jouw medewerking niet tot stand gekomen. Bovendien veel dank voor het beschikbaar stellen van de monoclonale antilichamen tegen de diverse keratines.

Prof. dr. E.M. McDowelll of the University of Maryland (USA), Department of Pathology. I would like to thank you for encouraging me to write an article on proliferating ciliated cells (Chapter 7) and for helpful discussions during my visit to your department.

R.M.L. Zwijsen, E.P.C. Kilsdonk en A.P.M. Wolterbeek, beste Renate, Liesbeth en André, mijn dank voor jullie pioniersonderzoek gedurende jullie stageperiode.

Voorts dank ik eveneens alle andere collega's voor hun bijdrage aan dit proefschrift.

Tenslotte, dank ik jou Dian voor je steun bij het voltooien van dit proefschrift. Bovendien dank ik je voor de orde die jij hebt geschapen in het literatuursysteem.

Fons Rutten

The investigations reported in this thesis were carried out at the Department of Biological Toxicology, TNO-CIVO Toxicology and Nutrition Institute, Zeist, Netherlands, in collaboration with the Department of Toxicology, Agricultural University Wageningen, Netherlands, and the Department of Pathology, University Hospital Nijmegen, Netherlands. Financial support was given by the Scientific Advisory Committee on Smoking and Health (Dutch Cigarette Industry Foundation), the Ministry of Welfare, Health and Cultural Affairs, and the Netherlands Organization for Applied Scientific Research (TNO).

Contents page

PART I

INTRODUCTION

CHAPTER 1

General introduction

The respiratory system in mammals is designed to transport air from the upper part to the lower part of the system where oxygen and carbon dioxide are exchanged between blood and air. The conducting part of the respiratory system conditiones the air for exchange, traps some of the air-borne particles, and is exposed to many environmental chemical contaminants including complex mixtures of combustible materials. Tobacco smoke is an example of the latter group and its uptake is associated with myocardial infarction, peripheral vascular disease, chronic obstructive lung disease, and cancer of the lung.

The evidence for a causal relationship between tobacco smoking and tracheobronchial cancer emerged first in humans (Doll and Hill 1950; Levin et al. 1950; Mills and Porter 1950; Schrek et al. 1950; Wynder and Graham 1950; Doll and Peto 1976). Sömmering suggested already in 1795 that smoking might be related to cancer (Sömmering 1795). Nowadays lung cancer among men is the predominant cause of mortality in affluent societies (e.g. Europe and USA).

In the beginning of this century many epidemiological studies showed an higher relative risk of cancer for the smoking population than for the non-smoking controls (for a review, see IARC 1986 and WHO 1986). Experimental studies also showed a positive relationship between tobacco smoking and respiratory tract cancer in animals. However, the delivery of smoke to obligatory nose breathers (rodents) and the toxicity of nicotine and carbon monoxide made the human inhalation pattern hard to reproduce. Despite these problems, data on the genotoxic, co-carcinogenic, carcinogenic and tumor-promoting activity of whole smoke and/or cigarette smoke condensate were obtained in several in vivo and in vitro model systems (IARC 1986; WHO 1986).

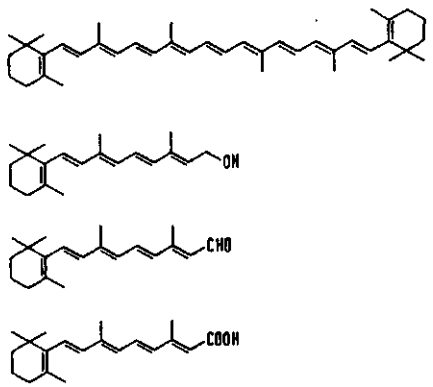
Several epidemiological studies have shown an inverse relationship between intake of retinoids and incidence of lung cancer, and therefore retinoids have become valuable tools in cancer research far beyond their classic roots in the study of vision and nutrition. The property of retinoids to control the cellular proliferation and differentiation have linked these compounds to the problem of malignant transformation (Sporn et al. 1984).

In the next sections aspects of retinoids are discussed in connection with cellular proliferation, differentiation, intercellular communication and intermediate filament expression of respiratory tract epithelium.

Role of retinoids in cancer research

Retinoids, a class of compounds consisting of four isoprenoid units conjoined in a head-to-tail manner, are generally used as a name for naturally occurring compounds with vitamin A activity (Fig. 1) as well as synthetic analogs. Because animals and man are not capable of de novo synthesis of vitamin A, they depend on plants, photosynthetic microorganisms and other animals as sources of vitamin A or provitamin A (carotenoids).

Fig. 1. Structural formulas of vitamin A-active compounds and β-carotene.



β-carotene

all-trans retinol

all-trans retinaldehyde

all-trans retinoic acid

Source: Sporn et al. (1984).

The fat-soluble vitamin A has been found to promote growth, to prevent against xerophthalmia and night blindness, and to be essential for vision and reproduction (Sporn et al. 1984). Further, retinoids play an important role in the control of cellular differentiation and proliferation of epithelial tissue. These cells manifest biochemical, morphological and functional changes in the absence or in the presence of physiological or pharmacological concentrations of retinoids (Wolbach and Howe 1925; Boren et al. 1974; McDowell et al. 1984b; Sporn et al. 1976; Sporn and Roberts 1984; Shapiro 1986).

In the past 20 years, several epidemiological and experimental studies have

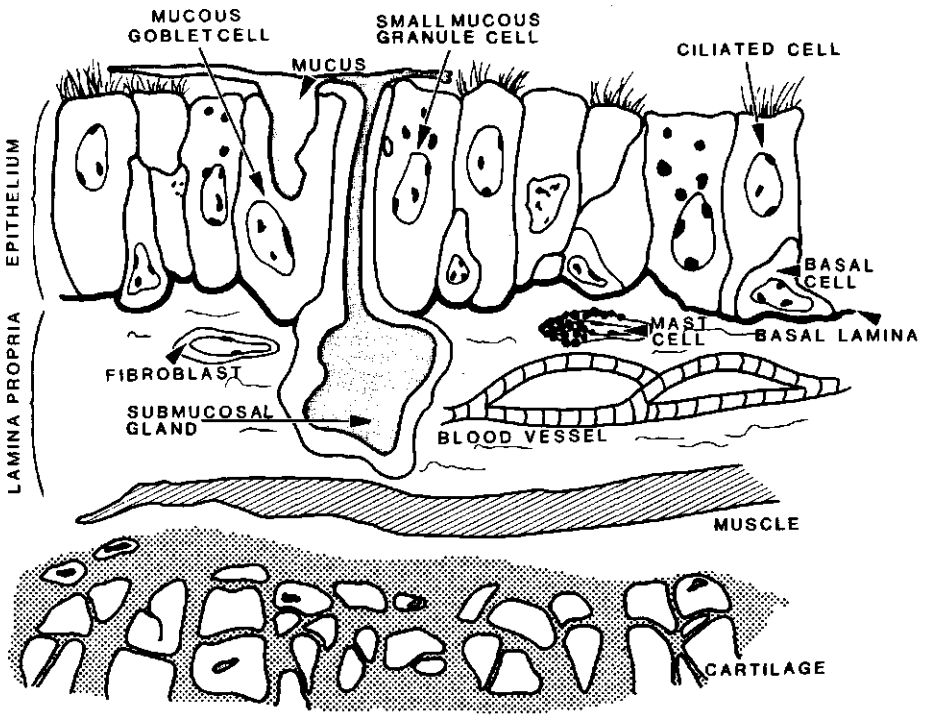
shown an inverse relationship between vitamin A consumption, especially provitamin A from green and yellow vegetables, and the risk of lung cancer (Bjelke 1975; Metlin et al. 1979; Gregor et al. 1980; Wald et al. 1980; Kark et al. 1981; Peto et al. 1981; Shekelle et al. 1981; for a review, see also Moon and Itri 1984). Bjelke (1975) was the first to report epidemiologic data that suggested a protective effect of vitamin A against lung cancer. In another prospective study an inverse relationship was found between the incidence of lung cancer and the intake of carotenoids (Shekelle et al. 1981). These findings support the hypothesis of Peto et al. (1981) who suggested that β-carotene, rather than retinyl esters, have a protective effect on cancer. A role for vitamin A in cancer was already suggested many years ago in the classic study of Wolbach and Howe (1925) that showed histopathological resemblances between the epithelia of vitamin A-deficient organs and neoplastic tissues. Vitamin A has also been found to be essential for maintenance of mucociliary activity in respiratory epithelium. Furthermore, many reports demonstrate that consumption or application of vitamin A and provitamin A protects against (pre)neoplastic lesions and cancer of the respiratory tract (Saffiotti et al. 1967; Port et al. 1974; Nettesheim et al. 1976; Peto et al. 1981). Lasnitzki and Bollag (1982; 1987) and Wille and Chopra (1988) showed that retinoids prevent the development of hyperplasia and metaplasia induced by benzo[a]pyrene or cigarette smoke condensate in rat tracheal organ cultures. In view of these findings, vitamin A is believed to play an important preventive role in the development of cancer. However, some contradictory observations have been reported with respect to the protective effect of vitamin A against respiratory cancer (Smith et al. 1975; Nettesheim and Williams 1976; Beems 1984 and 1986; Moon and Itri 1984). This might be related to differences in: stock diets, retinoid dose level, retinoid metabolism, time-point of carcinogen exposure compared with retinoid administration, and vitamin A storage in target organ(s) and other tissues. In spite of the large number of studies showing more or less clear effects of retinoids, little is known about the mode of action. However, the important finding that vitamin A (retinol and retinoic acid) binds to DNA, mediated by a lipoprotein receptor, explains many of the vitamin A effects in eukaryotic systems (Petkovich et al. 1987; Giguere et al. 1987; Ferrari et al. 1988). This recent finding is a great step forward towards the elucidation of the central questions of vitamin A research: How does vitamin A mediate gene regulation, alter cellular metabolism, and modify phenotype expression? In all three stages involved in the process of chemical

carcinogenesis (initiation, promotion and progression) nuclear DNA is altered. Therefore, interaction between vitamin A and DNA is considered to be important for the prevention of cancer.

Cytokinetics and histogenesis of tracheal epithelium

In Chapter 4 (Figs. 1-8) light micrographs of hamster tracheal epithelium from control and vitamin A-depleted tracheas are shown with detailed histomorphological characteristics of the various cells and tissues. The trachea is made up of three main structures: pseudostratified epithelium, fibroelastic lamina propria, and several C-shaped rings of cartilage (Rhodin 1974).

Fig. 2. Basic organization of the tracheal epithelium.



The lamina propria contains blood vessels, mast cells, fibroblasts, muscle cells, serous glands with excretory ducts and loose fibrous connective tissue (see Fig. 2) (Rhodin 1974). The tracheal epithelial 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 (Fig. 2); and some cell types that are less frequently observed: intermediate cells, brush cells, serous cells, and neuroendocrine cells (Rhodin 1974; Breeze and Wheeldon 1977; McDowell 1982).

Ciliated cells, small mucous granule cells, mucous goblet cells and ciliated cells are columnar cells, approximately 30 μm long and 7 μm wide, that rest on the basal lamina, a network of collagenous fibrils, and reach the tracheal lumen. Basal cells are ovoid cells, approximately 7 μm long and 7 μm wide, that rest on the basal lamina without reaching the luminal surface.

The tracheal epithelial cells are connected with complex interdigitations, desmosomes and gap junctions at the lower lateral cell surface, with tight junctions at the luminal surface. All tracheal epithelial cells are connected with the fibrous basal lamina by hemidesmosomes (Breeze and Wheeldon 1977; Rhodin 1974; McDowell 1982). The main function of the small mucous granule cells, and mucous goblet cells, and of the submucosal glands is secreting a viscous glycoprotein (mucins) mixture onto the luminal surface which covers the epithelium as a blanket. The major function of ciliated cells is moving the secreted mucus layer together with trapped particles towards the pharynx.

To understand the histogenesis of morphologic alterations in respiratory epithelium and its differentiation potential, it is essential to know which cells of the epithelium can divide, and thus are capable of inducing hyperplastic, metaplastic, dysplastic, and (pre)neoplastic states (McDowell 1982; McDowell and Trump 1983). Basal cells and small mucous granule cells are generally considered capable of proliferating and are held responsible for the renewal of cells in the respiratory epithelium. These cell types have been shown to incorporate [methyl-^{3}H]thymidine into the DNA during the S-phase prior to cell division. Furthermore, several studies using colchicine-induced metaphase arrest have shown that basal and small mucous granule cells do proliferate (Schultze and Oehlert 1960; Bindreiter et al. 1968; Harris et al. 1973; Boren and Paradise 1978; Chopra 1983; McDowell et al. 1984a,b).

In vitro or in vivo stimulation by carcinogens, tumor promoters, cigarette smoke condensate or mechanical trauma increases cell proliferation (Gordon and Lane 1977; Chopra and Cooney 1985; Lasnitzki and Bollag 1982 and 1987; Wille

and Chopra 1988), and cigarette smoke condensate and vitamin A deficiency induce well characterized morphological changes both in vivo and in vitro (Lasnitzki 1968; Clamon et al. 1974). In a serum-free, vitamin A-deficient CMRL-1066 medium, foci of squamous metaplasia with or without keratinization develop in the tracheal epithelium. Vitamin A and other biologically active retinoids prevent squamous metaplasia developing and promote the reestablishment of a pseudostratified mucociliary epithelium (Clamon et al. 1974; Clark et al. 1980). These morphological changes controlled by vitamin A in vitro are comparable to those found in vivo (Harris et al. 1972; Port et al. 1974; McDowell et al. 1984a, b; Sigler et al. 1987). The histogenesis and morphogenesis of squamous metaplasia in respiratory epithelium remain prone to controversy despite the great number of recent studies regarding this issue (Sigler et al. 1988). Several studies have suggested that the changes responsible for producing the metaplastic transformation take place at the level of basal cells (Palekar et al. 1968; Wong and Buck 1971; Mossman and Craighead 1975; Lane and Miller 1976; Lane and Gordon 1979; Chopra 1982; Chopra and Cooney 1985; Inayama et al. 1988), whereas several other studies have reported that these lesions derive largely from hyperplastic secretory (mucous) cells (Wang and Ying 1977; Trump et al. 1978; Keenan et al. 1982; McDowell et al. 1984a; Sigler et al. 1988).

Intermediate filament expression patterns

The cytoplasm of all mammalian cells is well organized by an intracellular complex structure of protein filaments which are involved in intracellular transport, intracellular organization of enzymes and cell organelles, exo- and endocytosis, protoplasmic streaming, locomotion, cellular polarity, anchorage, cell division and cell differentiation (for a review, see Franke et al. 1981; Moll et al. 1982; Shay 1986). The cytoskeleton is commonly divided into four fibrillar systems on the basis of their diameter: microtrabecular filaments (2-3 nm), microfilaments (5-7 nm), intermediate filaments (7-11 nm) and microtubules (22-25 nm) (Schliwa and van Blerkom 1981). The group of intermediate filaments is classified into five tissue specific proteins: keratins (epithelial cells), vimentin (mesenchymal cells), desmin (muscle cells), neurofilament proteins (neuronal cells) and glial fibrillary acid protein (astrocytes). The intermediate filaments desmin, vimentin and glial types

normally consist of one type of subunit protein, whereas keratin filaments are a complex family of various polypeptides (Franke et al. 1981; Moll et al. 1982; Ramaekers et al. 1983; Cooper et al. 1985; Fuchs et al. 1986). The cytokeratins are expressed in different epithelia and in different combinations of polypeptides ranging in their isoelectric pH (5 to 8) and molecular weight (40-68 kDa). Cellular differentiation, cell growth, cell type, stage of development and disease influence the expression of keratins (Cooper et al. 1985). Keratins are also characterized on the basis of their immunological properties (Ramaekers et al. 1983; 1987).

Retinoids change the keratin expression patterns in various types of mammalian epithelial cells (Fuchs and Green 1981; Eckert and Green 1984; DeLuca et al. 1985; Huang et al. 1986; Wu and Wu 1986). In human bronchial epithelial cells retinoids inhibit the synthesis of the 48 kDa and 50 kDa keratin proteins and stimulate the synthesis of the 40 kDa and 52-54 kDa keratin proteins (Wu and Wu 1986). Vitamin A-depleted tracheal epithelium expresses the 45, 46.5, 48, 50, 52, 55, 56 and 60 kDa keratin proteins, whereas normal mucociliated epithelium cultured in the presence of retinoic acid does not express these keratins (DeLuca et al. 1985; Huang et al. 1986).

Control of intercellular communication

Communication between cells in tissues and organs is essential for the control of proliferation and differentiation (Loewenstein 1979; Bennet and Spray 1985), and can take place over a long distance by hormones and growth factors, or over a short distance via cell-to-cell channels (e.g. transport of ions, amino acids, small peptides, interferons, nucleotides, carbohydrates and metabolites). These cell-to-cell channels are called "gap junctions" and are characterized by double-membrane plaques of protein-lipid structures in each of the adjacent cell membranes joined at a distance of 2 to 3 nm. Each gap junction contains six subunits leaving a central opening with a diameter of about 2 nm through which small hydrophilic molecules pass in the 200-1500 Da range (Loewenstein 1979; Zampighi and Simon 1985). Conformational changes of the six subunits induced by Ca^{2+} or H^{+} binding are responsible for changes in permeability (Peracchia and Girsch 1985). An example of gap junction-mediated control is the cell-cell communication between embryonic cells (Potter et al. 1966). During embryogenesis both inhibition and stimulation of cell-to-cell

communication regulates compartmentation and the development of germ layers (Warner and Lawrence 1973; Warner et al. 1984).

In the past 10 years, several in vitro methods and model systems have been developed to measure communication between cells in monolayer. Intercellular electric coupling was the first method used to measure coupling between nerve and heart cells (Bennett et al. 1963; Bennett 1966; Heppner and Plonsey 1970; Woodbury and Crill 1970). Kanno and Loewenstein (1964) reported that not only small ions but also a larger hydrophilic molecule, fluorescein, pass through gap junctions. Recently, Lucifer Yellow CH, a polar fluorescent that does not pass through membranes, was used to determine the dye-coupled intercellular communication (Bennett and Spray 1985; Yamasaki 1986). The metabolic cooperation assay is based on the transfer via gap junctions of the phosphorylated metabolite of purine or pyrimidine (e.g. 6-thioguanine) from a wild-type V79 cell to a hypoxanthine-guanine phosphoribosyltransferase-deficient V79 cell (Subak-Sharpe et al. 1969; Yotti et al. 1979; Trosko et al. 1982; 1983). In a cocultivation model system, Jongen et al. (1986 and 1988) found that benzo[a]pyrene metabolites are also transferred via gap junctions from chick embryo hepatocytes to V79 cells. Recently, two new techniques, fluorescence recovery after photobleaching (Wade et al. 1986) and the scrape-loading dye transfer assay (El-Fouly et al. 1987), have been applied to measure intercellular communication.

Dysfunction of the cell-to-cell short-range homeostatic control mechanism has been associated with tumor promotion (Murray and Fitzgerald 1979; Yotti et al. 1979; Trosko et al. 1983; Yamasaki 1986) and teratogenesis (Loch-Caruso and Trosko 1985). Inhibition of intercellular communication and the reduction of formation or maintenance of gap junctions by tumor promoters (e.g. TPA, PCB, DDT, cigarette smoke condensate, phenobarbital, saccharin, butylated hydroxytoluene) have been observed in several studies (e.g. Trosko and Chang 1984). Furthermore, decreased gap junctional communication was observed in neoplastic cells transformed by the oncogenes v-ras and v-src (Atkinson and Sheridan 1985; Azarnia and Loewenstein 1984; Chang et al. 1985), and loss of cell-to-cell communication was found to correlate with the metaplastic potential of cells (Nicolson et al. 1988).

In the past 50 years, several studies have reported effects of retinoids on cell growth and differentiation, and on the genesis of preneoplastic and neoplastic lesions (Sporn et al. 1984). Retinoids mostly promote differentiation, for instance in epithelial cells, but in some cases, for instance

mesodermally derived cells types, retinoids inhibit differentiation (Shapiro 1986). Recently, it was found that retinoic acid (Pitts et al. 1981; Walder and Lutzelschwab 1984; Davidson et al. 1985; Pitts et al. 1986), retinol, retinal, and retinyl acetate (Davidson et al. 1985) at pharmacological concentrations inhibited the gap-junction-mediated communication between animal cells, whereas some synthetic retinoids showed only minor effects (Pitts et al. 1986). However, in other studies no inhibitory effects of retinoic acid in metabolic cooperation assays were observed (Morel-Chaney et al. 1986; Trosko et al. 1981).

Scope of this thesis

The studies reported in this thesis primarily deal with the influence of vitamin A (all-trans retinol) and cigarette smoke condensate on cellular proliferation, and differentiation and intercellular communication in tracheal epithelium. The experiments were carried out with Syrian Golden hamster tracheas and primary tracheal epithelial cells maintained in serum-free, hormone-supplemented media. All-trans retinol was used to regulate cellular proliferation and differentiation, whereas cigarette smoke condensate was used to induce alterations.

This thesis is divided into three parts: General introduction (Chapters 1 and 2), Experiments (Chapters 3-8) and Conclusion (Chapters 9 and 10).

In Chapters 1 and 2 of the current knowledge of retinoids, tracheal epithelium, intermediate filaments, intercellular communication and in vitro model systems with tracheal epithelium are summarized.

In Chapters 3 and 4 the effects of all-trans retinol and cigarette smoke condensate on vitamin A-depleted hamster tracheal epithelium in organ culture are discussed with emphasis on cellular proliferation and differentiation.

Chapter 5 deals primarily with the role of proliferating basal cells in untreated tracheal epithelium, and in vitamin A-depleted tracheal epithelium treated with a physiological all-trans retinol concentration. The effects on tracheal epithelial cells were observed by means of monoclonal antibodies against various keratins. The information obtained in this Chapter was used to study the effects of cigarette smoke condensate and vitamin A depletion on intermediate filament expression patterns in tracheal epithelium (Chapter 6).

In Chapter 7 experimental evidence is given that ciliated tracheal

epithelial cells can divide in hamster tracheal organ cultures treated with all-trans retinol or cigarette smoke condensate.

Chapter 8 deals with the role of vitamin A (all-trans retinol and retinoic acid) and cigarette smoke condensate on dye-coupled intercellular communication between primary hamster tracheal epithelial cells.

In Chapters 9 and 10 a summary of the results is given with concluding remarks in both English and Dutch.

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CHAPTER 2

In vitro model systems with tracheal epithelium

Introduction

In the past few decades several in vitro techniques for culturing respiratory tract epithelium have been applied to study the effect of respiratory infections (viruses, mycoplasma and bacteria), environmental chemicals (such as ozone and nitrogen dioxide) or occupational chemicals (polycyclic aromatic hydrocarbons, such as benzo[a]pyrene), mucus synthesis and ciliary activity, and newly synthesized retinoids (for a review, see Lane 1978; Scott et al. 1986; Schiff 1986). Four model systems for in vitro research with respiratory epithelium have been developed: monolayer cell cultures, feeder layer cell cultures, explant or segment cultures, and organ cultures (Table 1).

Primary tracheal epithelial cells, obtained after enzymatic dissociation, can be cultured on a feeder layer of fibroblasts (Gray et al. 1983), or on a coating of collagen, fibronectin and/or bovine serum albumin (Lechner et al. 1981), and collagen gels (Lee et al. 1984). Explant cultures obtained by outgrowth of primary explants or tissue segments (Kennedy and Ranyard 1983) have

Table 1. Culture systems available for respiratory tract epithelium.

	Cell culture	Explant culture	Organ culture
Definition	Culture of dissociated cells	Cell outgrowth from tissue segments	Culture of intact organ segments
Advantage	Increased number of cells Individual cell types Microinjection of cells	Increased number of cells No enzyme exposure Relatively easy	Cell interactions Good morphology No enzyme exposure Close to in vivo
Limitation	Enzyme exposure Variable morphology Functional changes Artifical substratum	Variable morphology Variable cell growth and differentiation Nonepithelial cells	Supply of nutrient and oxygen No phase contrast microscopy Nonepithelial cells

Modified from Scott et al. 1986

several disadvantages such as variability of initial outgrowth, influence of the size and shape of the segment on cellular proliferation and differentiation, and contamination with nonepithelial cells (e.g. fibroblasts and endothelial cells). However, this system is advantageous in that it is relatively easy to initiate and that a high percentage of epithelial cells can be obtained.

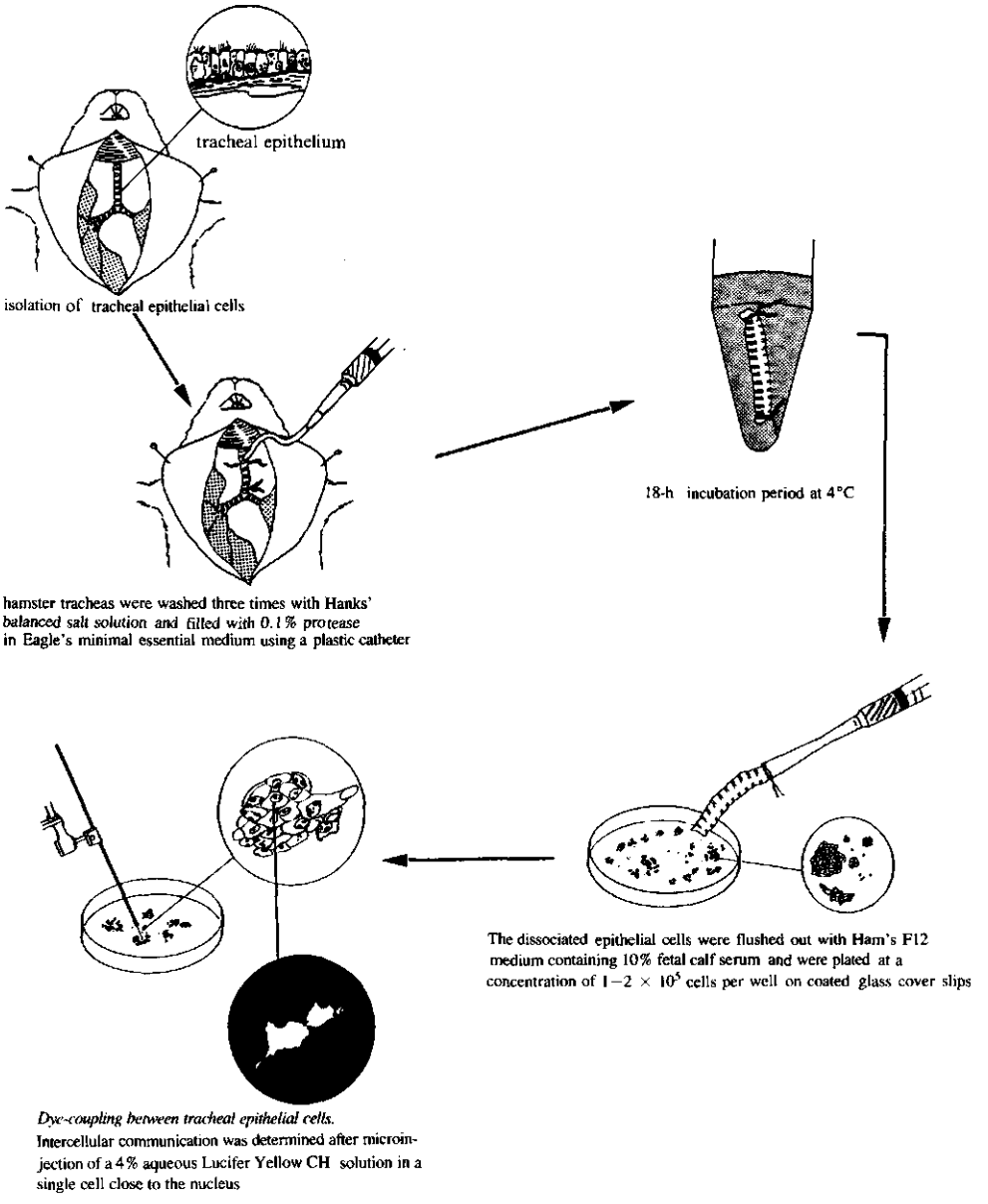
In tracheal organ cultures tissue organization and morphology, control of differentiation, cell population kinetics and cell-to-cell interactions are present in a way almost similar to the in vivo situation, whereas immune responses and inflammation reactions are absent (Table 1) (Lane 1978; Mass and Kaufman 1978; Schiff 1986). However, oxygen and nutrient diffusion may be affected by tissue size, and results of biochemical analyses may be limited due to the relatively small number of tracheal epithelial cells. Initial studies were carried out with tracheal segment outgrowth and dissociated epithelial cells cultured in serum-supplemented medium. Recently, however, tracheal organ and primary cell culture methods have been developed with serum-free hormone-supplemented chemically defined medium (Clamon et al. 1974; Lechner et al. 1981; Wu and Smith 1982; Wu et al. 1985). These culture methods have the advantage that effects of growth factors such as retinoids present in serum can be studied when the culture system is fairly well defined, and that selective culture of various cell types is possible. The next two sections present in detail two of the above in vitro methods used in the experiments reported in this thesis.

Primary hamster tracheal epithelial cell cultures

Tracheal epithelial cells obtained from Syrian Golden hamsters, used for the experiments reported in Chapter 8, were isolated according to the method described by Wu and Smith (1982), Lee et al. (1984) and Wu et al. (1985), with some modifications. The method for primary cell isolation is illustrated in Fig. 1. The distal end of the trachea was tied off just above the bifurcation, and the laryngeal end of the trachea was incised. The internal surface of the trachea was washed with Hanks' balanced salt solution (HBSS) and filled with 0.1% protease in Eagle's minimal essential medium via the laryngeal end with a plastic catheter (Fig. 1).

The excised trachea was immersed in HBSS, and incubated for 18 h at 4°C,

Fig. 1. Isolation and culture of hamster tracheal epithelial cells. The cells were used to study the effects of retinoids and cigarette smoke condensate on intercellular communication (Chapter 8).



after which the closed end of the trachea was cut off and the epithelial cells were flushed out with 10 ml Ham's F12 medium containing 10% fetal calf serum. Serum was added to the medium to inactivate the protease activity. Cell suspensions were centrifuged at 150xg for 10 min, and washed threetimes with Ham's F12 medium supplemented with cofactors. The dissociated epithelial cells (10^4 cells/cm^2) were cultured on coated glass cover slips (1.8 x 1.8 cm) or on tissue culture dishes (35 mm diameter) coated with 7.5 $\mu g/cm^2$ collagen, 2.5 $\mu g/cm^2$ bovine serum albumin and 2.5 $\mu g/cm^2$ human fibronectin. Ham's F12 medium with cofactors contained the following supplements: L-glutamine 2 mM, hydrocortisone 1 μM, bovine pancreatic insulin 5.0 mg/l, human transferrin 5 mg/l, epidermal growth factor 25 $\mu g/l$ and gentamycin 50 mg/l. The primary tracheal cell cultures were maintained at 37°C in a humidified incubator which was gassed with 95% air and 5% CO_2. The culture medium was changed every two days. Using this culture method, we found that tracheal epithelial cells do proliferate *in vitro* and that in addition to basal cells also differentiated cells such as ciliated and mucous cells are present during the culture period of 3 to 4 days.

