

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 Keratinocytes¹

Ingeborg Klaassen, Ruud H. Brakenhoff,
Serge J. Smeets, Gordon B. Snow, and
Boudewijn J. M. Braakhuis²

Department of Otolaryngology/Head and Neck Surgery, Section
Tumor Biology, University Hospital, Vrije Universiteit, 1007 MB
Amsterdam, the Netherlands

ABSTRACT

Retinoids show promise in the treatment of various (pre)malignancies, including head and neck squamous cell carcinoma (HNSCC). Previous studies have shown that the metabolic pathways of retinoids are important in the anti-cancer 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 high-performance liquid chromatography and conducted growth inhibition assays. We demonstrate here that in contrast to normal oral keratinocytes, HNSCC cell lines: (a) had averaged a 17-fold greater turnover rate of all-*trans*-retinoic acid (RA); (b) had a 1.9-fold less RA-induced growth inhibition; (c) were able to form polar metabolites; and (d) 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 an 11-fold higher turnover rate of RA and a 12-fold increase in the amount of polar metabolites, but it 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 (1, 2). When it was found that vitamin A deficiency resulted in hyperplasia and hyperkeratosis of oral mucosa (3), the interest in retinoids in relation to oral carcinogenesis increased. Retinoids were demonstrated to reverse malignant growth *in vitro* and *in vivo* (4, 5) and were effective as chemopreventive agents (6). Retinoids could successfully be used to treat oral leukoplakia (7), a potentially premalignant mucosal lesion, and the occurrence of second primary tumors following HNSCC³ could be inhibited or delayed (8). These second primary tumors, which occur at an incidence rate of 2–3% per year, are a major cause of death after surgical resection of early-stage head and neck cancer (9). 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 (10). 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 (11). In the plasma of the patients with remission, increased levels of oxidized retinoid metabolites like 4-OH-RA could be detected. Recently a RA-inducible *CYP* gene, *CYP26A1*, was discovered (12, 13) that has high specificity for all-*trans*-RA (14). 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 (13). These findings suggest that *CYP26A1* is the key enzyme for catabolism of RA to 4-hydroxylated products,

Received 9/26/00; revised 11/20/00; accepted 1/22/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This study was supported by the Dutch Cancer Society, Grant 95-926.

² To whom requests for reprints should be addressed, at Section Tumor Biology, Department of Otolaryngology/Head and Neck Surgery, University Hospital, Vrije Universiteit, P.O. Box 7057, 1007 MB Amsterdam, the Netherlands. Phone: 31-20-4440905; Fax: 31-20-4440983; E-mail: bjm.braakhuis@azvu.nl.

³ The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; CYP, cytochrome P450; RA, all-*trans*-retinoic acid; 4-OH-RA, 4-hydroxy-all-*trans*-retinoic acid; 18-OH-RA, 18-hydroxy-all-*trans*-retinoic acid; 4-oxo-RA, 4-oxo-all-*trans*-retinoic acid; 4-oxo-cRA, 4-oxo-13-*cis*-retinoic acid; 5,6-epoxy-RA, 5,6-epoxy-all-*trans*-retinoic acid; KGM, keratinocyte growth medium; RAR, retinoic acid receptor; RXR, retinoid X receptor; OKC, oral keratinocyte culture; HPLC, high performance liquid chromatography.

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 (14, 15). Earlier work of Randolph and Simon (16) 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 (17) 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. Ulf W. Wiegand (Hoffmann-la Roche, Basel, Switzerland); 4-hydroxy-RA and 18-hydroxy-RA were gifts from Dr. Louise H. Foley (Roche Pharmaceuticals, Nutley, NJ). All compounds were dissolved in 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 $<0.1\%$ and did not affect cell growth. $[11, 12\text{-}^3\text{H(N)}]\text{all-}trans\text{-RA}$ (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 by plastics (18).

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 (19) in six-well culture plates in KGM (Life Technologies, Inc., Paisley, United Kingdom) to which growth supplements, gentamicin sulfate (final concentration, 5 $\mu\text{g/ml}$), and amphotericin B (final concentration, 0.5 $\mu\text{g/ml}$) were added (all from Life Technologies, Inc.). Primary cultures of keratinocytes were subcultured at 70% confluence in a dilution of 10^5 cells/well and used for experiments at passage 3. Human HNSCC cell lines UM-SCC-35, -22A and -14C were provided by Dr. Thomas E. Carey (University of Michigan, Ann Harbor, MI) and are described elsewhere (20). 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 (21). The cell line VU-SCC-OE was established from human HNSCC xenografts in our laboratory (22). HNSCC cells were maintained in DMEM (Life Technologies, Inc.) supplemented with 50 units/ml penicillin, 50 $\mu\text{g/ml}$ streptomycin (penicillin/streptomycin; Life Technologies, Inc.), and 5% FCS (ICN Biomedicals, Irvine, United Kingdom).

