Expression of Retinoic Acid Receptor β in Human Renal Cell Carcinomas Correlates with Sensitivity to the Antiproliferative Effects of 13-cis-Retinoic Acid

Anthony D. Hoffman, Dov Engelstein, Thomas Bogenrieder, Christos N. Papandreou, Eric Steckelman, Ami Dave, Robert J. Motzer, Ethan Dmitrovsky, Anthony P. Albino, and David M. Nanus

Genitourinary Oncology Service, Division of Solid Tumor Oncology [T. B., E. S., A. D., A. P. A.], the Laboratory of Molecular Medicine, Oncology Research Laboratory [C. N. P.. D. M. N.], Department of Medicine, Memorial Sloan-Kettering Cancer Center, Cornell University Medical College, New York, New York 10021

ABSTRACT

The differentiation and growth suppressive effects of retinoic acid are mediated through retinoic acid nuclear receptors (RARs and RXRs), which are ligand-activated transcription factors. Recent data suggest that both altered and regulated expression of RARs are linked to retinoic acid response in a cell context-dependent manner. This study examined the antiproliferative effects of 13-cis-retinoic acid (cRA) on 12 renal cancer cell lines and correlated these findings with the basal and induced expression of RAR-α, -β and -γ. Eleven of 12 renal cancers that were either resistant to or only minimally inhibited by cRA did not basally express RAR-β as determined by Northern blot analysis. In these cells, cRA treatment did not induce RAR-β expression. In contrast, 1 of 12 cell lines (SK-RC-06) was >90% inhibited by cRA and basally expressed RAR-β. Furthermore, RAR-β mRNA in SK-RC-06 cells was up-regulated by cRA treatment. Amplification of cDNA using PCR and RAR-β isoform-specific primer pairs revealed that only SK-RC-06 cells expressed the RAR-β isoform. Expression of RAR-α transcripts was abundant in all 12 cell lines examined, whereas low levels of RAR-γ transcripts were detectable in 6 of 10 renal cancers. Expression of RAR-α and RAR-γ was not affected by cRA. These data showing that the majority of renal cancer cell lines are resistant to cRA suggest that: (a) resistance to the antiproliferative action of cRA correlates with repressed RAR-β mRNA expression; and (b) the antiproliferative effects of cRA in renal cancer cells are mediated through RAR-β.

INTRODUCTION

The retinoids (derivatives of vitamin A) exert potent growth and differentiation effects on normal, neoplastic, and embryonic tissues (1, 2). Numerous studies have shown that retinoids induce differentiation and/or inhibit tumor cell growth in a variety of in vitro model systems (3–5). Clinical investigations have substantiated these studies, demonstrating that all-trans-RA1 can induce remissions in patients with acute promyelocytic leukemia (6) and that cRA prevents second aerodigestive tract cancers and inhibits progression of the pre-malignant lesion, oral leukoplakia (7, 8). Retinoid effects are mediated through RARs, which are members of the steroid receptor superfamily of ligand-dependent transcriptional factors (9). Two distinct retinoid nuclear receptor systems exist, the RARs (RAR-α, -β, and -γ) and the RXRs (RXR-α, -β, and -γ; Ref. 10). The RARs and RXRs can heterodimerize after RA binding and transcriptionally activate or repress other genes that mediate the growth and differentiation effects of RA (10, 11).

Recent studies in a variety of human tumors indicate that retinoid effects in different cell types are linked to the expression of specific retinoid receptors (12–15). For example, we reported that RA response in human teratocarcinomas is mediated directly through RAR-γ (15, 16) and that the presence of a rearranged RAR-α (PML/RAR-α) in acute promyelocytic leukemia correlates with clinical sensitivity to RA treatment (17). Analogous observations are reported in lung cancer (13) and breast cancer (18). These data suggest that both altered and regulated expression of retinoid receptors are involved in response and resistance to RA effects and that the specific receptor subtype that mediates RA signaling is probably tumor cell specific.

