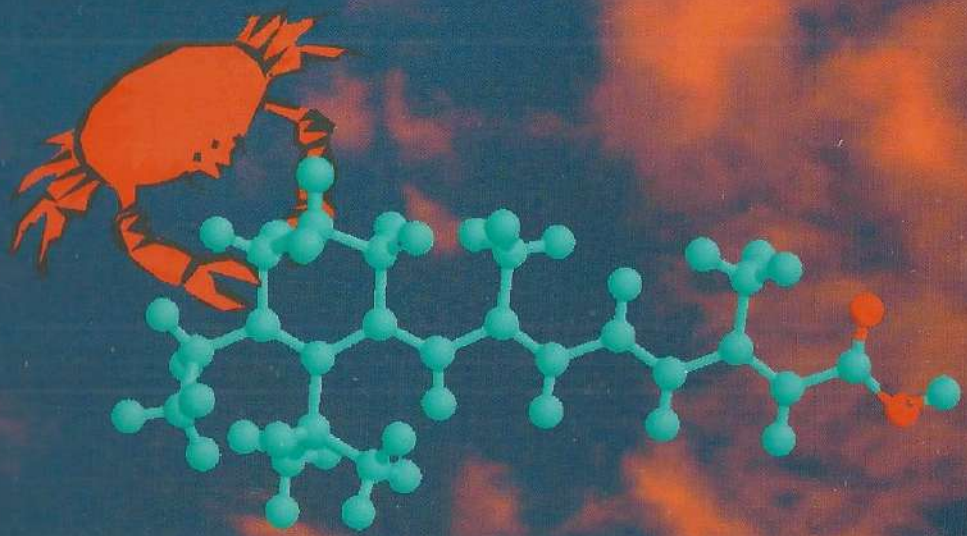
Determinants of retinoid sensitivity in head and neck squamous cell carcinoma



Ingeborg Klaassen

Determinants of retinoid sensitivity in head and neck squamous cell carcinoma Ingeborg Klaassen

Determinants of retinoid sensitivity in head and neck squamous cell carcinoma

The research described in this thesis was performed at the Section Tumor Biology of the Department of Otolaryngology, Vrije Universiteit Medical Center, Amsterdam, the Netherlands. The research was supported by the Dutch Cancer Society (grant: VU 95-926).

Publication of this thesis was financially supported by:

Dutch Cancer Society
BioWhittaker
Acros Organics

Cover design: Dennis Hilgers and Ingeborg Klaassen

Printed by: Ponsen & Looijen bv, Wageningen

Thesis Vrije Universiteit, Amsterdam
ISBN 90-64646589

VRIJE UNIVERSITEIT

Determinants of retinoid sensitivity in head and neck squamous cell carcinoma

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. T. Sminia,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der Geneeskunde
op maandag 15 oktober 2001 om 15.45 uur
in het hoofdgebouw van de universiteit,
De Boelelaan 1105

door

Ingeborg Klaassen

geboren te Rotterdam

promotor:	prof.dr. G.B. Snow
copromotoren:	dr. B.J.M. Braakhuis dr. R.H. Brakenhoff

aan mijn ouders

Contents

Chapter 1

General introduction

Head and Neck Squamous Cell Carcinoma

Head and neck squamous cell carcinoma (HNSCC) arises from the mucous membrane of the upper aerodigestive tract, including the oral cavity, larynx and pharynx. HNSCC is the fifth and seventh most common malignancy worldwide in males and females, respectively. In 1996, an estimated 575,000 new cases were diagnosed worldwide for cancer of the mouth or pharynx, and an estimated 190,000 for cancer of the larynx (WHO 1997). The total number of new incidence patients with cancer in mouth or pharynx in the Netherlands amounts to 1200 a year and the number of new patients with laryngeal cancer to 750 per year. Incidence and mortality rates are rising in most parts of the world, especially in the developing world, where nearly 80% of cases occur. At present, 5-year survival rates are approximately between 50% and 65% (WHO 1997).

Current treatment modalities of surgery, radiotherapy, and chemotherapy are limited in their success and improvement in long-term cure rates with these modalities has reached a plateau (Vokes *et al.* 1993, WHO 1997). The major recent successes in the treatment of head and neck cancer are advances in radiotherapeutic and surgical reconstructive techniques, resulting in improved quality of life. However, the overall survival rates have only marginally increased (Muir and Weiland 1995).

Multiple Primary Tumors

One of the major causes of treatment failure in early stage HNSCC is the occurrence of second primary tumors (SPTs). Patients with early-stage head and neck cancers are often cured from their original tumor, but have a 2% to 4% per year risk of developing a SPT cancer in the same organ or organ system: the respiratory and upper digestive tract, including the esophagus. SPTs usually carry a bad prognosis because they often occur either at notoriously bad sites, like the lung or the esophagus, or within previously treated areas within the head and neck (Snow 1992, Lippman *et al.* 1994).

The development of head and neck epithelial cancer is biologically characterized by two basic concepts: "multistep carcinogenesis" (Farber 1984) and "field cancerization" (Slaughter *et al.* 1953). The process of carcinogenesis occurs over years and involves multiple genetic and epigenetic alterations that lead to invasive cancer (Califano *et al.* 1996). The concept of field cancerization addresses the multifocal epithelial injury caused by exposure to carcinogens such as tobacco and alcohol that could explain the development of multiple primary tumors (Slaughter *et al.* 1953).

Strong evidence for multistep carcinogenesis came from molecular studies in tissues with a variable degree of malignancy measuring loss of heterozygosity (LOH) (Califano *et al.* 1996). In premalignant tissue LOH at chromosome loci 9p and 3p can occur and the presence of these alterations correlated with further development into

HNSCC (Mao *et al.* 1996). The frequency of LOH, not only at 3p and 9p, but also at 8p, 17p and 18q, increases at each histopathological step from dysplasia to carcinoma in situ to invasive cancer (Califano *et al.* 1996).

Evidence for Slaughter's hypothesis of field carcinogenesis as an explanation for SPT development was supported by studies of the p53 tumor suppressor gene. Chung *et al.* (1993) provided evidence that the development of first and second primary tumors can be genetically independent events, since p53 mutations found in the first tumors were different from those found in corresponding SPTs. Other studies have also found discordant p53 mutations between primary tumors and SPTs (Nees *et al.* 1993, el Naggar *et al.* 1995, Homann *et al.* 2001). Some studies, however, do not support the hypothesis of independent fields as the source for SPT and suggest that at least a proportion of primary tumors and associated SPTs may arise from a single clone (Bedi *et al.* 1996). This evidence was found in patients with multiple primary head and neck tumors exhibiting similar patterns of allelic loss on chromosome 9p and 3p. Local expansion or micrometastatic spread of (pre-)malignant cells may be responsible for the development of SPTs. It was hypothesized that metastatic foci arise from cells migrated from the original primary site or floated away with the saliva, re-implanting at a secondary site (Carey 1996). Molecular analysis of normal tissue surrounding a primary HNSCC indicated that lateral displacement of normal mucosa by premalignant cells certainly is the most realistic explanation for developing clonally related SPTs (Tabor *et al.* 2001).

Epidemiology of head and neck cancer

Tobacco, alcohol and poor diet are well recognized as major risk factors of head and neck cancer. Notwithstanding, many heavy smokers and alcohol drinkers never develop these malignancies. Recent studies suggest that the development of HNSCC is also dependent on the genetical "make-up" of an individual. Genetic polymorphisms of carcinogen-activating enzymes or carcinogen-inactivating enzymes, are associated with an increased risk for tobacco-related cancers (Bartsch *et al.* 2000). Recent reports indicate that the risk of HNSCC in those exposed to mutagens may depend not only on the level of exposure but also on inherent cancer susceptibility that is most likely related to the individual's ability to repair DNA damage induced by mutagens (Spitz *et al.* 1994, Cloos *et al.* 1996, Schantz *et al.*1997). Using the model compound bleomycin, Cloos *et al.* (1999) were able to show that this sensitivity to mutagens has a hereditary basis.

Chemoprevention

From epidemiologic studies that showed an association between low intake of certain nutrients or fibers and an increased risk of oral cancer (reviewed by Garewal, 1995), a

new approach in the efforts to control HNSCC was developed: chemoprevention. This is defined as the effort to arrest or reverse premalignant cells during their progression to invasive malignancy, using physiological mechanisms that are not cytotoxic (Sporn 1976). Chemoprevention is based on the premise that intervention is possible during the multiple steps of carcinogenesis through the use of agents that are able to block mutagenic carcinogens, thereby preventing DNA damage by free radicals, suppressing epithelial cell hyperproliferation, and/or modulating epithelial cell differentiation and apoptosis (Hong and Lippman 1995, Lotan 1996). Additional rationales for chemoprevention come from animal studies and basic studies of carcinogenesis (Willet and MacMahon 1984, Moon *et al.* 1994, DiGiovanni 1991).

In the last few years, many chemopreventive agents have been or are being studied in preclinical studies and phase I, II and III clinical trials in specific disease sites (CancerNet 2001: http://cancernet.nci.nih.gov/). A chemoprevention trial is characterized by a long duration, as the end-point of a trial is the development of cancer and/or survival. Another consequence is that drug administration has a chronic character, being often a daily intake for more than one year. An important issue is the level of toxicity that can be tolerated. It has to be realized that many individuals will be treated who will not develop a tumor at all. In general, it can be stated that a higher level of toxicity is acceptable when the risk for cancer increases.

Two major types of chemoprevention trials can be performed. The primary prevention trial addresses a general population or a population with a higher risk, such as long term smokers. The secondary chemoprevention trials applies to patients with precancerous lesions or patients at very high risk for a SPT. The cancer risk is much higher in secondary prevention trials and therefore a higher level of toxic side effects is acceptable in the study population.

It is clear that chemoprevention trials require long-term follow-up and large study populations. One of the most important goals of prevention research is to identify morphological, genetic and biochemical markers that can accurately detect early changes associated with a phase of carcinogenesis that precedes the final end point, cancer. This task of characterization and validation of the markers should be an integral part in all chemoprevention research. Markers should be used to identify the individuals who have the highest cancer risk and to monitor and predict the outcome of intervention. With validated markers trials will be performed in a shorter time, at reduced costs and less patients will be over-treated.

A recent review described the outcome of a large number of chemoprevention trials, showing that most trials have shown a lack of effect (Lippman *et al.* 1998). A few positive, but also negative outcomes have been reported.

With respect to HNSCC, chemoprevention has the potential to stop the progression of premalignant lesions (e.g. oral leukoplakia) and to reduce the incidence and mortality of SPTs. Because SPTs develop at the relatively low annual rate of three percent, HNSCC chemoprevention trials also need large number of subjects and a long-term follow-up, when cancer incidence is the study end-point (Shin *et al.* 1994).

Retinoids

Presently, the most studied chemopreventive agents are the retinoids, which are the natural and synthetic derivatives of vitamin A. An overview of the chemical structures of β-carotene, vitamin A and some of the synthetic retinoids that have been tested in chemoprevention trials are shown in Figure1.

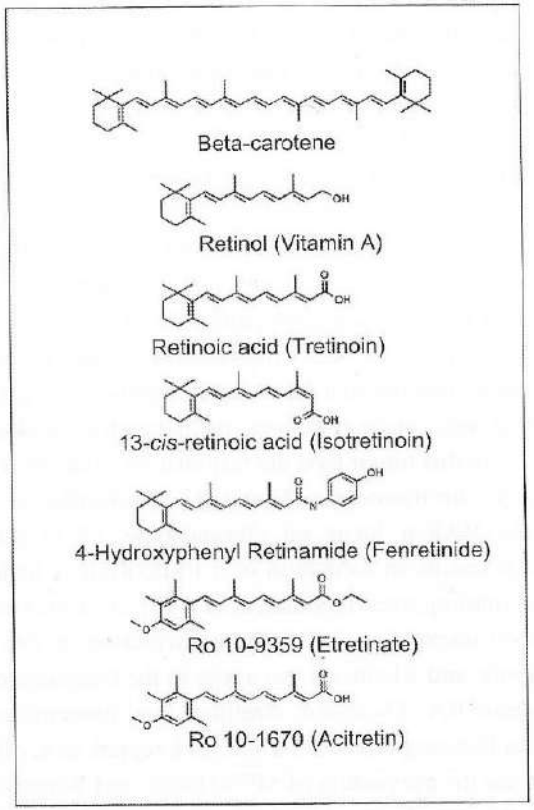


Figure 1. Structures of beta-carotene, vitamin A and a number of synthetic retinoids that have been tested in chemoprevention trials. Trivial names are given in parentheses.

Retinoids are required for several essential life processes, including vision, reproduction, metabolism, growth, differentiation, hematopoiesis, immunological processes, bone development, and pattern formation during embryogenesis (Sporn and Roberts 1984). Epidemiologic studies have addressed the relationships between retinoids and cancer. It was found that serum levels of β-carotene (provitamin A) and vitamin A (retinol) were significantly higher in control subjects than in oral cancer patients (Kune *et al.* 1993, Marshall *et al.* 1992, de Vries *et al.* 1990). Furthermore, studies of diet with questionnaires have reported that a lower intake of vitamin A is related to a higher risk for cancer. There is considerable evidence that retinoids have potent antiproliferative effects, and may be effective in the treatment of a variety of human diseases including cancer (Ong and Chytil 1983, Sporn and Roberts 1984). Retinoids can also suppress the proliferation of cells by inducing differentiation, as was reported for human HL-60 promyelocytic leukemia cells and for murine F9 embryo carcinoma cells (Warell 1993, Strickland and Mahdavi, 1987). Retinoids can act as immunostimulants (Prabhala *et al.* 1991, Lippman *et al.* 1987) and counteract oncogenic effects of HPV-16 in skin keratinocytes (Eckert *et al.* 1995).

Treatment with retinoids caused regression of dysplastic nevi or complete or partial remissions in 10% of the patients with basal cell carcinoma of the skin (Hill and Grubbs 1992). Squamous cell carcinoma of the skin (Lippman *et al.* 1992a) and the cervix (Lippman *et al.* 1992b) can be effectively treated with the combination of 13-*cis*-RA and α-interferon, resulting in remissions in 68% and 50% of patients, respectively. In an adjuvant setting, retinyl palmitate given at a high dose daily for a 12-month period could diminish the development of recurrent lung cancer (Pastorino *et al.* 1993). Interestingly, acute promyelocytic leukemia can be effectively treated with a low-dose of all-*trans*-RA (Hill and Grubbs 1992). In this tumor type the response to retinoids is associated with a translocation involving chromosomes 15 and 17 (Kakizuka *et al.* 1991). This translocation fuses the RAR-α locus on chromosome 17 to the PML gene on chromosome 15, which results in formation of a PML-RAR-α fusion protein which contains DNA and RA binding sites (Goddard *et al.* 1991, de Thé *et al.* 1991, Kakizuka *et al.* 1991). It is as yet unexplained what the contribution of this protein is in the development of leukemia, and whether it has a role in the treatment response. Retinol, retinyl palmitate, all-*trans*-RA, 13-*cis*-RA, etretinate, and fenretinide (4HPR) were all tested in clinical studies focusing on the head and neck region, either for reversal of oral preinvasive lesions or for the prevention of SPTs (Issing and Wustrow 1996, Lippman *et al.* 1994). Although the complexity of the biological responses of vitamin A suggests that several metabolites may exist, by the mid-1980s an abundance of evidence had led

researchers to believe that most actions were due to one specific metabolite, retinoic acid (RA), and more specifically all-*trans*-RA.

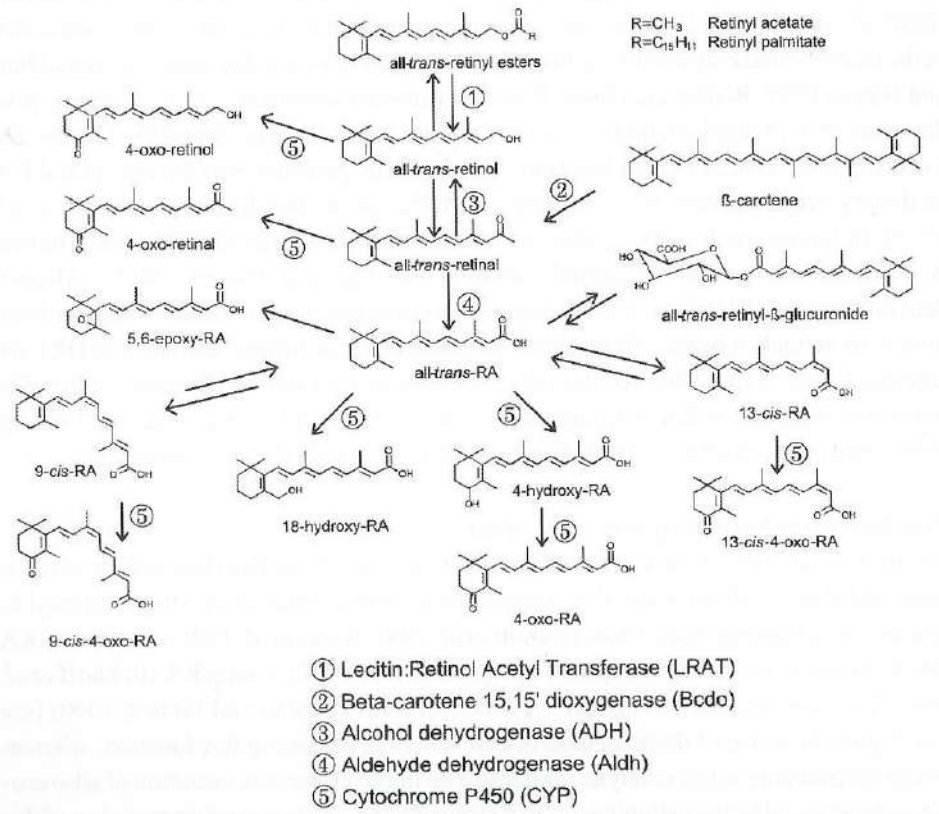


Figure 2. Structures of natural occurring retinoids with their proposed pathways of retinoid metabolism and enzymes involved.

Uptake, Transport and Metabolism of Retinol and Retinoic Acid

All retinoids in the body originate from retinyl-esters, carotenoids and retinol in the diet. The dietary carotenoids and preformed retinoids undergo a series of metabolic conversions, extracellularly in the lumen of the intestine and intracellularly in the intestinal mucosa. The absorbed retinol, along with other dietary lipids in the intestinal mucosa, is packaged as retinyl ester in nascent chylomicrons. The chylomicrons are

secreted into the lymphatic system, and the bulk of the chylomicron retinoids are eventually taken up by the liver, where the majority of the body's retinoids are stored (Blomhoff *et al.* 1990). In liver parenchymal cells retinyl esters are hydrolyzed to retinol and enter the circulation through the portal system, bound to retinol binding protein (RBP) (Hendriks *et al.* 1987). A very small portion of dietary retinoid is converted to RA in the intestine and is absorbed via the portal system as RA bound to serum albumin (Nau and Blaner 1999, Blaner and Olson 1994). The plasma level of RA is very low and is in the range of 4-14 nmol/l in humans, which is about 0.2-0.7% of plasma retinol levels (De Leenheer *et al.* 1982, Eckhoff and Nau 1990). A large percentage of the retinol and RA of dietary origin appears to be removed from the circulation by tissues (Smith *et al.* 1973). In tissues, such as the epithelial mucosa of the upper aerodigestive tract, retinol is oxidized via retinal to retinoic acid (Edenberg and Brown 1992). Alcohol dehydrogenases (ADH) have been proposed to catalyze the reversible oxidation from retinol to retinal, whereas short chain dehydrogenase/reductase enzymes (SDR) are proposed to be responsible for the reduction of retinal to retinol (Duester 1996). The oxidation of retinal to RA is catalyzed by aldehyde dehydrogenases (ALDHs) and by members of the cytochrome P450 family (CYP1A1 and CYP1A2) (Figure 2).

Further Metabolism of Retinoic Acid

Metabolites of all-*trans*-RA generated *in vivo* include 13-*cis*-RA (Eckhoff *et al.*1991, Tang and Russell 1990), 9-*cis*-RA (Heyman *et al.* 1992, Levin *et al.* 1992), retinoyl ß-glucuronide (Dunagin *et al.* 1966, Eckhoff *et al.* 1991, Barua *et al.* 1991), 5,6-epoxy-RA (Mc Cormick *et al.* 1978), 4-hydroxy-RA (Skare *et al.* 1982), 4-oxo-RA (Eckhoff *et al.* 1991, Tang and Russell 1991), and 3,4-didehydro-RA (Thaller and Eichele 1990) (see also Figure 2). Some of these metabolites are active in mediating RA function, whereas others are probably solely catalytic products. The intracellular concentration of all-*trans*-RA is determined by the cellular uptake of retinoids, the synthesis and degradation of this molecule, and its availability. The availability is also dependent on the concentration of a number of proteins in particular that of cellular retinoic acid binding protein (CRABP). Metabolic pathways of RA include isomerization, decarboxylation and glucuronidation processes (Rockley *et al.* 1980, Roberts *et al.* 1980, Leo *et al.* 1984, Sass *et al.* 1994). Another important route of RA metabolism consists of hydroxylation at position 4 of the cyclohexenyl ring to form 4-hydroxy-RA, which is readily oxidized to 4-oxo-RA. *In vitro*, 4-hydroxylation of RA is mediated by a cytochrome P450 (CYP)-dependent monooxogenase system that requires NAPDH and oxygen (Roberts *et al.* 1980, Leo *et al.* 1984). Hepatic 4-hydroxylation of RA has been shown to be catalyzed *in vitro* by different CYP subfamilies, depending on the species studied. The main isozymes

involved in humans are CYP26A1 and CYP2C8 (Leo *et al.* 1989, Ray *et al.* 1997, White *et al.* 1997). CYP26A1 is a recently discovered enzyme (Ray *et al.* 1997, White *et al.* 1997), which is RA-inducible and possesses strong RA 4-hydroxylating activity (Marikar *et al.* 1998).

Retinoic acid receptors

In contrast to retinol and β-carotene, the various forms of retinoic acid are thought to be active through direct interaction with specific nuclear receptors. The effects of retinoids are mediated by retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which act as ligand-activated transcription factors (Mangelsdorf *et al.* 1994, Heyman *et al.* 1992) regulating retinoid signal transduction (Figure 3). Each receptor class includes three subtypes (α, β, and γ), and of each subtype several isotypes exist arising from alternative promotors or splicing (Leid *et al.* 1992). The RARs have almost equal affinity for all-*trans*-RA and 9-*cis*-RA, whereas the RXRs preferentially bind 9-*cis*-RA. *In vitro* studies have shown that RXRs can act as homodimers or as heterodimeric partners of RARs or several other nuclear receptors including thyroid hormone receptors, vitamin D3 receptors, peroxisomal proliferator-activated receptors and several orphan receptors. Moreover, transactivation of genes by the retinoid receptors is also dependent on the action of coactivators and corepressors (reviewed by Xu *et al.* 1999).

Retinoids not only regulate transcription via the activation of specific retinoid receptors (transactivation activity), but also appear to suppress the activity of other transcription factors, such as AP-1, that are critical mediators of cellular proliferative activity (transrepression activity) (Fanjul *et al.* 1994, Chen *et al.* 1995, Nagpal *et al.* 1995). Although some researchers do not agree on mechanisms involved in transrepressive activity, recent studies suggest that AP-1 and other transcription factors compete with retinoid receptors for common "coupling factors" (proteins that control the activity of the RNA polymerase complex that transcribes genes) (Fanjul *et al.* 1994, Nagpal *et al.* 1995, Chen *et al.* 1995). When retinoids bind to their receptors, they can sequester these coupling factors so that they are inaccessible to other transcription factors (Kamei *et al.* 1996). Therefore, retinoids can turn down the activity of whole signaling pathways involved in either proliferative or inflammatory responses, including the pathway of AP-1, which mediates both proliferation and inflammation.

Hence, the action of retinoids is a fine-tuned mechanism dependent on the level of expression of specific receptor isotypes as well as on the type and concentration of retinoid compounds present in the cell.

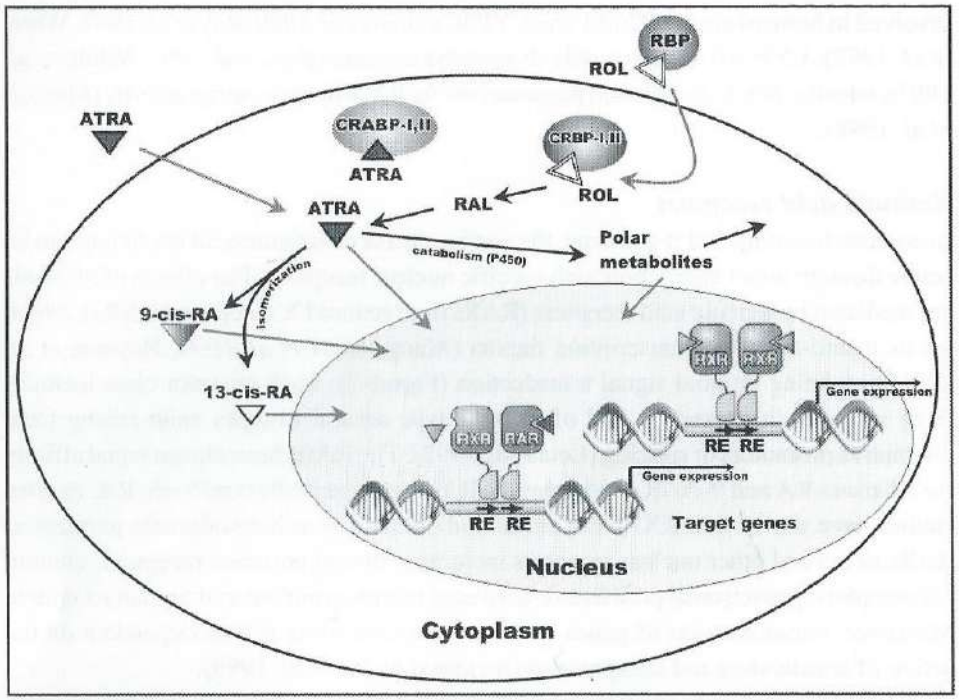


Figure 3. Schematic presentation of the retinoid signal transduction pathway. All-*trans*-retinoic acid (ATRA) enters the cell by passive diffusion or is intracellularly generated by conversion from retinol (ROL), via retinal (RAL). Retinol is transported extracellularly by RBP (retinol binding protein) and intracellularly by CRBP (cellular retinol binding protein); ATRA is transported intracellularly by CRABP (cellular retinoic acid binding protein). ATRA can be isomerized to 9-*cis*-RA or 13-*cis*-RA or catabolized to polar metabolites. ATRA, its isomers and some of the polar metabolites can bind with different binding affinities to the nuclear receptors, RAR (retinoic acid receptor) and RXR (retinoid X receptor). RARs preferentially bind with ATRA, RXRs with 9-*cis*-RA. The retinoid receptors can form homo-(RXR-RXR) or heterodimers (RXR-RAR) and bind on specific response elements (RE) in the promotor region of target genes, hence activating gene expression. Depending on the concentration of retinoids and receptors different signal transduction pathways can be activated.

Vitamin A and retinoids in HNSCC prevention

Vitamin A

To access the chemopreventive effects of vitamin A (retinyl palmitate) and N-acetylcysteine, a large randomized intervention study, the EUROSCAN trial, was undertaken (Van Zandwijk *et al.* 2000). In preclinical studies the cancer preventive activity of these compounds was proven, but this was never tested in a clinical trial. In the EUROSCAN trial 2592 patients were randomly assigned to receive retinyl palmitate

(300,000 IU daily for 1 year followed by 150,000 IU for a 2nd year), N-acetylcysteine (600 mg daily for 2 years), both compounds, or no intervention. A 2-year supplementation of retinyl palmitate and/or N-acetylcysteine resulted in no benefit - in terms of survival, event-free survival, or second primary tumors - for patients with head and neck cancer or with lung cancer, most of whom were previous or current smokers (Van Zandwijk *et al.* 2000).

Retinoids

Retinoids could successfully be used in the treatment of oral leukoplakia, a premalignant mucosal lesion (Hong *et al.* 1986, Lippman *et al.* 1988). Oral leukoplakia is a premalignant lesion that frequently develops into invasive HNSCC (Silverman *et al.* 1984, Schepman *et al.* 1998). Surgery has been considered the standard therapy for this condition, but is often not possible. The effects of retinoids on epithelial differentiation and proliferation led to investigate the efficacy of retinoids in reversing oral leukoplakia (Shklar *et al.* 1980). The most effective and least toxic form of retinoid therapy has not yet been established; among the studies in this area, the trial reported by Hong *et al.* (1986) showed promising results. The investigators tested the activity of 13-*cis*-RA in a randomized, placebo-controlled, double-blind trial involving 44 leukoplakia patients. 67% of the patients had an objective clinical response to the therapy, and 54% had a histological response. In contrast, the patients receiving placebo had only a 10% objective response rate. However, substantial toxicity and a high rate of relapse after discontinuation of the treatment presented major clinical limitations to this high-dose trial. Treatment with 13-*cis*-RA was associated with a distinct toxicity profile typical of hypervitaminosis A that includes mucocutaneous reactions (dry eyes, cheilitits, dry and itching skin), liver toxicity and myalgia.

In a subsequent study, Lippman *et al.* (1993) investigated a low dose of 13-*cis*-RA to address the toxicity and relapse problems of the first randomized trial. 90% of the patients showed regression of the lesion or stable disease and this low dose 13-*cis*-RA was well tolerated, with no patients dropping out because of toxicity. Other retinoids, including all-*trans*-RA and retinol also showed activity in leukoplakia (Issing *et al.* 1994, Stich *et al.* 1988).

Every year, patients have a 2% to 4% risk of developing SPTs following definitive treatment of early HNSCC. To alleviate this risk, Hong *et al.* (1990) conducted a 12-month randomized, double-blind, placebo-controlled trial of high-dose 13-*cis*-RA (50 to 100 mg/m^2/d) as adjuvant therapy following curative surgery and/or radiation therapy of primary HNSCC. Of the 103 patients studied, significantly fewer 13-*cis*-RA-treated patients (4%) than placebo patients (24%) developed SPTs after 32 months of follow-up

($P = 0.005$). Of the 14 SPTs that developed, 13 (93%) occurred in the tobacco-smoke exposed field of the upper aerodigestive tract, lungs, and esophagus. This study showed substantial toxicity in the high-dose 13-*cis*-RA arm: one third of retinoid patients needed dose reductions or discontinued therapy. The toxicity symptoms were similar as observed in the leukoplakia trial as mentioned above. Upon re-analysis after a median follow-up of 4.5 years (Hong *et al.* 1994), 13-*cis*-RA patients continued to have significantly fewer total SPTs: seven (14%) vs 16 (31%) in the placebo arm ($P = 0.042$). With respect to only SPTs in tobacco-exposed sites, only 7% of 13-*cis*-RA patients vs 33% of placebo patients developed these SPTs ($P = 0.008$). These long-term data suggest 13-*cis*-RA exerted a protective effect lasting several years after the completion of therapy.

The most important limitations to the effect of chemopreventive therapy are the presence of intrinsic or acquired resistance to retinoids and the occurrence of unwanted toxic side effects (Hong *et al.* 1994). In addition, the most active retinoid 13-*cis*-RA was marginally active in the more advanced forms of premalignant disease (Shin *et al.* 2000). At last, lesions may recur after the drug is stopped, implying that the user will require lifetime treatment for protection (Hong *et al.* 1995).

Despite many attempts, retinoids show limited activity in the treatment of various types of solid tumors in the more advanced stages. Treatment of 13-*cis*-RA in combination with IFN-α has shown positive therapeutic effects in patients with skin or cervical cancer (Lippman *et al.* 1992a, Lippman *et al.* 1992b). The combination of 13-*cis*-RA with IFN-α did however not work in advanced HNSCC (Cascinu *et al.* 1996).

Other retinoic acid derivatives are being developed and tested, hoping that these derivatives may lack the negative effects of 13-*cis*-RA and be able to provide similar or higher efficacy (Armstrong and Meyskens 2000). An understanding of the basic components of the cellular pharmacology of retinoids is essential if they are to be used efficiently and safely.

Role of retinoids, RARs and CYPs in Cancer

RAR-β mRNA was found to be reduced, compared with that in normal squamous epithelium, in 65% of HNSCC tumors, 30% in adjacent tissue and in 60% of oral dysplasias (Xu *et al.* 1994, Lotan *et al.* 1995). These results indicate that the decreased expression of RAR-β is related to the development of HNSCC, and suggests a role for RAR-β as a tumor suppressor (Lotan 1996). In premalignant oral lesions a clinical response was observed after up-regulation of RAR-β by 13-*cis*-RA (Lotan *et al.* 1995). There is some evidence that the mRNA expression of RAR-β is related to RA-sensitivity. Esophageal and lung cancer cell lines with reduced and inducible RAR-β mRNA expression, as compared to normal epithelial cells, were insensitive for the growth

inhibitory effects of retinoids (Xu *et al.* 1999, Sun *et al.* 2000). Furthermore, introduction of RAR-β increased growth inhibition by RA in cervical carcinoma (Frangioni *et al.* 1994) and breast carcinoma cells (Liu *et al.* 1996, Li *et al.* 1995, Seewaldt *et al.* 1995). In HNSCC the role of RAR receptors appears however to be different: it may not be RAR-β, but RAR-γ that is related with RA-sensitivity. RAR-γ is the major RAR type as judged from the low mRNA expression level in HNSCC cell lines compared to normal squamous cells (Hu *et al.* 1991). The relation between RAR-γ mRNA expression and RA-sensitivity in HNSCC was shown by experiments making use of mRNA over-expression and gene deletion (Oridate *et al.* 1996) and by measuring the effect of RAR-γ selective retinoids on growth inhibition (Le *et al.* 2000).

Reduction of RAR-β or RAR-γ mRNA expression could be a suitable explanation for the acquired resistance that is observed in patients during clinical trials with retinoids. From investigations in acute promyelocytic leukemia (APL) another explanation for acquired clinical resistance to retinoids was proposed, that is the increased induction of oxidative catabolism by CYPs (Muindi *et al.* 1994, Kizaki *et al.* 1997). In the plasma of the patients with remission increased levels of oxidized retinoid metabolites like 4-hydroxy-RA could be detected, which is an indication of CYP activity. For APL the increased activity of RA metabolizing enzymes is a plausible explanation for the occurrence of retinoid resistance.

Scope of this thesis

The pleiotropic effects of retinoids can be controlled by mechanisms at different levels. A large diversity of retinoid metabolites exist, which are converted from one to another by various enzymes and the resulting metabolites bind with different affinities to multiple RARs and RXRs, thereby activating specific retinoid signaling pathways. The work presented in this thesis aims to get more insight in the mechanisms that are involved in the retininoid induced growth inhibition of nonmalignant and (pre)malignant keratinocytes. It is investigated whether the differences as observed in response and toxicity in clinical trials with retinoids might be explained by differences between individuals in retinoid sensitivity and turnover. A comparison was made between normal and malignant oral squamous cells concerning retinoid metabolism (turnover rates and type of metabolites formed) and retinoid induced growth inhibition. The purpose of these studies is to obtain more knowledge about the determinants regulating retinoid metabolism between individuals. In addition, we were interested in the alteration in retinoid metabolism during the process of carcinogenesis. An important part of this study was the analysis of retinoid receptors in relation to RA-induced growth inhibition and turnover rate of RA. Since reduced receptor expression was often reported in HNSCC

cells, it may possibly also explain the lack of response in patients receiving retinoid treatment. These results may lead to a safer and more effective treatment with retinoids

In Chapter 2, an investigation on the stability and availability of RA and its isomers 13-*cis*-RA and 9-*cis*-RA during experimental procedures is described. Chapter 3 deals with the measurement of serum retinoid levels in HNSCC patients after one month treatment with retinyl palmitate compared with control persons. In Chapter 4, a study of RA-induced growth inhibition and RA turnover in three HNSCC cell lines is outlined. Chapter 5 reports on an investigation of growth inhibition and RA turnover and formation of metabolites in a larger panel of HNSCC cell lines ($n = 11$) and normal oral keratinocytes and the involvement of CYP26A1 in the metabolic route. An important goal was to elucidate whether RA turnover in these cell lines is a primary or secondary event in relation to its growth inhibiting effect. The discovery of the RA-specific cytochrome P450 enzyme, CYP26A1, provided the possibility to investigate its significancy for RA metabolism in normal and malignant head and neck cells. Chapter 6 outlines the investigation of mRNA expression of retinoic acid receptors (RAR-α, -β and -γ and RXR-β) and the relation with RA-sensitivity in five HNSCC cell lines. In Chapter 7 a study is described in which growth inhibition and turnover rate of four retinoid compounds (retinol, RA, 13-*cis*-RA and 9-*cis*-RA) are analyzed in four HNSCC cell lines and normal oral keratinocytes. Finally, Chapter 8 describes the difference in turnover rate of RA between normal oral keratinocytes of cancer patients and non-cancer patients.

References

Armstrong WB and Meyskens FL Jr (2000) Chemoprevention of head and neck cancer. *Otolaryngol Head Neck Surg* 122: 728-35

Bartsch H, Nair U, Risch A, Rojas M, Wikman H, and Alexandrov K (2000) Genetic polymorphism of CYP genes, alone or in combination, as a risk modifier of tobacco-related cancers. *Cancer Epidemiol Biomarkers Prev* 9: 3-28

Barua AB, Gunning DB, and Olson JA (1991) Metabolism in vivo of all-trans-[11-^{3}H]retinoic acid after an oral dose in rats. Characterization of retinoyl beta-glucuronide in the blood and other tissues. *Biochem J* 277: 527-31

Bedi GC, Westra WH, Gabrielson E, Koch W, and Sidransky D (1996) Multiple head and neck tumors: evidence for a common clonal origin. *Cancer Res* 56: 2484-7

Blaner WS and Olson JA (1994) Retinol and retinoic acid metabolism. *In:* The Retinoids, Sporn MB, Roberts AB, and Goodman DS (eds) pp 229-255. Raven Press: New York

Blomhoff R, Green MH, Berg T, Norum KR (1990) Transport and storage of vitamin A. *Science* 250: 399-404

Califano J, van der Riet P, Westra W, Nawroz H, Clayman G, Piantadosi S, Corio R, Lee D, Greenberg B, Koch W, and Sidransky D (1996) Genetic progression model for head and neck cancer: implications for field cancerization. *Cancer Res* 56: 2488-92

Carey TE (1996) Field cancerization: are multiple primary cancers monoclonal or polyclonal? *Ann Med* 28:183-8

Cascinu S, Del Ferro E, Ligi M, Graziano F, Castellani A, and Catalano G (1996) Phase II trial of 13-cis retinoic acid plus interferon-alpha in advanced squamous cell carcinoma of head and neck, refractory to chemotherapy. *Ann Oncol* 7: 538

Chen JY, Penco S, Ostrowski J, Balaguer P, Pons M, Starrett JE, Reczek P, Chambon P, and Gronemeyer H (1995) RAR-specific agonist/antagonists which dissociate transactivation and AP1 transrepression inhibit anchorage-independent cell proliferation. *EMBO J* 14: 1187-97

Chung KY, Mukhopadhyay T, Kim J, Casson A, Ro JY, Goepfert H, Hong WK, and Roth JA (1993) Discordant p53 gene mutations in primary head and neck cancers and corresponding second primary cancers of the upper aerodigestive tract. *Cancer Res* 53: 1676-83

Cloos J, Nieuwenhuis EJ, Boomsma DI, Kuik DJ, van der Sterre ML, Arwert F, Snow GB, and Braakhuis BJ (1999) Inherited susceptibility to bleomycin-induced chromatid breaks in cultured peripheral blood lymphocytes. *J Natl Cancer Inst* 91: 1125-30

Cloos J, Spitz MR, Schantz SP, Hsu TC, Zhang ZF, Tobi H, Braakhuis BJ, and Snow GB (1996) Genetic susceptibility to head and neck squamous cell carcinoma. *J Natl Cancer Inst* 88: 530-5.

de Vries N and Snow GB (1990) Relationships of vitamins A and E and beta-carotene serum levels to head and neck cancer patients with and without second primary tumors. *Eur Arch Otorhinolaryngol* 247: 368-70

de Thé H, Lavau C, Marchio A, Chomienne C, Degos L, and Dejean A (1991) The PML-RAR alpha fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. *Cell* 66: 675-84

De Leenheer AP, Lambert WE, and Claeys I (1982) All-trans-retinoic acid: measurement of reference values in human serum by high performance liquid chromatography. *J Lipid Res* 23: 1362-7

DiGiovanni J (1991) Modification of multistage skin carcinogenesis in mice. *Prog Exp Tumor Res* 33: 192-229

Duester G (1996) Involvement of alcohol dehydrogenase, short-chain dehydrogenase/reductase, aldehyde dehydrogenase, and cytochrome P450 in the control of retinoid signaling by activation of retinoic acid synthesis. *Biochemistry* 35: 12221-7

Dunagin PE Jr, Zachman RD, and Olson JA (1991) The identification of metabolites of retinal and retinoic acid in rat bile. *Biochim Biophys Acta* 124: 71-85

Eckert RL, Agarwal C, Hembree JR, Choo CK, Sizemore N, Andreatta-van Leyen S, and Rorke EA (1995) Human cervical cancer. Retinoids, interferon and human papillomavirus. *Adv Exp Med Biol* 375: 31-44

Eckhoff C, Collins MD, and Nau H (1991) Human plasma all-trans-, 13-cis- and 13-cis-4-oxoretinoic acid profiles during subchronic vitamin A supplementation: comparison to retinol and retinyl ester plasma levels. *J Nutr* 121: 1016-25

Eckhoff C and Nau H (1990) Identification and quantitation of all-trans- and 13-cis-retinoic acid and 13-cis-4-oxoretinoic acid in human plasma. *J Lipid Res* 31: 1445-54

Edenberg HJ and Brown CJ (1992) Regulation of human alcohol dehydrogenase genes. *Pharmacogenetics* 2: 185-96

el-Naggar AK, Lai S, Luna MA, Zhou XD, Weber RS, Goepfert H, and Batsakis JG (1995) Sequential p53 mutation analysis of pre-invasive and invasive head and neck squamous carcinoma. *Int J Cancer* 64: 196-201

Fanjul A, Dawson MI, Hobbs PD, Jong L, Cameron JF, Harlev E, Graupner G, Lu XP, and Pfahl M (1994) A new class of retinoids with selective inhibition of AP-1 inhibits proliferation. *Nature* 372: 107-11

Farber E (1984) The multistep nature of cancer development. *Cancer Res* 44: 4217-23

Frangioni JV, Moghal N, Stuart-Tilley A, Neel BG, and Alper SL (1994) The DNA binding domain of retinoic acid receptor beta is required for ligand-dependent suppression of proliferation. Application of general purpose mammalian coexpression vectors. *J Cell Sci* 107: 827-38

Garewal HS and Schantz S (1995) Emerging role of beta-carotene and antioxidant nutrients in prevention of oral cancer. *Arch Otolaryngol Head Neck Surg* 121: 141-4

Goddard AD, Borrow J, Freemont PS, and Solomon E (1991) Characterization of a zinc finger gene disrupted by the t(15;17) in acute promyelocytic leukemia. *Science* 254: 1371-4

Hendriks HF, Brekelmans PJ, Buytenhek R, Brouwer A, de Leeuw AM, and Knook DL (1987) Liver parenchymal cells differ from the fat-storing cells in their lipid composition. *Lipids* 22: 266-73

Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, and Thaller C (1992) 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* 68: 397-406

Hill DL and Grubbs CJ (1992) Retinoids and cancer prevention. *Annu Rev Nutr* 12: 161-81

Homann N, Nees M, Conradt C, Dietz A, Weidauer H, Maier H, and Bosch FX (2001) Overexpression of p53 in tumor-distant epithelia of head and neck cancer patients is associated with an increased incidence of second primary carcinoma. *Clin Cancer Res* 7: 290-6

Hong WK, Endicott J, Itri LM, Doos W, Batsakis JG, Bell R, Fofonoff S, Byers R, Atkinson EN, Vaughan C, and others (1986) 13-cis-retinoic acid in the treatment of oral leukoplakia. *N Engl J Med* 315: 1501-5

Hong WK, Lippman SM, Itri LM, Karp DD, Lee JS, Byers RM, Schantz SP, Kramer AM, Lotan R, Peters LJ, Dimery IW, Brown BW, and Goepfert H (1990) Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N Engl J Med* 323: 795-801

Hong WK and Itri LM (1994) Retinoids and human cancer. *In:* The Retinoids, Sporn MB, Roberts AB, and Goodman DS (eds) pp 597-630. Raven Press: New York

Hong WK, Lippman SM (1995) Cancer chemoprevention. *J Natl Cancer Inst Monogr* 49-53

Hong WK, Lippman SM, Hittelman WN, and Lotan R (1995) Retinoid chemoprevention of aerodigestive cancer: from basic research to the clinic. *Clin Cancer Res* 1: 677-86

Hu L, Crowe DL, Rheinwald JG, Chambon P, and Gudas LJ (1991) Abnormal expression of retinoic acid receptors and keratin 19 by human oral and epidermal squamous cell carcinoma cell lines. *Cancer Res* 51:9 72-81

Issing WJ and Wustrow TP (1996) Expression of retinoic acid receptors in squamous cell carcinomas and their possible implication for chemoprevention. *Anticancer Res* 16: 2373-7

Kakizuka A, Miller WH Jr, Umesono K, Warrell RP Jr, Frankel SR, Murty VV, Dmitrovsky E, and Evans RM (1991) Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor. *Cell* 66: 663-74

Kamei Y, Xu L, Heinzel T, Torchia J, Kurokawa R, Gloss B, Lin SC, Heyman RA, Rose DW, Glass CK, and Rosenfeld MG (1996) A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* 85: 403-14

Kizaki M, Ueno H, Matsushita H, Takayama N, Muto A, Awaya N, and Ikeda Y (1997) Retinoid resistance in leukemic cells. *Leuk Lymph* 25: 427-34

Kune GA, Kune S, Field B, Watson LF, Cleland H, Merenstein D, and Vitetta L (1993) Oral and pharyngeal cancer, diet, smoking, alcohol, and serum vitamin A and beta-carotene levels: a case-control study in men. *Nutr Cancer* 20: 61-70

Le Q, Dawson MI, Soprano DR, and Soprano KJ (2000) Modulation of retinoic acid receptor function alters the growth inhibitory response of oral SCC cells to retinoids. *Oncogene* 19: 1457-65

Leid M, Kastner P, and Chambon P (1992) Multiplicity generates diversity in the retinoic acid signalling pathways. *Trends Biochem Sci* 17: 427-33

Leo MA, Iida S, and Lieber CS (1984) Retinoic acid metabolism by a system reconstituted with cytochrome P- 450. *Arch Biochem Biophys* 234: 305-12

Leo MA, Lasker JM, Raucy JL, Kim CI, Black M, and Lieber CS (1989) Metabolism of retinol and retinoic acid by human liver cytochrome P450IIC8. *Arch Biochem Biophys* 269: 305-12

Levin AA, Sturzenbecker LJ, Kazmer S, Bosakowski T, Huselton C, Allenby G, Speck J, Kratzeisen C, Rosenberger M, Lovey A, and others (1992) 9-cis retinoic acid stereoisomer binds and activates the nuclear receptor RXR alpha. *Nature* 355: 359-61

Li XS, Shao ZM, Sheikh MS, Eiseman JL, Sentz D, Jetten AM, Chen JC, Dawson MI, Aisner S, Rishi AK, and others (1995) Retinoic acid nuclear receptor beta inhibits breast carcinoma anchorage independent growth. *J Cell Physiol* 165: 449-58

Lippman SM, Kessler JF, and Meyskens FL Jr (1987) Retinoids as preventive and therapeutic anticancer agents (Part I). *Cancer Treat Rep* 71: 391-405

Lippman SM, Kessler JF, Al Sarraf M, Alberts DS, Itri LM, Mattox D, Von Hoff DD, Loescher L, and Meyskens FL (1988) Treatment of advanced squamous cell carcinoma of the head and neck with isotretinoin: a phase II randomized trial. *Invest New Drugs* 6: 51-56

Lippman SM, Parkinson DR, Itri LM, Weber RS, Schantz SP, Ota DM, Schusterman MA, Krakoff IH, Gutterman JU, and Hong WK (1992a) 13-cis-retinoic acid and interferon alpha-2a: effective combination therapy for advanced squamous cell carcinoma of the skin. *J Natl Cancer Inst* 84: 235-41

Lippman SM, Kavanagh JJ, Paredes-Espinoza M, Delgadillo-Madrueno F, Paredes-Casillas P, Hong WK, Holdener E, and Krakoff ICH (1992b) 13-cis-retinoic acid plus interferon alpha-2a: highly active systemic therapy for squamous cell carcinoma of the cervix. *J Natl Cancer Inst* 84: 241-5

Lippman SM, Batsakis JG, Toth BB, Weber RS, Lee JJ, Martin JW, Hays GL, Goepfert H, and Hong WK (1993) Comparison of low-dose isotretinoin with beta carotene to prevent oral carcinogenesis. *N Engl J Med* 328: 15-20

Lippman SM, Spitz M, Trizna Z, Benner SE, and Hong WK (1994) Epidemiology, biology, and chemoprevention of aerodigestive cancer. *Cancer* 74: 2719-25

Lippman SM, Lee JJ, Sabichi AL (1998) Cancer chemoprevention: progress and promise. *J Natl Cancer Inst* 90: 1514-28

Liu Y, Lee MO, Wang HG, Li Y, Hashimoto Y, Klaus M, Reed JC, and Zhang X (1996) Retinoic acid receptor beta mediates the growth-inhibitory effect of retinoic acid by promoting apoptosis in human breast cancer cells. *Mol Cell Biol* 16: 138-49

Lotan R (1995) Squamous differentiation and retinoids. *Cancer Treat Res* 74: 43-72

Lotan R, Xu XC, Lippman SM, Ro JY, Lee JS, Lee JJ, Hong WK (1995) Suppression of retinoic acid receptor-beta in premalignant oral lesions and its up-regulation by isotretinoin. *N Engl J Med* 332: 1405-10

Lotan R (1996) Retinoids in cancer chemoprevention. *FASEB J* 10: 1031-9

Mangelsdorf DJ (1994) Vitamin A receptors. *Nutr Rev* 52: S32-44

Mao L, Lee JS, Fan YH, Ro JY, Batsakis JG, Lippman S, Hittelman W, and Hong WK (1996) Frequent microsatelite alterations at chromosomes 9p21 and 3p14 in oral premalignant lesions and their value in cancer risk assessment. *Nat Med* 2: 682-5

