Reduced Levels of Retinyl Esters and Vitamin A in Human Renal Cancers

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ABSTRACT

Clinical and preclinical studies suggest that retinoids can inhibit the growth of a small percentage of human renal cancers (RCs), although the majority of RCs both in vitro and in vivo are retinoid resistant. Our recent studies indicate that the metabolism of retinol to retinyl esters is greatly reduced in human carcinoma cell lines of the oral cavity, skin, and breast as compared with their normal epithelial counterparts, suggesting that human carcinoma cells are retinoid deficient relative to normal epithelial cells. We considered whether retinoid resistance in RCs was related to an abnormality in retinoid metabolism. The metabolism of [3H]retinol and of [3H]retinoic acid (RA) was examined in RC cell lines and normal human kidney (NK) epithelial cell cultures in media, in RA, or in RA plus IFN-α. The expression of LRAT (lecithin:retinol acyltransferase) was assessed by Northern and Western analysis. Retinol and retinyl ester levels were determined in tissue samples of normal human kidney and renal cell carcinoma. NK cells esterified all of the 50 nM [3H]retinol in which they were cultured. In contrast, six of the seven RC cell lines metabolized only 10–30% of 50 nM [3H]retinol to [3H]retinyl esters. Consistent with this relative lack of [3H]retinol esterification by the tumor cells, the tumor cells exhibited LRAT transcripts of aberrantly low sizes relative to those in normal epithelial cells. Moreover, the NK cells expressed abundant levels of LRAT protein by Western analysis, whereas the RC cell lines did not express LRAT protein. When samples of human kidney tumor tissue were compared with samples of normal kidney tissue from patients who had undergone surgery for primary RC, the normal kidney tissues contained much higher levels of retinol and retinyl esters (approximately 0.5–2 μg/gram wet weight) than the tumor tissues in all seven patients examined. Culture of the RC lines in IFN-α plus all-trans-RA, a combination therapy used clinically, resulted in higher intracellular levels of [3H]retinol and [3H]retinyl esters. The metabolism of [3H]RA was also examined in these RC lines versus NK cells. Although the NK epithelial cells metabolized [3H]RA, the majority of the RC lines metabolized [3H]RA at a much slower rate. Most of the RC lines metabolized only 10–30% of the 50 nM [3H]RA over 6 h of culture. These data indicate that RCs both in vitro and in vivo are retinol and retinyl ester deficient relative to the normal human kidney, and they suggest that the aberrant differentiation of the neoplastic renal cells results in part from a defect in retinoid metabolism.

INTRODUCTION

Retinoids, retinol (vitamin A), and its derivatives and metabolites, act as cancer chemopreventive and chemotherapeutic agents (1–7). It is likely that retinoids exert these cancer chemopreventive and cancer chemotherapeutic effects at least in part through their actions in the regulation of cell growth and differentiation (8). Retinol (vitamin A) can be metabolized to a variety of structurally related compounds, such as retinyl esters, RA, retinaldehyde, 4-oxoretinol, 14-hydroxy-4,14-retro-retinol, and anhydroretinol in many cell types (9–15). Esterification of retinol is mediated by two enzymes, LRAT and ARAT (reviewed in Ref. 9). A human LRAT cDNA has been identified (16), whereas the ARAT gene has not yet been cloned. We have shown recently that the metabolism of retinol to retinyl esters is greatly reduced in various types of human carcinoma lines, such as carcinomas of the oral cavity, skin, and breast, as compared with normal human cultured epithelial cell strains from these same human tissues (17–19). These data suggest that human carcinoma cells are retinoid deficient relative to normal epithelial cells.

The actions of retinol and RA are mediated by their binding proteins (CRBP I and II and cellular RA binding proteins I and II) and by two different families of nuclear RA receptors, RARs and RXRs, each consisting of three receptor types α, β, and γ (reviewed in Ref. 20). The RARs and RXRs act as ligand-activated trans-acting transcription factors, which regulate the expression of a number of retinoid-responsive genes and through this regulation, alter the growth and differentiation of normal and tumor cells. In addition, the RARs and RXRs act in concert with other proteins known as transcriptional coactivators and transcriptional corepressors. Although all of the interactions among retinoid receptors and transcriptional activators and corepressors are not yet well understood, in general it is known that transcriptional corepressors recruit histone deacetylases to promoters, whereas transcriptional coactivators either exhibit histone acetyltransferase activity or aid in recruiting histone acetylasules to promoters (Refs. 21 and 22; reviewed in Ref. 23).

