Retinoid-induced Suppression of Squamous Cell Differentiation in Human Oral Squamous Cell Carcinoma Xenografts (Line 1483) in Athymic Nude Mice


ABSTRACT

Retinoids are promising agents for therapy of squamous cancers. In vitro, retinoids decrease expression of differentiation markers in head and neck squamous carcinoma cells. Little information is available on effects of retinoids on head and neck squamous cell carcinoma xenograft growth in vivo. To address this issue, head and neck squamous carcinoma cells (line 1483) were established as xenografts in nude mice. Control tumors grew rapidly with doubling times of 4–6 days to mean volumes of 1696 mm$^3$ after 24 days. Histological analyses indicated the formation of well-differentiated squamous carcinoma cells exhibiting pronounced stratification (basal and suprabasal cells) and keratinization (keratin pearls) with abundant stroma. Cytokeratin 19 expression was restricted to the basal cell layer and the cytokeratin 4 expression was abundant in suprabasal cells. Mice were treated daily with 30 mg/kg 9-cis retinoic acid, 20 mg/kg all-trans retinoic acid, or 60 mg/kg 13-cis retinoic acid by p.o. gavage on a schedule of 5 days/week over 4 weeks. Low micromolar (1.48–3.67 μM) and nanomolar (200–490 nM) concentrations of 9-cis retinoic acid and all-trans retinoic acid were measured in plasmas and xenografts, respectively, 30 min after dosing. Retinoid treatment produced a marked suppression of the squamous cell differentiation of tumor cells manifest by decreased stratification, loss of stratification, and accumulation of basal cells. This was accompanied by large decreases in the number of CK4-positive cells and concomitant increases of CK19-positive cells. Retinoic acid receptor-β expression was also increased by 2.9–9.7-fold after chronic retinoid treatment. 9-cis retinoic acid and all-trans retinoic acid decreased tumor volumes by 23 ± 5 (SE) and 19 ± 3%, respectively (P ≤ 0.05); 13-cis retinoic acid was inactive. These retinoids did not decrease the rate of exponential tumor growth but increased the latent period until exponential growth began. These studies demonstrate that retinoids do not universally decrease tumor growth but profoundly suppress squamous cell differentiation in vivo in this xenograft model.

INTRODUCTION

Retinoids are promising agents for therapy of squamous cancers (1, 2). Clinically, 13-cis-RA$^3$ suppresses oral leukoplakia and prevents or delays reoccurrence of second primary head and neck tumors (3–5). ATRA also reverses cervical dysplasia (6), confirming the sensitivity of premalignancies to retinoids. In contrast, 13-cis-RA is not effective against primary head and neck tumors (5) or other solid neoplasms (1, 2, 7). However, the combination of 13-cis-RA and IFN has produced very efficacious results in treating squamous carcinomas of the skin and cervix (8, 9). Mechanisms by which retinoids modulate malignancy involve retinoid receptors, members of the steroid/thyroid superfamily of intracellular receptors (10). Improvements in therapy may come by gaining a better understanding of the basic mechanisms by which retinoic acid receptors modulate growth of squamous epithelial cell cancers.

The rationale for the use of retinoids in therapy of epithelial cancers is based primarily on their ability to modulate cellular differentiation and growth. Early studies demonstrated that vitamin A deficiency induces squamous cell hyperplasia that is reversible by vitamin A supplementation (11). This has been shown biochemically by inhibition of terminal squamous cell differentiation markers such as loricrin, involucrin, type I transglutaminase, CK1, and CK10, in normal and neoplastic epithelial cells in vitro (12–15). Retinoids can also modulate cellular proliferation. ATRA decreases cellular proliferation and cloning efficiency in many tumor cell lines in vitro, although effects are generally not >50% (15, 16). Another mechanism suggested by Sacks et al. (17) is induction of apoptosis. In an in vitro spheroid model (886Ln), ATRA inhibited spheroid growth without inhibiting thymidine incorporation into spheroids (17). This observation has been extended to other squamous carcinoma cell lines in which ATRA increased thymidine incorporation (18). These data suggest that retinoid regulation of any or all of these cellular processes, such as altered differentiation, decreased proliferation, or increased apoptosis, could contribute to antineoplastic activities of retinoids in squamous cancers.