Growth Inhibition Assays. The dose response effect on cell proliferation was determined with the SRB-assay. Details of the assay have been described previously (23). In short, cells ($1000\text{--}4000$ per well) were plated in 96-well plates in DMEM/5% FCS or KGM/0.1% BSA for HNSCC cell lines or OKCs, 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 a 72-h incubation (the "log phase") by staining the cellular protein with sulforhodamine B (SRB; Sigma) 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

Keratinocytes and HNSCC cells were plated at a density of 10^5 cells/well in six-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 (18) 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 PBS 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 (24). In short, to each sample, 50 μl of 1 M sodium acetate buffer (pH 4.0) and 600 μl of acetonitrile were added, and after vortex-mixing, the samples were centrifuged for 2–5 min at $3000 \times g$. The 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 of 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 HPLC (Gynkotek, Munich, Germany) 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 (100×4.6 mm) from Phase Separations (Deeside, United Kingdom) maintained at 30°C .

Composition of the mobile phases and the binary gradient used were as described by Eckhoff and Nau (25). UV detection was performed at 340 nm. Retinoids were identified using external standards. RA concentrations were quantified on the basis of 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; Ref. 26).

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 >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/mg protein/h.

Northern Blotting

Of each sample, 20 μ g of total RNA was separated on a 1% (w/v) formaldehyde agarose gel in 3-(*N*-morpholino)propane-sulfonic acid buffer and transferred to a Qiabrine 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 $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ (pH 7.2), and 1 mM EDTA and hybridized overnight at 65°C in the same solution after the addition of 25 ng of 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. A 28S rRNA oligo was labeled with [γ - ^{32}P]ATP, as described by Sambrook *et al.* (27), to monitor the amount of RNA loaded on the gel. The blot was washed twice with $2\times$ SSC-0.1% SDS and twice with $0.2\times$ 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 six-well plates in DMEM containing 10% FCS. Transfection was performed using Lipofectin reagent (Life Technologies, Inc.) following the manufacturer's protocol. To prevent clonal selection, transient CYP26A1 transfectants were generated. Cells were transfected at 50–70% confluence with 10 μ g of pcDNA3/P450RAI expression vector (a gift of Dr. M. Petkovitch, Cancer Research Laboratories, Queens University, Kingston, Ontario, Canada), designated as pCYP26 and, as a control, 10 μ g of pcDNA3 expression vector (Invitrogen, Carlsbad, CA). After 4 h, medium was refreshed, and 24 h later geneticin (500 μ g/ml; G418; Life Technologies, Inc.) was added to the cells. Resistant colonies were pooled 2 weeks later and cultured under prolonged G418 selection.

RESULTS

Turnover Rate and Response to RA in HNSCC Cell Lines and Oral Keratinocytes. The turnover rate of RA of 11 HNSCC cell lines and 11 OKCs obtained from healthy individ-

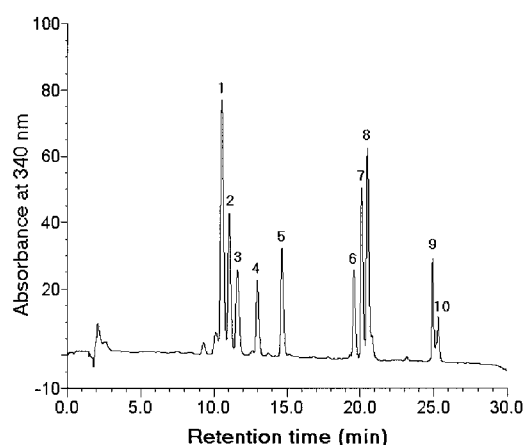


Fig. 1 HPLC profile of retinoid standard dissolved in water. Peak 1, 4-oxo-RA; peak 2, 4-oxo-cRA; peak 3, 4-OH-RA; peak 4, 18-OH-RA; peak 5, 5,6-epoxy-RA; peak 6, 13-cis-RA; peak 7, 9-cis-RA; peak 8, all-trans-RA; peak 9, retinol; and peak 10, retinal.

uals was determined subsequent to treatment with 1 μ M RA for 4–72 h. Retinoids were analyzed by reverse-phase HPLC with UV detection at 340 nm and identified using external standards (Fig. 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 Fig. 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. 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 OKCs, the rate of RA metabolism was, on average, ~ 17 -fold greater in the HNSCC cell lines (mean, 245.4 pmol/mg protein/h) compared with 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 μ M RA exposure for each cell line. On average, HNSCC lines were ~ 1.9 -fold less sensitive to growth inhibition by RA than normal 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 Oral Keratinocytes. In the medium, a difference in RA metabolites was found between HNSCC cell lines and