MarikarY, Wang Z, Duell EA, Petkovich M, Voorhees JJ, and Fisher GJ (1998) Retinoic acid receptors regulate expression of retinoic acid 4-hydroxylase that specifically inactivates all-*trans* retinoic acid in human keratinocyte HaCaT cells. *J Invest Dermatol* 111: 434-9

Marshall JR, Graham S, Haughey BP, Shedd D, O'Shea R, Brasure J, Wilkinson GS, and West D (1992) Smoking, alcohol, dentition and diet in the epidemiology of oral cancer. *Eur J Cancer B Oral Oncol* 28B: 9-15

McCormick AM, Napoli JL, Schnoes HK, DeLuca HF (1978) Isolation and identification of 5, 6-epoxy-retinoic acid: a biologically active metabolite of retinoic acid. Biochemistry 17: 4085-90

Moon RC, Mehta RG, and Rao KV (1994) Retinoids and cancer in experimental animals. *In:* The Retinoids, Sporn MB, Roberts AB, Goodman DS (eds) pp 573–596. Raven Press: New York

Muindi JR, Young CW, and Warrell RP Jr (1994) Clinical pharmacology of all-trans retinoic acid. *Leukemia* 8: 1807-12

Muir C and Weiland L (1995) Upper aerodigestive tract cancers. *Cancer* 75: 147-53

Nagpal S, Athanikar J, and Chandraratna RA (1995) Separation of transactivation and AP1 antagonism functions of retinoic acid receptor alpha. *J Biol Chem* 270: 923-7

Nees M, Homann N, Discher H, Andl T, Enders C, Herold-Mende C, Schuhmann A, and Bosch FX (1993) Expression of mutated p53 occurs in tumor-distant epithelia of head and neck cancer patients: a possible molecular basis for the development of multiple tumors. *Cancer Res* 53: 4189-96

Nau H and Blaner WS (1999) *In:* Retinoids: the biochemical and molecular basis of Vitamin A and retinoid action, Nau H and Blaner WS (eds) pp 32-95. Springer-Verlag, Berlin-Heidelberg

Ong DE and Chytil F (1983) Vitamin A and cancer. *Vitam Horm* 40: 105-44

Oridate N, Esumi N, Lotan D, Hing WK, Rochette-Egly C, Chambon P, and Lotan R (1996) Implication of retinoic acid receptor gamma in squamous differentiation and response to retinoic acid in head and neck SqCC/Y1 squamous carcinoma cells. *Oncogene* 12: 2019-28

Pastorino U, Infante M, Maioli M, Chiesa G, Buyse M, Firket P, Rosmentz N, Clerici M, Soresi E, Valente M, and others (1993) Adjuvant treatment of stage I lung cancer with high-dose vitamin A. *J Clin Oncol* 11: 1216-22

Prabhala RH, Garewal HS, Hicks MJ, Sampliner RE, and Watson RR (1991) The effects of 13-cis-retinoic acid and beta-carotene on cellular immunity in humans. *Cancer* 67: 1556-60

Ray WJ, Bain G, Yao M, and Gottlieb DI (1997) CYP26, a novel mammalian cytochrome P450, is induced by retinoic acid and defines a new family. *J Biol Chem* 272: 18702-8

Roberts AB, Lamb LC, and Sporn MB (1980) Metabolism of all-trans-retinoic acid in hamster liver microsomes: oxidation of 4-hydroxy- to 4-keto-retinoic acid. *Arch Biochem Biophys* 199: 374-83

Rockley NL, Halley BA, and Nelson EC (1980) Identification of a decarboxylation product of retinoic acid. *Biochim Biophys Acta* 627: 270-5

Sass JO, Forster A, Bock KW, and Nau H (1994) Glucuronidation and isomerization of all-trans- and 13-cis-retinoic acid by liver microsomes of phenobarbital- or 3-methylcholanthrene-treated rats. *Biochem Pharmacol* 47: 485-92

Schantz SP, Zhang ZF, Spitz MS, Sun M, and Hsu TC (1997) Genetic susceptibility to head and neck cancer: interaction between nutrition and mutagen sensitivity. *Laryngoscope* 107: 765-81

Schepman KP, van der Meij EH, Smeele LE, van der Waal I (1998) Malignant transformation of oral leukoplakia: a follow-up study of a hospital-based population of 166 patients with oral leukoplakia from The Netherlands. *Oral Oncol* 34: 270-5

Seewaldt VL, Johnson BS, Parker MB, Collins SJ, and Swisshelm K (1995) Expression of retinoic acid receptor beta mediates retinoic acid-induced growth arrest and apoptosis in breast cancer cells. *Cell Growth Differ* 6: 1077-88

Shin DM, Hittelman WN, and Hong WK (1994) Biomarkers in upper aerodigestive tract tumorigenesis: a review. *Cancer Epidemiol Biomarkers Prev* 3: 697-709

Shin DM, Mao L, Papadimitrakopoulou VM, Clayman G, El-Naggar A, Shin HJ, Lee JJ, Lee JS, Gillenwater A, Myers J, Lippman SM, Hittelman WN, and Hong WK (2000) Biochemopreventive therapy for patients with premalignant lesions of the head and neck and p53 gene expression. *J Natl Cancer Inst* 92: 69-73

Shklar G, Flynn E, Szabo G, and Marefat P (1980) Retinoid inhibition of experimental lingual carcinogenesis: ultrastructural observations. *J Natl Cancer Inst* 65: 1307-16

Silverman S Jr, Gorsky M, and Lozada F (1984). Oral leukoplakia and malignant transformation. A follow-up study of 257 patients. *Cancer* 53: 563-8

Skare KL, Schnoes HK, and DeLuca HF (1982). Biliary metabolites of all-trans-retinoic acid in the rat: isolation and identification of a novel polar metabolite. *Biochemistry* 21: 3308-17

Slaughter DP, Southwick HW, and Smejkel W (1953) "Field cancerization" in oral stratified squamous epithelium: clinical implications of multicentric origin. *Cancer* 6: 963-8

Smith JE, Milch PO, Muto Y, and Goodman DS (1973) The plasma transport and metabolism of retinoic acid in the rat. *Biochem J* 132: 821-7

Snow GB (1992) Follow-up in patients treated for head and neck cancer: how frequent, how thorough and for how long? *Eur J Cancer* 28: 315-6

Spitz MR, Hoque A, Trizna Z, Schantz SP, Amos CI, King TM, Bondy ML, Hong WK, and Hsu TC (1994) Mutagen sensitivity as a risk factor for second malignant tumors following malignancies of the upper aerodigestive tract. *J Natl Cancer Inst* 86: 1681-4

Sporn MB (1976) Approaches to prevention of epithelial cancer during the preneoplastic period. *Cancer Res* 36: 2699-702

Sporn MB and Roberts AB (1984) Role of retinoids in differentiation and carcinogenesis. *J Natl Cancer Inst* 73: 1381-7

Stich HF, Hornby AP, Mathew B, Sankaranarayanan R, and Nair MK (1988) Response of oral leukoplakias to the administration of vitamin A. *Cancer Lett* 40: 93-101

Strickland S and Mahdavi V (1978) The induction of differentiation in teratocarcinoma stem cells by retinoic acid. *Cell* 15: 393-403

Sun SY, Wan H, Yue P, Hong WK, and Lotan R (2000) Evidence that retinoic acid receptor beta induction by retinoids is important for tumor cell growth inhibition. *J Biol Chem* 275: 17149-53

Tabor MP, Brakenhoff RH, Van Houten VMM, Kummer JA, Snel MHJ, Snijders PJF, Snow GB, Leemans CR, and Braakhuis BJM (2001) Persistence of Genetically Altered Fields in Head and Neck Cancer Patients: Biological and Clinical Implications. *Clin Cancer Res* 7: 1523-32

Tang GW and Russell RM (1990) 13-cis-retinoic acid is an endogenous compound in human serum. *J Lipid Res* 31: 175-82

Tang G, Russell RM (1991) Formation of all-trans-retinoic acid and 13-cis-retinoic acid from all-trans-retinyl palmitate in humans. *J Nutr Biochem* 2: 201-13

Thaller C and Eichele G (1990) Isolation of 3,4-didehydroretinoic acid, a novel morphogenetic signal in the chick wing bud. *Nature* 345: 815-9

Van Zandwijk N, Dalesio O, Pastorino U, de Vries N, van Tinteren H (2000) EUROSCAN, a randomized trial of vitamin A and N-acetylcysteine in patients with head and neck cancer or lung cancer. For the EUropean Organization for Research and Treatment of Cancer Head and Neck and Lung Cancer Cooperative Groups. *J Natl Cancer Inst* 92:977-86

Vokes EE, Weichselbaum RR, Lippman SM, and Hong WK (1993) Head and neck cancer. *N Engl J Med* 328: 184-94

Warell RP (1993) Retinoid resistance. *Lancet* 341: 126

White JA, Beckett-Jones B, Guo YD, Dilworth FJ, Bonasoro J, Jones G, and Petkovich M (1997) cDNA cloning of human retinoic acid-metabolizing enzyme (hP450RAI) identifies a novel family of cytochromes P450. *J Biol Chem* 272: 18538-41

WHO (1997) The World Health Report 1997, Conquering suffering Enriching humanity. World Health Organisation, Geneve.

Willett WC and MacMahon B (1984) Diet and cancer--an overview. *N Engl J Med* 310: 633-8

Xu XC, Clifford JL, Hong WK, and Lotan R (1994) Detection of nuclear retinoic acid receptor mRNA in histological tissue sections using nonradioactive in situ hybridization histochemistry. *Diagn Mol Pathol* 3: 122-31

Xu L, Glass CK, and Rosenfeld MG (1999) Coactivator and corepressor complexes in nuclear receptor function. *Curr Opin Genet Dev* 9: 140-7

Chapter 2

Considerations for *in vitro* retinoid experiments: importance of protein interaction

Ingeborg Klaassen, Ruud H Brakenhoff, Serge J Smeets, Gordon B Snow, Boudewijn JM Braakhuis

Section Tumor Biology, Department of Otolaryngology, Vrije Universiteit Medical Center, Amsterdam, the Netherlands

Biochim Biophys Acta 1999; 1427: 265-75

Abstract

Retinoids, natural and synthetic substances structurally related to vitamin A, are important modulators of cell proliferation and differentiation, and have proven activity in cancer therapy. Experiments to reveal the mechanism of action of retinoids are routinely performed in *in vitro* models. As retinoids are relatively hydrophobic and unstable, we hypothesized that the composition of culture media is of critical importance for the stability and bioavailability of these compounds. Various culture media were incubated with all-*trans*-, 13-*cis*- and 9-*cis*-retinoic acid (RA). Without fetal calf serum (FCS) or bovine serum albumin (BSA) in the medium, the concentration of these retinoids was found to decrease to considerably low levels. This excessive loss of retinoids was due to absorption to culture plates, reaction tubes and pipet tips. Binding of retinoids to BSA was demonstrated to have attenuating effects on uptake and metabolism of all-*trans*-RA, as studied in oral keratinocytes and head and neck cancer cells, indicating that a balance exists between the bioavailability and the aspecific loss of retinoids. In this study we demonstrate that the type of culture medium and especially the presence of protein in the medium is of paramount importance to perform reproducible experiments with retinoids.

Introduction

Retinoids are natural and synthetic substances structurally related to vitamin A. They exert antiproliferative and differentiation-inducing effects on cancer cells and are used in the prevention and therapy of certain types of human cancer and precancerous lesions (Gudas *et al.* 1994, Lotan 1996). All-*trans*-retinoic acid is a natural vitamin A metabolite formed from beta-carotene by enterocytes and from retinol and retinaldehyde in target cells. Intracellular all-*trans*-RA can be isomerized to 13-*cis*-RA and 9-*cis*-RA, metabolized to products like 4-oxo-RA and 4-hydroxy-RA, which are finally catabolized to glucuronidized products. Interestingly, some of these metabolites are believed to play a role in cellular processes. Retinoids are thought to be active by binding to specific nuclear receptors that act as ligand-dependent transcription factors (Mangelsdorf and Evans 1995). The retinoid acid receptors (RARs) have almost equal affinity for all-*trans*-RA and 9-*cis*-RA, whereas the retinoid X receptors (RXRs) preferentially bind 9-*cis*-RA (Heyman *et al.* 1992, Levin *et al.* 1992). *In vitro* studies have shown that RXRs can act as homodimers or as heterodimeric partners of RARs or several other nuclear receptors including thyroid hormone receptors, vitamin D3 receptors, peroxisomal proliferator-activated receptors and several orphan receptors (Kastner *et al.* 1995). Hence, the action of retinoids is a fine-tuned mechanism dependent on the level of expression of specific receptor isotypes as well as on the type and concentration of retinoid compounds present

in the cell.

With regard to cancer therapy, retinoids have proven to be active in leukoplakia, a premalignant lesion in the mucosa of the oral cavity (Hong *et al.* 1986, Lippman *et al.* 1993) and 13-cis-RA could successfully prevent the occurrence of second primary tumors in the upper aerodigestive tract following head and neck squamous cell carcinoma (HNSCC) (Honge et al. 1990). A decreased level of retinol was found in serum of HNSCC patients (Bichler et al. 1983) and the mRNA level of RAR-ß is decreased in carcinoma and leukoplakia (Xu *et al.* 1994, Lotan *et al.* 1995). These results indicate that a dysregulated retinoid homeostasis is involved in the development of HNSCC.

We are interested to establish the role of retinoids in the malignant progression of oral keratinocytes toward HNSCC. The cancer cell lines must be cultured in serum-containing DMEM medium and primary keratinocytes in special medium containing growth factors but no serum. When measuring retinoid levels in low-protein keratinocyte medium we encountered, however, severe problems. Incubation of retinoids in this medium without cells resulted in very low retinoid concentrations, suggesting an artefact. Aim of the present study was to assess in detail the influence of the type of medium with or without protein on *in vitro* retinoid experiments and the consequences for retinoid action. We found a dramatic decrease in retinoid concentrations when serum or albumin was omitted from the medium. Our data indicate not only an important role for protein on the bioavailability of retinoids, but also on the reproducible standardized measurement of retinoids in *in vitro* experiments.

Materials and methods

Cell culture media

Two different types of Dulbecco's modified Eagle's media were used: powder DMEM (DMEM-P, Cat No. 52100, GIBCO Life Technologies B.V., Breda, the Netherlands), and liquid DMEM (DMEM-L, Cat. No. 12-709, Bio Whittaker, Verviers, Belgium). DMEM-P was prepared by dissolving powdered DMEM in sterile water, with 3.7 g/l $NaHCO_3$ and 5.9 g/l HEPES, adjusted with NaOH to pH 7.4. Both DMEM media were further supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin sulphate (all reagents from Life Technologies B.V., Breda, the Netherlands). Powder RPMI (Cat. No. 51800 from Bio Whittaker) was supplemented with 3 g/l $NaHCO_3$, 100 IU/ml penicillin, and 100 µg/ml streptomycin sulphate. Keratinocyte Growth Medium (KGM) was supplemented with gentamycin sulphate (final concentration 5 µg/ml) and amphotericin B (final concentration 0.5 µg/ml) (all from Life Technologies). FCS (Fetal Calf Serum) was obtained from Flow Laboratories (Irvine, Scotland, UK) and BSA (Bovine Serum Albumin) from Sigma (St. Louis, MO, USA).

Cell culturing

Primary oral keratinocytes were obtained from the uvulas of patients who underwent uvulopalatopharyngoplasty. Cells were isolated and cultured in keratinocyte growth medium (KGM) as

described previously (Reid *et al.* 1997). The HNSCC cell line UM-SCC-35 originating from a hypopharyngeal tumor was obtained from Dr TE Carey, University of Michigan, Ann Arbor, MI, USA and is described elsewhere (Carey 1985). These cells, normally cultured in DMEM with 5% FCS, were after adaptation routinely cultured in KGM medium. Primary cultures of keratinocytes were subcultured when they reached 70% confluence in a dilution of 10^5 cells/ml and were used at passage 3; tumor cell line UM-SCC-35 was subcultured once weekly with a split ratio of 1:5 and was used between passage 33 and 37.

Chemicals

All-*trans*-RA was obtained from Acros Chimica (Geel, Belgium), 4-oxo-*trans*- and *cis*-RA were kind gifts of Hoffmann-la Roche Basel, Switzerland; retinol and 13-*cis*-RA were obtained from Sigma (St. Louis, MO, USA). All compounds were dissolved in a 10^{-3} M stock in dimethyl sulfoxide (DMSO: JT Baker, Deventer, the Netherlands) and stored at -80°C. For each experiment, working dilutions were freshly prepared in the relevant cell culture medium. Final DMSO concentrations during incubation were always lower than 0.1% and did not affect cell growth. [11,12-^{3}H(N)]All-*trans*-RA (35.8 Ci/ mmol) was obtained from NEN (NEN Life Science Products, Boston, MA, USA). All procedures with retinoids were performed in subdued light.

Retinoid extraction and HPLC analysis

Retinoids were determined by reversed-phase HPLC after extraction with acetonitrile, as described previously (Teerlink *et al.* 1997). In short, two samples of 350 µl were taken from the medium and after removal of the residual medium the cells were washed once with PBS, collected by centrifugation after trypsin/EDTA treatment and subsequently lysed in 350 µl distilled water. Then 50 µl of 1 M sodium acetate buffer (pH 4.0) and 600 µl acetonitrile was added to the 350 µl samples (medium or lysed cell pellets), and after vortex-mixing the samples were centrifuged for 2-5 min at 3000 *g*. In total 720 µl of the clear supernatant was transferred to a 2 ml glass autosampler vial (Bekers, Amstelveen, the Netherlands), and after addition of 240 µl water the vials were capped, mixed by inversion and immediately put in the sample compartment of the autoinjector, which was kept at 4°C. A Separations (Hendrik Ido-Ambacht, the Netherlands) HPLC system was used, consisting of a Basic Marathon automatic sample injector, a Model M480G gradient pump (Gynkotek HPLC, Munich, Germany), a Model UVD 170S UV detector, and a column heater (Mistral). The mobile phase was degassed online using a model GT103 degasser. Chromelion software from Gynkotek (Munich, Germany) was used for instrument control and data acquisition. Separation was performed on a Spherisorb ODS2 3 mm column (100 * 4.6 mm) from Phase Separations (Deeside, UK) maintained at 30°C. Composition of the mobile phases and the binary gradient used were as described by Eckhoff and Nau (1990). UV detection was performed at 340 nm and retinoids were identified using external standards: 4-oxo-*trans*-RA, 4-oxo-*cis*-RA, 13-*cis*-RA, all-*trans*-RA, and retinol. Concentrations of 9-*cis*-RA were calculated using the extinction coefficient of 13-*cis*-RA.

Concentration of retinoids in different culture media

To four different culture media, 10^{-6} M all-*trans*-RA was added and distributed over 6 wells plates. Culture media used were DMEM-P, DMEM-L, RPMI and KGM. The first three culture media were tested with or without 5% FCS and the KGM medium with or without 1 mg/ml BSA. At several time points (0, 4, 24, 48, 72 h) samples of 750 µl were taken and stored after N_2 flushing at -80°C until further

analysis. Retinoids were quantified on the basis of peak height using external standardization. The protein content of the different media was determined by the Bio-Rad protein assay (Bio-Rad Laboratories B.V., Veenendaal, the Netherlands).

Assessment of retinoid loss by the extraction procedure

In a reaction tube (Sarstedt, Nümbrecht, Germany) with 750 µl distilled water, [^{3}H]all-*trans*-RA (ca. 2 x 10^6 cpm) was added. After pipetting the solution up and down, one sample of 350 µl was immediately transferred to a scintillation vial and a second sample of 350 µl to a fresh reaction tube. The pipet tip used for these procedures was put in a separate scintillation vial. The second sample was processed following the normal extraction procedure used for HPLC analysis. The total extraction solution was collected in a scintillation vial and the pipet tip in a separate vial. Finally the reaction tube used in the extraction and the initial reaction tubes were also put in two separate scintillation vials. The same experiment was done in distilled water containing 1 mg/ml BSA. To each scintillation vial Ultima Gold scintillation fluid was added and the radioactivity was measured. Each experiment was performed in duplicate.

Binding of retinoids to plastic materials

In a 24-wells plate (Costar, Acton, MA, USA) wells were filled either with 350 µl of distilled water or with distilled water containing 1 mg/ml BSA. Subsequently, [^{3}H]all-*trans*-RA (ca. 2 x 10^6 cpm) was added, the solutions were mixed by pipetting up and down and the content of each well was immediately collected in a scintillation vial. The used pipet tip (Sarstedt) was put in a second scintillation vial. Next, the wells were washed by adding 350 µl of distilled water, 50 µl of sodium acetate (1M, pH 4.0) and 600 µl of acetonitrile. This solution was similar to the solution used in the extraction, which is known to dissolve the retinoids with high efficiency (Teerlink *et al.* 1997). After pipetting up and down, again the total content of each well and the pipet tip were collected in separate scintillation vials. Finally, the wells were cut out the plates and put in separate scintillation vials. To each vial Ultima Gold scintillation fluid (Packard, Groningen, the Netherlands) was added and the radioactivity was measured. Each experiment was performed in triplicate.

Uptake of 10^{-6} M all-trans-RA by primary oral keratinocytes

Keratinocytes were cultured in 6 wells plates up to 70% confluence and exposed to KGM medium containing different amounts of BSA (0, 0.01, 0.1, 1, 10 mg/ml) and 10^{-6} M all-*trans*-RA. After 24 hours samples of 750 µl were taken, the cells were washed once with PBS, trypsinized and collected by centrifugation. Medium fractions and cell pellets were stored at -80°C under nitrogen until extraction for HPLC analysis. Retinoids were quantified on the basis of peak height using external standardization.

Metabolism in UM-SCC-35 with increasing amounts of BSA

Cells were cultured to near confluence in 6 well plates with 2 ml medium (KGM plus 1 mg/ml BSA) per well. Then the medium was replaced by 2 ml KGM and 10^{-6} M all-*trans*-RA with no, 1, 10 or 20 mg/ml BSA (final concentration). After several time points (0, 4, 24, 48 and 72 h), samples of 750 µl were taken and stored after N_2 flushing at -80°C. Since we intended also to measure unknown retinoid metabolites, the results were expressed as a percentage of the total area under the curve of the relevant part of the chromatogram (retention time between 6 and 30 min).

Results

Concentration of retinoids in different culture media

Retinoid concentrations in three different culture media with or without FCS, and in KGM with or without BSA were followed for 72 h after the addition of all-*trans*-RA (Figure 1). Without FCS or BSA in the medium all-*trans*-RA concentrations were much lower. However, the fractions remaining differed between the various media: in KGM medium without BSA the levels were considerably higher than in DMEM medium without FCS (20% vs. 0% at t=72 h); in RPMI without FCS the retinoid concentration remained higher as compared with the DMEM media without FCS (10% vs. 0% at t=72 h). In one of the DMEM media (DMEM-P$^+$) the retinoid concentrations decreased very rapidly during the incubation period, even in the presence of FCS. Isomerization, the conversion of all-*trans*-RA into 13- and 9-*cis*-RA, was observed for all media and varied from 20 to 50 %. Except for the peaks related to the known retinoids mentioned above, no other peaks could be detected. As a comparative analysis the two isomers of all-*trans*-RA, 13-*cis*-RA and 9-*cis*-RA, were investigated as well for their isomerization and concentration over time. All-*trans*-RA and 13-*cis*-RA were investigated in all medium types; 9-*cis*-RA was investigated in DMEM-L plus or minus FCS and KGM plus or minus BSA. The decline of 13-*cis*-RA and 9-*cis*-RA concentrations was comparable to that of all-trans-RA in all media, except for 13-*cis*-RA in DMEM-P + FCS, in which it was less stable (t=72 h: 13-*cis*-RA, 22.3%; all-*trans*-RA, 42.4%; data not shown). Figure 2 shows the isomerization of the three retinoic compounds in the various media, at t=0 and t=72 h. It has to be noted, however, that concentrations at t=72 h in serum- or albumin-free media are very low and could therefore give less accurate results. Isomerization of all three retinoids was found to be comparable, although 9-cis-RA appeared to be more stable than the other two retinoids in DMEM-L minus FCS and KGM minus BSA at t=0.

It appeared that protein content (5% FCS or 1 mg/ml albumin) is crucial to keep retinoids in solution. Albumin is an effective retinoid binder and transporter and forms the largest protein fraction in FCS. From the manufacturer's product information it is known that 5% FCS contains 1.3 mg/ml BSA (0.13% w/v) and 1.8 mg/ml total protein, whereas KGM medium contains about 42 µg protein/ml. No protein was detected in the DMEM and RPMI media. This variation in protein levels could largely explain the differences between the serum/albumin-free and -containing media assuming that albumin binding prevents the decrease in retinoid concentration. In principle, three reasons could explain the disappearance of retinoids in protein deficient

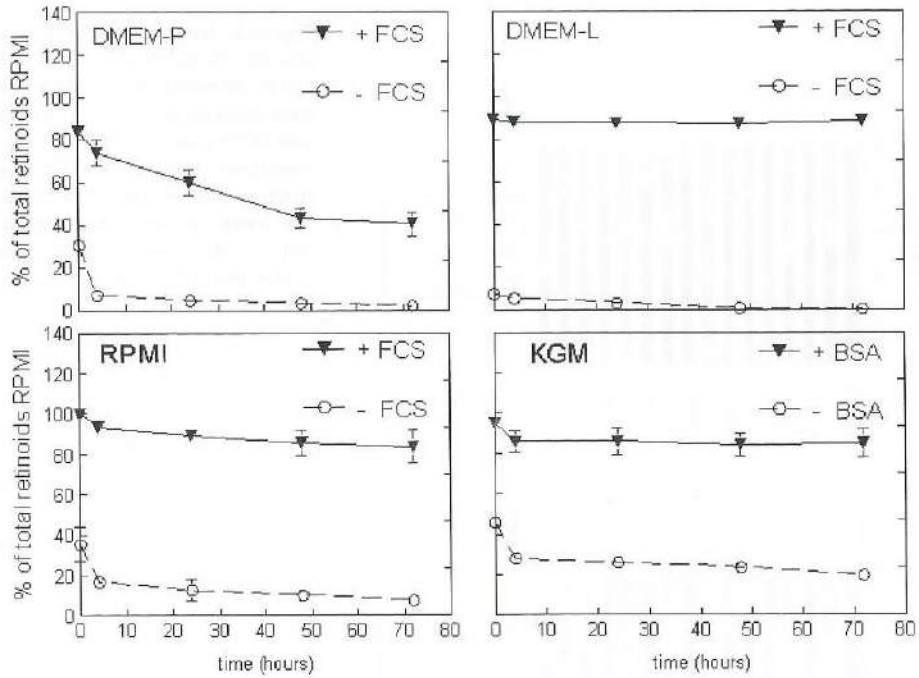


Figure 1. Concentration of retinoids in different culture media, after an exposure to 1 µM all-*trans*-RA during various incubation periods. Values of all-*trans*-RA were expressed as a percentage of the total amount of retinoids at t=0 in RPMI, which was set at a 100%. Except for the peaks of all-*trans*-RA, 13-*cis*-RA and 9-*cis*-RA, no other peaks could be detected. Values are presented as mean ± SD of three separate experiments. BSA (end concentration 1 mg/ml) was added to KGM, and FCS (end concentration 5%) to the other media.

medium 1) retinoids could be lost by physicochemical degradation, 2) the retinoids could be lost as a result of the extraction procedure for HPLC, and 3) retinoids could bind to the plastic materials like culture plates, pipet tips and reaction tubes.

Physicochemical degradation

To follow the physicochemical degradation, we analyzed the retinoid profile for breakdown products other than the isomeric forms of all-*trans*-RA, namely 13- and 9-*cis*-RA. In all media as described in Figure 1 we could not detect any other peaks, suggesting that no breakdown had taken place (data not shown).

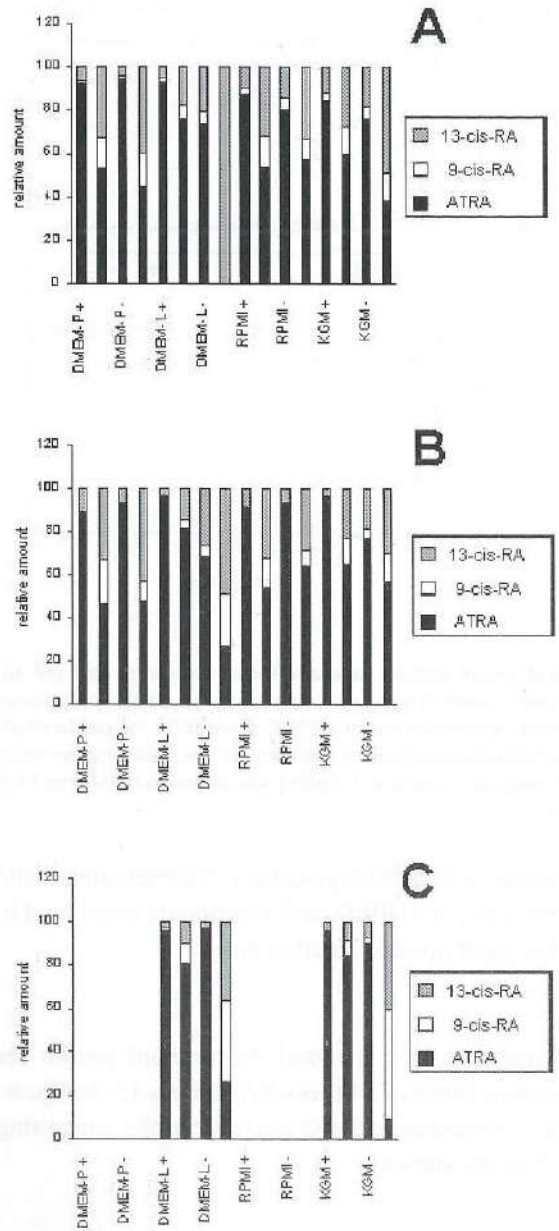


Figure 2. Isomerization of all-*trans*-RA (A), 13-*cis*-RA (B) and 9-*cis*-RA (C) in different culture media at t=0 (first stacked bar of each medium) and t=72h (second stacked bar), as described in Figure 1. For each medium and time point the total of the three isomers was set at a 100%. All experiments were performed in triplicate.

Assessment of retinoid loss by the extraction procedure

To study potential loss of retinoids by the extraction procedure, [^{3}H]all-*trans*-RA was measured before and after the extraction in albumin-free and albumin-containing distilled water (Figure 3). The results showed that the differences are not due to the extraction method and are independent on the presence of albumin. However, in this experiment we noted that considerably more radioactive material stuck to the pipet tips used for albumin-free solutions.

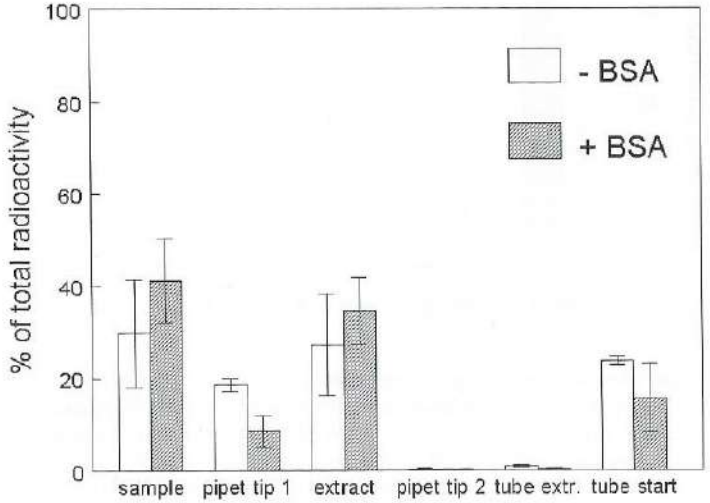


Figure 3. Potential loss of retinoids by the extraction procedure. [^{3}H]all-*trans*-RA was measured before and after the extraction in albumin-free and -containing distilled water (end concentration BSA 1 mg/ml). Sample = unextracted sample; extract = extracted sample; pipet tip 1 = pipet tip used with the transfer of the sample and the extracted sample; pipet tip 2 = pipet tip used with the transfer of the extraction solution to a scintillation vial; tube extr. = reaction tube in which the extraction was performed; tube start = reaction tube from which both samples were distributed. Values are presented as mean ± SD of two separate experiments.

Binding of retinoids to plastic materials

To investigate the loss of retinoids by binding to the used plastics, [^{3}H]all-*trans*-RA was added to 24-wells plates containing distilled water with or without 1 mg/ml BSA. After sampling, the wells were rinsed with the solvent used in the extraction procedure. In Figure 4 the results are shown of the relative amounts (in cpm) of [^{3}H] in the samples, the extraction solvent and other materials that came in contact with the retinoids. The effect of adding albumin to the solvent was striking: while in the albumin-containing samples more than 80% of the total cpms were found, on average only 25% was detected

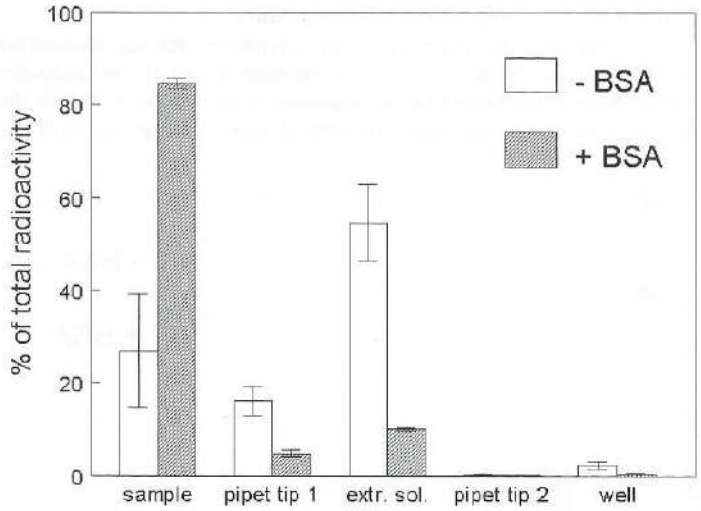


Figure 4. Binding of retinoids to plastic materials. [^{3}H]All-*trans*-RA was added to 24-wells plates containing distilled water with or without BSA (end concentration 1 mg/ml). Samples were taken and the wells were rinsed with extraction solvent. Distribution of [^{3}H] in the different fractions: sample = total content of a well containing water (+/- BSA) and a trace of [^{3}H]all-*trans*-RA; pipet tip 1 = pipet tip used with the sampling; extr. sol. = extraction solvent; pipet tip 2 = pipet tip used with the transfer of the extraction solution to a scintillation vial; well = well, in which experiment was performed. Values are presented as mean ± SD of two separate experiments.

in the samples without albumin. A considerable part of the labeled retinoids from the albumin-free samples was found in the pipet tips, and about 50% could be recovered by the extraction solvent, showing indeed that the retinoids bound to all plastics used. To check the extracted sample (from the albumin-free plates) on the presence of intact retinoids, it was separated on an HPLC column. From Figure 5 it can be seen that all-*trans*-RA that could be recovered from the plastic is not degraded or isomerized. Thus, the decrease in all-*trans*-RA concentration was due to the sticking to plastic and not due to degradation or isomerization in the medium.

Uptake of 10^{-6} M all-trans-RA by primary oral keratinocytes

We have shown that without albumin retinoids attach to hydrophobic entities, including plastic materials and only with the extraction buffer could re-dissolve them efficiently. As a consequence of its binding capacity, the presence of albumin in the media could have dramatic effects on the availability and uptake of retinoids in cultured cells.

As a model to test this hypothesis, we analyzed the uptake of retinoids in human primary oral keratinocytes in the presence of BSA concentrations ranging from 0 to 10 mg/ml. The cell-associated fractions and the cell-conditioned medium were analyzed by HPLC (Figure 6). With increasing amounts of BSA the concentrations of retinoids in the cell-associated fractions decreased, while in the medium the retinoid concentrations increased.

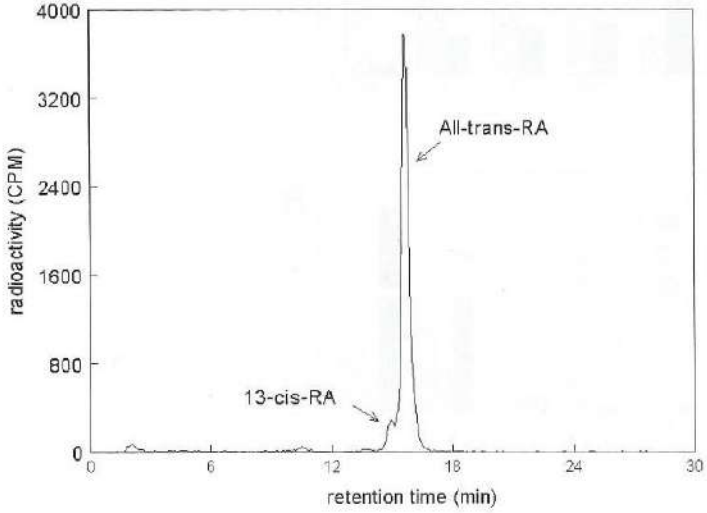


Figure 5. Lack of degradation and isomerization of all-*trans*-RA after binding to plastic material. Chromatogram of the rinsing solution from an experiment without BSA. A high peak corresponding to all-*trans*-RA and a small peak corresponding to 13-*cis*-RA could be observed. Further details of this experiment are shown in the legends of Figure 4 and in Materials and Methods .

Metabolism in UM-SCC-35 with increasing amounts of BSA

The final step was to determine if the presence of BSA had also consequences for the metabolism of all-*trans*-RA. To this end, the HNSCC cell line UM-SCC-35 was chosen as 1) we knew that the metabolism in this cell line is reproducible, and 2) the metabolism of UM-SCC-35 is fast (within about 24 h all-*trans*-RA is completely broken down) and polar metabolites can easily be detected in the medium (Braakhuis *et al.* 1997). Increasing the concentrations of albumin in the medium lead to lower levels of metabolites and higher levels of parental compound in the medium, indicating that retinoid turnover had decreased (Figure 7). In addition, a change in the type of metabolite was detected. For instance, without BSA relatively more 9-*cis*-RA and unidentified compounds were formed.

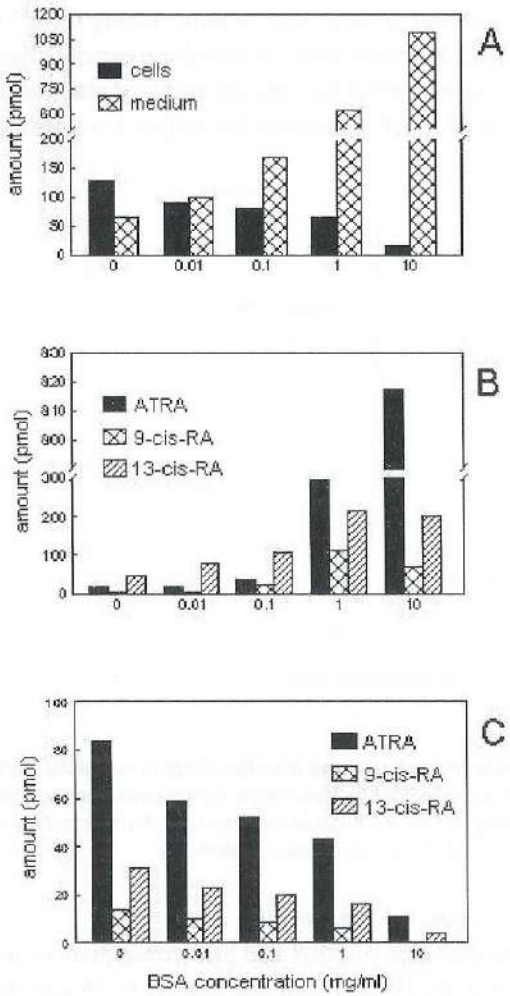


Figure 6. Uptake of 1 µM all-trans-RA by primary oral keratinocytes. Uptake of retinoids by primary oral keratinocytes was analyzed by HPLC after an exposure of 1 µM all-*trans*-RA for 24 h in KGM medium containing various concentrations of BSA. (A) Retinoid amounts in cell-associated fractions and in conditioned medium, presented in pmol (in 350 µl sample, dissolved cell pellet or medium) and are the sum of the amounts of all-*trans*-RA, 13-*cis*-RA and 9-*cis*-RA, determined by peak height. (B) Distribution of retinoids in the conditioned medium; Panel C: Distribution of retinoids in the cell-associated fractions. Experiments were performed in duplicate. The coefficients of variation were less than 15%.

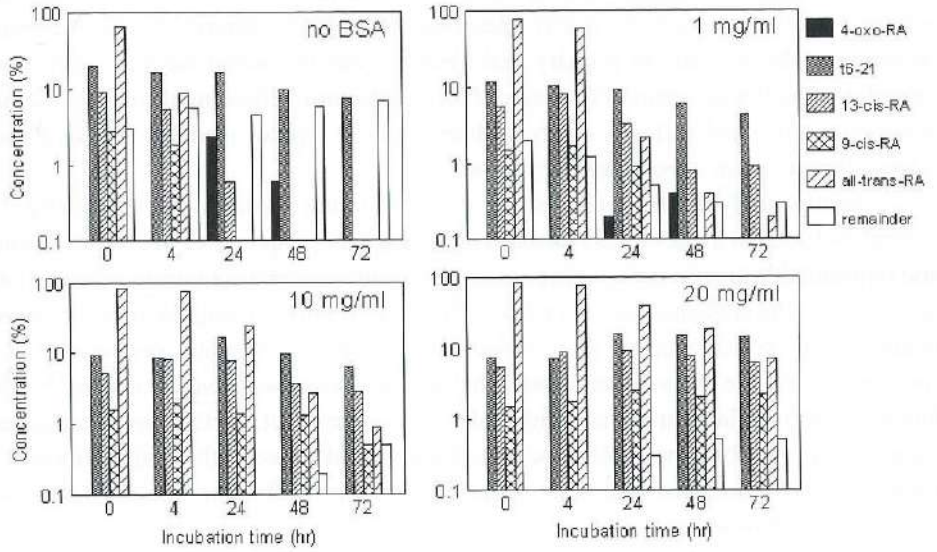


Figure 7. Metabolism in UM-SCC-35 with increasing amounts of BSA. In the medium of UM-SCC-35 retinoids were analyzed after various periods of exposure to 1 µM all-*trans*-RA. Cells were cultured in KGM medium in the presence of various concentrations of BSA. Values are expressed as a percentage of the area under the peaks of the indicated peaks or fractions; the sum of the values at t=0 has been set at a 100%. Experiments were performed in duplicate. The coefficients of variation were less than 12%. t6-21: total value of all peaks with retention times between 6 and 21 minutes. Remainder: sum of values of unidentified peaks and not with retention times between 6 and 21 minutes. NB: Note the logarithmic scale of the y-axis.

Discussion

Our initial aim was to compare retinoid metabolism in two different cell types, namely head and neck squamous cell carcinoma (HNSCC) cells and primary oral keratinocytes, in order to establish the role of retinoids in the malignant progression towards squamous cell carcinoma in the head and neck. While doing these experiments we encountered, however, some serious problems concerning the reproducibility. Moreover, in the keratinocyte medium unexpected low retinoid concentrations were found. From literature and our own experience we knew that retinoids are unstable compounds, sensitive for parameters such as light, oxygen, heat and other nonenzymatic factors (Blaner and Olson 1994, Urbach and Rando 1994, Shih *et al.* 1997). Furthermore, it has been demonstrated that serum albumin is a protein for which retinoic acid has a high binding affinity (Nerli and Pico 1994, Avis *et al.* 1995). In earlier experiments we had already reduced the variables light, oxygen and heat to a minimum. Another important

variable, to which we had payed no attention yet, was the type of culture medium. Both cell types were cultured in their own specified medium, the cancer cell lines in serum containing DMEM and the primary oral keratinocytes in special medium containing growth factors but no serum. For that reason, and the strong binding capacity of serum albumin, we decided to focus on the influence of the type of medium plus or minus serum albumin on *in vitro* retinoid experiments.

In this study we showed that the type of culture medium, but especially the presence of protein in the culture medium is of paramount importance to enable relevant and reproducible *in vitro* retinoid experiments. In culture media containing 5% fetal calf serum (FCS) the concentrations of all-*trans*-retinoic acid (RA) remained close to those predicted. In the absence of FCS, however, we found a dramatic decrease of RA concentrations. We demonstrated here that this decrease was mainly caused by the binding of RA to plastic materials with which it was in contact. Further, we investigated what influence the binding of RA to serum albumin would have on the availability of RA for the cells. We demonstrated that the presence of serum albumin reduced the uptake and metabolism of RA in cells.

Comparing methods of others in the field, we did not find a consensus in the setup of retinoid metabolism experiments concerning serum albumin content. Researchers who do add FCS or albumin may be aware of retinoid loss by sticking to plastics, while others who do not add serum or serum albumin, may have other reasons for this. First, some researchers may be unaware of the sticking of RA to plastics and may just have assumed that the RA added to the culture medium is in complete solution and uniformly distributed throughout the medium. Second, some researchers, although aware, ignored the sticking of RA to plastics, but were lucky to find comparative results since they have studied only one type of cells in one type of medium. Finally, researchers have deliberately omitted serum or serum albumin from the medium to exploit the increased intracellular accumulation of retinoids in order to obtain, for instance, high expression levels of retinoid inducible genes (Avis *et al.* 1995).

It has already been demonstrated by Noy (1992) that retinoic acid at micromolar concentrations is not uniformly distributed in aqueous solutions, but may exist as micelles. At physiological pH in aqueous environments, a predominant fraction of RA is ionized, and thereby more soluble. The presence of serum albumin or a change of pH had an effect on the solubility of RA. It can be imagined that additional factors like osmolality and buffering capacity, have an influence on the solubility of RA by changing its ionization. Our results indicated indeed that not only serum albumin, but also other proteins and other factors had an influence on the concentration of RA in the medium. In the DMEM media (DMEM-L and DMEM-P), which do not contain protein, the RA

concentrations decreased during the incubation period of 72 h to almost zero. This was not seen in the KGM and RPMI media. KGM appeared to contain a small amount of protein, while RPMI did not contain any protein at all. The unchanged RA concentration in RPMI suggested that other factors than protein content are important. Further, although the two DMEM media had the same composition, the stability of RA in the presence of FCS in these culture media differed considerable. The fact that they had been prepared by different manufacturers suggests that the use of different source components or possible impurities, might have played a role. We have not investigated this in more detail, but we judge the loss in retinoid concentrations in the DMEM-P medium unacceptable and therefore this medium does not seem useful for retinoid research. Although above described problems may only be minor as compared to BSA concentrations we think researchers working on *in vitro* retinoid experiments should take these medium dependent variations under consideration as well. Nevertheless, more research has to be done to uncover the importance and the real magnitude of these problems.

In the last part of this study it was shown that in the presence of serum albumin the uptake and turnover of RA in cells was reduced. In line with these results, several others have reported the diminishing effects of RA in the presence of serum as compared to serum-free conditions (Mouawad *et al.* 1996, Hodam and Creek 1996, Takatsuka *et al.* 1996). When albumin or FCS is not added to the medium artificially high intracellular concentrations of RA are reached, which could have tremendous impact on the studied effects. The consequence of the presence of serum albumin is a slower release from the albumin and uptake into the cells of RA. The above referred researchers, however, leave undecided whether this phenomenon is a problem or not. We propose that serum albumin concentrations that are not too limiting on retinoid actions, *i.e.*, up to 2 mg/ml in total solution, would be acceptable. Moreover, the attenuating effects are in fact representations of a more physiological condition as retinoids are in plasma bound to albumin. Furthermore, leaving out serum albumin from the medium will result in irreproducible results, since parameters such as cell concentration and the number of times and duration RA is in contact with plastics is hardly controllable.

In summary, this study demonstrated that the absence of serum albumin in the culture media during retinoid *in vitro* experiments resulted in unreliable and hardly reproducible concentrations of all-*trans*-retinoic acid, since much of the RA disappeared by binding to plastics. Further, uptake and metabolism of RA in the presence of serum albumin was attenuated, which prevented an artificially high accumulation of RA in the cells. In fact the presence of serum albumin enables a good balance between reproducibility and availability of retinoic acid.

Based on these conclusions we would like to recommend, with *in vitro* retinoid experiments, a certain level of serum albumin in the culture medium. In order to make a better comparison between retinoid experiments in HNSCC cells and primary oral keratinocytes, the addition of 1 mg/ml serum albumin to the KGM seemed a good choice, since this concentration is approximately the same as in DMEM with 5% FCS.

Acknowledgments

This study was supported by Grant 95-926 from the Dutch Cancer Society.

