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Biological Assay for Activity and Molecular Mechanism of Retinoids in Cervical Tumor Cells[1]

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The composition and response of the retinoid signaling pathway in a human cell line (CC-1), representative of a low grade cervical carcinoma, were evaluated. Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis demonstrated expression of cytoplasmic retinol binding protein, CRBPI, cytoplasmic retinoic acid binding protein, CRABPII, and nuclear retinoic acid receptors, RARα, RARγ, RXRα, and RXRβ, but not CRABPI or RARβ. This pattern is similar to that of the ectocervix. Activation of endogenous nuclear receptors was evaluated in a reporter subline of CC-1, called CC-B, containing a reporter gene controlled by a retinoic acid responsive element (RARE) and thymidine kinase promoter. Retinoid treatment of CC-B resulted in dose-dependent increases in reporter gene expression. Retinoids inhibited growth at concentrations greater than 100 n*M*. 9-*cis* retinoic acid (1 n*M*) significantly stimulated growth. Immunohistochemical analysis of CC-B organotypic cultures demonstrated a high level of epidermal growth factor receptor (EGF-R) expression that was decreased by retinoids. The degree of RARE transactivation induced by retinoids significantly correlated with the degree of inhibition of growth ($R = {}^{-}0.96$) and EGF-R expression ($R = {}^{-}0.92$). The dose-dependent and retinoid-specific responses of CC-1 at the molecular and biological levels demonstrate the utility of this reporter cell line for evaluation of retinoid activities.

INTRODUCTION

Retinoids are a class of drugs, modeled after retinoic acid, that have demonstrated promise as chemotherapeutic agents for the prevention and treatment of several types of cancer including cancer of the cervix [1–7]. World-wide, cervix cancer is the number one cause of cancer death in women, but in the United States, Canada, and Western Europe early detection of precursor lesions with Papincolaou (PAP) smear screening programs has greatly decreased the mortality of this disease [8]. The human papillomavirus (HPV) has been identified in the vast majority of cervical tumors and is believed to be an etiologic agent in the development of this disease. HPV types 16, 18, 31, and 33 are most frequently found in advanced cancer and are considered to be of the high risk type [9].

Transfection of type 16 or 18 HPV DNA into normal epithelial cells induces immortalization (unlimited growth potential) and increased epidermal growth factor receptor (EGF-R) expression [10–12]. In normal epithelium, EGF-R is expressed in the proliferating basal cells and is decreased or not expressed with increasing differentiation of cells toward the mucosal surface [13, 14]. An increase in the number of cells expressing EGF-R is observed in squamous intraepithelial lesions (SIL) of the cervix [13, 14]. The higher grade of SILs exhibit a greater percentage of proliferating cells and a corresponding increase in the numbers of EGF-R-expressing cells. This results in a positive correlation between grade of tumor and EGF-R expression. In invasive squamous cervical carcinoma, overexpression of EGF-R has been associated with biological aggressiveness of the tumor in some studies [15, 16], but not in others [17, 18].

Retinoic acid either decreases or increases EGF-stimulated growth and EGF-R expression depending on the cell line and culture conditions [19–23]. HPV-containing cell lines overexpress EGF-R and are more sensitive to retinoids than normal cells [24–27]. In human ectocervical epithelial (ECE) cells immortalized with HPV 16 (ECE-16), retinoids inhibit growth and decrease EGF-R numbers to levels similar to that observed in normal ECE cells [24]. Increased sensitivity of HPV-containing cells may explain the reversal of premalignant lesions and dysplasias of the cervix by topical retinoic acid [3–7] as well as the regression of cervical tumors in patients treated with a combination of systemic retinoic acid and interferon [1, 2]. The clinical effectiveness

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of retinoic acid however is hindered by significant toxicity [28]. Attempts to improve the therapeutic index of retinoid treatment have been made by developing selective retinoids that specifically activate individual retinoic acid receptors. The effectiveness of this approach can be improved by increasing our understanding of the receptors expressed in cervical tumor cells and their response to retinoid treatment.

The cellular molecules which bind retinoids consist of cytoplasmic retinol and retinoic acid binding proteins, CRBP and CRABP, respectively, as well as two kinds of nuclear retinoic acid receptors, RAR and RXR [29, 30]. Both RARs and RXRs have three subtypes, α, β, and γ, that are encoded by separate genes. The natural ligands which bind to these receptors are all-*trans* retinoic acid, which binds to all three RARs but not the RXRs, and 9-*cis* retinoic acid, which binds to both the RARs and the RXRs [31, 32]. RARs function as heterodimers with RXRs, while RXRs can form homodimers with other RXR molecules and heterodimers not only with RARs [29, 30, 33], but also with other homologous nuclear ligand receptors including the thyroid hormone receptors (TRα and TRβ) [29], the vitamin D receptor (VDR) [29], the peroxisome proliferator-activated receptor α [34], and several orphan receptors [35, 36].

The retinoic acid receptor dimers regulate gene expression by binding to specific DNA sequence elements found in retinoid-responsive gene promoters. Each retinoic acid receptor appears to regulate a different subset of genes [37, 38]. Retinoids modulate the activity of the receptors and thereby the expression of genes containing the specific DNA sequence elements termed retinoic acid responsive elements (RAREs). The RAREs consist of direct repeats of the consensus sequence purine GG(T/A)CA separated by 1 to five base pairs [29, 30]. Several natural *cis*-acting DNA elements which confer responsiveness to retinoic acid have been identified in mammalian genes [29, 30, 39].

The objective of this study was to determine if cultured cells representative of a low grade cervical carcinoma, CC-1, can display biologically relevant responses to retinoids that correlate with receptor activity [40].