Tracheal organ cultures of vitamin A-deprived hamsters

Extensive work has been carried out to develop sensitive *in vivo* and *in vitro* methods for the detection of retinoids by chemical analysis and bioassays (Sporn and Roberts 1984). Chemical analysis can provide more accurate quantitative data, whereas bioassays have the advantage that they provide information about functional aspects of retinoids (Sporn and Roberts 1984). Hamster trachea from vitamin A-deficient hamsters, cultured in serum-free hormone-supplemented medium, can be used to measure the ability of retinoids to reverse keratinization in a sensitive bioassay (Clamon et al. 1974; Sporn et al. 1976; Newton et al. 1980; Chopra 1982). This bioassay offers the possibility to detect retinoid activity at levels of 10^{-10} to 10^{-12} M. The biological parameters measured are the presence of keratohyaline granules and keratin in the vitamin A-deprived situation. These effects of vitamin A deficiency and the reversal of the keratinization process induced by retinoids have been described for *in vivo* (Wolbach and Howe 1925) and *in vitro* (Clamon et al. 1974) model systems.

Fig. 2 shows the time schedule of the *in vivo* vitamin A deprivation. Syrian

Table 2. Composition of the semipurified vitamin A-deficient diet for hamsters (prepared at TNO-CIVO)

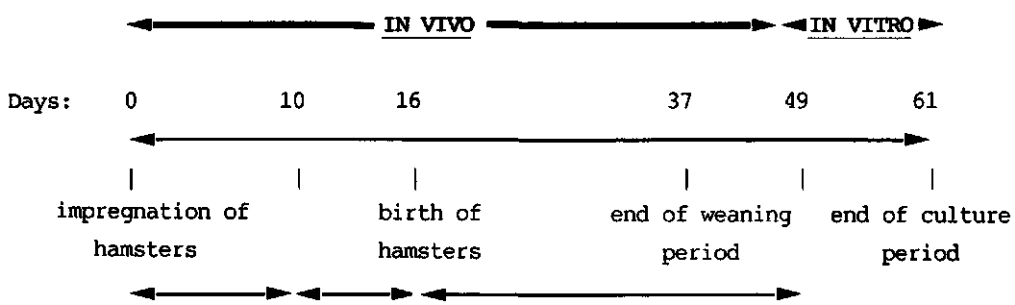
Ingredient	Unit	Amount per kg diet[a]
Casein	g	80.0
Isolated soy protein	g	120.0
dl-Methionine	mg	2000.0
Wheat starch	g	545.0
Pregelatinized wheat starch	g	50.0
Sucrose	mg	2574.0
purified cellulose	g	100.0
Soybean oil	g	50.0
Mineral mixture[b]	g	45.0
Thiamin	mg	30.0
Riboflavin	mg	22.5
Vitamin B_6	mg	9.0
Niacin	mg	135.0
Pantothenate (Ca)	mg	60.0
Biotin	mg	0.9
Folic acid	mg	3.0
Vitamin B_{12}	µg	15.0
Inositol	mg	150.0
Choline chloride	mg	2000.0
p-Aminobenzoic acid	mg	60.0
Cholecalciferol	IU	3750.0
α-Tocopherol	mg	4.5
Vitamin K_3	mg	120.0

[a] According to the estimated nutrient requirements of laboratory animals, Vol 10, pp 70-79, The National Research Council (1978).

[b] Mineral mixture (g/kg): KH_2PO_4 (354), $CaCO_3$ (380), NaCl (130), $MgSO_4$ (100), $FeSO_4$ (30), $ZnCl_2$ (1.0), $CuSO_4.5H_2O$ (0.45), $MnSO_4.H_2O$ (4.0), $CoCl_2.6H_2O$ (0.10), KI (0.8).

golden hamsters, ten days after impregnation, were fed a mixed diet comprising one part of the normal cereal-based diet and one part of a semipurified vitamin A-deficient diet. The ingredients used for the semipurified vitamin A-deficient diet (about 40 IU vitamin A per kg diet) are specified in Table 2. The mixed diet contained about 2600 IU vitamin A per kg. After parturition, the mothers were fed the vitamin A-deficient diet (Table 2; Fig. 2). The neonates were weaned at 3 weeks of age and henceforth fed the vitamin A-deficient diet moistened with water to a thick paste, and as pellets. Isolation of the tracheas was started when the young hamsters were 28 to 33

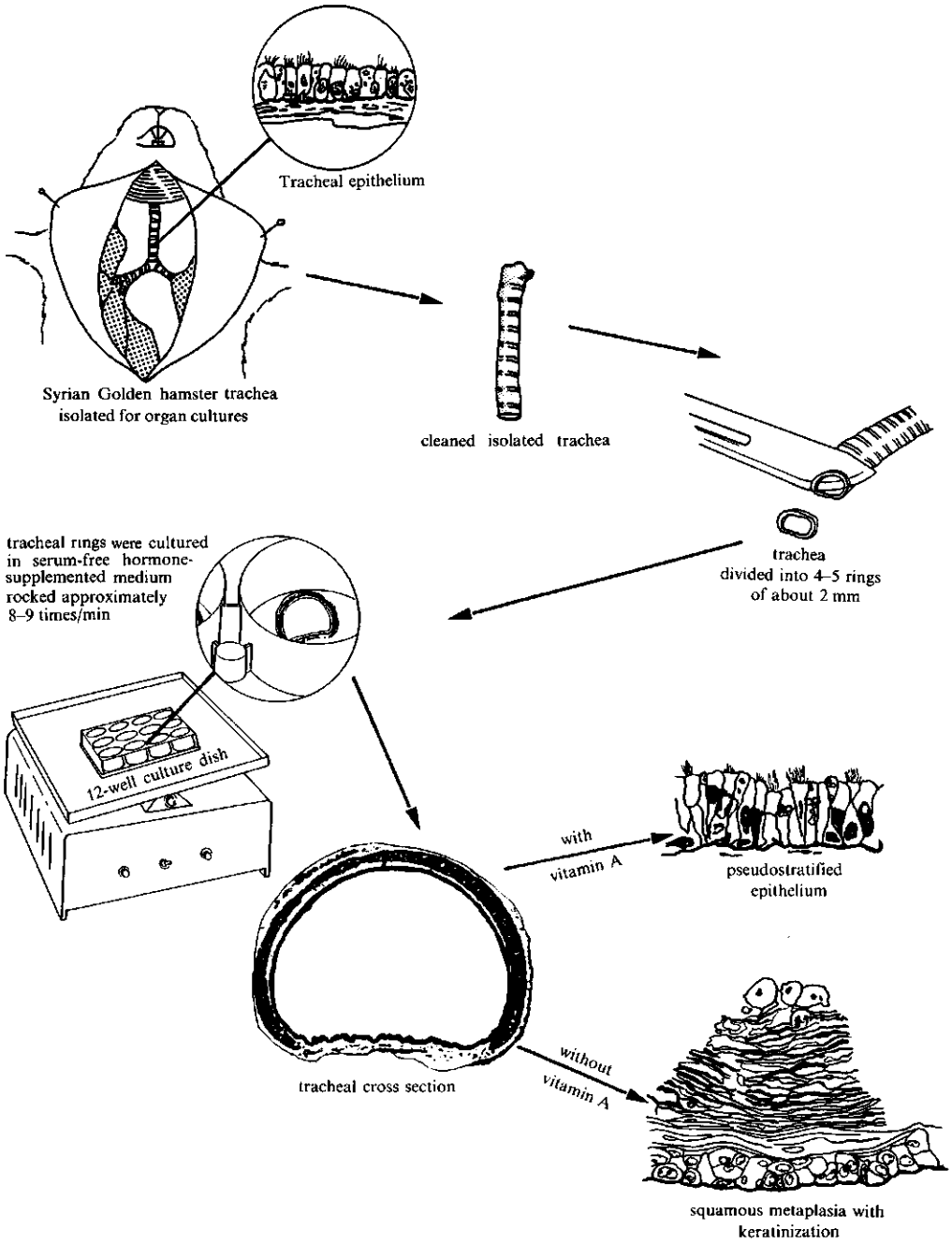
Fig. 2. Time schedule for in vivo vitamin A deprivation and in vitro tracheal organ culture



days of age. At that time the animals did not show signs of severe vitamin A deficiency and their tracheal epithelium was still pseudostratified containing basal, ciliated and mucous cells. However, the number of basal cells increased and the number of ciliated cells decreased after in vivo vitamin A deprivation. Table 3 shows the proportions of the cell population in control and vitamin A-deprived epithelium reported in the literature.

The in vitro tracheal organ culture system used for the experiments reported in this thesis was a modification of the systems described by several groups (Clamon et al. 1974; Sporn et al. 1976; Newton et al. 1980; Chopra 1982). Fig. 3 illustrates the isolation procedure and the culture method. Briefly, the external surface of the isolated trachea was cleaned to remove adherent tissue, and intact tracheas were then divided into 4-5 rings of about 3 mm. (In other studies the tracheas were opened along the membranous dorsal wall; Clamon et al. 1974; Sporn et al. 1976; Newton et al. 1980).

Fig. 3. Isolation of vitamin A-depleted tracheal rings for organ culture. The tracheal rings were used to study cellular proliferation and differentiation (Chapters 3-7).



The intact tracheal rings were cultured in 12-well culture dishes for at most 12 days in 1 ml serum-free hormone-supplemented CMRL-1066 medium containing: L-glutamine 2 mM, hydrocortisone 0.1 mg/l, bovine pancreatic insulin 1.0 mg/l and gentamicin 50 mg/l. Cultures were gassed with 50% O_2, 45% N_2 and 5% CO_2 in a humidified incubator at 37°C. The dishes were rocked approximately 9 times per minute to allow contact of the tracheas with both gas and medium. At the end of the 12-day culture period about 80% of the cultures that did not received vitamin A showed keratin or keratohyaline granules, whereas in cultures that had received all-trans retinol at a concentration of 10^{-7} or 10^{-8} M no keratinization was found (see Fig. 3).

Table 3. Mean proportion of the three main cell types as a percentage of the total number of counted cells in control and vitamin A-deprived tracheal epithelium.

Cell type	Boren et al. 1974		McDowell et al. 1984		Rutten et al. 1988	
	Control	Vitamin A-deprived	Control	Vitamin A-deprived	Control	Vitamin A-deprived
Basal	22.2	31.8	28.8	39.8	31.4	40.5
Mucous	48.4	50.9	59.3	53.1	51.2	50.7
Ciliated	26.9	14.8	11.0	6.8	19.8	9.5

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PART II

EXPERIMENTS

CHAPTER 3

Effects of all-trans retinol and cigarette smoke condensate on hamster tracheal epithelium in organ culture

I. A cell proliferation study

Virchows Archiv B Cell Pathology (1988) 55, 167-175

Rutten, AAJJL, JWGM Wilmer, RB Beems

Effects of all-trans retinol and cigarette smoke condensate on hamster tracheal epithelium in organ culture.
I. A cell proliferation study.

Summary

The effects of cigarette smoke condensate (CSC) and all-trans retinol on the cell proliferative activity of vitamin A-deprived hamster tracheal epithelium have been studied in vitamin A-deficient, serum-free, hormone-supplemented medium in organ culture. In the absence of retinol, CSC induced a dose-dependent increase in labeling index (LI) during 12 days of culture. The basal cells were more sensitive to CSC exposure than non-basal cells during the first 6 to 8 culture days. However, in squamous metaplastic foci developing after culture day 6, both basal and non-basal cells in the mid-part of the epithelium were labeled. Physiological concentrations of all-trans retinol stimulated the non-basal LI and inhibited the basal cell LI. Compared with dimethylsulfoxide (DMSO), all retinol concentrations used in the present study inhibited the basal cell LI at each time point examined (4-12 days culture). Exposure of tracheal rings to retinol, either before or after exposure to CSC, or simultaneous exposure to retinol and CSC, clearly decreased the CSC-induced basal cell proliferative activity depending on the retinol concentration used.

It is concluded from the present study that squamous metaplasia induced by vitamin A-deficiency or by CSC originates mainly from basal cells and that for the maintenance of these lesions, both basal and non-basal cells play a role. Furthermore, all-trans retinol inhibited the CSC-induced basal cell proliferation.

Introduction

In the past 15 years a variety of in vitro methods for tracheal organ, explant and cell cultures has been developed (Scott et al. 1986). Tracheal organ cultures, obtained from hamsters at an early stage of vitamin A deficiency (Clamon et al. 1975), have been widely used as an experimental model for studying the biological activity of retinoids (Sporn et al. 1976; Newton et

al. 1980). In a serum-free, vitamin A-deficient CMRL-1066 medium, foci of squamous metaplasia with or without keratinization develop in the tracheal epithelium. This metaplastic epithelium does not require vitamin A as a nutritional factor for the survival of the proliferative cells. Vitamin A and other biologically active retinoids prevent the development of squamous metaplasia or, in its presence, promote the reestablishment of a pseudostratified mucociliary epithelium (Clamon et al. 1974; Clark et al. 1980). These morphological changes controlled by vitamin A *in vitro* are comparable with those found *in vivo* (Harris et al. 1972; Port et al. 1974; McDowell et al. 1984a, b; Sigler et al. 1987). In hamster tracheal organ cultures from partially vitamin A-deficient animals, vitamin A regulates epithelial cellular proliferation and differentiation at physiological concentrations (Chopra 1982; Chopra 1983).

The hamster tracheal pseudostratified epithelium, composed of a heterogeneous phenotypically different population of mainly basal, ciliated and mucous cells, has a very low cell proliferative activity both under normal conditions and in vitamin A deficiency (McDowell et al. 1984b). *In vitro* or *in vivo* stimulation by carcinogens, tumour promoters, CSC or mechanical trauma increases cell proliferation (Chopra and Cooney 1985; Gordon and Pane 1977; Lasnitzki and Bollag 1982, 1987).

The objective of the present study was to investigate the ability of all-trans retinol to influence cellular proliferation in tracheal epithelial organ cultures of Syrian Golden hamsters. Furthermore, the effects of CSC were investigated with respect to the morphologic and cell dynamic alterations in the same model (Rutten et al. 1988).

Materials and methods

Animals and diet. Ten-day pregnant Syrian Golden hamsters were obtained from the TNO Central Institute for the Breeding of Laboratory Animals, Zeist, Netherlands. They were individually housed in macrolon cages on sterile saw dust under conventional laboratory conditions. To obtain vitamin A-deprived tracheal organ cultures, pregnant hamsters were fed a mixed diet (2600 IU vitamin A/kg), one half cereal-based (Van Eck, Cothen, Netherlands) (5000 IU vitamin A/kg), and the other half semi-synthetic and vitamin A-deficient (Clamon et al. 1975; Sporn et al. 1976). After birth, the mothers and the

young hamsters were fed only a vitamin A-deficient diet (44 IU vitamin A/kg). The young hamsters were weaned at an age of 21 days and their tracheas were isolated for organ cultures when they were 28-33 days old. The livers of the young hamsters fed the cereal-based diet contained 789000 IU vitamin A/kg, whereas those of the young hamsters fed the semi-sythethic vitamin A-deprived diet contained only 334 IU vitamin A/kg.

Culturing of tracheal rings. The external surface of the isolated trachea was cleaned to remove adherent tissue and then it was divided into 4-5 rings of about 2 mm. The tracheal rings were cultured in 12-well culture dishes (Costar, Cambridge, U.S.A.) for a maximum of 12 days in a serum-free hormone-supplemented CMRL-1066 medium (Flow Laboratories, Herts, England) containing L-glutamine 2 mM (Flow), hydrocortisone 0.1 μg/ml, bovine pancreatic insulin 1.0 μg/ml (Sigma Chemicals, St. Louis, U.S.A.) and gentamicin 50 μg/ml (Schering, Kenilworth, U.S.A.). Cultures were gassed with 50% O_2, 45% N_2 and 5% CO_2 in a humidified incubator at 37°C. The dishes were rocked approximately 9 times per minute to allow contact between the tracheal rings and both gas and medium.

Experimental protocol. The tracheal rings were randomly allocated to the wells. Two independent experiments were carried out in which the tracheal rings were treated for 12 days with all-trans retinol (Fluka AG, Buchs, Switzerland) at final concentrations ranging from 10^{-11} to 10^{-7}M, or with CSC (for preparation of CSC see Rutten and Wilmer 1986) at final concentrations ranging from 1.5 to 24.0 μg/ml. All test substances were dissolved in DMSO and added to medium at a final DMSO concentration of 0.1%. The culture medium was changed every 2 days, using frozen (-30°C) stock solutions for each concentration of the test substances. The purity of all-trans retinol was checked by high performance liquid chromatography (HPLC). Some tracheal rings were exposed either to CSC or retinol for 6 days followed by treatment with retinol or CSC for another 6 days or simultaneously to CSC and retinol during the whole culture period.

Cell proliferation. After varying periods of culture [methyl-^{3}H]thymidine (Amersham, Houten, Netherlands; specific activity 1.48 and 1.92 TBq/mmol), diluted in CMRL-1066 medium, was added to the culture medium at a final concentration of 74 kBq/ml, 18 h before the end of the culture period. Tracheal

rings were washed three times with Hanks balanced salt solution (HBSS), fixed in a 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) of the tracheal rings on microscope slides were dipped in Kodak NTB-2 emulsion (Eastman Kodak, New York, USA) diluted 1:1 with a 2% aqueous glycerol solution. Autoradiograms were exposed in dry, light-tight boxes for 4-6 weeks at -30°C, developed in Kodak D19, stained with 0.01% Toluidine Blue (TB) (Gurr, Chadwell Heath, England) solution and embedded in DePex.

Quantitative measurements. At days 4, 6, 8, 10 and 12 of the culture period, the cell proliferation activity in the tracheal epithelium was assessed by counting the number of labeled basal cells (cells in contact with the basal lamina and with a cytoplasm not reaching the tracheal lumen), and labeled non-basal cells (cells in contact with the basal lamina and with a cytoplasm reaching the tracheal lumen or epithelial cells which lost their contact with the basal lamina). A cell containing more than 10 silver grains above the nucleus was considered to be labeled. The background labeling was less than 1 grain per cell (see Figs. 6 to 9). The mean values for cell proliferation are given by a labeling index (LI) which is defined as the percentage dividing cells amongst the total number of epithelial cells counted. For each explant the LI was determined around the whole circumference (800 to 1600 cells) of two different cross-sections, separated by several cell layers (McDowell et al. 1984a).

Statistical analysis. The method used for the determination of cell proliferation activity had a mean coefficient of variation of 30.7% $\pm$ 14.4 (number of independent measurements was 114).

Results

The values obtained for the LI at days 4, 8 and 12 in the two experiments were combined because no differences were observed between them. Measurements on culture days 6 and 10 were made only in the second experiment.

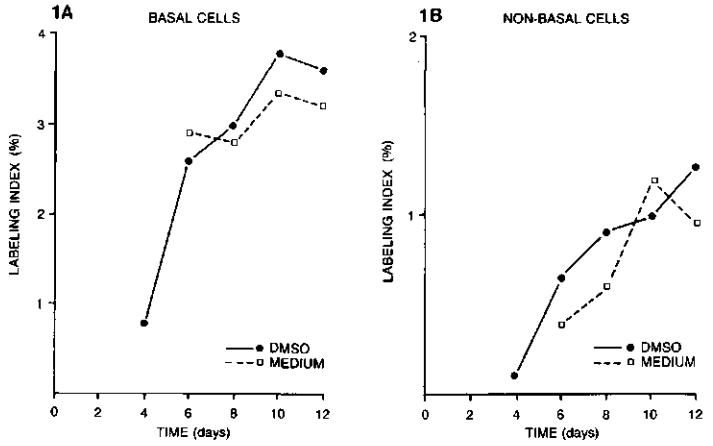

Fig. 1. Time-dependent effect on the epithelial labeling indices for basal **A** and non-basal **B** cells in vitamin A-deprived tracheal organ cultures treated with 0.1% DMSO in CMRL-1066 culture medium and without DMSO

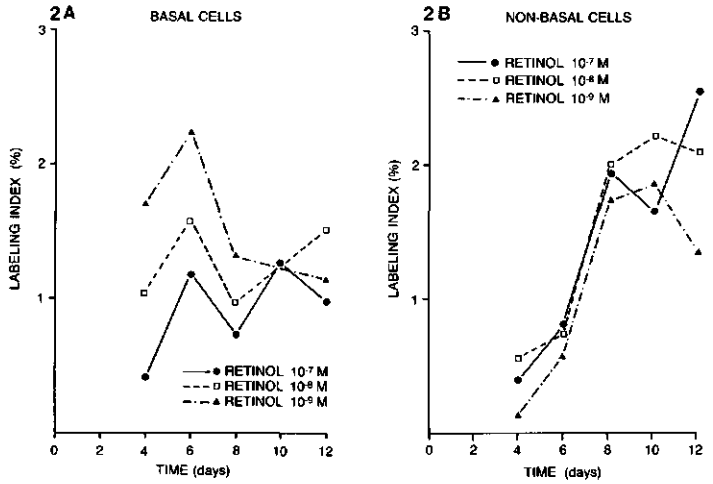

Fig. 2. Time-dependent effect on the epithelial labeling indices for basal **A** and non-basal **B** cells in vitamin A-deprived tracheal organ cultures treated with retinol

DMSO

DMSO (0.1%) did not influence the epithelial LIs either in basal (Fig. 1A) or non-basal cells (Fig. 1B) compared to the medium control. The LIs for non-basal cells were smaller than for basal cells at all time points measured.

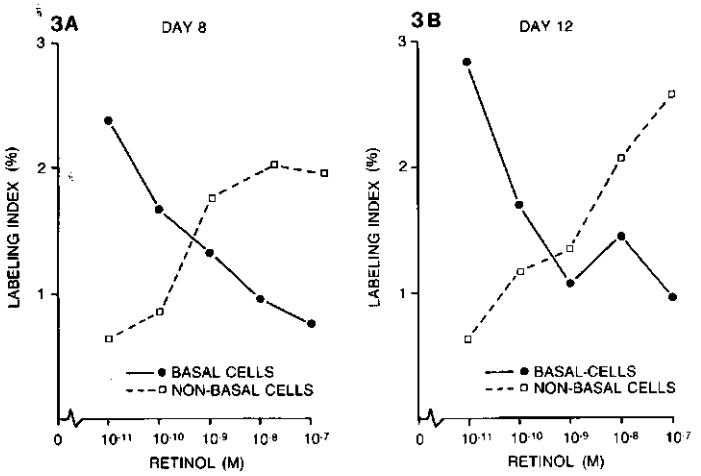

Fig. 3. Dose-dependent effect of retinol on the labeling index of basal and non-basal vitamin A-deprived tracheal epithelial cells after 8 **A** and 12 **B** days culture

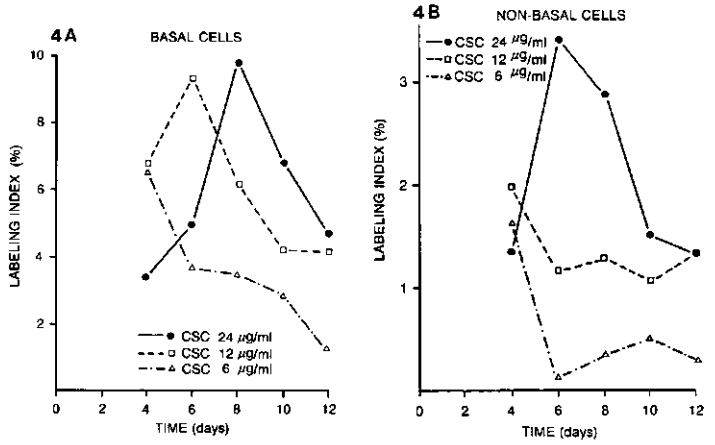

Fig. 4. Time-dependent effect on the epithelial labeling indices for basal **A** and non-basal **B** cells in vitamin A-deprived tracheal organ cultures treated with cigarette smoke condensate (CSC)

Retinol

In the presence of retinol, a slight time-dependent increase in basal cell proliferation was observed up to day 6 (Fig 2A). With non-basal cells, a more pronounced increase in cell proliferation was seen up to day 8 (Fig. 2B). However, for all retinol concentrations used, LIs of basal cells were lower than those of controls at the different culture days (Figs. 1A, 2A), whereas non-basal cell LIs where higher than controls (Figs. 1B, 2B). Increasing

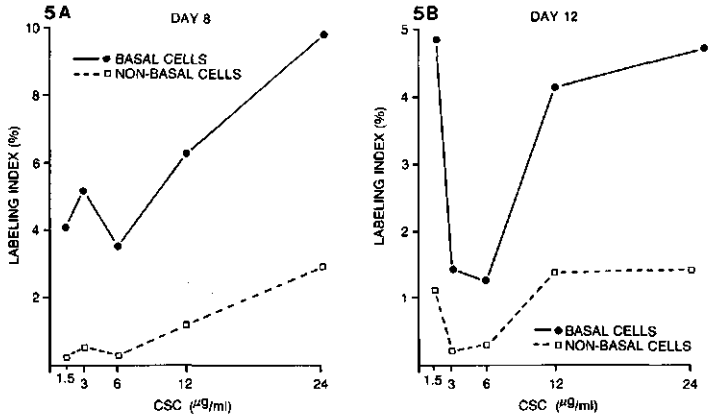

Fig. 5. Dose-dependent effect of cigarette smoke condensate (CSC) on the labeling index of basal and non-basal vitamin-A-deprived tracheal epithelial cells after 8 **A** and 12 **B** days culture

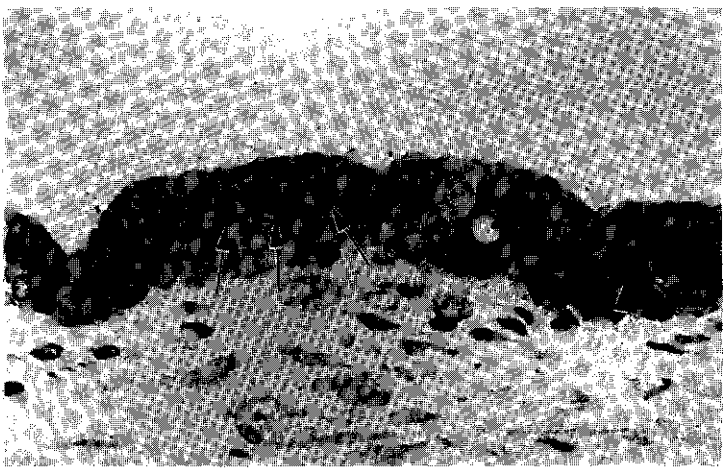

Fig. 6. Vitamin A-deprived tracheal organ culture treated with retinol 10^{-8} M for 12 days, showing three non-basal cells (*arrows*) with silver grains over the nuclei. The mid-epithelial part shows one dividing cell (*arrowhead*). Light micrograph of 1 µm section; Toluidine Blue staining. ×**425**

retinol concentrations (10^{-11} to 10^{-7} M) inhibited basal cell LIs and stimulated non-basal cell LIs (Fig. 3A, B). These results clearly show the ability of all-trans retinol to influence in vitro cell proliferation of heterogenous epithelial cells in different ways. Figure 6 shows dividing non-basal epithelial cells which were treated with retinol.

Cigarette smoke condensate.

