Model examination of chemoprevention with retinoids in squamous cell carcinomas of the head and neck region and suitable biomarkers for chemoprevention

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Abstract. The prognosis for patients with head and neck tumors (HNSCC) is poor, due among other things to the high-risk factor for locoregional recurrence and/or second primary tumors. Extensive studies on chemoprevention of oral pre-cancers to stop carcinogenesis and to prevent recurrence and/or second primary tumors have failed to reach the desired effects. The toxicity of retinoids (RA) for example limits their dosage. Biomarkers are used to evaluate the duration of therapy. In this study, cell culture models are used to demonstrate immunocytochemical expression of RA receptors (RAR, RXR), Ki-67 and p53 before and after all-trans retinoic acid (ATRA) treatment. Telomerase activity in PCR is used to assess the effectiveness of ATRA. Along with an RA-sensitive HNSCC cell line UM-SCC-35 we employed cell lines UM-SCC-14C and HaCaT. Our immunocytochemical examination produced no proof of a statistically significant change in expression of RARα, RARβ or RXRγ receptors after ATRA treatment, either in the cells of the sensitive UM-SCC-35 line or in HaCaT cells. The RARβ and RXRγ receptors showed increased expression after brief cell treatment of UM-SCC-14C. The reduced telomerase activity after prolonged treatment of the UM-SCC-35-cells with ATRA (as well as the reduced p53 expression) proved to be a biomarker for evaluating the success of therapy. Although XTT and MTT tests demonstrated that cell proliferation in UM-SCC-35 cells was inhibited after brief and extended RA influence, the immunocytological Ki-67 scores failed to confirm the inhibition. No reduction of p53 expression, of telomerase activity or of cell proliferation in the XTT and MTT test was detected in the RA-insensitive cell line UM-SCC-14C or in HaCaT cells. We also demonstrated the parameters used in examining the models in sections of carcinoma tissue and in control tissues from the head and neck region, so they can be examined in clinical chemopreventive studies on biopsy tissue.

Introduction

Field cancerization (1) in the direct vicinity of the tumor of squamous cell carcinoma in the head and neck region (HNSCC) (2-4) and even a minimal residual tumor disease in the tumor margin (5-8) lead to locoregional recurrence and second primary tumors. In numerous studies researchers have been trying for years to influence carcinogenesis and postoperative outcome of disease (second field tumors, second primary tumors or minimal residual cancers) with chemoprevention (9-23). Patients with a field cancerization are potential target groups for the study of cancer prevention (22). The concept of chemoprevention was coined by Sporn et al. in 1976 for the use of natural or synthetic chemical substances to prevent the occurrence of carcinomas or to inhibit or slow down the carcinogenic process (24,25).

For many years, natural or synthetic retinoids (26) have been the most common means for the chemoprevention of oral carcinomas in the head and neck region (9,14,17,18,27-31). They inhibit the cell proliferation of normal and malignant cells, modulate cell differentiation and trigger apoptosis (14,17,29,32-34). The cells are triggered in a G0/G1 cell cycle arrest thus prolonging the effectivity of the retinoids. This was demon-
strated by Ran et al (33), Giannini et al (35), Hayashi et al (36), Masuda et al (37), Xiao et al (38) and Wang et al (39) on different HNSCC cell lines. According to Masuda et al (37) the portion of cells of the lines YCU-N861 (established from a nasopharynx carcinoma) and YXU-H891 (established from a hypopharynx carcinoma) increased after treatment with retinoids in the GI phase from 63 to 86% and from 67 to 81%. After treatment with retinoids the doubling time of HNSCC cell lines is prolonged, as Giannini et al (35) established in 7 further HNSCC cell lines (FADU, Hep-2, CCL, SCC-9, SCC-15, SCC 25 and HN-212).

In clinical studies the effectivity of chemoprevention was compared to placebo in the treatment of oral leukoplasia or in postoperative reduction of a second primary tumor in the head and neck region (9,10,13-19,27,31,40,41). In some studies chemoprevention was started with an initially higher dose and the therapy was continued with a lower less toxic dose (15,16,27,31,42-44). Results of the studies were contradictory and effectivity was not confirmed with a large enough number of patients (11,14,30,40,42,44-48). The effect was often temporary (49) and waned markedly after the chemoprevention had been discontinued (16,44). In already existent carcinomas or in metastases, Bolla et al (11) and Sun and Lotan (50) among others were unable to demonstrate an effect. Other factors such as not smoking or having stopped smoking or reduced alcohol consumption can also be significant for the outcome of oral carcinomas (11,41,42,51-53). For this reason no binding recommendations have been made for chemoprophylaxis of head and neck tumors.

Some human oral squamous cell carcinoma cell lines were demonstrated to have a certain resistance to retinoids when their growth is only minimally or not at all inhibited (28,36,37,54-56). This resistance could explain why chemoprevention has no effect in some studies. The effectivity of retinoids depends on the dosage. The toxicity of higher retinoid doses constitutes a restriction on its effectiveness and cannot be used for patients with preneoplasias (46). Khuri et al (43) however established no reduction of the second primary tumor in an extensive placebo-controlled phase III study on 1190 patients with low doses of isotretinoin (13-cis-RA), and Toma et al (30) found no effect of chemoprevention with lower doses of 13-cis-RA.

Further clinical studies should be performed to test the use of a chemoprevention particularly on risk groups. To improve the effect of therapy and reduce the side-effects of chemoprevention efforts are being made to develop receptor-selective synthetic retinoids (14,28,50,57-65). According to Sun et al (61) synthetic retinoids are more effective and less toxic than natural retinoids.