A primary limitation of studies of retinoid action in vitro is attributed to the nonphysiological situation of malignant cells cultured in vitro. HNSCC lines, in particular, are noted for their dependence on stromal cells and have been difficult to clone from primary tumors (19, 20). Culture conditions in vitro do not replicate those in vivo in humans or animals. Cells are deprived of their normal stromal support, and this may affect squamous cell differentiation or growth (19, 20). Furthermore, proliferation or differentiation in vitro may be influenced by growth factors supplied in FCS (19, 20). To better replicate the biology of squamous cell cancer in vivo, we have used a xenograft model of HNSCC. In a xenograft model, stromal elements, vascularization, three-dimensional tumor cytoarchitecture, and squamous cell differentiation are allowed to develop more closely to that observed in clinical cancer (21). In addition, pharmacokinetics and metabolism of retinoids may be more faithfully replicated to those observed clinically. Thus, we chose to characterize an in vivo model to study HNSCC using line 1483 because it expresses ATRA-sensitive squamous cell differentiation markers (13, 15).

In this study, the 1483 HNSCC xenograft model has been characterized, and the effects of three naturally occurring retinoid isomers, 9-cis-RA, 13-cis-RA, and ATRA, on squamous cell differentiation and tumor growth have been compared. This report demonstrates a prominent inhibition of squamous cell differentiation by all three retinoids in this xenograft model. However, only 9-cis-RA and ATRA decreased tumor growth.


donated 3/24/95; accepted 5/16/95.
1 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.
2 Portions of this work have been presented at the Federation of American Societies for Experimental Biology Summer Conference on Retinoids, Copper Mountain, CO, June 1994, and the 86th Annual Meeting of the American Association for Cancer Research, Toronto, Ontario, Canada, March 1995. This work was supported by the Allergan-Ligand Joint Venture.
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4 The abbreviations used are: 13-cis-RA, 13-cis retinoic acid; 9-cis-RA, 9-cis retinoic acid; ATRA, all-trans retinoic acid; HNSCC, head and neck squamous cell carcinoma; CK4, suprabasal cell cytokeratin 4; CK19, basal cell cytokeratin 19, synonymous with keratin 10; CK10, synonymous with keratin 10; MTD, maximum tolerated dose; RAR, retinoic acid receptor; RXR, retinoic acid "X" receptor.

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MATERIALS AND METHODS

Reagents and Chemicals. 9-cis-RA (lot LG100947-000X004) was synthesized at Ligand Pharmaceuticals, Inc. (San Diego, CA). ATRA (Schweizerhall, South Plainfield, NJ) and 13-cis-RA (Sigma Chemical Co., St. Louis, MO) were purchased and recrystallized to >99% purity. Compound purity was verified by elemental analysis, nuclear magnetic resonance spectra, and HPLC-UV assay.

Cell Line. The 1483 cell line was derived from a patient with squamous cell cancer of the retromolar trigone. Histopathology showed that line 1483 was aggressive and had metastasized to one lymph node (22). Cells were routinely cultured in DMEM:F-12 media supplemented with 10% FCS, 2 mM L-glutamine, and 5 units/ml penicillin-5 µg/ml streptomycin (GIBCO-BRL, Grand Island, NY) in 95% air-5% CO2. Cells were found to be free of mycoplasma.

In Vivo Tumor Xenograft Studies. Cells in log phase were harvested by trypsinization, resuspended in DMEM:F-12 media (Biofluids, Rockville, MD), centrifuged at 1000 rpm for 10 min, and resuspended in culture media at a concentration of 1 x 10^6 cells/ml before s.c. implantation into mice.