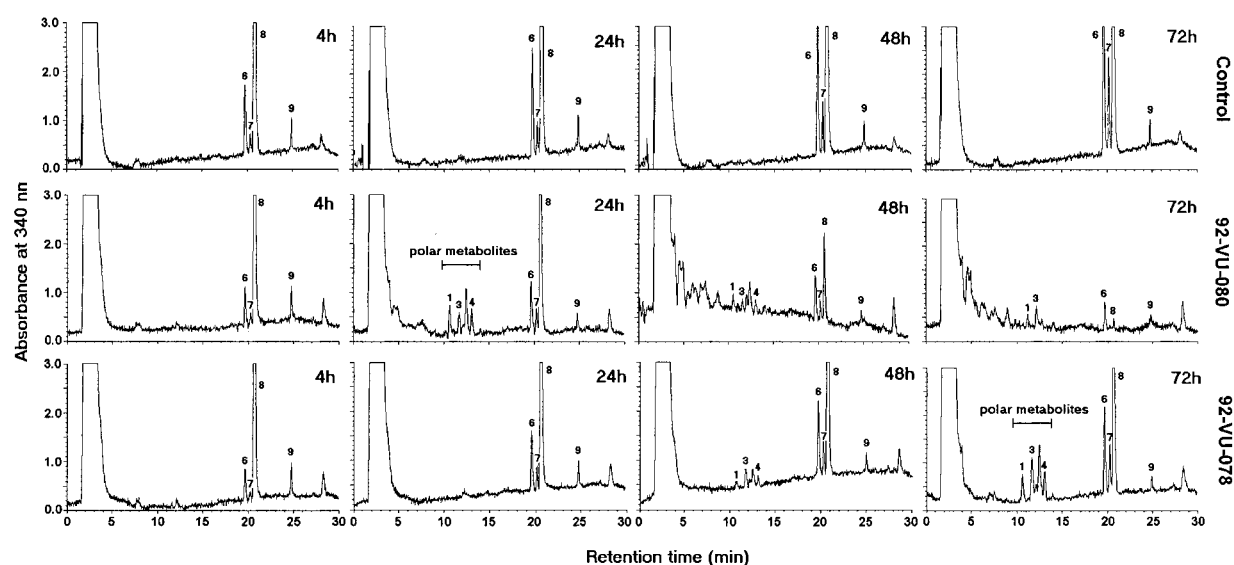


Fig. 2 The HPLC profiles of RA metabolites in medium of two representative HNSCC cell lines. Cells were cultured in medium containing $1 \mu\text{M}$ RA for the indicated time periods. Retinoids were extracted and separated by reverse-phase HPLC. The experiments were done at least three times, and very 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.

normal oral keratinocytes. Fig. 3 shows the typical profiles of metabolites in the medium of keratinocytes and HNSCC lines after various times of exposure to $1 \mu\text{M}$ RA. In the HNSCC cell lines, polar metabolites were found, at retention times between 10 and 15 min, that were identified as 4-oxo-RA, 4-oxo-cRA, 4-OH-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 of the peaks described above were not found in the medium of the control samples without cells. In the normal OKCs, no polar metabolites were detected in the medium. In the cellular fractions of HNSCC cell lines and OKCs, no major differences in RA metabolites were found. RA was the predominant metabolite found in the cellular fraction of both normal keratinocytes and HNSCC cells (Fig. 4). The lack of formation of polar metabolites in keratinocyte cultures was likely not attributable to unfavorable culture conditions during the experiments, because 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 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 $1 \mu\text{M}$ RA. No difference in metabolism was found in these cell lines as compared with metabolism in DMEM with FCS (data not shown). Growing the OKCs 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, sensitivity was increased by exposing the same OKC and HNSCC cell line shown in Fig. 3 for various incubation periods to 10 nM [^3H]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 (Fig. 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 catabolization of RA in normal keratinocytes takes place through an alternative pathway. To explore the first possibility, we added $1 \mu\text{M}$ 4-oxo-RA to the medium of HNSCC or oral keratinocytes and documented its loss from the medium (Fig. 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

Table 1 Turnover and growth inhibition after exposure to 1 μ M RA

In metabolic studies, 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 OKCs. For growth inhibition studies, growth was measured with the SRB-assay. These data are from at least two separate experiments.