The effects of retinoids are mediated by their nuclear receptors (retinoic acid receptors RARα, RARβ, RARγ and retinoid X receptors RXRα, RXRβ and RXRγ). The activation and the affinity of the receptors are dependent on the particular retinoids being used: ATRA prefers a ligand for RAR receptors while 9-cis-RA prefers RXRs receptors but will also bind RAR receptors (66-73). The selectivity of the retinoids and rexinoids to particular receptors is probably also the explanation for their differing therapeutic effects. The discovery of the RA receptors in 1987 (66-69,74-76) was followed shortly by the development of synthetic retinoids, above all of synthetic receptor-selective retinoids (70,71). In retinoic therapy, the growth and differentiation of cancer cells is modulated, presumably through the activation of gene transcription by nuclear RA receptors RAR and RXR (61). The expression of nuclear RA receptors RAR and RXR (74,77-83) has been demonstrated in several studies in oral leukoplasias and oral squamous cell carcinomas (18,19,84-93) with various methods. RARβ expression is already downregulated when carcinogenesis begins in oral carcinomas and is often completely absent in them (19,47,50,88,89,93-95). Following successful retinoid treatment, RARβ is again upregulated (18,50,60,88-90). A failed treatment is reflected in lower or absent RARβ upregulation (50,88).

Long-range studies with different biomarkers were performed to determine the endpoint of a chemoprophylaxis (60,96,97). The development of intermediate markers is crucial for chemoprevention trials (29). To determine a preliminary endpoint, genetic, cellular, biochemical or immunological surrogate biomarkers are being used and validated so that chemoprevention can be evaluated before a recurrence becomes evident. This requires an understanding of the biology of carcinogenesis to find biomarkers which reveal particular steps in tumor development (16,18,19,29,45,50,90,94,97-109). The demonstration of RARβ (as an mRNA or as a protein in immunohistochemistry) was used in some studies as a sensitive biomarker for chemoprevention (13,17,18,84,88,89,95).

The use of prognostic markers (e.g. p53, telomerase) is also helpful in developing chemopreventative strategies (17,27,29,89,100,101,107,109,110). Geyer et al (17) found chemoprevention effective if the wild-type p53 had not yet mutated, and used p53 as a biomarker. Shin et al (89) used it as a biomarker for deciding whether to continue or discontinue a chemoprevention, and Niles recommends that it be validated in animal models (111).

In carcinoma-free tumor margin tissue from HNSCC, telomerase activation indicates the occurrence of field cancerization in this tissue and the first steps of carcinogenesis (110,112-117). Geyer et al (17), Hong et al (27), Lieberman et al (100), Koch (101), Kelloff et al (102) and Smith and Saba (107) recommended telomerase as a biomarker for assessing chemoprevention in tumors in the head and neck tumors region. Proof of telomerase as a biomarker for cancer incidence (102) has yet to be validated. Tsao et al (29) recommend e.g. using proof of hTERT, the catalytic subunit of telomerase. We have demonstrated hTERT in carcinoma-free adjacent tissues (118), whereby the immunohistochemical hTERT proof did not correlate in all cases with telomerase activity in the same tissue (PCR-ELISA).

In our model investigations of three cell lines we investigated whether proof of telomerase activity can be used as a biomarker for the effectiveness of chemoprevention. On the same model cells we compared the proliferation before and after retinoid therapy as well as the expression of several retinoid receptors, from Ki-67 and p53. We used the two HNSCC cell lines UM-SCC-14C and UM-SCC-35 (119,120) and controlled with the spontaneously immortalized aneuploid human keratinocyte cell line HaCaT (121). The doubling time of UM-SCC-35 is between 38 (122) and 52 h (123), of UM-SCC-14 C it is ~26 h (119,120,123). HaCaT has a mean doubling time of ~30 h (121). From the studies by Braakhuis et al (55), Copper et al (123) and Klaassen et al (124-126) we know that ATRA, depending on its dosage, inhibits the growth of cells of the line UM-SCC-35.
Copper et al (123) and Klaassen et al (124), however, found that ATRA (124) and other retinoids (125) had no, or very little inhibiting effect on cells of the line UM-SCC-14C. Chen et al (127) investigated the restriction on growth of HaCaT exerted by various retinoids applied in increasing concentrations: Growth was inhibited strongest by increased concentration of retinoids, particularly by ATRA. Schroeder and Zouboulis (128) were also able to demonstrate dose-dependent inhibition of growth in HaCaT-cells, more clearly by 13-cRA than by ATRA.

Materials and methods

Cell lines UM-SCC-35, UM-SCC-14C and HaCaT. Carey et al (119,120) isolated the tumor cell lines from fresh head and neck cancer tissues: UM-SCC-35 for a primary tonsil carcinoma (T4N1M0, moderately well differentiated) and UM-SCC-14C for a skin metastasis of a floor and mouth carcinoma (poorly differentiated) after chemotherapy. The spontaneously immortalized aneuploid human keratinocyte cell line HaCaT was established by Boukamp et al (121) from the tumor-free skin area surrounding a melanoma. All cells were cultured in Dulbecco's modified Eagle's medium/NUT MIX F-12 (Gibco, Scotland, code 31 330-038), supplemented with 10% fetal calf serum (Biochrom KG, Berlin, Germany, code S 3113), 50 U/ml penicillin and 50 µg/ml streptomycin (Biochrom KG, Berlin, Germany, code A 2213) and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Following incubation of the cell lines with or without retinoid acid they were washed in medium three times. Cytological slide preparations for the immunocytochemical investigations were made with the help of a Shandon Centrifuge Cytospin3 (Life Science International GmbH, Germany, code A 2213) and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Following incubation of the cell lines with or without retinoid acid they were washed in medium three times. Cytological slide preparations for the immunocytochemical investigations were made with the help of a Shandon Centrifuge Cytospin3 (Life Science International GmbH, USA; 850 RPM, 5 min) with single or double Cytofunnels. The preparations were then air-dried and stored ready for immunocytochemical dyeing at -20°C. Carcinoma tissue and control tissues were examined immunohistochemically. Cell proliferation before and after the effects of ATRA was also performed on the three cell lines UM-SCC-35, UM-SCC-14C (119,120) and HaCaT (121) in 96-well plates (Sigma-Aldrich, USA, code L-9756). Against RAR α-antibody clone 4E9.