References

Avis I, Mathias A, Unsworth EJ, Miller MJ, Cuttitta F, Mulshine JL, Jakowlew SB (1995) Analysis of small cell lung cancer cell growth inhibition by 13-cis- retinoic acid: importance of bioavailability. *Cell Growth Differ* 6: 485-92

Bichler E, Daxenbichler G, Marth C (1983) Vitamin A status and retinoid-binding proteins in carcinomas of the head and neck region. *Oncology* 40: 336-9

Blaner WS, Olson JA (1994) Retinol and retinoic acid metabolism. In *The Retinoids*, Sporn MB, Roberts AB, Goodman DS (eds) pp 229-55. Raven Press: New York

Braakhuis BJ, Klaassen I, van der Leede BM, Cloos J, Brakenhoff RH, Copper MP, Teerlink T, Hendriks HF, van der Saag PT, Snow GB (1997) Retinoid metabolism and all-*trans* retinoic acid-induced growth inhibition in head and neck squamous cell carcinoma cell lines. *Br J Cancer* 76: 189-97

Carey TE (1985) Establishment of epidermoid carcinoma cell lines. In *Head and Neck Cancer*, Wittes RE (ed) pp 287-314. John Wiley and Sons: New York

Eckhoff C, Nau H (1990) Identification and quantitation of all-trans- and 13-cis- retinoic acid and 13-cis-4-oxoretinoic acid in human plasma. *J Lipid Res* 31: 1445-54

Gudas LJ, Sporn MB, Roberts AB (1994) Cellular biology and biochemistry of the retinoids. In *The Retinoids* , Sporn MB, Roberts AB, Goodman DS (eds) pp 443-520. Raven Press: New York

Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, Thaller C (1992) 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* 68: 397-406

Hodam JR, Creek KE (1996) Uptake and metabolism of [^{3}H]retinoic acid delivered to human foreskin keratinocytes either bound to serum albumin or added directly to the culture medium. *Biochim Biophys Acta* 1311: 102-10

Hong WK, Endicott J, Itri LM, Doos W, Batsakis JG, Bell R, Fofonoff S, Byers R, Atkinson EN, Vaughan C and others (1986) 13-cis-retinoic acid in the treatment of oral leukoplakia. *N Engl J Med* 315: 1501-5

Hong WK, Lippman SM, Itri LM, Karp DD, Lee JS, Byers RM, Schantz SP, Kramer AM, Lotan R, Peters LJ, Dimery IW, Brown BW, Goepfert H (1990) Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N Engl J Med* 323: 795-801

Kastner P, Mark M, Chambon P (1995) Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell* 83: 859-69

Levin AA, Sturzenbecker LJ, Kazmer S, Bosakowski T, Huselton C, Allenby G, Speck J, Kratzeisen C, Rosenberger M, Lovey A, et al (1992) 9-cis retinoic acid stereoisomer binds and activates the nuclear receptor RXR alpha. *Nature* 355: 359-61

Lippman SM, Batsakis JG, Toth BB, Weber RS, Lee JJ, Martin JW, Hays GL, Goepfert H, Hong WK (1993) Comparison of low-dose isotretinoin with beta carotene to prevent oral carcinogenesis. *N Engl J Med* 328: 15-20

Lotan R (1996) Retinoids in cancer chemoprevention. *FASEB J* 10: 1031-9

Lotan R, Xu XC, Lippman SM, Ro JY, Lee JS, Lee JJ, Hong WK (1995) Suppression of retinoic acid receptor-beta in premalignant oral lesions and its up-regulation by isotretinoin. *N Engl J Med* 332: 1405-10

Mangelsdorf DJ, Evans RM (1995) The RXR heterodimers and orphan receptors. *Cell* 83: 841-50

Mouawad R, Urien S, Soubrane C, Khayat D, Brandt R, Tillement JP, Bastian G (1996) Comparison of the uptake of retinoids 13-cis-retinoic acid and Ro 13-6298 delivered to HL-60 cells by serum albumin or low-density lipoprotein. *Cancer Chemother Pharmacol* 38: 198-201

Nerli B, Pico G (1994) Use of retinoic acid as a fluorescent probe to study conformational change of the albumin molecule. *Biochem Mol Biol Int* 32: 781-8

Noy N (1992) The ionization behavior of retinoic acid in aqueous environments and bound to serum albumin. *Biochim Biophys Acta* 1106: 151-8

Reid CB, Cloos J, Snow GB, Braakhuis BJ (1997) A simple and reliable technique for culturing of human oral keratinocytes and fibroblasts. *Acta Otolaryngol* 117: 628-33

Shih TW, Lin TH, Shealy YF, Hill DL (1997) Nonenzymatic isomerization of 9-cis-retinoic acid catalyzed by sulfhydryl compounds. *Drug Metab Dispos* 25: 27-32

Takatsuka J, Takahashi N, de Luca LM (1996) Retinoic acid metabolism and inhibition of cell proliferation: an unexpected liaison. *Cancer Res* 56: 675-8

Teerlink T, Copper MP, Klaassen I, Braakhuis BJ (1997) Simultaneous analysis of retinol, all-trans- and 13-cis- retinoic acid and 13-cis-4-oxoretinoic acid in plasma by liquid chromatography using on-column concentration after single-phase fluid extraction. *J Chromatogr B Biomed Sci Appl* 694: 83-92

Urbach J, Rando RR (1994) Isomerization of all-trans-retinoic acid to 9-cis-retinoic acid. *Biochem J* 299 : 459-65

Xu XC, Ro JY, Lee JS, Shin DM, Hong WK, Lotan R (1994) Differential expression of nuclear retinoid receptors in normal, premalignant, and malignant head and neck tissues. *Cancer Res* 54:3580-7

Chapter 3

Plasma retinoid levels in head and neck cancer patients: a comparison with controls without cancer, and the effect of retinyl palmitate treatment

Marcel P Copper[1], Ingeborg Klaassen[1], Tom Teerlink[2], Gordon B Snow[1]
Boudewijn JM Braakhuis[1]

[1]Departments of Otolaryngology and [2]Clinical Chemistry,
Vrije Universiteit Medical Center, Amsterdam, The Netherlands

Oral Oncol 1999; 35: 40-4

Abstract

Vitamin A and related compounds, also known as retinoids are thought to play a role in the development of head and neck cancer. We measured levels of the major retinoids, retinol, all-*trans*-retinoic acid, 13-*cis*-retinoic acid and 13-*cis*-4-oxo-retinoic acid in plasma of head and neck cancer patients in comparison with controls without cancer. No differences were found between plasma levels of these retinoids between 25 head and neck cancer patients and 21 controls. Mean baseline levels for the patients were 2458, 6.0, 6.4 and 8.6 nM for retinol, all-*trans*-retinoic acid, 13-*cis*-retinoic acid and 13-*cis*-4-oxo-retinoic acid, respectively. In addition, we selected 10 patients from the chemoprevention trial EUROSCAN and measured the effect on retinoid levels of 300,000 IU daily retinyl palmitate intake during 1 month. Medication caused significant elevations in retinol levels (1.2 fold), all-*trans*-retinoic acid (2.2 fold) and its metabolites 13-*cis*-retinoic acid (5.8 fold) and 13-*cis*-4-oxo-retinoic acid (8.9 fold). Because of its high increase in levels, 13-*cis*-4-oxo-retinoic acid seems a good candidate to serve as a suitable marker to monitor patient compliance in future chemoprevention trials involving retinoids. No relations were found between the occurrence of side-effects of retinyl palmitate and retinoid levels during treatment. However, the two patients who developed side-effects had the highest pre-treatment levels of 13-*cis*-retinoic acid and 13-*cis*-4-oxo-retinoic acid, suggesting that retinoid toxicity is associated with relatively high basal retinoid metabolism.

Introduction

Vitamin A or retinol is an essential micronutrient for several processes in man including growth, differentiation, vision and reproduction. The relation between dietary intake of retinol and the development of squamous cell cancer in several tissues of the body was recognized during the last two decades (Bjelke 1975, Fontham 1990, Byers *et al.* 1984). Epidemiological studies on the relation between low serum concentrations of retinol and subsequent cancer risk were not conclusive. A number of these studies showed a positive correlation between low serum retinol concentrations and the development of cancer (Wald *et al.* 1980, Zheng *et al.* 1993), but other studies failed to confirm this (Knekt *et al.* 1990, Coates *et al.* 1988, Wald *et al.* 1986, Willet *et al.* 1984). From the clinical point of view it was recognized that vitamin A has activity in leukoplakia, the most common premalignant form of oral cancer. Oral supplementation of retinoic acid (RA) in the form of 13-*cis*-RA was also able to prevent the formation of second primary tumors in head and neck cancer patients (Hong *et al.* 1990). At this moment it is not clear how retinol and its metabolites (termed 'retinoids') are able to inhibit the development of cancer. It is very likely that in addition to the availability of retinoid receptors (discussed below),

the concentrations of the specific retinoids are important (Mangelsdorf *et al.* 1994). Many studies in the past have put emphasis on serum retinol levels and the subsequent risk of cancer development, but not much is known about normal plasma levels of all-*trans*-RA and its metabolites 13-*cis*-RA and 13-*cis*-4-oxo-RA in cancer patients. The first aim of this study was to analyze these retinoids in the plasma of head and neck cancer patients in comparison with controls. In the late 1980s more insight into the molecular mechanisms of the action of vitamin A was acquired by the discovery of the RA receptors (RARs) and the retinoid X receptors (RXRs) (Petkovich *et al.* 1987, Giguere *et al.* 1987, Mangelsdorf *et al.* 1990). Natural metabolites of retinol, like all-*trans*-RA and 9-*cis* RA bind to these factors and form hetero- or homodimers. These dimers are transcription factors and regulate gene expression after binding to a specific DNA sequence (Yu *et al.* 1992) in the promotor region of various genes. Both receptor type as well as type of retinoid are of importance in this regulation (Mangelsdorf *et al.* 1994). For instance, all-*trans*-RA binds to RARs and not to RXRs, whereas 9-*cis*-RA binds to both classes of receptors.

The epidemiologic data supporting a protective effect of retinoids on cancer development and the data of animal studies which showed several retinoids to prevent cancer, resulted in the initiating of clinical chemoprevention trials in the last decade. One of the largest head and neck cancer and lung cancer chemoprevention trials conducted at the moment is the EUROSCAN trial (De Vries *et al.* 1991, De Vries *et al.* 1993). Cured head and neck cancer or lung cancer patients were randomized in a 2 x 2 factorial design to receive either retinyl palmitate (RP) or N-acetylcysteine or both or nothing during a period of 2 years. We were treating these patients in our clinic and we were interested to know whether: (1) RP treatment had influence on the plasma levels of the several retinoids; (2) retinoid levels could be used to monitor patient compliance; and (3) it was possible to predict retinoid-induced toxic side-effects.

Materials and methods

Patients and controls

Plasma was obtained from 25 patients (20 males and five females, mean age of 60 years) admitted in our clinic with a squamous cell carcinoma of the mucous membranes of the head and neck: eight patients with oral cancer, three patients with oropharyngeal cancer and 14 patients with laryngeal cancer (T_1-T_4, N_0-N_{2c}, according to the TNM classification of the UICC (Hermanek and Sobin 1987)). Twenty-one patients (12 males and nine females, mean age of 47 years) without cancer in their history and who were admitted at our department or the Department of Maxillofacial Surgery served as control group. These patients were admitted for various reasons: four patients underwent ear surgery, four sinus surgery, three septorhinoplasty, three reposition of maxillary fractures, two uvulopalatopharyngoplasty, and five patients were admitted for other surgery related to benign disease.

The mean values of the smoking habits of the cancer patients were 8.0 cigarettes per day (range: 0-40) versus 7.2 cigarettes per day (range: 0-30) for the control population. The mean values of alcohol consumption per day of the cancer patients were 2.3 (range: 0-7) versus 1.1 (range: 0-6) for the control population.

For the evaluation of the influence of high dose RP administration on retinoid levels, plasma samples of 10 patients participating in the chemoprevention trial EUROSCAN were examined. Patients used 300,000 IU RP per day during the first year of treatment, followed by a dose of 150,000 IU RP per day during the second year. The objective of the EUROSCAN trial is to study the effect of RP, N-acetylcysteine or both in preventing or delaying the occurrence of second primary tumours in patients curatively treated for invasive laryngeal cancer, carcinoma in situ of the larynx, oral and lung cancer. Patients were randomized in agreement with the EUROSCAN protocol of the European Organization for Research and Treatment of Cancer (EORTC study number 24871). Patients were seen every month in the outpatient clinic where side-effects were recorded. The most common side-effects were itching, dyspepsia, skin dryness and desquamation (De Vries *et al.* 1993). Routine liver function tests were performed (serum levels of serum glutamic oxaloacetic transaminase, serum glutamic pyruvate transaminase, lactate dehydrogenase, gamma-glutamyl transpeptidase and bilirubin) as well as serum cholesterol levels. Blood for retinoid analysis was collected immediately following randomization and 1 month afterwards when patients were visiting our outpatient clinic and 13 months afterwards ($n = 4$). Samples were taken from non-fasting individuals 2-5 h after RP intake. No correction for this difference in time interval was performed, since no correlation was found between this time period and the plasma retinoid levels.

Blood collection

Blood was obtained by venepuncture using vacuum tubes containing EDTA. These tubes were covered with aluminum foil to protect them from light and stored in a 4°C refrigerator. Within 1 h after venepuncture, plasma was obtained by centrifugation (10 min at 3000 *g* and 4°C).
Subsequently, plasma was collected in cryovials that were flushed with nitrogen, before closing. Cryovials were again covered with aluminum foil and then stored in the dark at -70°C.

Sample preparation and HPLC procedure

Sample preparation and HPLC procedure were performed as described earlier (Teerlink *et al.* 1997). This way of analysis is characterized by a high sensitivity and reproducibility.

Table 1. Plasma retinoid levels in head and neck cancer patients and healthy controls.

Group	*n*	Retinol	all-trans-RA	13-cis-RA	13-cis-4-oxo-RA
Patients	25	2458 ± 851	6.0 ± 2.5	6.4 ± 2.2	8.6 ± 5.3
Controls	21	2244 ± 798	4.9 ± 2.2	6.1 ± 2.6	7.4 ± 4.5
P-value[a]		0.40	0.16	0.66	0.39

RA, retinoic acid. Mean values ± SD are shown in nM.
[a] Two-sample *t*-test with equal variance.

Results

Cancer patients and controls

No differences were observed between retinoid levels of cancer patients as compared with control individuals (Table 1).

Patients using daily RP

Plasma retinol levels in patients increased slightly, but significantly after 1 month treatment with 300,000 IU RP per day. Mean values of plasma levels of all-*trans*-RA and its metabolites 13-*cis*-RA and 13-*cis*-4-oxo-RA were significantly increased two to nine times (Table 2). After 1 year of treatment with 300,000 IU RP the daily dose of RP was reduced to 150,000 IU RP, according to the protocol of the EUROSCAN trial. The plasma retinoid levels of four patients were evaluated in this second year. After 1 month of treatment with the lower dose RP (which is 13 months after randomization for the trial) plasma samples were investigated. As expected, retinoid levels in the second year were lower than in the first year, when patients were using the double dose, but higher than before treatment. Surprisingly, in three of the four evaluated patients, retinol levels were even lower at this time point than before treatment (Table 3); this difference was not significant ($P = 0.18$, two-sample t-test with equal variance).

Table 2. Plasma retinoid levels before and after retinyl palmitate (RP) intake.

Patients	Retinol	all-*trans* RA	13-*cis* RA	13-*cis*-4-oxo RA
Pre-treatment	2608 ± 622	5.7 ± 2.1	6.6 ± 2.0	10.3 ± 5.3
During treatment	3242 ± 445	12.8 ± 8.9	38.2 ± 12.9	92.1 ± 22.0
p-value[a]	0.01	0.01	$<10^{-5}$	$<10^{-5}$

RA, retinoic acid. Mean values ± SD are shown in nM from 10 patients before and 1 month after starting a daily intake of 300,000 IU RP.
[a] Paired two-sample *t*-test.

Table 3. Plasma retinoid levels before and 1 and 13 months after retinyl palmitate (RP) intake.

Patients	Retinol	all-*trans* RA	13-*cis*-RA	13-*cis*-4-oxo RA
Pre-treatment	2235 ± 632	5.2 ± 1.7	7.1 ± 2.2	11.8 ± 4.3
After 1 month	3077 ± 375	13.9 ± 7.0	40.0 ± 4.5	100.1 ± 21.8
After 13 months	1709 ± 687	8.7 ± 4.6	21.3 ± 11.4	82.7 ± 35.1

RA, retinoic acid. Mean ± SD are shown in nM from four patients before treatment, after 1 month daily treatment with 300,000 IU RP, and after 13 months treatment with 300,000 IU followed by 1 month treatment of 150,000 IU.

Side-effects and safety parameters

Two of the 10 patients using RP reported side-effects. These side-effects were well-tolerated complaints of mucosal dryness (one patient) and poorly tolerated skin desquamation (one patient). In this last patient, the daily dose of RP was reduced to 150,000 IU, resulting in moderate and bearable side-effects. The additional eight patients did not report to have side-effects. The pre-treatment levels of 13-*cis*-RA and 13-*cis*-4-oxo-RA were noted to be the highest in the patients with side-effects. The levels of 13-*cis*-RA in the patients with side-effects were 10.8 and 9.6 nM compared to a mean level of 5.8 nM (range: 4.1-7.2 nM) in the patients without side-effects. The levels of 13-*cis*-4-oxo-RA in the patients with side-effects were 18.9 and 20.4 nM compared to a mean level of 7.9 nM (range: 3.3-11.6 nM) in the patients without side-effects. The levels of retinol and all-*trans*-RA showed no differences between the patient groups with and without side-effects. None of the 10 patients had liver function disturbances as checked by blood tests. One patient had an increase in cholesterol level from 4.7 to 10.2 mM. Plasma retinoid levels after treatment of this patient were within the range of those of the other patients without cholesterol level increase.

Discussion

During the last decades epidemiological studies revealed a relationship between vitamin A intake levels and subsequent cancer risk. With the discovery of the retinoid receptors a molecular basis was provided for a possible working mechanism of retinol which could explain the epidemiological observations. Retinol itself does not bind to the RARs or RXRs and it has to be converted into all-*trans*-RA or 9-*cis*-RA before it can induce or inhibit gene expression. In most epidemiological studies no or minor differences in blood retinol concentrations were found in cancer patients compared with controls, not explaining an increased cancer risk of specific individuals. Besides these findings, oral supplementation of 13-*cis*-RA was shown to have a protective effect on the development of second primary tumors in head and neck cancer patients (Hong *et al.* 1990) and a therapeutic effect was found of this RA metabolite and also of all-*trans*-RA on the development of oral leukoplakia (Lippman *et al.* 1993). These two findings suggest a metabolic defect which could be restored by supplementing 13-*cis*- and all-*trans*-RA rather than retinol. We were, therefore, interested in the concentrations of all-*trans*-RA and its metabolites 13-*cis*-RA and 13-*cis*-4-oxo-RA of head and neck cancer patients, assuming that plasma levels reflect intracellular processes.

The present study showed no difference in the plasma levels of retinol, all-*trans*-RA, 13-*cis*-RA and 13-*cis*-4-oxo-RA between head and neck cancer patients and healthy controls. As for all-*trans*-RA and 13-*cis*-RA, these results were in agreement

with the findings of a Swedish group (Wahlberg and Fex 1996), although these investigators reported somewhat lower values for the patient and control groups. So, the combined data of both studies show that a relatively poor nutritional status, that may be expected in head and neck cancer patients, is not reflected in an alteration of the plasma levels of the most important retinoids. Consequently, it seems that the beneficial effect of 13-*cis*-RA on tumor-development and leukoplakia advancement can not be explained by correcting a deficiency of this metabolite. Apparently, the levels of retinoids in the plasma are too crude an indicator of the intracellular levels influencing the process of gene transcription.

We investigated the plasma levels of several retinoids in samples of patients randomized to use RP. We found a considerable increase of blood retinoids in all patients using RP. The RA metabolite 13-*cis*-4-oxo-RA showed the highest increase in plasma concentration of about nine times the pre-treatment levels. 13-*cis*-RA showed a mean plasma level increase of about six times the pre-treatment levels. Another study showed stationary plasma concentrations of 13-*cis*-4-oxo-RA during the day independent of the interval after retinoid intake, whereas 13-*cis*-RA showed considerable fluctuations of plasma levels during the day after retinoid intake (Eckhoff *et al.* 1991). A well-known problem in clinical chemoprevention trials is that it is troublesome to monitor patient compliance adequately. This monitoring is in most trials realized by questioning patients about taking their chemopreventive medication or by pill count. Both methods are subjective and the reliability of these methods is questionable. Earlier studies already showed increasing levels of RA and its metabolites after supplementation of retinol or RP (Eckhoff and Nau 1990, Tang and Russell 1991). Because of its high increase of levels 13-*cis*-4-oxo-RA seems a very suitable candidate to serve as an objective plasma marker to monitor patient compliance in future chemoprevention trials involving RP and possibly other retinoids.

Four patients were evaluated more than 1 year after the onset of RP treatment. Patients randomized in one of the RP arms of the EUROSCAN trial begin to use 300,000 IU RP daily during 1 year. In the second year this dose is reduced to 150,000 IU RP daily. As expected, plasma levels of all-*trans*-RA and its metabolites 13-*cis*-RA and 13-*cis*-4-oxo-RA were lower in comparison with the plasma levels in the first year of treatment. Surprisingly, retinol levels in the patients still receiving the relatively high dose of 150,000 IU RP daily were reduced compared with the pre-treatment levels in three of the four patients, suggesting an elevated metabolism of RP in the body after a prolonged period of RP supplementation.

A well-known problem in chemoprevention trials using retinoids is the occurrence of serious side-effects in a number of patients. In our group of patients, using 300,000

IU RP daily, side-effects were present in two patients. Plasma levels during RP treatment of patients who experienced side-effects were comparable with the levels of the patients without side-effects. An explanation of this observation could be that plasma levels of retinoids do not correlate with the intracellular retinoid levels of epithelial cells. The pre-treatment levels of 13-*cis*-RA and 13-*cis*-4-oxo-RA were the highest in the two patients with side-effects. Both are metabolites of all-*trans*-RA, which suggests an increased basal retinoid metabolism in these two patients. The numbers of patients with side effects is small in this study and more research is required to confirm this observation.

Acknowledgments

This study was supported by Grant 95-926 from the Dutch Cancer Society.

References

Bjelke E (1975) Dietary vitamin A and human lung cancer. *Int J Cancer* 15: 561-5

Byers T, Vena J, Mettlin C, Swanson M, Graham S (1984) Dietary vitamin A and lung cancer risk: an analysis by histologic subtypes. *Am J Epidemiol* 120: 769-76

Coates RJ, Weiss NS, Daling JR, Morris JS, Labbe RF (1988) Serum levels of selenium and retinol and the subsequent risk of cancer. *Am J Epidemiol* 128: 515-23

de Vries N, Van Zandwijk N, Pastorino U (1993) The EUROSCAN study: a progress report. *Am J Otolaryngol* 14: 62-6

de Vries N, van Zandwijk N, Pastorino U (1991) The EUROSCAN Study. EUROSCAN Steering Committee. *Br J Cancer* 64: 985-9

Eckhoff C, Collins MD, Nau H (1991) Human plasma all-trans-, 13-cis- and 13-cis-4-oxoretinoic acid profiles during subchronic vitamin A supplementation: comparison to retinol and retinyl ester plasma levels. *J Nutr* 121: 1016-25

Eckhoff C, Nau H (1990) Identification and quantitation of all-trans- and 13-cis- retinoic acid and 13-cis-4-oxoretinoic acid in human plasma. *J Lipid Res* 31: 1445-54

Fontham ET (1990) Protective dietary factors and lung cancer. *Int J Epidemiol* 19 Suppl 1: S32-42

Giguere V, Ong ES, Segui P, Evans RM (1987) Identification of a receptor for the morphogen retinoic acid. *Nature* 330: 624-9

Hermanek P, Sobin LH (1987) In *TNM Classification of malignant tumours, 4th Edition*, pp 13-26. Springer: Berlin

Hong WK, Lippman SM, Itri LM, Karp DD, Lee JS, Byers RM, Schantz SP, Kramer AM, Lotan R, Peters LJ, Dimery IW, Brown BW, Goepfert H (1990) Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N Engl J Med* 323: 795-801

Knekt P, Aromaa A, Maatela J, Aaran RK, Nikkari T, Hakama M, Hakulinen T, Peto R, Teppo L (1990) Serum vitamin A and subsequent risk of cancer: cancer incidence follow- up of the Finnish Mobile Clinic Health Examination Survey. *Am J Epidemiol* 132: 857-70

Lippman SM, Batsakis JG, Toth BB, Weber RS, Lee JJ, Martin JW, Hays GL, Goepfert H, Hong WK (1993) Comparison of low-dose isotretinoin with beta carotene to prevent oral carcinogenesis. *N Engl J Med* 328: 15-20

Mangelsdorf DJ, Ong ES, Dyck JA, Evans RM (1990) Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* 345: 224-9

Mangelsdorf DJ, Umesono K, Evans RM (1994) The retinoid receptors. In *The Retinoids*, Sporn MM, Roberts AR, Goodman DS (eds) pp 319-49. Raven Press: New York

Petkovich M, Brand NJ, Krust A, Chambon P (1987) A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* 330: 444-50

Tang G, Russell RM (1991) Formation of all-*trans*-retinoic acid and 13-*cis*-retinoic acid from all-*trans*-retinyl palmitate in humans. *J Nutr Biochem* 2: 210-3

Teerlink T, Copper MP, Klaassen I, Braakhuis BJ (1997) Simultaneous analysis of retinol, all-trans- and 13-cis- retinoic acid and 13-cis-4-oxoretinoic acid in plasma by liquid chromatography using on-column concentration after single-phase fluid extraction. *J Chromatogr B Biomed Sci Appl* 694: 83-92

Wahlberg P, Fex G (1996) Retinoic acid concentrations in patients with squamous cell carcinoma of the head and neck. *Eur J Cancer* 32A: 366-7

Wald N, Boreham J, Bailey A (1986) Serum retinol and subsequent risk of cancer. *Br J Cancer* 54: 957-61

Wald N, Idle M, Boreham J, Bailey A (1980) Low serum-vitamin-A and subsequent risk of cancer. Preliminary results of a prospective study. *Lancet* 2: 813-5

Willett WC, Polk BF, Underwood BA, Stampfer MJ, Pressel S, Rosner B, Taylor JO, Schneider K, Hames CG (1984) Relation of serum vitamins A and E and carotenoids to the risk of cancer. *N Engl J Med* 310: 430-4

Yu VC, Naar AM, Rosenfeld MG (1992) Transcriptional regulation by the nuclear receptor superfamily. *Curr Opin Biotechnol* 3: 597-602

Zheng W, Blot WJ, Diamond EL, Norkus EP, Spate V, Morris JS, Comstock GW (1993) Serum micronutrients and the subsequent risk of oral and pharyngeal cancer. *Cancer Res* 53: 795-8

Chapter 4

Retinoid metabolism and all-*trans*-retinoic acid induced growth inhibition in head and neck squamous cell carcinoma cell lines

Boudewijn JM Braakhuis[1], Ingeborg Klaassen[1], Bas-jan M van der Leede[2], Jacqueline Cloos[1], Ruud H Brakenhoff[1], Marcel P Copper[1], Tom Teerlink[3], Henk FJ Hendriks[4], Paul T van der Saag[2] and Gordon B Snow[1]

Departments of [1]Otolaryngology and [3]Clinical Chemistry, Vrije Universiteit Medical Center, Amsterdam, [2]Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Utrecht, [4]Department of Physiology and Kinetics, TNO Nutrition and Food Research Institute, Zeist, The Netherlands

Br J Cancer 1997; 76: 189-97

Abstract

Retinoids can reverse potentially premalignant lesions and prevent second primary tumors in patients with head and neck squamous cell carcinoma (HNSCC). Furthermore, it has been reported that acquired resistance to all-*trans*-retinoic acid (RA) in leukemia is associated with decreased plasma peak levels, probably the result of enhanced retinoid metabolism. The aim of this study was to investigate the metabolism of retinoids and relate this to growth inhibition in head and neck squamous cell carcinoma (HNSCC). Three HNSCC cell lines were selected on the basis of a large variation in the all-*trans*-RA induced growth inhibition. Cells were exposed to 9.5 nM (radioactive) for 4 and 24 h, and to 1 and 10 μM (non-radioactive) all-*trans*-RA for 4, 24, 48 and 72 h, and medium and cells were analyzed for retinoid metabolites. At all concentrations studied, the amount of growth inhibition was proportional to the extent at which all-*trans*-, 13- and 9-*cis*-RA disappeared from the medium as well as from the cells. This turnover process coincided with the formation of a group of as yet unidentified polar retinoid metabolites. The level of mRNA of cellular RA binding protein-II (CRABP-II), involved in retinoid homeostasis, was inversely proportional to growth inhibition. These findings indicate that for HNSCC retinoid metabolism may be associated with growth inhibition.

Introduction

Retinoids are a class of compounds that consists of the natural vitamin A derivatives, such as retinol, retinal, retinoic acid (RA) and their various metabolic products, and the synthetic derivatives that are structurally related to these natural compounds (Dawson and Hobbs 1994). Natural retinoids are important for normal epithelial cell differentiation. A low vitamin A (retinol) plasma level and a low dietary intake of retinoids have been proven to be risk factors in various carcinomas (Hong and Itri 1994). Many studies report inhibiting effects of exogenous retinoids on the induction and progression of cancer in various tissues (Lotan 1993). As for solid tumours, retinoids are particularly important for head and neck squamous cell carcinoma (HNSCC) (Benner *et al.* 1993). Three retinoids, 13-*cis*-retinoic acid (13-*cis*-RA) (Hong *et al.* 1986), retinyl palmitate (Stich *et al.* 1988) and all-*trans*-retinoic acid (all-*trans*-RA) (Koch 1978) cause responses in 40 to 70 % of patients with leukoplakia, the most common premalignant lesion of the mucosa of the oral cavity (Van der Waal 1992). It has also been demonstrated that administration of 13-*cis*-RA could successfully prevent and/or delay the occurrence of second primary tumors in the upper aerodigestive tract (Hong *et al.* 1990). In a chemotherapeutic approach, single agent 13-*cis*-RA has limited activity in advanced squamous cell carcinoma of the head and neck (Lippman *et al.* 1988). Thus far retinoids appear to be active in early stage HNSCC but their utility is limited by the inter-patient variability not only with respect to

response and but also to side effects. Another characteristic of treatment of HNSCC with retinoids is that discontinuation of treatment leads invariably to recurrence of the lesion (Hong and Itri 1994).

It is not known how retinoids are actually able to regulate growth control. The association between vitamin A deficiency and the development of cancer suggests that the intracellular retinoid dependent pathways play a role in cancer development. Most of the actions of retinoids are thought to result from changes in gene expression mediated by nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Mangelsdorf *et al.* 1994). Retinoids bind to these receptors, which act as transcription factors upon dimerization. The expression of one such receptor, RAR-β, as determined by *in situ* hybridization is selectively absent in 31 of 52 leukoplakia cases and can be restored by treatment with 13-*cis*-RA (Lotan *et al.* 1995). Restoration of expression is associated with a clinical response to 13-*cis*-RA. Pretreatment levels of RAR-β, however, did not predict the clinical response (Lotan *et al.* 1995). Sixty-five percent of clinical HNSCC samples show a lack of RAR-β expression, as judged by *in situ* hybridization (Xu *et al.* 1994). No association, however, was found in HNSCC cell lines between all-*trans*-RA sensitivity and the expression of RARs, RXRs and the cellular retinoic acid binding proteins (CRABP) (Zou *et al.* 1994).

All-*trans*-RA induces complete remission in most patients with acute promyelocytic leukemia. However, relapses are frequent and resistance to the drug is usually developing. This resistance is associated with unexpectedly low plasma levels of retinoids despite continued treatment (Muindi *et al.* 1994). The inter-individual variation in retinoid pharmacokinetics was already known from studies of others (Lee *et al.* 1993, Adamson *et al.* 1993a, Eckhoff *et al.* 1991) and it is hypothesized that the variability for the pharmacokinetics of all-*trans*-RA may result from differences in catabolic rates determined or influenced by genetic or environmental factors. Thus, a poor metabolism may be associated with a response, while enhanced retinoid metabolism is associated with acquired therapy resistance (Muindi *et al.* 1994). CRABP-II levels may be the cause of this resistance, since increased levels of this enzyme have been found in tumor cells of relapse patients treated with all-*trans*-RA (Delva *et al.* 1993). Also the oxidative breakdown via the cytochrome p450 enzyme system is a possible explanation (Rigas *et al.* 1993). Two lines of evidence for the latter possibility have been provided: a 10-fold increase of the 4-oxo-all-*trans*-RA glucuronide has been found in the urine of the relapse patients (Muindi *et al.* 1994) and ketoconazole and liarozole, inhibitors of the cytochrome P450 system, are able to attenuate this catabolism (Rigas *et al.* 1993, Wouters *et al.* 1992).

In vitro, a variation in growth inhibition after an exposure of HNSCC to all-*trans*-RA has been reported (Jetten *et al.* 1990, Sacks *et al.* 1995) and thus far this variation can not be explained (Zou *et al.*, 1994). The aim of this study was to investigate the presence or absence of a variation in retinoid metabolism between HNSCC cell lines and whether this is related to the degree of growth inhibition by all-*trans*-RA.

Materials and methods

Cell lines

HNSCC cell lines were obtained from Dr TE Carey, University of Michigan, Ann Arbor, MI, USA and are described elsewhere (Carey 1985). UM-SCC-14C originated from a local recurrence of the floor of the mouth, UM-SCC-22A and -35 from hypopharyngeal tumors. Cells were routinely cultured in DMEM (Dulbecco's modified Eagle's Medium, ICN Biomedicals, Ltd, Irvine, UK) with 5% Fetal Calf Serum (FCS, Flow Laboratories) in 75 cm^2 flasks (Nunc, Roskilde, Denmark). Cellular doubling times were 26 h for UM-SCC-14C, 52 h for UM-SCC-35 and 34 h for the UM-SCC-22A cell line.

Chemicals

All-*trans* RA was obtained from Acros Chimica (Geel, Belgium), 4-oxo-*trans*- and -*cis*-RA were kind gifts of Hoffmann-la Roche, Basel, Switzerland; retinol and 13-*cis*-RA were obtained from Sigma (St Louis, MO, USA). All compounds were dissolved as a 10^{-2} M stock in dimethylsulfoxide (DMSO, JT Baker, Deventer, the Netherlands) and stored at -80°C. For each experiment freshly prepared solutions were made, the first (10^{-3} M) being made with DMSO. Subsequent dilutions were prepared in cell culture medium. All handling with retinoids was performed in subdued light, tubes were wrapped in aluminum foil and oxidation was prevented by replacing the air by N_2.

Cell growth inhibition studies

Effects on the growth of HNSCC cells were determined by applying the Sulforhodamine B (SRB) assay. Details of the assay that measures the cellular protein content reflecting the actual cell number, has been previously described (Braakhuis *et al.* 1993). In short, cells were plated at a concentration of 1500 (UM-SCC-14C), 2000 (UM-SCC-22A) and 3000 (UM-SCC-35) cells/well in 150 µl DMEM and 5% FCS, and were allowed to attach and grow for 72 h (the "lag-phase"). After this phase, it was found that control (only incubated with culture medium) cell growth was logarithmic for a period up to 96 h. Consequently, all-*trans*-RA was added in 50 µl medium resulting in a final concentration that varied between 10^{-5} and 10^{-9} M. Growth was assessed after 72 h (the "log-phase"), by staining the cellular protein with SRB (Sigma) and spectrophotometric measurement of the absorption at 540 nm with a microplate reader. IC_{50}-values were estimated based on the absorption values and defined as the concentration that corresponded to a reduction of growth by 50% as compared to values of untreated control cells. When using the highest concentration of all-*trans*-RA, a 1% DMSO solution was present in the cell culture medium. Control experiments showed that exposing the cells to this level of DMSO without all-*trans*-RA leads to a growth inhibition of between 10 and 25 %.

In a separate set of experiments the effect of conditioned medium was tested on the growth rate of UM-SCC-14C and -35 cells. For that purpose near-confluent flasks with UM-SCC-35 and -14C cells were exposed to 10^{-6} and 10^{-8} M all-*trans*-RA for 24 h. These conditioned media were added to cells growing in 96-well plates that were about to start their log phase. The cells were exposed to this conditioned medium for another 72 h and the level of growth inhibition was determined by the "SRB-assay", as described.

Exposure to radioactive all-trans RA

Near-confluent cultures, growing under normal conditions, were treated for 4 and 24 hr with 9.5 nM [11,12-^{3}H]all-*trans*-RA (Dupont NEN Research Products, Dordrecht, the Netherlands, specific activity 52.1 Ci/mmol). After incubation the medium was removed and saved at -80°C. Cells were rinsed with phosphate buffered saline (pH 7.4, PBS), scraped in 1 ml PBS and collected by centrifugation. Cell pellets were stored at -80°C until extraction. Retinoids were extracted and analyzed by reverse-phase reversed-phase high-performance liquid chromatography (HPLC) as described (Pijnappel *et al.* 1993). The following standards were included: 13- and 9-*cis*- and all-*trans*-RA. The experiment was performed in duplicate.

Exposure to non-radioactive all-trans-RA

Cells were cultured to near confluence in 75 cm^2 flasks with 5 ml medium (DMEM plus FCS). The cells were exposed to 10 and 1 µM all-*trans*-RA. At each time point (0, 4, 24, 48 and 72 h) 500 µl of the supernatant was taken from a separate flask and stored at -80°C in the dark until analysis. For the analysis of the intracellular concentration of retinoids, the flasks were washed two times with fresh phosphate buffered saline (PBS, pH 7.4), and the cells were trypsinized. The number of living cells (determined with trypan blue) was calculated, cell pellets were washed two times with PBS and stored at -80°C in the dark.

Non-radioactive retinoids were determined by reversed-phase HPLC after extraction with acetonitrile (Teerlink *et al.*, submitted). A Waters (Milford, MA, USA) HPLC system was used, consisting of a Model 717 plus automatic sample injector, a Model 616 gradient pump, a Model 486 UV detector, and a temperature control module and column heater. Mobile phase was degassed online using a model DG2410 degasser from Uniflows (Tokyo, Japan). Millennium 2010 software from Waters was used for instrument control and data acquisition. Separation was performed on a Spherisorb ODS2 3 µm column (100 * 4.6 mm) from Phase Separations (Deeside, UK) maintained at 30°C. Composition of the mobile phases and the binary gradient used were as described by Eckhoff and Nau (1990). UV detection was performed at 340 nm and retinoids were identified using external standardization. We included the following standards: 4-oxo-*trans*-RA, 4-oxo-*cis*-RA, 13-*cis*-RA, all-*trans*-RA, and retinol. Since we intended to measure also the levels of unknown retinoid metabolites, the results were expressed as a percentage of the total area under the curve of the relevant part of the chromatogram (retention time between 6 and 30 min).

Measurement of CRABP mRNA levels

Total RNA was isolated from cultured cells according to Gough (1988). 20 µg of total RNA was loaded on an 1% agarose formaldehyde gel and electrophoresed in 3-(N-morpholine)propane sulfonic acid (MOPS) buffer essentially as described by Sambrook *et al.* (1989). The RNA was Northern blotted by capillary transfer in 10x saline sodium citrate (SSC) (Sambrook *et al.*, 1989) onto genescreen plus filters (Dupont NEN). The filter was baked for 2 h at 80°C, prehybridized in 7% sodium dodecyl sulfate (SDS), 0.5 M sodium phosphate buffer, 2 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0 for 2 h at 65°C, and after addition of the denatured probe hybridized at 65°C for 16 hr. The probes were made by labeling

the isolated 0.6 kb XbaI/BamHI fragment containing the human CRABP-I cDNA (Åström *et al.* 1991), the 1 kb EcoRI fragment containing the human CRABP-II cDNA (Åström *et al.* 1991), and the 0.2 kb fragment containing part of 18S rRNA cDNA with [α-^{32}P]dCTP to a specific activity of approximately 10^9 dpm/µg by multiprimed elongation (Feinberg and Vogelstein 1983). After hybridization the filters were washed twice with 2x SSC, 0.2% SDS and twice with 0.2x SSC, 0.2% SDS at 65°C for 15 min, and the bands visualized by autoradiography with Kodak X-AR 5 film using intensifying screens. 18S rRNA was used as an internal standard to correct for the amount of RNA loaded on the gel.

Results

Inhibition of cell proliferation

The three HNSCC cell lines were selected for their considerable difference in their response to all-*trans*-RA (Figure 1A). UM-SCC-35 was the most sensitive line with an IC_{50}-value of 6.8 nM. UM-SCC-14C hardly showed any response, even after exposure to the relatively high concentration of 10^{-5} M. The third line, UM-SCC-22A showed an intermediate type of response, with a moderate growth inhibition at the broad concentration range from 10^{-5} through 10^{-9} M.

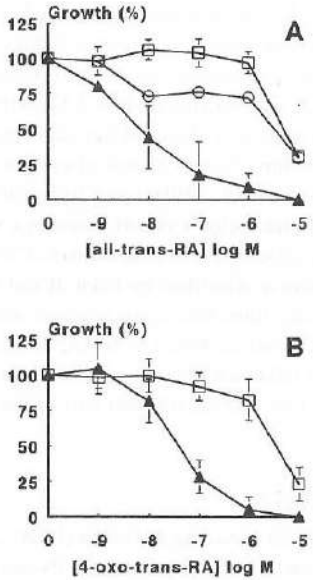


Figure 1. The SRB-test was used to assess the antiproliferative effect for all-*trans*-RA in three HNSCC cell lines (panel A). The growth inhibiting effect of 4-*trans*-oxo-RA on two HNSCC cell lines is shown in panel B. Results of three separate experiments are indicated (mean ± SD)

Metabolism of 9.5 nM ^{3}H-all-trans-RA

The fate of ^{3}H-all-*trans*-RA was studied in the media and the cell pellets of all three cell lines. The cells were exposed to 9.5 nM all-*trans* RA, a concentration that is in the range found in human plasma (Eckhoff *et al.* 1991). The concentration of ^{3}H-all-*trans*-RA decreased in the medium and the time dependency of this effect differed between the cell lines (Figure 2). In UM-SCC-35 this decrease started at 4 h and lead to a total loss at 24 h exposure (Figure 2, panels G and H). A similar decrease was seen for 9- and 13-*cis*-RA. For this cell line the contribution of all-*trans*-RA to the total amount of retinoids was relatively low, being 22% at 4 h and 1.4% at 24 h. For the insensitive line, UM-SCC-14C, the concentration of all-*trans*-RA in the medium was the highest (19.5%) of all three cell lines at 24 h (Figure 2, panels C and D) and for the UM-SCC-22A a somewhat lower value (9.4%) was observed (Figure 2, E and F). The HPLC-method of analysis enabled us to measure retinoid metabolites. In the media of the cell cultures a number of peaks could be detected after 4 and 24 h exposure to ^{3}H-all-*trans*-RA, corresponding to retention times between 2 and 20 min (Figure 2). For UM-SCC-35 these peaks formed at 24 h the majority (86 %) of the total of labeled retinoids. These polar metabolites were less prevalent in the two other cell lines, being 49% and 68% for UM-SCC-14C and 22A, respectively.

Since the intracellular recovery was rather low, varying between 0.2 and 2.5% of the total amount of radioactivity added, only estimations of retinoid levels could be made. Intracellular retinoid levels decreased in the course of time. UM-SCC-35 had the lowest levels of intracellular retinoids, most of them being polar metabolites. The differences between the lines, however, were not as large as seen in the media.

The observed decrease of retinoid levels in the cell culture media is mainly due to retinoid turnover by the cells. Without cells the decrease in retinoid levels in the medium was minimal (Figure 2, panels A and B).

Metabolism of 1 and 10 µM unlabelled all-trans-RA

When exposed to 1 µM all-*trans*-RA, retinoid metabolites were measured after various time points (Figure 3, Table I). The pattern is in general similar as was seen with the exposure to the lower concentration, but the effect was less dramatic. Because now the exposure time is longer, the kinetics of disappearance of the retinoids can be studied in more detail. The disappearance of the major retinoids from the medium was highest for the UM-SCC-35 cell line. After 24 h this is already very significant and reaches an apparent plateau at 48 h with 10% of the original level. The disappearance of retinoids from the other two cell lines is more gradual, UM-SCC-14C being the slowest (Figure 3). With regard to the composition of the retinoids a shift could be observed: the

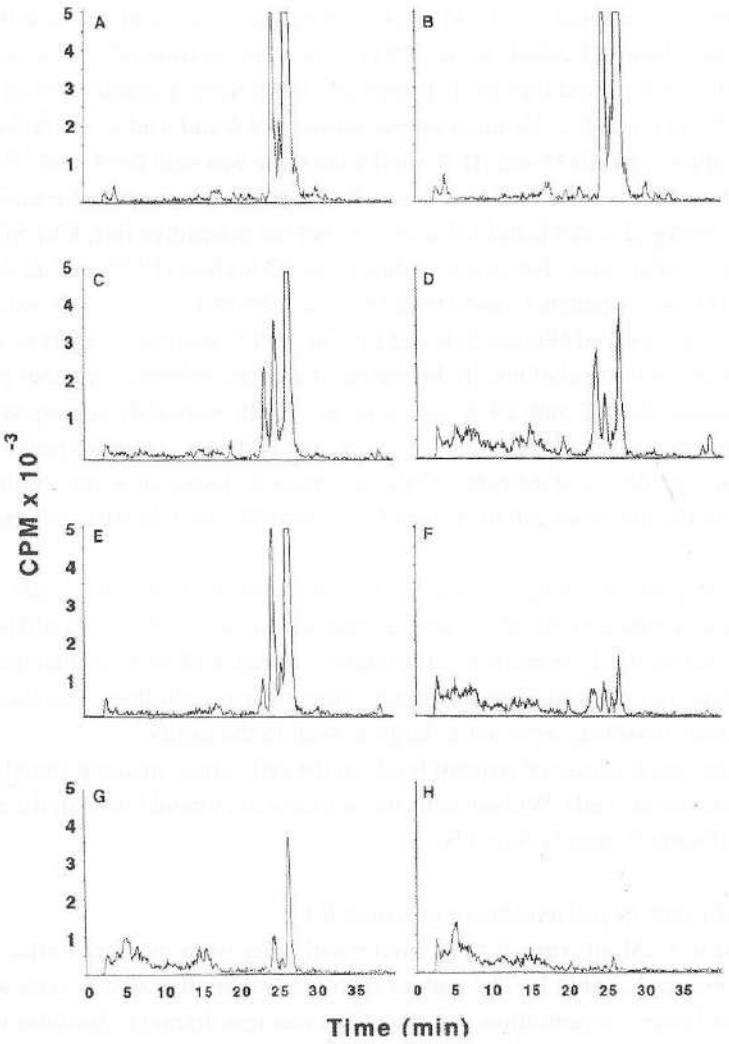


Figure 2. HPLC chromatogram after 4 (left) and 24 h (right) exposure to 9.5 nM ^{3}H-all-*trans*-RA. Results are expressed in cpm on the Y-axis after multiplication with 10^{-3}. Note the difference in disappearance of retinoids and formation of metabolites between the cell lines. Data are shown of the culture media. We included the following standards with the corresponding retention times in minutes: 13-*cis*-RA (24.3), 9-*cis*-RA (25.5), all-*trans*-RA (26.2). A and B: medium without cells, C and D: UM-SCC-14C, E and F: UM-SCC-22A and G and H: UM-SCC-35. This experiment was performed in duplicate, a representative experiment is shown.

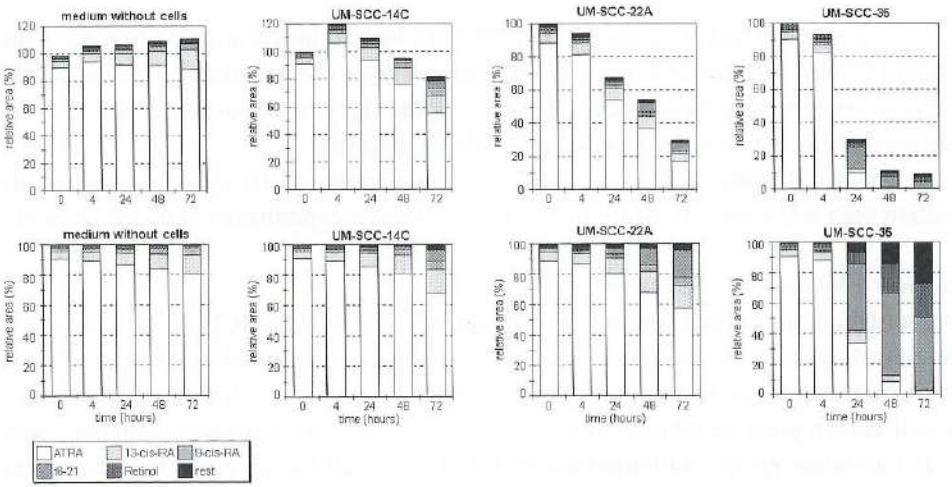


Figure 3. Measurement of retinoids in cell culture media after various periods of exposure to 10^{-6} M all-*trans*-RA. Upper panel: values are expressed as a percentage of the total area under the curve of the relevant part of the chromatogram (retention time between 6 and 30 min, see Fig. 4); T=0 has been set at 100%. Lower panel: values are expressed as a percentage of the total area under the curve of the total chromatogram at that given time point.

contribution of the unidentifiable polar metabolites, visible as a number of peaks with retention times between 6 and 21 min actually increased. These metabolites could be detected for all three cell lines, but was most remarkable for UM-SCC-35, followed in order by UM-SCC-22A and -14C. An example of a HPLC-chromatogram of UM-SCC-35 after 24 h exposure is given in Figure 4.

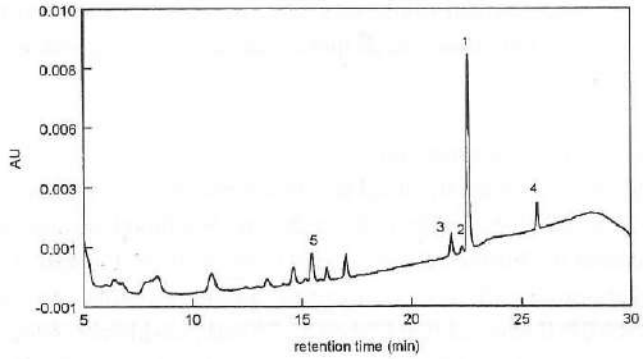


Figure 4. HPLC chromatogram of the cell culture medium of UM-SCC-35, 24 hr after exposure to 10^{-6} M all-*trans*-RA. Peaks: 1: all-*trans*-RA; 2: 9-*cis*-RA; 3: 13-*cis*-RA; 4: retinol; 5: 4-oxo-13-*cis*-RA. Note the unidentified peaks between 6 and 21 min.

After exposure to 1 μM all-*trans*-RA, UM-SCC-35 had the lowest and UM-SCC-22A the highest levels of intracellular retinoids. The intracellular concentrations were too low to account for accurate measurements. Generally speaking, retinoid levels decreased in the course of time, but no polar metabolites could be detected.

When the cells of the three cell lines were exposed to 10 μM all-*trans*-RA, the pattern seen was similar to that with the 1 μM exposure experiments (data not shown).

Growth inhibition by retinoid metabolites

We wished to investigate whether growth inhibition in the UM-SCC-35 cell line could be caused by the excessive formation of one or more toxic retinoid metabolites. To test this hypothesis we performed two types of experiments. Two cell lines were exposed to a well known polar metabolite of all-*trans*-RA, 4-oxo-*trans*-RA. This compound was found to induce growth inhibition in the UM-SCC-35 cell line (Figure 1B), although to a lesser extent when compared with all-*trans* RA, IC_{50}-values being 39.0 and 6.8 nM, respectively (Figure 1A). This metabolite was not active in the UM-SCC-14C cell line.