MATERIALS AND METHODS

Cell Culture and Retinoid Treatment

CC-1 cultures were maintained in Eagle's minimal essential media (MEM) containing Earle's salts and L-glutamine (Cellgro, Mediatech, Herndon, VA) supplemented with nonessential amino acids, sodium pyruvate, and 10% fetal bovine serum. Only lots of fetal bovine serum that contain undetectable concentrations (less than 10^{-8} *M*) of retinoic acid as determined by high-performance liquid chromatography (HPLC) were used.

All-*trans* and 13-*cis* retinoic acid (both from Sigma, St. Louis, MO) were dissolved in ethanol, and 9-*cis* retinoic acid was dissolved in dimethyl sulfoxide (DMSO). Retinoids were added to the media from 1000× stocks. Control cultures were treated with the same volume of the appropriate solvent. The final concentration of solvent in all cultures was 0.1%, which is not cytotoxic. Media and retinoids were replenished every 2 days.

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA was isolated using the Chomczynski/Sacchi method [41] and cDNA was synthesized with SuperScript reverse transcriptase (GibcoBRL, Gaithersburg, MD) according to manufacturer's instructions. Except for the annealing temperatures and primers listed in Fig. 1, all PCR were performed under the same conditions in a 50-μl reaction containing 5 μl cDNA from reverse transcription, 1.25 U of *Taq* DNA polymerase, and the buffer provided. Initial denaturation was performed at 94°C for 5 min; followed by 35 cycles of 45 sec at 94°C, 45 sec at the indicated annealing temperature, and 1 min at 72°C; and ending with one 5-min cycle at 72°C. The primer sequences were manually selected to amplify contiguous regions of the genes separated by introns. Except for the retinoid X receptor α (RXRα) primers, all sense primers anneal to the C (DNA binding) domain and all anti-sense primers anneal to the D (hinge) domain. The reaction conditions that eliminated amplification of nonspecific receptor sequences were determined by varying the annealing temperatures in reactions containing 5 ng of cDNA clones. Several RXRα primers designed in this manner exhibited nonspecific amplification of other receptor sequences; therefore, a different strategy was used to design specific primers for this gene. The primer set for RXRα was designed to amplify a 400-base pair unique region spanning from the initiation codon to the C domain; this is a region which is not homologous with other receptor genes. The amplified product was tested for specificity by digestion of the PCR product with the *Pvu*II restriction enzyme which cuts the specific band at one unique site resulting in two bands of 142 and 258 base pairs. All experiments were repeated at least twice and the same results were obtained. The primers and reaction conditions for specific amplification of the cytoplasmic proteins previously described were used [42].

Establishment of CC-B Reporter Cell Line and Southern Analysis

CC-1 cells were cotransfected with 10 μg of a reporter plasmid containing the coding sequence of the chloramphenicol acetyl transferase (CAT) gene driven by the RARE from the RARβ gene and the thymidine kinase (tk) promoter (β RARE-tk-CAT) plasmid along with 1 μg of a plasmid containing the neomycin resistance gene pXT1 (Stratagene, La Jolla, CA). Resistant clones were selected in 400 μg/ml

G418 and isolated. Genomic DNA isolated from G418 resistant clones was digested with restriction enzymes at *Kpn*I and *Sph*I sequences that flank the 1850-bp region containing the CAT gene, tk promoter, and RARE. Digested DNA and known concentrations of parent CAT plasmid also digested with *Kpn*I and *Sph*I were electrophoresed through a 1% agarose gel and transferred to a nylon membrane. The membrane was crosslinked with ultraviolet light, prehybridized, and subsequently hybridized with a ^{32}P-labeled CAT coding sequence. The blot was washed and autoradiographed.

Transactivation Assay

The CC-B cell line, at a concentration of 4×10^5 cells per well, was used to inoculated six-well tissue culture dishes. The cells were allowed to attach and then treated with retinoic acid or solvent the next day. After 48 hr of treatment, cell extracts were prepared and CAT activity was assayed as previously described [43] with the exception that [^{3}H]acetyl CoA was used. Protein concentrations of the extracts were determined using the Bio-Rad protein assay (Bio-Rad, Richmond, CA). In each experiment, the protein concentrations varied by less than 10% between extracts. Relative CAT activities were derived by dividing the CAT activity by the protein concentration. Fold induction was derived by dividing the relative CAT activity of the treated culture by that of the control culture. The results presented are the averages of three independent experiments.

Growth Assay

Six-well tissue culture dishes were inoculated with a concentration of 2×10^4 CC-1 or CC-B cells per well. Twenty-four hours after plating, the media were replenished and treatment was initiated. After 7 days, the number of cells per well in the treated and control cultures was determined using a particle counter (Coulter ZM, Miami, FL). At this time, neither the cells in the treated nor those in control cultures had reached saturation density. Percentage growth was determined by dividing the number of cells in the treated cultures by that in the control cultures and multiplying by 100. The results presented are the averages of three independent experiments. Proliferation of retinoic acid-treated cultures compared to control cultures was statistically evaluated with a one-way analysis of variance using SPSS version 6.1 software.

Organotypic Cultures

A modification to the previously published procedure for preparing organotypic cultures was made to lessen the technical difficulty associated with manipulating the collagen gels [40]. Instead of transferring the gels from six-well plates onto metal grids, they were allowed to form in 25-mm Falcon cell culture inserts containing filters that have 0.80-μm pores (Becton–Dickinson, Franklin Lakes, NJ). When wet, these filters are sufficiently transparent to allow visualization of the cultures with light microscopy. Inserts containing the collagen gels were placed in individual wells of six-well tissue culture dishes containing media. The gels then remained in the inserts throughout the culturing, fixing, and dehydrating procedures and were removed from the inserts prior to paraffin embedding. All procedures were performed by hand to avoid damage to the cultures which often occurs with automated procedures. Media and drugs were replenished every 2 days and processed after 7 days of treatment. All experiments were repeated twice and evaluated using immunohistochemistry as described below with similar results.