Chemicals. All-trans-RA (ATRA; Sigma-Aldrich, USA, code R-2625) was dissolved in the dark into dimethyl sulfoxide (DMSO; Merck KGaA, Darmstadt, Germany, code 109678) as a stock for 10⁻² M ATRA. Aliquots were stored at -20°C. For each experiment with 10⁻³ M and/or 10⁻⁸ M ATRA fresh working dilutions were prepared in the serum-free cell culture medium supplemented with 1 mg/ml bovine serum albumin (Sigma; code A-9647). With every change of medium 10 µl ATRA was added to 10 ml of the cell cultures, either ~6 days (short incubation) or ~26 days (long incubation). DMSO (10 µl) was added to the controls without RA. The final DMSO concentration was always 0.1% or lower and did not inhibit cell growth.

Immunocytochemical and immunohistochemical proof of the retinoid receptors RAR and RXR as well as of Ki-67 and p53. Before beginning with the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique (129), the cytopsin prepara-
Table I. Overview of primary antibodies used.

<table>
<thead>
<tr>
<th>Ab-code</th>
<th>Antibodies</th>
<th>Source; code</th>
<th>Characterization</th>
<th>Working dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab 1</td>
<td>Polyclonal rabbit anti-RARα-antibody (C-20)</td>
<td>Santa Cruz Biotechnology, Inc.; sc-551</td>
<td>Affinity purified IgG-antibody against a peptide mapping at the C-terminus of RARα1 and RARα2 of human origin</td>
<td>1:75 (cytospins 1:50)</td>
</tr>
<tr>
<td>Ab 2</td>
<td>Polyclonal rabbit anti-RARβ-antibody</td>
<td>Abcam (USA); ab15515</td>
<td>Affinity purified IgG-antibody against a synthetic peptide (N-terminal)</td>
<td>1:15</td>
</tr>
<tr>
<td>Ab 3</td>
<td>Monoclonal mouse anti-RXRα-antibody 4E9</td>
<td>Abnova (Novus Biologicals, Inc.); H00006256-M01 (NR2B1)</td>
<td>Purified IgG2a-κ antibody against partial recombinant RXRA</td>
<td>1:25</td>
</tr>
<tr>
<td>Ab 4</td>
<td>Polyclonal rabbit anti-RXRβ1-antibody (L-20)</td>
<td>Santa Cruz Biotechnology, Inc.; sc-556</td>
<td>Affinity purified IgG-antibody raised against a peptide mapping at the N-terminus of RXRβ1 of mouse origin</td>
<td>1:20</td>
</tr>
<tr>
<td>Ab 5</td>
<td>Monoclonal mouse anti-RXRγ-antibody clone 1373</td>
<td>Cascade Bioscience; ABM-4821</td>
<td>Purified IgG2a-κ antibody against a ~60-kDa peptide derived from the hinge region of human RXR-γ. Detects hRXR-γ; does not recognize α or β isotypes or hRAR.</td>
<td>1:10</td>
</tr>
</tbody>
</table>

Positive control antibodies

| Ab 6    | Monoclonal mouse anti-Ki-67 antibody clone MIB-1 | Dako, Denmark M7240 | Immunogen human recombinant peptide anti-human Ki-67 IgG1-κ antibody; corresponding to a 1002-bp Ki-67 cDNA | 1:100 |
| Ab 7    | Monoclonal mouse anti-mutated p53 antibody Ab-3 (OP 29-1) clone PAb240 | Calbiochem (Oncogene), USA; OP29 | Purified IgG1-antibody against a ~53 kDa mutant p53 protein under non-denaturing conditions, recognizes both the mutant and the wild-type p53 protein under denaturing conditions | 1:20 |

Ab, antibody; RAR, retinoic acid receptors; RXR, retinoid X receptors.

and obtained distinctly better results with the former. Table I lists the five antibodies Ab 1 to Ab 5 used for further experiments with cytospins and sections. We compared the results with a control using an antibody against Ki-67 (Ab 6) and an antibody against p53 (Ab 7). With some of the antibodies, only a limited number of tissues or cytospides stained. The antibodies were diluted in S2022 (DakoCytomation, ready to use), incubated for 60 min at 37°C in the drying oven and then again for 30 min at room temperature.

To ensure the reliability of our immunohistochemical stainings we ran a parallel assay with a negative control for each staining (negative control sera mouse Dako code N1698, negative control sera rabbit Dako code N1699; ready for use). These control sera were negative. All incubation steps taken in the immunohistochemical staining process were performed in a humidified chamber. To block any non-specific reactions we incubated sections at room temperature for 20 min with the ready-made X0909 blocking buffer (DakoCytomation).

All score values given for anti-RAR/RXR-receptors, Ki-67 and p53 refer exclusively to nuclear stainings in culture cells, in the carcinoma tissues and in control epithelial tissues. Based on experience gained in our previous immunohistochemical studies with other antibodies (118) we used the immunohistochemical evaluation by Remmele et al (130). Evaluation of cytospins and tissue sections was performed three times at different time points by an independent examiner and was based on estimated values for staining intensity (SI: 0, no staining; 1+, weak; 2+, moderate; 3+, strong and 4+, very strong staining) and percentage of positive staining in tissue sections (PP: 0, no positive cells; 1, 1-25%; 2, 26-50%; 3, 51-75% and 4, 76-100% positive cells). The immunohistochemical score value (IRS) was then calculated by multiplication of SI and PP.

Tumor tissues and tissues without tumors with control tissue. All tissues examined were taken from the head and neck area with previous consent of the patients in our clinic in the context of diagnostics and therapy. The squamous cell carcinoma specimens were collected from 18 patients in the period from 1994 to 1997 (Table IIA). The entire frozen section series was examined.
by a tumor pathologist (G.-P.W.) with conventional hemalaun-eosin staining to ensure that carcinoma was present in all tumor tissues. We examined tissue of different localizations from 16 males and 2 female patients aged 59±10 years. Thirteen patients had oral and 5 extraoral squamous cell carcinomas. TNM classification (131) and degree of differentiation were established by the Institute of Pathology in our hospital. The immunohistochemical detection of RA-receptors as well as of Ki-67 and p53 was described previously. For a critical evaluation of RA-receptor expressions in carcinoma tissues we examined oral and extraoral control tissue from different localizations in 17 patients without tumor from our clinic in the period from 1994

Table II. Characterization of the patients with and without a head and neck squamous cell carcinoma (HNSCC) and localization of tissues.