RC is a frequent cause of cancer morbidity and mortality with >10,000 deaths/year in the United States (24, 25). This fact reflects a lack of effective chemotherapeutic or biological treatment modalities for patients who develop metastatic disease. Recent preclinical and clinical data suggest that retinoids may play a role in inhibiting RC growth. Although we reported that RA alone inhibited the growth of only 1 of 12 RC cell lines (26) and no major responses were observed in 25 patients with advanced RC treated with 13-cis-RA (27), we did show that RA can significantly augment the growth-inhibitory effects of IFN-α in vitro (28, 29) and the antitumor effects of IFN-α in patients (29). Furthermore, our analysis of retinoid receptor expression in RC lines showed that although RARβ could not be detected or induced by 13-cis-RA treatment in all 11 RA-resistant RC cell lines, retinoid-sensitive SK-RC-06 cells basally expressed RARβ transcripts, and RARβ mRNA expression was up-regulated by 13-cis-RA treatment (26). Moreover, an analysis of sequential tumor biopsies from patients treated with 13-cis-RA and IFN-α demonstrated a significant increase in the intensity of RARB mRNA expression in RC cells only in patients who achieved a major clinical response (30). Taken together, these data suggest that RC cells that are responsive to retinoid therapy, as indicated by an increase in RARB expression, are also growth inhibited. Nevertheless, the vast majority of RCs both in vitro and in vivo are resistant to growth inhibition mediated by retinoids.
We considered whether the retinoid resistance to growth inhibition in RC cells was related in part to an inability to metabolize retinol to retinyl esters. We therefore examined RC cell lines and short-term cultures of normal human kidney proximal tubule cells (NK cells), the normal cellular counterpart of RCs (31, 32), for differences in: (a) the metabolism of retinol; (b) the metabolism of retinoic acid; and (c) the expression of genes involved in retinol and RA metabolism. For example, we examined the levels of both LRAT and CYP26, a gene that encodes a cytochrome P-450 enzyme responsible for metabolism of RA to more oxidized derivatives (33–36). We also measured the retinol and retinyl ester contents in human RC tissue and in autologous normal kidney from patients undergoing nephrectomy. We report major differences in the content and metabolism of retinol in RC as compared with NK.

MATERIALS AND METHODS

Materials. Radiolabeled retinol (all-trans-11,12-[^3]Hretinol); specific activity in the range of 27–47 Ci/mmol) was purchased from New England Nuclear (Boston, MA).[^3]HRA was also purchased from New England Nuclear. All other chemicals used, unless specified, were purchased from Sigma Chemical Co. (St. Louis, MO). IFN-γ was kindly provided by Schering Corp. (Kenilworth, NJ).

Cells and Culture Conditions. Short-term cultures of NK cells and RC lines were derived and maintained as described previously (37). Cell lines were negative for Mycoplasma contamination. The human NT-2 embryonal carcinoma cell line was cultured in DMEM plus 10% fetal bovine serum.

Growth Assays. Growth assays were performed as described previously (26). Briefly, ~10,000 cells/well were plated in 24-well tissue culture plates in MEM/7.5% FBS for 18 h, counted using a Coulter Count ZBI (Coulter, Hialeah, FL; day 0), and refed with MEM/7.5% FBS plus or minus 1 × 10–6 M RA (Sigma). RA was maintained as a 1 × 10–3 M stock dissolved in 100% ethanol, and control cells also received an equal concentration of ethanol. Cells were refed on day 3 and counted on day 6. All retinoid preparations and incubations were performed under dim light. All assays were performed at least two independent times in triplicate.

[^3]HRetinol and[^3]HRA Radiolabeling. Cells were plated at 1 × 10⁶ cells/60-mm dish 24 h prior to[^3]Hretinol or[^3]HRA addition. Cells were washed three times with PBS prior to radiolabeling and cultured for various time periods in 2 ml of labeling medium containing either 50 nM[^3]Hretinol (~2 μCi/ml) or 50 nM[^3]HRA (~1 μCi/ml). A separate control consisting of labeling medium without cells was included during the incubation period. Cells and one-fourth of the medium were collected. Cells were washed once with 0.5 ml of PBS and removed from the monolayer in 0.5 ml of PBS by scraping. Samples were stored at −70°C until retinoid extraction. The cell numbers were counted from parallel dishes for each treatment at the time of cell harvest.