Mice were quarantined for 1 week before the study and allowed access to food (Teklad LM-485 mouse/rat sterilizable diet; Harlan Teklad, Madison, WI) and water ad libitum. Female athymic NCI-nu mice, 6-7 weeks old (20 ± 2 g; Taconic Labs, Germantown, NY), were implanted with 1 x 10^6 cells bilaterally into the left and right axial regions with a 24-gauge needle/1 cc concentration of 1 x 10^7 cells/ml before s.c. implantation into mice. Animals were randomized into treatment groups 48 h after tumor implantation. Each group consisted of 5-6 animals bearing two tumors/animal. Tumors were measured 18-24 days after tumor implantation as described in the text. Experiments were repeated 3-7 times to ensure reproducibility. Retinoids were administered p.o. beginning 2 days after tumor cell implantation with a 20-gauge intragastric feeding tube (Popper & Sons, New Hyde Park, NY) daily, 5 days/week, in 0.1 ml of super-refined sesame oil (Croda, Inc., Parsippany, NJ) at doses of 20-60 mg/kg. The MTD was determined for each retinoid in nude mice based on effect on weight and mucocutaneous toxicities, and doses approximating 50-60% of the MTD were administered. Doses used in xenograft studies were 30, 20, and 60 mg/kg for 9-cis-RA, ATRA, and 13-cis-RA, respectively. Tumors were measured with electronic calipers (Mitutoyo, Japan) once or twice weekly, and tumor volumes were calculated with the use of the formula (length x width x height). Animal weights were recorded weekly with the use of a balance (O’Haus Model C305-S; Florham Park, NJ). Clinical signs such as overall health status and potential mucocutaneous toxicities were recorded weekly.

Xenograft Tumor Biomarker Analyses. Tumors were surgically excised, and cross-sections were cut with the use of sterile scalpels. Samples were fixed in 10% neutral buffered formalin for 24-48 h before embedding in paraffin blocks. Hematoxylin and cosin, CK4, and CK19 staining were performed on 5-µm cross-sections cut from these blocks. Antibodies to CK4 and CK19 were purchased from Sigma Chemical Co. and Dako Corp. (Carpintería, CA), respectively. Immunohistochemical staining was preceded by antigen retrieval in a microwave oven. Biotinylated Ho/AMo IgG or Gt/ARb IgG (Vector Laboratories, Burlingame, CA) was used at 1:150 and visualized by horseradish peroxidase-conjugated avidin (Vector Laboratories) at 1:1000 (12, 23, 24).

Portions of tumors were also snap-frozen in liquid nitrogen for RNA isolation.

RNA Protection Assay. RNA protection assays using a human probe for RAR-ß were conducted on RNA isolated from 1483 xenograft tumor tissues. Total cytoplasmic RNA was isolated as described (25) or with the use of Stat-60 phenol extraction (Tel Test “B,” Inc., Friendswood, TX). RNA protection assays were performed as described (26, 27). Hybridization of the RNA probe was carried out at 45°C overnight, followed by the addition of 300 µl of RNase digestion buffer containing 40 µg/ml of RNase A and 700 units/ml of RNase T1. RNase digestion was performed at 25°C for 1 h. RNase-resistant fragments were resolved by electrophoresis on 6% polyacrylamide sequencing gels. To control for RNA loading, a glyceraldehyde 3-phosphate dehydrogenase probe was included in all samples. As approximate size markers, 32P-labeled MspI-digested fragments of pBR322 were run on all gels.