In a second set of experiments we argued that UM-SCC-35 cell might produce toxic metabolites and that these were released into the medium. Therefore, UM-SCC-35 cells were exposed for 24 h to 10^{-6} and 10^{-8} M all-*trans*-RA and it was found that this medium, when added to virgin UM-SCC-14C cells, minimally affected growth of these cells (Figure 5). Incubation of UM-SCC-35 cells with conditioned medium of UM-SCC-14C cells (after treatment with 10^{-8}M), however, caused a stronger antiproliferative effect when compared to the medium derived from UM-SCC-35 cell cultures. It appears that newly formed retinoids are less potent with respect to growth inhibition than the parent compounds they were derived from, but it must be added that the concentration of these metabolites is significantly lower on an individual basis as compared to the parent retinoid (table 1).

Measurement of CRABP expression

CRABP-I and -II are proteins involved in retinoid homeostasis and they may be important with respect to metabolism and growth inhibition. We therefore analyzed the CRABP-I and -II expression by Northern blotting and hybridization. CRABP-I had undetectable transcript levels in all cell lines, confirming previous results (Zou *et al.* 1994). The analysis of CRABP-II showed that UM-SCC-35 had considerably lower transcript levels than the other two cell lines (Figure 6). Exposure of 24 h with 10^{-6} M all-*trans*-RA had an apparent down regulating effect on CRABP-II mRNA levels in UM-SCC-14C and -22A.

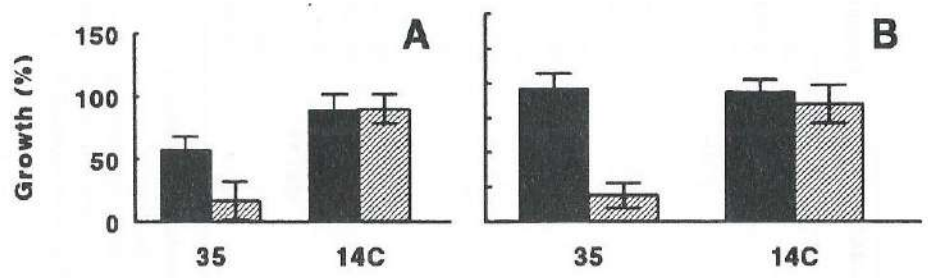


Figure 5. The results of the effects of conditioned media are expressed as a percentage of the control (untreated) cellular growth. Cells grown in culture flasks were exposed for 24 h with 10^{-8} M (solid bars) or 10^{-6} M (hatched bars) all-*trans*-RA to provide the conditioned medium (derived from UM-SCC-14C [panel A] and UM-SCC-35 [panel B]). The conditioned medium was added to cell cultures set up in 96 well plates and after a period of 72 h exposure the proliferation rate of the cells was measured. Cell growth was measured with the SRB-assay (see Materials and Methods). The mean of two experiments with SD is shown. For the sake of clarity the UM-SCC- prefixes in the designations of the cell lines were omitted.

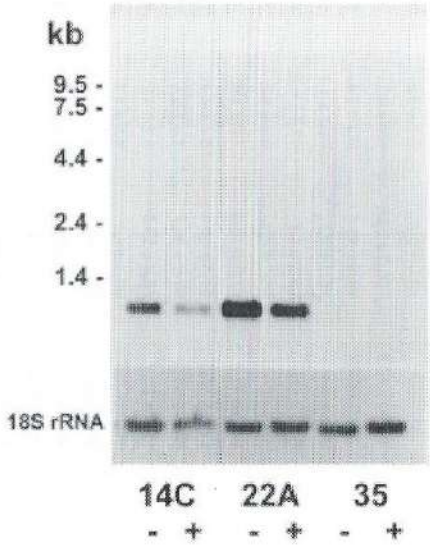


Figure 6. mRNA expression of CRABP-II was determined with Northern blot in three HNSCC cell lines. Cells were untreated (-) or treated with 10^{-6} M all-*trans*-RA (+) for 24 hr. 18S rRNA was used as a reference to correct for the amount of RNA loaded on the gel. For the sake of clarity the UM-SCC- prefixes in the designations of the cell lines were omitted.

Table 1. HPLC-analysis of cell culture medium after exposure to 10^{-6} M all-*trans*-retinoic acid

Cell line	Exposure time (h)	Recovery[a]	all-*trans*-RA[a]	13-*cis*-RA[a]	9-*cis*-RA[a,b]	retinol [a]	4-oxo-*cis*-RA [a]	Polar metabolites[a,c]	Remainder[a,d]
None	0	100	89.4 ± 3.1	5.1 ± 1.8	1.8 ± 0.8	2.6 ± 1.2	u.d.l.	0.1 ± 0.2	u.d.l.
	4	105.7 ± 8.1	93.6 ± 8.8	6.2 ± 2.5	2.1 ± 0.8	2.3 ± 1.1	u.d.l.	0.2 ± 0.5	1.0 ± 1.2
	24	106.7 ± 4.7	91.7 ± 7.2	8.4 ± 2.4	2.8 ± 0.9	2.3 ± 1.1	u.d.l.	0.1 ± 0.3	1.3 ± 1.3
	48	108.3 ± 5.6	90.7 ± 6.7	10.7 ± 1.5	3.5 ± 0.6	2.0 ± 0.9	u.d.l.	0.1 ± 0.3	1.4 ± 1.4
	72	110.9 ± 11.4	88.1 ± 11.5	14.4 ± 1.8	4.8 ± 0.7	1.7 ± 0.5	u.d.l.	0.1 ± 0.2	1.2 ± 1.3
UM-SCC-14C	0	100	90.8 ± 0.6	4.7 ± 0.3	1.5 ± 0.6	2.2 ± 0.4	u.d.l.	0.3 ± 0.4	0.5 ± 0.7
	4	119.6 ± 42.2	106.3 ± 39.0	6.9 ± 0.3	1.9 ± 0.1	2.7 ± 1.1	u.d.l.	0.8 ± 1.2	1.0 ± 0.8
	24	109.5 ± 52.7	93.5 ± 49.1	9.3 ± 1.4	2.6 ± 0.3	0.7 ± 1.0	0.6 ± 0.4	1.9 ± 1.2	1.6 ± 0.2
	48	95.6 ± 0.1	76.3 ± 3.7	11.7 ± 1.7	3.5 ± 1.1	0.5 ± 0.3	0.4 ± 0.1	1.9 ± 0.8	1.0 ± 1.5
	72	81.8 ± 7.5	55.1 ± 1.5	13.2 ± 5.5	4.5 ± 2.4	0.6 ± 0.1	1.1 ± 0.9	5.8 ± 2.2	2.6 ± 0.3
UM-SCC-22A	0	100	88.2 ± 4.2	6.1 ± 2.2	1.9 ± 1.2	1.7 ± 0.8	u.d.l.	u.d.l.	2.1 ± 1.0
	4	94.5 ± 12.9	81.5 ± 10.3	7.0 ± 1.9	1.9 ± 0.6	1.4 ± 0.3	u.d.l.	u.d.l.	2.7 ± 0.4
	24	67.6 ± 18.3	54.3 ± 15.1	6.7 ± 2.4	1.8 ± 0.7	0.8 ± 0.2	0.2 ± 0.2	2.4 ± 0.4	1.7 ± 0.5
	48	54.5 ± 49.5	37.0 ± 35.7	7.4 ± 8.4	2.3 ± 2.4	0.5 ± 0.3	0.9 ± 0.9	5.8 ± 2.5	1.5 ± 1.8
	72	29.4 ± 26.7	16.9 ± 19.3	4.5 ± 4.2	1.6 ± 1.5	0.3 ± 0.2	0.8 ± 0.7	5.1 ± 2.0	1.0 ± 1.0
UM-SCC-35	0	100	90.2 ± 1.5	4.6 ± 1.4	1.6 ± 0.5	2.8 ± 2.0	u.d.l.	u.d.l.	0.7 ± 0.8
	4	93.7 ± 21.0	82.2 ± 17.6	4.7 ± 3.1	1.6 ± 0.6	2.4 ± 1.5	0.4 ± 0.5	1.8 ± 1.1	1.0 ± 0.7
	24	29.8 ± 18.9	10.0 ± 8.0	2.0 ± 1.9	0.5 ± 0.5	2.3 ± 0.8	0.6 ± 0.8	12.8 ± 7.5	2.1 ± 2.9
	48	10.6 ± 6.2	0.9 ± 0.9	0.4 ± 0.4	0.1 ± 0.1	2.0 ± 0.9	0.6 ± 0.7	5.7 ± 3.6	1.5 ± 1.9
	72	9.0 ± 9.1	0.2 ± 0.4	u.d.l.	u.d.l.	2.0 ± 0.6	0.3 ± 0.2	4.2 ± 5.1	2.5 ± 3.0

Cell culture media were analyzed with HPLC after exposure for various times (mean of three separate experiments ± S.D. is shown). [a] Values are expressed as a percentage of the total area under the curve of the relevant part of the chromatogram (retention time between 6 and 30 min, see Figure 4). T = 0 has been set at 100%. [b] A peak was observed between all-*trans*-RA and 13-*cis*-RA and based on literature data this peak was identified to represent 9-cis RA. [c] This refers to compounds identified by peaks with a retention time between 6 and 21 min (see Figure 4). [d] This refers to compounds identified by peaks with a retention time between 21 and 30, with the exception of the known retinoids, 13-, 9-*cis*-, all-*trans*-RA and retinol. u.d.l., under detection limit.

Discussion

In this study of three HNSCC cell lines, it was shown that the extent of all-*trans*-RA induced growth inhibition is proportional to a decrease of retinoid levels in cells and the corresponding culture medium. In the culture medium without cells which was taken as a control, the extent of retinoid disappearance was minimal, leading to the interpretation that the removal of retinoids from the medium is a cell mediated process. This disappearance of retinoids coincided with the production of retinoid metabolites, detectable in the medium. Thus, the major finding of this study is that the extent of metabolism is proportional to the degree of growth suppression. This suggests that retinoid metabolism is associated with growth inhibition. Interestingly, this relation has also been found for breast carcinoma cell lines (Takatsuka *et al.* 1996, van der Leede *et al.* 1997)

It has been reported that in a proportion of patients treated continuously for leukemia with all-*trans*-RA have lower blood plasma retinoid levels at 28 days than during the first cycles of treatment (Muindi *et al.* 1992). This lowering of retinoid plasma levels is proposed to be the result of an induced metabolism that eventually leads to the development of therapy resistance (Martini and Murray 1993). So, hypothetically, two phenotypes of "rapid" and "slow catabolizers" can be discriminated in the population of leukaemia patients (Rigas *et al.* 1993). Our present data, however, indicate, that the ability of HNSCC cells to metabolize is not related to resistance but rather to a growth inhibitory effect. This paradox may be explained by the fact that various cell types could differ in retinoid requirement, turnover and induction of growth inhibition, as has been shown for the first two aspects to be the case for a variety of tissue types (Kurlandsky *et al.* 1995).

Membrane transport is most likely not an important variable in explaining a difference in growth inhibition. Retinol and retinoic acid are preferably bound to proteins and passive diffusion most likely determines cellular uptake (Blaner and Olson 1994). Differences in uptake between the cell lines that have been studied in the present report, however, can not be excluded, and a 63 kD receptor, recently described to be involved in retinol uptake (Bavik *et al.* 1993), may be involved in this process.

An important question which remains to be answered is whether metabolism is the cause or the consequence of growth modulation. First, the hypothesis can be formulated that intracellular turnover of retinoids is the driving force causing growth inhibition. In case of sensitivity, as soon as retinoic acid enters the cell in either the *cis*- or the *trans*-form, it is very efficiently metabolized to one or more cytotoxic polar compounds. In the most sensitive line studied here turnover is very efficient; at the physiological concentration of 9.5 nM the majority of the original concentration of all detectable retinoids has disappeared from the medium after 24 h. The remainder of the labeled

compound may have become too polar to be detected with the presently used eluent. In the culture medium of this sensitive cell line the majority of the detectable retinoids is present in the form of 'polar metabolites'. It is conceivable that a sensitive cell line has a relatively high expression of the enzymes involved in metabolism, like oxidative enzymes like the cytochrome P450s (Rigas *et al.*1993, Martini and Murray 1993). The question arises whether unidentified metabolites also have growth inhibitory activity. Although it has been reported that the 4-oxo-retinoic acid derivatives are considered breakdown products in humans (Eckhoff *et al.* 1991), these molecules have also a transcription activating capacity (Pijnappel *et al.* 1993). In addition, 4-oxo-derivation of retinal to 4-oxo-retinaldehyde and the subsequent conversion to 4-oxo-retinoic acid and 4-oxo-retinol is suggested to be an important step during Xenopus embryogenesis (Blumberg *et al.* 1996). This latter study also showed that all these 4-oxo products had were able to bind to and transactivate RARs. Our results show that also oxo-derivatives have growth inhibiting capacity (Figure 1B). The experiments with conditioned medium provided no evidence that the sensitive UM-SCC-35 cells produced a "suicide" retinoid. The lack of effect of the conditioned medium to produce growth inhibition, however, could be attributed to the fact that the levels of the specific metabolite were too low, perhaps because of further degradation. The peaks corresponding to the levels of the known 4-oxo-oxidation products were rather low.

A second hypothesis can be formulated on the relation between growth inhibition and metabolism. Retinoid metabolism is a secondary event and is an attempt of the cell to neutralize the growth inhibiting effect. In this scenario all-*trans*-RA and/or 13-*cis*-RA are the key retinoids that cause growth inhibition and the other metabolites must be considered as breakdown products. This hypothesis is further supported by the notion that conditioned medium of the insensitive cell line containing high levels of all-*trans*- and 13-*cis*-RA, appeared to be still very growth inhibitory for the UM-SCC-35 cells. Also the expression of CRABP-II mRNA is in further favor of this hypothesis. It has been suggested that CRABP form an intracellular buffer if the RA concentration exceeds a certain level (Delva *et al.* 1993, Åström *et al.* 1991, Blaner and Olson 1994, Bavik *et al.* 1993, Adamson *et al.* 1993b, Napoli *et al.* 1995, Griffiths *et al.* 1993). The present study supports this theory, since the most sensitive cell line was found to have the lowest levels of CRABP-II, and the addition of more RA failed to increase its synthesis. In contrast, Zou *et al.* (1994) reported that CRABP-II expression was not related to retinoid sensitivity in four HNSCC cell lines. An argument against the relation between high CRABP-II levels and insensitivity is the fact that all-*trans*-RA treatment does not up- but rather downregulate CRABP-II mRNA expression. This phenomenon has already been observed for other epithelial cell lines (Zou *et al.* 1994 and Sanquer *et al.* 1993). The data

altogether suggest that CRABP expression can be important, but that no general rule can be formulated. Further studies should elucidate the importance of these molecules and it cannot be excluded that the amount of protein is more decisive than the level of transcription. The positive correlation between an CRABP expression and metabolism as has been found for CRABP-I in F9 teratocarcinoma stem cells (Boylan and Gudas 1992) is in contrast with the presently reported results. The differences in the types of cells and the function of these proteins may explain this discrepancy.

The levels of retinol in the medium of UM-SCC-35 cells remain high during the course of the exposure and deserve special attention. The relatively low consumption of retinol from the medium of UM-SCC-35 suggests that the cells are not able to use retinol as a retinoid source to produce retinoic acid. A low activity of one or more enzymes of the group of alcohol-dehydrogenases, involved in the conversion of retinol to retinoic acid may be responsible for such an effect (Napoli *et al.* 1995, Harding and Duester 1992). As a consequence the cells may have adapted themselves to low intracellular levels of all-*trans*-RA. Theoretically, any excess of all-*trans*-RA is not adequately buffered and may lead to cell death. This concept is in favor of the hypothesis that metabolism is a secondary event.

One can only speculate about the mechanism responsible for retinoid induced growth inhibition. Studies on leukoplakia by Lotan *et al.* (1995) suggest that the induction of expression of RAR-β is important for growth suppression. The same group, however, could not find such a correlation when studying malignant HNSCC cell lines (Xu *et al.* 1994). We have also found no indication that the expression per se or induction by retinoic acid of RAR-α and -γ, and RXR-α is related to sensitivity of the cell lines used (Copper *et al.* 1998). RAR-β mRNA levels were too low to be measured. A recent *in vitro* study on breast cancer cells showed that RAR-α antagonists were as efficient in inhibiting growth as agonists (Dawson *et al.* 1995). This indicates that binding of a retinoid to a RAR may be important but that transcriptional activation on a retinoic acid responsive element (RARE) is not a prerequisite. It is not clear whether this finding can be extrapolated to the *in vivo* situation and whether it can be extended to other tumour types. No indications are available that RAR-α is important in growth inhibition of HNSCC.

Acknowledgments

We thank Hoffmann-la Roche for providing retinoids, Dr A Åström, University of Michigan, Ann Arbor, MI, USA for supplying the cDNA clones of the CRABPs, and Ivar Steen and T Schoemaker for their technical assistance.

References

Adamson PC, Boylan JF, Balis FM, Murphy RF, Godwin KA, Gudas LJ, Poplack DG (1993a) Time course of induction of metabolism of all-trans-retinoic acid and the up-regulation of cellular retinoic acid-binding protein. *Cancer Res* 53: 472-6

Adamson PC, Pitot HC, Balis FM, Rubin J, Murphy RF, Poplack DG (1993b) Variability in the oral bioavailability of all-trans-retinoic acid. *J Natl Cancer Inst* 85: 993-6

Åström A, Tavakkol A, Pettersson U, Cromie M, Elder JT, Voorhees JJ (1991) Molecular cloning of two human cellular retinoic acid-binding proteins (CRABP). Retinoic acid-induced expression of CRABP-II but not CRABP-I in adult human skin *in vivo* and in skin fibroblasts *in vitro*. *J Biol Chem* 266: 17662-6

Bavik CO, Levy F, Hellman U, Wernstedt C, Eriksson U (1993) The retinal pigment epithelial membrane receptor for plasma retinol- binding protein. Isolation and cDNA cloning of the 63-kDa protein. *J Biol Chem* 268: 20540-6

Benner SE, Lippman SM, Hong WK (1993) Retinoids in head and neck cancer. In *Retinoids in oncology*, Hong WK, Lotan R (eds) pp 203-23. Marcel Dekker Inc: New York

Blaner WS, Olson JA (1994) Retinol and retinoic acid metabolism. In *The Retinoids*, Sporn MB, Roberts AB, Goodman DS (eds) pp 229-55. Raven Press: New York

Blumberg B, Bolado J Jr, Derguini F, Craig AG, Moreno TA, Chakravarti D, Heyman RA, Buck J, Evans RM (1996) Novel retinoic acid receptor ligands in Xenopus embryos. *Proc Natl Acad Sci USA* 93: 4873-8

Boylan JF, Gudas LJ (1992) The level of CRABP-I expression influences the amounts and types of all-trans-retinoic acid metabolites in F9 teratocarcinoma stem cells. *J Biol Chem* 267: 21486-91

Braakhuis BJ, Jansen G, Noordhuis P, Kegel A, Peters GJ (1993) Importance of pharmacodynamics in the in vitro antiproliferative activity of the antifolates methotrexate and 10-ethyl-10-deazaaminopterin against human head and neck squamous cell carcinoma. *Biochem Pharmacol* 46: 2155-61

Carey TE (1985) Establishment of epidermoid carcinoma cell lines. In *Head and Neck Cancer*, Wittes RE (ed) pp 287-314. John Wiley and Sons: New York

Dawson MI, Chao WR, Pine P, Jong L, Hobbs PD, Rudd CK, Quick TC, Niles RM, Zhang XK, Lombardo A, Ely KR, Schroot B, Fontana JA (1995) Correlation of retinoid binding affinity to retinoic acid receptor alpha with retinoid inhibition of growth of estrogen receptor-positive MCF-7 mammary carcinoma cells. *Cancer Res* 55: 4446-51

Dawson MI, Hobbs PD (1994) The synthetic chemistry of retinoids . In *The retinoids*, Sporn MB, Roberts AR, Goodman DS (eds) pp 5-178. Raven Press: New York

Delva L, Cornic M, Balitrand N, Guidez F, Miclea JM, Delmer A, Teillet F, Fenaux P, Castaigne S, Degos L and others (1993) Resistance to all-*trans* retinoic acid (ATRA) therapy in relapsing acute promyelocytic leukemia: study of in vitro ATRA sensitivity and cellular retinoic acid binding protein levels in leukemic cells. *Blood* 82: 2175-81

Eckhoff C, Collins MD, Nau H (1991) Human plasma all-trans-, 13-cis- and 13-cis-4-oxoretinoic acid profiles during subchronic vitamin A supplementation: comparison to retinol and retinyl ester plasma levels. *J Nutr* 121: 1016-25

Eckhoff C, Nau H (1990) Identification and quantitation of all-trans- and 13-cis- retinoic acid and 13-cis-4-oxoretinoic acid in human plasma. *J Lipid Res* 31: 1445-54

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 132: 6-13

Gough NM (1988) Rapid and quantitative preparation of cytoplasmic RNA from small numbers of cells. *Anal Biochem* 173: 93-5

Griffiths CE, Elder JT, Bernard BA, Rossio P, Cromie MA, Finkel LJ, Shroot B, Voorhees JJ (1993) Comparison of CD271 (adapalene) and all-*trans* retinoic acid in human skin: dissociation of epidermal effects and CRABP-II mRNA expression. *J Invest Dermatol* 101: 325-8

Harding PP, Duester G (1992) Retinoic acid activation and thyroid hormone repression of the human alcohol dehydrogenase gene ADH3. *J Biol Chem* 267: 14145-50

Hong WK, Endicott J, Itri LM, Doos W, Batsakis JG, Bell R, Fofonoff S, Byers R, Atkinson EN, Vaughan C and others (1986) 13-cis-retinoic acid in the treatment of oral leukoplakia. *N Engl J Med* 315: 1501-5

Hong WK, Itri LM (1994) Retinoids and human cancer. In *The Retinoids*, Sporn MB, Roberts AB, Goodman DS (eds) pp 597-630. Raven Press: New York

Hong WK, Lippman SM, Itri LM, Karp DD, Lee JS, Byers RM, Schantz SP, Kramer AM, Lotan R, Peters LJ, Dimery IW, Brown BW, Goepfert H (1990) Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N Engl J Med* 323: 795-801

Jetten AM, Kim JS, Sacks PG, Rearick JI, Lotan D, Hong WK, Lotan R (1990) Inhibition of growth and squamous-cell differentiation markers in cultured human head and neck squamous carcinoma cells by beta-all-trans retinoic acid. *Int J Cancer* 45: 195-202

Koch HF (1978) Biochemical treatment of precancerous oral lesions: the effectiveness of various analogues of retinoic acid. *J Maxillofac Surg* 6: 59-63

Kurlandsky SB, Gamble MV, Ramakrishnan R, Blaner WS (1995) Plasma delivery of retinoic acid to tissues in the rat. *J Biol Chem* 270: 17850-7

Lee JS, Newman RA, Lippman SM, Huber MH, Minor T, Raber MN, Krakoff IH, Hong WK (1993) Phase I evaluation of all-trans-retinoic acid in adults with solid tumors. *J Clin Oncol* 11: 959-66

Lippman SM, Kessler JF, Al Sarraf M, Alberts DS, Itri LM, Mattox D, Von Hoff DD, Loescher L, Meyskens FL (1988) Treatment of advanced squamous cell carcinoma of the head and neck with isotretinoin: a phase II randomized trial. *Invest New Drugs* 6: 51-6

Lotan R (1993) Retinoids and squamous cell differentiation. In *Retinoids in oncology*, Hong WK, Lotan R (eds) pp 43-73. Marcel Dekker Inc: New York

Lotan R, Xu XC, Lippman SM, Ro JY, Lee JS, Lee JJ, Hong WK (1995) Suppression of retinoic acid receptor-beta in premalignant oral lesions and its up-regulation by isotretinoin. *N Engl J Med* 332: 1405-10

Mangelsdorf DJ, Umesono K, Evans RM (1994) The retinoid receptors. In *The Retinoids*, Sporn MM, Roberts AR, Goodman DS (eds) pp 319-49. Raven Press: New York

Martini R, Murray M (1993) Participation of P450 3A enzymes in rat hepatic microsomal retinoic acid 4-hydroxylation. *Arch Biochem Biophys* 303: 57-66

Muindi J, Frankel SR, Miller WH Jr, Jakubowski A, Scheinberg DA, Young CW, Dmitrovsky E, Warrell RP Jr (1992) Continuous treatment with all-trans retinoic acid causes a progressive reduction in plasma drug concentrations: implications for relapse and retinoid "resistance" in patients with acute promyelocytic leukemia. *Blood* 79: 299-303

Muindi JF, Scher HI, Rigas JR, Warrell RP Jr, Young CW (1994) Elevated plasma lipid peroxide content correlates with rapid plasma clearance of all-trans-retinoic acid in patients with advanced cancer. *Cancer Res* 54: 2125-8

Napoli JL, Boerman MH, Chai X, Zhai Y, Fiorella PD (1995) Enzymes and binding proteins affecting retinoic acid concentrations. *J Steroid Biochem Mol Biol* 53: 497-502

Pijnappel WW, Hendriks HF, Folkers GE, van den Brink CE, Dekker EJ, Edelenbosch C, van der Saag PT, Durston AJ (1993) The retinoid ligand 4-oxo-retinoic acid is a highly active modulator of positional specification. *Nature* 366: 340-4

Rigas JR, Francis PA, Muindi JR, Kris MG, Huselton C, DeGrazia F, Orazem JP, Young CW, Warrell RP Jr (1993) Constitutive variability in the pharmacokinetics of the natural retinoid, all-trans-retinoic acid, and its modulation by ketoconazole. *J Natl Cancer Inst* 85: 1921-6

Sacks PG, Harris D, Chou TC (1995) Modulation of growth and proliferation in squamous cell carcinoma by retinoic acid: a rationale for combination therapy with chemotherapeutic agents. *Int J Cancer* 61: 409-15

Sambrook J, Fritsch EF, Maniatis T (1989) In *Molecular cloning: a laboratory manual*, Cold Spring Harbor Laboratory Press: New York

Sanquer S, Eller MS, Gilchrest BA (1993) Retinoids and state of differentiation modulate CRABP II gene expression in a skin equivalent. *J Invest Dermatol* 100: 148-53

Stich HF, Hornby AP, Mathew B, Sankaranarayanan R, Nair MK (1988) Response of oral leukoplakias to the administration of vitamin A. *Cancer Lett* 40: 93-101

Takatsuka J, Takahashi N, de Luca LM (1996) Retinoic acid metabolism and inhibition of cell proliferation: an unexpected liaison. *Cancer Res* 56: 675-8

van der Waal I (1992) Oral precancerous lesions - present knowledge. *Deut Zahnartzl Z* 47: 860-4

Wouters W, van Dun J, Dillen A, Coene MC, Cools W, De Coster R (1992) Effects of liarozole, a new antitumoral compound, on retinoic acid- induced inhibition of cell growth and on retinoic acid metabolism in MCF-7 human breast cancer cells. *Cancer Res* 52: 2841-6

Xu XC, Ro JY, Lee JS, Shin DM, Hong WK, Lotan R (1994) Differential expression of nuclear retinoid receptors in normal, premalignant, and malignant head and neck tissues. *Cancer Res* 54: 3580-7

Zou CP, Clifford JL, Xu XC, Sacks PG, Chambon P, Hong WK, Lotan R (1994) Modulation by retinoic acid (RA) of squamous cell differentiation, cellular RA-binding proteins, and nuclear RA receptors in human head and neck squamous cell carcinoma cell lines. *Cancer Res* 54: 5479-87

Chapter 5

Enhanced turnover of all-*trans*-retinoic acid and increased formation of polar metabolites in head and neck squamous cell carcinoma lines compared to normal oral keratinocytes

Ingeborg Klaassen, Ruud H. Brakenhoff, Serge J. Smeets, Gordon B. Snow, Boudewijn J.M. Braakhuis

Section Tumor Biology, Department of Otolaryngology, Vrije Universiteit Medical Center, Amsterdam, the Netherlands

Clin Cancer Res 2001; 7:1017-25

Abstract

Retinoids show promise in the treatment of various (pre)malignancies, including head and neck squamous cell carcinoma (HNSCC). Previous studies have shown that metabolic pathways of retinoids are important in the anticancer effect of retinoids, and that these pathways may change during carcinogenesis. In the present study, we analyzed HNSCC cell lines (n = 11) and normal oral keratinocyte cultures (n = 11) by reverse-phase HPLC and conducted growth inhibition assays. We demonstrate here that in contrast to normal oral keratinocytes, HNSCC cell lines: 1) had a on average 17-fold greater turnover rate of RA, 2) had a 1.9-fold less RA-induced growth inhibition, 3) were able to form polar metabolites, and 4) were able to catabolize 4-oxo-RA. Furthermore, the mRNA expression of the RA specific 4-hydroxylase, CYP26A1, was dramatically increased after RA-induction in the two HNSCC cell lines with the highest metabolism, was undetectable in normal keratinocytes, and was not inducible by RA. Next, introduction of CYP26A1 cDNA in a low-metabolizing HNSCC cell line resulted in a 11-fold higher turnover rate of RA and a 12-fold increased amount of polar metabolites, but did not change sensitivity to RA. These observations point to fundamental changes in RA metabolism pathways during HNSCC carcinogenesis and may provide clues to a more rational approach for RA-mediated intervention.

Introduction

Over the years, basic and clinical research have improved the understanding of the role of retinoids in normal, premalignant and malignant growth. Retinoids, the natural and synthetic analogues of vitamin A, exert a wide range of biological actions, and are crucial for the regulation of epithelial growth and differentiation (Lotan 1980, De Luca 1991). When it was found that vitamin A deficiency resulted in hyperplasia and hyperkeratosis of oral mucosa (Graham 1984), the interest in retinoids in relation to oral carcinogenesis increased. Retinoids were demonstrated to reverse malignant growth *in vitro* and *in vivo* (Lotan 1996, Moon *et al.* 1994) and were effective as chemopreventive agents (Hong *et al.* 1990). Retinoids could successfully be used to treat oral leukoplakia (Lippman *et al.* 1988), a potentially premalignant mucosal lesion, and the occurrence of second primary tumors following HNSCC could be inhibited or delayed (Hong *et al.* 1995). These second primary tumors, which occur at an incidence rate of 2 to 3% per year, are a major cause of death following surgical resection of early-stage head and neck cancer (Snow 1992). Although retinoids exhibit clinical activity, some limitations became apparent: they are active in only a proportion of individuals with a high cancer risk, they are marginally active in

advanced cancer, and they can have serious side effects (Hong and Itri 1994). These variations in positive and negative effects might be explained by differences in the metabolic pathways either between individuals or between normal *versus* malignant tissues. To enhance the efficacy of retinoids, the basis of these differences needs to be elucidated.

The emergence of acquired clinical resistance to retinoids in acute promyelocytic leukemia has been related to the induction of oxidative catabolism by CYPs (Muindi and Young 1993). In the plasma of the patients with remission, increased levels of oxidized retinoid metabolites like 4-OH-RA could be detected. Recently an RA-inducible CYP gene, CYP26A1, was discovered (Ray *et al.* 1997, White *et al.* 1997) that has high specificity for all-*trans*-RA (Marikar *et al.* 1998). This enzyme is responsible for the oxidation of RA to 4-OH-RA, 18-OH-RA, and 4-oxo-RA. Analysis of CYP26A1 mRNA expression in a number of human tumor cell lines showed that it is variably expressed in a constitutive manner, and that it can be induced by RA treatment (White *et al.* 1997). These findings suggest that CYP26A1 is the key enzyme for catabolism of RA to 4-hydroxylated products, and its differential expression might explain differences in RA metabolism and growth inhibition between cell types.

Little is known about retinoid metabolism and the activity of CYP26A1 in normal and malignant oral keratinocytes. Studies on epidermal keratinocytes show that, except for the immortalized human keratinocyte HaCaT cell line, no or low mRNA levels of CYP26A1 were found, and it was not inducible by RA (Marikar *et al.* 1998, Popa *et al.* 1997). Earlier work of Randolph and Simon (1997) demonstrated that human keratinocytes form products that are more polar than 4-OH-RA and 4-oxo-RA, and carbon four metabolites were not detected. These authors suggested that the incapacity of detecting carbon four metabolites could be obscured by a very active RA metabolic pathway in these cells.

Recently, the work of Guo and Gudas (1998) indicated that retinol metabolism is different between normal and malignant oral epithelial cells. These authors reported that cultured normal epithelial cells esterify retinol to a much greater extent than the carcinoma cells. The reduced ability of the carcinoma cells to esterify retinol could reflect a phenotypic change that is required for malignant transformation.

In the present study we, related RA metabolism quantitatively and qualitatively to the extent of growth inhibition by RA and the mRNA expression of CYP26A1 in HNSCC cell lines and normal oral keratinocytes obtained from non-cancer patients. Our results show that RA metabolism is fundamentally different in normal and malignant epithelial cells of the head and neck region.

Materials and methods

Chemicals

All-*trans*-RA was obtained from Acros Chimica (Geel, Belgium), retinol, retinal and 13-*cis*-RA from Sigma (St. Louis, MO); 5,6-epoxy-RA, 4-oxo-all-*trans*-RA and 4-oxo-13-*cis*-RA were gifts from Dr UH Wiegand (Hoffmann-la Roche, Basel, Switzerland); 4-hydroxy-RA and 18-hydroxy-RA were gifts from Dr L Foley (Roche Pharmaceuticals, Nutley, NJ). All compounds were dissolved in dimethyl sulfoxide (DMSO) and stored as 10^{-3} M stock at -80°C under nitrogen. For each experiment, working dilutions were prepared freshly in the appropriate cell culture medium. Final DMSO concentration was always lower than 0.1% and did not affect cell growth. [11,12-^{3}H(N)]all-*trans*-RA (35.8 Ci/mmol) was obtained from NEN (Boston, MA). All handling with retinoids was performed in subdued light and in the presence of 0.1% BSA to prevent absorption to plastics (Klaassen *et al.* 1999).

Cells and culture conditions

Primary oral keratinocytes were obtained from the uvulas of non-cancer patients who underwent uvulopalatopharyngoplasty. Cells were isolated and cultured as described previously (Reid *et al.* 1997) in 6-well culture plates in keratinocyte growth medium (KGM) (Life Technologies, Paisley, UK) to which growth supplements, gentamycin sulphate (final concentration 5 µg/ml), and amphotericin B (final concentration 0.5 µg/ml) were added (all from Life Technologies). Primary cultures of keratinocytes were subcultured at 70% confluence in a dilution of 10^5 cells/well and were used for experiments at passage 3. Human HNSCC cell lines UM-SCC-35, -22A and -14C were provided by Dr TE Carey (University of Michigan, Ann Harbor, MI) and are described elsewhere (Carey *et al.* 1990). Cell lines 92-VU-041, 92-VU-059, 92-VU-078, 92-VU-080, 93-VU-094, 93-VU-096, and 93-VU-147 were established at the laboratory of Human Genetics, *Vrije Universiteit*, Amsterdam, the Netherlands (Hermsen *et al.* 1996). The cell line VU-SCC-OE was established from human HNSCC xenografts in our laboratory (Welters *et al.* 1997). HNSCC cells were maintained in Dulbecco's modified essential medium (DMEM) (Life Technologies) supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin (pen/strep, Life Technologies) and 5% FCS (ICN Biomedicals, Irvine, UK).

Growth inhibition assays

The dose response effect on cell proliferation was determined with the sulphorhodamine B (SRB) assay. Details of the assay have been described previously (Braakhuis *et al.* 1993). In short, cells (1000- 4000 per well) were plated in 96-well plates in DMEM/5% FCS or KGM/0.1% BSA for HNSCC cell lines or oral keratinocyte cultures, respectively, and were allowed to grow for 72 h (the "lag phase"). After this phase, the medium was replaced by medium containing RA, with a final concentration ranging from 10^{-9} to 10^{-6} M. Growth was assayed after 72 h incubation (the "log phase"), by staining the cellular protein with SRB (Sigma) and spectrophotometric measurement of the absorption at 540 nm with a microplate reader (Labsystems Multiskan, Helsinki, Finland). Only experiments were included in which the untreated cells had doubled in cell number during the log phase.

Metabolism of all-trans-retinoic acid

Keratinocytes and HNSCC cells were plated at a density of 10^5 cells per well in 6-well plates. Upon 70 - 80% confluence, the medium was removed and replaced by medium containing 1 µM RA, being KGM with 0.1% BSA for keratinocytes (Klaassen *et al.* 1999) or DMEM with 5% FCS for HNSCC cells. As a control, RA containing medium without cells was included during the incubation period. From the medium, two samples of 350 µl were taken; and after removal of the residual medium the cells were washed once with phosphate-buffered saline and collected in 350 µl trypsin/EDTA. The samples were stored at -80°C under nitrogen until retinoid extraction.

Retinoid extraction and HPLC analysis

Retinoids were analyzed by reverse-phase HPLC after extraction with acetonitrile, as described previously (Teerlink *et al.* 1997). In short, to each sample, 50 µl of 1 M sodium acetate buffer (pH 4.0) and 600 µl acetonitrile was added, and after vortex-mixing, the samples were centrifuged for 2 to 5 min at 3,000 *g*. Protein content of cell extracts was determined on the pellet with the Bio-Rad protein assay (Bio-Rad, Hercules, CA). In total, 720 µl of the clear supernatant was transferred to a 2 ml glass autosampler vial, and after addition of 240 µl water, the vials were capped, mixed by inversion and put in the sample compartment of the autoinjector, which was cooled at 4°C, or stored at −80°C under nitrogen. A Gynkotek (Gynkotek HPLC, Munich, Germany) HPLC system was used, consisting of a Basic Marathon automatic sample injector, a Model M480G gradient pump, a Model UVD 170S UV detector, and a column heater (Mistral, Spark Holland, Emmen, the Netherlands). The mobile phase was degassed online using a model GT103 degasser (Gastorr, Bad Honnef, Germany). Chromelion software (Gynkotek) was used for instrument control and data acquisition. Separation was performed on a 3-µm Spherisorb ODS2 column (100 x 4.6 mm) from Phase Separations (Deeside, UK) maintained at 30°C. Composition of the mobile phases and the binary gradient used were as described by Eckhoff and Nau (1990). UV detection was performed at 340 nm. Retinoids were identified using external standards. RA concentrations were quantified based on peak height. Concentrations of RA in the standards was determined spectrophotometrically by measurement at λ_{max} (350 nm) and use of the molar extinction coefficients (45,300) (Furr *et al.* 1994).

Determination of the turnover rate of RA

The results of the HPLC analysis were submitted to stringent selection. Experiments were excluded when medium controls (without cells) showed a decrease in RA of more than 60% in 48 h. The amount of RA was calculated relative to the amount of the medium control at the start of the experiment. The turnover rate was taken as the difference between 4 and 24 h, corrected for the decrease in the medium controls during this period. Next, this relative amount was converted to absolute amounts in pmol by use of the external standards. The turnover rate was expressed in pmol per mg protein per hour.

Northern blotting

Of each sample 20 µg total RNA was separated on an 1% (w/v) formaldehyde agarose gel in 3-(N-morpholino-)propanesulphonic acid (MOPS) buffer and transferred to a Qiabrane plus filter (Westburg, Leusden, the Netherlands). RNA was cross-linked to the blot by UV, prehybridized for 1 h at 65°C in 7% SDS, 0.5 M Na_2HPO_4/NaH_2PO_4 (pH 7.2), and 1 mM EDTA and hybridized overnight

at 65°C in the same solution after addition of 25 ng denatured labeled probe. The 1.9-kb SalI-EcoRI fragment containing human CYP26A1 cDNA (13) was labeled with [α-^{32}P]dCTP by random primed elongation. An 28S rRNA oligo was labeled with [γ-^{32}P]ATP, as described by Sambrook *et al.* (1989), to monitor the amount of RNA loaded on the gel. The blot was washed twice with 2 x SSC, 0.1% SDS and twice with 0.2 x SSC, 0.1% SDS for 15 min at 55°C and autoradiographed at −80°C with intensifying screens and/or phosphor screens at room temperature. Hybridizing bands were quantified by a phosphor imager (Molecular Dynamics, Krefeld, Germany).

DNA transfection

UM-SCC-14C cells were cultured in 6-well plates in DMEM containing 10% FCS. Transfection was performed using Lipofectin reagent (Life Technologies) following the manufacturer's protocol. In order to prevent clonal selection transient CYP26A1 transfectants were generated. Cells were transfected at 50-70% confluence with 10 µg pcDNA3/P450RAI expression vector (a gift of Dr M Petkovitch, Cancer Research Laboratories, Queens University, Kingston, Ontario, Canada), further designated as pCYP26, and as a control 10 µg pcDNA3 expression vector (Invitrogen, Carlsbad, CA). After 4 h, medium was refreshed, and 24 h later geneticin (500 µg/ml; G418; Life Technologies) was added to the cells. Resistant colonies were pooled 2 weeks later and cultured under prolonged G418 selection.

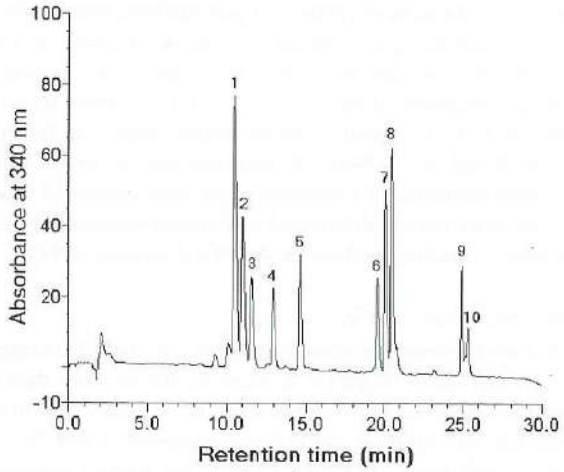


Figure 1. HPLC profile of retinoid standard dissolved in water. *Peak 1*, 4-oxo-RA; *peak 2*, 4-oxo-cRA; *peak 3*, 4-OH-RA; *peak 4*, 18-OH-RA; *peak 5*, 5,6-epoxy-RA; *peak 6*, 13-*cis*-RA; *peak 7*, 9-*cis*-RA; *peak 8*, all-*trans*-RA; *peak 9*, retinol; and *peak 10*, retinal.

Results

Turnover rate and response to RA in HNSCC cell lines and oral keratinocytes

The turnover rate of RA of eleven HNSCC cell lines and eleven OKCs obtained from healthy individuals was determined following treatment with 1 µM RA for 4 to 72 h. Retinoids were analyzed by reverse-phase HPLC with UV detection at 340 nm and identified using external standards (Figure 1). The rate at which RA disappeared from the medium and the formation of polar metabolites differed among the HNSCC cell lines. As an example, in Figure 2 the HPLC profiles of RA and its metabolites found in the medium after various incubation times are depicted for two HNSCC cell lines. In 93-VU-080, RA had almost completely disappeared after 72 h, and polar metabolites were observed after 24 h, in 92-VU-078 polar metabolites were not found before 48 h. The type of polar metabolites appeared to be the same based on the retention times.

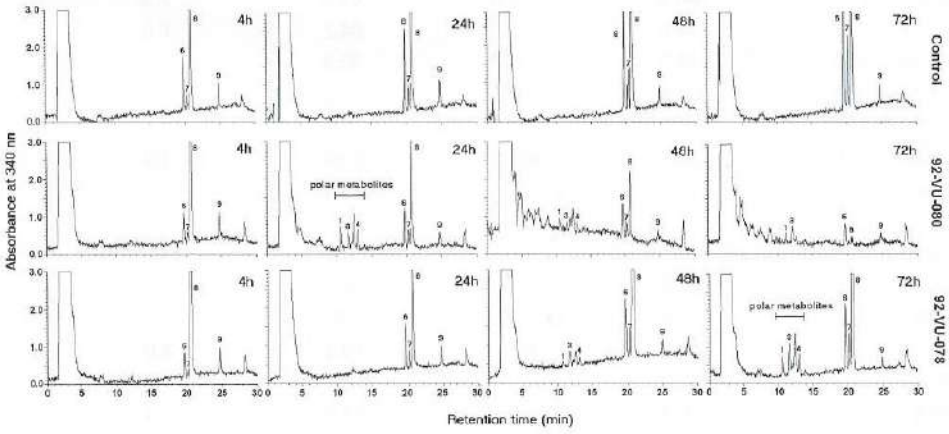


Figure 2. The HPLC profiles of RA metabolites in medium of two representative HNSCC cell lines. Cells were cultured in medium containing 1 µM RA for the indicated time periods. Retinoids were extracted and separated by reverse-phase HPLC. The experiments were done at least three times, and very similar results were obtained. Peaks were identified by external standardization: *Peak 1*, 4-oxo-RA; *peak 3*, 4-OH-RA; *peak 4*, 18-OH-RA; *peak 6*, 13-*cis*-RA; *peak 7*, 9-*cis*-RA; *peak 8*, all-*trans*-RA; *peak 9*, retinol. Compounds corresponding to peaks with retention times between 10 and 15 min are designated as polar metabolites. Note that scale is chosen as such that not all peaks are visible in full length.

Table 1. Turnover en growth inhibition after exposure to 1 µM RA.
Metabolic studies: the turnover was determined by measuring the decrease in RA concentration during the 4 and 24 h exposure period. The experiments were performed at least twice for the HNSCC lines and once for the oral keratinocyte cultures. Growth inhibition studies: the growth was measured with the "SRB-assay". These data are from at least two separate experiments.

	Turnover RA (pmol/mg protein/h)		Growth (%)[a]	
Cell Line/ Culture	Mean	SD	Mean	SD
HNSCC Cell Lines				
92-VU-041	141.0	8.6	92.8	5.8
92-VU-059	124.1	8.2	95.8	9.2
92-VU-078	104.3	15.2	87.5	0.6
92-VU-080	421.5	73.9	91.2	3.5
93-VU-094	30.7		91.5	3.5
93-VU-096	168.5	3.9	63.6	7.3
93-VU-147	43.8		64.7	7.0
UM-SCC-14C	13.3	9.2	96.5	5.2
UM-SCC-22A	104.8	67.9	72.9	3.7
UM-SCC-35	1016.0	413.2	16.4	10.5
VU-SCC-OE	558.9	152.5	56.9	3.3
Mean	*246.7*		*75.4*	
Oral Keratinocyte Cultures				
K669	29.5		ND[b]	
K670	0.0		ND	
K671	16.4		10.4	3.0
K672	7.9		ND	
K673	0.0		53.0	18.4
K674	0.0		27.9	4.5
K675	61.3		58.3	10.0
K679	0.0		ND	
K704	29.9		32.9	3.6
K709	9.5		36.9	5.1
K906	8.8		64.7	2.9
Mean	*14.8*		*40.6*	

[a] Growth is expressed as a percentage of the growth of untreated cells
[b] ND, not determined

Table 1 shows a summary of the turnover of RA for both HNSCC cell lines and OKCs. Although a considerable variation in the rate of RA metabolism was observed among individual HNSCC cell lines and oral keratinocyte cultures, the rate of RA metabolism was, on average, about 17-fold greater in the HNSCC cell lines (mean, 245.4 pmol/mg protein/h) compared to normal keratinocytes (mean, 14.8 pmol/mg protein/h).

To relate RA turnover rate with the response to RA, growth inhibition assays were performed in which the cells were exposed to increasing RA concentrations for 72 h. In all cell lines, a concentration-dependent inhibition of growth was demonstrated. A summary of the growth assays are presented in Table 1 and expressed as the percentage of growth at 1 µM RA exposure for each cell line. On average,

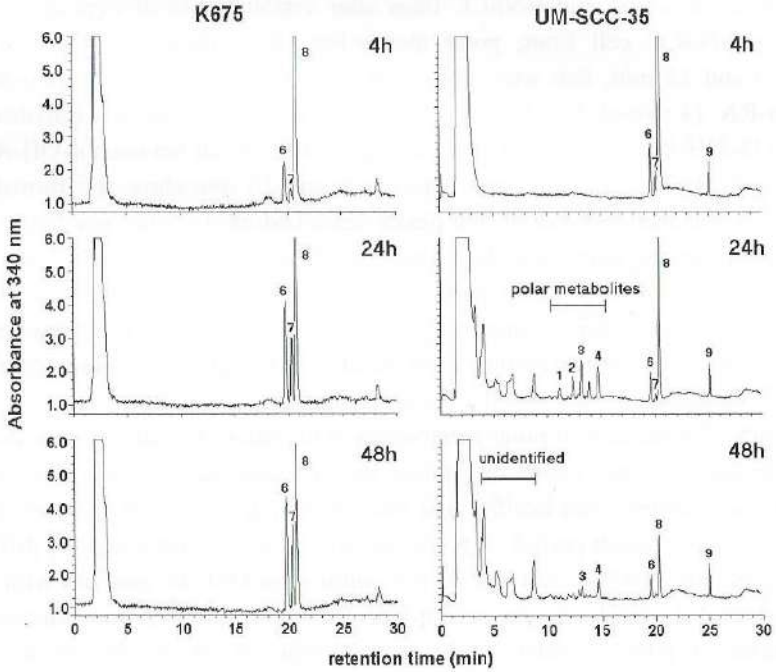


Figure 3. HPLC profiles of retinoid metabolites. The medium of an HNSCC cell line (right) and an oral keratinocyte culture (left) are shown, exposed to 1 µM RA for indicated periods of time. In contrast to the normal cells, the tumor cell lines are characterized by a high turnover and the occurrence of polar metabolites. The experiments were done at least three times and very similar results were obtained. *Peak 1*, 4-oxo-RA; *peak 2*, 4-oxo-cRA; *peak 3*, 4-OH-RA; *peak 4*, 18-OH-RA; *peak 5*, 5,6-epoxy-RA; *peak 6*, 13-*cis*-RA; *peak 7*, 9-*cis*-RA; *peak 8*, all-*trans*-RA; *peak 9*, retinol; *polar metabolites*, metabolites corresponding with peaks with retention times between 10 and 15 min; *unidentified*, unidentified polar metabolites corresponding with peaks with retention times between 5 and 10 min. Note that scale is chosen as such that not all peaks are visible in full length.

HNSCC lines were about 1.9-fold less sensitive to growth inhibition by RA than OKCs. In addition, in HNSCC cell lines a highly significant correlation (Pearson correlation coefficient (R) = 0.78; P = 0.002) was found between RA turnover and growth inhibition: a high growth inhibition was associated with a high turnover; normal keratinocyte cultures did not show any significant correlation between RA turnover rate and growth inhibition (data not shown).