A. Characterization of the 18 patients with a HNSCC.

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex/age</th>
<th>Localization</th>
<th>TNM&lt;sup&gt;b&lt;/sup&gt;</th>
<th>S</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/39</td>
<td>Floor of mouth</td>
<td>pT3N1</td>
<td>III</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>M/50</td>
<td>Floor of mouth</td>
<td>pT2N2b</td>
<td>IVA</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>M/50</td>
<td>Floor of mouth</td>
<td>pT4N1</td>
<td>IVA</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>M/62</td>
<td>Floor of mouth</td>
<td>pT2N2</td>
<td>IVA</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>M/50</td>
<td>Floor of mouth</td>
<td>pT4N1</td>
<td>IVA</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>M/52</td>
<td>Floor of mouth</td>
<td>pT4N2c</td>
<td>IVB</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>M/60</td>
<td>Floor of mouth</td>
<td>pT4N1</td>
<td>IVA</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>M/58</td>
<td>Floor of mouth/tongue</td>
<td>pT3N2</td>
<td>IVA</td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td>M/57</td>
<td>Floor of mouth/tongue</td>
<td>pT4N0</td>
<td>IVA</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>F/65</td>
<td>Floor of mouth/tongue</td>
<td>pT2N0</td>
<td>II</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>M/55</td>
<td>Tongue/floor of mouth</td>
<td>pT4N0</td>
<td>IVA</td>
<td>3</td>
</tr>
<tr>
<td>12</td>
<td>F/76</td>
<td>Buccal mucosa</td>
<td>pT3N1</td>
<td>III</td>
<td>2</td>
</tr>
<tr>
<td>13</td>
<td>M/53</td>
<td>Alveolar process</td>
<td>pT4N2</td>
<td>IVA</td>
<td>2</td>
</tr>
<tr>
<td>14</td>
<td>M/75</td>
<td>Lip</td>
<td>pT2N0</td>
<td>II</td>
<td>3</td>
</tr>
<tr>
<td>15</td>
<td>M/57</td>
<td>Ear</td>
<td>pT4N1</td>
<td>IVA</td>
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<tr>
<td>16</td>
<td>M/64</td>
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<tr>
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<td>IVA</td>
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</tr>
<tr>
<td>18</td>
<td>M/65</td>
<td>Ear</td>
<td>pT4N1</td>
<td>IVA</td>
<td>2</td>
</tr>
</tbody>
</table>

B. Characterization of the 17 patients without tumor and localization of control tissues

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex/age</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/20</td>
<td>Gingiva</td>
</tr>
<tr>
<td>2</td>
<td>F/56</td>
<td>Gingiva</td>
</tr>
<tr>
<td>3</td>
<td>F/22</td>
<td>Gingiva</td>
</tr>
<tr>
<td>4</td>
<td>M/36</td>
<td>Oral mucosa</td>
</tr>
<tr>
<td>5</td>
<td>F/36</td>
<td>Oral mucosa</td>
</tr>
<tr>
<td>6</td>
<td>F/61</td>
<td>Oral mucosa</td>
</tr>
<tr>
<td>7</td>
<td>F/64</td>
<td>Oral mucosa</td>
</tr>
<tr>
<td>8</td>
<td>F/48</td>
<td>Buccal mucosa</td>
</tr>
<tr>
<td>9</td>
<td>M/61</td>
<td>Tongue</td>
</tr>
<tr>
<td>10</td>
<td>M/60</td>
<td>Tongue</td>
</tr>
<tr>
<td>11</td>
<td>M/46</td>
<td>Nose</td>
</tr>
<tr>
<td>12</td>
<td>M/30</td>
<td>Ear</td>
</tr>
<tr>
<td>13</td>
<td>F/18</td>
<td>Ear</td>
</tr>
<tr>
<td>14</td>
<td>M/20</td>
<td>Chin</td>
</tr>
<tr>
<td>15</td>
<td>F/55</td>
<td>Eyelid</td>
</tr>
<tr>
<td>16</td>
<td>M/55</td>
<td>Scalp</td>
</tr>
<tr>
<td>17</td>
<td>F/10</td>
<td>Scalp</td>
</tr>
</tbody>
</table>

Sex: M, male; F, female; *Age at tissue harvesting in years; †TNM-classification of malignant tumors (131); T, tumor; N, node; M, metastasis; S, staging; G, grading.
Examination of growth inhibition in XTT and MTT tests. To test the growth inhibition effect by 10^{-5} M and 10^{-8} M ATRA, we used two colorimetric assays for non-radioactive quantification of cell proliferation and viability, the Cell Proliferating Kit II, XTT-based (cat. no. 11 465 015 001) and for comparison in separate cell culture assays the Cell Proliferating Kit I, MTT-based (cat. no. 11 465 007 001) from Roche Diagnostics (Mannheim, Germany). From a larger volume we took 100 µl cells in medium without phenol red with (10^{-5} and 10^{-8} M ATRA) and without RA treatment, taking 2.5x10^5 cells/well of each, and deposited this on microplates (tissue culture grade, 96 wells, flat bottom). These were incubated for 24 h at 37°C and 5% CO_2. In the XTT test after adding 50 µl of the XTT labelling mixture, we incubated the microplates once again for 4 h at 37°C and 5% CO_2, then took readings. In the MTT test, 10 µl of the MTT labelling reagent was added and the cells were incubated 4 h at 37°C and 5% CO_2. Afterwards 100 µl solubilization solution was added before the cells were again incubated overnight. To measure the spectrophotometrical absorbance of both tests we used a microplate reader (Dynatech MR 5000): XTT assay at a wavelength of 570 nm and reference wavelength of 690 nm. Determining telomerase activity in cell cultures before and after treatment with retinoids and in tissues. As described above (117), we used the test kits TelotAGGG Telomerase PCR ELISA (Roche Diagnostics, Applied Science; cat. no. 11854666910) and TelotAGGG Telomerase PCR ELISA^{Plus} (Roche Diagnostics, Applied Science, cat. no. 12013789001) to demonstrate telomerase activity. The frozen tissues from tumor and control patients (Table II) were lysed and the lysate adjusted with the addition of 0.5 and 5 µg protein or, if necessary, amounts under 0.5 µg per PCR trial. The cells were lysed before and after brief (~6 days) and extended (~26 days) treatment with 10^{-5} ATRA; we used 0.005-0.1 (with retinoic acid to 0.5 µg) protein per PCR. Protein concentration in the cell lysates was determined in accordance with Lowry et al (132) using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, USA). With a subsequent ELISA it was then possible to detect telomerase activity in mOD.