RCC is a frequent cause of cancer morbidity and mortality with over 10,000 deaths per year in the United States (19). This fact reflects a lack of effective chemotherapeutic or biological treatment modalities for patients who develop metastatic disease. We reported recently that the addition of cRA to IFN-α in the treatment of patients with advanced RCC resulted in a major response proportion of 30% (20), which is appreciably better than our previous experience with IFN-α alone (21). Although

1 The abbreviations used are: RA, retinoic acid; cRA, 13-cis-RA; RCC, renal cell carcinoma; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RAR and RXR, retinoic acid nuclear receptors.
preclinical studies suggested that cRA augments the antiproliferative effects of IFN (20, 22), the mechanism of beneficial interaction between cRA and IFN-α is unknown and under investigation (23). Moreover, although we and others have examined the antitumor effects of IFN-α on RCCs (24–26), the potential differentiation and antiproliferative effects of RA on RCCs have not been fully explored. To advance the understanding of the antitumor action of retinoids, the cRA-mediated growth effects and regulation of RARs were examined in RCCs.

MATERIALS AND METHODS

Cell Lines. Normal human kidney cultures and RCC cell lines were derived as described previously (27). Caki cells were obtained from the American Type Culture Collection (Rockville, MD), and the NTERA clone D1 (NT2/D1) teratocarcinoma cell line was established as described (28). Cultures were maintained in Eagle’s MEM supplemented with 2 mM glutamine, 1% nonessential amino acids, 100 units/ml streptomycin, 100 units/ml penicillin, and 7.5% FBS. Cell lines were negative for Mycoplasma contamination.

Growth Assays. Growth assays were performed as described previously (24). Briefly, approximately 10,000 cells/well were plated in 12-well tissue culture plates (Becton Dickinson, Cockeysville, MD) in MEM/7.5% FBS for 18 h, counted using a Coulter counter ZM (Coulter Electronics, Hialeah, FL) on day 0, and refed with MEM/7.5% FBS containing cRA (Sigma Chemical Co., St. Louis, MO). RA was maintained as a 10^{-2} M stock dissolved in DMSO (Sigma Chemical Co.), and all cells received an equal concentration of DMSO. Cells were refed on day 3 and counted on day 6. All retinoid preparation and incubations were performed in the dark. Results represent an average of two independent experiments performed in triplicate.

Northern Analysis. Total RNA was extracted from logarithmically growing cells using RNAzol B (Cinna/Biotecs Laboratories, Houston, TX) according to the manufacturer’s recommendations. Twenty μg of RNA per lane were electrophoresed in 1.2% agarose/formaldehyde gels, transferred to nitrocellulose membranes, and hybridized with a 1.4-kb EcoRI/BamHI-cut human RAR-β cDNA (29), a 1.8-kb EcoRI-cut RAR-α cDNA (30), or a full-length human RAR-γ cDNA (31) end-labeled with ^32P as described (Stratagene, La Jolla, CA; Refs. 32 and 33). Membranes were also hybridized with a PstI/XbaI-cut 0.78-kb GAPDH cDNA (34) to confirm similar amounts of RNA per lane. The RAR probes were kindly provided by Dr. Pierre Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, College de France, C. U. de Strasbourg, France). Membranes were exposed to XAR film for 5–7 days.

Southern Analysis. DNA was extracted from cells as described (35); digested with BamHI, EcoRI, or MspI restriction enzymes; electrophoresed on an agarose gel; transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH); and hybridized with RAR probes as described (35).