RESULTS

1483 Xenograft Model

Tumor Growth Conditions. Conditions for optimizing tumor growth were initially determined. 1 x 10^6, 2.5 x 10^6, 5 x 10^6, and 1 x 10^7 cells were suspended in culture medium and injected s.c. into mice. Matrigel, a basement membrane matrix used to enhance tumor take (30), was co-implanted with tumor cells to determine its effect on tumor growth. Tumor cells were not dependent on Matrigel for growth, but growth was dependent on the number of cells implanted (Fig. 1). There was a lag phase observed in all groups; good tumor growth was only observed in mice implanted with at least 0.5 or 1 x 10^6 cells. The best growth was observed after implanting 1 x 10^6 cells and resulted in emergence of solid tumors that readily became vascularized. Tumors had doubling times of 4-6 days achieving mean volumes of 1696 ± 222 mm^3 after 24 days. However, areas within tumors were cystic or necrotic after reaching volumes of ≥1500 mm^3.

PLASMA ANALYSES. Analytical procedures were carried out under subdued light at room temperature. Plasma concentrations of 9-cis-RA and ATRA were quantified by HPLC-UV detection with the use of external standardization. Duplicate standards at seven concentrations were prepared by spiking control mouse plasma with serially diluted ethanolic 9-cis-RA or ATRA solutions to achieve final concentrations ranging from 0.03-17 µM. Plasma samples (200 µl) and standards were extracted with 5 volumes of methanol, chilled to -20°C for 1 h, and centrifuged. Supernatants were evaporated in vacuo. After reconstitution in the 300-µl mobile phase (acetoni-trile:10 mM ammonium acetate:glacial acetic acid, 80:20:1), samples were centrifuged and resulting supernatants were transferred into inserts within amber autosampler vials for HPLC analysis.

Tumor Analyses. 9-cis-RA or ATRA was added to excised control tumors at the same concentrations as described for plasma standards (0.03-17 µM; 1 g = 1 ml, assumed). Tumors were minced individually with scissors. Water and methanol were added to achieve a (tumor + water):methanol of 1:5 (w/v). A Brinkmann Polytron was used to homogenize tumor-liquid mixtures for 20 s at 20,000 rpm. Homogenates were stored at -20°C overnight and centrifuged at 0-4°C for 10 min at 3200 g. Supernatants were evaporated in vacuo and processed for HPLC analysis as described below.

HPLC Analysis. One hundred-µl volumes were injected onto an Alttech Spring-Connect 5-µm C18 guard column connected to a Rainin Microsorb-MV 5-µm C18, 4.6 x 250-mm column. Run times were 17 min with the use of a 1 ml/min flow rate. Column temperature was maintained at 40°C. UV absorbance of 9-cis-RA or ATRA was monitored at 348 nm with a bandwidth of 60 nm; chromatographic peaks were integrated and quantified with the use of the peak area and the standard curve of the corresponding tissue. Raw tumor concentration data were normalized to individual tumor weights.

Data Analysis. Mean ± SEM values are shown for all data unless otherwise indicated. Statistical analyses of data were performed with the use of unpaired Student’s t test with two-tail comparison. Differences of P < 0.05 were considered significantly different from control.

RESULTS

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Tumor Growth Conditions. Conditions for optimizing tumor growth were initially determined. 1 x 10^6, 2.5 x 10^6, 5 x 10^6, and 1 x 10^7 cells were suspended in culture medium and injected s.c. into mice. Matrigel, a basement membrane matrix used to enhance tumor take (30), was co-implanted with tumor cells to determine its effect on tumor growth. Tumor cells were not dependent on Matrigel for growth, but growth was dependent on the number of cells implanted (Fig. 1). There was a lag phase observed in all groups; good tumor growth was only observed in mice implanted with at least 0.5 or 1 x 10^6 cells. The best growth was observed after implanting 1 x 10^6 cells and resulted in emergence of solid tumors that readily became vascularized. Tumors had doubling times of 4-6 days achieving mean volumes of 1696 ± 222 mm^3 after 24 days. However, areas within tumors were cystic or necrotic after reaching volumes of ≥1500 mm^3.
Implantation of $1 \times 10^6$ cells was chosen as a standard condition for additional study.