Immunohistochemistry

Sections (5 μm) of the paraffin-embedded organotypic culture blocks were stained with a commercially available anti-EGF-R kit (Biopath, Oklahoma City, OK) according to manufacturer's instructions. Tissue sections from an EGF-R positive undifferentiated adenocarcinoma of the breast which showed membrane staining were used as positive control. The negative control consisted of the same breast carcinoma incubated with normal mouse IgG and no primary antibody.

Image Analysis

Quantification of EGF-R immunostaining was performed with the CAS 200 Image Analysis System (Cell Analysis Systems, Inc., Elmhurst, IL), equipped with a Quantitative DNA Analysis software module [44]. The software was calibrated using the quantitative EGF-R assay (Oncogene Science, Uniondale, NY). The intensity of staining was measured in two solid-state image-sensing channels, one set to identify nuclei stained with hematoxylin and the other set for EGF-R stained with fast red. For each slide, 10 separate visual fields were analyzed and the results presented as averages of picograms of EGF-R protein per cell. All of the specimens were evaluated in one session with the same calibration settings. The repeat experiment was evaluated separately and similar results were obtained. All determinations were performed in a blinded fashion.

RESULTS

Expression of Nuclear Receptors and Cytoplasmic Binding Proteins

The specificity of the primers and reaction conditions used to amplify each nuclear receptor sequence are demonstrated in Fig. 1. These reaction conditions were used in RT-PCR analysis of RNA isolated from CC-1 cells cultured in the presence or absence of 1 μM all-*trans* retinoic acid (Fig. 2A) or 9-*cis* retinoic acid (data not shown). RNA isolated from another cervical tumor cell line, SiHa, which is representative of a high grade tumor [40], was also evaluated

(Fig. 2). Expression of RARα, RARγ, RXRα, and RXRβ was observed in the presence and absence of 9-*cis* or all-*trans* retinoic acid in both cell lines. Expression of RARγ was low in CC-1 in comparison to SiHa. Expression of RARβ was not detected (data not shown).

Expression of CRBPI and CRABPII was observed in both CC-1 and SiHa cells in the presence and absence of 9-*cis* or all-*trans* retinoic acid (Fig. 2B). Expression of CRBPI was not detected (data not shown). Expression of CRBPII could not be evaluated because this gene has not yet been cloned from human DNA.

Establishment of a Reporter Cell Line

The RARE found in the RAR β gene was chosen for this study because it is transactivated by all-*trans* retinoic acid in the presence of RAR homodimers, RAR/RXR heterodimers, and the RXR homodimer [33, 39]. Our first attempts to evaluate retinoid activation of endogenous receptors using the βRARE-tk-CAT reporter plasmid in transient transfection assays resulted in low levels of CAT induction. These levels did not provide adequate sensitivity to evaluate differences in transactivation efficiency between retinoids. Since only a small fraction of cells in transient assays take up the transfected DNA [45], it was hypothesized that a subline of

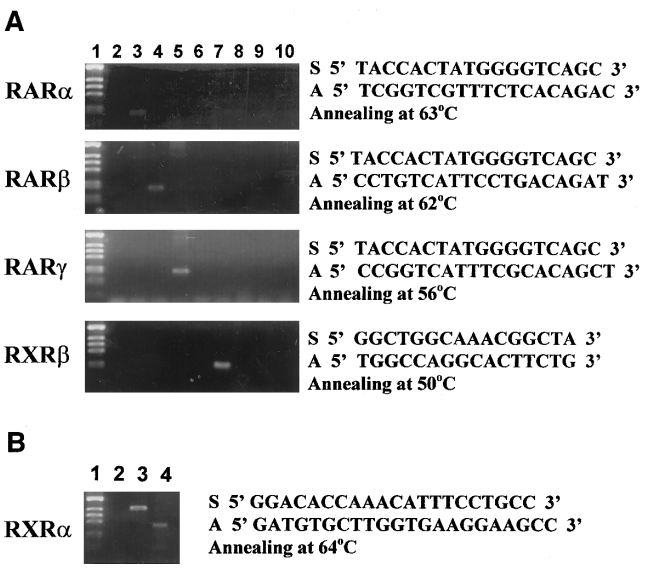

FIG. 1. Specificity test of PCR primers. The sense (S) and antisense (A) primers and annealing temperatures used are listed to the right of each picture. (A) PCR products obtained with cDNA clones were electrophoresed through 1.5% agarose gels containing ethidium bromide and photographed. The expected size of the specific bands is approximately 200 bps. Lane 1, molecular weight markers (1-kb DNA ladder, GibcoBRL, Gaithersburg, MD; the top band corresponds to 505 bps followed by 396, 344, 288, 220, 201, 154, 134, and 75); lane 2, no DNA negative control; lane 3, RARα; lane 4, RARβ; lane 5, RARγ; lane 6, RXRα; lane 7, RXRβ; lane 8, RXRγ; lane 9, VDR; lane 10, TRα. (B) Lane 1, molecular weight markers; lane 2, no RNA control; lane 3, specific RXRα band amplified from CC-1 cDNA; lane 4, specific RXRα band amplified from CC-1 cDNA digested with *Pvu*II.