Statistical analysis. Statistical evaluation (133) was performed with PASW Statistics 18 Version 18.0.0. Apart from descriptive statistics, we compared median values using the Mann-Whitney U test or, if distribution was normal, we compared mean values using the t-test. Test results with p≤0.05 were rated statistically significant.

Results

Immunocytological proof of RAR and RXR before and after brief and extended RA treatment in cell cultures in comparison to Ki-67 and p53. After the cytospins had been pretreated in the steamer (compare Fig. 1), we examined with different antibodies (Table I) the expression of the RAR and RXR receptors as well as of Ki-67 and p53 on the cells in all three cell lines both before and after exposure to 10^{-5} M and 10^{-8} M ATRA. We ascertained median nuclear score values according to Remmele et al (130). With the one antibody against RARβ available to us (Santa Cruz Biotechnology Inc.; sc-7397) results were not evaluable in immunocytology or immunohistochemistry. We therefore abandoned its further use in this study. For repeated examinations for receptor expression in at least three separate cell culture assays we were constrained to use proof of RARα, RARβ and RXRβ with the antibodies Ab 1, Ab 2 and Ab 5 (Table I) for statistical assessment: Fig. 2 presents the median score values (130) before and after brief and extended treatment with 10^{-5} M ATRA. We also demonstrated the receptors RXRα (Ab 3) and RXRβ (Ab 4) in the cell lines.

The score values for RARα, RARβ and RXRβ in UM-SCC-35 cells were not significantly altered after ATRA treatment: without RA/brief RA Abl: p=0.667, Ab 2: p=0.667,
Ab 5: p=0.333; without RA/extended RA Ab 1 (Ab 2, Ab 5): p=0.333. The growth inhibiting effect of retinoid treatment on UM-SCC-35 cells which was demonstrated in the XTT and MTT assay (Fig. 7) was not reflected in the parameter Ki-67 (Figs. 3 and 4A1-A3): without RA/brief RA exposure (~6 days): Ki-67: p=0.963; without RA/extended treatment with RA (~26 days): Ki-67: p=0.789. By contrast, the expression of p53 was lower after extended RA treatment in the cells of UM-SCC-35 (Figs. 3 and 4A4-A6), with the reduction just reaching significance (p=0.058). Brief treatment with ATRA altered p53 expression only minimally: p=0.797 (Fig. 3).

After brief treatment with RA, RARα expression on the cells of UM-SCC-14C (Ab 1, Fig. 2) was significantly higher than on the untreated cells (p=0.019). This was not the case after extended RA treatment. In the same cells after only a brief treatment with RA, the expression of RXRγ was distinctly
higher than on the untreated cells (Ab 5, p=0.062, Fig. 2), reaching borderline significance. The score values of the other parameters showed no significant differences: without RA/brief RA: Ab 2: p=1.000, Ki-67: p=0.919; without RA/extended RA: Ab 1: p=0.333, Ab 2: p=0.667, Ab 5: p=1.000; Ki-67: p=1.000 (Fig. 4B1-B3), p53: p=0.724 (Fig. 4B4-B6).

On the cells of the immortal keratinocyte cell line HaCaT, expression of the RA receptors after brief exposure with 10^{-5} M ATRA did not significantly change (Fig. 2): Ab 1: p=0.667; Ab 2: p=0.333, Ab 5: p=0.33; nor were the parameters Ki-67 or p53 altered after brief RA treatment: Ki-67: p=0.200, p53: p=0.974 (Figs. 3 and 4C1-C6).

**Immunohistochemical proof of RAR and RXR receptors as well as of Ki-67 and p53 in tissue.** We demonstrated all the parameters examined in the squamous cell carcinomas (Table IIA) and in the squamous epithelium of the control tissue with the methods described and in the same way as in the cytologic preparations. Fig. 5 summarizes the median nuclear score values in the tissues according to Remmele et al (130). These values are elucidated with the example of patient no. 15 (Table IIA) in Fig. 6. Receptors RARα (Ab 1), RARβ (Ab 2), RXRα (Ab 3), RXRβ (Ab 4) and RXRγ (Ab 5) were demonstrated both in squamous cell carcinoma and in squamous epithelia in the control tissues. There was no significant difference between the median score values for receptor expression on the carcinoma tissues and on the squamous epithelium in the control tissues: Ab 1: p=0.252; Ab 2: p=0.956; Ab 3: p=0.831; Ab 4: p=0.609, Ab 5: p=0.182. In contrast, the median Ki-67 (Ab 6) and p53 scores (Ab 7) were significantly higher in the carcinoma tissues than in the squamous epithelium of the control tissues (Ki-67: p=0.028, p53: p=0.003) (Fig. 5).

**Proof of inhibition by retinoid treatment in three cell lines.** The growth inhibiting effect of retinoid treatment was examined in the three cell lines UM-SCC-35, UM-SCC-14C and HaCaT in the XTT test and in the MTT test in several separate assays shown in Fig. 7. Brief treatment (~6 days) with both 10^{-5} and 10^{-8} M ATRA incurred growth inhibition of the cells of UM-SCC-35 as demonstrated both in the XTT test and in the distinctly lower extinction values in the MTT test. Comparison to the untreated cells reveals no significant difference (XTT: p=0.109, MTT: p=0.100). Growth in cells

![Figure 5. Expression of RA receptors and of Ki-67 and p53 in tissue sections (Tables I and II).](image)

![Figure 6. Expression of RA receptors as well as of Ki-67 and p53 in tissues from patient no. 15 (Table IIA): antibodies (Ab) Table I; bar, 20 µm.](image)
of the lines UM-SCC-14 (p=0.667) and HaCaT (p=0.333) is inhibited only minimally or not at all by ATRA.