CSC induced a marked cell proliferation in tracheal epithelium during the 12 culture days (Fig. 4A, B). Furthermore, CSC increased the basal and non-

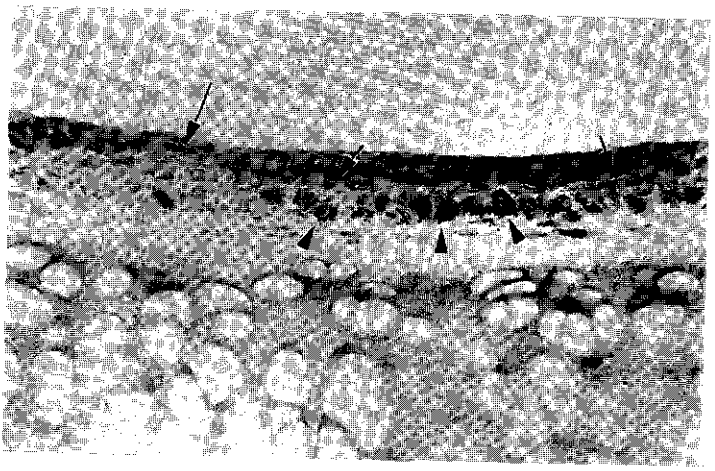

Fig. 7. Vitamine A-deprived tracheal epithelium cultured for 4 days in the presence of 24 µg CSC/ml. The labeled basal cells (*arrowheads*) are shown. The apical epithelial side (*large arrow*) and the mid-epithelial part (*small arrows*) consists of flattened cells, with rotated nuclei. Light micrograph of a 1 µm section; Toluidine Blue staining. × **425**

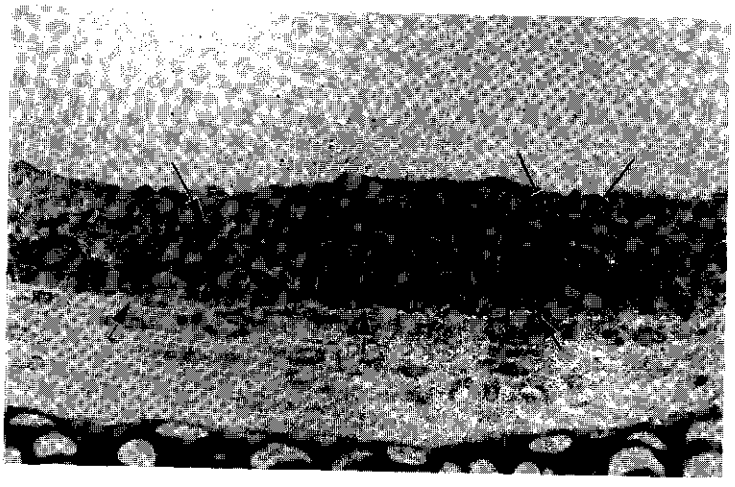

Fig. 8. Vitamin A-deprived tracheal organ culture exposed to 24 µg CSC/ml for 8 days. The epithelium shows a great number of basally situated cells (*small arrows*) with silver grains over the nuclei and a few non-basal cells (*large arrows*). In the mid-epithelium (arrowhead) cells become flattened and show rotated nuclei. Light micrograph of 1 µm section; Toluidine Blue staining. × **425**

basal LI at days 8 and 12 in a dose-dependent way (Fig. 5A, B). The maximal LI, both for basal and non-basal cells, was observed with 24 µg CSC/ml at day 8. After exposure to 12 µg CSC/ml the basal cells showed a maximal LI at culture day 6. From day 4 to 8, proliferating basal cells were seen in relatively small foci after exposure to 24 µg CSC/ml (Fig. 7), whereas from day 8 to 12 a nearly continuous row of mainly labeled basal- and some labeled non-basal cells was observed (Fig. 8). The number of labeled non-basal cells in the later period was smaller than the number of labeled basal cells (Figs. 4, 8). Basal epithelial cells seem to be more sensitive to CSC exposure than

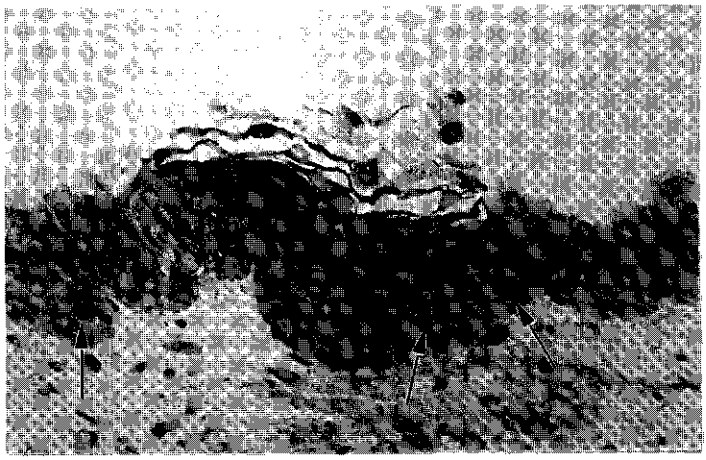

Fig. 9. Vitamin A-deprived tracheal epithelium treated in vitro with 24 μg CSC/ml for 8 days, showing basal hyperplasia and squamous metaplasia with keratinization. Dividing cells (*arrows*), mostly basally situated cells, can be seen by silver grains covering the nuclei. Light micrograph of 1 μm section; Toluidine Blue staining. ×**425**

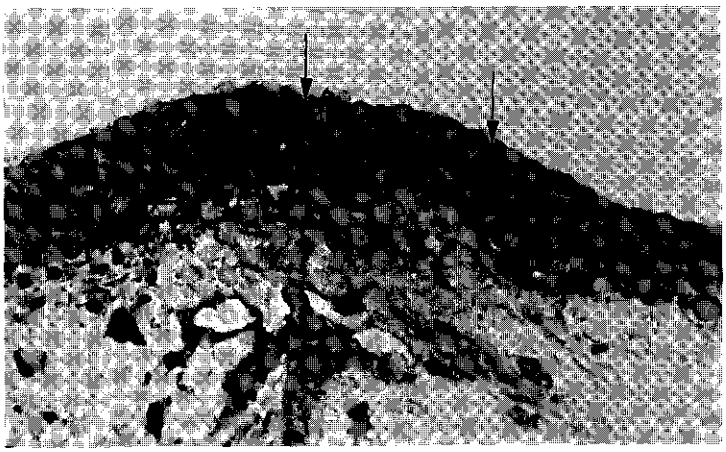

Fig. 10. Vitamin A-deprived tracheal epithelium, showing two non-basal dividing cells with mitotic figures (*arrows*). Organ culture treated with retinol 10^{-7} M for 6 days followed by CSC 24 μg/ml for 6 days. Light micrograph of 1 μm section; Multiple Stain-Hematoxylin staining. ×**425**

non-basal cells. In squamous, metaplastic foci, both basal and non-basal cells in the mid-epithelium were labeled (Fig. 9). Labeled non-basal cells found in the mid-epithelium were characteristic of CSC exposure. Remarkably, the process of keratinization was faster after treatment with CSC (Fig. 9) than in vitamin A deficiency (Fig. 11), but in the two experimental situations both basal and non-basal cells were labeled. Furthermore, after CSC treatment the tracheal lumen contained cell debris consiting of many exfoliated cells with pyknotic nuclei and irregular keratin sheets (Fig. 9).

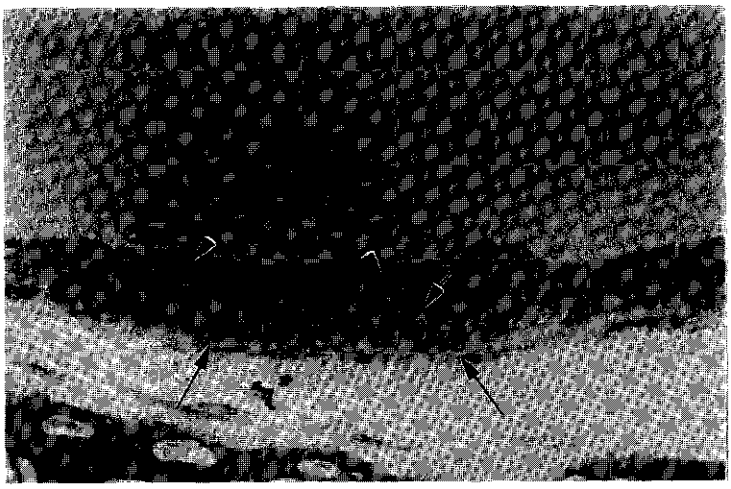

Fig. 11. Squamous metaplasia in vitamin A-deprived tracheal epithelial treated with retinol 10^{-11} M for 12 days. Basal cells (*large arrows*) and non-basal (*small arrow*) dividing epithelial cells which are covered by flattened keratinizing cells (*arrowheads*). Light micrograph of 1 µm section; Toluidine Blue staining. ×425

Table 1. Effects of single, combined and simultaneous exposure to retinol and cigarette smoke condensate (CSC) on the epithelial labeling indices of basal and non-basal cells

First treatment[a]	Second treatment[b]	*n*	Labeling indices (%)	
			Basal cells	Non-basal cells
			Mean SD	Mean SD
Single exposure				
DMSO 0.1%	DMSO 0.1%	6	3.58±0.55	1.27±0.24
CSC[c] 24 µg/ml	CSC 24 µg/ml	5	4.70±0.95	1.42±0.63
RET[d] 10^{-11} M	RET 10^{-11} M	3	2.81±0.46	0.61±0.14
RET 10^{-7} M	RET 10^{-7} M	5	0.96±0.40	2.57±0.42
Combined exposure				
CSC 24 µg/ml	RET 10^{-11} M	3	2.31±0.60	1.63±0.79
CSC 24 µg/ml	RET 10^{-7} M	3	0.91±0.47	1.34±0.45
RET 10^{-11} M	CSC 24 µg/ml	3	2.34±0.87	1.59±0.69
RET 10^{-7} M	CSC 24 µg/ml	3	0.46±0.12	0.95±0.33
Simultaneous exposure[e]				
RET 10^{-11} M +	CSC 24 µg/ml	3	4.12±1.07	1.39±0.49
RET 10^{-7} M +	CSC 24 µg/ml	3	2.00±0.43	2.37±0.44

[a] Exposure to the test substance during the first 6 culture days
[b] Exposure to the test substance during the second 6 culture days
[c] CSC is cigarette smoke condensate; [d] RET is all-trans retinol
[e] Simultaneous exposure to all-trans retinol and CSC during 12 culture days
n is number of tracheal cultures

Combined and simultaneous exposure to retinol and cigarette smoke condensate

Exposure of tracheal rings to retinol (6 days), either before or after exposure to CSC (6 days), or simultaneous exposure to retinol and CSC (12 days), clearly decreased the CSC-induced basal cell proliferative activity

depending on the retinol concentration (Table 1). However, a similar decrease in basal cell LI was also observed after a single exposure to retinol (10^{-7} compared to 10^{-11} M). Remarkably, after simultaneous exposure to retinol and CSC for 12 days, the basal cell LI was increased compared with the LI of the combined exposures (Table 1). This effect can be ascribed to CSC.

Combined exposure to retinol and CSC did not apparently influence non-basal cell proliferation. After simultaneous exposure to retinol (10^{-7} M) and CSC, a slight increase in non-basal cell proliferation was seen, which is comparable with the increase seen after single exposure to retinol. Figure 10 shows two metaphases of non-basal tracheal epithelial cells after 6 days exposure to 10^{-7} M retinol followed by 6 days treatment with 24 μg CSC/ml.

Discussion

Effects of vitamin A-deficiency and all-trans retinol in tracheal epithelium

McDowell et al. (1984a) showed that in vivo squamous metaplasia in vitamin A-deficiency results from proliferation and changes in mucous-producing cells. Although in vivo vitamin A deprivation decreased the epithelial proliferative activity of basal (3-4 fold) and mucous (14 fold) cells in non-metaplastic tissue compared with the normal epithelium, the basal cells in vitamin A deficient epithelium had a 2-fold higher LI than non-basal cells. However, in squamous metaplastic epithelium and stratified lesions about 70-75% of the dividing cells were non-basal cells (McDowell et al. 1984a). Squamous metaplasia developing as a regenerative response to mechanical injury showed the same features regarding the mucous cells during the process of epithelial restoration compared with those observed with vitamin A-deficiency (Keenan et al. 1983). Earlier reports by Chopra (1982, 1983) stressed the importance of proliferating basal cells in the development of squamous metaplasia induced by vitamin A deficiency in vitro. The results of the present study showed that, if no differences were made between normal, early and late metaplastic epithelium, the basal LI increased and the non-basal LI decreased with decreasing retinol concentrations. These results are in agreement with those obtained after ß-retinoic acid treatment of non-metaplastic epithelium (Chopra 1983). However, in squamous metaplastic foci induced by vitamin A deficiency, we found both basal (35-40%) and non-basal (60-65%) proliferating cells. This

was not observed in previous in vitro studies reported by Chopra (1982, 1983). These conflicting results may be explained by the use of different criteria for the classification of basal and non-basal cells present in the lower and mid-epithelium in metaplastic lesions.

It can be concluded from the present study that in in vitro vitamin A-deficiency, the metaplasia induced originates mainly from basal cells and that for maintenance of squamous metaplasia, both basal and non-basal cells are important. This conclusion is based first on the higher basal than non-basal LIs in normal and in pseudostratified vitamin A-deprived epithelium prior to the development of metaplastic lesions, and secondly on the presence of higher non-basal than basal cell LIs in established metaplastic foci.

Effects of cigarette smoke condensate on tracheal epithelium

Because of the marked cell proliferation and the corresponding hyperplasia after CSC exposure, it is easier to discriminate between basal and non-basal labeled cells (Figs. 7, 8 and 9) than in vitamin A deficiency (Fig. 11). It is interesting to note, that the maximum basal cell LI shifted from culture day 8 to day 6 at a lower CSC concentration. Rasmussen et al. (1981) reported that in mice during the first week of exposure to cigarette smoke, DNA-replication and unscheduled DNA-synthesis (UDS) increased in lung tissue. Longer exposure (up to 17 weeks) resulted in a 50% inhibition of the UDS and a twofold stimulation of the DNA-replication compared with the sham-exposed controls. In the present study decreased basal and non-basal LIs were found after 12 culture days, which is in agreement with the reported threefold increase in DNA-replication after 1 week and the nearly twofold increase in DNA-replication after 6 weeks exposure to cigarette smoke in vivo (Rasmussen et al. 1981).

Chopra and Cooney (1985) reported increased basal and non-basal cell proliferation after in vitro exposure to benzo(a)pyrene. The basal mitotic index remained high whereas the mitotic index of mucous cells decreased at the end of the 10 day culture period. In the present study tracheal organ cultures treated with CSC clearly showed the important role of both basal and non-basal cells in the development and maintenance of squamous metaplasia (Fig. 4A and 4B, 7, 8 and 9). Obviously, the CSC-induced development of metaplastic lesions differs from that produced by benzo(a)pyrene with respect to the different cell types involved.

Lasnitzki and Bollag (1982, 1987) reported an inhibitory effect of the synthetic retinoids etretinate and Ro 15-0778 on the benzo(a)pyrene- and CSC-induced epithelial mitotic index in cultured rat and mouse respiratory tissue. In the present study using tracheal organ cultures from vitamin A-deprived hamsters, physiological concentrations of the naturally occurring all-trans retinol or its metabolites inhibited or prevented the CSC-induced changes in the LIs of basal epithelial cells. However, if the tracheal cultures were simultaneously exposed to a physiologically active retinol concentration and CSC, cell proliferation activity of CSC was still present but to a higher level compared with the combined exposure. This may be due to the longer incubation period with CSC (12 days) compared with the combination experiments in which the tracheas were incubated for only 6 days in the presence of CSC.

Acknowledgements

The authors would like to thank Dr. V.J. Feron and Ing. B.J. Spit for helpful discussions, Prof. dr. J.H. Koeman for reviewing the manuscript, Miss I. de Rooy for animal care, Mr W.G. Rovers for preparing the histologic sections and Mr W.R. Leeman for preparing the autoradiograms. The study was supported by grants from the Scientific Advisory Committee on Smoking and Health (Dutch Cigarette Industry Foundation) and the Ministry of Welfare, Health and Cultural Affairs.

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CHAPTER 4

Effects of all-trans retinol and cigarette smoke condensate on hamster tracheal epithelium in organ culture

II. A histomorphological study

Virchows Archiv B Cell Pathology (1988) 55, 177-186

Rutten, AAJJL, RB Beems, JWGM Wilmer

Effects of all-trans retinol and cigarette smoke condensate on hamster tracheal epithelium in organ culture.
II. A histomorphological study.

Summary

The effects of all-trans retinol and cigarette smoke condensate (CSC) on tissue morphology and cellular differentiation were investigated in vitamin A-deprived tracheal epithelium cultured in vitamin A- and serum-free hormone-supplemented medium. Physiological retinol concentrations prevented the development of hyperplasia and squamous metaplasia with or without keratinization, and induced differentiation to mucous cells. Squamous metaplastic foci with keratinization were observed during 12 days of culture with low retinol concentrations and with dimethylsulfoxide (DMSO) which was accompanied by an increased number of basal and indeterminate cells. CSC induced dose-related hyperplasia and irregularly shaped foci of squamous metaplasia with atypical epithelial proliferation. In non-metaplastic epithelium, CSC exposure increased the number of ciliated cells. Hyperplasia and squamous metaplasia were inhibited if the tracheal rings were first treated with retinol followed by CSC exposure, or if the tracheas were simultaneously treated with retinol and CSC. CSC-exposure prior to retinol treatment induced similar histomorphological alterations as CSC alone.

Introduction

Tracheal organ cultures obtained from vitamin A-deprived hamsters are very sensitive to in vitro addition of biologically active retinoids (Sporn et al. 1976; Newton et al. 1980; Chopra 1983a). Cigarette smoke condensate (CSC) (Lasnitzki 1968) and vitamin A deficiency (Clamon et al. 1974) induced well characterized morphological changes in tracheal organ cultures. Lasnitzki and Bollag (1982; 1987) found a protective effect of the synthetic retinoids etretinate and Ro 15-0778 against CSC-induced changes in tracheal and lung organ cultures. Furthermore, compared with cell cultures, tracheal organ cultures have the advantage that the morphology and differentiation resemble

the normal in vivo situation, in that the pseudostratified columnar epithelium consists of basal, ciliated and mucus-producing cells attached to the basal lamina (Sigler et al. 1987). In this in vitro test-system, CSC and polycyclic aromatic hydrocarbons induce hyperplasia and squamous metaplasia in a similar way to that observed in vivo. Whether this phenotypic transformation of tracheal epithelial tissue is irreversible and progressive, or in some way related to the reversible alterations observed with vitamin A deficiency, is not clearly understood. However, in vivo squamous metaplasia of the respiratory tract is generally considered to represent a preneoplastic lesion (Harris et al. 1972; Becci et al. 1978; McDowell and Trump 1983).

In a previous paper we reported effects of CSC and all-trans retinol on cell proliferation in cultured tracheal epithelial rings (Rutten et al. 1988). In the present study the cellular and tissue alterations in tracheal epithelium organ cultures with physiological concentrations of retinol and CSC-induced changes have been investigated with respect to differentiation and morphology.

Materials and methods

Animals and diet. Ten-day pregnant Syrian Golden hamsters were obtained from the TNO Central Institute for the Breeding of Laboratory Animals, Zeist, Netherlands and fed a vitamin A-reduced diet during the last 6 days of the gestation. After birth, mothers and newborn hamsters received a vitamin A-deficient diet for 28 to 33 days (Rutten et al. 1988).

Tracheas, culture medium and experimental protocol. The tracheas of the young hamsters were isolated under aseptic conditions, divided into 4-5 rings and cultured for a period of 12 days in serum-free hormone-supplemented CMRL-1066 medium (Flow Laboratories, Herts, England) containing L-glutamine 2 mM (Flow), hydrocortisone 0.1 μg/ml, bovine pancreatic insulin 1.0 μg/ml (Sigma Chemicals, St. Louis, U.S.A.) and gentamicin 50 μg/ml (Schering, Kenilworth, U.S.A.). Tracheal rings were treated with cigarette smoke condensate (CSC) (for preparation of CSC see Rutten and Wilmer 1986), 1.5 to 24 μg/ml, or all-trans retinol (Fluka AG, Buchs, Switzerland), 10^{-11} to 10^{-7}M up to 12 days. At 4, 6, 8, 10 and 12 culture days tracheas were fixed to investigate treatment-related histomorphological changes. Detailed information on the trachea

culture system has been reported in a previous paper (Rutten et al. 1988).

Histology. The formaldehyde-fixed tracheal organs were dehydrated and embedded in Technovite 7100 plastic (Kulzer, Wehrheim, F.R.G.). Semi-thin sections were cut at 1 μm with Widia knives on a Autocut-1140 rotary microtome (Reichert-Jung, Vienna, Austria) and stained with Alcian Blue 8-GX (AB) (Gurr, Chadwell Heath, England) in combination with periodic acid Schiff (PAS) (Merck, Darmstadt, F.R.G.) or with Multiple Stain (MS) (Polysciences, Warrington, U.S.A.). All semi-thin sections were counter-stained with Gill's Hematoxylin activity 3 (Polysciences, Warrington, U.S.A.).

Morphologic observations and quantitative measurements. Cell differentiation was assessed by counting the number of indeterminate, basal, mucous and ciliated cells by light microscopy (x630 and x1000), using the definitions for the characterization of different epithelial cells described by McDowell et al. (1984a) and Chopra and Cooney (1985). Cells of the normal pseudostratified epithelium were scored as basal if their cytoplasm did not reach the tracheal lumen and was PAS-negative, as mucous if their cytoplasm was PAS-positive and reached the tracheal lumen, as ciliated if the cytoplasm was PAS-negative and reached the tracheal lumen with cilia located on the apex, and as indeterminate if their cytoplasm was PAS-negative and reached the tracheal lumen without clearly visible cilia on the apex. PAS-negative epithelial cells which lost their contact with the basal lamina, mostly situated in the mid-epithelium, were also classified as indeterminate. Squamous metaplasia was scored according to the criteria reported by Clamon et al. (1974).

Results

Vitamin A-deprived epithelium

Non-cultured tracheal epithelium of 33-days-old vitamin A-deprived (44 IU vitamin A/kg diet) and control (5000 IU vitamin A/kg diet) hamsters showed significant differences in the distribution of the heterogeneous cell population (Fig. 1A, B). In vitamin A-deprived epithelium the number of basal cells increased (control: 31.4 $\pm$ 1.67 and vitamin A-deprived: 40.5 $\pm$ 2.59),

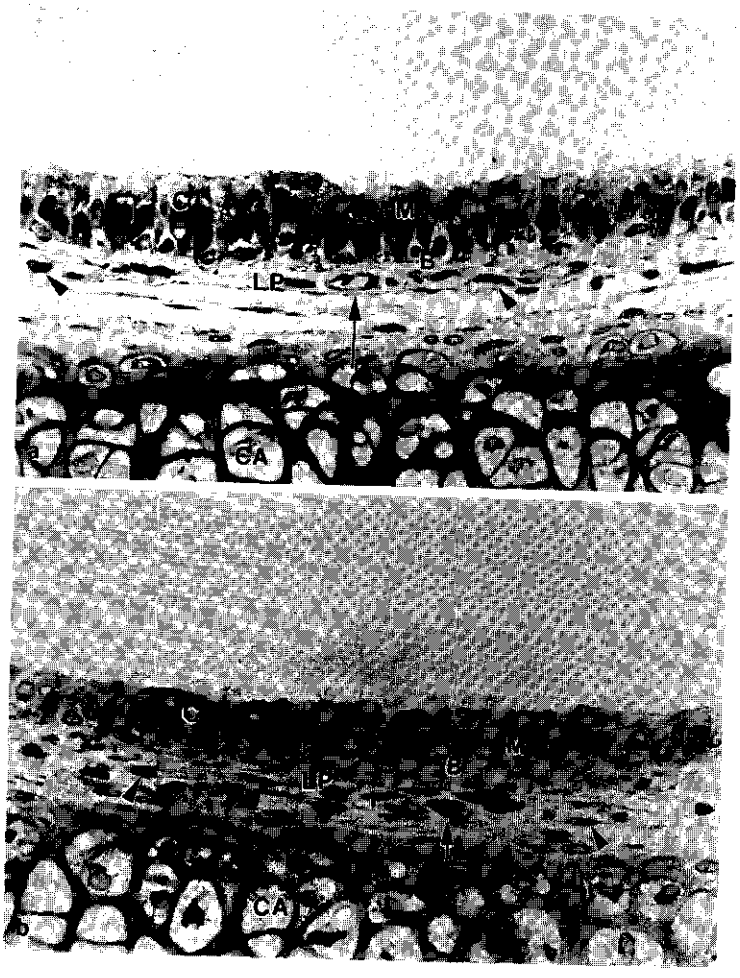

Fig. 1. Semi-thin cross section of a non-cultured trachea from hamster (33 days old) on a control diet **a** and a vitamin A-deprived diet **b**, showing the pseudostratified columnar epithelium consisting in a discontinuous row of basal (*B*), ciliated (*C*) and mucus-producing (*M*) cells. At the apical part cilia are clearly seen. The lamina propria (LP), containing fibroblasts (*arrowheads*), a blood vessel (large arrow), a mast cell (*small arrow*), and cartilage (*CA*) support the tracheal epithelium. Multiple Stain × **425**

that of ciliated cells decreased (control: 19.8 ± 2.28 and vitamin A-deprived: 9.5 ± 1.76) and that of mucous cells was not altered (control: 51.2 ± 4.77 and vitamin A-deprived 50.7 ± 2.42).

Effects of DMSO, retinol and cigarette smoke condensate

Morphology. The addition of 0.1% DMSO to the culture medium did not significantly influence the tissue morphology (Tables 1 and 2). Retinol prevented the development of squamous metaplasia, hyperplasia and keratinization in the tracheal epithelium both in the early (4, 6 and 8 days) *in vitro* (Table 1) and

Table 1. Cumulative (day 4, 6 and 8) early morphologic effects of DMSO, all-trans retinol and cigarette-smoke condensate (CSC) on the degree of hyperplasia, squamous metaplasia and keratinization in tracheal epithelium

Treatment	Concentration	*n*	% of tracheal organ cultures with								
			Hyperplasia				Squamous metaplasia				Keratinization and keratohyaline granules
			None	Mild	Marked	Severe	None	Mild	Marked	Severe	
DMSO	0.1%	13	30	70			38	46	15		46
Medium		6	17	83			67	33			33
Retinol	10^{-11} M	3	100				67	33			33
	10^{-10} M	3	67	33			67	33			0
	10^{-9} M	8	87	13			63	37			13
	10^{-8} M	10	100				100				0
	10^{-7} M	14	93	7			93	7			0
CSC	1.5 µg/ml	3		100			67	33			33
	3.0 µg/ml	3	33	33	33		33	67			67
	6.0 µg/ml	9	56	33	11		33	67			11
	12.0 µg/ml	8		37	50	13	13	87			38
	24.0 µg/ml	13		22	39	39	23	69	8		69

Hyperplasia of the tracheal epithelium is scored as severe if more than 20% of the total epithelial length is hyperplastic; as marked between 5–20%; as mild if less than 5%. Squamous metaplasia is graded as severe if more than 40% of the total epithelial length has squamous metaplasia; as marked between 10–40%; as mild if less than 10%. *n* is the number of tracheal organ cultures

Table 2. Cumulative (day 10 and 12) late morphologic effects of DMSO, all-trans retinol and cigarette-smoke condensate (CSC) on the degree of hyperplasia, squamous metaplasia and keratinization in tracheal epithelium

Treatment	Concentration	*n*	% of tracheal organ cultures with								
			Hyperplasia				Squamous metaplasia				Keratinization and keratohyaline granules
			None	Mild	Marked	Severe	None	Mild	Marked	Severe	
DMSO	0.1%	9	33	67			11	22	66	11	78
Medium		6	50	50				33	67		83
Retinol	10^{-11} M	3	33	67				34	33	33	67
	10^{-10} M	6	67	33			34	33	33		66
	10^{-9} M	5	80	20			80	20			20
	10^{-8} M	6	83	17			100				0
	10^{-7} M	8	100				100				0
CSC	1.5 µg/ml	6	67	33				83	17		100
	3.0 µg/ml	5	100				20	60	20		80
	6.0 µg/ml	9	78	22				22	67	11	67
	12.0 µg/ml	7		43	43	14		14	43	43	100
	24.0 µg/ml	8	12	50	13	25			87	13	87

Hyperplasia of the tracheal epithelium is scored as severe if more than 20% of the total epithelial length is hyperplastic; as marked between 5–20%; as mild if less than 5%. Squamous metaplasia is graded as severe if more than 40% of the total epithelial length has squamous metaplasia; as marked between 10–40%; as mild if less than 10%. *n* is the number of tracheal organ cultures

in the late (10 and 12 days) in vitro period (Table 2). The effects of retinol were most pronounced in the late in vitro period (Figs. 2 and 3). The squamous foci with keratinization due to vitamin A deprivation consisted of regular layers of flattened cells containing keratohyaline granules and these were accompanied by a few exfoliated cells (Fig. 2).

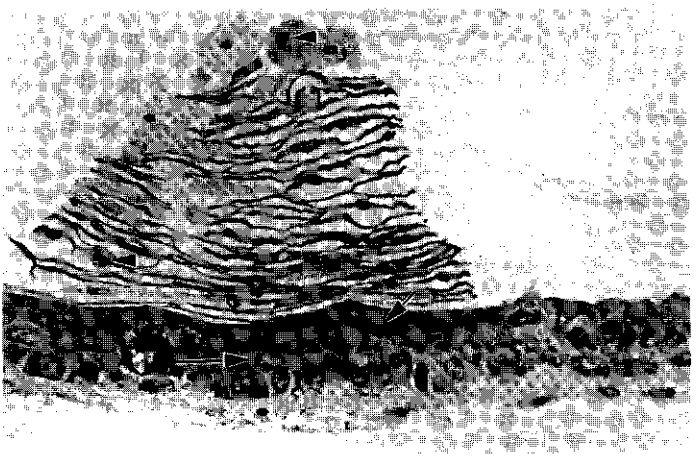

Fig. 2. Vitamin A-deficient trachea treated with retinol 10^{-11} M for 12 culture days, showing squamous metaplasia with keratinization. The luminal part of the epithelium is covered by stratified cells (*small arrow*) and cells with rotated nuclei (*large arrow*). A few exfoliated cells with pyknotic nuclei (*arrowheads*) are present in the tracheal lumen. Multiple Stain × **425**

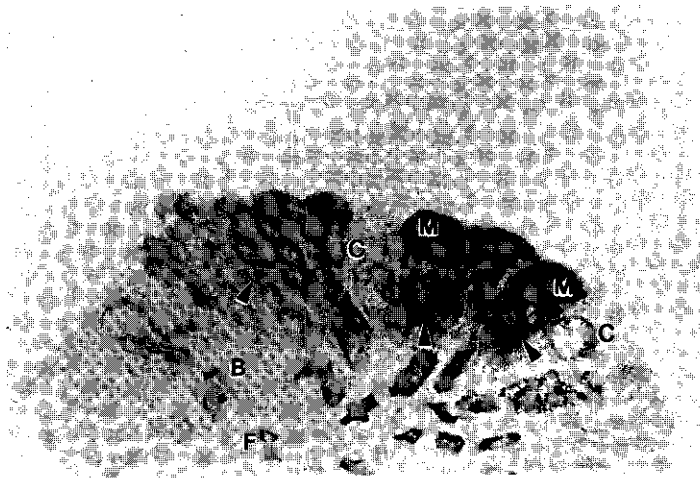

Fig. 3. Tracheal epithelium cultured for 12 days in the presence of 10^{-7} M retinol showing PAS-positive mucus droplets (*arrowheads*) and PAS-positive mucus producing cells (*M*). The ciliated cells (*C*), basal cells (*B*) and the fibroblasts (*F*) in the lamina propria are PAS-negative. Alcian Blue-Periodic Acid Schiff (AB-PAS) × **800**

CSC induced squamous foci with keratinization showing irregularly shaped squamous cells at the apical side and many exfoliated cells containing pyknotic nuclei (Figs. 4, 5 and 6). The latter change was not observed in vitamin A-deficient tracheas. Furthermore, protruding atypical epithelial proliferations covered with squamous and necrotic cells were regularly observed in CSC-treated tracheas (Fig. 6). The basally located epithelial cells were polygonal and have prominent nucleoli, whereas nearer the lumen epithelium nuclei were observed only infrequently. In the metaplastic foci the basal cells were more columnar in shape and contained oval nuclei (Figs. 4 and 7). CSC also induced hyperplasia in metaplastic foci (Figs. 4, 5 and 6) and in

Fig. 4. Squamous metaplasia with keratinization in tracheal epithelium induced by cigarette smoke condensate (12 µg/ml) after 12 culture days. In the tracheal lumen many exfoliated cells with pyknotic nuclei (*small arrows*) are present. The basally situated cells (large arrows) are enlarged and contain oval-shaped nuclei. Multiple Stain × **425**

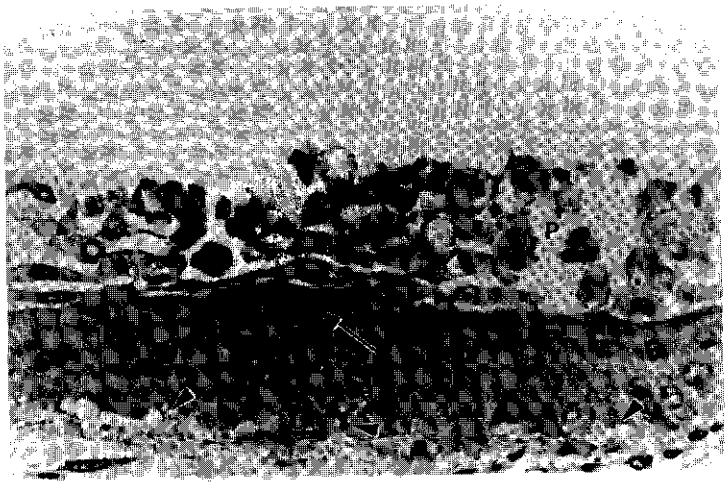

Fig. 5. Squamous metaplasia with keratinization in trachea treated with cigarette smoke condensate (12 µg/ml); parakeratotic changes are visible (P). The necrotic cells are sloughed from the surface and the underlying stratified cells cover the apical epithelial side (*arrow*). Some of these cells have almost lost their contact with the epithelium. In the basal part of the epithelium many vacuoles (*arrowheads*) and irregularly shaped nuclei are present. Multiple Stain × **425**

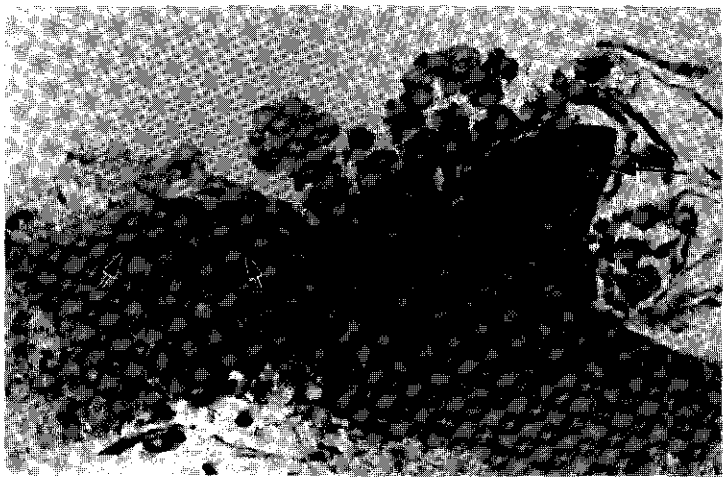

Fig. 6. Tracheal epithelium cultured for 12 days in the presence of 24 μg CSC/ml. Extensive squamous metaplasia with atypia is visible. The superficial cells are flattened (*small arrows*) and protrude into the lumen (*large arrows*). Necrotic cells with pyknotic nuclei occur in the tracheal lumen. Multiple Stain × 425

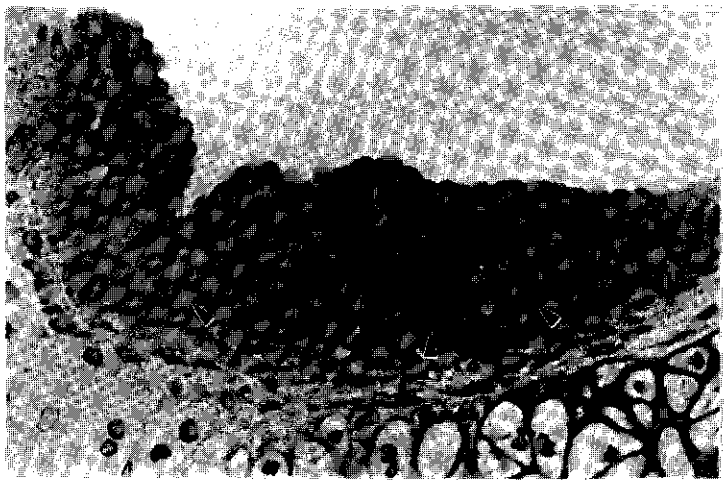

Fig. 7. Tracheal organ culture exposed to 12 μg CSC/ml for 12 days. The epithelium shows hyperplastic and metaplastic changes. The cells in contact with the basal lamina are enlarged. The superficial cells, which have lost their cilia, and the mucous cells have contact with the underlying epithelial cells. Multiple Stain × 425

non-squamous areas (Fig 7) of the epithelium. The early CSC-induced changes included dose-related hyperplasia, a slight increase in squamous metaplasia and a slight increase in keratinization compared with controls and retinol-treated tracheas (Table 1). In the late in vitro period, CSC induced the same morphological alterations as in the earlier period, but the changes in the late period were more severe and more clearly dose-related (Table 2). Squamous metaplasia without keratinization was observed more frequently in CSC-exposed than in vitamin A-deficient tracheas (Table 2).