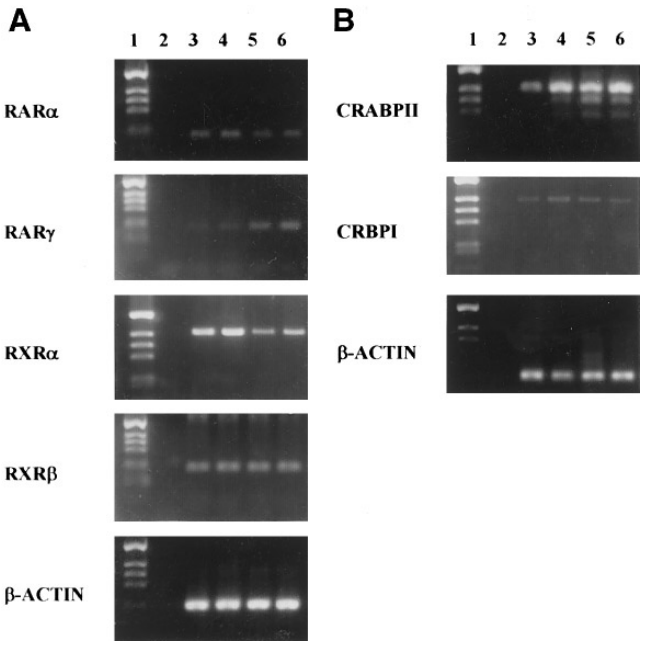

FIG. 2. Expression of retinoid nuclear receptors and binding proteins in CC-1 and SiHa cell lines. RT-PCR products obtained with cellular cDNA were electrophoresed through 1.5% agarose gels containing ethidium bromide and photographed. Specific primers that amplify nuclear receptors (A) and cytoplasmic binding proteins (B) were used. In both panels the lanes are designated as follows: lane 1, 1-kb DNA ladder; lane 2, no RNA negative control; lane 3, untreated CC-1; lane 4, CC-1 treated with 1 μM all-*trans* retinoic acid; lane 5, untreated SiHa; lane 6, SiHa treated with 1 μM all-*trans* retinoic acid.

CC-1 permanently transfected with the reporter gene would provide a more sensitive assay because 100% of the cells would contain the transfected DNA. In addition, use of a reporter cell line would eliminate the variability due to cellular trauma resulting from the transfection procedure. Therefore, the βRARE-tk-CAT plasmid was permanently transfected into CC-1 cells and the presence of the CAT reporter gene in two of five established clones was demonstrated with Southern analysis (Fig. 3). Clone 2 (called CC-B) was arbitrarily chosen for this study and monitored out to passage 35 with no significant increases or decreases in growth rate or basal level CAT expression observed (data not shown).

Retinoid treatment of the CC-B cell line resulted in a dose-dependent induction of CAT reporter activity (Fig. 4). The degree of induction depended on the isomer and concentration used. At concentrations lower than 10^{-9} *M*, there was no significant induction by any of the isomers. The 9-*cis* isomer was the most potent of the three retinoids at concentrations greater than 10^{-9} *M* and less than 10^{-6} *M*. At the 10^{-5} *M* concentration, the activities of the 9-*cis* and all-*trans* isomers were similar. The 13-*cis* isomer exhibited the weakest activity at all concentrations tested.

Regulation of Growth

The growth rate of CC-1 monolayer cultures treated with all-*trans*, 9-*cis*, or 13-*cis* retinoic acid was dependent on the

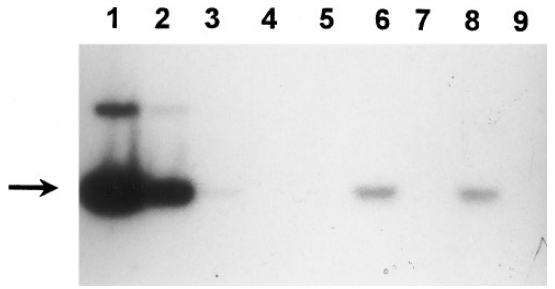

FIG. 3. Presence of the reporter plasmid in isolated transfectant clones of CC-1 as demonstrated by Southern analysis. The βRARE-tk-CAT reporter plasmid was used as positive control at 10 ng (lane 1), 1 ng (lane 2), and 0.1 ng (lane 3). Genomic DNA was loaded at a concentration of 10 μg from parental CC-1 cultures (lane 4) and from G418 resistant clones clone 1 (lane 5), clone 2 (lane 6), clone 3 (lane 7), clone 4 (lane 8), and clone 5 (lane 9). The arrow indicates the location of the 1850-bp band expected. The upper band is nonspecific.

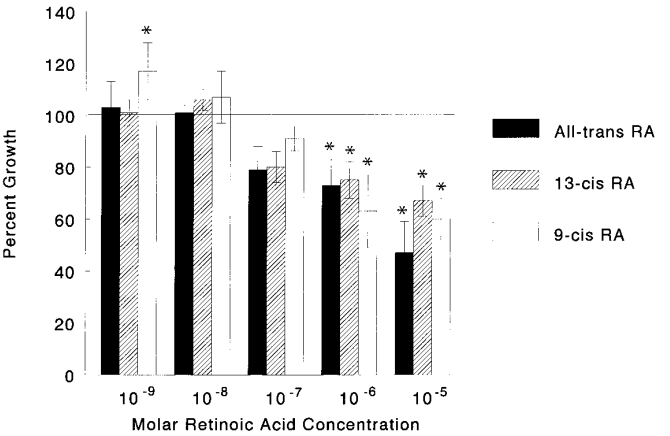

FIG. 5. Regulation of CC-1 growth by retinoids. Cultures were treated with a range of retinoid concentrations or a solvent control. After 6 days the number of cells per milliliter was counted and percentage growth of treated in comparison to control cultures determined. (*Growth is statistically different than the growth of untreated cultures. $P < 0.05$.)

concentration used (Fig. 5). At 10^{-9} *M*, the 9-*cis* isomer significantly stimulated growth ($P < 0.05$). At concentrations greater than 10^{-7} *M*, growth was significantly inhibited by all three isomers ($P < 0.05$). CC-B monolayer cultures exhibited the same growth rate and response to retinoic acid as the parent CC-1 line (data not shown).