**Tumor Morphology.** To characterize development of squamous cell differentiation, tumors were excised beginning 16 days after cell implantation, and sections were stained with hematoxylin and eosin. Fig. 2 shows tumors collected after 20, 22, 24, and 26 days of growth ($\times 40$). After 20 days, tumors were histologically and cytologically poorly differentiated as shown by lack of cellular stratification and a monotypic cellular composition. Accordingly, keratinization (keratin pearl formation) was confined to small areas. Increasing keratinization and organization of cells into distinct compartments with cytological characteristics of suprabasal and basal cells became maximal after 24 days. Keratin pearls are denoted by the letter "K," long arrows point toward basal cells, short arrows point toward suprabasal cell layers, and stroma surrounding epithelial nests is indicated by the letter "S." Parallel to changes consistent with a more differentiated state in the tumor parenchyma, the stroma became more prominent. A representative well-differentiated squamous cell carcinoma at high magnification ($\times 200$) is shown in Fig. 3.

**Cytokeratin Markers of Squamous Cell Differentiation.** To monitor squamous cell differentiation, cytokeratin expression was determined in tumor sections. CK4 was chosen to characterize the differentiating (suprabasal) cell compartment whereas CK19 is a marker of the basal, proliferating cells (31-33). In normal tissues, CK4 expression is confined to squamous epithelia of mucous membranes, whereas CK19 is expressed in basal keratinocytes of the epidermis as well as in mucous membranes (31-33). Control staining of CK4 and CK19 at low magnification ($\times 40$) is shown in Fig. 4. CK4 was localized to suprabasal cells and CK19 was localized to basal cells in an organized manner. Widespread CK4 staining indicates the presence of differentiating cells in 1483 tumors.

**Effects of Retinoids on 1483 Xenografts**

**Determination of MTD.** MTD was defined as the dose that produced mild, tolerable mucocutaneous toxicities commonly associated with hypervitaminosis A and body weight losses ($\leq 7\%$). To assess MTD, body weight and symptoms such as dry or red skin were scored biweekly on a scale of 0–3 where 0 = normal, 1 = mildly affected, 2 = moderately affected, and 3 = severely affected. Dose ranging data are shown in Table 1. 9-cis-RA produced dose-dependent increases in mucocutaneous toxicities and decreases in body weight with an MTD of 60 mg/kg. ATRA was less well tolerated and was assigned an MTD of 20 mg/kg. 13-cis-RA was better tolerated, and its MTD was set at 120 mg/kg based on decreases in body weight. To study effects of retinoids at comparably well-tolerated doses, doses approximating 50–60% of the MTD were used. These doses were 30 mg for 9-cis-RA, 60 mg/kg for 13-cis-RA, and 20 mg/kg for ATRA and produced only mild retinoid-related symptoms.

**Effects of Retinoids on Tumor Growth.** Fig. 5 shows effects of 9-cis-RA, 13-cis-RA, and ATRA on growth of 1483 tumors. 9-cis-RA decreased tumor volumes by 23 ± 5% after 24 days of growth ($P < 0.05; n = 7$). In comparison, 20 mg/kg ATRA decreased volumes by 19 ± 3% ($P = 0.05; n = 3$) after 24 days, whereas 60 mg/kg 13-cis-RA had no effect when studied up to 18 days ($n = 3$). 13-cis-RA was also studied at 30 mg/kg for 24 days with no effects on growth (data not shown). 9-cis-RA and ATRA did not decrease the rate of exponential tumor growth but increased the latent period until exponential growth began. To determine whether 9-cis-RA might have greater effects against a lower tumor burden, 0.5 × 10⁵ cells were implanted into mice and treated with this retinoid. After 32 days, tumor growth was decreased by 18% by 9-cis-RA relative to controls (data not shown), indicating that tumor sensitivity was unchanged with decreasing tumor burden. Thus, 9-cis-RA and ATRA decreased tumor growth by up to 23%, and 13-cis-RA had no effect on growth as assessed by tumor volume measurements.