**Proof of telomerase in cell cultures before and after retinoid treatment in the three cell lines and in tissues.** In the model experiment only the UM-SCC-35 cells proved to be RA-sensitive after extended treatment with 10^{-5} M ATRA, as evidenced in a significantly reduced telomerase activity (Fig. 8) compared to the cells without RA: without RA/brief RA p=0.686; without RA/extended RA p<0.001; brief RA/extended RA p=0.133. The telomerase activity of the other cell lines did not change statistically after RA treatment (p=0.109 and 0.180). In the tissue sections of head and neck tumors a significantly higher telomerase activity was detected than that in the control tissue from the same localization (Table II): p<0.001.

**Discussion**

Given the poor prognosis for squamous cell carcinomas in the head and neck region prophylactic measures are of vital importance for these patients. Reducing the risk factors tobacco and alcohol is the most effective measure in this regard (11,41,43,51-53). Numerous clinical studies have administered chemoprevention in the attempt to influence precancerous tissue changes or the postoperative course of disease. Their success is yet unclear (9-11,13-19,27-31,40-48). Most of these studies used retinoids administered in different doses and for differing periods of time. Often, however, the effects waned when the therapy was ended (49). To increase effectiveness, synthetic receptor-selective retinoids have been developed (14,28,50,57-65). Various biomarkers were applied to determine the optimal end point of chemoprophylactic treatment (16,18,19,29,45,50,60,90,94,96-109).

In our model study we examined the effects of chemoprophylaxis with ATRA in cell cultures and the suitability of several biomarkers.

**Growth inhibition of three cell lines by ATRA.** Copper et al (123) investigated growth inhibition effected by 10^{-4} and 10^{-8} M ATRA on UM-SCC-11B, UM-SCC-14A, UM-SCC-14C, UM-SCC-22A, UM-SCC-22B and UM-SCC-35 (55,123). The authors (123) used a cell proliferation assay based on staining cellular protein with sulpho-rhodamine B. They determined that cells of UM-SCC-35 are most affected in their growth: a growth reduction from 100% without RA to 9.0±1.0% by 10^{-6} M RA compared to reduction from 100% to 44±22% by 10^{-8} M ATRA), while other cell lines such as UM -SCC-14C showed no growth inhibition by ATRA (97±8.0% vs. 106±7.3%). The disparate behavior observed with doses up to 10^{-6} M ATRA was confirmed in studies by Klaassen et al (124,125) with the same method of proof. These authors also included a resistant cell line UM-SCC-35R which they had cultured for 8 months with increasing doses of ATRA. Le et al (134) demonstrated that ATRA inhibited growth in SCC-25 cells.

In our study we applied both the RA-sensitive cell line UM-SCC-35 and the cell line UM-SCC-14C, which is insensitive to ATRA (Fig. 7). In the XTT and MTT test we confirmed the results described by Copper et al (123) with varying growth inhibition after brief exposure (~6 days) to 10^{-5} and 10^{-8} M ATRA. The growth inhibition that we demonstrated in UM-SCC-35 after treatment by ATRA was not significant when compared to its growth without RA. In monolayer cultures and colony counts before and after exposure to RA under an inverted microscope Jetten et al (135) confirmed our results for inhibition of proliferation of UM-SCC-35 cells in the XTT and MTT test. They also included two further cell lines, 1483 (from an untreated patient with a well-differentiated SCC T2N1M0 of the retromolar trigone) and 183 (from an untreated patient with a poorly differentiated SCC T3NOMO of the tonsil). Both these cell lines were also examined by Lotan et al (136), who in cell counts demonstrated growth inhibition after ATRA treatment. Both Jetten et al (135) and Lotan et al (136) found growth inhibition after ATRA in varying degrees and in a dose-dependent fashion. While cell line 1483 was RA-sensitive, the more aggressive cell line 183 was not inhibited in growth. Since the RA-sensitive cell line UM-SCC-35 which we used was also established after chemotherapy for a primary tonsillar carcinoma (T4N1M0, moderately well
differentiated) and the RA-insensitive cell line UM-SCC-14C for a skin metastasis of a floor and mouth carcinoma (poorly differentiated), it may be supposed that not tumor localization but rather tumor aggressiveness relates to RA-sensitivity. This would correspond with the findings by Zou et al (137), who also demonstrated only a minimal inhibition in cells of the cell line 183 after 10^{-4} M ATRA exposure (19±3% growth inhibition) using electronic cell counts and calculating with the equation (1−N_{controls}/N_{treated}) x 100, while the HNSCC cell line 1483 proved to be clearly inhibited by ATRA (growth inhibition 64±7%).

For the cell line HaCaT (121), the XTT and MTT test evidenced no growth inhibition comparable to that of UM-SCC-14C after brief exposure to 10^{-3} or 10^{-4} M ATRA (Fig. 7). This result corresponds with the investigations by Schroeder and Zouboulis (128) who used a 4-methylumbel liferyl heptanoate (MUH) fluorescence assay to examine growth inhibition and with the results of Borland et al (138) who determined the number of cells before and after 10^{-4} to 10^{-5} M ATRA treatment. By contrast, Jang et al (139) proved growth inhibition in HaCaT cells with up to 10^{-4} M ATRA in the MTT test, and Wanner et al (140) with 10^{-6} M ATRA by determining the cell counts. Chen et al (127) had investigated the impairment to growth in HaCaT by different retinoids in increasing concentration in the MUH fluorescence assay by up to 0.5x10^{-6} M ATRA. By contrast, the growth of HaCaT cells in our findings was particularly inhibited by ATRA.