The concentration of 10^{-6} *M* was chosen for further analysis because it exhibited the greatest differential in RARE transactivation by individual retinoids and was significantly growth inhibitory. To test the efficacy of these assays in differentiating between retinoids, the activity of 10^{-6} *M* synthetic retinoid [46] in the growth and RARE transactivation assays was compared to the activities observed for the three retinoic acid isomers at this concentration. A scatter graph of retinoid effects on growth versus RARE transactivation for each retinoid demonstrated a significant correlation of the two activities with a correlation coefficient of $^{-}0.96$ (Fig. 6).

Inhibition of EGF-R Expression in Organotypic Cultures

CC-B organotypic cultures were grown in the presence or absence of 10^{-6} *M* all-*trans*, 13-*cis*, or 9-*cis* retinoic acid

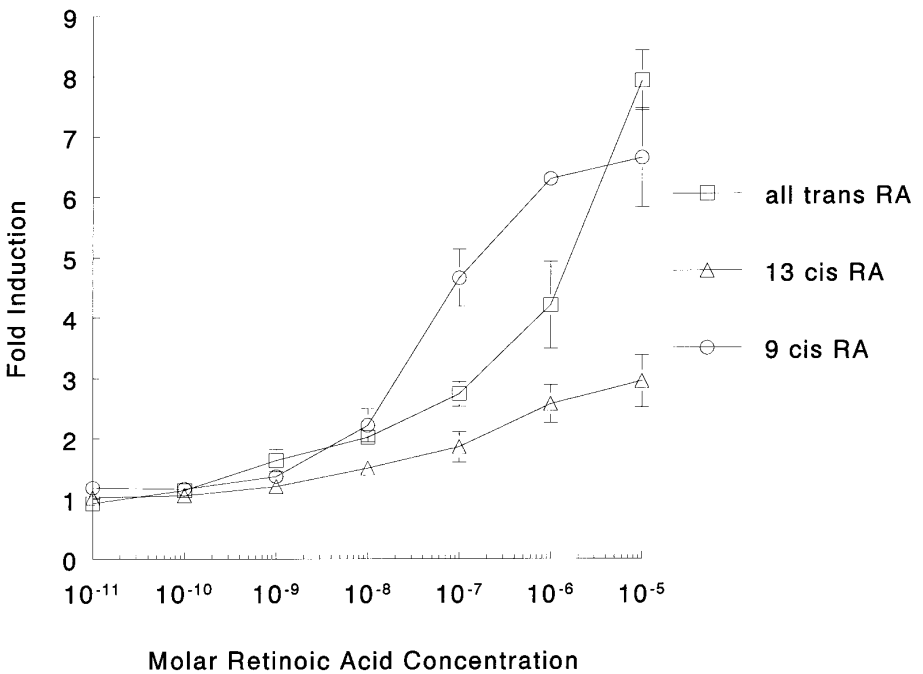

FIG. 4. Transactivation of RARE reporter by endogenous receptors in CC-B cultures treated with retinoic acids. Cultures were treated with a range of retinoid concentrations or solvent control for 48 hr. Cell lysates were then assayed for CAT activity and the fold induction was determined by dividing the CAT activity in the treated cultures by that in the control cultures.

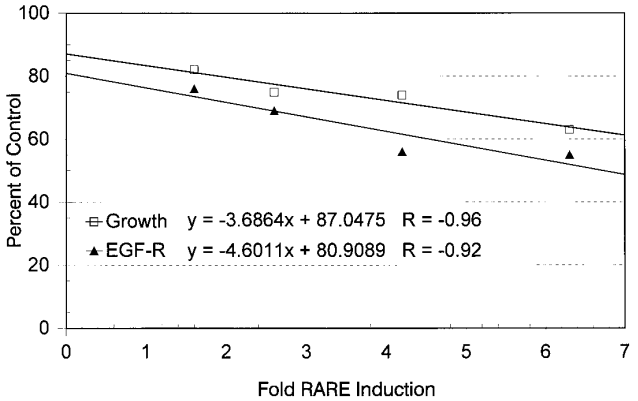

FIG. 6. Correlation of retinoid activities in the RARE transactivation assay with effects on growth and EGF-R expression. Scatter graphs of the percentage growth or EGF-R expression versus RARE transactivation exhibited by cultures treated with each retinoid were drawn and the equations and correlation coefficients derived using Harvard Graphics 3.0.

or the synthetic retinoid. Expression of EGF-R protein was measured in these cultures with immunohistochemical and CAS analysis. Retinoid treatment decreased EGF-R expression in these cultures to different extents depending on the retinoid used. A scatter graph of retinoid effects on EGF-R expression versus RARE transactivation demonstrated a statistically significant correlation of the two activities with a correlation coefficient of $^{-}0.92$ (Fig. 6). Retinoid activities in growth and EGF-R assays also correlated although with a lower, less significant correlation coefficient of 0.83.

DISCUSSION

In this study, we demonstrate the biologically relevant composition and response of the retinoid signaling pathway in the CC-1 cervical tumor cell line. In normal cervical epithelia, CRBPI is expressed throughout all cell layers, and CRABPI expression is inversely correlated with differentiation [47, 48]. Although CRABPII expression has not been evaluated in cervical epithelia, its expression in skin equivalent was observed to be enhanced with differentiation [49]. Therefore, CRBPI and CRABPII expression, but lack of CRABPI expression in CC-1 is consistent with what would be expected of a cell line capable of differentiation [40].

The expression patterns of nuclear retinoic acid receptors in a given cell type will dictate its specific response to retinoid treatment. Alterations in the expression pattern could result in deregulation of growth and differentiation which would contribute to tumorigenic progression. In agreement with this, loss or decreased expression of RARβ has been observed in tumors of the lung, oral cavity, and mammary gland [50–53]. In normal ectocervical epithelium, however, RARβ is not expressed, while other receptors, RARα, RXRα, and RXRβ, and to a lesser extent RARγ are expressed [54]. The expression profile of nuclear retinoic acid receptors that we observed in CC-1 cells is the same as that observed in normal ectocervix [54]. This similarity to normal cervix and to another cervical tumor cell line, SiHa, demonstrates that CC-1 exhibits a biologically relevant pattern of receptor expression.