**Effects of Retinoids on Tumor Morphology and Squamous Cell Differentiation.** Fig. 6 shows that retinoids markedly inhibited keratin pearl formation and decreased cellular stratification so that, in contrast to sesame-oil treated control tumors (Figs. 2 and 3), distinct suprabasal and basal cell layers could not be discerned. The tumor:parenchyma ratio significantly increased after retinoid treatment, and the tumor morphology was that of a solid carcinoma without features of obvious squamous cell differentiation. Furthermore, Fig. 7 shows that 9-cis-RA, ATRA, and 13-cis-RA markedly decreased CK4 staining and increased CK19 staining as compared to control (Fig. 4). Expression of these molecular markers of differentiation were in agreement with morphological effects produced by retinoids and demonstrated suppression of epidermoid squamous cell differentiation.

**Modulation of RAR-β Expression by Retinoids.** Table 2 shows results of RNA protection assays conducted to test effects of retinoids on RAR-β expression in xenografts. Tumors were collected after 24 days of growth following 5 day/wk treatment with each retinoid. RAR-β mRNA was expressed at low levels in xenografts isolated from animals dosed with sesame oil. 9-cis-RA and ATRA increased RAR-β expression by 5.9- ($n = 5$) and 9.7-fold ($n = 1$), respectively, whereas 13-cis-RA increased it by 2.9-fold ($n = 2$). These data demonstrate that retinoids increased RAR-β mRNA message after p.o. dosing (ATRA ≥ 9-cis-RA > 13-cis-RA).

**Tumor/Plasma Concentrations of 9-cis-RA or ATRA after p.o. Dosing.** Mice treated for 28 days with 9-cis-RA, and naive control mice were acutely dosed with 9-cis-RA to assess tumor and plasma concentrations of 9-cis-RA. Tissues were collected 30 min after dosing. Plasma and tumor concentrations of 9-cis-RA in mice pretreated with 9-cis-RA were 1.48 ± 0.30 μM and 0.20 ± 0.11 μM, respectively (Table 3). These concentrations were approximately one-half the mean values of 3.25 and 0.37 μM measured in plasma and tumor concentrations, respectively, in naive animals. Naive mice were also acutely dosed with 30 mg/kg ATRA to assess plasma and tumor concentrations of ATRA. Plasma and tumor concentrations of ATRA were 3.67 ± 1.72 μM and 0.49 ± 0.40 μM, respectively, 30 min after dosing. 13-cis concentrations were not studied. No areas under the curve could be determined from this study.
Fig. 2. Development of well-differentiated squamous carcinoma xenografts over time. Tumors are shown at A, 20 days; B, 22 days; C, 24 days; and D, 26 days by hematoxylin and eosin staining. K, keratin pearls; large arrows point to basal cells; small arrows point to suprabasal cells; S, stroma surrounding epithelial nests (× 40).

Fig. 3. High magnification photomicrograph of control tumor after 24 days (× 200).
DISCUSSION

The main goals of this study were to characterize the HNSCC 1483 xenograft model for studying effects of retinoids in vivo. Growth and differentiation of HNSCC 1483 xenografts were characterized. Cells implanted into nude mice readily formed tumors. Tumor incidence was >95%, and tumors were well vascularized with reproducible growth kinetics. Xenografts formed well-differentiated epidermoid cancer with prominent keratinization and abundant connective tissue stroma. The time course of tumor differentiation was very similar to that observed during maturation of the epidermis (32). The presence of basal and suprabasal cells and keratin pearls is consistent with a differentiated phenotype, whereby basal cells mature into suprabasal cells before terminally differentiating into squames (keratin pearls; Refs. 31–33). This morphology is often observed in patients with oral cancers deriving from nonkeratinizing mucosal surfaces (31, 33) such as HNSCC 1483 (22). Thus, tumors replicated a well-differentiated phenotype commonly observed clinically in oral cancer patients.