Renal Tissue Specimens. RC and normal kidney tissue specimens were obtained from radical nephrectomy specimens. Tissue acquisition was performed through the Tumor Procurement Service at Memorial Sloan-Kettering Cancer Center. Tissue was immediately brought to the Pathology Department, and samples from the tumor as well as normal kidney distal to the tumor were frozen in liquid nitrogen and stored at −70°C until the time of extraction. H&E staining was used to confirm the malignant and benign phenotypes of the specimens. Histology is summarized in Table 1. Tumor tissue (1 g) was thawed and homogenized in 2.0 ml of PBS, followed by extraction with 1.4 ml of acetonitrile/butanol (50:50, v/v). After the addition of 1.2 ml of saturated K₂HPO₄ and thorough mixing and centrifuging, the upper organic layer was collected and dried in a Speed-Vac. The dried sample was resuspended in 350 μl of acetonitrile:butanol for HPLC analysis.

Extraction of Retinoids and HPLC Chromatography. Retinoids were extracted as described previously (38). Nonradioabeled standards were added to each sample before extraction. Briefly, 350 μl of acetonitrile:butanol (50:50, v/v) and 0.1% butylated hydroxytoluene were added to 0.5 ml of cells or medium samples. The mixtures were vortexed thoroughly for 30 s. After addition of 300 μl of a saturated K₂HPO₄ solution and thorough mixing, the samples were centrifuged for 10 min at 3000 × g. The upper organic layer was collected, and the samples were transferred to injector vials for automated HPLC analysis.

The HPLC analysis was performed using a Waters Millennium system (Waters Corp., Milford, MA) to separate the various retinoids. Samples were applied to an analytical 5 μm reverse-phase C₈ column (Vydac, Hesperia, CA) at a flow rate of 1.5 ml/min. The gradient consisted of a 35-min linear gradient from 15 mM ammonium acetate (pH 6.5) in water to 85% acetonitrile and a 10-min linear gradient from 85% acetonitrile to acetonitrile:dichloromethane (80:20), followed by a 15-min hold. Nonradioabeled retinoid standards were run concurrently and monitored at a wavelength of 340 nm while a Packard A-500 radiochromatography detector (Packard Instruments, Downers Grove, IL) was used to monitor the labeled retinoids. The[^3]HRA samples were separated using a 20-min linear gradient from 70% 15 mM ammonium acetate (pH 6.5) in water and 30% acetonitrile to 33% 15 mM ammonium acetate (pH 6.5) and 67% acetonitrile, followed by a 5-min linear gradient to 100% acetonitrile and a 10-min treatment with 100% 15 mM ammonium acetate. Retinoids were identified by HPLC based on at least two criteria: an exact match of the retention times of unknown peaks with those of authentic retinoid standards; and identical UV spectra (220–400 nm) of unknowns against spectra from authentic retinoid standards during HPLC by the use of the photodiode array detector.

RNA Isolation and Northern Blot Analysis. Total cellular RNA was isolated from cultured cells using RNA Stat-60 according to the manufacturer’s instructions (Tel-Test, Friendswood, TX). RNA was electrophoretically fractionated by size on 1% agarose/2% w/v formaldehyde gels, transferred to nylon filters by blotting, and attached to the filters using a UV Stratalinker 1800. The cDNA probes used in this analysis were radiolabeled with[^3]PdCTP using a random primer labeling kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer’s directions. Human CRBP I and GAPDH cDNAs were used as probes as described previously (17). The human LRAT cDNA was an EcoRI fragment of 840 bp of LRAT coding region indicated by Ruiz et al. (16). An expressed sequence tag cloned for the human CYP26 (P450 RAl or RA hydroxylase) was purchased from Genome Systems (St. Louis, MO). The identity of this clone was verified by restriction enzyme analysis and DNA sequencing.