Activation of the nuclear receptors was demonstrated by a retinoid-specific and dose-dependent transactivation of a RARE in a stable transfectant of CC-1. The use of a reporter cell line represents a more biologically relevant assay for receptor activation than transient transfection assays. In the reporter line, both the receptors and the DNA encoding the RARE are in a more natural state, because promoters located in chromatin and endogenous receptors are evaluated. On the other hand, in transient transfection assays the receptors are overexpressed from cDNA clones and the promoters are located in naked DNA.

The concentration-dependent stimulation versus inhibition of growth observed with 9-*cis* retinoic acid echoes previous reports. This growth pattern was also observed for anchorage-independent growth of normal human keratinocytes treated with retinoic acid [26]. A biphasic concentration-dependent effect of retinoic acid on growth was also observed in monolayer cultures of prostate carcinoma cell lines [55]. Therefore, the dose of retinoids given may be critical for clinical use of these agents. The *in vitro* data suggest that the traditional strategy of Phase I trials, to determine the maximally tolerated dose, may not be appropriate for optimizing retinoid chemotherapies.

The opposite effects of low and high concentrations of retinoic acid on CC-1 growth rate may be due to differing levels of RARE transactivation and AP1 transrepression. The ability of retinoids to inhibit transactivation of AP1 sequences is a potential mechanism for retinoid growth regulation. This inhibition occurs through a retinoid-dependent physical interaction of the retinoid receptors with the transcription factors that transactivate AP1 sequences, cJun and cFos [56]. Synthetic retinoids which retain AP1 transrepression activity but lack the RARE transactivation activity (dissociating retinoids) inhibit anchorage-independent growth of Ki-*ras*-transformed 3T3 cells [57]. This demonstrates that the anti-AP1 activity of retinoids contributes to the mechanism of growth inhibition. It does not, however, eliminate the possibility of a contribution by the RARE transactivation activity of retinoids to the mechanism of growth inhibition.

EGF-R is a potential candidate for the mediator of retinoid and HPV regulation of growth in cervical cells. This has been evaluated in the C4-1 cervical carcinoma cell line which expresses high levels of EGF-R [58]. The rapid growth rate of this line is dependent upon expression of the viral E6 and E7 genes [59]. EGF-R expression levels in C4-1 have been shown to correlate with the growth rate but not with expression of E6 and E7 [58]. Therefore, the high levels of EGF-R expression observed in HPV-infected cells may be a consequence of increased proliferation and not directly caused

by HPV. This is reminiscent of the correlation of EGF-R expression and proliferation in higher grades of SIL of the cervix [14, 15]. The CC-1 cell line expresses high levels of EGF-R that are decreased by treatment with 10^{-6} *M* retinoic acid. This demonstrates a biologically relevant response of CC-1 and indicates clinical potential for the use of retinoic acid to inhibit tumor growth.

In conclusion, the complex interplay of factors involved in growth regulation by retinoids needs to be evaluated in the target cells for which these drugs are being developed. The CC-1 cell line provides a representation of a low-grade cervical carcinoma that exhibits biologically relevant responses to retinoids. The degree of these biological responses correlates in a statistically significant manner with activities of the retinoids at a molecular level. Future studies utilizing this cell line will provide further insight into the mechanism of tumor cell growth inhibition by retinoic acid.