The major effect of retinoids in vivo was on differentiation. Marked and uniform suppression of squamous cell differentiation was produced by 9-cis-RA, 13-cis-RA, and ATRA. Retinoids produced effects in this xenograft model parallel to those observed in patients with squamous cell dysplasias, such as oral leukoplakia (4, 5) or cervical dysplasia (6). Furthermore, retinoids suppressed squamous cell differentiation in this xenograft model as would be predicted from effects on keratin expression and terminal differentiation markers reported in in vitro HNSCC models (12–15). Immunohistochemical evidence of retinoid-induced decreases in CK4 and increases in CK19 expression coincided with morphological changes. These data support in vitro results that show both down-regulation of keratins such as CK1 or CK4 (14, 15), and up-regulation of CK19 expression after retinoid treatment in HNSCCs (30, 34, 35), providing evidence that squamous cell differentiation was truly suppressed by retinoids in this xenograft model. Thus, this report confirms in vitro findings and extends them into an in vivo setting. The data suggest that retinoids suppress differentiation in squamous carcinomas unlike
their prodifferentiating effects in hematopoietic malignancies such as acute promyelocytic leukemia (36).

In contrast to marked effects on differentiation, 9-cis-RA and ATRA inhibited the growth of 1483 xenografts by 23% and 19%, respectively, whereas 13-cis-RA was inactive on growth. 9-cis-RA acute promyelocytic leukemia (36).

These results parallel clinical results in which 13-cis-RA has failed to decrease growth of squamous cancers as a single agent (5, 6) and in 9-cis-RA and ATRA did not decrease the rate of exponential tumor growth but increased the latent period until exponential growth began. It is possible that 9-cis-RA and ATRA indirectly affected tumor growth by altering factors that influence growth around the tumor microenvironment or angiogenesis. However, there were no changes in vascularity observed grossly, and other factors through which retinoids might have indirectly altered growth, such as mitogens, nutrients, or angiogenesis, were not addressed in this study. Although it may be argued that growth inhibition effects were small, they were statistically significant. It may not be unexpected that growth effects by retinoids would be small based on the aggressiveness of the clinical cancer (22) and the rapid growth observed as xenografts. It is notable that 13-cis-RA had no effect on growth at a dose that was equally active with 9-cis-RA and ATRA in suppressing squamous cell differentiation. These results parallel clinical results in which 13-cis-RA has failed to decrease growth of squamous cancers as a single agent (5, 6) and in vitro clonogenic results in which 13-cis-RA was observed to be the least potent of these three retinoids in inhibiting clonogenicity in soft agar assays. The fact that 9-cis-RA and ATRA can decrease both differentiation and growth suggests that they may have clinical utility in therapy of human squamous cell cancers.

Plasma concentrations of 9-cis-RA after p.o. dosing were similar to those reported by Achkar et al. (29). Measurement of tumor-associated retinoid content demonstrated that 9-cis-RA and ATRA entered tumors after p.o. dosing. This is supported by retinoid-induced changes in tumor morphology and changes in cytokeratin expression. Although plasma and tumor concentrations of 13-cis-RA were not measured, it can be reasoned that significant concentrations of 13-cis-RA were reached in these tissues based on dose-ranging data and effects on tumor morphologies and cytokeratin expression. This assumption is supported by data obtained in SENCAR mice after p.o. dosing with each of these retinoids; 13-cis-RA achieved 27.5 μM plasma concentrations 30 min after an acute 30 mg/kg dose, whereas 30 mg/kg ATRA and 9-cis-RA reached 12.8 and 3.5 μM plasma concentrations, respectively, 30 min after dosing. Thus, 13-cis-RA achieved greater plasma concentrations than did 9-cis-RA or ATRA in SENCAR mice. These data, along with morphological data reported herein, suggest that delivery of 13-cis-RA to tumors should have been at least comparable to 9-cis-RA and ATRA. On the basis of these assumptions, there is no clear explanation as to why 9-cis-RA and ATRA inhibited tumor growth and 13-cis-RA did not. It is possible that pharmacokinetics of 13-cis-RA were altered by pretreatment but this question was not addressed in these tumor biology studies. It can be concluded that total tumor concentrations of 0.2 μM 9-cis-RA or ATRA were sufficient to yield effects on growth and differentiation. Thus, p.o. dosing provided an effective route of retinoid administration in this model.