Cell Line/Culture	Turnover RA (pmol/mg protein/h)		Growth (%) ^a	
	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	
OKCs				
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.

left these products almost untouched. Failure to detect 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 suggests that turnover in oral keratinocytes takes place via another metabolic pathway.

Expression of CYP26A1 mRNA. Recently, an RA-inducible CYP 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 (Fig. 7). UM-SCC-35 and VU-SCC-OE, both of which have a relatively high metabolism, showed a very high level of *CYP26A1* mRNA after induction with 1 μ M RA for 24 h, whereas it is barely detectable in other cell lines and could not be induced 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/P450RAI 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 the turnover rate of RA was changed and whether polar metabolites were formed. In Fig. 8A, the relative amounts of the polar metabolites as compared with the total amount of retinoids added are depicted for the indicated incubation periods. Transfection with pCYP26 led to an increased concentration of polar metabolites, and the type of polar metabolites were similar to those found in other HNSCC cell lines (Fig. 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. 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 (Fig. 8B). No change of sensitivity toward RA was found in the different transfected populations as compared with 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 (28–30). 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 ~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 exposed longer to RA, which is in agreement with the higher sensitivity to RA relative to HNSCC cell lines.

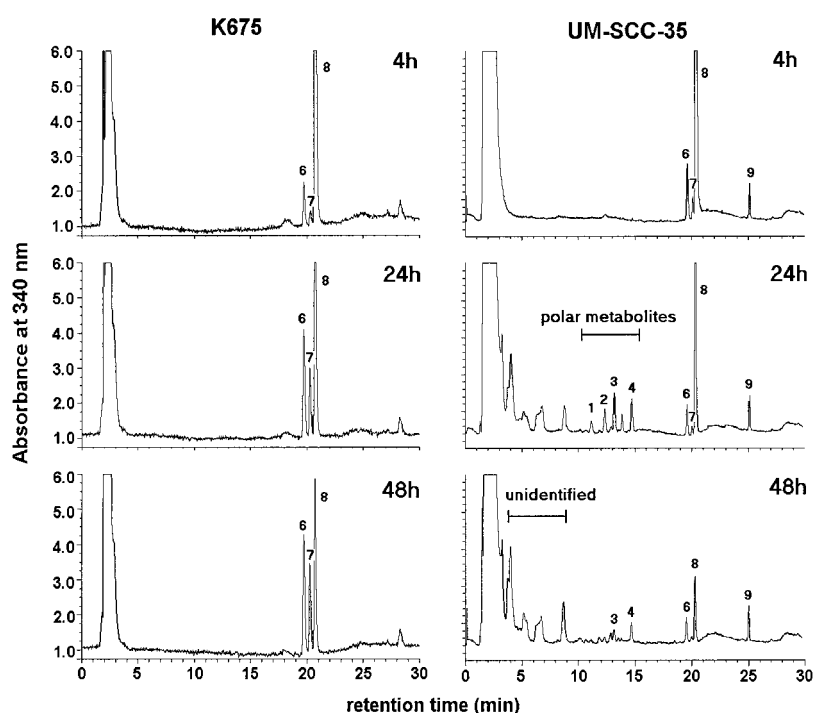


Fig. 3 HPLC profiles of retinoid metabolites. The medium of an HNSCC cell line (right) and an OKC (left) are shown, exposed to 1 μ M RA for the indicated periods of time. In contrast to the normal cells, the tumor cell lines are characterized by a high turnover and the occurrence of polar metabolites. 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.

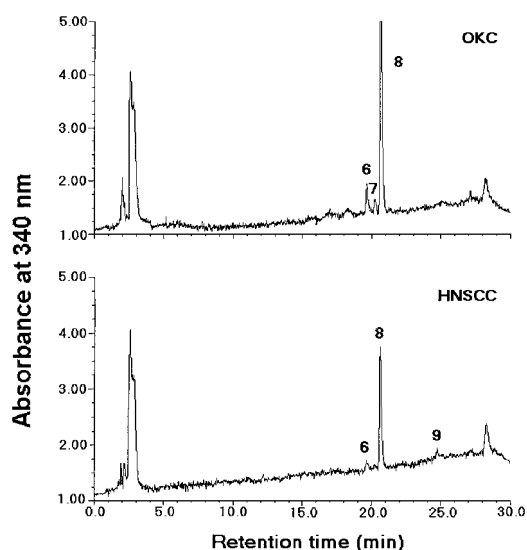


Fig. 4 HPLC profiles of retinoid metabolites in the cellular fractions. An OKC (top) and an HNSCC cell line (bottom) are shown, exposed to 1 μ M RA for 24 h. Peak 6, 13-cis-RA; peak 7, 9-cis-RA; peak 8, all-trans-RA; and peak 9, retinol. For both cell types no polar metabolites were detected in the cellular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