Table 3. Morphologic effects of all-trans retinol and cigarette-smoke condensate (CSC) on the degree of hyperplasia, squamous metaplasia and keratinization in tracheal epithelium in combination experiments

First[a] treatment	Second[b] treatment	% of tracheal organ cultures with[c]								
		Hyperplasia				Squamous metaplasia				Keratinization and keratohyaline granules
		None	Mild	Marked	Severe	None	Mild	Marked	Severe	
CSC 24 µg/ml[d]	RET 10^{-11} M[e]			100			33	33	33	100
CSC 24 µg/ml	RET 10^{-7} M	33	33	33			33	67		100
RET 10^{-11} M	CSC 24 µg/ml		100				67	33		100
RET 10^{-7} M	CSC 24 µg/ml		100			100				0
RET 10^{-11} M +	CSC 24 µg/ml[f]			100				100		100
RET 10^{-7} M +	CSC 24 µg/ml		33	67		67	33			0

Hyperplasia of the tracheal epithelium is scored as severe if more than 20% of the total epithelial length is hyperplastic; as marked between 5–20%; as mild if less than 5%. Squamous metaplasia is graded as severe if more than 40% of the total epithelial length has squamous metaplasia; as marked between 10–40%; as mild if less than 10%. *n* is the number of tracheal organ cultures.

[a] Exposure to the test substance for the first 6 (day 0–6) culture days
[b] Exposure to the test substance for the second 6 (day 6–12) culture days
[c] The number of tracheal organ cultures is 3 for each combination experiment
[d] CSC is cigarette-smoke condensate
[e] RET is all-trans retinol
[f] Simultaneous exposure to all-trans retinol and CSC during 12 culture days

Table 4. Cumulative (day 4, 6 and 8) early effects of all-trans retinol and cigarette smoke condensate (CSC) on the differentiation of cultured tracheal epithelium

Treatment	Concentration	*n*	Tracheal epithelial cell types[a]			
			Basal	Ciliated	Mucous	Indeterminate
Control[b]		19	39± 6	8±2	52±8	2.3±0.9
Retinol	10^{-11} M	3	36± 4	7±1	50±2	5.1±2.3
	10^{-10} M	3	33± 3	10±2	53±2	3.7±1.1
	10^{-9} M	8	35± 4	15±3	56±5	N.D.
	10^{-8} M	10	44± 3	13±3	49±4	N.D.
	10^{-7} M	14	40± 4	12±5	47±6	N.D.
CSC	1.5 µg/ml	3	40± 2	10±1	47±5	1.3±0.6
	3.0 µg/ml	3	47± 7	5±1	45±5	3.7±1.8
	6.0 µg/ml	9	43± 5	7±1	50±7	0.9±0.7
	12.0 µg/ml	8	42± 4	15±6	43±6	4.1±2.4
	24.0 µg/ml	13	44±10	18±4	34±5	2.8±1.3

[a] The epithelial cell types are given as mean percentage of the total number of epithelial cells
[b] Combined results of DMSO and medium. *n* is the number of tracheal rings. ND is not detectable, the number of indeterminate cells was smaller than 0.1%

Differentiation. Tracheal epithelial cell differentiation was not influenced by the addition of 0.1% DMSO compared with the control medium. Therefore, the results for cell differentiation are combined for culture medium with and without DMSO (Tables 4 and 5).

In the early *in vitro* period no distinct differences in cellular differentiation were found between the different retinol concentrations, with the

Table 5. Cumulative (day 10 and 12) late effects of all-trans retinol and cigarette smoke condensate (CSC) on the differentiation of cultured tracheal epithelium

Treatment	Concentration	*n*	Tracheal epithelial cell types[a]			
			Basal	Ciliated	Mucous	Indeterminate
Control[b]		15	41±4	7±2	42±4	6.0±1.1
Retinol	10^{-11} M	3	43±2	5±1	54±2	2.4±0.4
	10^{-10} M	6	42±1	8±2	46±6	2.5±0.7
	10^{-9} M	5	41±2	10±2	48±5	0.8±0.1
	10^{-8} M	6	36±2	9±1	54±5	1.4±0.3
	10^{-7} M	8	30±2	11±2	60±2	0.6±0.1
CSC	1.5 µg/ml	6	41±4	7±1	55±6	0.3±0.1
	3.0 µg/ml	5	44±4	4±1	49±2	1.6±0.4
	6.0 µg/ml	9	42±2	6±1	44±5	6.4±0.7
	12.0 µg/ml	7	46±2	5±1	39±6	11.3±2.0
	24.0 µg/ml	8	48±3	4±1	34±4	9.7±2.0

[a] The epithelial cell types are given as mean percentage of the total number of epithelial cells
[b] Combined results of DMSO and medium. *n* is the number of tracheal rings

Table 6. Effects of all-trans retinol and cigarette smoke condensate (CSC) on the differentiation of cultured tracheal epithelium in combination experiments

First[a] treatment	Second[b] treatment	Tracheal epithelial cell types[c]			
		Basal	Ciliated	Mucous	Indeterminate
CSC 24 µg/ml[d]	RET 10^{-11} M[e]	47±4	11±2	39±4	5.5±1.6
CSC 24 µg/ml	RET 10^{-7} M	40±3	10±2	45±4	2.9±1.0
RET 10^{-11} M	CSC 24 µg/ml	46±2	14±3	37±2	7.1±3.0
RET 10^{-7} M	CSC 24 µg/ml	33±4	6±1	61±5	2.3±0.9
RET 10^{-11} M +	CSC 24 µg/ml[f]	45±9	13±2	33±3	6.0±2.3
RET 10^{-7} M +	CSC 24 µg/ml	41±7	16±1	40±4	1.7±0.4

[a] Exposure to the test substance for the first 6 (day 0–6) culture days
[b] Exposure to the test substance for the second 6 (day 6–12) culture days
[c] The number of tracheal organ cultures is 3 for each combination experiment. The epithelial cell types are given as mean percentage of the total number of epithelial cells
[d] CSC is cigarette smoke condensate
[e] RET is all-trans retinol
[f] Simultaneous exposure to all-trans retinol and CSC during 12 culture days

exception of the presence of indeterminate cells at low retinol concentrations (Table 4). In the late in vitro period the number of basal and indeterminate cells decreased, while the number of mucus-producing cells increased with increasing retinol concentrations (Table 5 and Fig. 3). Furthermore, a relative low number of ciliated cells was observed with the lowest retinol concentration.

CSC treatment slightly stimulated the number of basal cells and reduced the number of mucous cells in both the early and the late period (Tables 4 and 5). A remarkable difference was observed in the number of ciliated cells, with an increase in the early period (Table 4) and a decrease in the late period

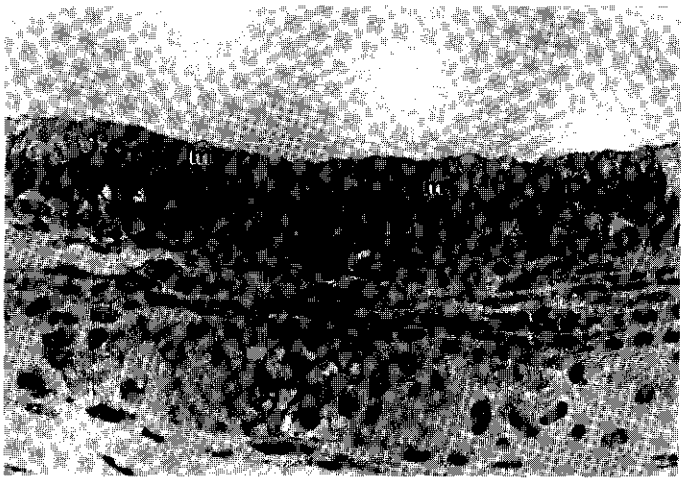

Fig. 8. Tracheal epithelium cultured for 6 days with retinol 10^{-7} M followed by treatment with 24 μg CSC/ml. Pseudostratified epithelium which consists of columnar mucous cells (M). No ciliated cells are present. Multiple Stain × **425**

(Table 5). Due to the CSC-induced hyperplasia and squamous metaplasia, the number of indeterminate cells in the late in vitro period also increased.

Effect of combined exposures to retinol and cigarette smoke condensate

Morphology. Treatment with CSC (24 μg CSC/ml) followed by retinol (10^{-7} and 10^{-11} M) induced keratinization and hyperplasia in all tracheal rings, but hyperplasia was less obvious in the presence of retinol 10^{-7} M compared with retinol 10^{-11} M (Table 3). However, if the tracheas were first treated with retinol 10^{-7} M followed by CSC, mild hyperplasia but no keratinization or squamous metaplasia was observed. Retinol 10^{-11} M followed by CSC treatment showed almost the same changes as the reversed treatment. Simultaneous exposure to retinol 10^{-7} M and CSC resulted in decreased metaplasia and the absence of keratin or keratohyaline granules compared with exposure to retinol 10^{-11} M and CSC.

Differentiation. In all the combination experiments a decreased number of indeterminate and basal cells and an increased number of mucus-producing cells were found with retinol 10^{-7}M (Table 6). The largest differences were observed after retinol exposure followed by the CSC treatment. Figure 8 shows pseudostratified tracheal epithelium treated with retinol 10^{-7} M (6 days) followed by CSC 24 μg/ml (6 days).

Discussion

Effects of retinol on cultured tracheal epithelium

The results obtained in the present study agree with those of Boren et al. (1974) and McDowell et al. (1984b), who reported increased numbers of basal cells and decreased numbers of ciliated cells in vitamin A-deprived epithelium. However, with respect to the mucous cells, the results are contradictory. McDowell et al. (1984b) reported a decreased number of mucous cells whereas in the present study and in that of Boren (1974), no significant changes in the number of mucous cells were found. The effects on cellular differentiation found in the present study are comparable with those reported by Chopra (1983b) during *in vitro* vitamin A deprivation, with the exception of the increased number of basal cells. The increased number of basal cells in Chopra's (1983b) study can be explained by different classification criteria used. According to our classification of cell types we scored basally located cells present in the mid-epithelium as indeterminate cells because they have no cellular characteristics.

Effects of cigarette smoke condensate in cultured tracheal epithelium

In the present study, dose-related hyperplasia and squamous metaplasia were observed with increasing CSC concentrations. During the early *in vitro* period only hyperplasia was found, which was followed by both hyperplasia and squamous metaplasia in the late *in vitro* period. Furthermore, the hyperplasia induced by CSC was more marked than that which occurred in vitamin A-deficient cultures, and seems to originate from basal cells.

Histologically, distinct differences exist between CSC-induced metaplastic changes and squamous metaplasia induced by vitamin A-deficiency. Most CSC induced lesions showed severe parakeratosis, atypia and exophytic growth which were comparable to observations reported after treatment with benzo(a)pyrene *in vivo* (Harris et al. 1972) and *in vitro* (Richter-Reichhelm et al. 1982a,b). Michiels et al. (1981) also reported a correlation between benzo(a)pyrene- and CSC-induced preneoplastic lesions in embryonic rat lung cultures.

Treatment of tracheal explants with CSC decreased the number of mucous cells and increased the number of ciliated cells in a dose-dependent way in the early *in vitro* period. In the late *in vitro* period the decreased number of

mucous cells was accompanied by a decrease in the number of ciliated cells. This decrease of ciliated cells in the late period is probably due to the presence of larger foci of squamous metaplasia without ciliated cells. In the non-squamous epithelium a larger proportion of the total cells is ciliated (about 30%). Chopra and Cooney (1985) also reported stimulation of ciliogenesis by benzo(a)pyrene treatment after 3 days culture and a decrease after 10 days culture.

Effects of retinol and CSC on cultured tracheal epithelium

Lasnitzki and Bollag (1982;1987) reported a reversion of benzo(a)pyrene- and CSC-induced epithelial hyperplasia and squamous metaplasia in cultured neonatal rat tracheas by synthetic retinoids. With respect to the epithelial differentiation, we found greater changes if the tracheas were first treated with a physiologically active retinol concentration followed by CSC treatment.

The sequence of adding retinol or CSC appeared to be important. As expected, a low retinol concentration (10^{-11} M) did not prevent hyperplasia, squamous metaplasia and keratinization respectively if tested in combination with CSC, whereas a higher retinol concentration (10^{-7} M) prevented or reduced these alterations if retinol was given prior to or simultaneously with CSC.

In conclusion, CSC and vitamin A have been shown to produce distinct effects on vitamin A-deprived epithelium in organ culture. Combination experiments with CSC and retinol showed that all-trans retinol or its metabolites can influence histomorphological alterations induced by CSC in this model system. However, the role of ciliated cells in CSC-induced alterations and the development of early metaplastic changes needs further investigation.

Acknowledgements

The authors would like to thank Dr. V.J. Feron and Ing. B.J. Spit for helpful discussions, Prof. dr. J.H. Koeman for reviewing the manuscript and Mr. W.G. Rovers for preparing the sections for histologic examination. The study was supported by grants from the Scientific Advisory Committee on Smoking and Health (Dutch Cigarette Industry Foundation) and the Ministry of Welfare, Health and Cultural Affairs.

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CHAPTER 5

Intermediate filament expression in normal and vitamin A-deprived cultured hamster tracheal epithelium as detected by monoclonal antibodies

A study with emphasis on histological changes

Virchows Archiv B Cell Pathology (1988) 56, 103-110

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Intermediate filament expression in normal and vitamin A-depleted cultured hamster tracheal epithelium as detected by monoclonal antibodies. A study with emphasis on histological changes

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Summary

Using immunohistochemical techniques, the keratin expression patterns in basal and columnar cells (mucus-producing and ciliated cells) were investigated in tracheal organ cultures. Tracheas were from either hamsters fed a control diet or from hamsters fed a vitamin A-deficient diet; tracheas from the latter group were treated in vitro with all-trans retinol. In tracheas from hamsters fed a control diet, basal cells generally reacted with the RCK102 antibody and columnar cells with the RGE53 and the HCK19 antibodies, and both basal and columnar cells were recognized by the RCK105 antibody. The squamous cell cytokeratin 10 (detected by the RKSE60 antibody) was not expressed in cultured tracheas from hamsters fed a normal or a vitamin A-deficient diet. In the course of the in vitro period a number of keratins were "switched on" or "switched off" in both basal and columnar cells. In tracheas from vitamin A-deprived hamsters the RCK102 antibody clearly recognized basal cells and cigarette smoke condensate (CSC)-induced proliferating basal cells, whereas the RGE53 antibody reacted with mucus-producing and ciliated cells. During organ culture foci of columnar epithelial cells expressed basal cell properties (detected with the RCK102 antibody) after all-trans retinol treatment and were found negative for the RGE53 antibody. Furthermore, it appeared that the RGE53-negative columnar cells contained periodic acid Schiff-positive mucous granules. These findings indicate that basal cells may differentiate into columnar cells. Tracheal epithelium did not appear to co-express vimentin next to keratins during organ culture, which may be due to the intact three-dimensional organization present in these organ cultures.

Introduction

The class of cytokeratins represents a morphologically homogeneous subgroup of intermediate-sized filament proteins specific to tissues of epithelial

origin (Moll et al. 1982). The keratin expression patterns depend on epithelial cell type and stage of development or differentiation, and changes in these expression patterns may be related to the differences in molecular weights, isoelectric points and immunological properties of the individual keratin proteins (Franke et al. 1981; Moll et al. 1982; Ramaekers et al. 1983).

Retinoids have an influence on proliferation and differentiation of epithelial cells and are essential for the maintenance of mucociliary activities in respiratory epithelium (Sporn and Roberts 1984). Previous studies showed that exposure to retinoids changes the cytokeratin expression patterns in different types of mammalian epithelial cells (Fuchs and Green 1981; Eckert and Green 1984; DeLuca et al. 1985; Huang et al. 1986; Wu and Wu 1986). Human keratinocytes cultured in medium supplemented with 10 to 20% serum containing vitamin A do not express the high molecular weight keratins (acidic 56.5 kDa and basic 65-67 kDa proteins). However, in the absence of serum these keratins are expressed (Fuchs and Green 1981). Wu and Wu (1986) reported that retinoids inhibit the synthesis of the 48 kDa and 50 kDa keratin proteins and stimulate the synthesis of the 40 kDa and 52-54 kDa keratin proteins in cultured human bronchial epithelial cells. Furthermore, when hamsters are kept on a vitamin A-deficient diet changes in the keratin biosynthesis of tracheal epithelium are induced in organ culture (DeLuca et al. 1985; Huang et al. 1986). The squamous vitamin A-depleted tracheal epithelium expressed the 45, 46.5, 48, 50, 52, 55, 56 and 60 kDa keratin proteins, whereas normal mucociliated epithelium cultured in the presence of retinoic acid does not express these keratins. It has been suggested that retinoids influence keratin expression at the transcriptional level (Fuchs and Green 1981; Eckert and Green 1984).

We studied intermediate filament protein expression in cultured tracheal epithelial rings, with emphasis on the cellular proliferation and differentiation processes. Furthermore, we investigated the role of basal and proliferating basal cells in the genesis of differentiated tracheal epithelial cells, and the expression of vimentin.

Materials and methods

Animals and diets. Syrian Golden hamsters were obtained from the TNO Central Institute for the Breeding of Laboratory Animals, Zeist, Netherlands.

Pregnant hamsters were either fed a control cereal-based diet (ca. 5000 IU vitamin A/kg) or a diet with a reduced vitamin A content (ca. 2500 IU vitamin A/kg) during the last 6 days of gestation. After birth, mothers and neonates of the vitamin A-reduced group received a vitamin A-deficient diet (ca. 40 IU vitamin A/kg), whereas the hamsters of the control group received the normal cereal-based diet. Tracheas were excised for organ culture when the hamsters were 32 days old (Rutten et al. 1988a).

Tracheal organ culture. Tracheal rings were cultured for up to 10 days in a serum-free CMRL-1066 medium (Flow Laboratories, Rickmansworth, UK) supplemented with L-glutamine 2 mM (Flow), hydrocortisone 0.1 mg/l, bovine pancreas insulin 1.0 mg/l (Sigma Chemicals, St. Louis, MO, USA) and gentamicin 50 mg/l (Flow). Cultures were rocked eight to nine times per min in a humidified incubator at 37°C in 50% O_2, 45% N_2 and 5% CO_2 (Rutten et al. 1988a). The vitamin A-depleted tracheas were treated with 1 ml/l dimethylsulphoxide (DMSO) or with all-trans retinol (Fluka AG, Buchs, Switzerland) dissolved in DMSO at concentrations of 10^{-11} and 10^{-7} M. Cigarette smoke condensate (CSC) was used at a final concentration of 24 mg/l to induce basal cell proliferation in vitamin A-depleted tracheal epithelium (for preparation of CSC, see Rutten and Wilmer 1986). The control tracheas were treated with 1 ml/l DMSO.

Characterization of the monoclonal intermediate filaments antibodies. The following monoclonal antibodies against intermediate filament proteins were used: RGE53 (against human cytokeratin (HCK) 18); RKSE60 (against HCK 10); RCK102 (against HCK 5 and HCK 8); RCK103 (which recognizes keratins and specifically stains basal cells in several organs; Ramaekers et al. 1987); RCK105 (against HCK 7); clone 4.62 (against HCK 19; Bio-Makor, Rehovot, Israel); and vimentin (against swine vimentin, recognizes human and rodent vimentin; Dakopatts, Glostrup, Denmark). The classification of the keratins used has been published by Moll et al. (1982). More detailed information on the characteristics and specificities of the monoclonal intermediate filaments antibodies has been published previously (Ramaekers et al. 1987).

Immunohistological staining. The tracheal rings were snap-frozen in isopentane at ca. -160° C, and stored in liquid nitrogen. Cryostat sections (7 µm) were mounted on glass slides which were pre-coated with poly-l-lysine, airdried and fixed for 10 min in acetone.

Table 1. Expression of intermediate filaments in cultured tracheal epithelium from control hamsters

Antibodies	Number of days in culture									
	2		4		6		8		10	
	B	M-C	B	M-C	B	M-C	B	M-C	B	M-C
RCK103	+++[a]	+++	+++	+++	+++	+++	+++	+++	+++	+++
RKSE60	0	0	0	0	0	0	0	0	0	0
RCK105	+++	+++	+++	+++	+++	+++	++	+++	+	+++
HCK19	+/−	+++	0	+++	0	+++	0	+++	0	+++
RGE53	0	+++	0	+++	0	+++	0	+++	0	+++
RCK102	+++	+/−	+++	+/−	+++	+/−	+++	0	+++	0
Vimentin	0	0	0	0	0	0	0	0	0	0

B: basal cells
M–C: mucus-producing and ciliated cells
[a] +++ proportion of peroxidase-positive cells greater than 90%
++ proportion of peroxidase-positive cells between 40 and 90%
+ proportion of peroxidase-positive cells between 10 and 40%
+/− proportion of peroxidase-positive cells between 1 and 10%
0 proportion of peroxidase-positive cells smaller than 1%

Table 2. Expression of intermediate filaments in cultured vitamin A-depleted hamster tracheal epithelium treated with retinol 10^{-7} M

Antibodies	Number of days in culture					
	2		6		10	
	B	M-C	B	M-C	B	M-C
RCK103	+++[a]	+++	+++	+++	+++	+++
RKSE60	0	0	0	0	0	0
RCK105	+	+++	+/−	+++	+++	+++
HCK19	+	+++	+	+++	+/−	+++
RGE53	+	+++	+++	+++	+/−	+++
RCK102	+++	+	+++	++	+++	+/−
Vimentin	0	0	0	0	0	0

B: basal cells
M–C: mucus-producing and ciliated cells
[a] +++ proportion of peroxidase-positive cells greater than 90%
++ proportion of peroxidase-positive cells between 40 and 90%
+ proportion of peroxidase-positive cells between 10 and 40%
+/− proportion of peroxidase-positive cells between 1 and 10%
0 proportion of peroxidase-positive cells smaller than 1%

The fixed cryostat sections were rinsed in phosphate-buffered saline (PBS, 0.01 M, pH7.4) and incubated with the primary antibody (undiluted culture supernatant, except for HCK19 which was diluted 1:40) at 20° C for 1 h in a moist chamber, rinsed for 30 minutes in PBS, then incubated for 30 min with a peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark), diluted 1:300 in PBS, rinsed in PBS followed by Tris/HCl (0.05 M, pH7.6), and finally incubated for 10 min with the chromogen diaminobenzidine as described by Polak and Noorden (1983). The sections were counterstained with Gill's Haematoxylin activity 3 (Polysciences, Warrington, FL, USA), or stained with periodic acid Schiff (PAS) (Merck, Darmstadt, FRG)

Results

The epithelium of cultured tracheal rings showed a pseudostratified columnar epithelium expressing different cytokeratins depending on the degree of differentiation (Figs. 1-3 and Tables 1, 2).

RCK103. The keratin(s) detected by the RCK103 antibody are present in all epithelial cell layers, in both control tracheas treated with 1 ml/l DMSO and in vitamin A-depleted tracheas treated with 10^{-7} M all-trans retinol. This expression pattern is maintained during the whole organ culture period (Tables 1, 2).

RKSE60. In control tracheas, and in vitamin A-depleted tracheas treated *in vitro* with all-trans retinol (10^{-7} M), the development of squamous metaplasia is inhibited. This histomorphological observation is supported by a RKSE60-negative reaction in the epithelium of all organ cultures indicating that no (single cell) keratinization occurs (Tables 1, 2).

HCK19. Immunostaining with the antibody against HCK19 in control tracheas shows that the basal epithelial cells are negative for this antibody, while the mucus-producing and ciliated cells are strongly positive (Table 1). In vitamin A-depleted tracheal epithelium cultured for 2 days in the presence of retinol (10^{-7} M), a significant number of basal cells become positive with this antibody, whereas the mucus-producing and ciliated cells seem to stain somewhat less intensely than those of control epithelium. After the addition of retinol (10^{-7} M) to these vitamin A-depleted tracheas, HCK19 expression occurs only in the mucus-producing and ciliated cells after 10 days, which is comparable to the HCK19 expression found in control tracheas on culture day 2 (Tables 1, 2).