In situ proof of RAR and RXR receptors and Ki-67 and p53 after brief and extended treatment of three cell lines with ATRA. Since the effects of retinoids are mediated via their nuclear RAR and RXR receptors, we examined RAR (RARα, RARβ, and RARγ) and RXR (RXRα, RXRβ, and RXRγ) on the three cell lines (Fig. 2). The results (RXRα, RXRβ, and RXRγ, not presented) were not statistically assessable. The only antibody against RARγ available to us did not yield evaluable findings.

For in situ proof of the receptors on the cell lines we chose immunocytochemistry after pretreatment of the cytosplins in the steamer. In our preliminary experiments the nuclear results had proved to be clearly better than those without pretreatment (Fig. 1). The expression of receptors RARα (Ab 1), RARβ (Ab 2) and RXRγ (Ab 5; Fig. 2) as demonstrated in immunocytoLOGY on the cells of the RA-sensitive UM-SCC-35 was low before and after brief (~6 days) and extended (~26 days) RA treatment, but not significantly reduced (Fig. 2). As opposed to this, Copper et al (123) demonstrated up-regulation of mRNA levels for RARα and RXRγ (from 12 to 24% and from 47 to 84%, respectively) on the RA-sensitive cells of UM-SCC-35 during retinoid therapy, while the receptor RXRα remained unchanged and low. In the study by Klaassen et al (124) the increase in RARα-mRNA was less than in the studies by Copper et al (123). Klaassen et al (124) found only very low concentrations of RARβ-mRNA in UM-SCC-35 cells, either before or after RA treatment. These in vitro findings could not be confirmed in head and neck cancer tissue samples: in these samples, RARβ expression was higher after retinoid treatment (18,50,60,88-90).

On the cells of the cell line UM-SCC-14C (Fig. 7), which was not affected by ATRA, we demonstrated RARα to be significantly higher in immunocytochemistry after brief exposure to ATRA (~6 days) (Fig. 2; Ab 1), and RXRγ (Fig. 2; Ab 5), bordering on significance. This could no longer be demonstrated after extended treatment by ATRA (~26 days). This increased expression of RARα is congruent with the findings by Copper et al (123), who after treating the cells with ATRA were able to measure an increase in mRNA-RARα from 94 to 100%, while RARγ was down-regulated (mRNA from 95 to 51%). Using the same method, Klaassen et al (124) demonstrated an even steeper increase in the expression of mRNA-RARα in UM-SCC-14C after ATRA treatment. These results were congruent with ours (Ab 1; Fig. 2). However, Klaassen et al (124), contrary to our results (Fig. 2), found no mRNA of RARβ in UM-SCC-14C cells, either before or after ATRA treatment. In other HNSCC cell lines (183, 886, 1483 and SqCC/Y1) Zou et al (137) detected an increase in expression of the RA receptors after 10^{-4} ATRA both in Northern blot and in immunocytoLOGY, whereby the authors did not perform quantitative evaluation of the immunocytological results.

ImmunocytoLOGY examination revealed no changes in the expression of Ki-67 (Ab 6; Table 1) in the cells of UM-SCC-35 (Fig. 4A1-A3) or in UM-SCC-14C (Fig. 4B1-B3) after brief (~6 days) or extended RA treatment. There appears to be a contradiction between the obvious growth inhibition of UM-SCC-35 by ATRA (Fig. 7) and Ki-67 as a proliferation marker (141). D'Ambrosio et al (142) however demonstrated inhibition of Ki-67 by several different retinoids, including ATRA, in quantitative Ki-67-ELISA.

By choosing an antibody as proof of p53 protein (Ab 7; Table 1) and pretreating the cytosplins and frozen sections in the steamer we proved both the expression of wt-p53 protein (wild-type of p53) and mtp-p53 protein (mutant form of p53) in our immunocytochemical investigations. After extended incubation with ATRA, the p53 expression in UM-SCC-35 cells was clearly reduced to borderline significance (Fig. 4A4-A6). In immunocytochemistry, Hauser et al (143) demonstrated p53 in untreated cells of UM-SCC-14C, as we also did in this cell line without and after RA treatment (Figs. 3 and 4B4). After brief and extended ATRA treatment, the p53 scores in the cells of UM-SCC-14C were demonstrated to be comparable to those previous to RA treatment (Figs. 3 and 4B4-B6). The absence of growth inhibition after brief RA incubation with 10^{-4} and 10^{-5} M ATRA (Fig. 7) is accompanied in our model study in the case of UM-SCC-14C by a non-reduction of p53 expression (Fig. 4B3-B6). In a quantitative p53 ELISA following RA treatment, D'Ambrosio et al (142) showed in 10 of 15 other HNSCC cell lines a decrease in mtp-p53 which was not the case in 5 of 15 cell lines. Bradford et al (144) demonstrated p53 mutations in the cell line UM-SCC-14A, established from a primary carcinoma of the floor of the mouth, in the sequence analysis of the p53 gene. In the PCR, Sun et al (61) detected p53 mutations in different HNSCC cell lines, among others in cells of UM-SCC-14B. This cell line had been established in the relapse of the same patient. This tumor then metastasized in the patient's skin, and from this metastasis was established the ATRA-resistant cell line UM-SCC-14C which we studied. We can assume that UM-SCC-14C contains p53 mutations as do UM-SCC-14A and UM-SCC-14B. Sun et al (61) also demonstrated p53 mutants in the cell line 183A, whose growth (like that of UM-SCC-14C) was not inhibited or altered after RA treatment (Fig. 7). These
The effects of ATRA are clearly connected with the absence of p53 mutants or, conversely, with non-sensitivity in the presence of p53 mutants. This is confirmed in the results by Nakashima et al (145), who used the HNSCC cell lines Tu138 and MDA686LN with p53 mutations. Their growth was not or only barely inhibited by 10⁻⁷ and 10⁻⁶ M ATRA administered in doses comparable to those used in our study, and only markedly inhibited by a dosage of 10⁻⁵ M ATRA.