The increased catabolic activity in HNSCC cell lines may reflect a phenotypic change that is involved in the progression of HNSCC toward malignancy. A change in normal and neoplastic epithelial cells was also found in the metabolism of retinol (17). 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 toward 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 (16, 31). 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 (15). 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 (32). In the present study, with a larger panel of HNSCC

Fig. 5 [^3H]-HPLC profiles of retinoid metabolites in the medium. An HNSCC cell line (right) and an OKC (left), were exposed to 10 nM [^3H]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 could 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; and peak 8, all-*trans*-RA. Note that scale is chosen as such that not all peaks are visible in full length.

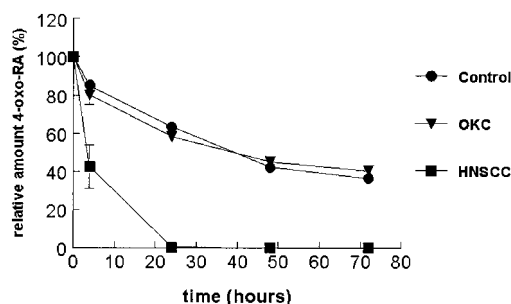
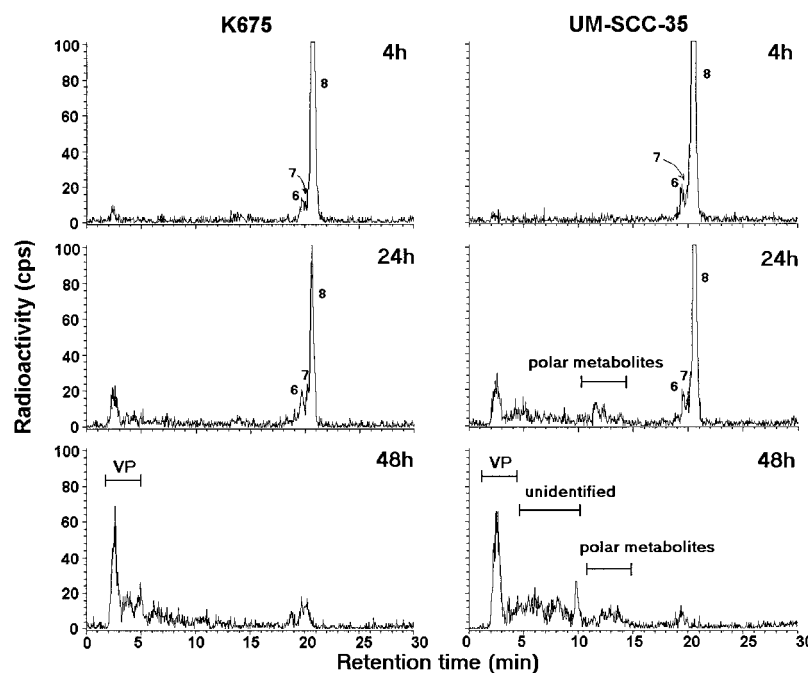


Fig. 6 Kinetics of 4-oxo-RA metabolism. Cells were grown in the presence of 1 μM 4-oxo-RA. Control, medium without cells. The HNSCC cell line catabolized 4-oxo-RA much more efficiently than OKC.

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 OKCs, 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 (33) and serum-free conditions (34).

Two hypotheses can be formulated to explain the relation

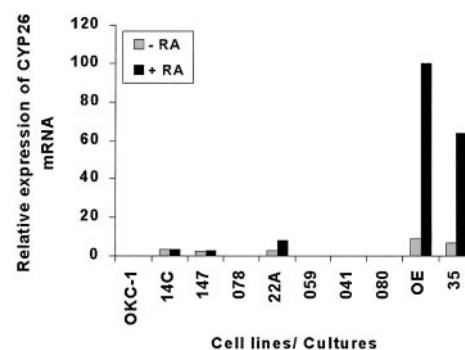


Fig. 7 Expression of *CYP26A1* mRNA determined by Northern blotting. Analysis was done for nine HNSCC cell lines and one OKC (OKC-1). Total RNA (20 μg) was isolated from the cells, untreated (–) and treated (+) with 1 μM RA for 24 h. The blots were quantitated by phosphorimaging. After correction for 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.

between growth inhibition and the 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 (32). 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