Differences in RA metabolites between HNSCC cell lines and OKCs

In the medium, a difference in RA metabolites was found between HNSCC cell lines and normal oral keratinocytes. Figure 3 shows the typical profiles of metabolites in the medium of keratinocytes and HNSCC lines after various times of exposure to 1 μM RA. In the HNSCC cell lines, polar metabolites were found, at retention times between 10 and 15 min, that were identified as 4-oxo-all-*trans*-RA (4-oxo-RA), 4-oxo-13-*cis*-RA (4-oxo-cRA), 4-hydroxy-all-*trans*-RA (4-OH-RA), 18-hydroxy-all-*trans*-RA (18-OH-RA), and one unidentified peak was found between 4-OH-RA and 18-OH-RA. Peaks at retention times between 5 and 10 min show the formation of unidentified polar metabolites. All the peaks described above were not found in the medium of the control samples without cells. In the normal oral keratinocyte cultures no polar metabolites were detected in the medium. In the cellular fractions of HNSCC cell lines and oral keratinocyte cultures no major differences in RA metabolites were found. All-*trans*-RA, was the predominant metabolite found in the cellular fraction of both normal keratinocytes and HNSCC cells (Figure 4).

The lack of formation of polar metabolites in keratinocyte cultures was likely not due to unfavorable culture conditions during the experiments, since we observed that the cells looked normal and healthy and increased at least 1.5 fold in cell number during the 48 h incubation period. A point that was of our concern was the difference in culture medium. HNSCC cell lines were cultured in DMEM medium with serum and normal oral keratinocytes in serum-free KGM medium. Serum contains low concentrations of retinol, which could be of influence on the formation of RA metabolites. The cell lines UM-SCC-14C, -22A, and -35 were cultured in KGM medium for several passages and analyzed by HPLC after exposure to 1 μM RA. No difference in metabolism was found in these cell lines as compared to metabolism in DMEM medium with FCS (data not shown). Growing the oral keratinocyte cultures in the presence of 2% FCS also did not result in the formation of polar metabolites.

We reasoned that the incapacity of observing polar metabolites in oral keratinocytes was hampered by a low sensitivity of the detection method. Hence,

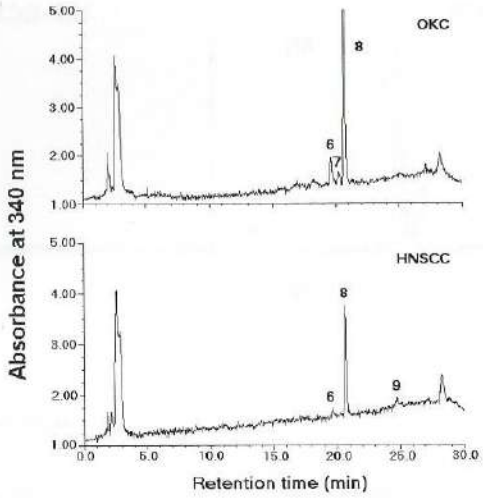


Figure 4. HPLC profiles of retinoid metabolites in the cellular fractions. An OKC (*top*) and an HNSCC cell line (*bottom*) are shown, exposed to 1 μM RA for 24 h. *Peak 6*, 13-*cis*-RA; *peak 7*, 9-*cis*-RA; *peak 8*, all-*trans*-RA; *peak 9*, retinol. For both cell types no polar metabolites were detected in the cellular fractions. Note that scale is chosen as such that not all peaks are visible in full length.

sensitivity was increased by exposing the same oral keratinocyte culture and HNSCC cell line shown in Figure 3 for various incubation periods to 10 nM [^{3}H]RA. An additional advantage of this method is that all visible peaks correspond to RA-derived metabolites. The HPLC profiles of the RA metabolites of the HNSCC cell line again show the formation of polar compounds (retention times between 10 and 15 min and 5 and 10 min), whereas these are not detectable in the normal keratinocytes (Figure 5) nor in the medium control samples. The HPLC profiles of the HNSCC cell line and the normal keratinocyte culture both show a peak at retention times between 0 and 5 min, suggesting the formation of very polar metabolites from RA.

Our results indicate that both OKCs and HNSCC cell lines can process RA, but that their intermediate catabolites are clearly different. Whereas both cell types are able to catabolize RA completely, in the normal keratinocyte cultures no polar 4-hydroxylated products can be observed. These results suggest that either a) keratinocytes catabolize these 4-hydroxylated products far more efficiently than HNSCC cell lines, making these products undetectable; or b) the catabolism of RA

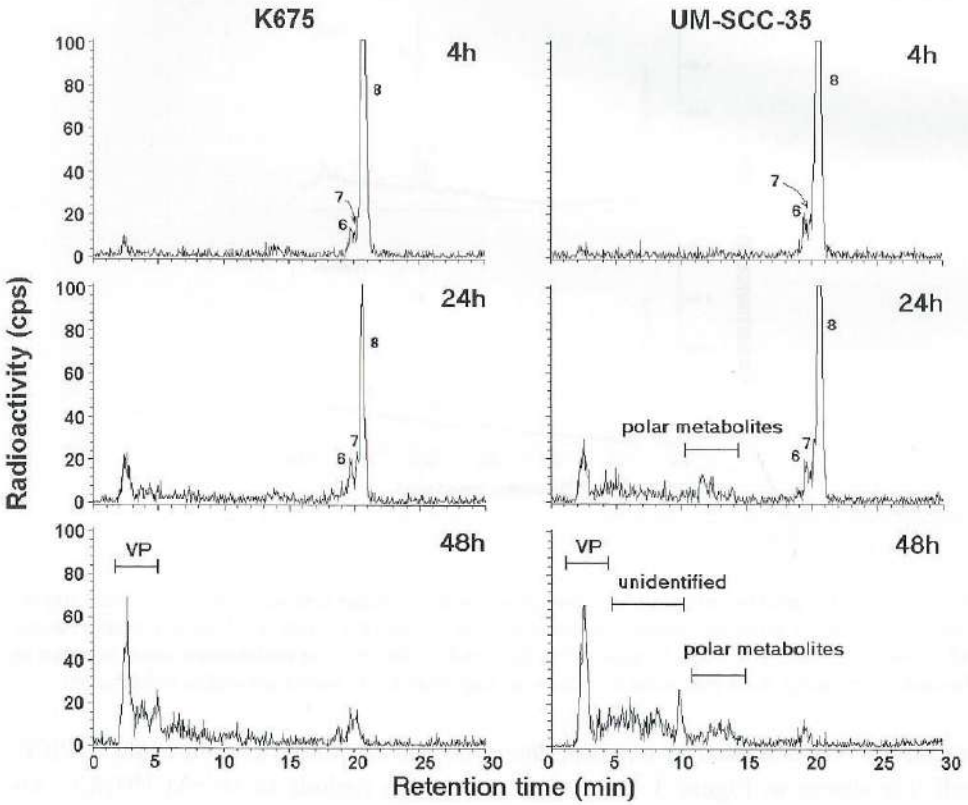


Figure 5. [^{3}H]-HPLC profiles of retinoid metabolites in the medium. An HNSCC cell line (*right*) and an oral keratinocyte culture (*left*), were exposed to 10 nM [^{3}H]RA for various times. The profiles of the HNSCC cell line showed the formation of polar metabolites (peaks corresponding to retention times between 10 and 15 min) and unidentified, more polar metabolites (retention times between 5 and 10 min), which could not or hardly be detected in the OKC. Both cell types indicate the formation of very polar metabolites (*VP*, retention times between 0 and 5 min). *Peak 6*, 13-*cis*-RA; *peak 7*, 9-*cis*-RA; *peak 8*, all-*trans*-RA. Note that scale is chosen as such that not all peaks are visible in full length

in normal keratinocytes takes place through an alternative pathway. To explore the first possibility, we added 1 μM 4-oxo-RA to the medium of HNSCC or oral keratinocytes and documented its loss from the medium (Figure 6). The HNSCC cell lines were found to catabolize 4-oxo-RA much more efficiently than the oral

keratinocytes. In HNSCC cell lines, the 4-oxo-RA had completely disappeared after 24 h, whereas the concentration in the medium of the OKCs was similar to the control (medium without cells). 4-oxo-RA was extensively isomerized to its cis-isoform, 4-oxo-cRA, even in medium without cells, and small amounts of 4-OH-RA were found in the conditioned medium. It was observed that HNSCC cell lines were able to break down polar products easily, whereas oral keratinocytes left these products almost untouched. Failure of detecting polar metabolites in the conditioned medium of OKCs after the addition of RA thus cannot be explained by the fast turnover of these polar metabolites and suggest that turnover in oral keratinocytes takes place via another metabolic pathway.

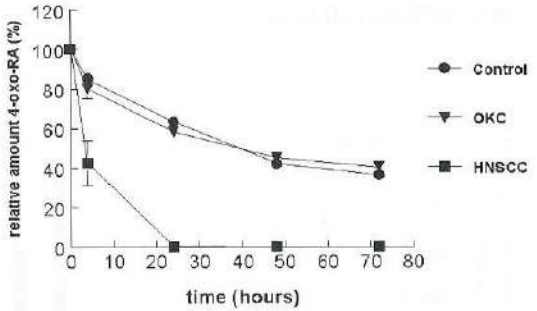


Figure 6. Kinetics of 4-oxo-RA metabolism. Cells were grown in the presence of 1 μM 4-oxo-RA. *Control*, medium without cells; *OKC*, oral keratinocyte culture; *HNSCC*, head and neck SCC line. The HNSCC cell line catabolized 4-oxo-RA much more efficiently than OKC.

Expression of CYP26A1 mRNA

Recently, an RA inducible cytochrome P450 enzyme, CYP26A1, has been identified that is highly specific for RA. To investigate whether the turnover rate of RA is related to CYP26A1 levels, we examined the expression of CYP26A1 mRNA by Northern blotting (Figure 7). UM-SCC-35 and VU-SCC-OE, both of which have a relative high metabolism, showed h a very high level of CYP26A1 mRNA after induction with 1 μM RA for 24, whereas it is barely detectable in other cell lines and not inducible by RA. In the normal keratinocytes, CYP26A1 was not detectable and not inducible by RA. These results suggest that in the high metabolizing cell lines,

VU-SCC-OE and UM-SCC-35, CYP26A1 is the major enzyme responsible for the conversion of RA to polar metabolites.

Transfection of CYP26A1 DNA

The pcDNA3/P450RA1 vector and, as a control, the pcDNA3 vector were transfected into UM-SCC-14C cells. Four transiently (G418-resistant) transfected populations of pCYP26 and four of pcDNA3s were used for additional analysis. Next, metabolism studies were performed to determine whether turnover rate of RA was changed and whether polar metabolites were formed. In Figure8A the relative amounts of the polar metabolites as compared to the total amount of retinoids added are depicted for the indicated incubation periods. Transfection with pCYP26 lead to an increased concentration of polar metabolites, and the type of polar metabolites were similar to those found in other HNSCC cell lines (Figure 2). Because the polar metabolites were similar in HNSCC cell lines as found in the transfected cells, it is most likely that CYP26A1 is also active in these cell lines.

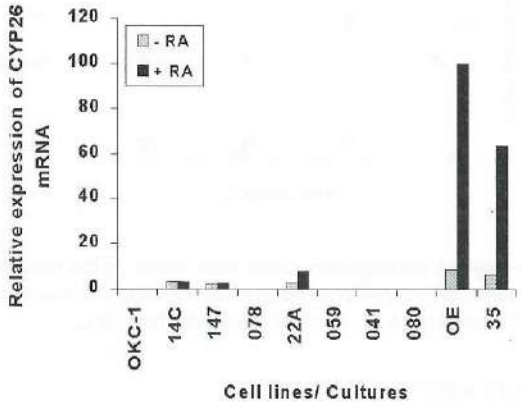


Figure 7. Expression of *CYP26A1* mRNA determined by Northern blotting. Analysis was done for nine HNSCC cell lines and one OKC (OKC-1). Total RNA (20 µg) was isolated from the cells, untreated (-) and treated (+) with 1 µM RA for 24 h. The blots were quantitated by phosphoimaging. After correction for the amount of RNA loaded on the gel (28S rRNA), CYP26A1 levels were expressed as relative amounts, with VU-SCC-OE (+) set as 100%. This experiment was performed twice with similar results; one experiment is shown. The cell lines were arranged according to their turnover capacity. After RA induction a dramatic increase of *CYP26A1* mRNA expression was seen in two cell lines that have the highest metabolism; no expression could be observed in the OKC.

The turnover of RA and the concentration of polar metabolites in pCYP26-transfected cells was, on average, 11- and 12-fold higher as compared with untransfected cells (Figure 8B). No change of sensitivity towards RA was found in the different transfected populations as compared to untransfected cells (data not shown).

Discussion

In this study we have demonstrated a number of differences in the metabolism of RA between normal oral keratinocytes and HNSCC cells. A) these cell types differed in turnover rate of RA and sensitivity to RA; b) the types of retinoid metabolites differed; and c) a correlation exists between growth inhibition and turnover rate within the group of HNSCC cell lines.

Difference in turnover rate of RA and sensitivity to RA between HNSCC cell lines and OKCs

Our results demonstrate that HNSCC cells are, on average, more efficient in removing and metabolizing RA from the medium and are, on average, less sensitive to growth inhibition by RA than normal oral keratinocytes. This increased ability of HNSCC cells relative to normal oral keratinocytes to catabolize RA can be explained by a higher activity of CYP enzymes, like CYP26A1. Relatively high mRNA levels of CYP26A1 were found after induction with RA in the cell lines with the highest metabolic rate, UM-SCC-35 and VU-SCC-OE. Although the CYP26A1 mRNA levels were much lower in the other HNSCC cell lines, metabolites were found in the medium that were identified as the typical oxidation products of RA, like 4-oxo-RA, 4-oxo-cRA, 4-OH-RA and 18-OH-RA. These so-called polar metabolites are the result of the activity of CYP26A1 or other CYP enzymes (Gubler and Sherman 1990, Nadin and Murray 1996, Blaner and Olson 1994). This suggests that either low CYP26A1 levels are sufficient for a high processing of RA, and that CYP26A1 possibly is overexpressed in UM-SCC-35 and VU-SCC-OE, or that other CYPs with CYP26-like activity are responsible for RA turnover. The idea that the enhanced RA turnover is caused by *CYP26A1* was supported by transfection of this gene in a low catabolizing HNSCC cell line, UM-SCC-14C. Introducing the *CYP26A1* DNA caused an increase of RA turnover of about 11-fold and a 12-fold higher production of the same polar metabolites as mentioned above were detected. In the OKCs, neither constitutive nor RA-inducible *CYP26A1* mRNA expression was observed and no polar products could be detected. The lack of CYP26A1 expression in the keratinocyte cultures could explain the lower turnover of RA. As a consequence these cells are

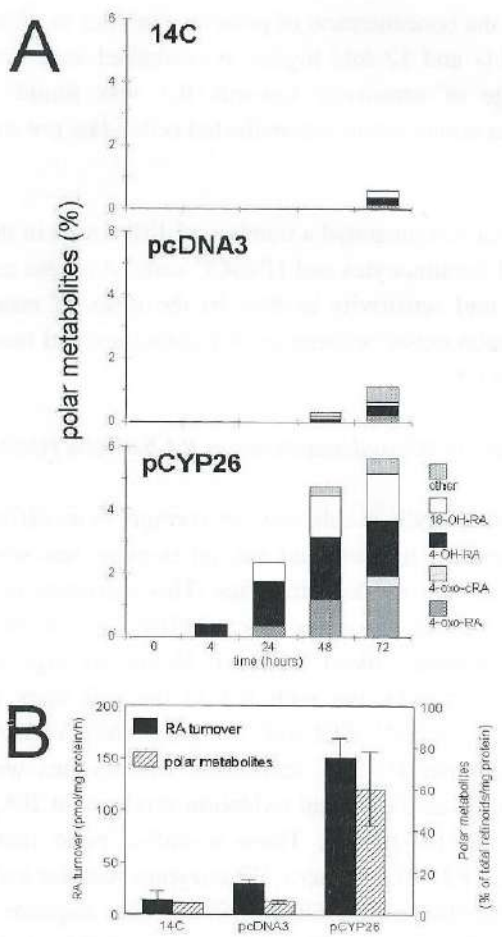


Figure 8. Transfection of CYP26A1 cDNA in an HNSCC cell line. UM-SCC-14C was transfected with pCYP26 expression vector or with pcDNA3 expression vector as a control. *A*, Relative amounts of the different polar metabolites formed after various incubation periods expressed as a percentage of total retinoids at the start of the experiment. Four separate experiments were done with similar results, one experiment is shown. *B*, Turnover rate of RA expressed in pmol/mg protein/h and the maximal amount of polar metabolites formed, expressed as a percentage of total retinoids at the start of the experiment per mg of protein. Results of four separate experiments are shown (mean ± SD). As compared with untransfected cells and cells transfected with control vector, RA turnover and the formation of polar metabolites was highly increased in the CYP26A1-transfected population.

longer exposed to RA, which is in agreement with the higher sensitivity to RA relative to HNSCC cell lines.

The increased catabolic activity in HNSCC cell lines may reflect a phenotypic change that is involved in the progression of HNSCC towards malignancy. A change in normal and neoplastic epithelial cells was also found in the metabolism of retinol (Guo and Gudas 1997). Normal epithelial cells are able to esterify retinol to a much greater extent than are SCC cell lines. These findings support the idea that tumor cells have selected for mechanisms that reduce intracellular retinoid concentrations. Selection may have taken place towards a phenotype that circumvents the antiproliferative and differentiation-inducing effects of RA.

Difference in metabolites formed between HNSCC cell lines and OKCs

The lack of formation of polar metabolites was consistent for all normal keratinocyte cultures we have tested. The formation of polar metabolites was not medium-dependent, because HNSCC cell lines were able to produce these polar metabolites in KGM medium with 0.1% BSA, and growing the keratinocytes in 2% FCS did not result in the formation of polar metabolites either. Others, investigating epidermal keratinocytes in culture, were also not able to observe these polar metabolites (Randolph and Simon 1997, Hodam and Creek 1996). This suggests that in keratinocytes, the polar metabolites are degraded very efficiently or not formed at all. When giving 4-oxo-RA as a substrate, it seemed that normal keratinocytes were not able to catabolize this compound, in contrast with HNSCC cell lines, which implied that in keratinocytes, RA is not converted to 4-oxo-RA at all. Furthermore, *CYP26A1*, which is responsible for the 4-hydroxylation of RA, was not expressed and not inducible in normal oral keratinocytes. The same observation was done recently in normal epidermal keratinocytes (Popa *et al.* 1999). Keratinocytes are capable to catabolize RA, which is also supported by the observation that they significantly synthesize very polar metabolites (retention times between 0 and 5 min), but apparently they use enzymes other than CYP26A1 for RA catabolism.

Turnover rate and response are correlated in HNSCC cell lines

Previously we have suggested a relationship between the level of growth inhibition and the rate at which RA disappears from the medium and the cells in three HNSCC cell lines (Braakhuis *et al.* 1997). In the present study, with a larger panel of HNSCC cell lines, we could confirm this relationship. The correlation between growth inhibition and RA turnover among tumor cell lines is unexpected, because we also found in this study that the average rate of turnover of RA by the HNSCC cell lines is

greater than that of the oral keratinocyte cultures, whereas the HNSCC cell lines are on average less susceptible to inhibition of growth by RA. It can, however, not be excluded that chance explains this relation between the levels of turnover and sensitivity in HNSCC lines. The correlation can be attributed completely to the results obtained with the UM-SCC-35 cell line. Without this cell line, the correlation is not significant ($R = 0.39$, $P = 0.26$). There are, however, no biological reasons to delete the results of this cell line, and moreover, others have also found a similar relation between growth inhibition and RA turnover in breast cancer cells and squamous cell carcinoma cell lines under serum-containing (Van der Leede *et al.* 1997) and serum-free conditions (Takatsuka *et al.* 1996).

Two hypotheses can be formulated to explain the relation between growth inhibition and metabolism in HNSCC cell lines. First, it might be that, in tumor cell lines ,specific catabolites of RA are responsible for the growth-inhibiting effects of RA. Evidence is, however, arguing against this hypothesis (Braakhuis *et al.* 1997). The second and most likely explanation is that retinoid metabolism is a secondary event and an attempt to neutralize the growth-inhibiting effects. This hypothesis is supported further by the notion in the present study that overexpression of *CYP26A1* in a low-metabolizing HNSCC cell line resulted in an increase of RA turnover from 13 to 150 pmol/mg protein/h but not in an increase of sensitivity towards RA.

We hypothesize that metabolism as well as growth inhibition are regulated by the RARs. After binding of ligands, like RA or 9-cis-RA, to these receptors, RAR-RXR heterodimers or RXR-RXR homodimers are formed which bind to specific RA-responsive elements on target genes. Depending on the concentration of certain receptors, different genes are activated or inhibited in their expression (Heyman *et al.* 1992, Zhang *et al.* 1992, Mangelsdorf *et al.* 1992). The involvement of RARs in the induction of CYP26A1 was demonstrated with receptor-specific synthetic retinoids (Marikar *et al.* 1998). Furthermore, transfection of either RAR-α , RAR-β, or RAR-γ were able to restore CYP26A1 expression upon RA treatment in HCT-116 cells (Sonneveld *et al.* 1998). These results and its high RA-inducibility (Ray *et al.* 1997, White *et al.* 1997, Sonneveld *et al.* 1998) suggested the presence of a RA-responsive element in the promotor region of *CYP26A1* and this has recently been identified (Loudig *et al.* 2000). Sensitivity to RA was also found to be related to mRNA expression levels of RARs by analyzing transcription levels (Kaiser *et al.* 1998) or performing transfection studies (Oridate *et al.* 1996, Sun *et al.* 1999). The assumption that RA-sensitivity as well as the rate to turnover RA are related to the presence of RARs may also explain why RA-sensitivity was not increased by overexpression of CYP26A1 alone. Thus, RA turnover and inhibition of growth by RA are independent

from each other and are possibly both dependent on RAR expression levels. Currently, we are testing the hypothesis whether RA-resistance and reduced RA turnover are related to reduced transcription levels of RARs in HNSCC cell lines. The expression of RAR-γ appears to be most important in this respect (Klaassen *et al.* 2001).

Altogether, the findings we report here may contribute to a better understanding of the fundamental changes of retinoid metabolism during malignant progression. It seems likely that tumor cells have selected against the growth-inhibiting and differentiation-regulating effects of retinoids by changing metabolism, and it might be interesting to direct future research on the underlying mechanisms that cause this switch between RA metabolism pathways.

Acknowledgments

We thank Drs L Foley and UL Wiegand for providing retinoids and Dr M Petkovich for the pcDNA3/P450RAI expression vector. This study was supported by the Dutch Cancer Society, grant 95-926.

References

Blaner WS, Olson JA (1994) Retinol and retinoic acid metabolism. In *The Retinoids*, Sporn MB, Roberts AB, Goodman DS (eds) pp 229-55. Raven Press: New York

Braakhuis BJ, Jansen G, Noordhuis P, Kegel A, Peters GJ (1993) Importance of pharmacodynamics in the in vitro antiproliferative activity of the antifolates methotrexate and 10-ethyl-10-deazaaminopterin against human head and neck squamous cell carcinoma. *Biochem Pharmacol* 46: 2155-61

Braakhuis BJ, Klaassen I, van der Leede BM, Cloos J, Brakenhoff RH, Copper MP, Teerlink T, Hendriks HF, van der Saag PT, Snow GB (1997) Retinoid metabolism and all-*trans* retinoic acid-induced growth inhibition in head and neck squamous cell carcinoma cell lines. *Br J Cancer* 76: 189-97

Carey TE, Wolf GT, Baker SR, Krause CJ (1990) Cell surface antigen expression and prognosis. 77-82

De Luca LM (1991) Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. *FASEB J* 5: 2924-33

Eckhoff C, Nau H (1990) Identification and quantitation of all-trans- and 13-cis- retinoic acid and 13-cis-4-oxoretinoic acid in human plasma. *J Lipid Res* 31: 1445-54

Furr HC, Barua AB, Olson JA (1994) Analytical methods. In *The Retinoids*, Sporn MB, Roberts AB, Goodman DS (eds) pp 179-209. Raven Press: New York

Graham S (1984) Epidemiology of retinoids and cancer. *J Natl Cancer Inst* 73: 1423-8

Gubler ML, Sherman MI (1990) Metabolism of retinoic acid and retinol by intact cells and cell extracts. *Methods Enzymol* 189: 525-30

Guo X, Gudas LJ (1998) Metabolism of all-*trans*-retinol in normal human cell strains and squamous cell carcinoma (SCC) lines from the oral cavity and skin: reduced esterification of retinol in SCC lines. *Cancer Res* 58: 166-76

Hermsen MA, Joenje H, Arwert F, Welters MJ, Braakhuis BJ, Bagnay M, Westerveld A, Slater R (1996) Centromeric breakage as a major cause of cytogenetic abnormalities in oral squamous cell carcinoma. *Genes Chromosomes Cancer* 15: 1-9

Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, Thaller C (1992) 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* 68: 397-406

Hodam JR, Creek KE (1996) Uptake and metabolism of [^{3}H]retinoic acid delivered to human foreskin keratinocytes either bound to serum albumin or added directly to the culture medium. *Biochim Biophys Acta* 1311: 102-10

Hong WK, Itri LM (1994) Retinoids and human cancer. In *The Retinoids*, Sporn MB, Roberts AB, Goodman DS (eds) pp 597-630. Raven Press: New York

Hong WK, Lippman SM, Hittelman WN, Lotan R (1995) Retinoid chemoprevention of aerodigestive cancer: from basic research to the clinic. *Clin Cancer Res* 1: 677-86

Hong WK, Lippman SM, Itri LM, Karp DD, Lee JS, Byers RM, Schantz SP, Kramer AM, Lotan R, Peters LJ, Dimery IW, Brown BW, Goepfert H (1990) Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N Engl J Med* 323: 795-801

Kaiser A, Wolf-Breitinger M, Albers A, Dorbic T, Wittig B, Riecken EO, Rosewicz S (1998) Retinoic acid receptor gamma1 expression determines retinoid sensitivity in pancreatic carcinoma cells. *Gastroenterology* 115: 967-77

Klaassen I, Brakenhoff RH, Smeets SJ, Snow GB, Braakhuis BJ (1999) Considerations for in vitro retinoid experiments: importance of protein interaction. *Biochim Biophys Acta* 1427: 265-75

Klaassen I, Brakenhoff RH, Smeets SJ, Snow GB, Braakhuis BJ (2001) Expression of retinoic acid receptor gamma correlates with retinoic acid sensitivity and metabolism in head and neck squamous cell carcinoma cell lines. *Int J Cancer* 92: 661-5

Lippman SM, Kessler JF, Al Sarraf M, Alberts DS, Itri LM, Mattox D, Von Hoff DD, Loescher L, Meyskens FL (1988) Treatment of advanced squamous cell carcinoma of the head and neck with isotretinoin: a phase II randomized trial. *Invest New Drugs* 6: 51-6

Lotan R (1980) Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells. *Biochim Biophys Acta* 605: 33-91

Lotan R (1996) Retinoids in cancer chemoprevention. *FASEB J* 10: 1031-9

Loudig O, Babichuk C, White J, Abu-Abed S, Mueller C, Petkovich M (2000) Cytochrome P450RAI(CYP26) promoter: a distinct composite retinoic acid response element underlies the complex regulation of retinoic acid metabolism. *Mol Endocrinol* 14: 1483-97

Mangelsdorf DJ, Borgmeyer U, Heyman RA, Zhou JY, Ong ES, Oro AE, Kakizuka A, Evans RM (1992) Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. *Genes Dev* 6: 329-44

Marikar Y, Wang Z, Duell EA, Petkovich M, Voorhees JJ, Fisher GJ (1998) Retinoic acid receptors regulate expression of retinoic acid 4-hydroxylase that specifically inactivates all-*trans* retinoic acid in human keratinocyte HaCaT cells. *J Invest Dermatol* 111: 434-9

Moon RC, Mehta RG, Rao KVN (1994) Retinoids and cancer in experimental animals. 573-94

Muindi JF, Young CW (1993) Lipid hydroperoxides greatly increase the rate of oxidative catabolism of all-trans-retinoic acid by human cell culture microsomes genetically enriched in specified cytochrome P-450 isoforms. *Cancer Res* 53: 1226-9

Nadin L, Murray M (1996) All-*trans*-retinoic acid 4-hydroxylation in human liver microsomes: *in vitro* modulation by therapeutic retinoids. *Br. J. Clin. Pharmacol.* 41: 609-12

Oridate N, Esumi N, Lotan D, Hing WK, Rochette-Egly C, Chambon P, Lotan R (1996) Implication of retinoic acid receptor gamma in squamous differentiation and response to retinoic acid in head and neck SqCC/Y1 squamous carcinoma cells. *Oncogene* 12: 2019-28

Popa C, Dicker AJ, Dahler AL, Saunders NA (1999) Cytochroom P450, CYP26AI, is expressed at low levels in human epidermal keratinocytes and is not retinoic acid-inducible. *Br J Dermatol* 141: 460-8

Randolph RK, Simon M (1997) Metabolism of all-*trans*-retinoic acid by cultured human epidermal keratinocytes. *J Lipid Res* 38: 1374-83

Ray WJ, Bain G, Yao M, Gottlieb DI (1997) CYP26, a novel mammalian cytochrome P450, is induced by retinoic acid and defines a new family. *J Biol Chem* 272: 18702-8

Reid CB, Cloos J, Snow GB, Braakhuis BJ (1997) A simple and reliable technique for culturing of human oral keratinocytes and fibroblasts. *Acta Otolaryngol* 117: 628-33

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning, A Laboratory Manual, 2nd.* Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY

Snow GB (1992) Follow-up in patients treated for head and neck cancer: how frequent, how thorough and for how long? *Eur J Cancer* 28: 315-6

Sonneveld E, van den Brink CE, van der Leede BM, Schulkes RK, Petkovich M, van der Burg B, van der Saag PT (1998) Human retinoic acid (RA) 4-hydroxylase (CYP26) is highly specific for all-*trans*-RA and can be induced through RA receptors in human breast and colon carcinoma cells. *Cell Growth Differ* 9: 629-37

Sun SY, Kurie JM, Yue P, Dawson MI, Shroot B, Chandraratna RA, Hong WK, Lotan R (1999) Differential responses of normal, premalignant, and malignant human bronchial epithelial cells to receptor-selective retinoids. *Clin. Cancer Res.* 5: 431-7

Takatsuka J, Takahashi N, de Luca LM (1996) Retinoic acid metabolism and inhibition of cell proliferation: an unexpected liaison. *Cancer Res* 56: 675-8

Teerlink T, Copper MP, Klaassen I, Braakhuis BJ (1997) Simultaneous analysis of retinol, all-trans- and 13-cis- retinoic acid and 13-cis-4-oxoretinoic acid in plasma by liquid chromatography using on-column concentration after single-phase fluid extraction. *J. Chromatogr. B Biomed. Sci. Appl.* 694: 83-92

Van der Leede BM, van den Brink CE, Pijnappel WW, Sonneveld E, van der Saag PT, van der Burg B (1997) Autoinduction of retinoic acid metabolism to polar derivatives with decreased biological activity in retinoic acid-sensitive, but not in retinoic acid-resistant human breast cancer cells. *J. Biol. Chem.* 272: 17921-8

Welters MJ, Fichtinger Schepman AM, Baan RA, Hermsen MA, van der Vijgh WJ, Cloos J, Braakhuis BJ (1997) Relationship between the parameters cellular differentiation, doubling time and platinum accumulation and cisplatin sensitivity in a panel of head and neck cancer cell lines. *Int J Cancer* 71: 410-5

White JA, Beckett-Jones B, Guo YD, Dilworth FJ, Bonasoro J, Jones G, Petkovich M (1997) cDNA cloning of human retinoic acid-metabolizing enzyme (hP450RAI) identifies a novel family of cytochromes P450. *J Biol Chem* 272: 18538-41

Zhang XK, Lehmann J, Hoffmann B, Dawson MI, Cameron J, Graupner G, Hermann T, Tran P, Pfahl M (1992) Homodimer formation of retinoid X receptor induced by 9-cis retinoic acid. *Nature* 358: 587-91

Chapter 6

Expression of retinoic acid receptor gamma correlates with retinoic acid sensitivity and metabolism in head and neck squamous cell carcinoma cell lines

Ingeborg Klaassen, Ruud H Brakenhoff, Serge J Smeets, Gordon B Snow, Boudewijn JM Braakhuis

Section Tumor Biology, Department of Otolaryngology, Vrije Universiteit Medical Center, Amsterdam, the Netherlands

Int J Cancer 2001; 92: 661-5

Abstract

Retinoids, analogues of vitamin A, can reverse premalignant lesions and prevent second primary tumors in patients with head and neck squamous cell carcinoma (HNSCC). The effects of retinoids are mediated by retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which act as ligand-activated transcription factors. The regulation of cell growth, differentiation and retinoid metabolism in normal, premalignant and malignant cells by retinoids is thought to be a result of their effects on gene expression. We investigated mRNA expression of RARs (α, β, and γ) and RXR-β by means of RNase protection and related this to retinoic acid (RA)-induced growth inhibition and RA turnover in four HNSCC cell lines (UM-SCC-14C, UM-SCC-22A, UM-SCC-35 and VU-SCC-OE). An RA-resistant subline of UM-SCC-35 was generated by exposure to increasing concentrations of RA for eight months (designated UM-SCC-35R). RA turnover was determined on the basis of decreasing RA levels in the cells and culture medium after exposure to 1 μM RA. We found that RAR-γ mRNA expression was strongly correlated with RA-induced growth inhibition ($P = 0.016$, $R = 0.92$) and RA turnover ($P = 0.041$, $R = 0.86$). RAR-β transcript levels were reduced in three of five cell lines as compared to normal mucosa, and these did not correlate with RA-induced growth inhibition and RA turnover. Expression of RAR-α and RXR-β was not substantially altered in any of the cell lines. These findings suggest that in HNSCC cell lines RAR-γ is the most important retinoid receptor for regulation of RA turnover rate and RA-induced growth inhibition.

Introduction

The vitamin A-derived retinoids play an important role in growth and differentiation of a variety of cell types (Gudas *et al.* 1994). Retinoids also inhibit the growth of cancer cells (Lotan 1980) and are useful in the treatment and prevention of human cancer (Bollag and Holdener 1992). In head and neck squamous cell carcinoma (HNSCC) retinoids were successful in the prevention of second primary tumors (Hong *et al.* 1990) and in the treatment of premalignant lesions such as oral leukoplakia (Lippman *et al.* 1988).

Chemoprevention with retinoids is however not optimal: some patients experience toxic side effects and some tumors are resistant (Hong and Itri 1994). To explain these effects, the mechanisms of retinoid action need to be elucidated. In leukemia, acquired resistance to retinoic acid (RA) was found to be a consequence of enhanced catabolism of RA (Muindi and Young 1993; Kizaki *et al.* 1997). The opposite was suggested for HNSCC, in which a statistical significant inverse relationship was found between RA resistance and RA catabolism (Braakhuis *et al.* 1997; Klaassen *et al.* 2001). This

paradoxical finding was also found for other solid tumor types and suggests that the molecular mechanisms underlying RA-resistance differ between solid tumors and leukemia.

Most actions of retinoids are mediated by the nuclear retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). RAR-RXR heterodimers and RXR-RXR homodimers interact with specific retinoic acid response elements, RAREs and RXREs respectively, in the promotors of a variety of target genes (De Luca 1991, Mangelsdorf 1994). Upon binding of certain retinoids to the receptor dimers, specific gene expression can be regulated. Three different types of RARs and RXRs were identified (α, β, and γ) (Mangelsdorf *et al.* 1994, Heyman *et al.* 1992). In HNSCC, RAR-β mRNA was found to be reduced in 65% of tumors compared with 30% in adjacent tissue and in 60% of oral dysplasias (Xu *et al.* 1994, Lotan *et al.* 1995). The relation between malignancy and RAR-β loss suggests a role for RAR-β as a tumor suppressor. RAR-γ, which is the major RAR type judged from the mRNA expression level in HNSCC cell lines, was also found to be frequently reduced compared to normal squamous cells (Hu *et al.* 1991).

Other *in vitro* studies have addressed the role of RARs in RA-mediated growth inhibition. Investigations in esophageal and lung carcinoma cells suggested that growth inhibition by RA is associated with selective loss of RAR-β (Xu *et al.* 1999, Sun *et al.* 1999). In HNSCC cell lines, it was demonstrated that not RAR-β (Wan *et al.* 1999) but RAR-γ (Oridate *et al.* 1996, Le *et al.* 2000) was related with RA-mediated growth inhibition; all these studies were performed by introducing sense or antisense RAR types or RAR-selective retinoids.

We aimed to investigate constitutive and RA-induced mRNA expression levels of RARs in a panel of HNSCC cell lines. RAR-α, -β and -γ and RXR-β transcription levels were measured and related to RA-sensitivity and RA turnover rate in five HNSCC cell lines that differed widely in their sensitivity to RA and their RA turnover rate. We included an RA-resistant cell line that was obtained by exposing RA-sensitive UM-SCC-35 cells to RA for eight months.

Material and methods

Chemicals.

All-*trans*-RA, obtained from Acros Chimica (Geel, Belgium), was dissolved in dimethyl sulfoxide (DMSO) and stored as 10^{-3} M stock at -80°C under nitrogen. For each experiment, working dilutions were freshly prepared in the appropriate cell culture medium. Final DMSO concentration was always lower than 0.1% and did not affect cell growth. All handling with retinoids was performed in subdued light and in the presence of 0.1% BSA to prevent absorption to plastics (Klaassen *et al.* 1999).

Cells and culture conditions.

Normal mucosal tissue and primary oral keratinocytes were obtained from the uvulas of non-cancer patients who underwent uvulopalatopharyngoplasty. The mucosal layer was stripped from the tissue as described previously (Reid *et al.* 1997). Human HNSCC cell lines UM-SCC-35, -22A and -14C were provided by Dr TE Carey (University of Michigan, Ann Harbor, MI) and are described elsewhere (Carey, 1990). An RA-resistant subline, UM-SCC-35R, was established from the RA-sensitive UM-SCC-35 cell line by exposing it to increasing concentrations of RA (10^{-8} to 10^{-6} M) during a period of eight months. VU-SCC-OE was established from human HNSCC xenografts in our laboratory (Welters *et al.*, 1997). HNSCC cells were maintained in Dulbecco's modified essential medium (DMEM) (Life Technologies) supplemented with 5% FCS (ICN Biomedicals, Irvine, UK) and 50 units/ml penicillin, 50 µg/ml streptomycin (Life Technologies).

Growth inhibition assays.

The dose-response effect on cell proliferation was determined with the sulphorhodamine B (SRB) assay. Details of the assay have been described previously (Braakhuis *et al.* 1993). In short, cells (1,000 to 4,000 per well) were plated in 96-well plates in DMEM/5% FCS and were allowed to grow for 72 h (the "lag phase"). After this phase the medium was replaced by medium containing RA with a final concentration ranging from 10^{-9} to 10^{-6} M. Growth was assayed after 72 h incubation (the "log phase"), by staining the cellular protein with SRB (Sigma, St. Louis, MO) and spectrophotometric measurement of the absorption at 540 nm with a microplate reader (Labsystems Multiskan, Helsinki, Finland). Only experiments in which the untreated cells had doubled in cell number during the log phase were included.

Metabolism of all-trans-retinoic acid.

HNSCC cells were plated at a density of 10^5 cells per well in 6-well plates. Upon 70 to 80% confluence, the medium was removed and replaced by medium containing 1 µM RA. As a control, RA-containing medium without cells was included during the incubation period. From the medium two samples of 350 µl were taken, and after removal of the residual medium the cells were washed once with phosphate-buffered saline and collected in 350 µl trypsin/EDTA. The samples were stored at - 80°C under nitrogen until retinoid extraction.

Retinoid extraction and HPLC analysis.

Retinoids were analyzed by reverse-phase HPLC after extraction with acetonitrile, with recovery of all retinoids >95% and reproducibility >92%, as described previously (Teerlink et al., 1997). In short, to each sample 50 µl of 1 M sodium acetate buffer (pH 4.0) and 600 µl acetonitrile was added and after vortex-mixing, the samples were centrifuged for 2 to 5 min at 3000 *g*. Protein content of cell extracts was determined on the pellet with the Bio-Rad protein assay (Life Science Research, Bio-Rad Laboratories B.V., the Netherlands).

In total 720 µl of the clear supernatant was transferred to a 2 ml glass autosampler vial; after addition of 240 µl water, the vials were capped, mixed by inversion and put in the sample compartment of the autoinjector, which was cooled at 4°C, or stored at -80°C under nitrogen. A Gynkotek (Munich, Germany) HPLC system was used, consisting of a Basic Marathon automatic sample injector, a model M480G gradient pump, a Model UVD 170S UV detector, and a column heater (Mistral, Spark Holland, Emmen, the Netherlands). The mobile phase was degassed online using a model GT103 degasser

(Gastorr, Bad Honnef, Germany). Chromelion software (Gynkotek) was used for instrument control and data acquisition. Separation was performed on a 3 µm Spherisorb ODS2 column (100 x 4.6 mm) from Phase Separations (Deeside, UK) maintained at 30°C. Composition of the mobile phases and the binary gradient used were as described by Eckhoff and Nau (1990). UV detection was performed at 340 nm.

Retinoids were identified using external standards. RA concentrations were quantified based on peak height. Concentrations of RA in the standards was determined spectrophotometrically by measurement at λ_{max} (350 nm) and use of the molar extinction coefficient (45,300) (Furr *et al.* 1994). Experiments were excluded when medium controls (without cells) showed a decrease in RA of more than 60% in 48 h. The amount of RA was calculated relative to the amount of the medium control at the start of the experiment. The turnover rate was taken as the difference between 4 and 24 h in medium plus pellets, corrected for the decrease in the medium controls during this period. Next, this relative amount was converted to absolute amounts in pmol by use of the external standards. The turnover rate was expressed in pmol per mg protein per hour.

RNase Protection analysis.

RNase protection assays utilizing human probes for RAR-α, RAR-β, RAR-γ and RXR-β (kindly provided by Dr BM Van der Leede, Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Utrecht) and GAPDH (kindly provided by Dr PAE Scott, Molecular Angiogenesis Laboratory, John Radcliffe Hospital, Oxford, UK; Scott *et al.* 1997) were conducted on RNA isolated from cells incubated for 24 h with or without 1 µM RA. As a control, RNA isolated from normal mucosal layer was used, which originated from uvulas of non-cancer patients.

Total cytoplasmic RNA was isolated and RNA protection assays were performed according to the protocol of the manufacturer (Ambion, Austin, TX). In brief, [α-^{32}P]-labeled riboprobes (for RAR/RXR-probes 2.5 x 10^5 cpm/sample and for GAPDH 2.0 x 10^4 cpm/sample) were hybridized to total RNA (3 µg, except for RAR-β: 15 µg) at 45°C overnight. Unbound probes were digested by the addition of 200 µl RNase digestion buffer containing 2.5 U/ml of RNase A and 100 U/ml of RNase T1. RNase digestion was performed at 37°C for 30 min. RNase-protected fragments were resolved by electrophoresis on 6% denaturating-polyacrylamide gels. To check for RNA loading, a GAPDH probe was included in all samples. Yeast RNA was used as a control for complete unbound probe digestion. As a positive control for RAR-β mRNA expression embryonic fibroblasts (cell line 293) were used in which RAR-β was clearly detectable with 3 µg total RNA. Gels were exposed to film over night at -80°C with intensifying screens and/or phosphor screens at room temperature. Hybridizing bands were quantified by a phosphor imager (Molecular Dynamics, Krefeld, Germany) or by a digital imaging system (IS1000 system, Biozym, Landgraaf, the Netherlands).

Results

Growth inhibition of HNSCC cell lines by RA is related to RA turnover rate.

To test the effect of RA on the growth of head and neck cancer cells, the HNSCC cells in monolayer culture were treated with RA (10^{-8} to 10^{-6} M) for three days (Figure.1). UM-SCC-35 was the most sensitive to the RA growth inhibitory effect, with 91% inhibition when it was treated with 10^{-6} M RA, whereas UM-SCC-22A and VU-SCC-OE

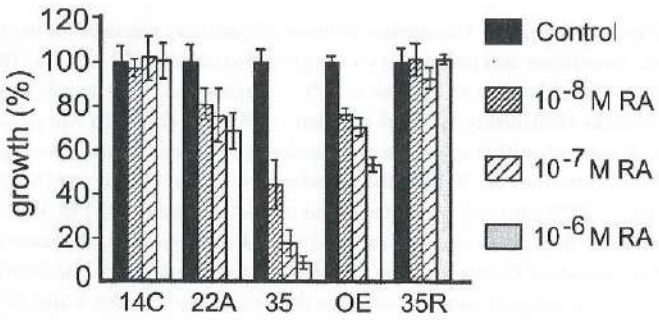


Figure 1. Growth inhibition of HNSCC cell lines. Relative growth of cells exposed to increasing concentrations of RA as compared to untreated cells. Experiments were performed at least three times. Mean ± SD are given.

displayed a moderate response, with 33% and 46% inhibition respectively, at this concentration. The other two cell lines, UM-SCC-14C and UM-SCC-35R, were completely resistant to RA. This latter line was obtained by exposing UM-SCC-35 cells to increasing concentrations of RA for eight months.

Subsequently, metabolism experiments were performed to test the turnover rate of RA in these cell lines. Cells were exposed to 1 μM RA, and RA concentrations were measured after 4, 24, 48 and 72 h. The capacity to metabolize RA differed widely among the cell lines and was highest in the most sensitive ones, UM-SCC-35 and VU-SCC-OE. By introducing the resistant subline UM-SCC-35R, an alternative approach was chosen

Table 1. Sensitivity and turnover after exposure to 1 μm RA in HNSCC cell lines.[1]

Cell line/Culture	Growth at 1 μM RA [2]		RA turnover [3]	
	mean	SD	mean	SD
UM-SCC-14C	96.5	5.2	13.3	9.2
UM-SCC-22A	72.9	3.7	104.8	67.9
UM-SCC-35	16.4	10.5	1016.0	413.2
UM-SCC-35R	101.1	5.0	52.7	25.4
VU-SCC-OE	56.9	3.3	558.9	152.5

[1] Some of the data are taken from Klaassen *et al.* 2001.

[2] Growth at 1 μM RA as compared to untreated cells (%).

[3] RA turnover (pmol/mg protein/h).

to test the relation between RA-induced growth inhibition and RA turnover rate. This RA-resistant subline was found to have a 19-fold reduced metabolism compared with that of UM-SCC-35 (Figure 1, Table 1). RA turnover was found to be significantly correlated with growth at 1 μM RA among all cell lines ($P = 0.003$; $R = 0.96$, Pearson correlation test) and was still significantly correlated when UM-SCC-35R was excluded ($P = 0.010$; $R = 0.97$).

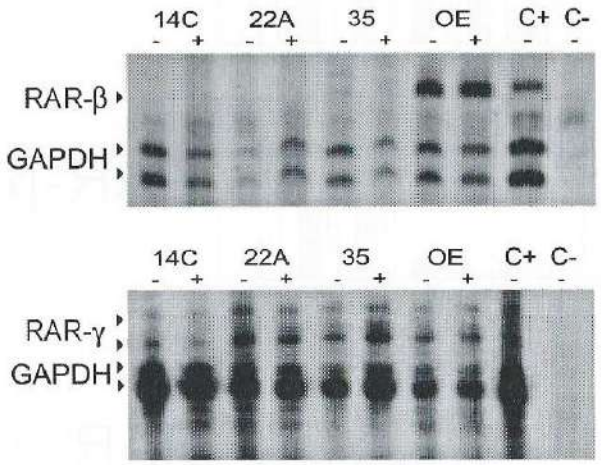


Figure.2. Representative examples of RNase protection. Expression of RAR-β and -γ was measured by RNase protection as indicated in Materials and Methods. The specific protected fragments are indicated in the figure. The experiments were performed on 3 μg (RAR-γ) or 15 μg (RAR-β) total RNA of cells, untreated (-) and treated (+) with 1 μM RA for 24 h. C+, positive control (RAR-γ: cultured oral keratinocytes; RAR-β: embryonal fibroblasts); C-, negative control (yeast). The GAPDH probe in the RNase protection of RAR-β was synthesized using [α-^{32}P]CTP 1:50 diluted in cold CTP. The blots were quantified by phosphoimaging and corrected for the amount of RNA loaded on the gel by GAPDH mRNA levels. In this figure single experiments are shown. The mean of all experiments after correction for GAPDH is presented in Figure 3.

Growth inhibition by RA and RA turnover rate is related to RAR-γ expression in HNSCC cell lines

HNSCC cell lines were analyzed on retinoid receptor mRNA expression by means of RNase protection assays. Cell lines were treated with and without 10^{-6} M RA for 24 h and evaluated for RA receptor expression as compared to normal mucosal tissue. We have chosen normal mucosal tissue as a reference for normal cells instead of cultured keratinocytes. The use of mucosal tissue reflects in our opinion a more physiological situation than proliferating keratinocytes, since keratinocytes hardly proliferate under normal physiological conditions.

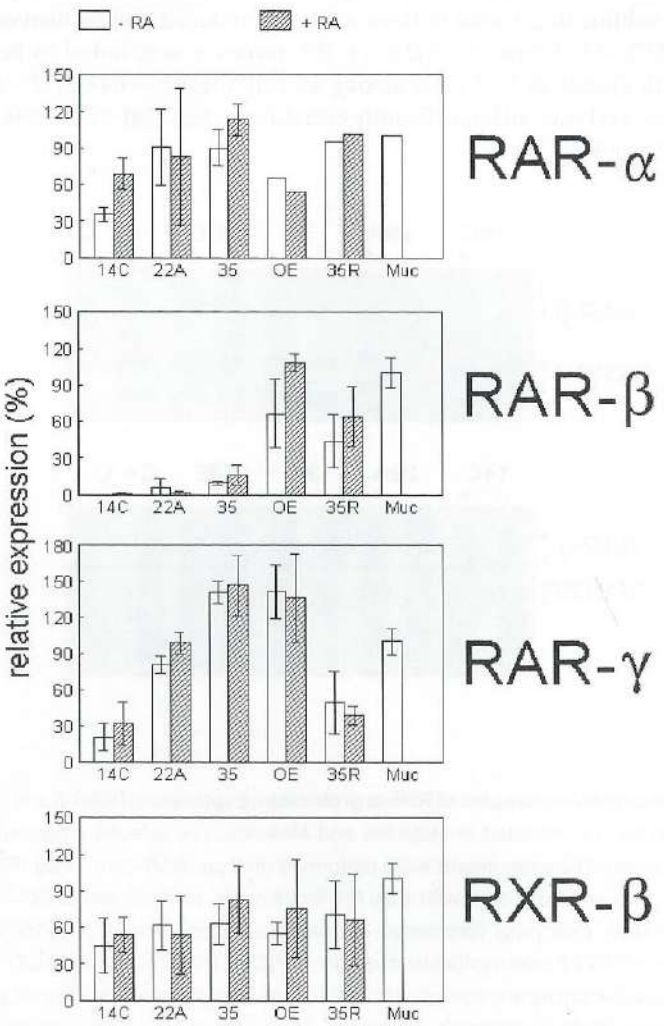


Figure 3. Relative expression levels of RAR-α, -β, -γ and RXR-β analyzed by RNase protection. Five HNSCC cell lines and mucosal tissue (Muc) were analyzed. Total RNA was isolated from the cells, untreated (- RA) and treated (+ RA) with 1 μM RA for 24 h. The blots were quantified by phosphoimaging. After correction for the amount of RNA loaded on the gel (GAPDH), mRNA levels were expressed as relative amounts, with mucosal tissue set as 100%. Presented are the mean ± SD for two or more experiments (for RAR-α in VU-SCC-OE and UM-SCC-35R one experiment).