RGE53 and RCK102. The expression of keratins detected by the antibodies RGE53 and RCK102 in control tracheal epithelium remains almost unchanged during the whole culture period (Table 1). RGE53 stains the mucus-producing and ciliated cells and is virtually negative in the basal cells, but RCK102 reacts mainly with basal cells and leaves most of the mucus-producing and ciliated cells unstained (Fig. 1A). In vitamin A-depleted tracheas treated *in vitro* with a biologically active retinol concentration, however, a number of changes are observed with respect to the staining patterns of RGE53 and RCK102 (Table 2; Figs. 1-3). The addition of retinol (10^{-7} M) to vitamin A-depleted tracheal rings increases the number of RCK102-positive mucus-producing and ciliated cells between culture days 2 and 6. On day 10 the number of RCK102-

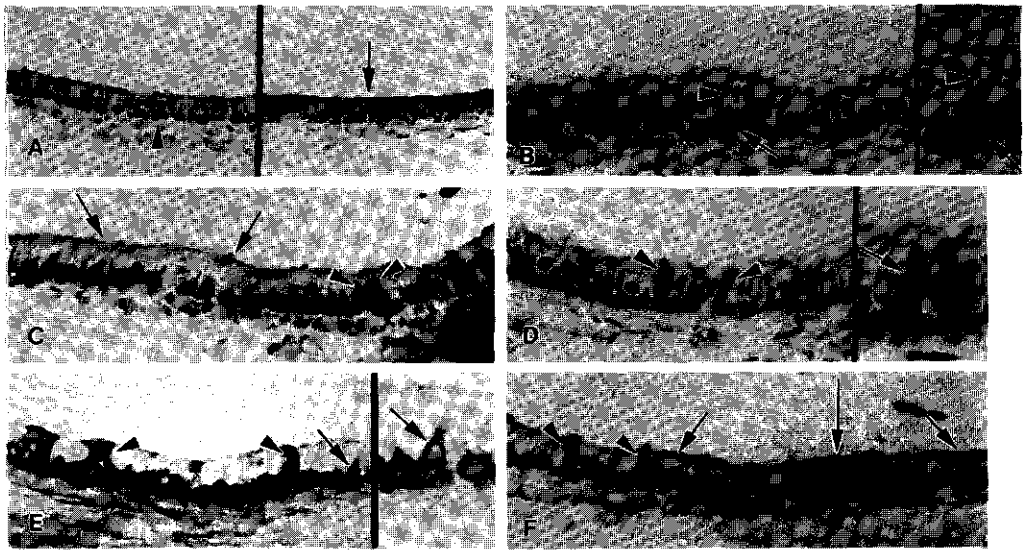

Fig. 1. Tracheal epithelium of vitamin A-deprived hamsters cultured in serum-free hormone-supplemented medium treated with DMSO, all-trans retinol or cigarette smoke condensate for several days. The tracheal cryostat sections were incubated with the monoclonal antibodies against intermediate filaments followed by an immunoperoxidase staining procedure (see Materials and methods). (**A**) Cultured tracheal epithelium treated with all-trans retinol (10^{-7} M) for 10 days. The left micrograph show RCK102-positive basal cells (*arrowhead*), and the right micrograph RGE535-positive mucus-producing and ciliated cells (*arrow*). Haematoxylin counter-staining, × **180**. (**B**) Tracheal epithelium treated in vitro with all-trans retinol (10^{-7} M) for 6 days showing two daughter basal cells (*arrow*) which are RCK102-positive. One of the two cells (the most apically situated cell) shows a thin line of cytoplasm that migrates towards the lumen (*arrowhead*). Haematoxylin counter-staining, × **180**. *Inset:* enlargement of the two daughter basal cells, × **400**. (**C**) Cryostat section of cultured normal tracheal epithelium treated with DMSO (1 ml/l) for 6 days showing RCK102-positive basal cells and some RCK102-positive cells in the mid-epithelial part containing lines of cytoplasm (*arrowheads*) that may migrate towards the tracheal lumen. Faint periodic acid-Schiff (PAS)-positive staining is present at the apical side of the epithelium (*arrows*). PAS counter-staining, × **180**. (**D**) Tracheal epithelium obtained from vitamin A-deprived hamster treated in vitro for 6 days with retinol 10^{-7} M showing several RCK102-positive mucus-producing and ciliated cells (*arrowheads*) and basal cells. × **180**. *Inset:* enlargement of RCK102-positive cells (*arrow*). Haematoxylin counter-staining, × **400**. (**E**) Cultured tracheal epithelium obtained from hamsters fed a control diet, treated with DMSO (1 ml/l) for 6 days showing two divided basally situated cells (*arrow*) which are RCK102-positive. Some tracheal epithelial cells (*arrowheads*) which are positive show a cytoplasm that reaches to the tracheal lumen. PAS counter-staining, × **180**. *Inset:* enlargement of the two basally situated cells, × **400**. (**F**) Vitamin A-depleted tracheal epithelium treated with cigarette smoke condensate (24 mg/l) for 10 days. Basal tracheal epithelial cell, two mucus-producing or ciliated cells (*arrowheads*) and a foci of mucus-producing or ciliated cells (*large arrow*) are RCK102-positive. Faint periodic acid-Schiff (PAS)-positive staining is present at the apical side of the epithelium (*small arrows*). PAS counter-staining, × **180**

positive mucus-producing and ciliated cells is decreased. On day 6 a significant increase in the number of RGE53-positive basal cells is seen compared with days 2 or 10 (Table 2). Fig. 1A (left) shows a lining of RCK102-positive basal epithelial cells and RGE53-positive mucus-producing and ciliated cells (Fig. 1A, right) in a vitamin A-depleted trachea that had been treated in vitro with retinol (10^{-7} M) for 10 days. The staining pattern of RCK102 is comparable with the basal cell staining reaction found in control tracheal epithelium (Table 1). The number of RCK102-positive mucus-producing and ciliated cells is increased on day 6 in vitamin A-depleted tracheal epithelium

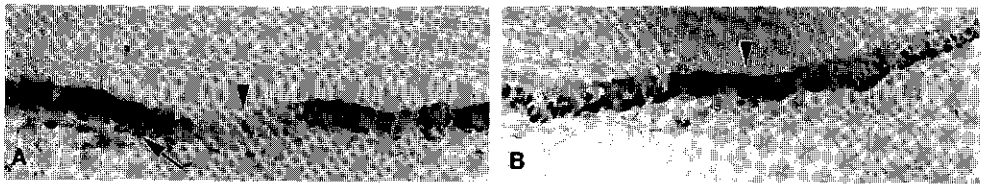

Fig. 2. Serial cryostat sections of vitamin A-depleted hamster tracheas treated with all-trans retinol 10^{-7} M for 10 days. (**A**) Section showing a foci of mucus-producing or ciliated cells that are negative for RGE53 (*arrowhead*) surrounded by RGE53-positive cells. Basal epithelial cells are negative (*arrow*). Haematoxylin counter-staining. ×180. (**B**) Cryostat section showing that the RGE53-negative epithelial cells shown in A are RCK102-positive (*arrowhead*). Haematoxylin counter-staining. ×180

Fig. 3. Serial cryostat sections of vitamin A-depleted tracheal epithelium treated with all-trans retinol (10^{-7} M) for 10 culture days. (**A**) The micrograph shows a line of basal RCK102-positive cells and a foci of mucus-producing and ciliated RCK102-positive cells (*arrow*). Note the small mucous granule cells which are PAS-positive (*arrowheads*). PAS counter-staining. ×180. (**B**) Serial section showing that the mucus-producing and ciliated RCK102-positive cells shown in A are RGE53-negtive (*arrow*) and are for the greater part small mucous granule cells which are PAS-positive (*arrowheads*). PAS counter-staining. ×180

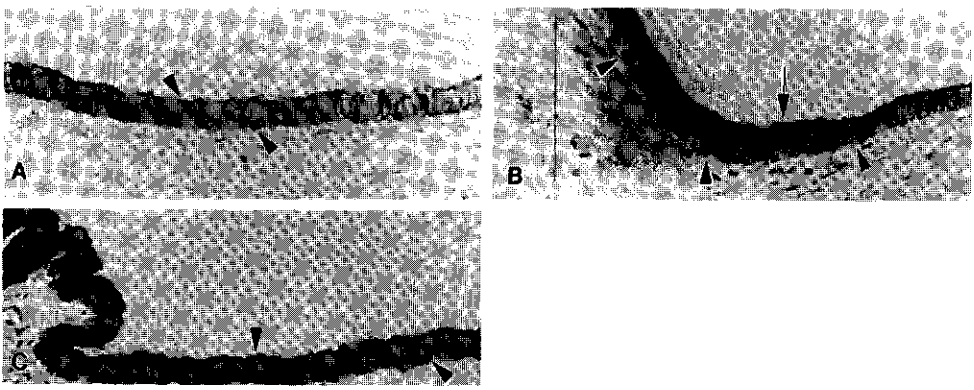

Fig. 4. (**A**) Normal tracheal epithelium cultured for 2 days showing RCK105-positive basal and mucus-producing and ciliated cells (*arrowheads*). Haematoxylin counter-staining, ×180. (**B**) Six day cultured tracheal epithelium of vitamin A-deprived hamsters treated with retinol 10^{-7} M showing basal cells that are RCK105-negative (*arrowheads*). The mucus-producing and ciliated cells are RCK105-positive (*arrow*). Haematoxylin counter-staining, ×180. (**C**) Vitamin A-depleted tracheal epithelium treated with all-trans retinol 10^{-7} M for 10 days showing both basal and mucus-producing and ciliated cells RCK105-positive tracheal epithelial cells (*arrowheads*). Haematoxylin counter-staining. ×180

treated with all trans retinol (10^{-7}M; Figs. 1B, 1D), DMSO (1 ml/l; Fig. 1C) or cigarette smoke condensate (24 mg/l; Fig. 1F). Interestingly, two basal epithelial daughter cells are both RCK102-positive, and the most apically situated cell shows a thin line of RCK102-positive cytoplasm reaching towards the tracheal lumen (Figs. 1B, 1D, 1E). In serial cryostat sections, areas with

RCK102-positive mucus-producing and ciliated cells are complementary to areas of RGE53-negative cells (compare Figs. 2A, 2B, and 3A, 3B). Most of these cell groups are found to contain faint PAS-positive small mucous granules (Figs. 3A, 3B). Upon electron microscopic examination of acetone-fixed tracheal rings a few of these RCK102-positive non-basal cells appear to be ciliated cells (data not shown).

RCK105. In control tracheas virtually all epithelial cells are RCK105-positive at the beginning of the culture period (Fig. 4A). However, during organ culture the number of RCK105-positive basal cells decreases (Fig. 4B), whereas the mucus-producing and ciliated cells remain RCK105-positive (Table 1). In contrast, vitamin A-depleted tracheal epithelium shows many RCK105-negative basal cells at the beginning of the culture period. The addition of retinol results in an increasing number of RCK105-positive basal cells during the organ culture period (Table 2; Fig. 4C). No changes are observed in the staining patterns of mucus-producing and ciliated cells with the RCK105 antibody during the 10-day culture period (Fig. 4A-C).

Epithelial cells of tracheal rings obtained from normal and vitamin A-deprived hamsters do not co-express the intermediate filament vimentin besides the various keratins during the 10-day culture period, whereas the mesenchymal cells (fibroblasts in the lamina propria and cartilage cells) are vimentin-positive (Tables 1, 2).

Discussion

There is wide agreement on the essential role of retinoids, especially retinol and retinoic acid, in the regulation of cellular proliferation and differentiation in tracheal epithelium (Sporn and Roberts 1984; McDowell et al. 1984a,b; Rutten et al. 1988a-c). The relationship between cytokeratin expression and vitamin A deprivation in hamster tracheal epithelium has been reported recently (Huang et al. 1986; DeLuca et al. 1985; Rutten et al. 1988d). Huang et al. (1986) found that keratin biosynthesis increases in vitamin A-depleted squamous epithelium as detected by immunoblot analysis with polyspecific antibodies. DeLuca et al. (1985) reported similar changes in keratin expression as a result of vitamin A deficiency and benzo[a]pyrene exposure. Other workers have reported that retinoids influence keratin expression at the transcriptional level (Fuchs and Green 1981; Eckert and Green 1984). Recently,

two studies reported the finding of a human retinoic receptor belonging to the family of nuclear receptors (Giguere et al. 1987; Petkovich et al. 1987). Moreover, a group of closely related retinoic acid receptor-related genes have been found to show also an affinity for retinol. Petkovich et al. (1987) suggested a conversion of retinol into retinoic acid before binding to the receptor. This important discovery shows a direct interaction of retinoids (retinol and retinoic acid) with DNA, and may indicate that retinoids influence keratin expression at the DNA level.

Concerning the monoclonal antibody RCK102 (which recognizes human keratins 5 and 8), there are major differences in staining patterns between human epithelium (Ramaekers et al. 1983) and cultured hamster tracheal epithelium as found in the present study. In human tracheal epithelium both keratins 5 and 8 are expressed (Moll et al. 1982). Therefore, in hamsters the RCK102-antibody is not necessarily specific for the hamster counter-part of the corresponding human keratin. It could be possible that antibody RCK102 recognizes only one of the human keratins in hamster tracheal epithelium.

The tracheal epithelium, both in vivo and in organ cultures, consists of a lining of pseudostratified epithelium. Although it is still unknown which pathways are important in the morphogenesis of differentiated tracheal epithelial cells, some authors indicate that both basal (Lane and Gordon 1979; Chopra 1982; 1983; Inayama et al. 1988) and small mucous granule cells (McDowell et al. 1984a,b; Sigler et al. 1988) play a role in this process. The results obtained in the present study indicate that, in addition to small mucous granule cells, dividing basal epithelial cells in the trachea give rise to columnar cells. Furthermore, in previous studies we have reported that ciliated cells in tracheal organ cultures can proliferate (Rutten et al. 1988c), and that CSC exposure and vitamin A-depletion produce distinct basal cell proliferation (Rutten et al. 1988b). The fact that vitamin A-depleted mucus-producing and ciliated cells express basal cell properties after retinol and CSC treatment (as concluded from the RCK102-positive and the RGE53-negative reaction) suggests: 1) an increased rate of differentiation of basal cells into mucus-producing and ciliated cells, or 2) a switch-on of the RCK102-keratin and a switch-off of the RGE53-keratin in genuine columnar cells under the conditions used. Furthermore, it appeared that there are two types of PAS-positive small mucous granule cells: 1) cells that are recognized by RCK102-positive/RGE53-negative staining, and 2) cells that are RCK102-negative/RGE53-positive. It is suggested that the first group of small mucous

granule cells are immature columnar cells still containing the basal cell keratin pattern, and that the secound group are more mature, terminally differentiated, small mucous granule cells which have lost the basal cell keratin. In view of these findings, we assume a role for basal cells in the morphogenesis of differentiated columnar tracheal epithelial cells.

In recent years several studies have reported co-expression of keratin and vimentin in certain normal tissues, in tumours, during embryonic development, and especially in cell cultures (Virtanen et al. 1981; Lane et al. 1983; Ramaekers et al. 1983; Emura et al. 1986: Gatter et al. 1986; Paranko and Virtanen 1986; Gröne et al. 1987). However, in the present study co-expression of vimentin and keratin was not observed in the tracheal epithelium of organ cultures, at least during the 10-day culture period of control epithelium, or in vitamin A-depleted epithelium treated with all-trans retinol. Moreover, neither CSC-treated nor vitamin A-depleted tracheal epithelium co-expresses vimentin and keratin as detected by immunohistological techniques (Rutten et al. 1988d). Respiratory epithelial cells in vitro may, however, co-express cytokeratin and vimentin (Emura et al. 1986), whereas vimentin is not detectable in non-cultured tracheal epithelium. A possible explanation for the absence of vimentin in the epithelium of tracheal organ cultures has been given by La Rocca and Rheinwald (1984), who suggested that cell-to-cell interactions in the three-dimensional organization of cells suppress the expression of vimentin in addition to keratin. Considering the mechanisms of action, experiments with cells in monolayers have pointed to a role for cellular proliferation or desmosomal disruption in co-expression (Ben-Ze'ev A 1986; Emura et al. 1986). Furthermore, our findings with tracheal organ cultures do not indicate that all-trans retinol influences the co-expression of vimentin in the epithelium. Therefore, we conclude that organized tissue structure along with cellular interactions and contacts may prevent the induction of vimentin expression in tracheal epithelium in organ culture.

In conclusion, the results obtained in the present study indicate that columnar tracheal epithelial cells may derive from basal cells. Furthermore, the RCK102 and the RGE53 antibodies seem to be useful tools for studing the role of basal cells in the restoration of mucociliary epithelium from vitamin A-depleted hamster tracheas. In tracheal organ cultures co-expression of cytokeratin and vimentin was not observed. This may be due to the intact three-dimensional organization present in these organ cultures.

Acknowledgements

This study was supported by grants from the Scientific Advisory Committee on Smoking and Health (Dutch Cigarette Industry Foundation) and the Ministry of Welfare, Health and Cultural Affairs, and partly by the Netherlands Cancer Foundation. We would like to thank Dr V.J. Feron and Dr J.W.G.M. Wilmer for helpful discussions, and Prof. Dr J.H. Koeman for critically reading the manuscript. We thank A. Huijsmans, G. Schaart, O. Moesker and K. Verrijp for excellent technical assistance in the preparation and specificity testing of the monoclonal antibodies.

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CHAPTER 6

Effect of vitamin A-deprivation and cigarette smoke condensate on keratin expression patterns in cultured hamster tracheal epithelium

An immunohistomorphological study using monoclonal antibodies to keratins

Virchows Archiv B Cell Pathology (1988) 56, 111-117

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Effect of cigarette smoke condensate and vitamin A depletion on keratin expression patterns in cultured hamster tracheal epithelium.
An immunohistomorphological study using monoclonal antibodies to keratins.

Summary

Keratin expression in hamster tracheal epithelium was investigated during organ culture in serum-free, hormone-supplemented medium using monospecific monoclonal antibodies. Generally, tracheal basal cells expressed keratins detected by antibodies RCK102 and RCK103, while columnar epithelial cells were stained positively by RGE53, RCK103, RCK105 and HCK19. Metaplastic squamous cell foci reacted with antibodies RKSE60, RCK103 and HCK19. Early metaplastic alterations were more clearly RKSE60-positive than the mature lesions. In the vitamin A-depleted tracheas basal cells were clearly RCK102-positive. Superficial cells in the central part of areas of squamous metaplasia induced by cigarette smoke condensate expressed the basal cell keratins, and were negative for the columnar cell keratin 18 detected by the RGE53 antibody. This finding suggests that in cigarette smoke condensate induced squamous metaplasia basal cells play an important role. The mucus-producing cells at the edges of metaplastic squamous cell foci expressed the keratins specific to columnar cells. Cigarette smoke condensate exposure accelerated epithelial keratinization compared to the vitamin A-depleted epithelium. It was concluded that not only small mucous granule cells but also basal cells are involved in the development and maintenance of induced squamous metaplasia in tracheal epithelium. Furthermore, in vitro vitamin A-depleted epithelium did not co-express vimentin in addition to the different keratins.

Introduction

In tracheal organ cultures derived from vitamin A-deprived Syrian Golden hamsters, retinoids are capable of influencing epithelial cell proliferation and differentiation, while vitamin A deficiency induces squamous metaplasia with or without keratinization (Clamon et al. 1974; Sporn et al. 1976; Newton et al. 1980; Chopra 1982, 1983; Rutten et al. 1988a,b; Sigler et al. 1987;

1988). The mechanism by which vitamin A regulates cell proliferation and differentiation is not well understood. However, in vitamin A-depleted tracheal organ cultures keratin pattern changes in the mucociliary epithelium preceding the development of early metaplastic lesions can be studied in vitro.

Tobacco smoke and cigarette smoke condensate (CSC) show carcinogenic (IARC 1986), genotoxic (DeMarini 1983), co-mutagenic (Rutten and Wilmer 1986) and tumour-promoting activities (Gelhorn 1958; Wynder and Hoffmann 1961; Van Duuren et al. 1966; Bock and Clausen 1980). Lasnitzki (1958; 1968) has demonstrated CSC-induced hyperplasia and squamous metaplasia in human lung, and in mouse and rat tracheal organ cultures. The observed morphological changes could be reversed by aromatic retinoids (Lasnitzki and Bollag 1982; 1987), or all-trans retinol (Rutten et al. 1988b). Furthermore, CSC was found to inhibit dye-coupled intercellular communication in primary tracheal epithelial cells (Rutten et al. 1988c). The arrangement of junctional complexes, communication channels and the communication across these channels may be related to intermediate filament organization, cell morphology and phenotype (Kopan et al. 1987). In view of these findings one might assume that cigarette smoke (condensate) plays an important role in tumour promotion, and may influence keratin expression patterns.

In a previous study, changes in keratin expression patterns in normal tracheal epithelium and vitamin A-depleted epithelium treated in vitro with all-trans retinol have been described (Rutten et al. 1988d). The objective of the present study was to investigate the changes in intermediate filament (especially keratin) protein expression during the development of metaplastic lesions in tracheal epithelium induced by vitamin A depletion and CSC.

Materials and methods

Animals. Syrian Golden hamsters were obtained from the TNO Central Institute for the Breeding of Laboratory Animals, Zeist, Netherlands. Hamsters were deprived of vitamin A by feeding a vitamin A reduced diet from 10 days of pregnancy until the neonates were 32 days old (see Rutten et al. 1988a).

Cultured tracheas. Tracheas were excised from 32-day-old vitamin A-deprived hamsters and cultured for 10 days in a serum-free CMRL-1066 medium (Flow Laboratories, Rickmansworth, UK) supplemented with co-factors as described

previously (Rutten et al. 1988a). The tracheal rings were treated with solvent (1 ml dimethylsulphoxide (DMSO)/l), all-trans retinol (Fluka AG, Buchs, Switzerland) (10^{-11} M), or cigarette smoke condensate (CSC) (24 mg CSC /l)(for preparation of CSC see Rutten and Wilmer 1986).

Characterization of monoclonal antibodies against intermediate filaments. The following monoclonal antibodies against intermediate filament proteins were used: RGE53, RKSE60, RCK102, RCK103, RCK105, HCK19 and vimentin. In previous studies more detailed information has been given on the characteristics and specificities of the monoclonal intermediate filament antibodies (Ramaekers et al. 1987; Rutten et al. 1988d).

Immunohistological staining. Detailed information on the immunohistochemical staining method has been described (Rutten et al. 1988d). Briefly, cryostat sections (5–7 μm) on glass slides were fixed in acetone, washed and incubated with the monoclonal antibody against the different intermediate filaments. Thereafter they were stained with a peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark) followed by incubation with diaminobenzidine. The sections were counterstained with Gill's Hematoxylin (Polysciences, Warrington, FL,).

Results

Initially, pseudostratified tracheal epithelium of vitamin A-deprived hamsters undergoes squamous metaplasia during organ culture in the absence of vitamin A, in the presence of very low all-trans retinol concentrations, or in the presence of CSC. Changes in intermediate filament expression patterns in vitamin A-depleted tracheal epithelium, cultured at concentrations of vitamin A insufficient for normal differentiation (Table 1) or treated with CSC (Table 2), is detected by various monoclonal antibodies. No significant differences in tracheal epithelial intermediate filament expression patterns are found between treatments of epithelium with DMSO or all-trans retinol (10^{-11}M). These results have therefore been combined (Table 1). Changes in keratin expression in both metaplastic squamous cell foci and the adjacent normal pseudostratified epithelium are shown in Fig. 1 for vitamin A-depleted tracheas and in Fig. 2 for CSC-treated tracheas.

Table 1. Expression of intermediate filaments in cultured vitamin A-depleted hamster tracheal epithelium. Combined results of DMSO (1 ml/l) and all-trans retinol (10^{-11} M) treatment.

Antibody	Number of days in culture									
	2		4		6		8		10	
	B	M–C	B	M–C	B	M–C	B	M–C	B	M–C
RCK103	+++[a]	+++	++	+++	+++	+++	+++	+++	+++	+++
RKSE60	0	0	0	+/−	0	+/−	0	+/−	0	+/−
RCK105	++	+++	++	+++	+/−	+++	+/−	+++	+	+++
RGE53	0	+++	+	+++	0	++	+	+++	0	+++
RCK102	+++	0	+++	+	+++	+	+++	+/−	+++	+/−
HCK19	0	+++	0	+++	0	++	0	+++	0	+++
Vimentin	0	0	0	0	0	0	0	0	0	0

B: basal cells
M–C: mucus-producing and ciliated cells
[a] +++ proportion of peroxidase-positive cells greater than 90%
++ proportion of peroxidase-positive cells between 40 and 90%
+ proportion of peroxidase-positive cells between 10 and 40%
+/− proportion of peroxidase-positive cells between 1 and 10%
0 proportion of peroxidase-positive cells smaller than 1%

Table 2. Expression of intermediate filaments in cultured vitamin A-depleted hamster tracheal epithelium treated with cigarette smoke condensate (24 mg/l)

Antibody	Number of days in culture									
	2		4		6		8		10	
	B	M–C	B	M–C	B	M–C	B	M–C	B	M–C
RCK103	+++[a]	+++	+/−	+++	+++	+++	+++	+++	+++	+++
RKSE60	0	+/−	0	+/−	0	+	0	+/−	0	+/−
RCK105	+/−	+++	+/−	+++	+/−	+++	+/−	+++	0	+++
RGE53	0	+++	+++	+++	+++	+++	+/−	+++	0	++
RCK102	+++	+/−	+++	+	+++	+/−	+++	+/−	+++	++
HCK19	+/−	+++	0	++	+/−	++	0	+++	0	+++
Vimentin	0	0	0	0	0	0	0	0	0	0

B: basal cells
M–C: mucus-producing and ciliated cells
[a] +++ proportion of peroxidase-positive cells greater than 90%
++ proportion of peroxidase-positive cells between 40 and 90%
+ proportion of peroxidase-positive cells between 10 and 40%
+/− proportion of peroxidase-positive cells between 1 and 10%
0 proportion of peroxidase-positive cells smaller than 1%

RCK103. Immunohistochemical staining with the RCK103 antibody shows that at the beginning and after 10 days of culture metaplastic squamous epithelium, superficial keratin and normal tracheal epithelium are positive for this antibody in both vitamin A–depleted (Fig. 1A) and CSC-treated epithelium (Fig. 2A). Interestingly, immunohistomorphological examination shows that on culture day 4 almost all basal epithelial cells in CSC-treated tracheas are RCK103-negative (Table 2), whereas in the untreated vitamin A–depleted tracheas a slight decrease of RCK103-positive basal cells is seen at day 4 (Table 1).

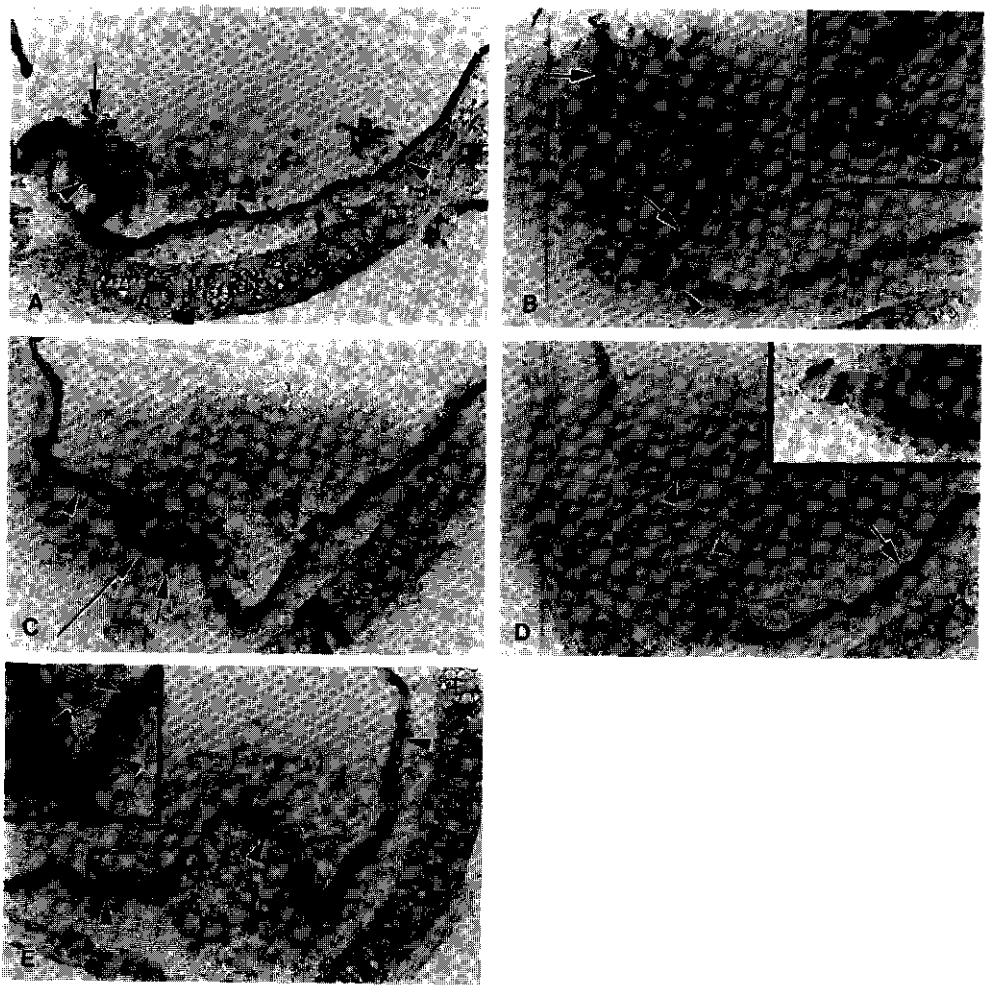

Fig 1. Trachea of vitamin A-deprived hamster *treated in vitro with all-trans retinol* (10^{-11} M) for 10 days. Immunohistochemical staining with different monoclonal antibodies against intermediate filaments in serial sections. The sections have been counterstained with haematoxilin. **(A)** Tracheal section stained with the RCK103 antibody showing a positive reaction in both normal and metaplastic squamous epithelium (*arrowheads*), and with keratinization (*arrow*). × **70**. **(B)** A cross-section stained with the RKSE60 antibody showing positive cells (*arrows*) in and around the metaplastic squamous cell foci. The normal pseudostratified tracheal epithelial cells are negative. × **70**. Inset-enlargement of the tracheal epithelium clearly showing RKSE60-positive superficial cells, and columnar cells which are in contact with the basal lamina (*arrowhead*), × **160**. **(C)** RCK105-positive cells along the normal epithelium (*arrowheads*) and a few positive cells (*small arrow*) in the metaplastic squamous cell foci which is mostly negative (*large arrow*). × **70** **(D)** RGE53-negative squamous metaplasia (*arrowheads*) with keratinization. The superficial cells of the normal epithelium are RGE53-positive (*arrow*). × **70**. *Inset:* enlargement of the epithelium showing RGE53-positive and negative mucus-producing and ciliated cells. × **160**. **(E)** RCK102-positive basal cells (*arrowheads*) in both the metaplastic squamous and normal epithelium. × **70**. *Inset:* enlargement showing hyperplastic RCK102-positive basal epithelial cells (*arrowhead*), and superficial RCK102-positive cells (*arrows*). × **160**

RKSE60. The hamster counterpart of human keratin 10 is detected by the RKSE60 antibody in the metaplastic squamous epithelium of both vitamin A-deficient (Fig. 1B) and CSC-treated (Fig. 2B) epithelium. CSC treatment induces more RKSE60-positive cells (i.e. keratinization) than vitamin A-depletion alone (compare Tables 1 and 2). Tracheal basal cells do not stain with RKSE60. In the present study, only a few columnar cells in contact with the basal lamina are slightly RKSE60-positive (Fig. 1B).

RCK105. Metaplastic squamous cell foci are RCK105-negative in tracheal epithelium (Figs. 1C and 2C). In CSC-treated vitamin A-depleted tracheas almost all basal epithelial cells are RCK105-negative during organ culture, whereas in tracheas treated with DMSO or all-trans retinol about 50% of the basal cells are RCK105-positive after 4 culture days (compare Tables 1, 2).

RGE53. In pseudostratified tracheal epithelium the RGE53 antibody stains mucus-producing and ciliated cells only, while metaplastic squamous cell foci are negative for RGE53. Fig. 2D shows that columnar cells at both edges of the metaplastic squamous cell foci are RGE53-positive. Periodic acid-Schiff (PAS) staining shows that these RGE53-positive cells do contain mucous granules (data not shown). Furthermore, it was found that these cells are negative for RKSE60. The number of RGE53-positive basal epithelial cells is increased after CSC treatment and in the vitamin A-depleted epithelium on culture days 4 and 6 (Tables 1, 2). This effect is more pronounced in the CSC-treated tracheas. On day 10 basal cells are no longer RGE53-positive (Fig. 1D and 2D). In some of the RGE53-positive columnar cells the intensity of the immunoperoxidase staining is decreased, especially after CSC treatment. This is clearly visible in the epithelium to the left in Fig. 2D.