Blots were prehybridized and hybridized at 42°C in 50% (w/v) formamide, 5 × SSC, 50 mM NaH₂PO₄ (pH 7.4), 5 mM EDTA, 0.08% polyvinylpyrrolidone, 10% (w/v) BSA, and 10% (w/v) salmon sperm DNA. After 10–16 h of hybridization, blots were washed twice in 2× SSC, 0.5% SDS for 20 min at room temperature and twice in 0.2× SSC, 0.1% SDS at 50°C. Autoradiographs were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Western Analysis. This procedure was carried out as described previously (16). Briefly, polyclonal antisera were generated in rabbits to a mixture of two different LRAT peptides. Total cell protein was used, and blot analysis on nitrocellulose filters was performed using antiserum diluted to 1:1000 for detection of LRAT. Protein bands were detected by the ECL system (Pierce, Rockford, IL).

RESULTS

Metabolism of[^3]HRetinol in Cultured NK and RC Cells. We first examined[^3]Hretinol metabolism in cultured NK and RC cells to ascertain whether metabolic differences between normal and tumor cells, such as those we reported previously in other types of tumors (17–19), were present. Cells were cultured in the presence of 50 nM[^3]Hretinol for various times (22 h is shown in Fig. 1A). Cells and medium were then harvested, retinoids were extracted, and the me-

Table 1. Clinicopathological characteristics of renal cancer tissue specimens

<table>
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tabolites of [3H]retinol were resolved by HPLC. Nonradiolabeled retinoid standards were added to each sample to allow the identification of many of the radiolabeled retinoids. The HPLC tracings of intracellular [3H]retinol and its metabolites from NK and from seven different RC cell lines at 22 h are shown in Fig. 1A. Whereas the NK cells esterified almost all of the 50 nM [3H]retinol, the ability to esterify [3H]retinol was greatly impaired in almost all of the RCs (Fig. 1).

Quantitation of the HPLC data shown in Fig. 1A and additional data from the media samples are shown in Fig. 1, B–D. The total [3H]retinol remaining both inside the cells and in the medium (Fig. 1B) is an indicator of the amount of retinol metabolism into all types of products. In Fig. 1B, it can be seen that even 23 h after 50 nM [3H]retinol addition to the medium, most of the tumor cell lines still have almost 50 nM [3H]retinol present in the medium plus cells, indicating almost no ability to metabolize retinol. This contrasts with the NK cells, which have metabolized almost all of the [3H]retinol. The time course of [3H]retinol metabolism is shown in Fig. 1C, whereas the intracellular concentrations of the [3H]retinyl esters synthesized from [3H]retinol are shown in Fig. 1D. Only the NK cells exhibit high intracellular levels of these [3H]retinyl esters. The SK-RC-06 cell line esterified approximately one-third as much [3H]retinol as the normal NK cells (Fig. 1, A and D), and this was shown previously to be the only RC line that was growth inhibited by 13-cis-RA (26). These results from the RC cell lines (Fig. 1) are similar to those that we obtained previously with human carcinoma lines from the oral cavity, skin, and breast; defects in the abilities of the carcinoma lines derived from these tissues to esterify retinol were observed (17–19).

It has been reported previously that pretreatment with RA can increase retinol esterification. To determine whether this was also true in renal cells, NK cells and RC cell lines were cultured in 10^6 cells. This experiment was performed three times with very similar results. Bars, SD.

### Retinol and Retinyl Ester Content in Tissue Samples of Normal Human Kidney and Renal Cell Carcinoma.

Because we had shown that the majority of RC cell lines were not able to metabolize [3H]retinol to [3H]retinyl esters (Fig. 1), we next wanted to determine whether this resulted in a lower retinyl ester content in tumor samples obtained from patients undergoing nephrectomy for a renal tumor compared with their own normal kidney. Samples from seven patients were compared by HPLC analysis using a photodiode array detector. The all-trans retinol peaks and peaks of retinyl esters, primarily retinyl palmitate, were observed in all of the normal kidney samples from the seven patients (Fig. 3, A–D). The all-trans retinol peaks and retinyl palmitate peaks were identified on the basis of their elution times, with the all-trans retinol standard at 30.8 min and the retinyl palmitate
standard at 55 min, and by their absorbance maxima at 326.3 nm by photodiode array detection (Fig. 3A).