Representative RNase protection experiments for RAR-β and RAR-γ are shown in Figure 2. RAR-β transcripts were clearly detectable when 15 μg total RNA was used (instead of 3 μg, with which the other RARs were easily detected) in the intermediate sensitive VU-SCC-OE cells, but were nearly undetectable in UM-SCC-14C, UM-SCC-22A and UM-SCC-35. RAR-γ transcripts (two bands) were detectable in all cell lines, but varied largely in expression (Figure 2).

Figure 3 shows the relative expression levels compared with mucosal tissue for each cell line for RAR-α, -β, -γ and RXR-β. RAR-α transcripts were detectable in all cell lines, with little variation in expression levels between cell lines, except for basal mRNA levels of cell line UM-SCC-14C and for basal and RA-induced mRNA levels of cell line VU-SCC-OE. In two of five cell lines RAR-β transcripts were clearly detected. In the other three, expression was very low. One of two cell lines that have detectable RAR-β mRNA expression is resistant (UM-SCC-35R), and the other is intermediately sensitive to RA (VU-SCC-OE). RAR-γ mRNA shows a large variation in expression between cell lines, and intra-individual variation is small. UM-SCC-14C and UM-SCC-35R which are the most RA-resistant, show the lowest, UM-SCC-35 and VU-SCC-OE, which are the most RA-sensitive, the highest expression of RAR-γ and UM-SCC-22A, which is intermediate sensitive to RA, an intermediate expression of RAR-γ. mRNA expression levels of RXR-β show little variation between cell lines and are somewhat lower as compared with normal oral mucosa.

Linear regression analysis showed that only expression of RAR-γ is strongly correlated with the level of growth inhibition and with RA turnover rate. Without RA treatment this was significant for growth inhibition ($P = 0.032$, $R = 0.88$) and turnover rate ($P = 0.033$, $R = 0.88$). With RA treatment, significant correlations were also found with growth inhibition ($P = 0.016$, $R = 0.92$) and turnover ($P = 0.041$, $R = 0.86$). When UM-SCC-35R was excluded, a rather strong, but borderline significant ($P = 0.073$, $R = 0.89$) correlation was still found between RA-induced growth inhibition and RAR-γ expression (Figure 4).

Discussion

A relationship was found between the level of growth inhibition by RA and the turnover rate of RA: HNSCC cell lines with a higher RA turnover rate were more sensitive to RA. The results of the subline UM-SCC-35R confirmed this relationship, since reduction in RA-sensitivity coincided with a decrease in RA turnover. The explanation that toxicity of RA catabolites is responsible for enhanced RA-sensitivity was unlikely, since we and others established that the anti-proliferative activity of several catabolites of RA was equal or even less potent than RA (Braakhuis *et al.* 1997, Reynolds *et al.* 1993, Van Heusden *et al.* 1998).

Other investigators have provided support for the hypothesis RA-related responses are directed by reduced expression of nuclear retinoid receptors (RARs and RXRs) (Heyman *et al.* 1992, Lotan *et al.* 1995). In the present study we discovered that growth

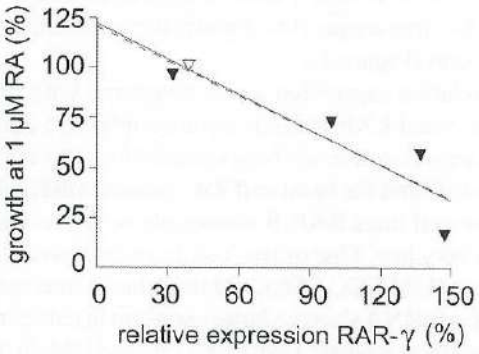


Figure 4. Correlation of RAR-γ expression with RA-sensitivity. RA-sensitivity is expressed as percentage of growth after addition of 1 µM RA as compared to untreated cells. RAR-γ mRNA levels are given as expression after 24 h exposure to 1 µM RA. ▽, UM-SCC-35R; ▼, other HNSCC cell lines (UM-SCC-35, UM-SCC-22A, UM-SCC-14C, VU-SCC-OE). The lines represent the regression lines: solid black line, growth vs RAR-γ mRNA expression of all five HNSCC cell lines (R = - 0.92, P = 0.016); dotted line, growth vs RAR-γ mRNA expression of HNSCC cell lines without UM-SCC-35R (R = - 0.89, P = 0.073).

inhibition and RA turnover are associated with the mRNA expression level of RAR-γ. Two RA-resistant cell lines with a low RA turnover showed a relative low RAR-γ mRNA expression, whereas two RA-sensitive cell lines (with a high RA turnover) showed a relative high expression of RAR-γ, and an intermediate sensitive cell line had an intermediate transcription level of RAR-γ. Furthermore, the subline UM-SCC-35R, derived from UM-SCC-35 which became RA-resistant after prolonged exposure to RA, showed a significant decrease in RAR-γ mRNA expression compared with UM-SCC-35.

Our results were obtained by comparing cell lines that varied in RA-sensitivity. Other reports, based on the use of receptor-selective retinoids or transfection of sense or antisense RARs are in concordance with our results (Oridate et al., 1996, Sun *et al.* 1999, Le *et al.* 2000). Transfection of sense or antisense RAR-γ in the HNSCC cell line SqCC/Y1 revealed that the growth inhibitory effect of RA was enhanced in the sense transfectants and decreased in the antisense ones (Oridate *et al.* 1996). Furthermore, with RAR-γ selective retinoids, it was recently shown that RAR-γ can suppress growth inhibition in an oral SCC cell line (Le *et al.* 2000).

Studies in esophageal and lung cancer cell lines, however, demonstrated a role for RAR-β in RA-induced growth inhibition (Xu *et al.* 1999, Sun *et al.* 2000). Furthermore, introduction of RAR-β increased growth inhibition by RA in cervical carcinoma (Frangioni *et al.* 1994) and breast carcinoma cells (Liu *et al.* 1996, Li *et al.* 1995, Seewaldt *et al.* 1995). In the present study, no correlation between RA-sensitivity and RAR-β expression was observed. Supportive evidence was found with cell line UM-SCC-35R, in which exposure to RA for eight months led to an increase of RAR-β.

The differences in results described in the reports above can be explained by differences in cell-type. RAR-γ is the predominant receptor type in HNSCC cells (Hu *et al.* 1991), and we found that RAR-β transcript levels were lower than that of the other transcripts, since we had to use five times more RNA to see detectable levels of RAR-β. This indicates that RAR-γ has a more prominent role in RA-related responses than other retinoid receptor types in HNSCC cells. Moreover, evidence has been provided that in HNSCC RAR-β is involved in suppression of terminal squamous differentiation and not in growth inhibition (Zou *et al.* 1999, Wan *et al.* 1999). Therefore the suppression of squamous differentiation may explain the clinical response which was observed after up-regulation of RAR-β by 13-*cis*-RA in premalignant oral lesions (Lotan *et al.* 1995). In our study we did not see up-regulation of RAR-β after RA exposure in HNSCC cell lines and this up-regulation may only be a characteristic of premalignant cells. Also from the point of squamous cancer development, RAR-γ was shown to be an important molecule. Using a mouse skin tumor carcinogenesis model, it was shown that RAR-γ expression decreases during tumor progression (Darwiche *et al.* 1995).

RA-induced growth inhibition is related to RA turnover rate among HNSCC cell lines, as was also observed by others and in our previous studies (Takatsuka *et al.* 1996, Braakhuis *et al.* 1997, Klaassen *et al.* 2001), and therefore it was not surprising to find that the expression level of RAR-γ was related to RA turnover rate. It has been suggested that the enzymes responsible for the turnover of RA are induced by RARs in the presence of RA (Isogai *et al.* 1997, Abu-Abed *et al.* 1998).

After the discovery and cloning of a highly RA-specific cytochrome P450 enzyme, CYP26A1 (Ray *et al.* 1997, White *et al.* 1997), a more direct approach could be followed by measuring its mRNA expression levels. With receptor-specific synthetic retinoids, the involvement of RARs in the induction of CYP26A1 could be demonstrated (Marikar *et al.* 1998). Furthermore, transfection of either RAR-α , RAR-β, or RAR-γ were able to restore CYP26A1 expression upon RA treatment in HCT-116 cells (Sonneveld *et al.* 1998). These results suggest the presence of one or more RAREs in the promotor region of that gene and may therefore explain the high RA-inducibility of CYP26A1. Indeed, a RARE, to which RAR-γ can bind, has recently been identified in the promotor region of CYP26A1 (Loudig *et al.* 2000). The variation of RA turnover among HNSCC cell lines in our study is related to the mRNA expression levels of RARs, and in particular that of RAR-γ. This suggests a role for RAR-γ in mediating RA turnover rate in HNSCC cell lines.

In summary, previously we found a strong correlation between RA-induced growth inhibition and RA turnover rate in HNSCC cell lines. In this study we selected five of these cell lines that differed widely in their sensitivity to RA and their capacity to catabolize RA and analyzed RAR mRNA expression in these cell lines. We found that the level of growth inhibition by RA and the turnover rate of RA are associated with the transcription level of RAR-γ.

Acknowledgments

We thank Dr P Chambon and his group for providing the cDNA clones encoding retinoid receptors. This work was supported by the Dutch Cancer Society, grant 95-926.

References

Abu-Abed SS, Beckett BR, Chiba H, Chithalen JV, Jones G, Metzger D, Chambon P, Petkovich M (1998) Mouse P450RAI (CYP26) expression and retinoic acid-inducible retinoic acid metabolism in F9 cells are regulated by retinoic acid receptor gamma and retinoid X receptor alpha. *J Biol Chem* 273: 2409-15

Bollag W, Holdener EE (1992) Retinoids in cancer prevention and therapy. *Ann Oncol* 3: 513-26

Braakhuis BJ, Jansen G, Noordhuis P, Kegel A, Peters GJ (1993) Importance of pharmacodynamics in the in vitro antiproliferative activity of the antifolates methotrexate and 10-ethyl-10-deazaaminopterin against human head and neck squamous cell carcinoma. *Biochem Pharmacol* 46: 2155-61

Braakhuis BJ, Klaassen I, van der Leede BM, Cloos J, Brakenhoff RH, Copper MP, Teerlink T, Hendriks HF, van der Saag PT, Snow GB (1997) Retinoid metabolism and all-*trans* retinoic acid-induced growth inhibition in head and neck squamous cell carcinoma cell lines. *Br J Cancer* 76: 189-97

Carey TE, Wolf GT, Baker SR, Krause CJ (1990) Cell surface antigen expression and prognosis. In *Head and neck cancer*, Fee WE, Goephert H, Johns ME, Strong EW, Ward PH (eds) pp 77-82. BC Decker: Toronto - Philadelphia

De Luca LM (1991) Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. *FASEB J* 5: 2924-33

Eckhoff C, Nau H (1990) Identification and quantitation of all-trans- and 13-cis- retinoic acid and 13-cis-4-oxoretinoic acid in human plasma. *J Lipid Res* 31: 1445-54

Frangioni JV, Moghal N, Stuart-Tilley A, Neel BG, Alper SL (1994) The DNA binding domain of retinoic acid receptor beta is required for ligand-dependent suppression of proliferation. Application of general purpose mammalian coexpression vectors. *J Cell Sci* 107: 827-38

Furr HC, Barua AB, Olson JA (1994) Analytical methods. In *The Retinoids*, Sporn MB, Roberts AB, Goodman DS (eds) pp 179-209. Raven Press: New York

Gudas LJ, Sporn MB, Roberts AB (1994) Cellular biology and biochemistry of the retinoids. In *The Retinoids*, Sporn MB, Roberts AB, Goodman DS (eds) pp 443-520. Raven Press: New York

Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, Thaller C (1992) 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* 68: 397-406

Hong WK, Itri LM (1994) Retinoids and human cancer. In *The Retinoids*, Sporn MB, Roberts AB, Goodman DS (eds) pp 597-630. Raven Press: New York

Hong WK, Lippman SM, Itri LM, Karp DD, Lee JS, Byers RM, Schantz SP, Kramer AM, Lotan R, Peters LJ, Dimery IW, Brown BW, Goepfert H (1990) Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N Engl J Med* 323: 795-801

Hu L, Crowe DL, Rheinwald JG, Chambon P, Gudas LJ (1991) Abnormal expression of retinoic acid receptors and keratin 19 by human oral and epidermal squamous cell carcinoma cell lines. *Cancer Res* 51: 3972-81

Isogai M, Chiantore MV, Haque M, Scita G, De Luca LM (1997) Expression of a dominant-negative retinoic acid receptor construct reduces retinoic acid metabolism and retinoic acid-induced inhibition of NIH-3T3 cell growth. *Cancer Res* 57: 4460-4

Kizaki M, Ueno H, Matsushita H, Takayama N, Muto A, Awaya N, Ikeda Y (1997) Retinoid resistance in leukemic cells. *Leuk Lymphoma* 25: 427-34

Klaassen I, Brakenhoff RH, Smeets SJ, Snow GB, Braakhuis BJ (2001) Enhanced turnover of all-trans-retinoic acid and increased formation of polar metabolites in head and neck squamous cell carcinoma lines compared with normal oral keratinocytes. *Clin Cancer Res* 7: 1017-25

Le Q, Dawson MI, Soprano DR, Soprano KJ (2000) Modulation of retinoic acid receptor function alters the growth inhibitory response of oral SCC cells to retinoids. *Oncogene* 19: 1457-65

Li XS, Shao ZM, Sheikh MS, Eiseman JL, Sentz D, Jetten AM, Chen JC, Dawson MI, Aisner S, Rishi AK and others (1995) Retinoic acid nuclear receptor beta inhibits breast carcinoma anchorage independent growth. *J Cell Physiol* 165: 449-58

Lippman SM, Kessler JF, Al Sarraf M, Alberts DS, Itri LM, Mattox D, Von Hoff DD, Loescher L, Meyskens FL (1988) Treatment of advanced squamous cell carcinoma of the head and neck with isotretinoin: a phase II randomized trial. *Invest New Drugs* 6: 51-6

Liu Y, Lee MO, Wang HG, Li Y, Hashimoto Y, Klaus M, Reed JC, Zhang X (1996) Retinoic acid receptor beta mediates the growth-inhibitory effect of retinoic acid by promoting apoptosis in human breast cancer cells. *Mol Cell Biol* 16: 1138-49

Lotan R (1980) Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells. *Biochim Biophys Acta* 605: 33-91

Lotan R, Xu XC, Lippman SM, Ro JY, Lee JS, Lee JJ, Hong WK (1995) Suppression of retinoic acid receptor-beta in premalignant oral lesions and its up-regulation by isotretinoin. *N Engl J Med* 332: 1405-10

Loudig O, Babichuk C, White J, Abu-Abed S, Mueller C, Petkovich M (2000) Cytochrome P450RAI(CYP26) promoter: a distinct composite retinoic acid response element underlies the complex regulation of retinoic acid metabolism. *Mol Endocrinol* 14: 1483-97

Mangelsdorf DJ (1994) Vitamin A receptors. *Nutr Rev* 52: S32-44

Marikar Y, Wang Z, Duell EA, Petkovich M, Voorhees JJ, Fisher GJ (1998) Retinoic acid receptors regulate expression of retinoic acid 4-hydroxylase that specifically inactivates all-*trans* retinoic acid in human keratinocyte HaCaT cells. *J Invest Dermatol* 111: 434-9

Muindi JF, Young CW (1993) Lipid hydroperoxides greatly increase the rate of oxidative catabolism of all-trans-retinoic acid by human cell culture microsomes genetically enriched in specified cytochrome P-450 isoforms. *Cancer Res* 53: 1226-9

Oridate N, Esumi N, Lotan D, Hing WK, Rochette-Egly C, Chambon P, Lotan R (1996) Implication of retinoic acid receptor gamma in squamous differentiation and response to retinoic acid in head and neck SqCC/Y1 squamous carcinoma cells. *Oncogene* 12: 2019-28

Ray WJ, Bain G, Yao M, Gottlieb DI (1997) CYP26, a novel mammalian cytochrome P450, is induced by retinoic acid and defines a new family. *J Biol Chem* 272: 18702-8

Reid CB, Cloos J, Snow GB, Braakhuis BJ (1997) A simple and reliable technique for culturing of human oral keratinocytes and fibroblasts. *Acta Otolaryngol* 117: 628-33

Reynolds NJ, Fisher GJ, Griffiths CE, Tavakkol A, Talwar HS, Rowse PE, Hamilton TA, Voorhees JJ (1993) Retinoic acid metabolites exhibit biological activity in human keratinocytes, mouse melanoma cells and hairless mouse skin in vivo. *J Pharmacol Exp Ther* 266: 1636-42

Scott PAE, Smith K, Bicknel R, Harris AL (1997) A reliable external control for ribonuclease protection assays. *Nucleic Acids Res* 25: 1305-6

Seewaldt VL, Johnson BS, Parker MB, Collins SJ, Swisshelm K (1995) Expression of retinoic acid receptor beta mediates retinoic acid- induced growth arrest and apoptosis in breast cancer cells. *Cell Growth Differ* 6: 1077-88

Sonneveld E, van den Brink CE, van der Leede BM, Schulkes RK, Petkovich M, van der Burg B, van der Saag PT (1998) Human retinoic acid (RA) 4-hydroxylase (CYP26) is highly specific for all-*trans*-RA and can be induced through RA receptors in human breast and colon carcinoma cells. *Cell Growth Differ* 9: 629-37

Sun SY, Kurie JM, Yue P, Dawson MI, Shroot B, Chandraratna RA, Hong WK, Lotan R (1999) Differential responses of normal, premalignant, and malignant human bronchial epithelial cells to receptor-selective retinoids. *Clin Cancer Res* 5: 431-7

Takatsuka J, Takahashi N, de Luca LM (1996) Retinoic acid metabolism and inhibition of cell proliferation: an unexpected liaison. *Cancer Res* 56: 675-8

Teerlink T, Copper MP, Klaassen I, Braakhuis BJ (1997) Simultaneous analysis of retinol, all-trans- and 13-cis- retinoic acid and 13-cis-4-oxoretinoic acid in plasma by liquid chromatography using on-column concentration after single-phase fluid extraction. *J Chromatogr B Biomed Sci Appl* 694: 83-92

van Heusden J, Wouters W, Ramaekers FC, Krekels MD, Dillen L, Borgers M, Smets G (1998) The antiproliferative activity of all-trans-retinoic acid catabolites and isomers is differentially modulated by liarozole-fumarate in MCF-7 human breast cancer cells. *Br J Cancer* 77: 1229-35

Wan H, Oridate N, Lotan D, Hong WK, Lotan R (1999) Overexpression of retinoic acid receptor beta in head and neck squamous cell carcinoma cells increases their sensitivity to retinoid-induced suppression of squamous differentiation by retinoids. *Cancer Res* 59: 3518-26

Welters MJ, Fichtinger Schepman AM, Baan RA, Hermsen MA, van der Vijgh WJ, Cloos J, Braakhuis BJ (1997) Relationship between the parameters cellular differentiation, doubling time and platinum accumulation and cisplatin sensitivity in a panel of head and neck cancer cell lines. *Int J Cancer* 71: 410-5

White JA, Beckett-Jones B, Guo YD, Dilworth FJ, Bonasoro J, Jones G, Petkovich M (1997) cDNA cloning of human retinoic acid-metabolizing enzyme (hP450RAI) identifies a novel family of cytochromes P450. *J Biol Chem* 272: 18538-41

Xu XC, Liu X, Tahara E, Lippman SM, Lotan R (1999) Expression and up-regulation of retinoic acid receptor-beta is associated with retinoid sensitivity and colony formation in esophageal cancer cell lines. *Cancer Res* 59: 2477-83

Xu XC, Ro JY, Lee JS, Shin DM, Hong WK, Lotan R (1994) Differential expression of nuclear retinoid receptors in normal, premalignant, and malignant head and neck tissues. *Cancer Res* 54: 3580-7

Zou CP, Hong WK, Lotan R (1999) Expression of retinoic acid receptor beta is associated with inhibition of keratinization in human head and neck squamous carcinoma cells. *Differentiation* 64: 123-32

Chapter 7

Metabolism and growth inhibition of four retinoids in head and neck squamous normal and malignant cells

Ingeborg Klaassen, Ruud H Brakenhoff, Serge J Smeets, Gordon B Snow, Boudewijn JM Braakhuis

Section Tumor Biology, Department of Otolaryngology, Vrije Universiteit Medical Center, Amsterdam, the Netherlands

Br J Cancer 2001; 85: 630-5

Abstract

Isotretinoin (13-*cis*-retinoic acid, 13-*cis*-RA) has proven to be active in chemoprevention of head and neck squamous cell carcinoma (HNSCC). Moreover, both all-*trans*-retinoic acid (ATRA) and 13-*cis*-RA induce objective responses in oral premalignant lesions. After binding of retinoids to retinoic acid receptors (RARs and RXRs) dimers are formed that are able to regulate the expression of genes involved in growth and differentiation. We compared the metabolism and level of growth inhibition of 13-*cis*-RA with that of ATRA, 9-*cis*-RA and retinol in four HNSCC cell lines and normal oral keratinocyte cultures (OKC). These retinoid compounds are known to bind with different affinities to the retinoic acid receptors. We observed that all retinoids were similar with respect to their capacity to induce growth inhibition. One HNSCC line could be ranked as sensitive, one as moderately sensitive and the remaining two were totally insensitivity; OKC were moderately sensitive. The rate at which the cells were able to catabolize the retinoid was similar for all compounds. Metabolism in HNSCC cells resulted in a profile of retinoid products which was unique for each retinoid. These metabolic profiles were different in OKC. Our findings indicate that differences in retinoid receptor selectivity of these retinoids do not influence the level of growth inhibition and rate of metabolism.

Introduction

Retinoic acid is an active metabolite of retinol (ROL, vitamin A) that plays an important role in growth and differentiation of a variety of cell types (Gudas *et al.* 1994). Retinoids also inhibit the growth of cancer cells (Lotan, 1980) and are useful in the treatment and prevention of human cancer (Bollag and Holdener 1992). In head and neck squamous cell carcinoma (HNSCC) 13-*cis*-retinoic acid (13-*cis*-RA) was successful in the prevention of second primary tumors (Hong *et al.* 1990). Retinoids demonstrate the ability to induce objective responses in oral leukoplakia (Hong and Itri 1994). This is a whitish or reddish plaque in the epithelial lining in the oral cavity that has a relatively high risk to become malignant. For 13-*cis*-RA a response rate (based on both partial and complete responses) varying between 55% and 87% has been reported. (Lippman *et al.* 1988, Hong and Itri 1994). All-*trans*-retinoic acid (ATRA) was shown to be active in 59% of leukoplakia patients (Koch 1978). The activity of retinoids, however, is associated with serious side effects and intrinsic and acquired resistance do occur (Hong and Itri 1994). Acquired resistance to ATRA in leukemia was found to be a consequence of enhanced catabolism of ATRA (Muindi *et al.* 1994). In contrast, an inverse relationship was found between intrinsic ATRA resistance and ATRA catabolism in HNSCC (Braakhuis *et al.* 1997, Klaassen *et al.* 2001a). This paradoxical finding suggests that the molecular mechanisms underlying RA-resistance in HNSCC are different. The possible mechanisms explaining

(the lack of) the effect of retinoids have not been elucidated but are thought to be mediated through retinoid binding to specific nuclear retinoid receptors, with modulation of the expression of genes involved in growth and differentiation (Oridate *et al.*, 1996). Recent data show that in HNSCC especially the expression of RAR-γ is important for retinoid induced growth inhibition (Le *et al.* 2000, Klaassen *et al.* 2001b).

ATRA is a naturally occurring compound that shows a much more avid binding to retinoic acid receptors than 13-*cis*-RA (Åström *et al.* 1990). Whereas ATRA preferentially binds retinoic acid receptors (RARs), its stereoisomer 9-*cis*-RA (9-*cis*-RA) has a high binding affinity for RARs as well as retinoid X receptors (RXRs) (Heyman *et al.* 1992). Details on their binding and transactivating activity has been reported earlier in full detail (Allenby *et al.* 1993, Sun *et al.* 1997). Related to differences in receptor binding affinities between the three stereoisomers, different effects on growth and metabolism may be expected.

Differences in metabolic rates and/or type of metabolites formed between the various retinoid compounds may determine the growth inhibiting capacities of these compounds. One important route of ATRA metabolism consists of hydroxylation at position 4 of the cyclohexenyl ring to form 4-hydroxy-RA, which is readily oxidized to 4-oxo-RA. Other metabolic pathways of ATRA include isomerization, decarboxylation and glucuronidation processes (Rockley *et al.* 1980, Roberts *et al.* 1980, Sass *et al.* 1994). *In vitro*, 4-hydroxylation of ATRA is mediated by a Cytochrome P450 (CYP)-dependent monooxogenase system that requires NAPDH and oxygen (Roberts *et al.* 1980).

The present study was undertaken to investigate to what extent a panel of retinoids, including 13-*cis*-RA, is able to inhibit growth of HNSCC cells and cultures of normal oral keratinocytes (OKC). Growth inhibition was related to retinoid turnover and the pattern of metabolites formed.

Materials and methods

Chemicals

13-*cis*-RA and retinol (ROL) were obtained from Sigma (St. Louis, MO), ATRA from Acros Chimica (Geel, Belgium); 4-oxo-all-*trans*-RA (4-oxo-RA) and 4-oxo-13-*cis*-RA were gifts from Dr UH Wiegand (Hoffmann-la Roche, Basel, Switzerland); 4-hydroxy-RA (4-OH-RA) and 18-hydroxy-RA ((18-OH-RA) were gifts from Dr L Foley (Roche Pharmaceuticals, Nutley, NJ). All compounds were dissolved in dimethyl sulfoxide (DMSO) and stored as 10^{-3} mol l^{-1} stock at -80°C under nitrogen. For each experiment, working dilutions were freshly prepared in the appropriate cell culture medium. Final DMSO concentration was always lower than 0.1% and did not affect cell growth. All handling with retinoids was performed in subdued light and in the presence of 0.1% BSA to prevent absorption to plastics (Klaassen *et al.* 1999).

Cells and culture conditions

Primary OKC were obtained from the uvulas of non-cancer patients who underwent uvulopalatopharyngoplasty. The mucosal layer was stripped from the tissue and cells were isolated and cultured as described previously (Reid *et al.* 1997) in 6-well culture plates in keratinocyte growth medium (KGM) (Life Technologies, Paisley, UK) supplemented with growth factors (BPE and rEGF), gentamycin sulphate (final concentration 5 µg/ml) and amphotericin B (final concentration 0.5 µg ml^{-1}) (all from Life Technologies). At 70% confluency, primary cultures of keratinocytes were subcultured and plated at a density of 10^5 cells/ well. Keratinocytes were used for experiments at passage 3, when they reached 70-80% confluence. Human HNSCC cell lines UM-SCC-35, -22A and -14C were provided by Dr TE Carey (University of Michigan, Ann Arbor, MI) and are described elsewhere (Carey, 1990). An RA-resistant subline, UM-SCC-35R, was established from the RA-sensitive UM-SCC-35 cell line by exposing it to increasing concentrations of ATRA (10^{-8} to 10^{-6} M) during a period of eight months. HNSCC cells were maintained in Dulbecco's modified essential medium (DMEM, Life Technologies) supplemented with 5% FCS (ICN Biomedicals, Irvine, UK) and 50 U/ml penicillin, 50 µg/ml streptomycin (Life Technologies).

Growth inhibition assays

The dose response effect on cell proliferation was determined with the sulphorhodamine B (SRB). Details of the assay have been described previously (Braakhuis *et al.*, 1993). In short, cells (1000- 4000 per well) were plated in 96-well plates in DMEM/5% FCS or KGM/0.1% BSA for HNSCC cell lines and oral keratinocyte cultures, respectively, and were allowed to grow for 72 h (the "lag phase"). After this phase the medium was replaced by medium containing the appropriate retinoid with a final concentration ranging from 10^{-9} to 10^{-6} M. Growth was assayed after another 72 h incubation (the "log phase"), by staining the cellular protein with SRB (Sigma) and spectrophotometric measurement of the absorption at 540 nm with a microplate reader (Labsystems Multiskan, Helsinki, Finland). Growth of untreated cells during the log phase must at least have doubled its initial value.

Metabolism experiments

Keratinocytes and HNSCC cells were plated at a density of 10^5 cells per well in 6-well plates. Upon 70-80% confluence, the medium was removed and replaced by medium containing 1 µM RA, being KGM with 0.1% BSA for keratinocytes (Klaassen *et al.* 1999) or DMEM with 5% FCS for HNSCC cells. As a control retinoid containing medium without cells was included during the incubation period. From the medium two samples of 350 µl were taken and after removal of the residual medium the cells were washed once with phosphate-buffered saline and collected in 350 µl trypsin/EDTA. The samples were stored at - 80°C under nitrogen until retinoid extraction.

Determination of the turnover rate

Retinoids were analyzed by reverse-phase HPLC after extraction with acetonitrile, as described previously (Teerlink *et al.* 1997, Klaassen *et al.* 2001a). The amount of retinoid compound at each time point was calculated relative to the amount of the medium control at the start of the experiment. The turnover rate was taken as the difference between 4 and 24 h, corrected for the decrease in the medium controls during this period. Next, this relative amount was converted to absolute amounts in pmol by use of the external standards. The turnover rate was expressed in pmol per mg protein per hour. Protein

content of cell extracts was determined on the pellet with the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Table 1. Retinoid metabolism in four HNSCC cells and OKC exposed to 1 mM of four different retinoids

HNSCC line	retinoid	metabolism*		metabolites**	
		mean	S.D.	pellet	medium
14C	ATRA	13.3	9.2	e	a,b
	13-*cis*-RA	78.7	27.2	d2	a,b
	9-*cis*-RA	59.3	13.2	e	a,b
	ROL	223.7	84.5	d1	d1
22A	ATRA	104.8	67.9	e	a,b
	13-*cis*-RA	154.1	76.3	d2	a,b
	9-*cis*-RA	139.3	45.9	e	a,b
	ROL	349.9	12.7	d1	d1
35	ATRA	1016.0	413.2	e	a,b
	13-*cis*-RA	949.5	53.0	e	a,b
	9-*cis*-RA	962.3	88.2	b	a,b,c
	ROL	1336.8	76.7	c1	a,b,c1,d1
35R	ATRA	52.7	25.4	b	a,b
	13-*cis*-RA	6.9	9.8	b, d2	a,b
	9-*cis*-RA	23.4	5.5	b	a,b
	ROL	111.7	2.9	c1, d1	c1, d1
OKC	ATRA	14.8	19.0	e	e
	13-*cis*-RA	38.8	20.5	d2	e
	9-*cis*-RA	12.0	12.5	e	e
	ROL	98.7	78.5	d1	d1

The profiles of 72 hr exposure are shown. Note that the names of the cell lines have been abbreviated; the prefix "UM-SCC-" has been omitted. Experiments were performed in triplicate, OKC cultures were from four different individuals *Metabolism determined from HPLC analysis expressed as relative decrease in medium and cells together of the corresponding retinoid between 4 and 24 h exposure as compared to medium controls at t= 0 h per mg protein per hour. ** Metabolites in medium and in cell pellets determined from HPLC analysis. Metabolites: a, between 5 and 10 min (retention time); b, between 10 and 15 min; c, between 15 min and 13-*cis*-RA; d, between ATRA and ROL; e, no metabolites. A more detailed explanation of the metabolites mentioned is given in Figure 2.

Results

Growth inhibition of various retinoid compounds in HNSCC cell lines

The relative growth of four HNSCC cell lines treated with ATRA, its isomers (13-*cis*-RA and 9-*cis*-RA), and its precursor ROL is illustrated in Figure 1. Two cell lines (UM-SCC-22A and UM-SCC-35) exerted a dose-dependent growth inhibition for all compounds, while the other two cell lines (UM-SCC-14C and UM-SCC-35R) were insensitive; UMSCC-35 was the most sensitive cell line. 4-oxo-metabolites of ATRA and 13-*cis*-RA were tested in four cell lines to find out whether these catabolites were able to induce growth inhibition and whether a lack of 4-hydroxylation could explain the lack of response in the insensitive cell lines. From Figure 1 it can be seen that the level of growth inhibition by 4-oxo-RA and 4-oxo-13-*cis*-RA was similar to that of the other retinoid compounds. In addition, OKC cultures from different individuals were exposed to four retinoids as well; a moderate dose dependent growth inhibition was observed that was not different between the various compounds (Figure 1).

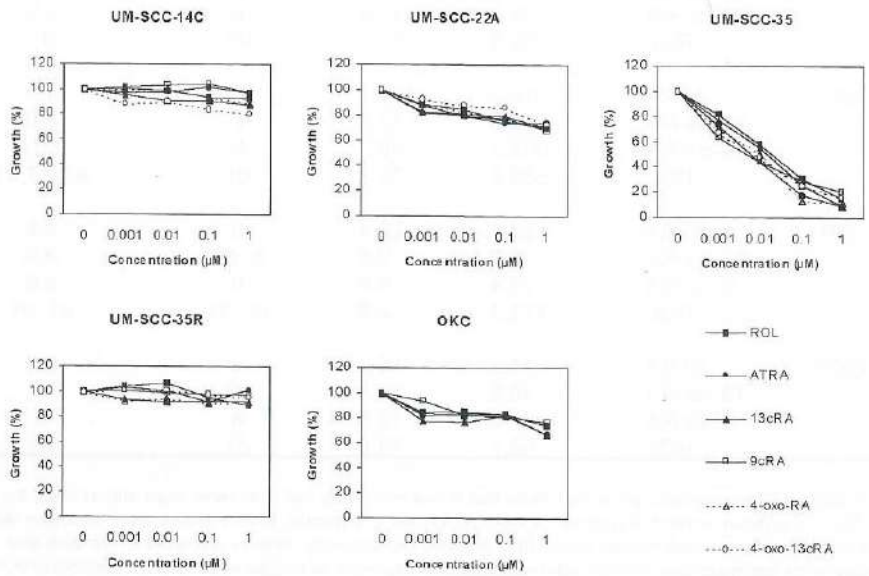


Figure 1. Relative growth of cells exposed to various concentrations of ATRA, 13-*cis*-RA, 9-*cis*-RA, ROL, 4-oxo-13-*cis*-RA and 4-oxo-RA as compared to untreated cells. OKC cultures (from four different individuals) were exposed to the first four drugs. Growth was assessed with the "SRB-assay" and experiments were performed at least three times. Average values are shown and coefficients of variation were less than 5% (HNSCC lines) and 13% (OKC cultures).

Metabolism of ATRA, 13-cis-RA, 9-cis-RA and ROL in HNSCC cells and OKC cultures

A summary of the turnover rate of all different compounds and the metabolites formed in the medium and pellets is given in Table 1 and Figure 2. The rate of retinoid turnover was more cell line than compound dependent. In general, UM-SCC-35 showed the highest turnover rate for all compounds, UM-SCC-35R and UM-SCC-14C the lowest and UM-SCC-22A an intermediate turnover rate; turnover rates in OKC were comparable to the turnover rates in UM-SCC-35R and UM-SCC-14C. The turnover rate of ROL was the highest of all compounds in all cell lines.

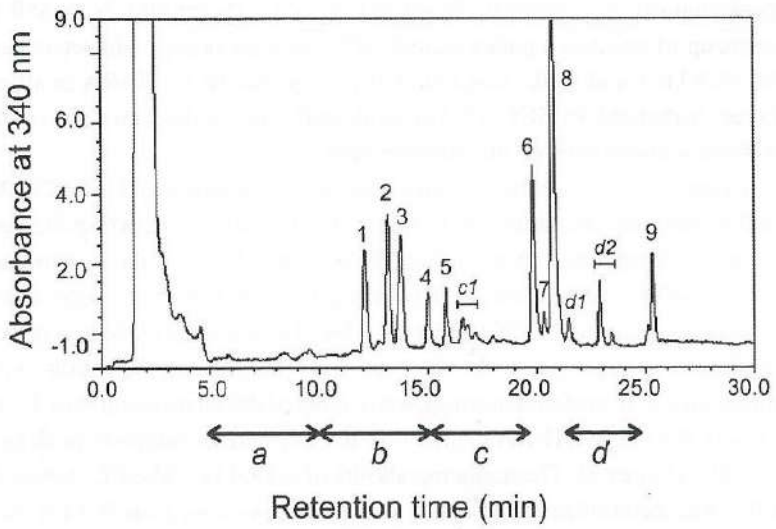


Figure 2. Compilation of all metabolites formed in cells and medium after addition of ATRA, 9-*cis*-RA, 13-*cis*-RA or ROL is shown. This chromatogram is necessary for the interpretation of Table 1 and shows 4-oxo-RA (peak 1), 4-oxo-cRA (2), 4-OH-RA (3), 18-OH-RA (4), 5,6-epoxy-RA (5), 13-*cis*-RA (6), 9-*cis*-RA (7), ATRA (8) and ROL (9). Furthermore, the chromatogram is divided in groups of peaks as follows: *a*, polar metabolites corresponding to peaks with retention times between 5 and 10 min; *b*, polar metabolites corresponding to peaks with retention times between 10 and 15 min; *c*, polar metabolites corresponding to peaks with retention times between 15 min and that of 13-*cis*-RA; *d*, peaks with retention times between that of ATRA and ROL.; *c1*, peaks found after exposure to ROL in medium and cells of UM-SCC-35 and UM-SCC-35R; *d1*, peak found after exposure to ROL in medium and cells of HNSCC cell lines and OKC; *d2*, peaks found in cells of HNSCC cell lines and OKC after exposure to 13-*cis*-RA. Peak 5 (5,6-epoxy-RA) was found only in medium of UM-SCC-35 after exposure to 9-*cis*-RA.

In general, the metabolites formed were different between HNSCC cell lines and OKC (Table 1). Polar metabolites in "group *b*" and metabolites in "group *a*" (Figure 2) were only found in HNSCC cell lines, but not in OKC. The widest variety of metabolites was found in the medium of cell line UM-SCC-35, probably due to its high turnover rate. 5,6-epoxy-RA was, for instance, only found in medium of UM-SCC-35. After 13-*cis*-RA and 9-*cis*-RA exposure, a relative high formation of polar metabolites in UM-SCC-35 and UM-SCC-35R was observed, similar as was reported for ATRA (Klaassen *et al.*, 2001a). Metabolism of ROL resulted in the formation of an unknown product, with a retention time a little longer than that of ATRA (Figure 2, "*d*1"), which was found in almost every medium and cell fraction of cancer cell lines as well as OKC, whereas a group of unknown peaks named "*c*1" was only found in UM-SCC-35 and UM-SCC-35R (Table 1). Another group of unknown peaks named "*d*2", corresponding with retention times between that of ATRA and ROL, was found after exposure to 13-*cis*-RA in all cellular fractions, except in that of UM-SCC-35. The unidentified peaks described above were not found in medium without cells or in untreated cells.

As an example, profiles of metabolites formed in the medium of UM-SCC-35 after exposure to four retinoids are shown in Figure 3. Each compound corresponds with an unique pattern of metabolites. In the other HNSCC cell lines generally similar peak profiles as in UM-SCC-35 were found. Following ROL and 9-*cis*-RA exposure some more peaks were detected in UM-SCC-35 and -35R (Table 1). Co-elution of peaks with authentic retinoids of absorption at the two wavelengths of detection with the corresponding ratios of the reference compounds enabled the identification of 4-oxo-RA (1), 4-oxo-13-*cis*-RA (2), 4-OH-RA (3), 18-OH-RA (4), and an unknown peak (A) after exposure to ATRA (Figure 3). The major metabolite of ATRA in UM-SCC-35 was 4-OH-RA. 13-*cis*-RA was metabolized to products identified as 4-oxo-13-*cis*-RA (2) and three unknown peaks (A, B and C), of which peak A and B were the highest (Figure 3). Metabolism of 9-*cis*-RA resulted in peaks corresponding with the same metabolites as found with 13-*cis*-RA metabolism, but with higher amounts of peak A and a substantially lower amount of peak B and an additional peak coeluting with 5,6-epoxy-RA (peak 5). The level of the polar metabolites (Figure 2, "group *b*") formed after 13-*cis*-RA or 9-*cis*-RA exposure was much higher than that of the group of polar metabolites formed from ATRA.

After ATRA exposure, as compared to other HNSCC cell lines a relatively high isomerization (up to 10%) was found to 13-*cis*-RA in UM-SCC-22A, UM-SCC-14C and OKC and a relatively high level of polar metabolites in UM-SCC-35R. In UM-SCC-22A, UM-SCC-14C and OKC a relatively high isomerization to ATRA after 13-*cis*-RA exposure (up to 10%) and a relatively high isomerization to ATRA and 13-*cis*-RA after

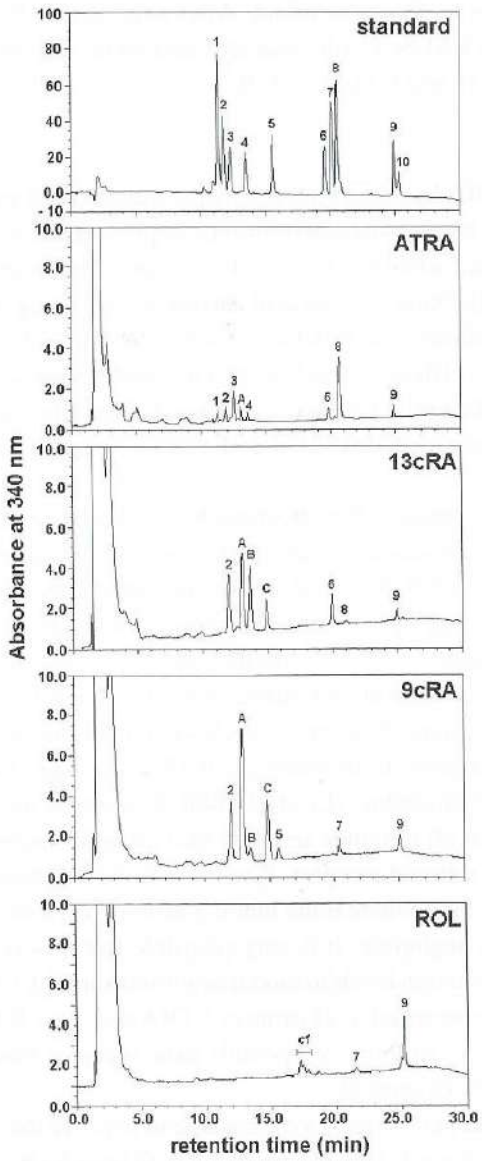


Figure 3. HPLC profiles of retinoid metabolites in the medium of of UM-SCC-35 are shown after exposure to 1 µM of various retinoid compounds for 24 h. The upper panel one is a HPLC profile of retinoid standards dissolved in water. For identification of the peaks see the legend of figure 2 and in addition peak 10 corresponds to retinal. Peaks A, B and C indicate unidentified compounds.

9-*cis*-RA exposure (up to 5%) was found. After exposure to ROL, a relatively high formation of ATRA in UM-SCC-14C was seen and no or very small amounts of other retinoids in UM-SCC-35 and UM-SCC-35R.

Discussion

Treatment with 13-*cis*-RA has shown several major drawbacks: it exhibited considerable toxicity at therapeutic levels, was active in only a proportion of the individuals with premalignant lesions and when the treatment is stopped the lesions recur (Hong *et al.* 1986, Hong *et al.* 1990). Other retinoic acid derivatives are being developed and tested, hoping that these derivatives may lack the negative effects of 13-*cis*-RA and be able to provide similar or higher efficacy, including activity against advanced cancer (Armstrong and Meyskens, 2000). Recently, a randomized Phase I chemoprevention trial with ATRA in patients with curatively treated HNSCC ended and a Phase II trial is on its way (Park *et al.* 2000).

The present study showed that all retinoids tested were equally active in HNSCC cell lines. Thus, although retinoids have different binding affinities for the nuclear receptors (Allenby *et al.* 1993, Sun *et al.* 1997), this apparently has no influence on the level of growth inhibition *in vitro*. This finding suggest that all these compounds induce growth inhibition through a common mechanism. Evidence has been provided that retinoids regulate cellular growth and differentiation through binding to retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Mangelsdorf *et al.* 1994, Heyman *et al.* 1992). In particular in HNSCC the binding to RAR-γ has suggested to be important for retinoid induced growth inhibition. (Le *et al.* 2000, Klaassen *et al.* 2001b). So the most likely explanation is that all retinoids are able, in a direct or indirect way to reach the downstream target, the retinoid receptor. It remains to be explained why 13-*cis*-RA is active in some HNSCC lines whereas the binding affinity of 13-*cis*-RA to both retinoic acid receptor classes is negligible. It is very plausible that 13-*cis*-RA is converted to retinoids that have high enough levels to modulate growth through binding to the retinoid receptors. 13-*cis*-RA is converted to its isomers ATRA and 9-*cis*-RA and its breakdown product 4-oxo-13-*cis*-RA; all these compounds have receptor binding property and a growth inhibiting activity (Figure 1).

The retinoids used in this study were not able to improve the sensitivity profile of the standard compound 13-*cis*-RA. In the insensitive cell lines ATRA, 9-*cis*-RA and ROL did not enhance the level of growth inhibition. It is possible that in these cell lines the receptor system is seriously disrupted and that it will be very hard to find a retinoid that will be active.

We tested turnover rates of 13-*cis*-RA, ATRA, 9-*cis*-RA and ROL to investigate whether these would explain differences in growth inhibition between HNSCC cell lines. In the individual cell lines, turnover rates of the three stereoisomers were in a narrow range, and that of ROL somewhat higher. The most likely explanation for the relatively higher turnover rate of ROL as compared to the other retinoids might be that ROL can be converted to at least four different compounds. Besides oxidation to ATRA via retinal, ROL can also be esterified to retinyl esters (Kurlandsky *et al.* 1996), 4-hydroxylated to 4-oxo-ROL (Leo and Lieber 1985), or metabolized to anhydroretinol (Bhat *et al.* 1979). Esterification has been studied in more detail by Guo and Gudas (1998). These authors showed that HNSCC cells have a reduced capacity to esterify ROL as compared to normal oral keratinocytes. This would explain the relatively lowt turnover rate of ROL as compared to tumor cell cultures, but whether this leads to differences in metabolites, remains to be established.

We also performed metabolic studies on normal OKC. These cells were slow with respect to the rate of retinoid turnover and were not able to synthesize polar metabolites. This confirms the results of a previous study with ATRA (Klaassen *et al.* 2001a).

The comparison of the retinoids with respect to metabolism showed two phenomena that needs further discussion. There was only a small difference between the compounds in turnover rate and each compound had its unique pattern of metabolites. With respect to the rate of turnover it is known that the CYP-related enzyme system is involved in the conversion (4-hydroxylation) to polar retinoids, like 4-OH-RA, 18-OH-RA and 4-oxo-RA. For instance, CYP26 was found to be responsible for the 4-hydroxylation of ATRA (Ray *et al.* 1997, White *et al.* 1997). CYP26 is highly substrate (ATRA) specific and can not metabolize 13-*cis*-RA or 9-*cis*-RA (Sonneveld *et al.* 1998). In the present study it was found that 13-*cis*-RA and 9-*cis*-RA were catabolized with similar turnover rates as ATRA, which suggests that one or more enzymes analogous to CYP26 are responsible for the catabolism of 13-*cis*-RA and 9-*cis*-RA. To a small degree, isomerization to ATRA can be involved, however the finding that each retinoid has its unique pattern of metabolites limits this possibility. Furthermore, other studies have shown evidence that 4-hydroxylated forms of 9-*cis*-RA and 13-*cis*-RA are directly formed from their parental compounds (Eckhoff and Nau 1990, Dzerk *et al.* 1998). This strongly suggests that specific CYPs exist that are responsible for the conversion of 9-*cis*-RA and 13-*cis*-RA to their corresponding 4-oxo-compounds. The CYP isoform that metabolizes *cis*- and *trans*-RA-isomers can be different as shown with isomers of retinal. Raner *et al.* (1996) demonstrated that *in vitro* 4-hydroxylation of 13-*cis*- and all-*trans*-retinal was predominantly catalyzed by the CYP1A1 isoform, whereas CYP2B4 and CYP2C3 are most active in the metabolism of 9-*cis*-retinal.

In conclusion, despite variation in their binding to retinoid receptors, all retinoids did not differ with respect to their capacity to induce growth inhibition. The rate at which the cells were able to catabolize the retinoid was similar for all compounds. Cellular metabolism in HNSCC cells leads to a metabolite profile that is unique for each retinoid.

Acknowledgments

This work was supported by the Dutch Cancer Society, grant 95-926.