RCK102. The basal cells are normally RCK102-positive, as shown in the epithelium to the right in Figs. 1E and 2E (Tables 1, 2). Proliferative basal cells in the metaplastic squamous cell foci are also RCK102-positive (Figs. 1E and 2E). In focal squamous cell metaplasia induced by CSC, some superficial cells neighbouring the basal cells are RCK102-positive (Fig. 2E). This finding is less frequently observed in vitamin A-depleted tracheal epithelium (Fig. 1E). Solitary RCK102-positive columnar cells or groups of these cells are observed after vitamin A-depletion, and especially in the presence of CSC, on culture day 10 (Table 2).

HCK19. The immunohistochemical staining patterns with HCK19 antibody show positive columnar cells and superficial cells of early metaplastic squamous cell foci in CSC-treated tracheas (Fig. 2F). Superficial cells in more mature

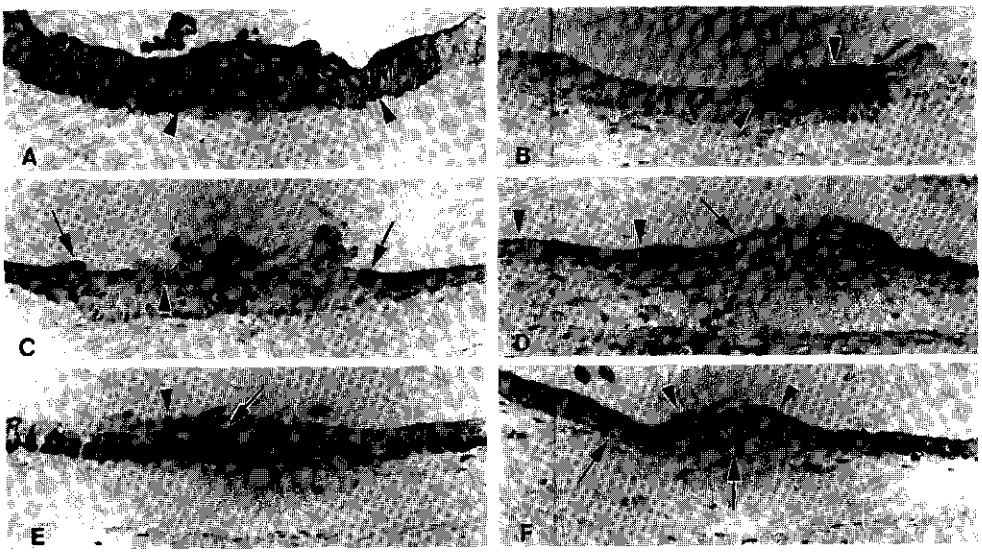

Fig. 2. Trachea of vitamin A-deprived hamster treated in vitro with cigarette smoke condensate (24 mg/l) for 10 days. Immunohistochemical staining with different monoclonal antibodies against intermediate filaments in serial sections. The sections have been counterstained with haematoxilin. **(A)** RCK103-stained tracheal epithelium showing that both normal and metaplastic squamous epithelium (with keratinization) are positive for this antibody (*arrowheads*). Some necrotic cells (*arrow*) present in tracheal lumen are also positively stained. × **180**. **(B)** RKSE60-positive cells in a developing metaplastic squamous cell foci (*arrowheads*). The basal epithelial cells of the metaplastic squamous cell foci and the normal tracheal epithelial cells are negative. × **180**. **(C)** RCK105-positive cells in the normal epithelium at both sides of the squamous metaplasia (*arrows*). The metaplastic squamous cell foci showing keratohyaline granules is negative (*arrowhead*). × **180**. **(D)** RGE53-negative squamous metaplasia with a few positive superficial cells (*arrow*) at both sides of the lesion. The superficial cells of the normal epithelium are generally RGE53-positive with a few negative cells (*arrowheads*), while the basal cells are negative. × **180**. **(E)** RCK102positive basal cells in both the metaplastic squamous and the normal epithelium. The proliferative basal cells and the superficial cells of te squamous metaplasia are RCK102-positive (*arrow*). The superficial squamous cells show keratohyaline granules (*arrowhead*). × **180**. **(F)** HCK19-positive superficial cells of the normal and metaplastic squamous epithelium (*arrowheads*). The proliferative basal epithelial cells in the squamous metaplasia and the basal cells in the normal epithelium are negative (*arrows*). × **180**

metaplastic squamous cell foci are negative for the HCK19 antibody in both vitamin A-depleted and CSC-treated tracheas.

Expression of vimentin in addition to various keratines is not observed in the tracheal epithelium (Tables 1, 2).

Discussion

Tracheal organ cultures derived from vitamin A-deprived hamsters have been shown to be a sensitive model system for studying the effects of retinoids on epithelial cell proliferation and differentiation (Sporn and Roberts 1984; Chopra 1983). Previously we have reported that the development of squamous metaplasia induced by vitamin A deficiency or cigarette smoke condensate can

be monitored in these organ cultures (Rutten et al. 1988a,b). Recently, Huang et al. (1986) showed changes in keratin biosythesis in cultured hamster tracheas during vitamin A depletion, as detected by immunoblot analysis. They paid little attention, however to the tissue morphology in combination with the expression of keratins in the various types of epithelial cells. The results of our study demonstrate that keratin expression in basal, columnar or squamous tracheal epithelial cells changes under the influence of vitamin A depletion or CSC treatment, and can be followed by immunohistological techniques using monoclonal antibodies against specific keratin proteins.

The monoclonal antibodies used in the present study have been shown to react with different keratins in human tissues (Ramaekers et al. 1983; 1987). The antibodies RGE53 (exclusively recognizing human keratin 18), RCK102 (specific for human keratin 5 and 8) and RKSE60 (specific for human keratin 10) also seemed to be useful for studying the in vitro histogenesis of squamous metaplasia in hamster tracheal epithelium. Immunohistochemical analysis with the RKSE60 antibody revealed an apparently accelerated epithelial keratinization during CSC treatment, which was also indicated by the disappearance of stainability of this epithelium by RCK105 and RGE53. Furthermore, it appeared that RKSE60 clearly recognizes early metaplastic cells, whereas the antibody is less reactive with mature metaplastic cells. The finding of a few columnar slightly RKSE60-positive tracheal epithelial cells might indicate a change in keratin expression before clear morphological alterations become apparent. These findings in tracheal epithelium correspond satisfactorily with the results of earlier studies on the changing keratin expression patterns in squamous metaplastic human cervical epithelium (Puts et al. 1985).

In human tracheas the broadly cross-reacting antibody RCK102 (Ramaekers et al. 1987) reacts with both basal and luminal epithelial cells, whereas in the present study and a previous one (Rutten et al. 1988d) this antibody appeared to react with basal cells in hamster tracheal epithelium. This finding makes the RCK102 antibody, in combination with the RGE53 antibody (recognizing columnar cells), a useful tool for time-sequence studies of basal cell proliferation and differentiation in hamster tracheal epithelium. One should, however, be aware of the possible masking or unmasking of the epitope(s) recognized by such monoclonal antibodies as a result of changes in biological activity, or of the bipotent (pluripotent) character of cells, e.g. the RGE-positive basal cells which under certain conditions expressed columnar cell keratin.

As discussed previously (Rutten et al. 1988a,b), basal cells seem to play a

role in the development or maintenance of squamous metaplasia, especially in CSC-treated epithelium or benzo[a]pyrene-treated epithelium (Chopra 1982; 1983; Chopra and Cooney 1985). However, study of the involvement of basal tracheal epithelial cells in the histogenesis of squamous metaplasia remains difficult. McDowell et al. (1984a,b) have presented evidence that vitamin A supplementation after vitamin A depletion induces mucous cell proliferation and that the small mucous granule cells play an important role in the genesis of ciliated cells, and in the histogenesis of squamous metaplasia. Recently, Sigler et al. (1988) reported that the histogenesis of metaplastic lesions in vitro and in vivo appears to be the same, whereas the morphogenesis of these lesions was different. The differences in morphogenesis were ascribed to the fact that in vitro nutrients are available not only from the submucosal side but also from the luminal surface. It was concluded that, both in vitro and in vivo small mucous granule cells make up the larger part of metaplastic lesions (Sigler et al. 1988). Whether or not basal cells play a role in the development of such differentiated cells is still to be investigated. However, the present study provides evidence that in rapidly developing squamous metaplasia the superficial cells may still express or contain intermediate filament proteins which normally occur in basal cells only (as recognized by the monoclonal antibody RCK102). This finding was most clearly seen in CSC-treated tracheas and suggests that morphologically columnar cells still possess basal cell keratins. DeLuca et al. (1985) have reported expression of 50 and 55 kD keratins in basal cells of normal and metaplastic squamous epithelium after vitamin A depletion or exposure to benzo[a]pyrene (detected by monospecific antibodies, using immunofluorescent staining). In squamous metaplasia induced by benzo[a]pyrene no superfical cells were shown to contain these basal cell keratins (DeLuca et al. 1985). In the present study, it was found that basal cells are involved in the genesis of CSC-induced squamous metaplasia based on the RCK102-positive superficial cells (see Fig. 2E).

In conclusion, it is suggested that as a result of CSC treatment the proliferating basal cells and the superficial cells in the central part of the metaplastic squamous cell foci show a basal cell keratin expression pattern. Furthermore, it is possible that small mucous granule cells are also be involved in the development of squamous metaplasia, because at the edges of the lesion RGE53-positive/PAS-positive cells are present. However, it is difficult to understand why two different cell types should give rise to one other cell type. Squamous metaplasia developing during vitamin A depletion shows a more

regular and slower process of cell flattening and keratinization (McDowell et al. 1984a,b; Chopra 1982; Rutten et al. 1988b). This is probably the reason why, in vitamin A-depleted tracheal epithelium, the superficial cells present in metaplastic squamous cell foci do not express the basal cell keratin compared to CSC-exposed tracheas. Finally, vitamin A-depleted tracheal organ cultures treated with DMSO, all-trans retinol (10^{-11}M) or CSC for 10 days did not co-express vimentin in addition to keratins. As discussed previously this may be due to the intact tissue configuration present in organ cultures (Rutten et al. 1988d).

Acknowledgements

This study was supported by grants from the Scientific Advisory Committee on Smoking and Health (Dutch Cigarette Industry Foundation), the Ministry of Welfare, Health and Cultural Affairs, and partly by the Netherlands Cancer Foundation. We would like to thank Dr V.J. Feron and Dr J.W.G.M. Wilmer for helpful discussions, and Prof. Dr J.H. Koeman for critically reading the manuscript. We wish to thank A. Huijsmans, G. Schaart, O. Moesker and K. Verrijp for excellent technical assistance in the preparation and specificity testing of the monoclonal antibodies used in this study.

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CHAPTER 7

Ciliated cells in vitamin A-deprived cultured hamster tracheal epithelium do divide

In Vitro Cellular & Developmental Biology (1988) 24, 931-935

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Ciliated cells in vitamin A-deprived cultured hamster tracheal epithelium do divide.

Summary

The pseudostratified tracheal epithelium, composed of a heterogeneous phenotypically varying cell population, was studied with respect to the in vitro cell proliferative activity of differentiated epithelial cells. Ciliated tracheal epithelial cells so far have been considered to be terminally differentiated nonproliferating cells. Tracheal organ cultures obtained from vitamin A-deprived Syrian Golden hamsters were cultured in a vitamin A-deficient, serum-free, hormone-supplemented medium. In vitamin A-deprived tracheal epithelium treated with physiologically active all-trans retinol and low cigarette smoke condensate concentrations it is possible to stimulate the cell proliferation of both basal and columnar cells. Therefore, the probability of finding proliferating columnar cells was increased compared with the in vivo and the vitamin A-deprived situation in which cell proliferative activity is relatively low. In the presence of cigarette smoke condensate in a non-cytotoxic concentration, basal, small mucous granule, ciliated and indifferent tracheal epithelial cells incorporated [methyl-^{3}H]thymidine into the DNA during the S phase. The finding that ciliated cells were labeled was supported by serial sections showing the same labeled ciliated cell in two section planes separated by 2 to 3 μm, without labeled epithelial cells next to the ciliated cell. Furthermore, a ciliated tracheal epithelial cell incorporating [methyl-^{3}H]thymidine into DNA was also seen in tracheal cultures of vitamin A-deprived hamsters treated with all-trans retinol in a physiologic concentration.

Introduction

The tracheal pseudostratified respiratory epithelium contains several phenotypically varying cell types, such as cells in contact with the basal lamina that do not reach the tracheal lumen (e.g., basal and endocrine cells) and cells in contact with the basal lamina that reach the tracheal lumen with

their apical side (e.g. small mucous granule cells, mucous goblet cells, ciliated-mucous cells, ciliated cells, and indifferent cells) (McDowell 1982).

Basal cells and small mucous granule cells are generally considered capable of proliferating and are held responsible for the renewal of cells in the respiratory epithelium. These cell types have been shown to incorporate [methyl-^{3}H]thymidine into the DNA during the S phase before cell division. Furthermore, several studies showed by colchicine-induced metaphase arrest that basal and small mucous granule cells do divide (Schultze and Oehlert 1960; Bindreiter et al. 1968; Kaufman et al. 1972; Harris et al. 1973; Boren et al. 1974; Gordon and Lane 1977; Boren and Paradise 1978; McDowell et al. 1979 and 1984a,b; Chopra 1983; Rutten et al. 1988a,b). The cell cycle in unstimulated tracheal epithelium was found to be 28 hours for basal cells and 25 hours for small mucous granule cells (Boren and Paradise 1978).

Proliferation of tracheal epithelial cells under various conditions has been reported. In vivo and in vitro studies showed that columnar cells (small mucous granule or mucous goblet cells) increased their mitotic activity when vitamin A was supplemented after vitamin A deprivation (McDowell et al. 1984a, b; Rutten et al. 1988a,b). Cigarette smoke condensate causes in vitro ciliostasis in human respiratory epithelium (Dalhamn 1959; Ballenger 1960), basal and mucous cell proliferation (Rutten et al. 1988a,b) and inhibition of intercellular communication across gap junctions in tracheal epithelial cells (Rutten et al. 1988c).

In a previous study we found that in tracheal organ cultures of vitamin A-deprived hamsters the columnar cell proliferation was stimulated by physiologically active all-trans retinol and relatively low cigarette smoke condensate concentrations (Rutten et al. 1988a). Because, to the best of our knowledge, no proliferative activity of tracheal epithelial cells other than basal or small mucous granule cells has ever been reported, we studied the proliferative activity of differentiated tracheal epithelial cells.

Materials and methods

Animals. Ten-day-pregnant Syrian golden hamsters were obtained from the TNO Central Institute for the Breeding of Laboratory Animals, Zeist, Netherlands. The hamsters were fed a vitamin A-reduced diet during the last 6 days of gestation. After birth, mothers and newborn hamsters received a vitamin A-

deficient diet for 28 to 33 days (Clamon et al. 1974; Rutten et al. 1988a). Detailed information about vitamin A levels of the diets and of the livers of the young hamsters were reported previously (Rutten et al. 1988a).

Tracheal organ cultures and medium. The tracheal rings were cultured in 12-well culture dishes (Costar, Cambridge, MA) in a serum-free, hormone-supplemented CMRL-1066 medium (Flow Laboratories, Herts, England) containing L-glutamine 2 mM (Flow), hydrocortisone 0.1 μg/ml, bovine pancreatic insulin 1.0 μg/ml (Sigma Chemicals, St. Louis, MO), and gentamicin 50 μg/ml (Flow). Cultures were gassed with 50% O_2, 45% N_2 and 5% CO_2 in a humidified incubator at 37°C (Rutten et al. 1988a). All-trans retinol was obtained from Fluka AG (Buchs, Switzerland), and the cigarette smoke condensate (CSC) used in the present study (6 μg/ml) was prepared according to the method described by Rutten and Wilmer (1986). Dimethylsulfoxide (DMSO) was used as solvent for all-trans retinol and CSC, and was used as control. The final DMSO concentration in medium was 0.1%.

Autoradiography. [Methyl-^{3}H]thymidine (Amersham, Houten, Netherlands; specific activity 1.48 and 1.92 TBq/mmol), diluted in CMRL-1066 medium supplemented with cofactors, was added to the culture medium to a final concentration of 74 kBq/ml, 18 hours before the end of the culture period. Tracheal rings were rinsed three times with cold Hanks' balanced salt solution (HBSS), fixed in a 4% aqueous phosphate buffered formaldehyde solution (pH7), and dehydrated by using an ethanol series, and embedded in Technovite 7100 plastic (Kulzer, Wehrheim, FRG). Semithin sections (1 μm) were dipped in Kodak NTB-2 emulsion (Eastman Kodak, New York, NY) diluted 1:1 with a 2% aqueous glycerol solution. Autoradiograms were exposed in dry light-tight boxes for 4 weeks at -30°C, developed in Kodak D19, and stained with a 0.01% Toluidine Blue (TB) (Gurr, Chadwell Heath, England) solution.

Quantitative measurements. Tracheal epithelial cells were scored a) as basal if their cytoplasm did not reach the tracheal lumen and showed a slightly stained cytoplasm; b) as small mucous granule if their cytoplasm was stained relatively dark, showed granules and reached the tracheal lumen; c) as ciliated if the cytoplasm was slightly stained and reached the tracheal lumen with cilia located on the apex; and d) as indifferent if their cytoplasm was slightly stained and reached the tracheal lumen without cilia on the apex.

Slightly stained epithelial cells that lost their contact with the basal lamina, mostly situated in the mid-epithelium, were classified as indeterminate. The cell proliferative activity of basal, small mucous granule, ciliated, and indifferent cells were assessed by light microscopy (x 400 and x1000), around the whole circumference (1200 to 1600 cells) of two different cross-sections per organ culture. Three tracheal organ cultures were used for each experimental point. Cells showing more than ten silver grains over their nucleus were considered to incorporate [methyl-^{3}H]thymidine during the S phase. Background labeling was less than 1 silver grain per cell (see Figs. 1 and 2). Mean values for cell proliferation of the various cell types were given by labeling indices. The labeling index was expressed as the percentage of the number of labeled cells among the total number of epithelial cells (Boren and Paradise 1978). The effects of vitamin A deprivation, all-trans retinol treatment, and CSC exposure with respect to cell proliferative activity, tissue morphology, and cell differentiation in the same model were studied previously (Rutten et al. 1988a,b).

Table 1. Labeling indices of various epithelial cell types in vitamin A-deprived tracheal organ cultures treated with DMSO 0.1%, all-trans retinol (10^{-7} M) and cigarette-smoke condensate (6 μg/ml).

Treatment	n	Labeling indices of various cells[a)]				
		Basal	Mucous	Ciliated	Indifferent	Indeterminate
DMSO 0.1 %[b)]	3	3.01 ± 0.45	0.10 ± 0.02	0.0	0.33 ± 0.10	0.55 ± 0.16
DMSO 0.1 %[c)]	3	3.97 ± 0.61	0.08 ± 0.02	0.0	0.25 ± 0.05	0.51 ± 0.17
Retinol 10^{-7}M[b)]	3	0.81 ± 0.14	2.16 ± 0.68	0.07 ± 0.06	0.23 ± 0.08	0.0
CSC 6 μg/ml[c)]	3	1.84 ± 0.27	0.30 ± 0.12	0.08 ± 0.07	0.07 ± 0.07	0.14 ± 0.10

n is number of tracheal cultures

a) The cell proliferation activity of basal, small mucous granule, ciliated, indifferent, and indeterminate cells (for definitions see Materials and Methods) were assessed by light microscopy (x400 and x1000), around the whole circumference (1200 to 1600 cells) of two different cross-sections per organ culture.

Labeling index = $\frac{\text{number of labeled cells}}{\text{total cells}} \times 100\%$

b) Tracheas were treated for 12 days

c) Tracheas were treated for 10 days

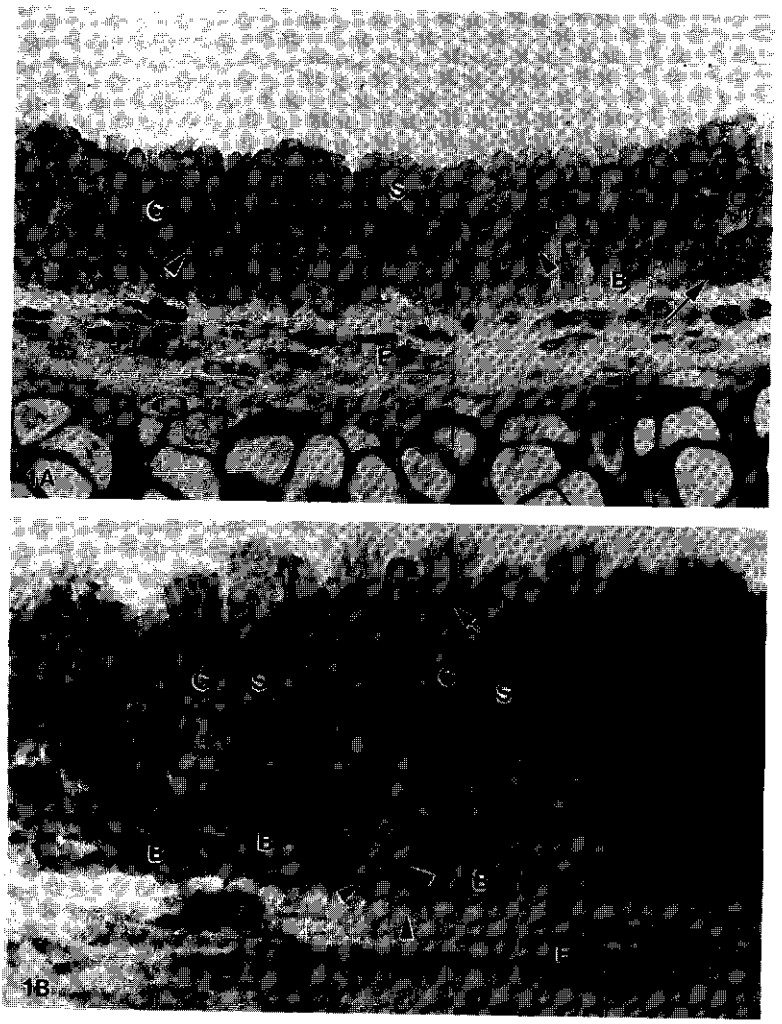
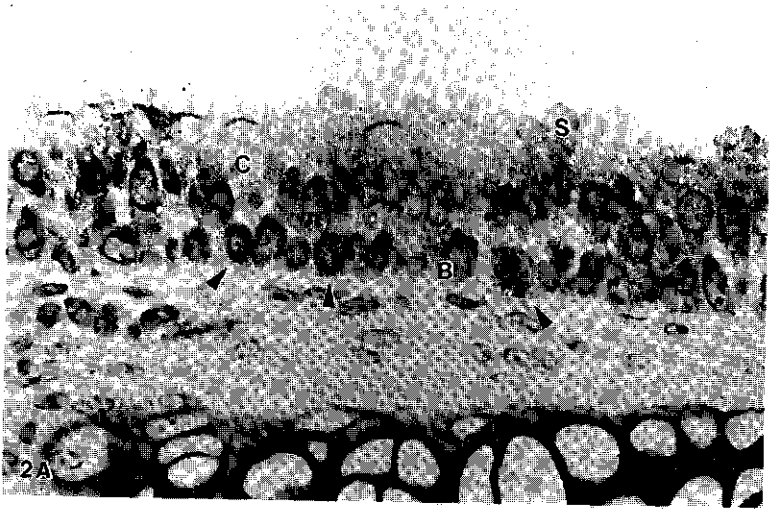


Fig. 1. Tracheal epithelium of a vitamin A-deprived hamster cultured for 12 days in the presence of 10^{-7} M all-trans retinol. Pseudostratified epithelium consists of basal cells (B), small mucous granule cells (S), and ciliated cells (C). Fibroblasts (F) are present in the lamina propria.

A. Autoradiograph showing several small mucous granule cells (arrowheads) and one basal cell (arrow) with silver grains over their nuclei.

B. This autoradiograph shows a ciliated cell with well-pronounced cilia (small arrow) and a lining of basal bodies on the apical side. The nucleus of the ciliated cell is covered with silver grains. Cytoplasm (arrowheads) of the ciliated cell which incorporated [methyl-^{3}H]thymidine clearly reaches the basal lamina (arrowheads). Light micrograph of 1 μm section. TB staining. x800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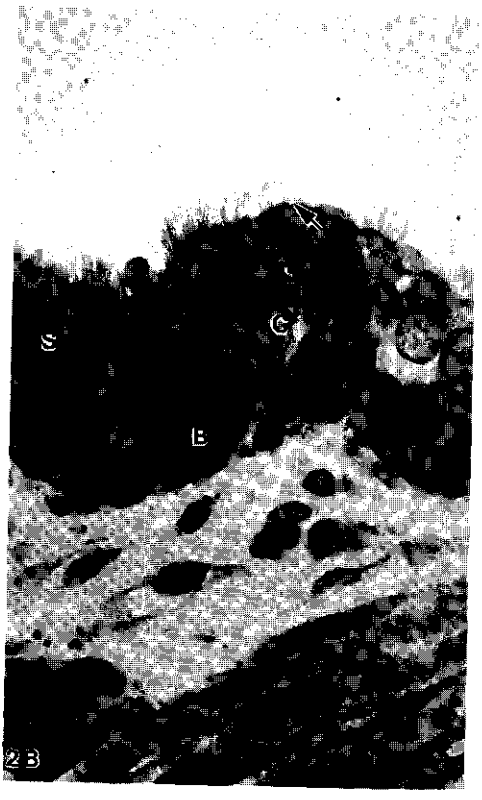
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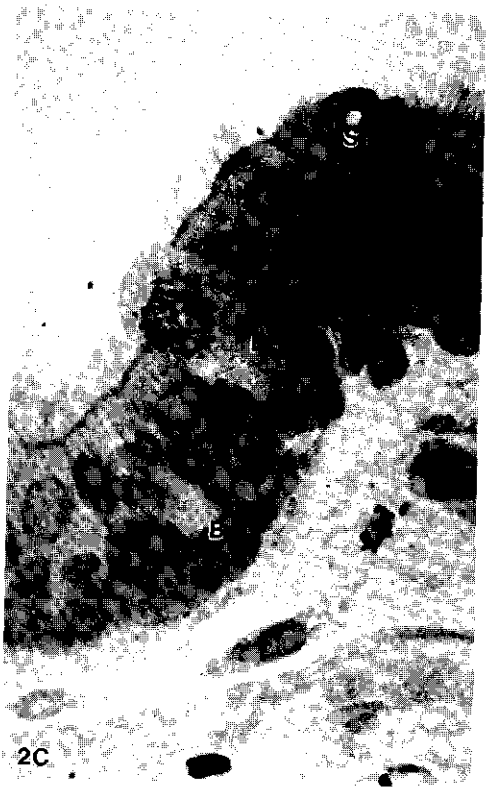
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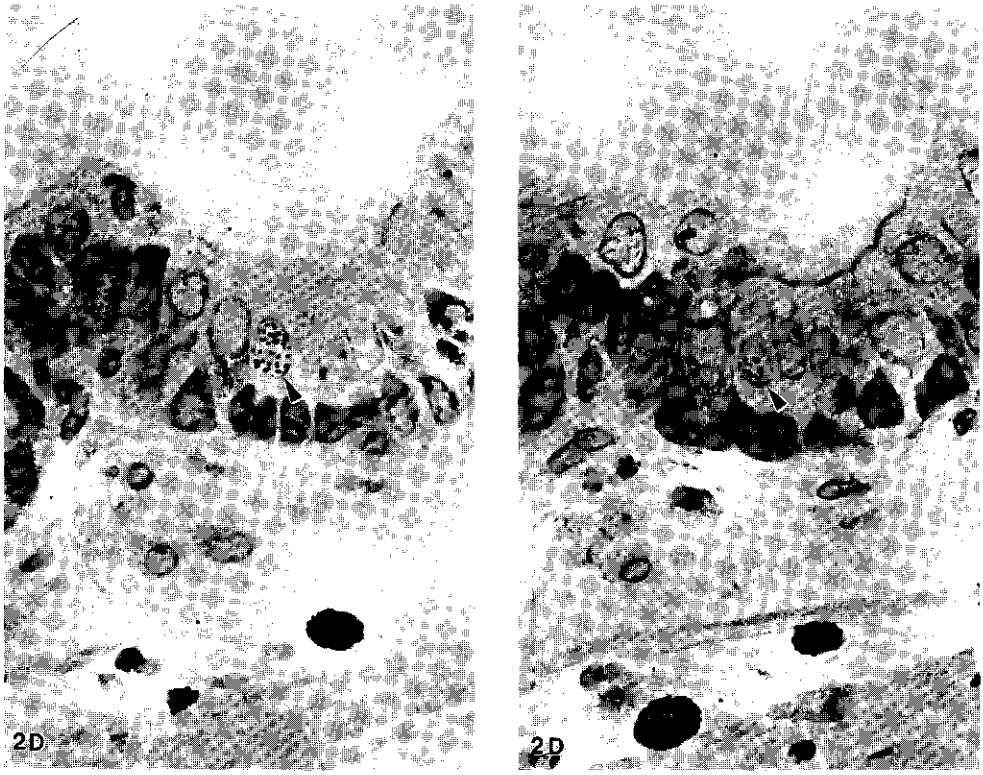

Fig. 2. Tracheal epithelium of a vitamin A-deprived hamster after 10 days in vitro exposure to cigarette-smoke condensate (6 μg/ml). Pseudostratified epithelium consists of basal cells (B), small mucous granule cells (S) with darker-stained cytoplasm, and ciliated cells (C) with faintly stained cytoplasm showing clearly visuable cilia (small arrow) on the apical side.

A. Autoradiograph showing three basal tracheal epithelial cells (arrowheads) which incorporated [methyl-^{3}H]thymidine into their DNA.

B. Incorporation of [methyl-^{3}H]thymidine into the DNA of the ciliated cell is shown in this autoradiograph by silver grain over the nucleus.

C. The labeled indifferent cell (I) is clearly shown by the silver grains above the nucleus in this semithin section (1 μm). This indifferent cell shows a slightly stained cytoplasm without mucous granules and cilia.

D. Autoradiograph showing ciliated epithelial cells in two serial sections (left and right), that are seperated by 2 to 3 μm, with silver grains above the nucleus (arrowhead). Light micrographs of a 1 μm section. TB staining. x800.

Results

The mucociliary pseudostratified tracheal epithelium is well conserved in organ cultures treated with relatively low CSC (Fig. 2) or physiologically active, all-trans retinol concentrations (Fig. 1). Moreover, it was found that vitamin A deficiency, and CSC at higher concentrations (12 and 24 μg/ml) induce hyperplastic and squamous metaplastic foci in the tracheal epithelium (Rutten et al. 1988b). In the present study, we investigated in more detail the cell types involved in cell proliferation, using material from our previous studies (Rutten et al. 1988a,b).