In contrast, none of the seven renal tumor samples (five clear cell carcinomas, one chromophobe carcinoma, and one transitional cell carcinoma of the renal pelvis; see Table 1) exhibited high levels of retinol and retinyl esters. For example, in patient number 1, the all-
trans retinol content in the normal kidney tissue was 0.7 µg/g of tissue, whereas in the tumor it was 0.09 µg/g of tissue, 8-fold lower than in the normal tissue. In one patient sample (patient 7), derived from a chromophobe carcinoma, which is generally more indolent than in the normal tissue. In one patient sample (patient 7) derived from a chromophobe carcinoma, which is generally more indolent than in the normal tissue.

The ability to metabolize [3H]RA to more polar metabolites in the SK-RC-06 RC line correlated with the expression of CYP26 (RA hydroxylase) transcripts (Fig. 2C). The cytochrome P-450 enzyme CYP26 uses retinoic acid as a substrate (33–36, 39–41). Only SK-RC-06 cells expressed significant levels of the CYP26 mRNA, and this was seen only after the cells were cultured in the presence of 1 µM RA for 48 h. Although the NK cells metabolized [3H]RA, these cells did not express CYP26 mRNA, suggesting that other enzymes in NK cells are responsible for RA metabolism.

Cell Growth Inhibition by Retinoids. We then compared the growth inhibition by RA and 4-oxoRA in testing of the RC lines, SK-RC-06, SK-RC-07, and SK-RC-08, to ascertain whether growth inhibition by these retinoids was correlated with the reduced ability to metabolize RA, because this would result in higher internal RA levels. The opposite was found to be the case. SK-RC-06 cells were growth inhibited by both RA and 4-oxoRA, whereas the other RC cell lines were not growth inhibited by these retinoids (Fig. 5). Thus, there was a correlation between the ability of the SK-RC-06 cells to induce CYP26 and metabolize RA and the inhibition of growth of these cells by all-trans-RA and 4-oxoRA.

Effects of IFN-α and RA on Retinol Metabolism. It has been shown for several tumors that the combination of RA and IFN-α results in additive or synergistic responses with respect to cell growth inhibition, although the molecular basis for these effects are not well understood (29, 42–48). We reasoned that IFN and RA could alter retinoid levels and/or metabolism in the RC cells. Thus, we examined the metabolism of [3H]retinol in RCs that were cultured in the presence or absence of either all-trans-RA alone or in the combination of all-trans-RA and IFN-α. We found (Fig. 6) that treatment of the RC cells with the combination of RA and IFN-α resulted in greater esterification of [3H]retinol and higher levels of [3H]retinyl esters in both the SK-RC-06 RC line, which can esterify some [3H]retinol (see Fig. 1) and in the SK-RC-39 line, which esterified only a small amount of [3H]retinol (Fig. 1). Although we have not proved that this increase in retinol esterification in the presence of IFN-α and RA can account for the growth-inhibitory effects of this drug combination, the combination of IFN-α plus RA is able to alter the metabolism and intracellular levels of [3H]retinol and [3H]retinyl esters in the RC lines.
DISCUSSION

In this report, we demonstrate that human RC cell lines exhibit a greatly reduced ability to metabolize [3H]retinol to [3H]retinyl esters, relative to NK cells (Fig. 1). These data extend previous research from this research group showing that the ability to esterify retinol is greatly decreased in carcinoma cells from the breast (18), the oral cavity, and skin (17). We also demonstrate that the RC lines express aberrantly smaller sized LRAT mRNA transcripts and undetectable LRAT protein, as compared with NK cells (Fig. 2). The mechanism by which the expression of the LRAT transcripts is altered in RC cells versus NK cells is not fully understood at this time. Because LRAT genomic clones containing the promoter region and other potential regulatory DNA elements are not yet available, we have not deter-

Fig. 3. Contour plots of the photodiode array analyses of retinoids present in cellular extracts from RC and normal epithelial tissues from human patients. Cellular retinoids were extracted and subjected to HPLC and photodiode array analysis. The contour plot shows a spectral window of 300–450 nm and a sensitivity of 0.001–0.01 absorbance units. The identification of retinoids is based on elution time and spectral analysis in comparison with known standards. A, standards for all-trans-RA, all-trans-retinol, and all-trans-retinyl palmitate. B, the same standards shown at one absorbance (325 nm). C, patient 1, normal tissue. D, patient 1, RC sample. E and F, quantitation of photodiode array data of retinol content of retinyl ester content, respectively, in seven pairs of normal human kidney versus kidney tumor samples; information about patients is in Table 1.