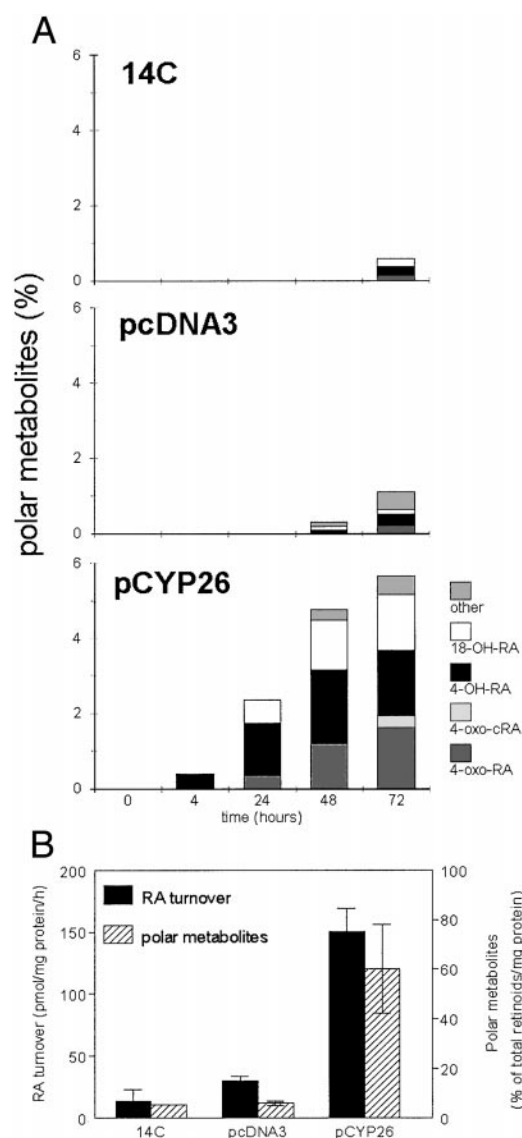


Fig. 8 Transfection of CYP26A1 cDNA in an HNSCC cell line. UM-SCC-14C was transfected with pCYP26 expression vector or with pcDNA3 expression vector as a control. **A**, relative amounts of the different polar metabolites formed after various incubation periods expressed as a percentage of total retinoids 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 \pm 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.

increase of RA turnover from 13 to 150 pmol/mg protein/h but not in an increase of sensitivity toward 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 concen-

tration of certain receptors, different genes are activated or inhibited in their expression (35–37). The involvement of RARs in the induction of CYP26A1 was demonstrated with receptor-specific synthetic retinoids (14). Furthermore, transfection of either RAR- α , RAR- β , or RAR- γ were able to restore CYP26A1 expression upon RA treatment in HCT-116 cells (38). These results and its high RA-inducibility (12, 13, 39) suggested the presence of a RA-responsive element in the promoter region of CYP26A1 and this has recently been identified (40). Sensitivity to RA was also found to be related to mRNA expression levels of RARs by analyzing transcription levels (41) or performing transfection studies (42, 43). 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 the inhibition of growth by RA are independent from one another and are possibly both dependent on RAR expression levels. Currently, we are testing the hypothesis that 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 (44).

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. Louise Foley and Ulf W. Wiegand for providing retinoids and Dr. Martin Petkovich for the pcDNA3/P450RAI expression vector.

REFERENCES

1. Lotan, R. Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells. *Biochim. Biophys. Acta*, 605: 33–91, 1980.
2. De Luca, L. M. Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. *FASEB J.*, 5: 2924–2933, 1991.
3. Graham, S. Epidemiology of retinoids and cancer. *J. Natl. Cancer Inst.*, 73: 1423–1428, 1984.
4. Lotan, R. Retinoids in cancer chemoprevention. *FASEB J.*, 10: 1031–1039, 1996.
5. Moon, R. C., Mehta, R. G., and Rao, K. V. N. Retinoids and cancer in experimental animals. In: M. B. Sporn, A. B. Roberts, and D. S. Goodman (eds.), *The Retinoids*, Ed. 2, pp. 573–594. New York: Raven Press, 1994.
6. Hong, W. K., Lippman, S. M., Itri, L. M., Karp, D. D., Lee, J. S., Byers, R. M., Schantz, S. P., Kramer, A. M., Lotan, R., Peters, L. J., Dimery, I. W., Brown, B. W., and Goepfert, H. Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N. Engl. J. Med.*, 323: 795–801, 1990.
7. Lippman, S. M., Kessler, J. F., Al Sarraf, M., Alberts, D. S., Itri, L. M., Mattox, D., Von Hoff, D. D., Loeschner, L., and Meyskens, F. L. Treatment of advanced squamous cell carcinoma of the head and neck with isotretinoin: a Phase II randomized trial. *Investig. New Drugs*, 6: 51–56, 1988.
8. Hong, W. K., Lippman, S. M., Hittelman, W. N., and Lotan, R. Retinoid chemoprevention of aerodigestive cancer: from basic research to the clinic. *Clin. Cancer Res.*, 1: 677–686, 1995.