Autoradiograms of semithin (1 μm) sections from cultured tracheal rings are presented in Figs. 1 and 2. Serial sectioning did not reveal evidence that labeled tracheal epithelial cells descended from labeled parent cells. All-trans retinol increased the cell proliferative activity of small mucous granule, ciliated, indifferent, and indeterminate cells compared to the vitamin A-deprived situation (DMSO) (Table 1). No labeled ciliated cells were observed in DMSO-treated tracheas after 10 or 12 culture days (Table 1). In Figure 1A several labeled small mucous granule cells are shown in tracheal epithelium, which was treated with all-trans retinol (10^{-7}M). Fig. 1B shows a ciliated tracheal epithelial cell that incorporated [methyl-^{3}H]thymidine into the DNA during the S phase, after 12 culture days in the presence of 10^{-7} M all-trans retinol. In CSC treated tracheal epithelium, basal cells are more frequently labeled than small mucous granule, ciliated, indifferent, and intermediate cells (Table 1, Figure 2A). Differences in labeling density were frequently found between the various epithelial cell types (Fig. 1 and 2), because the tracheal epithelial cell population divided asynchronously at the time of adding the [methyl-^{3}H]thymidine. In Figure 2 a labeled ciliated cell (2B) and an indifferent (2C) cell are shown after 10 days of <u>in vitro</u> exposure to 6 μg CSC/ml. Two serial sections of CSC-treated tracheal epithelium are showing the same labeled ciliated cell in both sectioning planes (Figure 2D). The micrograph at the right shows a cross section containing a smaller part of the labeled nucleus with fewer silver grains than the cross section presented at the left, Fig. 2D.

Ciliated cells are recognized by their slightly stained cytoplasm (Toluidine Blue staining) and by cilia accompanied by a dark line of basal bodies at the apical side; small mucous granule cells are characterized by mucous granules and a relatively dark-stained cytoplasm (Figs. 1 and 2). The indifferent cells

have a slightly stained cytoplasm without mucous granules and cilia or a line of basal bodies (Fig. 2C). In our in vitro system by far the most labeled cells consist of basal and small mucous granule cells. However, occasionally labeled indifferent cells (Fig. 2C) and labeled ciliated cells (Figs. 1B, 2B and 2D) were observed. Labeled indifferent cells were more frequently seen than labeled ciliated cells.

Discussion

Hamster tracheal organ cultures obtained from vitamin A-deprived hamsters have been widely used for studying the biological activity of retinoids (Sporn et al. 1976; Newton et al. 1980). Recently, we reported the effects of CSC and all-trans retinol on the cellular proliferation and differentiation and on tissue morphology in this model system (Rutten et al. 1988a,b). In the present study it was found that hamster tracheal epithelial ciliated cells incorporated [methyl-^{3}H]thymidine into the DNA during the S phase after treatment with all-trans retinol or exposure to CSC. The finding that ciliated cells incorporated [methyl-^{3}H]thymidine is not an artifact. First, in semithin sections (1 μm) of tracheal rings, it is possible to distinguish clearly between the various types of epithelial cells; second it is most likely that the silver grains over the nuclei of ciliated cells are descended from the β-rays of ^{3}H-incorporated [methyl-^{3}H]thymidine because they have a relative short range of about 1 μm. Third, the pulse labeling of 18 hours is shorter than the cell cycle of tracheal epithelial cells (25 to 28 hours (Boren and Paradise 1978). Fourth, the whole nuclear area of the ciliated cells was covered with silver grains. Fifth, serial sections of tracheal organ cultures, that are separated by 2 to 3 μm, showed labeled ciliated cells in both sectioning planes. Sixth, serial sectioning did not reveal evidence that labeled tracheal epithelial cells descended from labeled parent cells.

Many in vivo and in vitro studies showed that both basal and small mucous granule cells and indifferent cells can proliferate and give rise to differentiated cells (Schultze and Oehlert 1960; Bindreiter et al. 1968; Kaufman et al. 1972; Harris et al. 1973; Boren et al. 1974; Gordon and Pane 1977; Boren and Paradise 1978; McDowell et al. 1979 and 1984a,b; Chopra 1983; Rutten et al. 1988a,b). In vivo, the hamster tracheal epithelial labeling index (LI) and the mitotic index (MI) are relatively low: LI=0.1 and MI=0.043

for basal cells and LI=0.42 and MI=0.024 for small mucous granule cells (Boren and Paradise 1978). McDowell et al. (1984b) reported that the LI=0.83 (control) and LI=0.31 (vitamin A-deprived) for basal cells, and the LI=2.21 (control) and LI=0.14 (vitamin A-deprived) for mucous cells. The LIs found in vivo in control tracheas of hamsters are in agreement with the LIs found in the present study with all-trans retinol. In the present study with vitamin A-deprived tracheas the in vitro LIs are increased compared with in vivo vitamin A deprivation. This may be an effect of culture conditions. However, due to the relative high proliferative activity of columnar tracheal epithelial cells in vitro after exposure to CSC and all-trans retinol (Rutten et al. 1988a) the probability of finding a labeled ciliated cell is increased. Because, only a few dividing ciliated cells were found in this study, this pathway of proliferation may be of minor biological importance. However, because ciliated cells are often difficult to recognize, even in semithin sections, ciliated cells may divide more frequently than is generally assumed. Other investigators have indicated or found some evidence that ciliated cells may divide (EM McDowell, personal communication). From pulse labeling studies with [methyl-^{3}H]thymidine in rat tracheas, Bindreiter et al. (1968) concluded that ciliated cells infrequently divide, if ever. They based their conclusion on the observation that the number of labeled ciliated cells increased with time, but the number of grains above the nuclei of ciliated cells did not decrease with time. Boren and Paradise (1978) used pulse-labeling and double (^{3}H and ^{14}C) thymidine methods and showed that labeled tracheal epithelial cells can divide and give rise to daughter cells.

In Figure 3 the possible interrelationships are shown between the five major cells types present in the tracheal epithelium, i.e. basal, indifferent, small mucous granule, mucous goblet cells, ciliated-mucous, and ciliated cells. The figure represents a modification of the schemes published by McDowell (1982) and Boren and Paradise (1978). The addition to this scheme mainly concerns the introduction of dividing ciliated cells and the difference between major and minor pathways. Basal, indifferent, and small mucous granule cells are considered to be the most important cells for the maintainance and development of the normal pseudostratified tracheal epithelium in Syrian Golden hamsters. Ciliated and mucous goblet cells are considered the most terminally differentiated tracheal epithelial cells. Exposure to xenobiotics and vitamin A deficiency or recovery from vitamin A deficiency may influence these pathways.

Further studies are needed to investigate the role and the biological

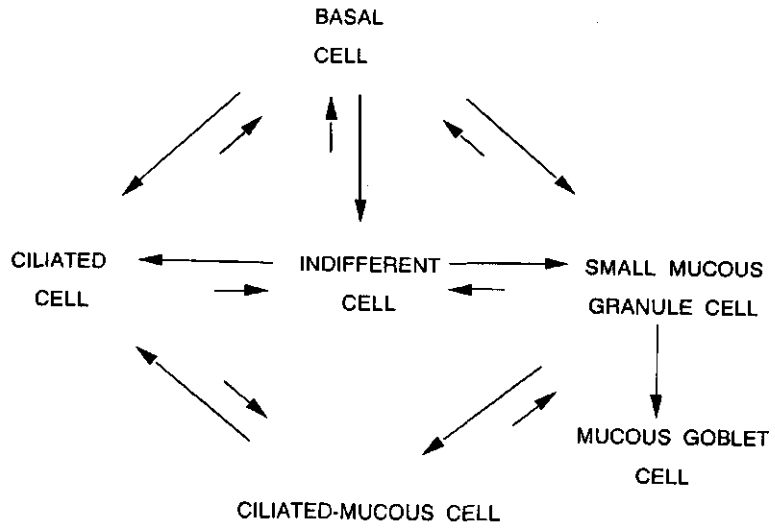

Fig. 3. Proposed pathway for interrelationships between tracheal epithelial cells *in vitro*. Modification of the scheme described by McDowell (1982) and Boren and Paradise (1978). (⟶ major pathway, -► minor pathway)

importance of dividing ciliated cells in tracheal epithelium.

In conclusion, the present study clearly shows that, besides basal and small mucous granule cells, ciliated and indifferent tracheal epithelial cells derived from vitamin A-deprived Syrian golden hamsters, at least under the experimental conditions applied, do divide.

Acknowledgements

The present study was financially supported by the Scientific Advisory Committee on Smoking and Health (Dutch Cigarette Industry Foundation) and the Ministry of Welfare, Health and Cultural Affairs. The authors thank Ms I. de Rooy for animal care, Mr W.G. Rovers for preparing the histologic sections, Mr W.R. Leeman for preparing the autoradiograms, and Prof. Dr J.H. Koeman for critically reviewing the manuscript.

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CHAPTER 8

Effects of retinol and cigarette smoke condensate on dye-coupled intercellular communication between hamster tracheal epithelial cells

Carcinogenesis (1988) 9, 315-320

Rutten, AAJJL, WMF Jongen, LHJ de Haan, EGJ Hendriksen, JH Koeman

Effect of retinol and cigarette smoke condensate on dye-coupled intercellular communication between hamster tracheal epithelial cells

Summary

The dye-coupled intercellular communication across gap junctions in primary hamster tracheal epithelial cells has been studied in serum-free, hormone-supplemented medium. In the absence of vitamin A, non-cytotoxic concentrations of cigarette smoke condensate (CSC) inhibited intercellular communication between tracheal epithelial cells in a concentration-dependent way. All-trans retinol and retinoic acid showed biphasic effects on intercellular communication depending on their concentration. Physiological concentrations of retinol and retinoic acid increased the dye-coupled transfer of Lucifer Yellow CH via gap junctions compared with the dimethylsulfoxide-(DMSO)-treated tracheal epithelial cells. At pharmacological concentrations retinol slightly increased the intercellular communication in the first 2 h of the exposure period, whereas upon longer treatment times with retinol and retinoic acid, gap-junction-mediated intercellular communication was inhibited almost completely. When retinol was given to tracheal epithelial cells before exposure to CSC or simultaneously with CSC-exposure, retinol counteracted the inhibitory potential of CSC on intercellular communication.

The results of the present study clearly indicate that both CSC and all-trans retinol influence the intercellular communication between primary hamster tracheal epithelial cells in serum-free, hormone-supplemented culture medium.

Introduction

Communication across gap junctions between adjacent cells within various tissues is considered to play an important role in the control of cellular growth and differentiation (Bennett and Spray 1985; Loewenstein 1979). Inhibition or stimulation of intercellular communication controls the short-range homeostasis in cell conglomerates. Dysfunction of this control mechanism has been suggested to be an important determinant in tumor promotion (Murray

and Fitzgerald 1979; Yotti et al. 1979; Trosko et al. 1983).

In the past 50 years, several studies have reported the influence of retinoids with respect to growth and differentiation, and the genesis of preneoplastic and neoplastic lesions (Sporn et al. 1984). In spite of the great number of studies showing more or less clear effects of retinoids, the mode of action is still not known. Recently, it was found that retinoic acid (Pitts et al. 1981; Walder and Lutzelschwab 1984; Davidson et al. 1985; Pitts et al. 1986), retinol, retinal and retinyl acetate (Davidson et al. 1985) at pharmacological concentrations inhibited the gap-junction-mediated communication between animal cells, whereas some synthetic retinoids showed only minor effects (Pitts et al. 1986). However, in other studies no inhibitory effects of retinoic acid in metabolic co-operation assays were observed (Morel-Chaney et al. 1986; Trosko et al. 1981).

Cigarette smoke (condensate) has been shown to induce hyperplasia and squamous metaplasia in respiratory epithelium, and was found to be positively correlated to lung cancer (IARC 1986). Furthermore, cigarette smoke condensate (CSC) is an active tumor promoter in the mouse skin painting assay (Van Duuren et al. 1966), and shows inhibition of the metabolic co-operation via gap junctions in cultured mammalian cells (Hartman and Rosen 1983; Jongen et al. 1985).

Cell cultures of human and rodent respiratory epithelial cells have been commonly used in many model systems for investigating functional aspects of airway epithelium and toxic effects of environmental chemicals (Scott et al. 1986; Schiff 1985). Recently, improved culture conditions were reported in which dissociated hamster tracheal epithelial cells divide and express differentiated characteristics of their in vivo functions in serum-free, hormone-supplemented medium (Lee et al. 1984; Wu et al. 1985). Intercellular communication across gap junctions in tracheal epithelial cells may be an essential factor for the maintenance of differentiated functions in these primary cell cultures. Previous ultrastructural studies showed the presence of gap junctions in primary dog tracheal epithelial cultures (Widdicombe et al. 1987; Welsh 1985).

In the present study, we investigated the effects of all-trans retinol and CSC on the dye-coupled intercellular communication across gap junctions in confluent clusters of primary Syrian golden hamster tracheal epithelial cells cultured in serum-free, hormone-supplemented medium.

Materials and methods

Animals and diet. Syrian golden hamsters were obtained from the SAVO-Ivanovas Institute for the Breeding of Laboratory Animals, Kisslegg, F.R.G. The hamsters were housed in Macrolon cages on sterile sawdust under conventional laboratory conditions, and were fed the standard laboratory diet RMH-B (Hope Farms, Woerden, Netherlands). The animals were killed with Nembutal, at an age of 7-10 weeks, and the tracheas were isolated aseptically and prepared for tissue culture.

Culture medium and conditions. The tracheal epithelial cells were isolated according to the method described by Wu et al. (1985). Isolated hamster tracheas were washed three times with Hanks' balanced salt solution (HBSS) (Flow Laboratories, Herts, UK), filled with 0.1% protease (Sigma Chemicals, St. Louis, MO) in Eagle's minimal essential medium (MEM) (Flow Laboratories, Herts, UK), using a 22G x 1 in. plastic catheter. After an 18-h incubation period at 4°C the epithelial cells were flushed out with Ham's F12 medium containing 10% fetal calf serum (Gibco, Breda, Netherlands), centrifugated at 150g, and washed three times with Ham's F12 medium (Flow) supplemented with co-factors. The dissociated epithelial cells were cultured on coated glass cover slips (1.8 x 1.8 cm) containing: 7.5 $\mu g/cm^2$ collagen (Serva Chemicals, Heidelberg, F.R.G.), 2.5 $\mu g/cm^2$ bovine serum albumin (BSA) (Sigma) and 2.5 $\mu g/cm^2$ human fibronectin (Sigma). The vitamin A-deficient Ham's F12 medium with co-factors contained the following supplements: L-glutamine 2 mM (Flow), hydrocortisone 1 μM (Sigma), bovine pancreatic insulin 5.0 $\mu g/ml$ (Sigma), human transferrin 5 $\mu g/ml$ (Sigma), epidermal growth factor (EGF) 25 ng/ml (Sigma) and gentamycin 50 $\mu g/ml$ (Flow).

The primary tracheal cell cultures were maintained in a humidified incubator at 37°C which was gassed with 5% CO_2. CSC (for preparation CSC, see Rutten and Wilmer 1986), all-trans retinol (Sigma), all-trans retinoic acid (Sigma) and 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma) were dissolved in dimethylsulfoxide (DMSO) and added to medium at a final DMSO concentration of 0.1%.

Cell proliferation. The cell proliferative activity of isolated tracheal epithelial cells was determined by [methyl-^{3}H]thymidine incorporation. At different culture days [methyl-^{3}H]thymidine (Amersham, Houten, Netherlands; specific activity 1.48 and 1.92 TBq/mmol), diluted in Ham's F12 medium supplemen-

ted with co-factors, was added to the culture medium at a final concentration of 74 kBq/ml, 18 h before the end of the culture period. The cells were washed three times with HBSS and preserved in a 4% aqueous phosphate buffered formaldehyde solution (pH 7.0). The formaldehyde-fixed tracheal cells on coated cover slips were dehydrated, mounted on microscope slides and dipped in Kodak NTB-2 emulsion diluted 1:1 with a 2% aqueous glycerol solution. Autoradiograms were exposed in dry, light-tight boxes for 2 weeks at -20°C, developed in Kodak D19, and stained with a 0.01% Toluidine Blue (TB) (Gurr, Chadwell Heath, UK) solution.

Electron microscopy. Cultured tracheal epithelial cells on collagen, fibronectin and BSA coated (see "Culture medium and conditions") Thermanox plastic cover slips (Miles Laboratories, Naperville, IL) were fixed with 2.5% cacodylate buffered (0.1 M, pH 7.35) glutaraldehyde solution, stained with ruthenium red and post-fixed with OsO_4, dehydrated with an acetone series and embedded in Epon-Araldite 1:1. The ultra-thin (60-100 nm) sections were post-stained with lead citrate and uranyl acetate.

Experimental protocol for the intercellular communication

Dye-coupling between tracheal epithelial cells. Tracheal epithelial cells were plated out at a concentration of 1-2 x 10^5 cells per well (2 cm diameter) on coated glass cover slips (1.8 x 1.8 cm) and cultured for 3 or 4 days in Ham's F12 medium with co-factors. On the third or fourth day after isolation the tracheal cells were transferred into a 6-cm-diameter Petri dish containing 5 ml Ham's F12 medium with co-factors and 20 mM Hepes (Flow), and exposed to the test substance for 1 to 2 h. Intercellular communication was determined after microinjection of a 4% aqueous Lucifer Yellow CH (Jansen Chemica, Beerse, Belgium) solution in a single cell close to the nucleus. In each trachea cell culture at least 20 individual cells were microinjected using a mechanically drived micro-manipulator (Narishige, Tokyo, Japan) with a dye-filled capillary glass tip (Clark, Pangbourne, UK). The glass capillary tip was prepared by an automatic magnetic puller (Narishige, Tokyo, Japan) with a tip diameter of < 1 μm (assessed with scanning electron microscopy). During the microinjection procedure (by light microscopy at a magnification of X320) and the determination of the number of communicating cells (by fluorescence microscopy at a magnification of X250) the cells were kept at 37°C in a Leitz-

Diavert Klimatbox with combined light and fluorescence microscopic facilities. The dye-filled cells were checked with light and fluorescence microscopy directly after microinjection. After the microinjection procedure was completed in at least 20 individual epithelial cells, approximately 20-30 min after the first microinjection, the number of communicating cells was determined. Photographs of the tracheal cells were made using the Leitz-Diavert photo microscope with a high-speed Ilford HP5 film.

Metabolic co-operation between V79 cells. The metabolic co-operation assay with mutant V79 cells (hypoxanthine-guanine phosphoribosyltransferase deficient, $HGPRT^-$) and wild-type V79 cells ($HGPRT^+$), as described by Yotti et al. (1979) and modified by Jongen et al. (1985), was used to test the inhibitory activity of CSC and the positive control TPA.

Results

Primary hamster tracheal epithelial cells manifested their mucociliary activity at least during four culture days. Figure 1 shows a confluent cluster of tracheal epithelial cells which still divide *in vitro* after 3 days. In the first 3 culture days the labeled cells were regularly distributed throughout the cell cluster, whereas after 4 culture days relatively more dividing cells were observed at the outer part of the cluster. A morphological study revealed the presence of gap junctions between these cells. Figure 2 shows a gap junction between adjacent tracheal epithelial cells. Ciliated cells lost their ciliary activity within a few minutes after micro-injection of the dye, whereas communicating dye-coupled ciliated cells did not show other visible changes. Lower concentrations (up to 0.004 %) of Lucifer Yellow CH showed the same effect.

In the metabolic co-operation assay with V79 Chinese hamster lung fibroblasts the inhibitory activity of the CSC batch used in the present study was comparable to previously reported activities (Hartman and Rosen 1983; Jongen et al. 1985) (Table I). Exposure of primary hamster tracheal epithelial cells to CSC resulted in a concentration-dependent decrease of the intercellular communication via gap junctions (Figure 3). The almost complete inhibition by CSC of the Lucifer Yellow CH transport across gap junctions was observed after 2 h of treatment (Figure 4 and 6C) and lasted for at least 19 h (Figure 4).

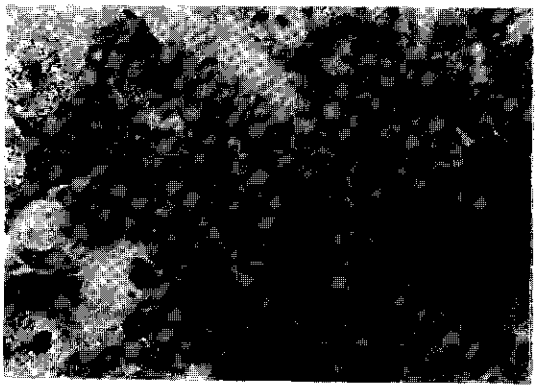

Fig. 1. Autoradiogram of non-exposed primary tracheal epithelial cell cluster (day 3). The dividing cells showing clear silver grains above their nuclei. Toluidine blue staining, × **380**.

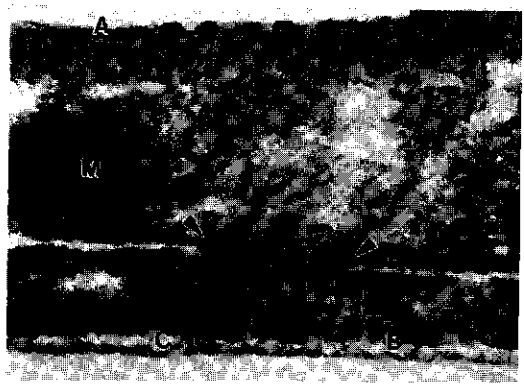

Fig. 2. Gap junction (arrowheads) between cultured tracheal epithelial cell M: mitochondrium, A: apical side, B: basal lamina, C: collagen–fibronectin–bovine serum albumin coating on the cover slip. ruthenium r staining, × **72000**.

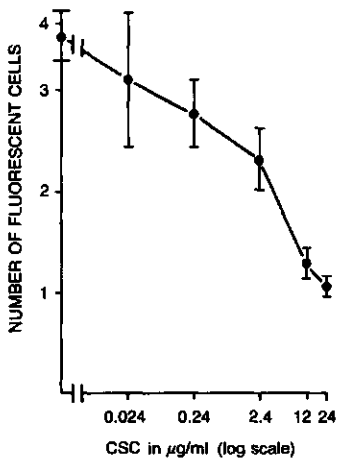

Fig. 3. Effect of CSC on the fluorescent dye transfer across gap junctions in primary hamster tracheal epithelial cell clusters.

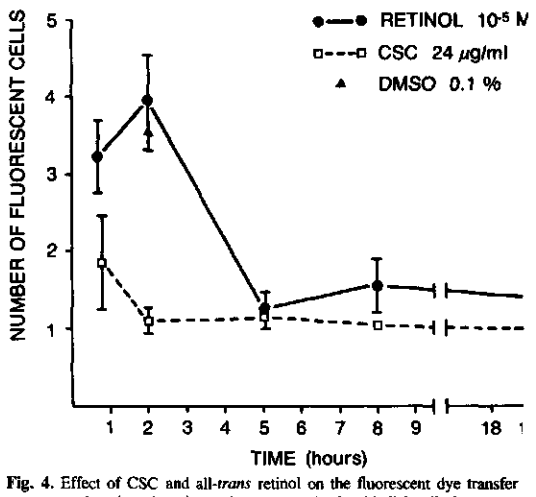

Fig. 4. Effect of CSC and all-*trans* retinol on the fluorescent dye transfer across gap junctions in primary hamster tracheal epithelial cell clusters.

The highest CSC concentration (24 µg/ml) used in the present study did not show adverse effects on the epithelial cells during the first 5 h exposure period. However, after 5 h exposure to CSC (24 µg/ml) a decreased ciliary activity was observed as compared with non-exposed cells, and after a 19 h exposure period most ciliated cells lost their beating activity. Furthermore, the cells showed a granular cytoplasm. Some solitary tracheal epithelial cells were rounder and detached from the coated cover slip. The metabolic cooperation

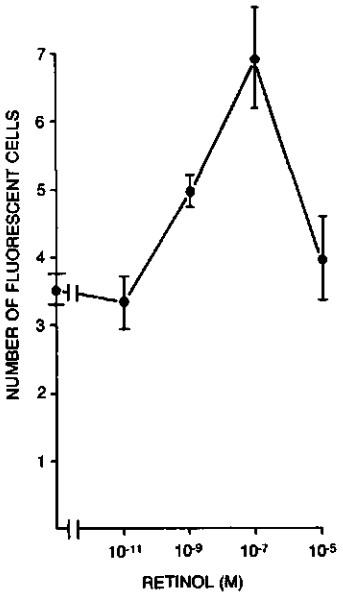

Fig. 5. Effect of all-*trans* retinol on the fluorescent dye transfer across gap junctions in primary hamster tracheal epithelial cell clusters.

assay with V79 cells also showed a CSC-induced inhibition of the intercellular communication; however, the inhibition was observed at higher CSC concentrations than in the primary tracheal epithelial cells.

All-trans retinol stimulated the dye-coupled intercellular communication of tracheal epithelial cells at physiological concentrations (10^{-9} and 10^{-7} M) compared with 0.1% DMSO and retinol 10^{-11} M (Figure 5). This stimulation of the Lucifer Yellow CH transport was observed after a 2-h incubation period (Figure 6A and 6B). No distinct changes in dye-coupled transport was found with retinol 10^{-5} M after 2 h (Figure 4 and 5). However, after 5 h incubation retinol 10^{-5} M inhibited the intercellular communication across gap junctions (Figure 4). This inhibition was still present at 8 and 19 h after treatment with retinol 10^{-5} M.

TPA (up to 1.0 ng/ml) completely inhibited and retinoic acid stimulated (10^{-7} M) or completely inhibited (10^{-5} M) the intercellular dye transport in primary tracheal epithelial cells after a 2-h exposure period.

Retinol treatment either before or after CSC exposure showed that retinol prevented the CSC-induced inhibition of the dye-coupled transport in tracheal epithelial cells relative to the effect of CSC exposure in the absence of

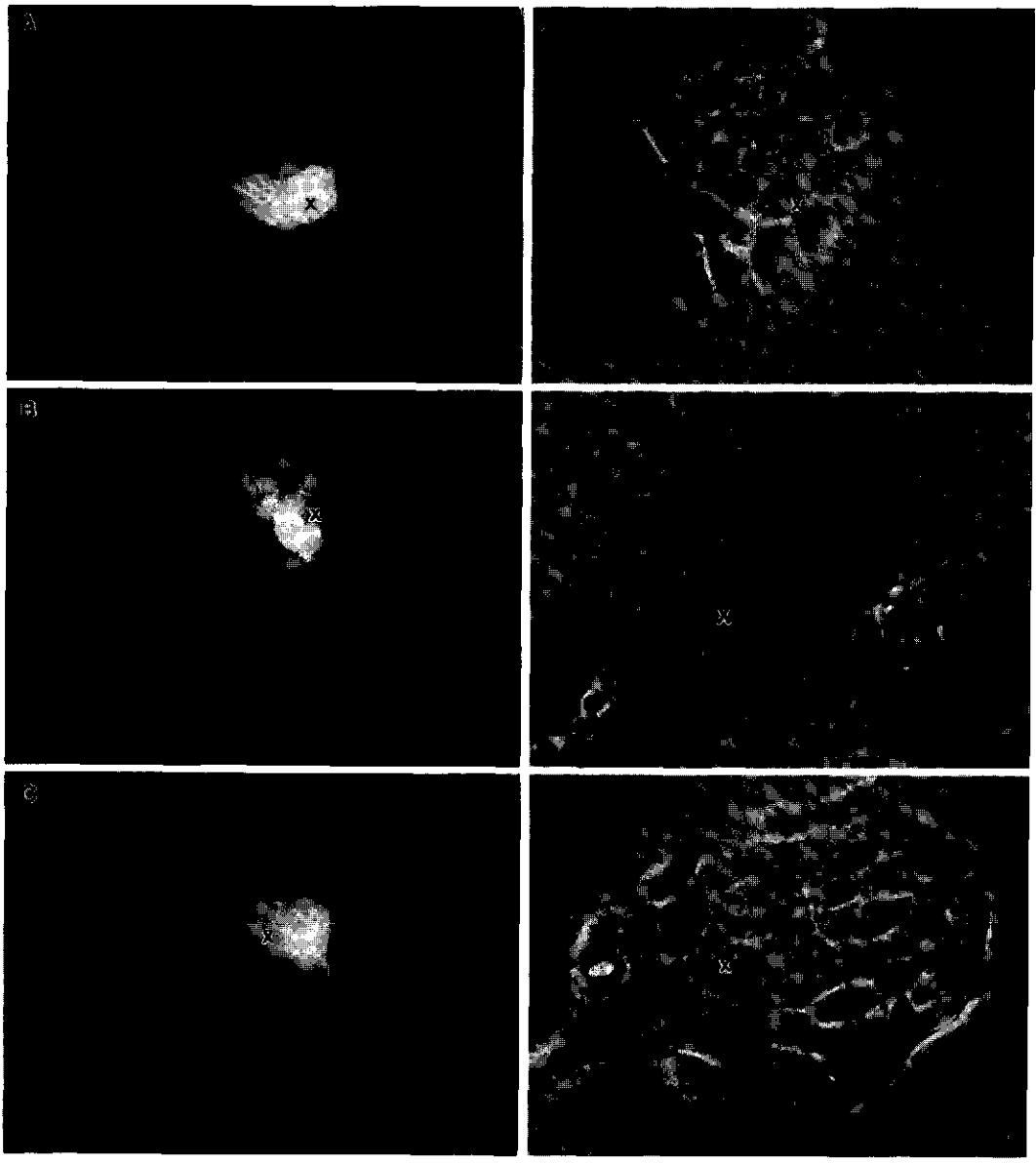

Fig. 6. Lucifer Yellow CH transfer patterns between primary hamster tracheal epithelial cells in confluent cell clusters. X indicates the microinjected cells. **Left**, fluorescent micrograph (×**300**); and **right**, phase-contrast micrograph (×**300**) of the same cell cluster. **A**, tracheal epithelial cells treated with retinol 10^{-7} M for 0.5–1 h (day 4); **B**, tracheal epithelial cells treated with retinol 10^{-9} M for 1–1.5 h (day 3); **C**, tracheal epithelial cells treated with CSC fo 1–1.5 h (day 3).

retinol (Table II). When retinol was given in a concentration of 10^{-7} M before the CSC treatment, a slightly higher number of communicating cells was found in comparison with retinol administration after CSC treatment (Table II).