RAR-β is another marker of retinoid exposure because it is upregulated in vitro by ATRA (15, 34, 35). Increased RAR-β message after dosing with 9-cis-RA, 13-cis-RA, and ATRA indicated that tumors were exposed and responded to retinoids after p.o. administration. It is interesting that 9-cis-RA and ATRA produced greater induction than did 13-cis-RA. A clear role for RAR-β has not been established in head and neck cancer. RAR-β expression has been reported to progressively decrease with malignant progression in patients with head and neck cancers (38). It has also been proposed that RAR-β expression is inversely related to squamous cell differentiation and that RAR-β and CK19 expression are "linked" (15, 35, 36).
Fig. 6. Representative effects of retinoids on morphology of tumors after 24 days of growth. A, 9-cis-RA; B, 13-cis-RA; C, ATRA decreased keratin pearl formation and loss of squamous cell differentiation.
If so, poorly differentiated tumors would express higher levels of RAR-β and CK19 than would well-differentiated control tumors. This was observed in this xenograft model but it is not clear whether increased RAR-β expression was due to increased transcription of the RAR-β gene or whether it could have been due to increased number of CK19-positive cells that express RAR-β.

There is interest in developing new preclinical models for identifying more effective retinoids for clinical study. This report identifies one model in which retinoids have pronounced effects on a classical mechanism of action of retinoids, modulation of squamous cell differentiation. Impetus for identifying more efficacious retinoids comes from the fact that after extensive clinical study, 13-cis-RA has had limited efficacy against primary cancers of the head and neck (1, 2, 5, 6). 9-cis-RA and ATRA, retinoids of increasing clinical interest, have been sparsely studied in human oral cancer. 9-cis-RA is a novel retinoid that binds to both RAR and RXR subtypes (40). Activation of RAR/RXR heterodimers may lead to improved anticancer efficacy over activation of RARs or RXRs alone. In this study, increased efficacy in the form of tumor growth inhibition was observed by 9-cis-RA and ATRA over 13-cis-RA, suggesting that pan agonist or RAR-selective retinoids should be studied clinically in cancers of the head and neck.

This report demonstrated that HNSCC 1483 xenografts mimic a moderately to well-differentiated phenotype commonly observed clinically in patients with oral squamous cancer (31, 33). The model is
useful for studying effects of retinoids or other agents on squamous cell carcinoma in vivo. The main conclusion from these studies is that 9-cis-RA, 13-cis-RA, and ATRA have greater effects on differentiation than on growth in this xenograft model. All three retinoids were equally efficacious in suppressing differentiation of HNSCC xenografts at comparable 50% MTD doses; however, 9-cis-RA and ATRA were more efficacious than 13-cis-RA in inhibiting tumor growth. The HNSCC xenograft model provides another tool for elucidating mechanisms of actions of retinoids in an in vivo cancer model that mimics the morphology of well-differentiated squamous cell cancer.

ACKNOWLEDGMENTS

We are grateful to Paula Sicurello for histological assistance; Kristin Spath for help in conducting xenograft studies; Dr. Stan Howell, Elaine Berger, and Lisa Sheeter for helpful comments; Dr. Marcus Boehm for synthesis and purification of retinoids; and Stephen Dixon for performing HPLC assays. We are also grateful to Dr. Reuben Lotan for supplying HNSCC 1483 cells.

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