9. Snow, G. B. Follow-up in patients treated for head and neck cancer: how frequent, how thorough and for how long? *Eur. J. Cancer*, 28: 315–316, 1992.
10. Hong, W. K., and Itri, L. M. Retinoids and human cancer. In: M. B. Sporn, A. B. Roberts, and D. S. Goodman (eds.), *The Retinoids*, Ed. 2, pp. 597–630. New York: Raven Press, 1994.
11. Muindi, J. F., and Young, C. W. 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: 1226–1229, 1993.
12. Ray, W. J., Bain, G., Yao, M., and Gottlieb, D. I. CYP26, a novel mammalian cytochrome P450, is induced by retinoic acid and defines a new family. *J. Biol. Chem.*, 272: 18702–18708, 1997.
13. White, J. A., Beckett-Jones, B., Guo, Y. D., Dilworth, F. J., Bonasoro, J., Jones, G., and Petkovich, M. cDNA cloning of human retinoic acid-metabolizing enzyme (hP450RAI) identifies a novel family of cytochromes P450. *J. Biol. Chem.*, 272: 18538–18541, 1997.
14. Marikar, Y., Wang, Z., Duell, E. A., Petkovich, M., Voorhees, J. J., and Fisher, G. J. 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: 434–439, 1998.
15. Popa, C., Dicker, A. J., Dahler, A. L., and Saunders, N. A. Cytochrome P450, CYP26A1, is expressed at low levels in human epidermal keratinocytes and is not retinoic acid-inducible. *Br. J. Dermatol.*, 141: 460–468, 1999.
16. Randolph, R. K., and Simon, M. Metabolism of all-*trans*-retinoic acid by cultured human epidermal keratinocytes. *J. Lipid Res.*, 38: 1374–1383, 1997.
17. Guo, X., and Gudas, L. J. 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: 166–176, 1998.
18. Klaassen, I., Brakenhoff, R. H., Smeets, S. J., Snow, G. B., and Braakhuis, B. J. Considerations for *in vitro* retinoid experiments: importance of protein interaction. *Biochim. Biophys. Acta*, 1427: 265–275, 1999.
19. Reid, C. B., Cloos, J., Snow, G. B., and Braakhuis, B. J. A simple and reliable technique for culturing of human oral keratinocytes and fibroblasts. *Acta Otolaryngol.*, 117: 628–633, 1997.
20. Carey, T. E., Wolf, G. T., Baker, S. R., and Krause, C. J. Cell surface antigen expression and prognosis. In: W. E. Fee, H. Goepfert, M. E. Johns, E. W., Strong, and P. H. Ward (eds.), *Head and Neck Cancer*, Ed. 2, pp. 77–82. Philadelphia: BC Decker, B. C. 1990.
21. Hermsen, M. A., Joenje, H., Arwert, F., Welters, M. J., Braakhuis, B. J., Bagnay, M., Westerveld, A., and Slater, R. Centromeric breakage as a major cause of cytogenetic abnormalities in oral squamous cell carcinoma. *Genes Chromosomes Cancer*, 15: 1–9, 1996.
22. Welters, M. J., Fichtinger Schepman, A. M., Baan, R. A., Hermsen, M. A., van der Vijgh, W. J., Cloos, J., and Braakhuis, B. J. 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: 410–415, 1997.
23. Braakhuis, B. J., Jansen, G., Noordhuis, P., Kegel, A., and Peters, G. J. 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: 2155–2161, 1993.
24. Teerlink, T., Copper, M. P., Klaassen, I., and Braakhuis, B. J. 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, 1997.
25. Eckhoff, C., and Nau, H. Identification and quantitation of all-*trans*- and 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid in human plasma. *J. Lipid Res.*, 31: 1445–1454, 1990.
26. Furr, H. C., Barua, A. B., and Olson, J. A. Analytical methods. In: M. B. Sporn, A. B. Roberts, and D. S. Goodman (eds.), *The Retinoids*, Ed. 2, pp. 179–209. New York: Raven Press, 1994.
27. Sambrook, J., Fritsch, E. F., and Maniatis, T. Extraction, purification, and analysis of messenger RNA from eukaryotic cells. In: Sambrook, E. F., Fritsch, E. F., and Maniatis (eds.), *Molecular Cloning*, A Laboratory Manual, Ed. 2, pp. 7.71–7.78. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989.
28. Gubler, M. L., and Sherman, M. I. Metabolism of retinoic acid and retinol by intact cells and cell extracts. *Methods Enzymol.*, 189: 525–530, 1990.