Table I. Effect of CSC and TPA on the metabolic co-operation between Chinese hamster lung fibroblasts (V79) cells

Treatment	Concentration	Cell type[a]		No. of colonies/dish[b]	Percentage inhibition
		$HGPRT^-$	$HGPRT^+$		
DMSO	0.1%	+	−	126 ± 15	100
	0.1%	+	+	73 ± 9	0
CSC	3 μg/ml	+	+	78 ± 22	9
	6 μg/ml	+	+	98 ± 16	47
	12 μg/ml	+	+	96 ± 21	43
	24 μg/ml	+	+	129 ± 34	106
TPA	100 ng/ml	+	+	108 ± 18	66

[a]For each dish 200 hypoxanthine-guanine phosphoribosyltransferase deficient ($HGPRT^-$) cells and 10^5 $HGPRT^+$ cells were plated.
[b]Mean and SD of five independent experimental points.

Table II. Effect of DMSO, retinol and CSC on the fluorescent dye transport across gap junctions in primary hamster tracheal epithelial cells

First treatment[a]		Second treatment[b]		No. of fluorescent cells (mean ± SD)
Compound	Concentration	Compound	Concentration	
Single exposures[c]				
DMSO	0.1%	None		3.52 ± 0.22
CSC	12 μg/ml	None		1.28 ± 0.11
CSC	24 μg/ml	None		1.08 ± 0.06
Retinol	10^{-7} M	None		6.92 ± 0.80
Combined exposures				
CSC	12 μg/ml	Retinol	10^{-7} M	3.05 ± 0.26
CSC	24 μg/ml	Retinol	10^{-7} M	3.14 ± 0.53
Retinol	10^{-7} M	CSC	12 μg/ml	4.75 ± 0.35
Retinol	10^{-7} M	CSC	24 μg/ml	4.41 ± 0.28

[a]Combined exposures; 2 h.
[b]Combined exposures; 1−2 h.
[c]1−2 h exposure.

Discussion

The present study demonstrated that CSC and TPA inhibited the dye-coupled intercellular communication across gap junctions among primary tracheal epithelial cells. Furthermore, all-trans retinol and retinoic acid, depending on the concentration, stimulated or inhibited the intercellular junctional communication. Since intercellular communication across gap junctions seems to have an important short-range regulatory function during the development and the homeostasis of organized multicellular tissues (Bennett and Spray 1985; Loewenstein 1979; Yamazaki 1986), altered communication may have serious consequences for growth and differentiation. There is accumulating evidence for a high degree of correlation between in vivo tumor promoters and in vitro inhibitors of the intercellular communication (Yotti et al. 1979; Murray and

Fitzgerald 1979; Trosko et al. 1981 and 1983; Yamazaki 1986); CSC-induced inhibition of the gap junction mediated dye transport underlines such correlations.

The results obtained in the present study with CSC are in agreement with previous studies (Hartman and Rosen 1983; Jongen et al. 1985), which also showed CSC-induced inhibition of gap junction mediated communication. These effects were found with a metabolic co-operation assay (Yotti et al. 1979) using medium supplemented with serum (Hartman and Rosen 1983; Jongen et al. 1985). It is noteworthy that inhibition of dye-coupled intercellular communication among tracheal epithelial cells in serum-free medium was accomplished at lower CSC concentrations than those effective in the metabolic co-operation assay with V79 cells (Table I and Figure 3).

A previous study showed influences of serum supplements on the results obtained in a metabolic co-operation assay (Trosko et al. 1982). It might be possible that, besides the differences in cell type and method, serum is an important factor for the observed differences in sensitivity in the present study. This is because medium supplemented with, for example 10% serum, contains normally about 0.5-1 x 10^{-7} M retinol (Long 1971) bound to its carrier protein the serum retinol binding protein. In the present study it has been found that such physiologically active concentrations increase intercellular communication. Recently, Miller et al. (1987) reported a lack of effect of the tumor promoter TPA on the intercellular communication in primary newborn mouse epidermal cells and Chinese hamster fibroblasts (V79) cultured under non-standard conditions in enriched Waymouth's medium supplemented with 10% horse serum. Therefore, it is recommended that the effects of retinoids and tumor promoters be studied using a chemically defined, serum-free, hormone-supplemented medium with in vitro assays. Moreover, serum retinol might be an important interfering factor explaining the conflicting results obtained with vitamin A in in vitro assays.

In the present study a biphasic effect of retinol and retinoic acid on intercellular communication was seen. This effect is partially in agreement with the retinol- and retinoic acid-induced inhibition of junctional communication found in human fibroblasts and V79 cells (Davidson et al. 1985), and the inhibition of labeled uridine transfer by high retinoic acid concentrations in V79 (Pitts et al. 1986) and rat liver epithelial cells (Walder and Lutzelschwab 1984; Pitts et al. 1986). Although no stimulation of intercellular communication was reported by Davidson et al. (1985), the figures

presented in this paper indicate a slight increase of the intercellular communication at low retinoic acid concentrations. Furthermore, Shuin et al. (1983) also reported a biphasic effect on metabolic cooperation depending on the retinoic acid concentration in V79 cells treated with TPA. Morel-Chany et al. (1986) concluded from their results in a metabolic co-operation assay with rat liver epithelial cells that at low concentrations retinoic acid acts as co-promoter and not as promoter, because retinoic acid inhibited synergistically the intercellular communication in the presence of TPA but was inactive when retinoic acid was tested alone. The latter observation was not confirmed in the present study.

The results obtained in the present study in combination experiments showed that retinol was able to restore the CSC-induced blocked intercellular communication. Retinol was also protective against CSC-induced inhibition of junctional communication. This restoration of the CSC-induced inhibition of the intercellular dye transfer seems to be a reversible process which can be influenced within a few hours by retinol. The tumor promoter TPA, which was tested in many experimental in vivo and in vitro test systems, can block junctional communication (Yotti et al. 1979; Murray and Fitzgerald 1979; Trosko et al. 1981 and 1983; Walder and Lutzelschwab 1984; Davidson et al. 1985; Jongen et al. 1985; Morel-Chaney et al. 1986). Furthermore, morphological studies showed that inhibition of labeled nucleotides transfer was followed by a decreased number of gap junctions after in vitro exposure to TPA in V79 cells and primary hepatocytes (Jongen et al. 1987). Yancey et al. (1982) reported also a decreased number of communicating channels after TPA exposure. High retinoic acid concentrations also inhibited intercellular communication across gap junctions (Pitts et al. 1981 and 1986; Walder and Lutzelschwab 1984; Davidson et al. 1985); however, in contrast to the effect of TPA, after 24 h exposure to retinoic acid the number of gap junctions was significantly increased (Pitts et al. 1981 and 1986). Furthermore, retinoic acid also increased the number of gap junctions in keratoacanthomas (Prutkin 1975). Obviously, retinoic acid, and may be retinol, alters the gap-junction-mediated intercellular communication via a mechanism different from that of TPA.

As shown in a previous study, effects of retinol on cell proliferation and differentiation in serum-free cultured hamster tracheal epithelium were clearly observed if the epithelium was first made sensitive to the in vitro retinol addition by vitamin A deprivation (Rutten et al. 1988a,b). The tracheal epithelial cell culture system seems also to be a sensitive model for

investigating effects of retinoids and respiratory toxins on intercellular communication, but further ultrastructural and biochemical studies with these retinoid sensitive cells are needed to elucidate the mechanism responsible for the vitamin A-induced alterations of the junctional communication.

Acknowledgements

The authors would like to thank Drs. V.J. Feron, J.W.G.M. Wilmer and B.J. Spit for helpful discussions, Dr. J.H.M. Temmink for the scanning electron microscopic investigations and Mr. J. de Haas for animal care. The present study was financially supported by the Scientific Advisory Committee on Smoking and Health (Dutch Cigarette Industry Foundation) and the Ministry of Welfare, Health and Cultural Affairs.

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PART III

CONCLUSION

CHAPTER 9

Summary and concluding remarks

Summary and concluding remarks

Inhalation of tobacco smoke is considered to be strongly related to pathological changes of the respiratory tract epithelium. Epidemiological studies show a strong positive relationship between tobacco use and the development of (pre)neoplastic changes in lung and tracheobronchial epithelium. Several experimental studies, both *in vivo* and *in vitro*, confirmed cellular alterations in tracheobronchial epithelium related to cigarette smoke (condensate) exposure.

Many reports demonstrate that consumption or application of vitamin A and/or provitamin A (carotenoids) protects against (pre)neoplastic lesions and cancer of the respiratory tract. However, some contradictory results have been reported with respect to the protective effect of vitamin A against cancer of the respiratory tract. Metabolism and absorption of carotenoids in animal studies, and concentration and type of vitamin A used in *in vitro* studies might be important factors for the modifying effects of retinoids. At physiological concentrations retinoids control proliferation and differentiation of epithelial cells and are essential to maintenance of mucociliary activity in respiratory epithelium. Furthermore, vitamin A deficiency, and benzo[a]pyrene or cigarette smoke condensate (CSC) treatment induce squamous metaplasia with or without keratinization in tracheal epithelium, which can be restored by vitamin A. Therefore, vitamin A is believed to play an important preventive role in the development of cancer.

This thesis deals with the study of the effects of vitamin A depletion and CSC on tracheal epithelium in organ and cell cultures. Effects were studied on cellular proliferation and differentiation, intermediate filament expression patterns in various cells and dye-coupled intercellular communication.

In Part I some aspects of the current knowledge of retinoids are summarized, in relation to tracheal epithelium cytokinetics and histogenesis, intermediate filaments, and intercellular communication (Chapter 1). *In vitro* model systems with tracheal organ cultures and tracheal epithelial cell cultures are described in Chapter 2.

In the experimental part (Part II) of this thesis the results of *in vitro* studies with tracheal organ cultures and tracheal epithelial cell cultures are discussed (Chapters 3-8).

Chapter 3 deals with the effects of CSC and all-trans retinol on the cell proliferative activity of vitamin A-deprived hamster tracheal epithelium in organ culture. CSC clearly increased cell proliferation during 12 days of culture. Furthermore, tracheal epithelial basal cells were found to be more sensitive to CSC exposure than mucus-producing and ciliated cells during the first 6 to 8 culture days. However, in squamous metaplastic foci, mainly developing after culture day 6, both basal and nonbasal cells in the mid-part of the epithelium were labeled. Physiological concentrations of all-trans retinol stimulated mucous cell proliferation and inhibited basal cell proliferation. Exposure of tracheal rings to retinol, either before or after exposure to CSC, or simultaneous exposure of tracheal rings to retinol and CSC, clearly decreased the CSC-induced basal cell proliferative activity depending on the retinol concentration used.

Chapter 4 describes the in vitro effects of all-trans retinol and CSC on tissue morphology and cellular differentiation in vitamin A-deprived tracheal epithelium. The development of hyperplasia or squamous metaplasia, with or without keratinization, was inhibited by physiological retinol concentrations. Keratinized squamous metaplastic foci were observed during 12 days of culture both with low retinol concentrations and with the solvent dimethylsulfoxide (DMSO). CSC induced, in a dose-dependent way, hyperplasia and irregularly shaped foci of squamous metaplasia with atypical epithelial proliferations. In non-metaplastic epithelium, CSC exposure increased the number of ciliated cells. Hyperplasia and squamous metaplasia were inhibited when the tracheal rings were treated with retinol before exposure to CSC, or when the tracheas were simultaneously treated with retinol and CSC. CSC exposure prior to retinol treatment induced histomorphological alterations of the same kind as CSC alone.

Intermediate filaments constitute a considerable part of the cytoskeleton of eukaryotic cells. Epithelial cells are characterized by keratin intermediate filaments. The keratin expression patterns depend on epithelial cell type and stage of development or differentiation, and changes in these expression patterns may be related to differences in molecular weights, isoelectric points and immunological properties of the individual keratin proteins. In Chapters 5 and 6 immunohistochemical techniques are described used to study the keratin expression patterns, both in basal and columnar (mucus-producing and ciliated) cells (Chapter 5) and in metaplastic squamous cell foci (Chapter 6).

In tracheas from hamsters fed a control diet, basal cells generally reacted

with the RCK102 antibody, and columnar cells (mucus-producing and ciliated cells) reacted with the RGE53 and the HCK19 antibodies. Basal and columnar cells were recognized by the RCK105 antibody. The squamous cell keratin 10 (detected by the RKSE60 antibody) was expressed in vitamin A-depleted tracheas and in tracheas treated with CSC. Furthermore, CSC exposure accelerated epithelial keratinization as compared to the vitamin A-depleted epithelium. In cultured tracheas from hamsters fed a normal diet keratin 10 was not expressed.

In the course of the in vitro period some keratins were "switched on" or "switched off" in both basal and columnar cells. In tracheas from vitamin A-deprived hamsters the RCK102 antibody evidently recognized basal cells and CSC-induced proliferating basal cells, whereas the RGE53 antibody reacted with mucus-producing and ciliated cells. During organ culture foci of columnar epithelial cells expressed basal cell properties (detected with the RCK102 antibody) after all-trans retinol treatment. Furthermore, it was found that these foci were negative for the RGE53 antibody. Metaplastic squamous cell foci reacted with antibodies RKSE60, RCK103 and HCK19. Early metaplastic alterations were more clearly RKSE60-positive than mature metaplastic lesions. Superficial cells in the central area of CSC-induced squamous metaplasia expressed the basal cell keratins, but not the columnar cell keratin 18 as detected by the RGE53 antibody. The mucus-producing cells at the edges of metaplastic squamous cell foci expressed the keratins specific to columnar cells. Tracheal epithelium did not appear to co-express vimentin, an intermediate filament expressed in cells of mesenchymal origin, next to keratins during organ culture.

Chapter 7 deals with in vitro cell proliferative activity of differentiated epithelial cells. Ciliated tracheal epithelial cells so far have been considered to be terminally differentiated nonproliferating cells. In vitamin A-deprived tracheal epithelium treated with physiological all-trans retinol concentrations and low concentrations of CSC it is possible to stimulate the cell proliferation of both basal and columnar cells (mainly mucus-producing cells). Therefore, the probability of finding proliferating columnar cells in vitro was increased compared with the in vivo situation, in which cell proliferation is relatively low. In the presence of CSC, at a noncytotoxic concentration, basal cells, small mucous granule cells, ciliated cells, and indifferent cells incorporated [methyl-^{3}H]thymidine into the DNA during the S-phase. The finding that ciliated cells were labeled was supported by serial sections showing the

same labeled ciliated cell in two section planes 2-3 μm apart, without labeled epithelial cells next to the ciliated cell. Furthermore, a ciliated tracheal epithelial cell incorporating [methyl-^{3}H]thymidine into DNA was also seen in tracheal cultures of vitamin A-deprived hamsters treated with a physiological concentration of all-trans retinol.

In Chapter 8 the dye-coupled intercellular communication across gap junctions was investigated in hamster primary tracheal epithelial cells in serum-free, hormone-supplemented medium. In the absence of vitamin A, noncytotoxic concentrations of CSC inhibited communication between tracheal epithelial cells in a concentration-dependent way. All-trans retinol and retinoic acid showed biphasic effects on intercellular communication depending on their concentration. Physiological concentrations of retinol and retinoic acid increased the dye-coupled transfer of Lucifer Yellow CH via gap junctions compared with the DMSO-treated tracheal epithelial cells. At pharmacological concentrations retinol slightly increased the intercellular communication in the first 2 h of the exposure period, whereas upon longer treatment with retinol and retinoic acid, gap-junction-mediated intercellular communication was inhibited almost completely. When retinol was given to tracheal epithelial cells before exposure to CSC or simultaneously with CSC exposure, retinol counteracted the inhibitory potential of CSC on intercellular communication.

The following conclusions can be drawn from the studies reported in this thesis.

1. All-trans retinol inhibits the CSC-induced basal cell proliferation. Squamous metaplasia induced by vitamin A deficiency or by CSC originates mainly from basal cells, and both basal and nonbasal cells in the mid-epithelium play a role in the maintenance of this lesion. Furthermore, it may be assumed that basal cells differentiate into columnar cells.
2. CSC and vitamin A exert clear effects on vitamin A-deprived epithelium in a organ culture system. Experiments with CSC as well as retinol showed that all-trans retinol or its metabolites can modulate histomorphological alterations induced by CSC in this system.
3. Unlike in cell culture tracheal epithelium in organ culture does not co-express vimentin next to keratins, which may be due to the interactions of the submucosa with the epithelium or to the intact three-dimensional organization present in organ cultures.

4. Besides basal and small mucous granule cells, ciliated and indifferent tracheal epithelial cells derived from vitamin A-deprived Syrian Golden hamsters, do divide under the experimental conditions applied.
5. CSC inhibits the dye-coupled communication between hamster primary tracheal epithelial cells in serum-free, hormone-supplemented culture medium. Furthermore, all-trans retinol and retinoic acid either stimulate or inhibit communication between tracheal epithelial cells, depending on their concentration.

The studies described in this thesis show that tracheal organ cultures from vitamin A-depleted hamsters is a useful *in vitro* model to study the effects of chemicals on proliferation, differentiation and intermediate filament expression patterns of epihelial cells.

Further experimental work should focus on the relationship between metabolism of chemical compounds, the damage caused and the subsequent repair in the various individual (tracheal) epithelial cells.

CHAPTER 10

Samenvatting en slotbeschouwingen

Inhaleren van sigaretterook gaat duidelijk gepaard met pathologische veranderingen in het epitheel van de ademhalingswegen. Zo zijn er uit epidemiologisch onderzoek sterke aanwijzingen verkregen dat er een positieve relatie bestaat tussen gebruik van tabak en de ontwikkeling van (pre)neoplastische veranderingen in het epitheel van de longen, de trachea en de bronchiën. Ook experimenteel onderzoek, zowel in vivo als in vitro, laat zien dat de veranderingen in het epitheel van de trachea en de bronchiën verband houden met de blootstelling aan sigaretterook(condensaat).

Uit een groot aantal studies blijkt dat de consumptie van vitamine A en/of provitamine A (carotenoïden) beschermend kan werken tijdens de ontwikkeling van (pre)neoplastische veranderingen in het trachea-epitheel en bij het onstaan van longkanker. In sommige studies is echter géén of juist een tegengesteld effect gevonden. De opname in het maagdarmkanaal en het metabolisme van carotenoïden in vivo, en de concentratie en de vorm waarin retinoïden in vitro worden toegediend, lijken een belangrijke rol te spelen bij het modulerend effect van vitamine A. In fysiologische concentraties, controleren retinoïden de delingsactiviteit en differentiatie van epitheelcellen en zijn verder noodzakelijk voor de instandhouding van het pseudomeerlagig trachea-epitheel. Vitamine A-deficiëntie, en blootstelling aan benzo[a]pyreen of sigaretterookcondensaat (SRC) kunnen leiden tot metaplasie naar plaveiselepitheel met of zonder verhoorning. Vitamine A kan deze veranderingen een halt toeroepen. Vanwege deze eigenschap wordt aan vitamine A een belangrijke preventieve rol toegeschreven bij het onstaan van kanker.

In dit proefschrift worden studies beschreven naar de effecten van vitamine A-deficiëntie en van blootstelling aan SRC op tracheaweefsel in orgaan- en celkweek. Met name worden beschreven de effecten op: 1) de delingsactiviteit van de epitheelcellen en 2) de differentiatie van de epitheelcellen, 3) de veranderingen in de intermediaire filamenten van het cytoskelet in de verschillende cellen, en 4) de communicatie via "gap junctions" tussen cellen.

In deel I worden enkele aspecten van de retinoïden samengevat in relatie tot de celkinetiek en de histogenese van trachea-epitheel, de intermediaire filamenten en de communicatie tussen cellen (hoofdstuk 1). De in vitro modellen waarbij gebruik wordt gemaakt van trachearingen en geïsoleerde trachea-

epitheelcellen worden in detail beschreven in hoofdstuk 2.

Het experimentele gedeelte (deel II) beschrijft de studies naar de effecten van all-trans-retinol en SRC in trachea-orgaankweken en in trachea-epitheelcelkweken (hoofdstukken 3-8).

In hoofdstuk 3 worden de effecten beschreven van SRC en all-trans retinol op de delingsactiviteit van trachea-epitheelcellen in orgaankweek. De trachea's werden verkregen van vitamine A-deficiënte hamsters. Blootstelling aan verschillende concentraties SRC verhoogde de celdelingsactiviteit gedurende 12 dagen. Verder bleek dat gedurende de kweekperiode basaalcellen gevoeliger zijn voor SRC-behandeling dan slijmproducerende en trilhaarcellen. In metaplasie naar plaveiselepitheel, die in vitro onstond na 6 dagen, prolifereerden echter zowel basaalcellen als niet-basaalcellen (in het midden van het epitheel). Fysiologische concentraties retinol stimuleerden de delingsactiviteit van slijmproducerende cellen terwijl die van basaalcellen werd geremd. Blootstelling van trachea-ringen aan retinol vóór of na de toediening van SRC, of tegelijk met de blootstelling aan SRC, verminderde de door SRC veroorzaakte delingsactiviteit van basaalcellen. De mate van remming was gecorreleerd aan de retinolconcentratie.

In hoofdstuk 4 worden de effecten beschreven van retinol en SRC op de weefselmorfologie en de celdifferentiatie van vitamine A-deficiënt trachea-epitheel. De ontwikkeling van hyperplasie, en metaplasie naar plaveiselepitheel met of zonder verhoorning, werd geremd door fysiologische concentraties retinol. Metaplasie naar plaveiselepitheel met verhoorning ontstond tijdens 12 dagen kweken bij zeer lage retinolconcentraties en bij gebruik van het oplosmiddel dimethylsulfoxide (DMSO). SRC induceerde een dosis-afhankelijke toename van hyperplasie en een grillige metaplasie naar plaveiselepitheel met atypische proliferaties. In het pseudomeerlagige epitheel verhoogde SRC het aantal trilhaarcellen. Hyperplasie en metaplasie naar plaveiselepitheel kwamen minder voor als de tracheae waren voorbehandeld met retinol of gelijktijdig werden behandeld met SRC en retinol. In de tracheae die met SRC werden behandeld en waaraan daarna retinol werd toegevoegd werden histomorfologische veranderingen waargenomen die overeen kwamen met de veranderingen gevonden na blootstelling aan SRC zonder retinol.

Intermediaire filamenten vormen een onderdeel van het cytoskelet van elke cel. Keratine is een intermediair filament dat specifiek is voor cellen van epitheliale herkomst. De expressie van keratines in de diverse cellen is afhankelijk van het stadium van de celontwikkeling of celdifferentiatie. De

veranderingen in de keratine-expressiepatronen zijn gerelateerd aan verschillen in molecuulgewicht, iso-elektrisch punt en immunologische eigenschappen van de individuele keratine-eiwitten. Bij het onderzoek beschreven in de hoofdstukken 5 en 6 worden immunohistochemische technieken gebruikt om de veranderingen in keratine-expressie te bestuderen van basaalcellen en cylindrische cellen (slijmproducerende cellen en trilhaarcellen) (hoofdstuk 5) en van metaplasie naar plaveiselepitheel (hoofdstuk 6).

In trachea-epitheel van hamsters op een normaal dieet werden basaalcellen aangetoond met het RCK102-antilichaam en waren cylindrische cellen positief voor zowel het RGE53- als het HCK19-antilichaam. Basaalcellen en cylindrische cellen werden ook herkend door het RCK105-antilichaam. Het plaveiselepitheel celkeratine 10 (aantoonbaar met het RKSE60-antilichaam) werd tot expressie gebracht in vitamine A-deficiënte tracheae en in de tracheae die blootgesteld waren aan SRC. Een versnelde epitheliale verhoorning werd gevonden na blootstelling aan SRC. In normaal pseudomeerlagig trachea-epitheel van hamsters op een normaal dieet werd keratine 10 niet tot expressie gebracht.

Tijdens de in vitro periode kwamen sommige keratines plotseling tot expressie of verdwenen ineens, zowel in basaalcellen als cylindrische cellen. In tracheae van vitamine A-deficiënte hamsters werden de normale basaalcellen en prolifererende basaalcellen duidelijk herkend door het RCK102-antilichaam, terwijl het RGE53-antilichaam juist de cylindrische cellen herkende. In trachea-orgaankweken ontstonden na behandeling met retinol groepjes cylindrische epitheelcellen die het keratine van basaalcellen tot expressie brachten (aangetoond met het RCK102-antilichaam). Deze groepjes cellen waren negatief voor het RGE53-antilichaam. Metaplasie naar plaveiselepitheel werd herkend door de antilichamen RKSE60, RCK103 en HCK19. Bij relatief vroeg ontwikkelde metaplasie naar plaveiselepitheel waren de cellen duidelijker RKSE60-positief dan in metaplastische veranderingen in een later stadium. De afgeplatte cellen, in het midden van de door SRC teweeggebrachte metaplasie naar plaveiselepitheel, bevatten eveneens het keratine van de basaalcellen (RCK102) en niet het keratine voor cylindrische cellen (RGE53). De slijmproducerende cellen aan de rand van metaplasie naar plaveiselepitheel waren positief voor het RGE53-antilichaam. Verder werd gevonden dat in het trachea-epitheel naast keratine expressie geen expressie plaats vond van vimentine, een intermediair filament van cellen met een mesenchymale oorsprong.

De delingsactiviteit van de diverse trachea-epitheelcellen wordt in hoofdstuk 7 beschreven. De trilhaarcellen van het trachea-epitheel zijn tot nu toe

steeds beschouwd als niet-delende gedifferentieerde cellen. In vitamine A-deficiënt trachea-epitheel dat in vitro wordt behandeld met fysiologische retinolconcentraties en met lage SRC-concentraties is het mogelijk de delingsactiviteit van zowel basaalcellen als slijmproducerende cellen te stimuleren. Hierdoor wordt de kans om een delende cel te vinden verhoogd, dit in tegenstelling tot de situatie in vivo waarin de celdelingsactiviteit laag is. In de aanwezigheid van relatief lage, niet-cytotoxische concentraties SRC werden delende basaalcellen, slijmproducerende cellen, trilhaarcellen en indifferente cellen gevonden met behulp van [methyl-^{3}H]thymidine inbouw. In seriecoupes, 2-3 μm van elkaar gescheiden, werd dezelfde delende ([methyl-^{3}H]thymidine gelabeld) trilhaarcel gevonden zonder delende aangrenzende cellen. Verder werd een delende trilhaarcel gevonden in vitamine A-deficiënt trachea-epitheel dat behandeld was met all-trans-retinol.

In hoofdstuk 8 wordt onderzoek beschreven naar de effecten van all-trans-retinol en SRC op de intercellulaire communicatie via "gap junctions" in trachea-epitheelcelkweken van hamsters in medium zonder serum waaraan cofactoren zijn toegevoegd. Een dosis-afhankelijke remming van de intercellulaire communicatie werd gevonden na blootstelling aan SRC in afwezigheid van vitamine A. Retinol en retinoïnezuur veroorzaakten een concentratie-afhankelijk bifasisch effect op de intercellulaire communicatie. In vergelijking met de trachea-epitheelcellen die met DMSO waren behandeld verhoogden fysiologische concentraties retinol en retinoïnezuur de intercellulaire communicatie. Twee uur na het toedienen van een farmacologische concentratie retinol werd een geringe stijging van de intercellulaire communicatie waargenomen, terwijl bij langere blootstelling aan retinol en retinoïnezuur de intercellulaire communicatie bijna volledig werd geremd. Wanneer retinol werd toegediend vóór of gelijktijdig met SRC, werkte retinol beschermend tegen de remming veroorzaakt door SRC.

Uit het in dit proefschrift beschreven onderzoek kunnen de hierna beschreven conclusies worden getrokken.

1. De door vitamine A-deficiëntie en SRC-behandeling veroorzaakte metaplasie naar plaveiselepitheel onstaat voornamelijk uit de differentiatie van basaalcellen. Basaalcellen en prolifererende basaalcellen in het midden van het epitheel zijn belangrijk voor het in stand houden van deze metaplasie. All-trans-retinol remt de door SRC in gang gezette delingsactiviteit van

basaalcellen. Basaalcellen zijn betrokken bij het onstaan van cylindrische cellen.

2. SRC en vitamine A hebben duidelijke effecten op vitamine A-deficiënt trachea-epitheel in orgaankweek. In een orgaankweekmodel kunnen zowel all-trans-retinol als metabolieten van retinol de door SRC en vitamine A-deficiëntie veroorzaakte veranderingen beïnvloeden.
3. Anders dan in celkweken vindt in tracheakweken geen gelijktijdige expressie van vimentine en keratine plaats. Interacties tussen de submucosa en het epitheel en de driedimensionale opbouw van het epitheel in orgaankweken spelen hierbij waarschijnlijk een belangrijke rol.
4. In vitamine A-deficiënte hamstertracheakweken kunnen naast delende basaalcellen en slijmproducerende cellen, ook delende trilhaar- en indifferente cellen worden aangetoond.
5. SRC remt de communicatie tussen trachea-epitheelcellen van hamsters in medium zonder serum en met cofactoren. All-trans-retinol en retinoïnezuur remmen of stimuleren, afhankelijk van de concentraties, de intercellulaire communicatie.

Op grond van de studies beschreven in dit proefschrift is het gebleken dat het in vitro-model met trachea-orgaankweken van vitamine A-deficiënte hamsters een goed model is voor het bestuderen van de effecten van chemische verbindingen die biologische processen, zoals celdeling en celdifferentiatie, beïnvloeden.

Toekomstig onderzoek met trachea-orgaankweken zou zich meer moeten richten op de relatie tussen het metabolisme, de veroorzaakte schade en het daarop volgend herstel in individuele (trachea)-epitheelcellen.

Curriculum vitae

Fons Rutten werd op 28 maart 1958 te Geldrop geboren. Na het behalen van de diploma's MAVO (1974) aan de Radboud MAVO te Asten en HAVO (1976) en VWO (1978) aan het Peelland College te Deurne begon hij in september 1978 met de studie Humane Voeding aan de Landbouwhogeschool te Wageningen. Zijn stage vervulde hij bij het Instituut CIVO-Toxicologie en Voeding TNO, afdeling Biologische Toxicologie, sectie Klinische Chemie en Metabolisme (dr. H.E. Falke). Het doctoraalexamen, met als afstudeervakken Toxicologie (prof. dr. J.H. Koeman) en Humane Voeding (prof. dr. J.G.A.J. Hautvast) legde hij af in november 1984. In maart 1984 begon hij, in een deeltijdfunctie, met onderzoek bij de afdeling Biologische Toxicologie, Instituut CIVO-Toxicologie en Voeding, Hoofdgroep Voeding en Voedingsmiddelen TNO, in Zeist. In maart 1985 startte hij het in dit proefschrift beschreven onderzoek in samenwerking met de Vakgroep Toxicologie van de Landbouwuniversiteit te Wageningen.