References

Allenby G, Bocquel MT, Saunders M, Kazmer S, Speck J, Rosenberger M, Lovey A, Kastner P, Grippo JF, Chambon P, Levin AE (1993) Retinoic acid receptors and retinoid X receptors: interactions with endogenous retinoic acids. *Proc Natl Acad Sci USA* 90: 30-4

Armstrong WB, Meyskens FL Jr (2000) Chemoprevention of head and neck cancer. *Otolaryngol Head Neck Surg* 122: 728-35

Åström A, Pettersson U, Krust A, Chambon P, Voorhees JJ (1990) Retinoic acid and synthetic analogs differentially activate retinoic acid receptor dependent transcription. *Biochem Biophys Res Commun* 173: 339-45

Bhat PV, De Luca LM, Adamo S, Akalovsky I, Silverman-Jones CS, Peck GL (1979) Retinoid metabolism in spontaneously transformed mouse fibroblasts (Balb/c 3T12-3 cells): enzymatic conversion of retinol to anhydroretinol. *J Lipid Res* 20: 357-62

Bollag W, Holdener EE (1992) Retinoids in cancer prevention and therapy. *Ann Oncol* 3: 513-26

Braakhuis BJ, Jansen G, Noordhuis P, Kegel A, Peters GJ (1993) Importance of pharmacodynamics in the in vitro antiproliferative activity of the antifolates methotrexate and 10-ethyl-10-deazaaminopterin against human head and neck squamous cell carcinoma. *Biochem Pharmacol* 46: 2155-61

Braakhuis BJ, Klaassen I, van der Leede BM, Cloos J, Brakenhoff RH, Copper MP, Teerlink T, Hendriks HF, van der Saag PT, Snow GB (1997) Retinoid metabolism and all-*trans* retinoic acid-induced growth inhibition in head and neck squamous cell carcinoma cell lines. *Br J Cancer* 76: 189-97

Carey TE, Wolf GT, Baker SR, Krause CJ (1990) Cell surface antigen expression and prognosis. 77-82

Dzerk AM, Carlson A, Loewen GR, Shirley MA, Lee JW (1998) A HPLC method for the determination of 9-cis retinoic acid (ALRT1057) and its 4-oxo metabolite in human plasma. *J Pharm Biomed Anal* 16: 1013-9

Eckhoff C, Nau H (1990) Identification and quantitation of all-trans- and 13-cis- retinoic acid and 13-cis-4-oxoretinoic acid in human plasma. *J Lipid Res* 31: 1445-54

Gudas LJ, Sporn MB, Roberts AB (1994) Cellular biology and biochemistry of the retinoids. In *The Retinoids* , Sporn MB, Roberts AB, Goodman DS (eds) pp 443-520. Raven Press: New York

Guo X, Gudas LJ (1998) Metabolism of all-*trans*-retinol in normal human cell strains and squamous cell carcinoma (SCC) lines from the oral cavity and skin: reduced esterification of retinol in SCC lines. *Cancer Res* 58: 166-76

Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, Thaller C (1992) 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* 68: 397-406

Hong WK, Endicott J, Itri LM, Doos W, Batsakis JG, Bell R, Fofonoff S, Byers R, Atkinson EN, Vaughan C and others (1986) 13-cis-retinoic acid in the treatment of oral leukoplakia. *N Engl J Med* 315: 1501-5

Hong WK, Itri LM (1994) Retinoids and human cancer. In *The Retinoids*, Sporn MB, Roberts AB, Goodman DS (eds) pp 597-630. Raven Press: New York

Hong WK, Lippman SM, Itri LM, Karp DD, Lee JS, Byers RM, Schantz SP, Kramer AM, Lotan R, Peters LJ, Dimery IW, Brown BW, Goepfert H (1990) Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N Engl J Med* 323: 795-801

Klaassen I, Brakenhoff RH, Smeets SJ, Snow GB, Braakhuis BJ (1999) Considerations for in vitro retinoid experiments: importance of protein interaction. *Biochim Biophys Acta* 1427: 265-75

Klaassen I, Brakenhoff RH, Smeets SJ, Snow GB, Braakhuis BJ (2001a) Enhanced turnover of all-trans-retinoic acid and increased formation of polar metabolites in head and neck squamous cell carcinoma lines compared with normal oral keratinocytes. *Clin Cancer Res* 7: 1017-25

Klaassen I, Brakenhoff RH, Smeets SJ, Snow GB, Braakhuis BJ (2001b) Expression of retinoic acid receptor gamma correlates with retinoic acid sensitivity and metabolism in head and neck squamous cell carcinoma cell lines. *Int J Cancer* 92: 661-5

Koch HF (1978) Biochemical treatment of precancerous oral lesions: the effectiveness of various analogues of retinoic acid. *J Maxillofac Surg* 6: 59-63

Kurlandsky SB, Duell EA, Kang S, Voorhees JJ, Fisher GJ (1996) Auto-regulation of retinoic acid biosynthesis through regulation of retinol esterification in human keratinocytes. *J Biol Chem* 271: 15346-52

Le Q, Dawson MI, Soprano DR, Soprano KJ (2000) Modulation of retinoic acid receptor function alters the growth inhibitory response of oral SCC cells to retinoids. *Oncogene* 19: 1457-65

Leo MA, Lieber CS (1985) New pathway for retinol metabolism in liver microsomes. *J Biol Chem* 260: 5228-31

Lippman SM, Kessler JF, Al Sarraf M, Alberts DS, Itri LM, Mattox D, Von Hoff DD, Loescher L, Meyskens FL (1988) Treatment of advanced squamous cell carcinoma of the head and neck with isotretinoin: a phase II randomized trial. *Invest New Drugs* 6: 51-6

Lotan R (1980) Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells. *Biochim Biophys Acta* 605: 33-91

Mangelsdorf DJ (1994) Vitamin A receptors. *Nutr Rev* 52: S32-44

Muindi JR, Young CW, Warrell RP Jr (1994) Clinical pharmacology of all-trans retinoic acid. *Leukemia* 8 Suppl 3: S16-21

Oridate N, Esumi N, Lotan D, Hing WK, Rochette-Egly C, Chambon P, Lotan R (1996) Implication of retinoic acid receptor gamma in squamous differentiation and response to retinoic acid in head and neck SqCC/Y1 squamous carcinoma cells. *Oncogene* 12: 2019-28

Park SH, Gray WC, Hernandez I, Jacobs M, Ord RA, Sutharalingam M, Smith RG, Van Echo DA, Wu S, Conley BA (2000) Phase I trial of all-trans retinoic acid in patients with treated head and neck squamous carcinoma. *Clin Cancer Res* 6: 847-54

Raner GM, Vaz AD, Coon MJ (1996) Metabolism of all-trans, 9-cis, and 13-cis isomers of retinal by purified isozymes of microsomal cytochrome P450 and mechanism-based inhibition of retinoid oxidation by citral. *Mol Pharmacol* 49: 515-22

Ray WJ, Bain G, Yao M, Gottlieb DI (1997) CYP26, a novel mammalian cytochrome P450, is induced by retinoic acid and defines a new family. *J Biol Chem* 272: 18702-8

Reid CB, Cloos J, Snow GB, Braakhuis BJ (1997) A simple and reliable technique for culturing of human oral keratinocytes and fibroblasts. *Acta Otolaryngol* 117: 628-33

Roberts AB, Lamb LC, Sporn MB (1980) Metabolism of all-trans-retinoic acid in hamster liver microsomes: oxidation of 4-hydroxy- to 4-keto-retinoic acid. *Arch Biochem Biophys* 199: 374-83

Rockley NL, Halley BA, Nelson EC (1980) Identification of a decarboxylation product of retinoic acid. *Biochim Biophys Acta* 627: 270-5

Sass JO, Forster A, Bock KW, Nau H (1994) Glucuronidation and isomerization of all-trans- and 13-cis-retinoic acid by liver microsomes of phenobarbital- or 3-methylcholanthrene-treated rats. *Biochem Pharmacol* 47: 485-92

Sonneveld E, van den Brink CE, van der Leede BM, Schulkes RK, Petkovich M, van der Burg B, van der Saag PT (1998) Human retinoic acid (RA) 4-hydroxylase (CYP26) is highly specific for all-*trans*-RA and can be induced through RA receptors in human breast and colon carcinoma cells. *Cell Growth Differ* 9: 629-37

Sun SY, Yue P, Dawson MI, Shroot B, Michel S, Lamph WW, Heyman RA, Teng M, Chandraratna RA, Shudo K, Hong WK, Lotan R (1997) Differential effects of synthetic nuclear retinoid receptor-selective retinoids on the growth of human non-small cell lung carcinoma cells. *Cancer Res* 57: 4931-9

Teerlink T, Copper MP, Klaassen I, Braakhuis BJ (1997) Simultaneous analysis of retinol, all-trans- and 13-cis- retinoic acid and 13-cis-4-oxoretinoic acid in plasma by liquid chromatography using on-column concentration after single-phase fluid extraction. *J Chromatogr B Biomed Sci Appl* 694: 83-92

White JA, Beckett-Jones B, Guo YD, Dilworth FJ, Bonasoro J, Jones G, Petkovich M (1997) cDNA cloning of human retinoic acid-metabolizing enzyme (hP450RAI) identifies a novel family of cytochromes P450. *J Biol Chem* 272: 18538-41

Chapter 8

Non-malignant oral keratinocytes of head and neck squamous cell carcinoma patients show enhanced metabolism of retinoic acid

Ingeborg Klaassen, Jacqueline Cloos, Serge J Smeets, Ruud H Brakenhoff, Maarten P Tabor, Gordon B Snow, and Boudewijn JM. Braakhuis

Section Tumor Biology, Department of Otolaryngology, Vrije Universiteit Medical Center, Amsterdam, the Netherlands

Submitted

Abstract

Retinoids show promise in the treatment of various (pre)malignancies, including head and neck squamous cell carcinoma (HNSCC). It has been shown that metabolic pathways of retinoids are important in the anticancer effect of retinoids and that these pathways may change during HNSCC carcinogenesis. We have previously reported that HNSCC cells have a 17-fold greater turnover rate of retinoic acid (RA) than normal oral keratinocytes of non-cancer controls, and formation of polar metabolites, such as 4-oxo-RA and 4-hydroxy-RA, was only seen in HNSCC cell lines. Objective: We aimed to establish whether this altered retinoid metabolism is an intrinsic characteristic of HNSCC patients. Methods: The normal mucosa of cancer and non-cancer patients was the source for keratinocyte cultures. The cells were exposed to RA for various time periods and levels of various retinoids were measured in the culture medium and cell pellets with reverse phase liquid chromatography. Results: Cells from cancer patients were morphologically normal and showed no genetic aberrations (loss of heterozygosity). RA turnover rate in normal oral keratinocytes of cancer patients was 15-fold higher (P = 0.003) than in normal oral keratinocytes of non-cancer controls, with average turnover rates of 218.6 and 14.8 pmol/mg protein/h, respectively. Specific profiles of RA metabolites were similar. Conclusions: The observed higher RA metabolism in non-cancer cells of HNSCC patients suggests that individuals with a relatively high RA turnover have an increased risk to develop HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) affects over 500,000 persons per year worldwide. It accounts for 10% of all cancer cases and more than 5% of all cancer deaths (Vokes et al. 1993). HNSCC arises in the mucosa of the oral cavity, pharynx and larynx, and develops as a result of exposure to carcinogens and cancer-promoting agents in a multistep process (Harris 1991). Exposure to carcinogens such as tobacco smoke and alcohol is a major risk factor for HNSCC. Multifocal epithelial injury, related to this continuous exposure, may lead to a risk for the development of multiple premalignant foci and multiple primary tumors (Cooper et al. 1989). These second primary tumors, which occur at an incidence rate of 2 to 3% per year are a major cause of death in HNSCC patients (Snow 1992). Exposure to carcinogens, however, does not explain all HNSCC cases. Recent evidence indicated that an hereditary susceptibility to DNA-damage plays an important role in the development of HNSCC (Cloos et al. 1996, Cloos et al. 1999).

Retinoids were found to inhibit or reverse the *in vitro* growth of malignant cells by its capacity to inhibit cell proliferation and to regulate cell differentiation (Lotan 1980,

De Luca 1991). Since *in vivo* models have shown that the processes of initiation and progression could be delayed or even reversed (Wille and Chopra 1988), retinoids have been selected as chemopreventive agents. Retinoids were successfully used in the treatment of oral leukoplakia (Lippman et al. 1988), a potentially premalignant mucosal lesion. Moreover, the development of second primary tumors following HNSCC could be prevented or delayed (Hong et al. 1995).

Although retinoids exhibit clinical activity some limitations are apparent: they are minimally active in advanced cancer, intrinsic as well as acquired resistance frequently occur and the toxic side effects can be serious (Hong and Itri 1994). Variations in retinoid effects might be explained by differences either between individuals or between normal and malignant cells. Some studies provide evidence that retinoid pathways are different in cancer tissues. For instance, expression of RAR-β, one of the nuclear receptors involved in retinoid signal transduction is lower in (pre-)malignant tissue as compared to normal squamous cells (Xu et al. 1994, Lotan et al. 1995). Further, differences in retinol esterification between malignant and non-malignant cells were found (Guo and Gudas 1998). Recently, we have found that RA metabolism is also different in HNSCC cell lines as compared to normal oral keratinocyte cultures (Klaassen et al 2001). In short, when exposed to all-*trans*-retinoic acid (RA) malignant cells showed a 17-fold greater turnover rate of RA and polar metabolites were formed. These data implicated that keratinocytes change their retinoid metabolism during the process of carcinogenesis or it means that keratinocytes of HNSCC patients have an intrinsic retinoid related defect that is associated with an increased cancer risk. In the present study we have investigated the mechanisms underlying the altered metabolism in HNSCC cells. We investigated RA metabolism in normal oral keratinocytes of head and neck cancer patients and compared these with normal oral keratinocytes of non-cancer patients and HNSCC cells. A genetic analysis was performed to proof the non-malignant character of the keratinocytes of the cancer patients. Our results suggest that an altered retinoid metabolism in oral keratinocytes is an intrinsic factor, associated with an increased cancer risk.

Materials and methods

Chemicals

All-*trans*-RA was obtained from Acros Chimica (Geel, Belgium). Retinoids used in HPLC standard solution, retinol and 13-*cis*-RA were obtained from Sigma (St. Louis, MO); 4-oxo-all-*trans*- and 4-oxo-13-*cis*-RA were gifts from Dr. U.H. Wiegand (Hoffmann-la Roche, Basel, Switzerland); 4-hydroxy-RA and 18-hydroxy-RA were gifts from Dr. L. Foley (Roche Pharmaceuticals, Nutley, NJ). All compounds were dissolved in dimethyl sulfoxide (DMSO) and stored as 10^{-3} M stock at -80°C under nitrogen. For each experiment, working dilutions were freshly prepared in the appropriate cell culture medium. Final

DMSO concentration was always lower than 0.1% and did not affect cell growth. [11,12-^{3}H(N)]all-*trans*-RA (30.0 Ci/mmol) was obtained from NEN (Boston, MA). All handling with retinoids was performed in subdued light and in the presence of 0.1% BSA to prevent absorption to plastics (Klaassen et al. 1999).

Cells and culture conditions

Normal primary oral keratinocytes (OKC-N) were obtained from the uvulas of non-cancer patients who underwent uvulopalatopharyngoplasty. Keratinocyte cultures of HNSCC patients (OKC-T) were obtained from macroscopically normal non-keratinizing mucosal tissue, more than 1 cm away from the tumor. Tumor locations and staging are listed in Table 1.

Table 1. Characteristics of tumors of patients from whom tissue was taken adjacent to the tumor

Patient code	Site	TNM staging [1]
1	vallecula fossa	T4N3
2	hypopharynx	T4N2b
3	oropharynx	T3N1
4	oropharynx	T2N2b
5	oropharynx	T3N0
6	oropharynx	T3N0
7	retromolar trigone	T1N0
8	oropharynx	T2N0
9	tongue	T1N0
10	retromolar trigone	T4N1
11	supraglottic larynx	T4N0
12	piriform sinus	T3N2b
13	supraglottic larynx	T3N2b

[1] TNM, tumor staging according to the American Joint Commission on Staging (Hermanek and Sobin 1987)

Cells were isolated and cultured in keratinocyte growth medium (KGM) (Life Technologies, Paisley, UK) in 6-well culture plates as described previously (Reid et al. 1997). At 70% confluence, primary cultures of keratinocytes were subcultured and plated at a density of 10^5 cells/ well. Keratinocytes were used for experiments at passage 3, when they had reached 70-80% confluence. In this study eleven human HNSCC cell lines were used. UM-SCC-35, -22A and -14C were provided by Dr. T.E. Carey (University of Michigan, Ann Harbor, MI) and are described elsewhere (Carey et al. 1990). Cell lines 92-VU-041, 92-VU-059, 92-VU-078, 92-VU-080, 93-VU-094, 93-VU-096, 93-VU-147 were established at the laboratory of Human Genetics, *Vrije Universiteit*, Amsterdam, the Netherlands (Hermsen et al. 1996). The cell line VU-SCC-OE was established from a human HNSCC xenograft in our laboratory (Welters et al. 1997)]. T9917, originating from a HNSCC in the trigonum retromolare was recently established in our laboratory and used for experiments at passage 9. The malignant character of this tumor culture was confirmed by LOH on three 3p (D3S1293, D3S1766, D3S1274) and three 9p loci (D9S157, D9S171, D9S1751) and a mutation in the TP53 gene . HNSCC cells were maintained in Dulbecco's Modified Essential Medium (DMEM) (Life Technologies) supplemented with 5% FCS (ICN Biomedicals, Irvine, UK) and 50 U/ml penicillin, 50 µg/ml streptomycin (Biowhittaker, Verviers, Belgium).

Approval for this study was obtained from the Institutional Review Board. Informed consent was obtained from all patients. From the patient records we collected information on age, gender, smoking and drinking habits (Table 2). Through questionnaires patients were asked whether they smoked (never, former or current), how many packs of cigarettes per day and for how many years. One pack-year (PY) of smoking is equivalent to having smoked one pack (25 cigarettes) per day for one year. Tobacco consumption of the subjects was stratified in three categories: non-smokers, moderate smokers (less than 30 PY) and heavy smokers (30 or more PY). For alcohol consumption patients were asked whether they drunk alcohol (never, former or current), how many consumptions (units) of alcohol per day and for how many years. One unit-year (UY) of drinking is equivalent to having consumed one unit of alcohol per day for one year. Alcohol consumption of subjects was stratified in three categories: non-drinkers, moderate drinkers (less than 100 UY) and heavy drinkers (100 or more UY).

Growth inhibition assays

The dose response effect on cell proliferation was determined with the Sulforhodamine B (SRB)-assay. Details of the assay have been described previously (Braakhuis et al. 1993). In short, cells (1000- 4000 per well) were plated in 96-well plates in DMEM/5% FCS or KGM/0.1% BSA for HNSCC cell lines or oral keratinocyte cultures, respectively, and were allowed to grow for 72 h (the "lag phase"). Then, the medium was replaced by medium containing RA with a final concentration ranging from 10^{-9} to 10^{-6} M. Growth was assayed after 72 h incubation (the "log phase"), by staining the cellular protein with sulphorhodamine B (SRB; Sigma-Aldrich) and spectrophotometric measurement of the absorption at 540 nm with a microplate reader (Labsystems Multiskan, Helsinki, Finland).

*Metabolism of all-*trans*-retinoic acid*

Keratinocytes and HNSCC cells were plated at a density of 10^5 cells per well in 6-well plates. Upon 70 - 80% confluence, the medium was removed and replaced by medium being KGM with 0.1% BSA for keratinocytes (Klaassen et al. 1999) or DMEM with 5% FCS for HNSCC cells containing 1 µM RA or 10 nM ^{3}H-RA. As a control RA containing medium without cells was included during the incubation period. From the medium two samples of 350 µl were taken and after removal of residual medium cells were washed once with phosphate-buffered saline and collected in 350 µl trypsin/EDTA. The samples were stored at - 80 °C under nitrogen until retinoid extraction.

Retinoid extraction and HPLC analysis

Retinoids were analyzed by reverse-phase 'high performance liquid chromatography' (HPLC) after extraction with acetonitrile as described previously (Teerlink et al. 1997). In short, to each sample 50 µl of 1 M sodium acetate buffer (pH 4.0) and 600 µl acetonitrile was added, and after vortex-mixing the samples were centrifuged for 2 min at 3000 *g*. After retinoid extraction, protein content of the pellet was determined with the Bio-Rad protein assay (Bio-Rad, Hercules, CA). In total 720 µl of the clear supernatant was transferred to a 2 ml glass autosampler vial, and after addition of 240 µl water the vials were capped, mixed by inversion and put in the sample compartment of the autoinjector (cooled at 4 °C), or stored at -80 °C under nitrogen. A Gynkotek (Gynkotek HPLC, Munich, Germany) HPLC system was used, consisting of a Basic Marathon automatic sample injector, a Model M480G gradient pump, a

Model UVD 170S UV detector, and a column heater (Mistral, Spark Holland, Emmen, the Netherlands). The mobile phase was degassed online using a model GT103 degasser (Gastorr, Bad Honnef, Germany). Chromelion software (Gynkotek) was used for instrument control and data acquisition. Separation was performed on a 3-µm Spherisorb ODS2 column (100 x 4.6 mm) from Phase Separations (Deeside, UK) maintained at 30 °C, and a 10 µm C_{18} guard column from Separations (H.I.Ambacht, The Netherlands). Composition of the mobile phases and the binary gradient used were as described by Eckhoff & Nau (1990). UV detection was performed at 340 nm. Retinoids were identified and RA concentrations were quantified using external standards. Concentrations of RA in the standards were calculated by measurement at λ_{max} (350 nm) and use of the molar extinction coefficient (45,300) (Furr et al. 1994). Radiolabelled retinoids were detected with a Packard/Radiomatic Flow-trough monitor (Packard Instruments, Meriden, CT).

Determination of the turnover rate of RA

The results of the HPLC analysis were submitted to stringent selection. Experiments were excluded when medium controls (without cells) showed a decrease in RA of more than 60% in 48 h. The amount of RA was calculated relative to the amount of the medium control at the start of the experiment. The turnover rate was taken as the difference between 4 and 24 h in medium plus cells, corrected for the decrease in the medium controls during this period. Next, this relative amount was converted to absolute amounts in pmol by use of the external standards. The turnover rate was expressed in pmol per mg protein per hour.

LOH analysis

Microsatellite markers were analyzed at loci on chromosomes 3p and 9p: 3p12 (D3S1274), 3p14 (D3S1766), 3p24 (D3S1293), 9p21 (D9S171, D9S1751) and 9p22 (D9S157). Primer sequences were obtained from the Genome Database for all of these markers (http://gdbwww.gdb.org). Before PCR amplification, crude cell extracts of about 1 x 10^5 were treated with 1mg/ml proteinase K for 24h at 52° C in 100 µl buffer containing 100 mM TRIS (pH 9.0), 10 mM NaCl, 5 mM EDTA, 1% SDS. The DNA was purified by phenol-chloroform extraction and collected by ethanol precipitation using 2 µg glycogen as carrier. The DNA was redissolved in LoTE-buffer (3 mM TRIS pH 7.4; 0.2 mM EDTA pH 7.5). Normal DNA was isolated from blood samples obtained at the time of surgery. The DNA concentration was measured by microfluorometry with the Hoefer Dynaquant (Amersham/Pharmacia Benelux NV, Roosendaal, the Netherlands). Microsatellite analysis was carried out on an automated ABI PRISM sequencer (310 Genetic Analyzer, PE Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). One primer (Isogen Bioscience, Maarssen, the Netherlands) of each marker was end-labeled with one of the fluorescent dyes FAM, HEX or NED. DNA (10 ng) was amplified by PCR in a total volume of 10 µl containing 2 pmol of each labeled and unlabeled primer. The PCR buffer included 10 mM TRIS-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate and 0.5 U of Taq DNA polymerase (AmpliTaq, Perkin Elmer, Gouda, the Netherlands). PCR amplifications of each primer set were performed for 35 cycles consisting of denaturation at 94 °C for 1 minute, annealing at 55 to 65 °C (depending on the primer set) for 1 minute, and extension at 72 °C for 2 minutes. The amplified product was diluted in sterilized water, usually 1:5-1:10 times. For analysis, 12 µl deionized formamide was combined with 0.5 µl Genescan-350 [ROX] size standard (PE Applied Biosystems) and 1 µl of diluted PCR product in a Genetic Analyzer sample tube. The samples were loaded on an ABI PRISM 310

Genetic Analyzer and run following the supplier's protocol. The data were analyzed with GeneScan Analysis software (version 1.2, PE Applied Biosystems). LOH was scored if one allele was decreased by greater than 50% in the cell line sample when compared with the same allele in normal DNA, isolated from peripheral blood lymphocytes.

Statistical analysis

Differences between cell types with respect to RA turnover rate, growth and age of the patients was calculated using the Student's t-test. The correlation between age and growth versus the RA turnover rate was established using linear regression analysis.

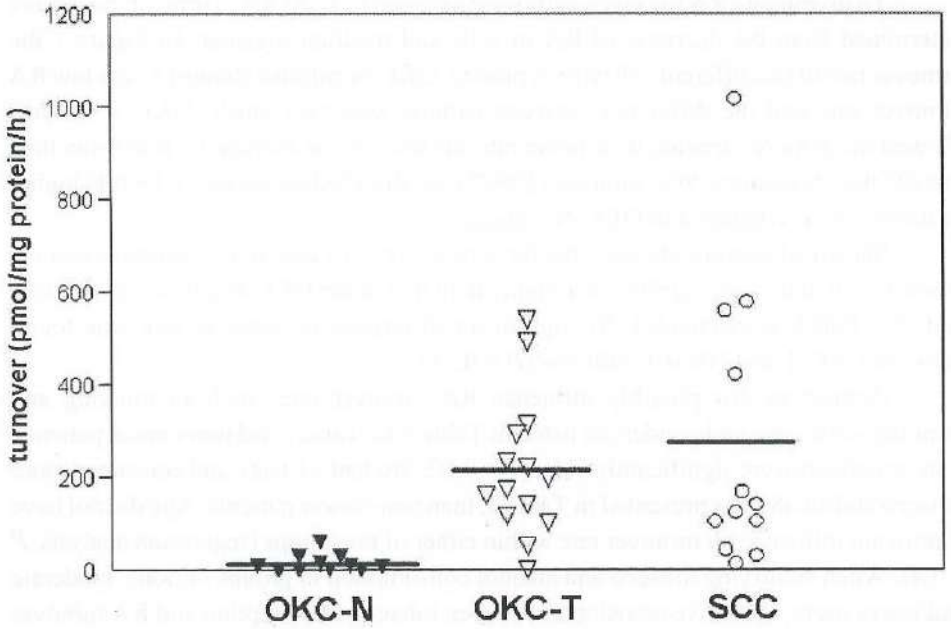


Figure1. Scatterplot of turnover rate (pmol RA/mg protein/h) for all cell cultures indicated in the time period between 4 and 24h after exposure to 1 mM RA. The mean is presented as a horizontal line (mean ± SD: OKC-N, 14.8 ± 19.0; OKC-T, 218.6 ± 158.6; SCC, 275.3 ± 305.8). OKC-N (▼, n = 11), oral keratinocyte cultures of non-cancer patients; OKC-T (▽, n = 13), oral keratinocyte cultures of cancer patients; SCC (○, n = 12), head and neck squamous cell carcinoma cell lines. RA Turnover rate of OKC-T and SCC was significantly higher than that of OKC-N (P = 0.003 and P = 0.014). Difference in turnover rate between OKC-T and SCC was not significant (P = 0.51)

Results

Normal oral mucosa of non-cancer patients and normal mucosal tissue, adjacent to the tumor from cancer patients were isolated and keratinocytes were cultured. The macroscopically normal mucosal tissue from cancer patients, appeared to be normal as judged from histopathological examination; also, the keratinocyte cultures derived from it showed a normal morphology. To confirm the non-malignant nature of the keratinocyte cultures they were subjected to LOH analysis of loci at 3p and 9p. Allelic loss of these loci is known to be associated with early steps of HNSCC carcinogenesis (Califano et al. 1996). None of the keratinocyte cultures showed genetic aberrations, confirming the non-malignant character of the cells.

To investigate RA turnover, cells were exposed to 1 μM RA. Turnover rates were determined from the decrease of RA in cells and medium together. In Figure 1 the turnover rate of the different cell types is plotted. OKC-N cultures showed a very low RA turnover rate and the difference between cultures was very small. OKC-T cultures showed much more variation in turnover rate and this was on average 15-fold faster than that of OKC-N cultures. SCC cultures (T9917 included) had on average a 19-fold higher turnover rate as compared to OKC-N cultures.

Statistical analysis showed that the turnover rate of the OKC-T cultures and the HNSCC cell lines were significantly higher than that of the OKC-N cultures ($P = 0.014$ and $P = 0.003$, respectively). No significant difference in turnover rate was found between OKC-T and HNSCC cultures ($P = 0.51$).

Parameters that possibly influence RA turnover rate, such as smoking and drinking status, age and gender are listed in Table 2 for cancer and non-cancer patients. Cancer patients were significantly older ($P = 0.02$, Student's t-test), and consumed more tobacco and alcohol, as presented in Table 2, than non-cancer patients. Age did not have significant influence on turnover rate within either of the groups (regression analysis, $P > 0.4$). When stratifying tobacco and alcohol consumption in groups of none, moderate and heavy users, a positive association between tobacco consumption and RA turnover could not be demonstrated (see Table 3); on the contrary, the results rather point to an inversed relationship. Turnover rate was also not related to tumor site or stage.

Growth inhibition tests were conducted on the cell cultures to investigate whether RA sensitivity is related to RA turnover rate. In both groups a large variation in growth after exposure to 1 μM RA was observed (Table 3), and a significant correlation with turnover rate or a difference between study groups could not be found.

Table 2. Overview of characteristics of non-cancer controls ($n = 11$) and cancer patients ($n = 13$)

	Non-cancer controls (%)	Cancer patients (%)
Age		
Mean	44.4 ± 11.0	54.7 ± 8.5
Range	26.5 - 65.3	40.8 - 72.8
Sex		
Male	11 (100)	11 (84.6)
Female	0 (0)	2 (15.4)
Smoking Status		
Never	2 (18.2)	0 (0)
Former	2 (18.2)	6 (46.2)
Current	5 (45.5)	6 (46.2)
Unknown	2 (18.2)	1 (7.7)
Tobacco consumption		
None	2 (18.2)	0 (0)
Moderate	5 (45.5)	3 (23.1)
Heavy	2 (18.2)	9 (69.2)
Unknown	2 (18.2)	1 (7.7)
Drinking Status		
Never	3 (27.3)	2 (15.4)
Former	1 (9.1)	4 (30.8)
Current	5 (45.5)	6 (46.2)
Unknown	2 (18.2)	1 (7.7)
Alcohol consumption		
None	3 (27.3)	2 (15.4)
Moderate	5 (45.5)	2 (15.4)
Heavy	1 (9.1)	8 (61.5)
Unknown	2 (18.2)	1 (7.7)

Table 3. Growth and turnover rate and the influence of tobacco and alcohol consumption in oral keratinocyte cultures of non-cancer patients (OKC-N) and cancer patients (OKC-T)

	OKC-N (*n* = 11)		OKC-T (*n* = 13)	
	Mean	SD	Mean	SD
Growth[1]	40.8	18.7	49.2	23.0
Turnover[2]				
Mean of all	14.8	19.0	218.6	158.6
Tobacco consumption				
None	19.2	14.6	n.a.	
Moderate	21.5	25.5	332.9	141.0
Heavy	4.0	5.6	186.9	162.2
Alcohol consumption				
None	2.0	5.1	360.8	183.1
Moderate	27.4	22.5	265.1	66.5
Heavy	7.9	n.a.	127.2	95.2

[1] Growth at 1 mM RA as compared to untreated cells (%). [2] RA turnover (pmol/mg protein/h), in the time period between 4 and 24h after exposure to 1 mM RA. Mean of all and separate values stratified by tobacco or alcohol consumption are given. n.a.= not applicable.

The metabolites formed after exposure to 1 μM RA were analyzed in all different cell types (Figure 2). HNSCC cell lines were found to catabolize RA to polar metabolites like 4-hydroxy-RA and 4-oxo-RA, while normal oral keratinocytes from either non-cancer and cancer patients bearing individuals did not. In addition, we exposed the cells to10 nM ^{3}H-RA to be able to recover all RA-derived products. Despite this sensitive method, polar metabolites were not detected in OKC-N and OKC-T (Figure 2). A more detailed presentation of the kinetics of 10 nM ^{3}H-RA is given in Table 4.

When the total of cells and medium is taken together, recovery per time point for each cell type is near to 100%, indicating that almost nothing of the initial radioactivity is lost by the experimental procedure. The decrease of RA over time coincided with an increase of peaks with a short retention time (0-10 min) in all cell types. In HNSCC cell lines also an increase of polar metabolites (10-15 min) could be observed.

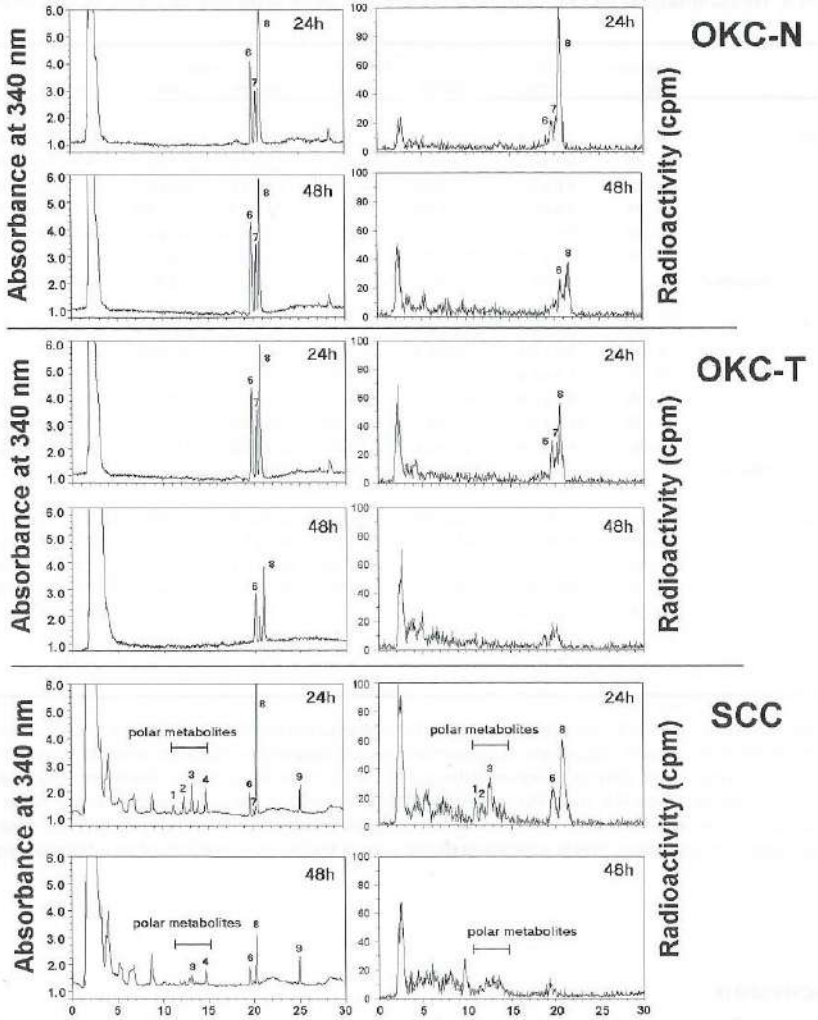


Figure 2. Representative HPLC profiles of retinoid metabolites in the medium of normal keratinocyte cultures of non-cancer patients (OKC-N) and cancer patients (OKC-T) and HNSCC cell lines (SCC) exposed to 1 mM RA (left) or 10 nM ^{3}H-RA (right) for 24 h and 48 h. In contrast to the keratinocyte cultures of cancer as well as non-cancer patients, the tumor cell lines are characterized by the occurrence of polar metabolites. *Peak 1*, 4-oxo-RA; *peak 2*, 4-oxo-cRA; *peak 3*, 4-OH-RA; *peak 4*, 18-OH-RA; *peak 5*, 5,6-epoxy-RA; *peak 6*, 13-*cis*-RA; *peak 7*, 9-*cis*-RA; *peak 8*, all-*trans*-RA; *peak 9*, retinol; *polar metabolites*, metabolites corresponding with peaks with retention times between 10 and 15 min. Note that scale is chosen to show all individual peaks and as such that not all peaks are visible in full length

Table 4. HPLC-analysis of cell culture medium and cells after exposure to 10 nM ^{3}H-RA.

Cell type	Retention time[1]	t = 0 h control	t = 4 h medium	t = 4 h cells	t = 24 h medium	t = 24 h cells	t = 48 h medium	t = 48 h cells
OKC-N	0 - 5	7.0 ± 1.5	7.1 ± 0.8	0.5 ± 0.1	10.7 ± 3.4	1.0 ± 0.1	28.0 ± 3.9	0.7 ± 0.1
	5 - 10	4.1 ± 0.5	4.4 ± 2.0	0.7 ± 0.1	6.8 ± 1.0	0.7 ± 0.1	9.5 ± 1.6	0.8 ± 0.1
	10 - 15	4.0 ± 0.2	4.1 ± 0.6	0.8 ± 0.1	5.4 ± 0.5	0.8 ± 0.1	5.3 ± 0.8	0.7 ± 0.1
	15 - 19	5.8 ± 0.5	6.2 ± 1.8	1.3 ± 0.2	8.1 ± 1.2	1.3 ± 0.3	5.7 ± 0.5	1.0 ± 0.1
	19 - 25	75.6 ± 11.4	63.6 ± 6.1	4.8 ± 0.7	57.2 ± 11.0	4.6 ± 0.9	39.5 ± 6.8	2.0 ± 0.3
	25 - 30	3.4 ± 0.3	2.8 ± 0.4	0.6 ± 0.1	3.4 ± 0.6	0.6 ± 0.1	4.6 ± 0.5	0.9 ± 0.2
	Recovery[2]	*100.0*	*88.3*	*8.6*	*91.7*	*9.0*	*92.6*	*6.0*
OKC-T	0 - 5	8.8 ± 0.2	12.0 ± 0.9	2.2 ± 0.1	20.1 ± 3.3	1.4 ± 0.5	33.3 ± 2.7	1.3 ± 0.2
	5 - 10	5.2 ± 0.2	6.9 ± 0.4	0.9 ± 0.0	9.1 ± 1.5	0.8 ± 0.0	15.7 ± 2.7	0.9 ± 0.1
	10 - 15	6.9 ± 1.4	6.8 ± 0.2	1.1 ± 0.0	7.1 ± 0.7	0.7 ± 0.1	7.4 ± 0.7	0.9 ± 0.1
	15 - 19	8.2 ± 0.9	8.7 ± 1.0	2.0 ± 0.1	9.1 ± 1.2	2.2 ± 0.3	8.2 ± 1.3	2.0 ± 0.1
	19 - 25	66.9 ± 5.5	51.1 ± 5.1	5.7 ± 0.2	37.7 ± 2.3	5.0 ± 0.8	23.0 ± 2.2	3.3 ± 0.7
	25 - 30	4.0 ± 0.9	3.3 ± 0.1	0.7 ± 0.1	2.9 ± 0.3	0.9 ± 0.1	3.7 ± 0.5	0.8 ± 0.2
	Recovery	*100.0*	*88.8*	*12.6*	*86.0*	*11.1*	*91.3*	*9.2*
HNSCC	0 - 5	7.1 ± 1.8	16.8 ± 3.3	1.2 ± 0.2	25.2 ± 2.9	0.7 ± 0.1	29.7 ± 3.5	0.7 ± 0.0
	5 - 10	3.9 ± 1.7	9.6 ± 1.0	0.6 ± 0.2	14.6 ± 1.7	0.5 ± 0.1	16.7 ± 2.6	0.4 ± 0.1
	10 - 15	4.8 ± 0.7	11.7 ± 3.3	0.9 ± 0.2	16.6 ± 3.4	0.7 ± 0.1	17.2 ± 1.3	0.5 ± 0.1
	15 - 19	4.9 ± 0.3	7.0 ± 1.6	0.5 ± 0.2	6.6 ± 0.5	0.5 ± 0.1	7.9 ± 0.3	0.6 ± 0.0
	19 - 25	77.1 ± 4.8	46.2 ± 11.5	3.2 ± 0.3	21.8 ± 2.4	1.8 ± 0.3	14.6 ± 1.0	0.9 ± 0.2
	25 - 30	2.0 ± 0.7	2.2 ± 0.4	0.7 ± 0.2	2.5 ± 0.1	0.9 ± 0.0	2.4 ± 0.1	0.9 ± 0.3
	Recovery	*100.0*	*93.4*	*7.2*	*87.3*	*5.1*	*92.5*	*3.9*

Cell culture medium and cells were analyzed with HPLC after exposure for various times (mean of three separate experiments ± SD is shown). Values are expressed as a percentage of the total area under the curve of the relevant part of the chromatogram. Control (medium without cells) at t = 0 h is set at 100%. [1]Retention time is expressed in minutes. For clarity, 13-*cis*-RA, 9-*cis*-RA and RA are taken together in the cluster with retention times 19 -25 min. The cluster with retention times 10 -15 min contains the polar metabolites. [2]Recovery is given for each time point, medium and cells separated, and is expressed as the sum of the relative amounts of all retention times together

Discussion

The main finding of this study is that RA turnover rate in normal oral cells of HNSCC patients is significantly (15-fold) higher than in normal oral cells of non-cancer patients. This is an intriguing finding, since turnover level is close to what was found in HNSCC cells, which metabolize RA 19-fold faster than normal cells of non-cancer patients. Although, the OKC-T and HNSCC cell lines show similar high RA turnover rates we have evidence that the cells isolated from cancer patients were not malignant. Microsatellite marker analysis showed no genetic alterations associated with HNSCC carcinogenesis. Moreover, the metabolic profile of retinoid metabolites in the medium

of the OKC-T was identical to that of OKC-N. In an earlier study we demonstrated that the catabolism of RA in HNSCC cell lines coincided with the formation of polar metabolites, such as 4-hydroxy-RA and 4-oxo-RA (Klaassen et al. 2001). These polar metabolites could not be detected in OKC-N and also not in OKC-T. The dramatic change in metabolic profiles appears most probably as an acquired genetic change during carcinogenesis.

In order to explain the differences in RA turnover rate between OKC-N and OKC-T we considered that environmental factors might have played a role. The direct influence of these factors on the outcome of the *in vitro* assays is expected to be minimal and all cells were cultured in the similar culture medium and under similar conditions. Nevertheless, between cancer patients and control persons there was a difference in the amount of alcohol and tobacco consumption. When comparing RA turnover rates in groups of none, moderate and heavy users it could, however, not be demonstrated that the level of RA turnover is proportional to tobacco and alcohol consumption (see Table 3); if any, there rather was an inverse relationship.

Other differences between cancer and control subjects were age and location of the tissue. Age was significantly higher in the cancer patient group, but within that group no correlation between age and turnover score was observed. Tissue from non-cancer patients was taken from uvulas only, while tissue from cancer patients was taken from various locations of the upper-aerodigestive tract. Distinction in tissue location is, however, also unlikely a reason for the difference in RA turnover rate, as we see no influence of the specific site on the turnover rate among these patients. Moreover, many samples originate from sites near the uvula and all tissues share a similar histology, being non-keratinized squamous epithelium.

Our study shows that an interindividual variation in RA turnover exists and that a high RA turnover is associated with an increased risk to develop HNSCC. These results are in line with those reported by Rigas et al. for squamous and large cell lung cancer (Rigas et al. 1996). The plasma concentration over time was measured after a single dose of RA and it was found that a rapid decrease of RA levels was linked with a higher risk for lung cancer. It appears that the phenotype of rapid retinoid catabolism is a risk factor for cancer of the respiratory and upper digestive tract. This is in line with the concept that relatively low intracellular RA levels, a possible result of a high turnover, has a promoting effect on the development of cancer. This concept confirms that retinoid supplementation can be used to inhibit or reverse the process of carcinogenesis (Lotan 1996). Moreover, epidemiological studies indicate that a decrease of ingestion of vitamin A is associated with an increased risk for oral cancer or cancer of the larynx (Graham 1984).

Based on all considerations above, it is most likely that the observed variation in RA turnover is due to an intrinsic variation between individuals. It is still to be elucidated what the underlying genetic basis of this intrinsic variation might be. We considered a genetic polymorphism of metabolic enzymes, like cytochrome P450 (CYP) enzymes, the most likely explanation. The most specific CYP for catabolism of RA is CYP26 (Ray et al. 1997, White et al. 1997). If a polymorphism in CYP26 is found to be responsible for a relatively high RA turnover, tests may be designed to identify cancer-prone individuals. On the basis of this cancer susceptibility marker it can be decided who should be selected for chemoprevention trials. To provide evidence for the genetic background of this retinoid turnover we performed experiments with cultured lymphocytes. Unfortunately, these cells can not be used with this currently used method since we have found that RA metabolism was very low in these cells and we could not found differences between individuals. This is in line with the study of Buck K et al. who reported a lack of synthesis of endogenous RA and a slow turnover of exogenous RA in cultured lymphoblasts (Buck et al. 1991). These authors suggested that lymphocytes use a retinoid metabolic pathway that does not involve the formation of RA.

In summary, in this study we report an enhanced catabolism of RA in normal cells of cancer patients, while the cells appeared morphologically and genetically normal. This suggests that intrinsic factors influenced the turnover rate of RA and that a high RA turnover is a risk factor for the development of HNSCC. The cause of this difference is unknown, but it is tempting to speculate that this will be due to genetic polymorphisms in metabolic enzymes.

Acknowledgments

This work was supported by the Dutch Cancer Society, grant 95-926.

References

Bjelke E (1975) Dietary vitamin A and human lung cancer. *Int J Cancer* 15: 561-5

Byers T, Vena J, Mettlin C, Swanson M, Graham S (1984) Dietary vitamin A and lung cancer risk: an analysis by histologic subtypes. *Am J Epidemiol* 120: 769-76

Coates RJ, Weiss NS, Daling JR, Morris JS, Labbe RF (1988) Serum levels of selenium and retinol and the subsequent risk of cancer. *Am J Epidemiol* 128: 515-23

De Vries N, Van Zandwijk N, Pastorino U (1993) The EUROSCAN study: a progress report. *Am J Otolaryngol* 14: 62-6

de Vries N, van Zandwijk N, Pastorino U (1991) The EUROSCAN Study. EUROSCAN Steering Committee. *Br J Cancer* 64: 985-9

Eckhoff C, Collins MD, Nau H (1991) Human plasma all-trans-, 13-cis- and 13-cis-4-oxoretinoic acid profiles during subchronic vitamin A supplementation: comparison to retinol and retinyl ester plasma levels. *J Nutr* 121: 1016-25

Eckhoff C, Nau H (1990) Identification and quantitation of all-trans- and 13-cis- retinoic acid and 13-cis-4-oxoretinoic acid in human plasma. *J Lipid Res* 31: 1445-54

Fontham ET (1990) Protective dietary factors and lung cancer. *Int J Epidemiol* 19 Suppl 1: S32-42

Giguere V, Ong ES, Segui P, Evans RM (1987) Identification of a receptor for the morphogen retinoic acid. *Nature* 330: 624-9

Hermanek P, Sobin LH (1987) In *TNM Classification of malignant tumours, 4th Edition*, pp 13-26. Springer: Berlin

Hong WK, Lippman SM, Itri LM, Karp DD, Lee JS, Byers RM, Schantz SP, Kramer AM, Lotan R, Peters LJ, Dimery IW, Brown BW, Goepfert H (1990) Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N Engl J Med* 323: 795-801

Knekt P, Aromaa A, Maatela J, Aaran RK, Nikkari T, Hakama M, Hakulinen T, Peto R, Teppo L (1990) Serum vitamin A and subsequent risk of cancer: cancer incidence follow- up of the Finnish Mobile Clinic Health Examination Survey. *Am J Epidemiol* 132: 857-70

Lippman SM, Batsakis JG, Toth BB, Weber RS, Lee JJ, Martin JW, Hays GL, Goepfert H, Hong WK (1993) Comparison of low-dose isotretinoin with beta carotene to prevent oral carcinogenesis. *N Engl J Med* 328: 15-20

Mangelsdorf DJ, Ong ES, Dyck JA, Evans RM (1990) Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* 345: 224-9

Mangelsdorf DJ, Umesono K, Evans RM (1994) The retinoid receptors. In *The Retinoids*, Sporn MM, Roberts AR, Goodman DS (eds) pp 319-49. Raven Press: New York

Petkovich M, Brand NJ, Krust A, Chambon P (1987) A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* 330: 444-50

Tang G, Russell RM (1991) Formation of all-*trans*-retinoic acid and 13-*cis*-retinoic acid from all-*trans*-retinyl palmitate in humans. *J Nutr Biochem* 2: 210-3

Teerlink T, Copper MP, Klaassen I, Braakhuis BJ (1997) Simultaneous analysis of retinol, all-trans- and 13-cis- retinoic acid and 13-cis-4-oxoretinoic acid in plasma by liquid chromatography using on-column concentration after single-phase fluid extraction. *J Chromatogr B Biomed Sci Appl* 694: 83-92

Wahlberg P, Fex G (1996) Retinoic acid concentrations in patients with squamous cell carcinoma of the head and neck. *Eur J Cancer* 32A: 366-7

Wald N, Boreham J, Bailey A (1986) Serum retinol and subsequent risk of cancer. *Br J Cancer* 54: 957-61

Wald N, Idle M, Boreham J, Bailey A (1980) Low serum-vitamin-A and subsequent risk of cancer. Preliminary results of a prospective study. *Lancet* 2: 813-5

Willett WC, Polk BF, Underwood BA, Stampfer MJ, Pressel S, Rosner B, Taylor JO, Schneider K, Hames CG (1984) Relation of serum vitamins A and E and carotenoids to the risk of cancer. *N Engl J Med* 310: 430-4

Yu VC, Naar AM, Rosenfeld MG (1992) Transcriptional regulation by the nuclear receptor superfamily. *Curr Opin Biotechnol* 3: 597-602

Zheng W, Blot WJ, Diamond EL, Norkus EP, Spate V, Morris JS, Comstock GW (1993) Serum micronutrients and the subsequent risk of oral and pharyngeal cancer. *Cancer Res* 53: 795-8

Chapter 9

General discussion

Part of this Chapter will be submitted as a review

General discussion

Retinoids have potential for the prevention of head and neck squamous cell carcinoma (HNSCC). Administration of retinoids has been shown to induce regression of premalignant lesions in the oral cavity and larynx. Furthermore, these compounds are capable of inhibiting the development of second primary tumors (SPTs) in patients curatively treated for HNSCC. Major problems in retinoid chemoprevention are the inter-individual differences in response and in toxicity as well as the transient character of the response. Over the last years it has become clear that inter-individual variation exists with respect to the metabolism of these retinoids. This variation may explain differences in response, in toxicity and in cancer risk. Moreover, the mechanism of action of retinoids involves changes in gene expression mediated by nuclear retinoic acid receptors (RARs and RXRs), which appear to function as transcription factors. Expression of these retinoid receptors may be important in the development, prevention and treatment of HNSCC.