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REFERENCES

1. Lippman SM, Kavanagh JJ, Paredes-Espinoza M, Delgadillo-Madrueno F, Paredes-Casillas P, Hong WK, Holdener E, Krakoff IH: 13-cis retinoic acid plus interferon α-2a: Highly active systemic therapy for squamous cell carcinoma of the cervix. J Natl Cancer Inst 84:241–245, 1992
2. Lippman SM, Kavanagh JJ, Paredes-Espinosa M, Delgadiool-Madrueno F, Paredes-Casillas P, Hong WK, Massimini G, Holdener EE, Krakoff IH: 13-cis retinoic acid plus interferon-α-2a in locally advanced squamous cell carcinoma of the cervix. J Natl Cancer Inst 85:499–500, 1993
3. Romney SL, Palan PR, Duttagupta C, Wassertheil-Smoller S, Lucido D: Retinoids and the prevention of cervical dysplasias. Am J Obstet Gynecol 141:890–894, 1981
4. Meyskins FL, Graham V, Chvapil M, Dorr RT, Alberts DS, Surwit EA: A phase I trial of β-all-trans-retinoic acid delivered via collagen sponge and cervical cap for mild or moderate intraepithelial cervical neoplasia. J Natl Cancer Inst 71:921–925, 1983
5. Graham V, Surwit ES, Weiner S, Meyskens FL: Phase II trial of β-all-trans-retinoic acid for cervical intraepithelial neoplasia delivered via collagen sponge and cervical cap. West J Med 145:192–195, 1986
6. Meyskens FL, Surwit ES: Clinical experience with topical tretinoin in the treatment of cervical dysplasia. J Am Acad Dermatol 15:826–829, 1986
7. Weiner SA, Surwit EA, Graham VE, Meyskens FL: A phase I trial of topically applied trans-retinoic acid in cervical dysplasia—Clinical efficacy. Invest New Drugs 4:241–244, 1986
8. Beral V, Hermon C, Munoz N, Devesa SS: Cervical cancer. Cancer Surveys 19–20:265–285, 1994
9. zur Hausen H: Papillomaviruses in human cancers. Mol Carcin 1:147–150, 1988
10. Pirisi L, Yasumoto S, Feller M, Doninger JK, di Paolo JA: Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. J Virol 61:1061–1066, 1987
11. Dürst M, Dzarlieva-Petrusevska RT, Boukamp P, Fusenig N, Gissmann L: Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. Oncogene 1:251–256, 1988
12. Kaur P, McDougall JK: Characterization of primary human keratinocytes transformed by human papillomavirus type 18. J Virol 62:1917–1924, 1988
13. Chapman WB, Lorincz AT, Willett GD, Wright VC, Kurman RJ: Epidermal growth factor receptor expression and the presence of human papillomavirus in cervical squamous intraepithelial lesions. Int J Gynecol Pathol 11:221–226, 1992
14. Berchuck A, Rodriguez G, Kamel A, Soper JT, Clarke-Pearson DL, Bast RC: Expression of epidermal growth factor receptor and HER-2/Neu in normal and neoplastic cervix, vulva, and vagina. Obstet Gynecol 76:381–387, 1990
15. Pfeiffer D, Stellwag B, Pfeiffer A, Borlinghaus P, Meier W, Scheidel P: Clinical implications of the epidermal growth factor receptor in the squamous cell carcinoma of the uterine cervix. Gynecol Oncol 33:146–150, 1989
16. Hale RJ, Buckley CH, Gullick WJ, Fox H, Williams J, Wilcox FL: Prognostic value of epidermal growth factor receptor expression in cervical carcinoma. J Clin Pathol 46:149–153, 1993
17. Tervahauta A, Syrjanen S, Syrjanen K: Epidermal growth factor receptor expression in human papillomavirus lesions of the uterine cervix. Int J Gynecol Pathol 13:234–240, 1994
18. Hayashi Y, Hachisuga T, Iwasaka T, Fukuda K, Okuma Y, Yokoyama M, Sugimori H: Expression of ras oncogene product and EGF receptor in cervical squamous cell carcinomas and its relationship to lymph node involvement. Gynecol Oncol 40:147–151, 1991
19. Roberts AB, Anzano MA, Lamb LC, Smith JM, Sporn MB: Antagonistic actions of retinoic acid and dexamethasone on anchorage independent growth and epidermal growth factor binding of normal rat kidney cells. Cancer Res 44:1635–1641, 1984
20. Chen JK, Li L, Mioh H: Differential responsiveness of normal and simian virus 40-transformed BALB/c 3T3 cell to retinoic acid: Rapid enhancement of epidermal growth factor receptor binding in a simian virus 40-3T3 variant. Cancer Res 47:4995–4999, 1987
21. Zheng ZS, Goldsmith LA: Modulation of epidermal growth factor receptors by retinoic acid in ME180 cells. Cancer Res 50:1201–1205, 1990
22. Piazza GA, Ritter JL: Involvement of transforming growth factor-alpha and its receptor in the growth response of cultured human epidermal cells to retinoic acid. Epithel Cell Biol 2:170–175, 1993
23. Grandis JR, Zeng A, Tweardy DJ: Retinoic acid normalizes the increased gene transcription rate of TGF-alpha and EGFR in head and neck cancer cell lines. Nature Med 2:237–240, 1995
24. Sizemore N, Rorke EA: Human papillomavirus 16 immortalization of normal human ectocervical epithelial cells alters retinoic acid regulation of cell growth and epidermal growth factor receptor expression. Cancer Res 53:4511–4517, 1993
25. Agarwal C, Rorke EA, Irwin JC, Eckert RL: Immortalization by human papillomavirus type 16 alters retinoid regulation of human ectocervical epithelial cell differentiation. Cancer Res 51:3982–3989, 1991
26. Pirisi L, Batova A, Jenkins GR, Hodam JR, Creek KE: Increased sensi-

tivity of human keratinocytes immortalized by human papillomavirus type 16 DNA to growth control by retinoids. Cancer Res 52:187–193, 1992