In our study HaCaT cells were found to be RA insensitive (Fig. 7). The expression of receptors RARα (Ab 1), RARβ (Ab 2) and RXRα (Ab 5) was reduced, but not significantly (Fig. 2), after brief ATRA treatment (~6 days). After a 24-h incubation of HaCaT cells using all-trans-RA, Boudjelal et al (146) were able to reduce RARγ and RXRα receptors at the protein level by one half. This result was not demonstrated at the level of mRNA. RAR-β was not demonstrated, even after 24 h stimulation of the HaCaT cells with RA. The expression of Ki-67 and p53 (Figs. 3 and 4C1-C6) which was demonstrated in our study did not change significantly after brief ATRA treatment. Soo Lee et al (147) were able to prove only a slight reduction in p53 in Western blot.

p53 has already been used as a biomarker in individual chemoprevention studies (17,29,60,89,107,109,110). The results of our model experiments show that proof of reduced protein expression in p53 as a biomarker indicates successful retinoid treatment. D'Ambrosio et al (142) arrive at the same conclusion. This needs confirmation in further RA sensitive cell lines. In clinical studies Geyer et al (17) and Lippman et al (87) only established effectiveness of chemoprevention with retinoids when the wild-type of p53 was not yet mutated and Shin et al (89) proved in chemoprevention studies after isotretinoin therapy (13-cis-RA) that the effects of retinoids were better in patients with a low p53 expression (response rate 65%) than in those with higher expression (response rate 27%).

In situ proof of RAR and RXR receptors and of Ki-67 and p53 on carcinomas in the head and neck region and on the control tissues. Parallel to the model experiments on cell lines we demonstrated on squamous cell carcinomas (Table IIA) and on control tissues in comparable localizations (Table IIB) with the same methods of proof and the same antibodies (Table I) the RA receptors RARα (Ab 1), RARβ (Ab 2), RXRα (Ab 3), RXRβ (Ab 4) and RXRγ (Ab 5) as well as Ki-67 (Ab 6) and p53 (Ab 7) (Figs. 5 and 6). This is congruent with the results by Lotan (32), Xu et al (85), Ralhan et al (92), Ornait et al (148), Sherman and Partridge (149) as well as Wan et al (150), who demonstrated RA receptor on normal, premalignant and malignant tissues from the head and neck region using various different methods. Although we demonstrated the RA receptors in carcinoma tissue (Fig. 5) and in the cell lines (Fig. 2). Our model experiments on cell lines show that this is not reliable enough for their use as biomarkers for a successful chemoprevention with ASTRA.

Proof of telomerase as a potential biomarker. Telomerase has been investigated and demonstrated in many studies in squamous cell carcinomas of the head and neck region (among others in 112), including in our investigations (117). Activation of telomerase points to an early carcinogenesis in this location (112-117). High telomerase activity can be demonstrated in the permanent cell lines established from head and neck tumors but also in the immortalized keratinocyte cell lines HaCaT (139). This raises the question of whether proof of telomerase might be a suitable biomarker for the chemoprevention of precancerous lesions or postoperatively (17,27,100-102,107). Fig. 8 illustrates the telomerase activity explored in our studies in several assays before and after ATRA treatment in PCR-ELISA in the RA-sensitive cell line UM-SCC-35, the RA-resistant cell line UM-SCC-14C and in the keratinocyte line HaCaT. In addition, we examined telomerase activity in the same tumor and control tissues (Table II) that we had used for the immunohistochemical proof of the RA receptors (Fig. 8). In none of the three cell lines did brief treatment (~6 days) of 10⁻⁵ ATRA lead to an inhibition of telomerase activity (Fig. 8), not even in the RA sensitive growth-inhibited cell line UM-SCC-35 (Fig. 7). Only after extended treatment (~26 days) with 10⁻⁵ ATRA could we demonstrate a distinct inhibition of telomerase activity in the cells of UM-SCC-35 (Fig. 8). No reduction in telomerase activity was to be found in the cell lines UM-SCC-14C (Fig. 8). The fact that there was no inhibition of telomerase activity in these cells is in keeping with the absence of growth inhibition after treatment with 10⁻⁵ and 10⁻⁶ M ATRA (Fig. 7). Zhang et al (151) studied the effect of both 10⁻⁵ and 5x10⁻⁶ M ATRA on cells of the HNSCC line Tca8113. Both cell growth and telomerase and hTERT were markedly suppressed in this line after a 9-day treatment (151).

HaCaT cells showed no significant growth inhibition in our experiments after brief RA treatment (Fig. 7). Nor was telomerase activity significantly reduced by RA treatment (Fig. 8). As opposed to our results, Jang et al (139) observed a dosis-dependent inhibition of telomerase activity in HaCaT cells after a 5-day treatment of 10⁻⁵, 10⁻⁴ and 10⁻³ M ATRA, which was accompanied by a parallel reduction in inhibition of proliferation as measured in an MTT cell survival assay. The investigation by You et al (152), however, yielded the finding that the telomerase activation of established normal oral keratinocytes from the gingiva was not inhibited by ATRA.

Although our model experiments on RA-sensitive cell line UM-SCC-35 show that growth inhibition can be demonstrated even after brief RA treatment (Fig. 7) and reduced telomerase activation only after extended RA treatment (Fig. 8), our results do support proof of telomerase as a biomarker for retinoid treatment. It is however necessary to corroborate proof of telomerase activation in other RA sensitive cell lines before and after retinoid treatment.

In conclusion, in our model experiments the immunocytochemical proof of p53 and the demonstration of telomerase activity in the PCR proved to be suitable for confirming the presence or absence of growth inhibition by ATRA (XTT, MTT). With all necessary precaution we conclude that both parameters are suitable biomarkers for testing the effectiveness of chemoprevention. In clinical chemoprevention studies Geyer et al (17), Hong et al (27), Kelloff et al (62), Liebermann et al (100), Koch (101), Kelloff et al (102) and Smith and Saba (107) also drew the conclusion that the investigation of p53 and the proof of telomerase activity can be suitable biomarkers for the chemoprevention of oral carcinomas. According to the observations made by Kelloff et al (102) the proof of telomerase activity could above all be used for applying surrogate end points in chemopreventive drug development.
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