Fig. 4. Metabolism of [3H]RA in various RC lines and in NK epithelial cells. A, HPLC tracings of cells that were cultured in the presence of 50 nM [3H]RA for varying times; 20 h is shown. Cells and one-fourth of the medium were harvested, and retinoids were extracted and separated by reverse-phase HPLC analysis as described in “Materials and Methods.” Data are normalized per 1 × 10^6 cells. This experiment was performed three times with very similar results. Data from one experiment are shown here. B, total (intracellular plus in the medium) [3H]RA remaining at 20 h, calculated as described previously (17–19). Bars, SD. C, total [3H]RA remaining at various times (3, 10, and 20 h) after addition of [3H]RA to the cells at time 0.
mined why the LRAT transcripts in the RC cells are of smaller sizes. However, aberrant splicing, leading to the abnormally sized transcripts of 3 and 1.5 kb in the tumor cells, is a likely explanation for our findings. We have found that all of the various types of carcinoma lines examined thus far, including those from breast, skin, and oral cavity, exhibit LRAT transcripts of altered sizes and a low to undetectable level of LRAT protein by Western analysis, as compared with all of the normal epithelial cell strains (19). Our results to date strongly suggest that a major alteration in LRAT transcripts and the lack of expression of LRAT protein are common features of cultured human carcinoma cells.

We have shown that most of the RC lines do not metabolize [3H]RA very rapidly (Fig. 4, A–C). Many laboratories have shown that rapid metabolism of RA to polar RA metabolites is correlated with sensitivity to growth inhibition by RA (39–41, 49, 50). Our data are consistent with these data from other types of carcinoma lines in that only the SK-RC-06 cell line metabolized [3H]RA rapidly (Fig. 4, A and B), and SK-RC-06 is the only RC cell line the growth of which was arrested by RA (Fig. 5; Ref. 26). Furthermore, SK-RC-06 cells, unlike all of the other RC cell lines tested, expressed CYP26 mRNA upon RA treatment (Fig. 2C), expressed the RARβ, and exhibited increased expression of RARβ in response to 13-cis-RA (26, 30). Many cultured carcinoma cell lines and biopsies taken from patients with various types of carcinomas exhibit low or undetectable levels of RARβ transcripts, a gene that is RA-inducible in many normal cell types (51–69). Experiments that show that reexpression of RARβ in malignant cell lines containing low levels of RARβ transcripts results in growth arrest have led to the conclusion that defects in RARβ are one of the causes of retinoid resistance. Consistent with this, recent data from this laboratory have shown that F9 teratocarcinoma cells with both alleles of the RARB2 gene disrupted via homologous recombination do not growth arrest in response to all-trans-RA (66).

Low levels of RARB mRNA expression generally reflect the low content of active retinoids in cells (70–72). We suggest that the impairment in the ability to convert retinol to retinyl esters in the RCs leads to their inappropriate growth and to the loss of normal differentiation responses, at least in part because of the lack of sufficient internal retinyl ester stores to regulate retinoid-responsive genes, including RARB.

The combination of IFNs and RA has demonstrated a significant antitumor effect in a variety of tumor types, including RC. How retinoids and IFNs interact to increase the antitumor effects in renal cell carcinoma has not been elucidated. The data presented here (Fig. 6) suggest that IFN may increase retinoid content in RC cells, possibly rendering some renal cell carcinomas more sensitive to RA-induced growth inhibition.

Previously, we had examined only cell lines derived from human carcinomas; in this report, we show that tumor specimens also exhibit extremely low levels of retinol and retinyl esters as compared with normal kidney samples from the same patients (Fig. 3). An earlier report of low levels of retinol and retinyl esters in tumors has been published, and these data were from an N-methyl-N-nitrosourea-induced mammary carcinoma rat model (73). These new data from human tumor versus normal kidney tissues validate the cell culture analyses we have performed for this study. Moreover, these data...
provision of the basis for the design of therapeutic strategies that increase the intracellular levels of retinyl esters in malignant (or premalignant) cells as a novel approach to treat patients with RC and other epithelial malignancies.

ACKNOWLEDGMENTS

We thank Yan Meng and Daniel Navarro for technical assistance, the staff at the Gudas and Nanus laboratories for helpful discussions, Dr. Victor Reuter for histological review, and Taryn Resnick for editorial assistance.

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