27. Agarwal C, Hembree JR, Rorke EA, Eckert RL: Interferon and retinoic acid suppress the growth of human papillomavirus type 16 immortalized cervical epithelial cells, but only interferon suppresses the level of the human papillomavirus transforming oncogenes. Cancer Res 54:2108–2112, 1994
28. Tallman MS, Wiernik PH: Retinoids in cancer treatment. J Clin Pharmacol 32:868–888, 1992
29. Giguère V: Retinoic acid receptors and cellular retinoid binding proteins: Complex interplay in retinoid signaling. Endocrin Rev 15:61–79, 1994
30. Petkovich M: Regulation of gene expression by vitamin A: The role of nuclear retinoic acid receptors. Annu Rev Nutr 12:443–471, 1992
31. Heyman R, Mangelsdorf P, Dyck J, Stien R, Eichele G, Evans R, Thaller C: 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. Cell 68:397–406, 1992
32. Levin A, Sturzenbecker L, Kazmer, Bosakowski T, Huselton C, Allenby G, Speck J, Kratzeisen C, Rosenberger M, Lovey A, Grippo J: 9-cis retinoic acid stereoisomer binds and activates the nuclear receptor RXR α. Nature 355:359–361, 1992
33. Zhang X-k, Lehmann J, Hoffman B, Dawson MI, Cameron J, Graupner G, Hermann T, Tran P, Pfahl M: Homodimer formation of retinoid X receptor induced by 9-cis retinoic acid. Nature 358:587–591, 1992
34. Palmer CM, Hsu MH, Muerhoff AS, Griffin KJ, Johnson EF: Interaction of the peroxisome proliferator-activated receptor alpha with the retinoid X receptor alpha unmasks a cryptic peroxisome proliferator response element that overlaps an ARP-1 binding site in the CYP4A6 promoter. J Biol Chem 269:18083–18089, 1994
35. Apfel R, Benbrook D, Lernhardt E, Ortiz MA, Salbert G, Pfahl M: A novel orphan receptor specific for a subset of thyroid hormone-responsive elements and its interaction with the retinoid/thyroid hormone receptor subfamily. Mol Cell Endocrin 14:7025–7035, 1994
36. Kliewer SA, Umesono K, Heyman R, Mangelsdorf DJ, Dyck JA, Evans RM: Retinoid X receptor–Coup–TF interactions modulate retinoic acid signaling. Proc Natl Acad Sci USA 89:1448–1452, 1992
37. Gianni M, Zanotta S, Terao M, Garattini S, Garattini E: Effects of synthetic retinoids and retinoic acid isomers on the expression of alkaline phosphatase in F9 teratocarcinoma cells. Biochem Biophys Res Commun 196:252–259, 1993
38. Boylan JF, Lohnes D, Taneja R, Chambon P, Gudas LJ: Loss of retinoic acid receptor γ function in F9 cells by gene disruption results in aberrant Hoxa-1 expression and differentiation upon retinoic acid treatment. Proc Natl Acad Sci USA 90:9601–9605, 1993
39. de The H, Vivanco-Ruiz M, Tiollais P, Stunnenberg H, Dejean A: Identification of a retinoic acid responsive element in the retinoic acid receptor beta gene. Nature 343:177–180, 1990
40. Benbrook DM, Rogers RS, Medlin MA, Dunn ST: Immunohistochemical analysis of proliferation and differentiation in organotypic cultures of cervical tumor cell lines. Tissue Cell 27:269–274, 1995
41. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem 162:156–159, 1987
42. Kindmark A, Törmä H, Johansson AG, Sverker L, Melhus H: Cellular retinoic acid-binding protein type II is expressed in adult human osteoblasts and in adult liver. Biochem Biophys Res Commun 189:1397–1403, 1992
43. Sleigh MJ: A nonchromatographic assay for expression of the chloramphenicol acetyltransferase gene in eukaryotic cells. Anal Biochem 156:251–256, 1986
44. Taylor SR, Titus-Ernstoff L, Stitely S: Central values and variation of measured nuclear DNA content in imprints of normal tissues determined by image analysis. Cytometry 10:382–387, 1989
45. Loyter A, Scangos GA, Ruddle FH: Mechanisms of DNA uptake by mammalian cells: Fate of exogenously added DNA monitored by the use of fluorescent dyes. Proc Natl Acad Sci USA 79:422–426, 1982
46. Benbrook DM, Madler MM, Spruce LW, Birckbichler P, Nelson EC, Subramanian S, Weerasekare GM, Gale JB, Patterson MK Jr, Wang B, Wang W, Lu S, Rowland TC, DiSilvestro P, Berlin KD, Lindamood C, Hill DL: Biologically active heteroarotinoids exhibit anticancer activity and decreased toxicity. Submitted
47. Hillemanns P, Tannous-Khuri L, Koulos JP, Talmage D, Wright TC: Localization of cellular retinoid-binding proteins in human cervical intraepithelial neoplasia and invasive carcinoma. Am J Pathol 141:973–980, 1992
48. Tannous-Khuri L, Hillemans P, Rajan N, Wright TC, Talmage DA: Expression of cellular retinol- and cellular retinoic acid-binding proteins in the rat cervical epithelium is regulated by endocrine stimuli during normal squamous metaplasia. Am J Pathol 144:148–159, 1994
49. Sanquer S, Eller MS, Gilchrest BA: Retinoids and state of differentiation modulate CRABPII gene expression in a skin equivalent. J Invest Dermatol 100:148–153, 1993
50. Hu L, Crowe DL, Rheinwald JG, Chambon P, Gudas LJ: Abnormal expression of retinoic acid receptors and keratin 19 by human oral and epidermal squamous cell carcinoma cell lines. Cancer Res 51:3972–3981, 1991
51. Gebert JF, Moghal N, Frangioni JV, Sugarbaker DJ, Neel BG: High frequency of retinoic acid receptor β abnormalities in human lung cancer. Oncogene 6:1859–1868, 1991
52. Swisshelm K, Ryan K, Lee X, Tsou HC, Peacocke M, Sager R: Down-regulation of retinoic acid receptor beta in mammary carcinoma cell lines and its up-regulation in senescing normal mammary epithelial cells. Cell Growth Diff 5:33–141, 1994
53. Zhang X-k, Liu Y, Mi-Ock L, Pfahl M: A specific defect in the retinoic acid response associated with human lung cancer cell lines. Cancer Res 54:5663–5669, 1994
54. Darwiche N, Celli G, De Luca LM: Specificity of retinoid receptor gene expression in mouse cervical epithelia. Endocrinology 134:2018–2025, 1994
55. Fong CJ, Sutkowski DM, Braun EJ, Bauer KD, Sherwood ER, Lee C, Kozlowski JM: Effect of retinoic acid on the proliferation and secretory activity of androgen-responsive prostatic carcinoma cells. J Urol 149:1190–1194, 1993
56. Pfahl M: Nuclear recptor/AP-1 interaction. Endocrine Rev 14:651–658, 1993
57. Chen J-Y, Penco S, Ostrowski J, Balaguer P, Pons M, Starrett JE, Reczek P, Chambon P, Gronemeyer H: RAR-specific agonists/antagonists which dissociate transactivation and AP1 transrepression inhibit anchorage-independent cell proliferation. EMBO J 14:1187–1197, 1995
58. von Knebel Doeberitz M, Gissman L, zur Hausen H: Growth-regulating functions of human papillomavirus early gene products in cervical cancer cells acting dominant over enhanced epidermal growth factor receptor expression. Cancer Res 50:3730–3736, 1990
59. Watanabe S, Kanda T, Yoshiike K: Growth dependence of human papillomavirus 16 DNA-positive cervical cancer cell lines and human papillomavirus 16-transformed human and rat cells on the viral oncoproteins. Jpn J Cancer Res 84:1043–1049, 1993