29. Nadin, L., and Murray, M. All-*trans*-retinoic acid 4-hydroxylation in human liver microsomes: *in vitro* modulation by therapeutic retinoids. *Br. J. Clin. Pharmacol.*, 41: 609–612, 1996.
30. Blaner, W. S., and Olson, J. A. Retinol and retinoic acid metabolism. In: M. B. Sporn, A. B. Roberts, and D. S. Goodman (eds.), *The Retinoids*, 2nd ed., pp. 229–255. New York: Raven Press, 1994.
31. Hodam, J. R., and Creek, K. E. Uptake and metabolism of [³H]retinoic acid delivered to human foreskin keratinocytes either bound to serum albumin or added directly to the culture medium. *Biochim. Biophys. Acta*, 1311: 102–110, 1996.
32. Braakhuis, B. J., Klaassen, I., van der Leede, B. M., Cloos, J., Brakenhoff, R. H., Copper, M. P., Teerlink, T., Hendriks, H. F., van der Saag, P. T., and Snow, G. B. Retinoid metabolism and all-*trans* retinoic acid-induced growth inhibition in head and neck squamous cell carcinoma cell lines. *Br. J. Cancer*, 76: 189–197, 1997.
33. Van der Leede, B. M., van den Brink, C. E., Pijnappel, W. W., Sonneveld, E., van der Saag, P. T., and van der Burg, B. 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: 17921–17928, 1997.
34. Takatsuka, J., Takahashi, N., and de Luca, L. M. Retinoic acid metabolism and inhibition of cell proliferation: an unexpected liaison. *Cancer Res.*, 56: 675–678, 1996.
35. Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A., Stein, R. B., Eichele, G., Evans, R. M., and Thaller, C. 9-*cis* retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell*, 68: 397–406, 1992.
36. Zhang, X. K., Lehmann, J., Hoffmann, B., Dawson, M. I., Cameron, J., Graupner, G., Hermann, T., Tran, P., and Pfahl, M. Homodimer formation of retinoid X receptor induced by 9-*cis* retinoic acid. *Nature (Lond.)*, 358: 587–591, 1992.
37. Mangelsdorf, D. J., Borgmeyer, U., Heyman, R. A., Zhou, J. Y., Ong, E. S., Oro, A. E., Kakizuka, A., and Evans, R. M. Characterization of three RXR genes that mediate the action of 9-*cis* retinoic acid. *Genes Dev.*, 6: 329–344, 1992.
38. Sonneveld, E., van den Brink, C. E., van der Leede, B. M., Schulkes, R. K., Petkovich, M., van der Burg, B., and van der Saag, P. T. 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: 629–637, 1998.
39. Sonneveld, E., van den Brink, C. E., van der Leede, B. M., Schulkes, R. K., Petkovich, M., van der Burg, B., and van der Saag, P. T. 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: 629–637, 1998.
40. Loudig, O., Babichuk, C., White, J., Abu-abad, S., Mueller, C., and Petkovich, M. Cytochrome P450RAI(CYP26) promoter: a distinct composite retinoic acid response element underlies the complex regulation of retinoic acid metabolism. *Mol. Endocrinol.*, 14: 1483–1497, 2000.
41. Kaiser, A., Wolf-Breitinger, M., Albers, A., Dorbic, T., Wittig, B., Riecken, E. O., and Rosewicz, S. Retinoic acid receptor γ 1 expression determines retinoid sensitivity in pancreatic carcinoma cells. *Gastroenterology*, 115: 967–977, 1998.
42. Oridate, N., Esumi, N., Lotan, D., Hing, W. K., Rochette-Egly, C., Chambon, P., and Lotan, R. Implication of retinoic acid receptor γ in squamous differentiation and response to retinoic acid in head and neck SqCC/Y1 squamous carcinoma cells. *Oncogene*, 12: 2019–2028, 1996.
43. Sun, S. Y., Kurie, J. M., Yue, P., Dawson, M. I., Shroot, B., Chandraratna, R. A., Hong, W. K., and Lotan, R. Differential responses of normal, premalignant, and malignant human bronchial epithelial cells to receptor-selective retinoids. *Clin. Cancer Res.*, 5: 431–437, 1999.
44. Klaassen, I., Brakenhoff, R. H., Smeets, S. J., Snow, G. B., and Braakhuis, B. J. M. Expression of retinoic acid receptor γ correlates with retinoic acid sensitivity and metabolism in head and neck squamous cell carcinoma cell lines. *Int. J. Cancer*, in press, 2001.