In this thesis retinoid metabolism is described in normal oral keratinocytes of cancer and non-cancer patients, and in HNSCC cells (Chapter 4, 5, 7 and 8). Our observations point to fundamental differences in retinoic acid (RA) metabolism between normal oral keratinocytes and HNSCC cells and may provide clues to a more rational approach for RA intervention during HNSCC carcinogenesis. Furthermore, the differences in RA metabolism between normal keratinocytes of cancer patients and non-cancer patients point to aberrant retinoid metabolism in cancer patients which may explain the higher cancer risk. Aberrations in expression of RAR-γ, which we found to be related to RA-sensitivity in HNSCC cell lines (Chapter 6), and expression of certain CYP26A1 (CYP = cytochrome P450 enzyme) polymorphisms may attribute to low success rates in chemopreventive treatment with retinoids (Chapter 8). In general, our results provide further knowledge about the mechanisms of RA metabolism and provide a basis for the application of retinoids in chemoprevention.

Retinoid blood plasma level as biomarker

In Chapter 3 we described the fate of plasma retinoid levels of patients that received retinyl palmitate (RP)during the EUROSCAN trial. RP is a precursor of retinol and it is in this form that retinoids are normally stored in the liver. Only when needed it is converted to retinol and released in the blood stream (Blomhoff *et al.* 1990). The EUROSCAN Study was designed to test whether RP and N-acethylcysteine could improve the prognosis of patients, most of whom had a history of smoking, treated for

HNSCC or for lung cancer by preventing SPTs (de Vries *et al.* 1991). An earlier, but relatively smaller, study with RP in the same group of patients suggested that this agent had protective activity (Pastorino *et al.* 1993). Further, RP administration can lead to increased 13-cis-RA levels in blood plasma (Tang and Russel 1991), and of this retinoid it is known that it can be active in preventing SPT in HNSCC patients (Hong *et al.* 1990). The outcome of the EUROSCAN trial was, however, disappointing, since no benefit - in terms of survival, event-free survival, or SPTs - in the treated as compared to the untreated patients was found (van Zandwijk *et al.* 2000). The small study described in Chapter 3 of this thesis showed that after one month of daily RP intake the plasma concentration of 13-*cis*-RA increased 5.8-fold and that of 4-oxo-13-*cis*-RA 8.9-fold in 10 patients. After twelve months plasma levels of these two compounds remained high. Thus, although significant amounts of 13-*cis*-RA and 4-oxo-13-*cis*-RA were found in the blood of patients treated with RP, no protective activity could be observed. Possibly intracellular levels of the target cells, which do not reflect those in blood, are too low. However, as stated in Chapter 3, 4-oxo-13-*cis*-RA is a suitable marker to monitor patient compliance of RP intake during chemoprevention trials.

Differences in RA metabolism between normal and malignant cells

In Chapter 5 we described several differences between normal oral keratinocytes and HNSCC cell lines with regard to RA-induced growth inhibition, RA turnover rate and metabolic pathways of RA.

In the medium of HNSCC cells, but not in that of normal keratinocytes, metabolites were found that were identified as the typical oxidation products of RA, such as 4-oxo-RA, 4-oxo-cRA, 4-OH-RA and 18-OH-RA. The presence of these so-called "polar metabolites" strongly indicates the involvement of CYP26A1 activity or that of other CYPs (Ray *et al.* 1997, White *et al.* 1997, Nadin and Murray 1996). Evidence of the involvement of CYP26A1 was found, since mRNA expression of this enzyme could be measured in the majority of the HNSCC cell lines, and not in normal keratinocytes. Furthermore, ectopic overexpression of CYP26A1 increased the formation of polar metabolites 12-fold. These results together suggest that CYP26A1 or analogous enzymes determine the formation of polar metabolites in HNSCC cells. This makes CYP26A1 an interesting target in the treatment of HNSCC. Prolonged high intracellular RA concentrations could be achieved by the inhibition of this enzyme.

The approach of blocking CYPs was already suggested by Van Wauwe and Janssen in 1989, but the clinical application has until now been unsuccessful. Liarozole, which was the first clinically tested RA metabolism blocking agent (RAMBA), demonstrated anti-tumor effects, but did not have an optimal P450 inhibition profile

(Miller 1998). A new RAMBA, called R115866, was recently tested in rodents and was found to inhibit CYP26-dependent RA conversion in a nanomolar range and was about three orders of magnitude more powerful than liarozole (IC_{50} = 3 μM) (Stoppie *et al.* 2000).

There are however be some potential drawbacks of blocking CYP that should be taken into account. At first, there may be a lack of selectivity; prolonged high RA concentrations may have toxic effects on nonmalignant epithelial and liver cells. Second, CYP26A1 may not be the only enzyme responsible for RA catabolism in HNSCC cell lines, since we found CYP26A1 mRNA expression in five of nine cell lines. Furthermore, as we described in Chapter 7, RA can be isomerized and consequently 13-*cis*-RA and 9-*cis*-RA can be catabolized by other enzymes. For these reasons retinoids should be found that are not easily catabolized in HNSCC cells, and have a better (selective) anti-tumor activity.

RA-sensitivity is related to RAR-γ expression.

In HNSCC RAR-γ mRNA expression was found to be frequently reduced compared to normal squamous cells (Hu *et al.* 1991). In Chapter 6, the mRNA expression of four receptor subtypes was investigated in HNSCC cell lines and revealed that RAR-γ expression was related to the level of RA-induced growth inhibition and the level of RA turnover. Our investigations in HNSCC cell lines confirmed other results with RAR-γ (Oridate *et al.* 1996; Sun *et al.* 1999; Le *et al.* 2000).

The results suggest that RA-sensitivity can be enhanced by restoring RAR-γ expression. Thus, one of the approaches could be local gene therapy with RAR-γ in tumor cells to re-activate RA-induced growth inhibition. Another approach may be the use of RAR-γ independent retinoids. It was found that inhibition of cell growth and/or anti-tumor activity could also be achieved through other receptors. LGD1069 (Targretin), for instance, is a retinoid X receptor agonist that modulates cell proliferation (Gottardis *et al.* 1996; Miller *et al.* 1997) and was shown to have an efficacy equivalent to that of tamoxifen in mammary carcinoma with no classic signs of "retinoid-associated" toxicities (Bischoff *et al.* 1998).

Intrinsic variation in RA turnover rate in normal oral keratinocytes between individuals

In Chapter 8 a study is described in which evidence was provided for intrinsic differences between individuals concerning RA turnover rate. Cultured normal oral keratinocytes from cancer patients appeared to have a much higher turnover of RA than normal oral keratinocytes of non-cancer patients. An increased metabolic activity could not be

attributed to permanent alterations as a consequence of exposure to tobacco carcinogens or alcohol, since no relation was found between exposure data and the turnover rate of RA. These results suggest that a genetic factor is involved in retinoid metabolism that may explain an increased risk to develop HNSCC. The variation in RA turnover is likely to be related to a genetic polymorphism of RA catabolizing enzymes of which the most likely candidates are the cytochrome P450 enzymes (CYPs). CYP enzymes represent a large family consisting of more than 100 enzymes in the human body and are involved in the metabolism of many xenobiotics, drugs and carcinogens as well as endogenous compounds, including steroids and retinoids (Guengerich 1989). Genetic polymorphism plays an important role modulating drug interactions with the CYP metabolic enzyme system and several mutant alleles have been described (Bartsch *et al.* 2000, Brockmoller *et al.* 2000, Nair and Bartsch 2001). Environmental factors such as smoking, diet and co-administration of medications might also influence the CYP enzyme activity. By the use of genotyping or phenotyping methods every individual can be classified as either a poor, an intermediate, an extensive or an ultrarapid metabolizer (Van der Weide and Steyns 1999, Tanaka 1999). Having such information will help in determining the appropriate dosage of certain drugs when treating patients with an inherited abnormality of a drug-metabolizing enzyme.

New strategies in retinoid chemoprevention

HNSCC chemoprevention studies have not resulted in a standard treatment protocol for the prevention of SPT. Although some success has been reported with the administration of 13-cis-RA, a high toxicity precluded its further use (Hong *et al.* 1990). A trial with a lower dose of 13-cis-RA will be evaluated in 2002. The administration of RP in the EUROSCAN trial was not able to prevent the occurrence of SPTs (van Zandwijk *et al.* 2000). About this lack of effect can only be speculated, but may be related to the pharmacodynamic shortcoming of the compound, namely to increase intra-cellular levels of the relevant retinoic acids (see chapter 3 of this thesis). A so-called second generation retinoid, etretinate did not prevent the occurrence of SPT, as published by the French GETTEC group in 1996 (Bolla *et al.* 1996). Altogether, it can be concluded that the search for new active retinoids needs to be continued.

A. New synthetic compounds

In addition to the natural retinoids ATRA, 9-*cis*-RA and 13-*cis*-RA, several novel retinoid compounds have been synthesized, and the hope is that these derivatives may be able to provide similar efficacy as 13-*cis*-RA with fewer side effects. Among these novel compounds, the best characterized to date is all-*trans*-N-(4-hydroxyphenyl)retinamide

(4HPR or fenretinide), which is currently under evaluation in clinical trials as a chemopreventive agent against head and neck, and breast cancer (Conley *et al.* 2000) and has been tested in lung, prostate, and bladder cancer (Kurie *et al.* 2000; Thaller *et al.* 2000; Torrisi *et al.* 2000). Fenretinide is a synthetic retinoid that is reported to have less toxic side effects compared to natural occurring retinoids such as RA and 9-*cis*-RA and has been shown to induce apoptosis even in RA-resistant cell lines (Ulukaya and Wood 1999). Other novel synthetic retinoids, such as CD437 and anhydroretinol also have potent receptor-independent apoptosis-inducing activity (Chen *et al.* 1999 , Oridate *et al.* 1997).

Some of the novel synthetic retinoids have selective retinoid receptor activity. ALRT1550, a high-affinity ligand for all three RARs, has shown to have potent anti-tumor activity against human oral squamous carcinoma xenografts in nude mice (Shalinsky *et al.* 1997). LGD1069 (Targretin) is a retinoid X receptor agonist that modulates cell proliferation (Gottardis *et al.* 1996; Miller *et al.* 1997). CD437, which acts in a receptor (RAR-γ) dependent manner, activates and upregulates the transcription factor AP-1, leading eventually to programmed cell death (Sun *et al.* 2000).

B. Combination therapies

Combinations of retinoids with cytokines, such as interferons, receive currently much attention. Studies in cervix cancer and squamous carcinoma of the skin have been successful, with 50% and 68% response rate, respectively (reviewed in Eisenhauer *et al.* 1994). There is evidence to suggest that interferons may modulate the retinoid-signaling pathways by inducing or increasing the expression of RARs or RXRs, rendering cells more sensitive to retinoid actions and even restoring retinoic acid sensitivity in RA-insensitive cells (Marth *et al.* 1986; Fanjul *et al.* 1996).

Another strategy is based on the cooperative effects with agents acting on other steroid hormone receptors, resulting in synergistic activation of the receptor heterodimeric complex. Besides dimerizing with RARs, RXRs can form dimers with many other nuclear receptors of the steroid/thyroid hormone receptor family. This family includes the androgen receptor (AR), the estrogen receptor (ER), the glucocorticoid receptor (GR), the mineralocorticoid receptor (MR), the peroxisome proliferator-activated receptor (PPAR), the progesterone receptor (PR), the thyroid hormone receptor (TR), the vitamin D receptor (VDR), and various orphan receptors. Thus, RXRs may participate in many different signaling pathways and act as important factors in the cross-talk between retinoids and other hormones.

Cooperative effects on growth inhibition using a combination of a retinoid with a vitamin D3 analogue have been observed in several experimental systems, including

lung cancer cells (Higashimoto *et al.* 1996), pancreatic cells (Zugmaier *et al.* 1996) and the HL-60 leukemic cells (Elstner *et al.* 1996). Other strategies are the use of selective estrogen receptor modulators (SERMs), polyamine biosynthesis inhibitors (e.g. difluoromethylornithine), cyclooxygenase-2 (COX) inhibitors and several other agent classes (Hong and Sporn, 1997; Lippman *et al.* 1998).

The discovery of new, synthetic retinoid analogues will enable more effective therapy with less toxicity than the natural occurring retinoids. The evaluation and characterization of their mechanism will also contribute to a better understanding of the head and neck carcinogenic process and is essential for a rational approach in chemopreventive intervention. New agents may be useful in advanced disease or in an adjuvant setting in combination with other steroid hormones, inhibitors of specific signal transduction pathways, or in combination with cytotoxic chemotherapy agents currently in use in the clinic.

References

Bartsch H, Nair U, Risch A, Rojas M, Wikman H, Alexandrov K (2000) Genetic polymorphism of CYP genes, alone or in combination, as a risk modifier of tobacco-related cancers. *Cancer Epidemiol Biomarkers Prev* 9: 3-28

Bischoff ED, Gottardis MM, Moon TE, Heyman RA, Lamph WW (1998) Beyond tamoxifen: the retinoid X receptor-selective ligand LGD1069 (TARGRETIN) causes complete regression of mammary carcinoma. *Cancer Res* 58: 479-84

Blomhoff R, Green MH, Berg T, Norum KR (1990) Transport and storage of vitamin A. *Science* 250: 399-404

Bolla M, Lefur R, Ton Van J, Domenge C, Badet JM, Koskas Y, Laplanche A (1994) Prevention of second primary tumours with etretinate in squamous cell carcinoma of the oral cavity and oropharynx. Results of a multicentric double-blind randomised study. *Eur J Cancer* 30A: 767-72

Brockmoller J, Kirchheiner J, Meisel C, Roots I (2000) Pharmacogenetic diagnostics of cytochrome P450 polymorphisms in clinical drug development and in drug treatment. *Pharmacogenomics* 1: 125-51

Chen Y, Buck J, Derguini F (1999) Anhydroretinol induces oxidative stress and cell death. *Cancer Res* 59: 3985-90

Conley B, O'Shaughnessy J, Prindiville S, Lawrence J, Chow C, Jones E, Merino MJ, Kaiser-Kupfer MI, Caruso RC, Podgor M, Goldspiel B, Venzon D, Danforth D, Wu S, Noone M, Goldstein J, Cowan KH, Zujewski J (2000) Pilot trial of the safety, tolerability, and retinoid levels of N-(4-hydroxyphenyl) retinamide in combination with tamoxifen in patients at high risk for developing invasive breast cancer. *J Clin Oncol* 18: 275-83

de Vries N, van Zandwijk N, Pastorino U (1991) The EUROSCAN Study. EUROSCAN Steering Committee. *Br J Cancer* 64: 985-9

Eisenhauer EA, Lippman SM, Kavanagh JJ, Parades-Espinoza M, Arnold A, Hong WK, Massimini G, Schleuniger U, Bollag W, Holdener EE, *et al.* (1994) Combination 13-cis-retinoic acid and interferon alpha-2a in the therapy of solid tumors. *Leukemia* 8: 1622-5

Elstner E, Linker-Israeli M, Umiel T, Le J, Grillier I, Said J, Shintaku IP, Krajewski S, Reed JC, Binderup L, Koeffler HP (1996) Combination of a potent 20-epi-vitamin D3 analogue (KH 1060) with 9-cis-retinoic acid irreversibly inhibits clonal growth, decreases bcl-2 expression, and induces apoptosis in HL-60 leukemic cells. *Cancer Res* 56: 3570-6

Fanjul AN, Bouterfa H, Dawson M, Pfahl M (1996) Potential role for retinoic acid receptor-gamma in the inhibition of breast cancer cells by selective retinoids and interferons. *Cancer Res* 56: 1571-7

Gottardis MM, Bischoff ED, Shirley MA, Wagoner MA, Lamph WW, Heyman RA (1996) Chemoprevention of mammary carcinoma by LGD1069 (Targretin): an RXR-selective ligand. *Cancer Res* 56: 5566-70

Guengerich FP (1989) Polymorphism of cytochrome P-450 in humans. *Trends Pharmacol Sci* 10: 107-9

Higashimoto Y, Ohata M, Nishio K, Iwamoto Y, Fujimoto H, Uetani K, Suruda T, Nakamura Y, Funasako M, Saijo N (1996) 1 alpha, 25-dihydroxyvitamin D3 and all-trans-retinoic acid inhibit the growth of a lung cancer cell line. *Anticancer Res* 16: 2653-9

Hong WK, Lippman SM, Itri LM, Karp DD, Lee JS, Byers RM, Schantz SP, Kramer AM, Lotan R, Peters LJ, et al (1990) Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N Engl J Med* 323: 795-801

Hong WK and Sporn MB (1997) Recent advances in chemoprevention of cancer. *Science* 278: 1073-7

Hu L, Crowe DL, Rheinwald JG, Chambon P, Gudas LJ (1991) Abnormal expression of retinoic acid receptors and keratin 19 by human oral and epidermal squamous cell carcinoma cell lines. *Cancer Res* 51: 3972-81

Kurie JM, Lee JS, Khuri FR, Mao L, Morice RC, Lee JJ, Walsh GL, Broxson A, Lippman SM, Ro JY, Kemp BL, Liu D, Fritsche HA, Xu X, Lotan R, Hong WK (2000) N-(4-hydroxyphenyl) retinamide in the chemoprevention of squamous metaplasia and dysplasia of the bronchial epithelium. *Clin Cancer Res* 6: 2973-9

Le Q, Dawson MI, Soprano DR, Soprano KJ (2000) Modulation of retinoic acid receptor function alters the growth inhibitory response of oral SCC cells to retinoids. *Oncogene* 19: 1457-65

Lippman SM, Lee JJ, Sabichi AL (1998) Cancer chemoprevention: progress and promise. *J Natl Cancer Inst* 90: 1514-28

Marth C, Daxenbichler G, Dapunt O (1986) Synergistic antiproliferative effect of human recombinant interferons and retinoic acid in cultured breast cancer cells. *J Natl Cancer Inst* 77: 1197-202

Miller WH (1998) The emerging role of retinoids and retinoic acid metabolism blocking agents in the treatment of cancer. *Cancer* 83: 1471-82

Miller VA, Benedetti FM, Rigas JR, Verret AL, Pfister DG, Straus D, Kris MG, Crisp M, Heyman R, Loewen GR, Truglia JA, Warrell RP Jr (1997) Initial clinical trial of a selective retinoid X receptor ligand, LGD1069. *J Clin Oncol* 15: 790-5

Nadin L and Murray M (1996) All-*trans*-retinoic acid 4-hydroxylation in human liver microsomes: *in vitro* modulation by therapeutic retinoids. *Br J Clin Pharmacol* 41: 609-12

Nair U and Bartsch H (2001) Metabolic polymorphisms as susceptibility markers for lung and oral cavity cancer. *IARC Sci Publ* 154: 271-90

Oridate N, Esumi N, Lotan D, Hing WK, Rochette-Egly C, Chambon P, Lotan R (1996) Implication of retinoic acid receptor gamma in squamous differentiation and response to retinoic acid in head and neck SqCC/Y1 squamous carcinoma cells. *Oncogene* 12: 2019-28

Oridate N, Higuchi M, Suzuki S, Shroot B, Hong WK, Lotan R (1997) Rapid induction of apoptosis in human C33A cervical carcinoma cells by the synthetic retinoid 6-[3-(1-adamantyl) hydroxyphenyl]- 2-naphtalene carboxylic acid (CD437). *Int J Cancer* 70: 484-7

Pastorino U, Infante M, Maioli M, Chiesa G, Buyse M, Firket P, Rosmentz N, Clerici M, Soresi E, Valente M, et al (1993) Adjuvant treatment of stage I lung cancer with high-dose vitamin A. *J Clin Oncol* 11: 1216-22

Ray WJ, Bain G, Yao M, Gottlieb DI (1997) CYP26, a novel mammalian cytochrome P450, is induced by retinoic acid and defines a new family. *J Biol Chem* 272: 18702-8

Shalinsky DR, Bischoff ED, Lamph WW, Zhang L, Boehm MF, Davies PJ, Nadzan AM, Heyman RA (1997) A novel retinoic acid receptor-selective retinoid, ALRT1550, has potent antitumor activity against human oral squamous carcinoma xenografts in nude mice. *Cancer Res* 57: 162-8

Stoppie P, Borgers M, Borghgraef P, Dillen L, Goossens J, Sanz G, Szel H, Van Hove C, Van Nyen G, Nobels G, Vanden Bossche H, Venet M, Willemsens G, Van Wauwe J (2000) R115866 inhibits all-trans-retinoic acid metabolism and exerts retinoidal effects in rodents. *J Pharmacol Exp Ther* 293: 304-12

Sun SY, Kurie JM, Yue P, Dawson MI, Shroot B, Chandraratna RA, Hong WK, Lotan R (1999) Differential responses of normal, premalignant, and malignant human bronchial epithelial cells to receptor-selective retinoids. *Clin Cancer Res* 5: 431-7

Sun SY, Yue P, Mao L, Dawson MI, Shroot B, Lamph WW, Heyman RA, Chandraratna RA, Shudo K, Hong WK, Lotan R (2000) Identification of receptor-selective retinoids that are potent inhibitors of the growth of human head and neck squamous cell carcinoma cells. *Clin Cancer Res* 6: 1563-73

Tanaka E (1999) Update: genetic polymorphism of drug metabolizing enzymes in humans. *J Clin Pharm Ther* 24: 323-9

Tang G, Russell RM (1991) Formation of all-trans-retinoic acid and 13-cis-retinoic acid from all-trans-retinyl palmitate in humans. *J Nutr Biochem* 2: 201-13

Thaller C, Shalev M, Frolov A, Eichele G, Thompson TC, Williams RH, Dillioglugil O, Kadmon D (2000) Fenretinide therapy in prostate cancer: effects on tissue and serum retinoid concentration. *J Clin Oncol* 18: 3804-8

Torrisi R and Decensi A (2000) Fenretinide and cancer prevention. *Curr Oncol Rep* 2: 263-70

Ulukaya E and Wood EJ (1999) Fenretinide and its relation to cancer. *Cancer Treat Rev* 25: 229-35

Van der Weide J and Steijns LS (1999) Cytochrome P450 enzyme system: genetic polymorphisms and impact on clinical pharmacology. *Ann Clin Biochem* 36: 722-9

Van Wauwe JP and Janssen PA (1989) Is there a case for P-450 inhibitors in cancer treatment? *J Med Chem* 32: 2231-9

van Zandwijk N, Dalesio O, Pastorino U, de Vries N, van Tinteren H (2000) EUROSCAN, a randomized trial of vitamin A and N-acetylcysteine in patients with head and neck cancer or lung cancer. For the EUropean Organization for Research and Treatment of Cancer Head and Neck and Lung Cancer Cooperative Groups. *J Natl Cancer Inst* 92: 977-86

White JA, Beckett-Jones B, Guo YD, Dilworth FJ, Bonasoro J, Jones G, Petkovich M (1997) cDNA cloning of human retinoic acid-metabolizing enzyme (hP450RAI) identifies a novel family of cytochromes P450. *J Biol Chem* 272: 18538-41

Zugmaier G, Jager R, Grage B, Gottardis MM, Havemann K, Knabbe C (1996) Growth-inhibitory effects of vitamin D analogues and retinoids on human pancreatic cancer cells. *Br J Cancer* 73: 1341-6

Summary

Summary

In **Chapter 1** a general introduction on retinoids and its potential role in HNSCC is provided.

In **Chapter 2** an investigation on the stability and availability of RA and its isomers 13cRA and 9cRA during experimental procedures are described. This study revealed the importance of the choice of culture medium and the addition of serum albumin to prevent loss by adherence of retinoids to plastics. From this study an approach for reliable and reproducible experiments was developed.

In **Chapter 3** a study is reported in which plasma retinoid levels were measured in HNSCC patients before and after one month treatment with retinyl palmitate as compared to control persons. Cancer patients did not differ from control persons with respect to retinoid levels in blood plasma. Medication caused significant elevations of retinol levels (1.2 fold), all-*trans*-RA (2.2 fold), 13-*cis*-RA (5.8 fold) and its metabolite 13-*cis*-4-oxo-RA (8.9 fold). Because of its high levels, 13-*cis*-4-oxo-RA seems a good candidate to assess compliance during chemoprevention trials involving retinoids.

In **Chapter 4** the results of three HNSCC cell lines show that a high turnover rate of RA is associated with a high level of RA-induced growth inhibition. The turnover process coincided with the formation of a group of polar retinoid metabolites, as was measured with high performance liquid chromatography (HPLC) using UV or radioactive detection. Evidence was found that these polar metabolites were not the cause of the RA-induced growth inhibition.

In **Chapter 5** the detailed analysis of RA metabolism and RA-induced turnover is presented for normal oral keratinocytes and HNSCC cell lines. A number of differences have been found between these different cell types. Turnover of RA in HNSCC cell lines was on average 17-fold faster than that of the oral keratinocytes and formation of polar metabolites was only observed in HNSCC cell lines. CYP26A1 (an enzyme known to be involved in retinoid catabolism) was highly inducible in two HNSCC cell lines with the highest RA turnover rates. These observations point to fundamental changes in RA metabolism pathways during HNSCC carcinogenesis and may provide clues to a more rational approach for RA-mediated intervention.

In **Chapter 6** a study is reported on the relation between mRNA expression of nuclear retinoid receptors (RAR-α, -β and -γ and RXR-β) and the RA-induced growth inhibition in five HNSCC cell lines. In some cell lines reduced expression of RAR-β and/or RAR-γ was observed, but only for RAR-γ an association was found with RA-induced growth inhibition in these cell lines. These findings suggest that in HNSCC cell lines RAR-γ is the most important retinoid receptor for regulation of RA turnover rate and RA-induced growth inhibition.

In **Chapter 7** a study is shown on the growth inhibition and turnover rate of four retinoid compounds (retinol, ATRA, 13-*cis*-RA and 9-*cis*-RA) in four HNSCC cell lines and normal oral keratinocyte cultures. All retinoids were similar with respect to their capacity to induce growth inhibition and the rate at which the cells were able to catabolize the retinoid. A cellular metabolism resulted in a pattern of metabolites that is unique for each retinoid and not cell line dependent. Our findings indicate that the differences in retinoid receptor selectivity of these compounds does not influence the level of growth inhibition or rate of metabolism.

In **Chapter 8** an analysis is described of RA turnover in normal oral keratinocytes cultures of cancer patients (OKC-T) versus that of non-cancer patients (OKC-N). LOH analysis confirmed the non-malignant character of the OKC-T cultures. Interestingly, turnover of RA in keratinocytes originating from cancer patients was on average 15 times faster. The type of metabolites was the same as observed in OKC-N. These results suggest the involvement of a genetic factor in retinoid metabolism, which may be related to an increased risk for HNSCC. A polymorphism of the CYP26A1 gene may form the basis of the observed variation.

Nederlandse samenvatting

Nederlandse samenvatting

Het onderzoek in dit proefschrift betreft de werking van vitamine A-zuur, een metaboliet van vitamine A, bij hoofd-hals plaveiselcelcarcinoom (HHPCC). Tabak en alcohol zijn de voornaamste risicofactoren voor het ontstaan van plaveiselcelcarcinomen in het slijmvlies van de bovenste lucht- en voedselweg, terwijl ook genetische aanleg een belangrijke rol speelt. Wanneer deze tumoren in een vroeg stadium worden ontdekt, kunnen zij met behulp van chirurgie of bestraling meestal curatief behandeld worden. Patiënten met tumoren in een meer gevorderd stadium hebben een slechte prognose: meer dan de helft van hen overlijdt binnen vijf jaar ten gevolge van lokaal/regionaal recidief of metastasen op afstand. Daarnaast bestaat bij patiënten, die genezen zijn van een HHPCC een relatief groot risico op het ontwikkelen van een tweede primaire tumor in hetzelfde anatomische gebied, de slokdarm of de longen. Gebleken is dat 2 à 3% van de patiënten per jaar zo'n tweede primaire tumor ontwikkelt. Omdat deze tweede primaire tumoren ontstaan in reeds behandeld gebied of op notoir ongunstige plaatsen, hebben deze patiënten een slechte prognose.

Het ontstaan van tweede primaire tumoren is mogelijk te voorkomen met chemopreventieve therapie (het toedienen van middelen die het ontstaan van kanker remmen of voorkomen). Omdat vele onderzoeken uitgewezen hebben dat het eten van groenten en fruit een beschermende werking tegen kanker heeft, werden vitaminen gezien als goede kandidaten voor het voorkomen van deze tweede primaire tumoren. Afgeleide stoffen van vitamine A, retinoïden genoemd, zijn in HHPCC patiënten als chemopreventieve middelen het meest onderzocht.

Hong en medewerkers publiceerden in 1990 dat een retinoïde in staat was de ontwikkeling van tweede primaire tumoren in hoofd-hals kanker patiënten te remmen. Patiënten die genezen waren van een primaire tumor kregen 13-*cis*-RA (een isomeer en analoog van vitamine A-zuur; retinoic acid, RA) voorgeschreven. Van de 49 behandelde patiënten kregen slechts 2 een tweede primaire tumor, terwijl dit het geval was voor 12 van de 51 personen van de onbehandelde controle groep. Er dient opgemerkt te worden dat er kritiek op deze studie was en dat deze behandeling niet algemeen ingang heeft gevonden. Hoe dan ook, uit deze studie blijkt dat deze therapie niet voor elke patiënt succesvol is en dat bij een relatief groot aantal patiënten ernstige bijwerkingen optreden. Er zijn ondertussen resultaten van ander klinisch onderzoek met retinoïden bekend geworden, weliswaar met een lagere toxiciteit, maar helaas zonder effect op het ontstaan van tweede primaire tumoren. Voor het veilig en effectief toepassen van retinoïden is het van belang om te weten wat het verschil in werking van

deze stoffen tussen patiënten veroorzaakt. Vervolgens zou er voor elke patiënt een individueel aangepaste therapie ontworpen kunnen worden. Carcinogenese is een meerstaps proces, waarbij fouten in het DNA zich ophopen gedurende de ontwikkeling van normale plaveiselcellen tot een kwaadaardige tumor. Om tot een efficiëntere behandeling te komen is het van belang om te bepalen gedurende welke stappen het nog mogelijk is met behulp van retinoïden het proces van carcinogenese te remmen.

In dit proefschrift wordt een onderzoek beschreven dat nagaat of er verschillen zijn in de werking van retinoïden tussen individuen en of deze verschillen veranderen gedurende het kankerproces. Om deze vragen te beantwoorden zijn cellen in kweek gebracht van HHPCC en van normale plaveiselcellen afkomstig van zowel HHPCC-patiënten als van patiënten zonder HHPCC. In deze cellen is bepaald: de gevoeligheid voor retinoïden, de snelheid van afbraak van retinoïden en de expressie van verschillende genen die hierbij betrokken zijn.

HOOFDSTUK 2

Een belangrijke voorwaarde voor *in vitro* onderzoek is dat experimenten betrouwbaar en reproduceerbaar gedaan kunnen worden. Bij de experimenten in normale cellen deden zich in vergelijking met tumorcellijnen nogal eens merkwaardige verrassingen voor die niet goed verklaard konden worden. Hierbij moest worden aangenomen dat retinoïden door plastic werden geabsorbeerd of niet stabiel bleven. In dit hoofdstuk wordt een studie beschreven naar de stabiliteit en beschikbaarheid tijdens de experimenten van all-*trans*-vitamine A-zuur (all-*trans*-retinoic acid, ATRA) en zijn isomeren (analoge stoffen) 13-*cis*-RA en 9-*cis*-RA. Hierin wordt onder meer aangetoond hoe belangrijk de keuze van het celkweekmedium is en dat de toevoeging van eiwit aan het medium essentieel is. Binding aan eiwitten, zoals albumine, zorgt ervoor dat retinoïden stabiel blijven en er geen verlies optreedt door binding aan plastic. Uit deze studie werd een aanpak ontwikkeld voor betrouwbare en reproduceerbare experimenten met retinoïden.

HOOFDSTUK 3

In dit hoofdstuk wordt een studie beschreven waarbij plasma concentraties van retinoïden werden gemeten bij HHPCC patiënten die in het kader van de EUROSCAN trial retinylpalmitaat (vitamine A) innamen. De EUROSCAN trial was een grote gerandomiseerde trial die het effect onderzocht van retinylpalmitaat, N-acetylcysteine of een combinatie van deze beide stoffen op het ontstaan van tweede primaire tumoren bij patiënten die recent curatief behandeld werden voor HHPCC (of longkanker). Patiënten die in deze trial retinylpalmitaat innamen, toonden na één maand behandeling

een opmerkelijke stijging in het plasma van de concentratie van 13-*cis*-4-oxo-retinoic acid, een product van ATRA. 13-*cis*-4-oxo-retinoic acid blijkt een marker te zijn die het mogelijk maakt te volgen of een patiënt trouw het geneesmiddel slikt.

HOOFDSTUK 4

Hoofdstuk 4 behandelt een studie waarin een relatie is gevonden tussen het groei-remmend effect van ATRA en de snelheid van afbraak van ATRA in drie HHPCC cellijnen. Gedurende de afbraak van ATRA worden producten gevormd die "polaire metabolieten" worden genoemd. Aanwijzingen werden gevonden dat de afbraak tot deze polaire metabolieten niet een oorzaak, maar een gevolg is van de groei-remmende activiteit van ATRA. Cellen die gevoelig zijn voor ATRA stellen zich - blijkbaar vergeefs - teweer door het af te breken.

HOOFDSTUK 5

In dit hoofdstuk wordt een onderzoek beschreven betreffende verschillen in metabolisme van retinoïden tussen normale mondholte-epitheelcellen (keratinocyten) van patiënten zonder HHPCC en HHPCC-cellen. Kankercellen bleken ATRA veel sneller om te zetten (17 keer) dan keratinocyten en tevens bleken keratinocyten geen polaire metabolieten te vormen. Een verklaring hiervoor kan zijn dat keratinocyten de polaire metabolieten zo snel omzetten dat ze niet waarneembaar zijn. Aanwijzingen werden gevonden dat normale keratinocyten een heel andere metabolische route voor de afbraak van ATRA gebruiken.

Recentelijk is een nieuw enzym ontdekt, CYP26, dat zeer specifiek ATRA afbreekt tot polaire metabolieten. Ook in de onderzochte cellen bleek dit enzym een grote rol te spelen. Zo kwam het enzym alleen voor in cellen waarbij polaire metabolieten detecteerbaar waren, zijnde de tumorcellen, en het meest in de cellijnen met het hoogste metabolisme.

Net als in het onderzoek met drie HHPCC cellijnen (hoofdstuk 4) werd in het onderzoek van dit hoofdstuk in elf HHPCC cellijnen een significante correlatie gevonden tussen de omzettingssnelheid van ATRA en het groeiremmende effect van ATRA. Introductie van CYP26 DNA in een HHPCC cellijn resulteerde in een sterke toename van de ATRA omzetting en de vorming van polaire metabolieten. Er trad echter geen verandering op in het groeiremmend effect van ATRA.

HOOFDSTUK 6

Omdat uit de resultaten van hoofdstuk 5 bleek dat de groeiremming van ATRA waarschijnlijk niet oorzakelijk gerelateerd is aan zijn metabolisme, werd naar een

andere verklaring gezocht voor het gevonden verschil tussen de HHPCC cellijnen. Het is bekend dat veel cellulaire processen, bijvoorbeeld groei en differentiatie, door ATRA via retinoïd-specifieke kernreceptoren gereguleerd worden. Er zijn twee typen receptoren, RAR en RXR. Van elk type zijn weer 3 subtypen (α, β, γ). Uit het in dit hoofdstuk beschreven onderzoek kan geconcludeerd worden dat de mate van groeiremming door ATRA en de snelheid van afbraak van ATRA gerelateerd zijn aan de expressie van RAR-γ. Dit suggereert dat de aanwezigheid van RAR-γ een belangrijke factor is voor de effectiviteit van de groeiremmende en daardoor chemopreventieve werking van retinoïden. Het bleek namelijk dat dit receptor-subtype alleen tot expressie kwam in de cellijnen, die sterk in de groei geremd werden en niet in de ongevoelige cellijnen. Gebruikmakend van deze kennis zou gentherapie met RAR-γ in tumorcellen kunnen resulteren in een herstelde gevoeligheid voor ATRA en zouden retinoïden zelfs als chemotherapeutica toegepast kunnen worden.

HOOFDSTUK 7

Om te onderzoeken of ook andere derivaten dan ATRA actief zijn of een andere omzettingssnelheid vertonen, werden vier aan ATRA gerelateerde retinoïden *in vitro* getest op zowel vier HHPCC cellijnen als op normale keratinocyten. Deze retinoïden binden elk met een verschillende affiniteit aan de retinoïd receptoren en zouden daardoor andere effecten kunnen veroorzaken. Er bleek echter geen verschil in activiteit of omzettingssnelheid te bestaan tussen de verschillende retinoïden. Wel werden er afbraakproducten gevonden die per retinoïde verschilden. Dit wijst erop dat er retinoïde isomeer-specifieke enzymen bestaan. Uit recentelijk onderzoek is gebleken dat CYP26 alleen ATRA kan afbreken en niet 13-*cis*-RA of 9-*cis*-RA; enzymen voor deze laatste twee zijn nog niet beschreven.

HOOFDSTUK 8

Eén van de hypothesen van dit onderzoeksproject was dat normale plaveiselcellen het mondslijmvlies van HHPCC-patiënten een andere ATRA gerelateerde groeiremming hebben dan die van personen zonder kanker en dat dit veroorzaakt wordt door variatie in retinoïd metabolisme. De keratinocyten van HHPCC-patiënten bleken gemiddeld een veel hoger metabolisme te hebben dan die van controle personen. Qua gevoeligheid was er nauwelijks een verschil tussen de twee populaties. Op grond van literatuur-gegevens mag aangenomen worden dat ATRA de ontaarding van normale cellen tot kankercellen onderdrukt. Wanneer ATRA sneller dan normaal afgebroken wordt, zou dit een verhoogd risico op het ontstaan van hoofd-hals kanker kunnen

verklaren. De basis van dit verschil in afbraak is mogelijk terug te voeren op een verschil in genetische aanleg en dit zou kunnen verklaren waarom slechts een gedeelte van de bevolking een HHPCC ontwikkelt.

Het onderzoek in dit proefschrift werd uitgevoerd om verschillen te identificeren tussen personen, in het bijzonder tussen individuen met en zonder kanker en tussen kankerpatiënten onderling met als doel betere strategieën te ontwerpen voor chemopreventie. De uitkomsten van dit onderzoek hebben voornamelijk geleid tot een beter begrip van de werking van retinoïden in de verschillende stadia in de ontwikkeling van hoofd-hals kanker. Als effectieve chemopreventieve middelen blijken retinoïden echter nog niet direct toepasbaar. De gegevens in dit proefschrift suggereren dat tumorcellen in staat zijn zich teweer te stellen tegen de groeiremmende effecten van retinoïden middels verschillende tactieken, zoals een verhoogd metabolisme of inactivering van retinoïde receptoren, beide leidend tot resistentie voor retinoïden. Het verhoogde ATRA metabolisme in normale cellen van kankerpatiënten ten opzichte van controle personen zonder kanker suggereert dat er een aanleg voor ATRA-resistentie bestaat. Het is nog te vroeg om over te gaan tot het ontwikkelen van geïndividualiseerde therapieën, gezien de grote complexiteit van het retinoïden metabolisme en de overlap met andere metabolische routes (bijvoorbeeld die van vitamine D en stikstofoxide) en zeker nu blijkt dat kankerpatiënten in feite resistenter tegen retinoïden zijn dan gezonde controles. Het gebruik van gentherapie met RAR-γ zou wel een aantrekkelijke aanpak kunnen zijn wanneer de voordelen ervan boven die van chirurgie en bestraling gaan uitstijgen. Ook zou het meten van de aanwezigheid van RAR-γ in premaligne weefsels van waarde kunnen zijn om een succesvolle behandeling met retinoïden te voorspellen. Nieuwe strategieën die gebruik maken van retinoïden chemopreventie richten zich tegenwoordig voornamelijk op synthetische varianten van retinoïden en op combinaties met andere steroïde hormonen, remmers van specifieke signaal transductie routes, of cytotoxische chemotherapeutica.

Dankwoord

Onderzoek is bijna nooit af, maar dit boekje nu gelukkig wel. Al met al heeft het iets langer geduurd dan vier jaar. "Better let as net", zoals de Friezen zeggen. Terugdenkend aan de afgelopen jaren realiseer ik mij dat dit boekje er nooit was geweest zonder de steun en inzet van velen. Daarom wil ik iedereen bedanken die heeft meegeholpen aan de totstandkoming van dit proefschrift. Een aantal mensen wil ik graag in het bijzonder noemen.

Mijn promotor prof. Snow, bedankt voor het vertrouwen dat u in mij hebt gesteld en de mogelijkheid die u mij hebt gegeven om binnen de afdeling KNO te mogen promoveren en voor het altijd zeer nauwkeurige nakijken van mijn manuscripten.

Mijn directe begeleider Boudewijn Braakhuis. Boudewijn, bedankt voor je jarenlange steun, vertrouwen en kritische blik. Door die combinatie van kritisch analyseren en je vrolijke gemoed (giegel giegel, wat zat er nou eigenlijk in die koffie?) heb ik het altijd erg prettig gevonden met je samen te werken. Ook de contacten die je door de jaren in het wetenschappelijk gebied had opgebouwd zijn goed van pas gekomen. Alhoewel het vroegtijdig doen uitlekken van onderzoeksresultaten soms niet zo handig was (OK, het is je vergeven...).

Mijn begeleider op moleculair biologisch gebied Ruud Brakenhoff. Bedankt voor het altijd kritisch beoordelen van het onderzoek, voor je grote expertise en kennis en het altijd weer opnieuw aanleveren van nieuwe ideeën. Voor alle geduld en tijd die je hebt genomen om in urenlange discussies (en monologen) de onderzoeksresultaten te beoordelen en je enthousiasme ("Dit wordt een Nature-artikel!!!").

Mijn analist en paranimf Serge Smeets. Serge, aan jou ben ik de meeste dank verschuldigd. Door jou fantastische inzet en alle overuren die je gemaakt hebt zou het boekje zeker niet zo dik zijn geworden. Het doorzettingsvermogen dat je hebt getoond om alle technieken aan de praat te krijgen, de toewijding aan het verzorgen van de celletjes en het steeds weer opnieuw herhalen van experimenten (want één experiment is geen experiment, en twee experimenten is een half experiment), daar heb ik erg veel respect voor! Aan onze vriendschappelijke samenwerking en je ongelofelijke verhalen denk ik met veel plezier terug.

Jacqueline Cloos, bedankt voor al je hulp en interesse in mijn onderzoek (Sodeju! Wat ingewikkeld), voor je pep-talks en je relativeringsvermogen. Met veel ontzag heb ik kunnen meemaken hoe je altijd voor mij en andere mensen klaar stond, ondanks dat je vaak ook zelf teleurstellingen moest verwerken.

Binnen de afdeling KNO heb ik veel mensen zien komen en gaan. Het is onmogelijk om iedereen te noemen. Marijke wil ik bedanken voor de logistiek op de celkweek en de immunohistochemie en ik wens Viola, Iris, Maarten T en Maarten V veel succes met promoveren. In het algemeen wil ik zeggen: iedereen bedankt voor de leuke tijd op het lab en tijdens de labuitjes, de etentjes het schaatsen en de zeilweekenden.

Natuurlijk wil ik ook mijn studenten Nynke, Jorrit en Geeske bedanken Jullie hebben elk goed werk verricht en belangrijk bijgedragen aan dit proefschrift. Bedankt voor jullie inzet.

De resultaten in dit proefschrift zijn in belangrijke mate mede tot stand gekomen door samenwerking met andere afdelingen binnen de VU en daarbuiten. Tom Teerlink wil ik bedanken voor het opzetten van een specifiek voor retinoïden bedoelde HPLC-methode, die voor erg mooie plaatjes heeft gezorgd; Bas-Jan van der Leede en Edwin Sonneveld, van het Hubrecht Lab, voor de radioactieve HPLC-analyse van retinoïde metabolieten. Henk Dekker en Paul Noordhuis voor het delen van de Radiomatic. Kim Creek (University of South Carolina School of Medicine, Columbia), Rueben Lotan (MD Anderson Cancer Center, Houston), Luigi DeLuca (NIH, Bethesda): thanks for your visits, invitations, revision of manuscripts and interest in our projects. It was very nice to discuss retinoid research with experts.

Ik ben prof.dr. G. Giaccone, prof.dr. R. J. Scheper, prof.dr. P. E. Postmus, dr. G.J. Peters, dr. T. Teerlink en dr. P. J. F. Snijders en zeer erkentelijk voor hun bereidheid zitting te nemen in mijn promotiecommissie en voor hun interesse in mijn proefschrift.

Special thanks to the "crazy german" Ralphiii for the opportunity to work as a post-doc in Potsdam and for his hospitality. Great retinoid research and friendship ("I hate you!" ;-)). Greetings to all the people of the lab (I'll be back).

Naast mijn werk heb ik ook een hele goede tijd gehad in Amsterdam, dankzij vele vrienden, vriendinnen, kennissen en vage bekenden. Mijn paranimfe Suzan (zal zij de commissie weten af te leiden?), Annelies (lekker belangrijk), Marcel, Ieke-Wally (biertje?), Mariëtte, Hadewey, Nynke (butter, brea en griene tsiis?) Ilonka en Dennis (bedankt voor het maken van de mooie foto's voor de omslag!) en anderen die dit boekje van mij hebben ontvangen: bedankt voor de gezelligheid en de interesse die jullie af en toe hadden voor mijn onderzoek, ook al begrepen jullie er geen hout van...

De belangrijkste mensen heb ik bewaard voor het laatst: Alie, Ben, Friso en Anton. Jullie belangstelling en hulp waardeer ik zeer. Papa en Mama, bedankt voor jullie steun en dat jullie altijd achter mij hebben gestaan.

Lieve Anton, bedankt voor alle interesse en geduld die je hebt gehad en voor het koken en afwassen de laatste tijd. We hebben een mooi huisje en het samenwonen bevalt prima, and you know what: I'm happy!!!

Curiculum vitae

Ingeborg Klaassen werd geboren op 13 juni 1970 te Rotterdam. In 1988 behaalde zij haar VWO diploma aan het Nassau College te Heerenveen, waarna zij begon aan haar studie Biologie aan de Rijks*universiteit* Groningen.

Tijdens haar studie voltooide zij drie stages. Als eerste stage deed ze onderzoek naar de opsporing van Small Cell Lung Cancer (SCLC) bij de afdeling Immunologie aan het Academisch Ziekenhuis Groningen (prof.dr. L. de Leij). In haar tweede stage bij de afdeling Biochemie aan de Rijksuniversiteit Groningen bestudeerde ze signaaltransductie in Dictyostelium *discoideum* (prof.dr. P.J. Van Haastert). Haar derde stage werd uitgevoerd bij TRANSGENE Biotechnology in Straatsburg (Frankrijk) waar ze onderzoek verrichtte aan de ontwikkeling van melkzuurbacteriën als 'live vaccine vehicle' voor immunisatie tegen het HIV virus (dr. A. Mercenier).

Na haar afstuderen heeft ze eerst een half jaar bij de afdeling Moleculaire Genetica aan de Rijksuniversiteit Groningen gewerkt om de signaaltransductie in Bacillus *subtilis* te bestuderen (prof.dr. G. Venema).

In juni 1995 is ze begonnen bij de sectie Tumorbiologie van de afdeling Keel- Neus- en Oorheelkunde van het *Vrije Universiteit* Medisch Centrum te Amsterdam met haar promotieonderzoek beschreven in dit proefschrift. Het onderzoek werd uitgevoerd in het kader van het KWF-project "Determinants of retinoid sensitivity in the prevention and treatment of head and neck squamous cell carcinoma" (VU 95-926).

Gedurende de eerste helft van 2001 heeft ze een half jaar als (pre)post-doc in Potsdam (Duitsland) gewerkt bij het Institut für Ernährungswissenschaft aan de Universiteit van Potsdam, waar ze onderzoek verrichtte aan de effecten van retinoïden op embryonale ontwikkeling en reproductieve organen.

Publications

Braakhuis BJ, **Klaassen I**, van der Leede BM, Cloos J, Brakenhoff RH, Copper MP, Teerlink T, Hendriks HF, van der Saag PT, Snow GB (1997) Retinoid metabolism and all-trans retinoic acid-induced growth inhibition in head and neck squamous cell carcinoma cell lines. *Br J Cancer* 76: 189-97

Cooper MP, **Klaassen I**, Brakenhoff RH, Cloos J, Snow GB, Braakhuis BJ (1997) All-trans retinoic acid induced gene expression and growth inhibition in head and neck cancer cell lines. *Oral Oncol* 33: 270-4

Teerlink T, Copper MP, **Klaassen I**, Braakhuis BJ (1997) Simultaneous analysis of retinol, all-trans- and 13-cis-retinoic acid and 13-cis-4-oxoretinoic acid in plasma by liquid chromatography using on-column concentration after single-phase fluid extraction. *J Chromatogr B Biomed Sci Appl* 694: 83-92

Klaassen I, Copper MP, Brakenhoff RH, Smeets SJ, Snow GB, Braakhuis BJ (1998) Exfoliated oral cell messenger RNA: suitability for biomarker studies. *Cancer Epidemiol Biomarkers Prev* 7: 469-72

Copper MP, **Klaassen I**, Teerlink T, Snow GB, Braakhuis BJ (1999) Plasma retinoid levels in head and neck cancer patients: a comparison with healthy controls and the effect of retinyl palmitate treatment. *Oral Oncol* 35: 40-4

Klaassen I, Brakenhoff RH, Smeets SJ, Snow GB, Braakhuis BJ (1999) Considerations for in vitro retinoid experiments: importance of protein interaction. *Biochim Biophys Acta* 1427: 265-75

Klaassen I, Brakenhoff RH, Smeets SJ, Snow GB, Braakhuis BJ (2001) Enhanced turnover of all-trans-retinoic acid and increased formation of polar metabolites in head and neck squamous cell carcinoma lines compared with normal oral keratinocytes. *Clin Cancer Res* 7: 1017-25

Klaassen I, Brakenhoff RH, Smeets SJ, Snow GB, Braakhuis BJ (2001) Expression of retinoic acid receptor gamma correlates with retinoic acid sensitivity and metabolism in head and neck squamous cell carcinoma cell lines. *Int J Cancer* 92: 661-5

Klaassen I, Brakenhoff RH, Smeets SJ, Snow GB, Braakhuis BJ (2001) Metabolism and growth inhibition of four retinoids in head and neck squamous normal and malignant cells. *Br J Cancer* 85: 630-5

Klaassen I, Cloos J, Smeets SJ, Brakenhoff RH, Tabor MP, Snow GB, Braakhuis BJM. Non-malignant oral keratinocytes of head and neck squamous cell carcinoma patients show enhanced metabolism of retinoic acid. *Submitted*

Klaassen I, Brakenhoff RH, Braakhuis BJM. Determinants of Retinoid Sensitivity in Head and Neck Squamous Cell Carcinoma. *In preparation*