Preparation of cDNA and PCR. cDNA was generated from total RNA as described previously (36). All cDNA was first amplified with primers specific for β-actin to confirm the quality of cDNA in a separate reaction. PCR amplification of cDNA was performed using gene-specific oligonucleotide primers as reported previously (33, 36). Briefly, cDNA was heated to 95°C for 10 min and cooled on ice for 5 min; then 1 μl was added to a 25-μl reaction mixture [2.5 μl of 10X PCR reaction buffer (Perkin-Elmer, Norwalk, CT); 9.6 μM each of dATP, dCTP, dGTP, and dTTP (Pharmacia Fine Chemicals, Piscataway, NJ); 150 ng of each priming oligomer; 1.0 unit Taq polymerase (Perkin-Elmer); and H₂O]. Reaction mixes were prepared for multiple samples and aliquoted. A negative control consisting of a 25-μl aliquot without the addition of cDNA was included in each amplification. Twenty-five μl of mineral oil (Fisher Scientific) were layered over the aqueous phase to prevent evaporation. Amplification was performed using a DNA thermal cycler (M. J. Research Inc., Watertown, MA). A cycle profile consisted of 1 min at 95°C for denaturation, 1 min at 61°C for annealing, and 1 min at 72°C for primer extension. Electrophoresis of 20 μl of reaction mixture on a 1% SeaKem agarose (FMC Bioproducts, Rockland, ME) gel containing ethidium bromide was performed to evaluate amplification and size of fragments generated.

PCR Oligonucleotide Primers. Oligonucleotide primers were synthesized using a DNA synthesizer 380A (Applied Biosystems, Foster City, CA). Gene sequences used to construct oligonucleotide primers were from published sources. All primer pairs used were designed to bracket cDNA sequences that in genomic DNA cross an intron-exon boundary. Primer sequences were as follows: β-actin, 5’-GTGGGGGCGCCCGGCACCAA and 3’-TCTCTTAAATGTCAAGCGGATTCC (37); and RAR-β, 5’-CCAGCAATGGAACGGACACAT (RAR-β specific sense; Ref. 38), 5’-CTGGATTTCTACACTGCGAG (RAR-β specific sense), and 5’-CCCCACTTCAGAGCAGCTTCTG-3’ (RAR-β antisense; Ref. 29). Four different methods were used to confirm the identity of the DNA product generated by sequence specific primers: (a) PCR-amplified DNA was analyzed by electrophoresis in agarose and compared to fragment size predicted by the location of the primers used; (b) PCR-amplified DNA was sequenced and the results were compared to the known DNA sequence of the gene (method used for β-actin, Ref. 36); (c) PCR-amplified DNA was digested with restriction endonucleases and then fractionated by electrophoresis in agarose; the resultant fragments were compared to predicted sizes based on location of internal cleavage sites (method used for RAR-β, and RAR-β); and (d) PCR fragments were transferred to a Nytran membrane and hybridized with a RAR-β-specific probe (method used for RAR-β).
### Table 1  
RAR expression in RCC cell lines correlated to cRA sensitivity

<table>
<thead>
<tr>
<th>Cell line</th>
<th>RAR-α</th>
<th>RAR-β</th>
<th>RAR-γ</th>
<th>cRA sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caki</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>&lt;20</td>
</tr>
<tr>
<td>SK-RC-02</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>&lt;20-30</td>
</tr>
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<td>SK-RC-06</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>&lt;20</td>
</tr>
<tr>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>&lt;20</td>
</tr>
<tr>
<td>SK-RC-18</td>
<td>+</td>
<td>-</td>
<td>ND</td>
<td>&lt;20</td>
</tr>
<tr>
<td>SK-RC-35</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>ND</td>
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<td>&lt;20</td>
</tr>
<tr>
<td>SK-RC-49</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

a Summary of Northern analysis of total cellular RNA expression in RCC cells. Expression was scored after 5-7 days of exposure to film. Data was confirmed on representative blots by densitometric scanning. Results represent the average of at least two experiments. ++, abundant transcripts; +, low levels of transcripts; −, not detected; ND, not determined.

b Percentage of growth inhibited. RA sensitivity was determined in proliferation assays (see “Materials and Methods”).

in all 12 RCC cell lines. RAR-β transcripts could only be detected in retinoid-sensitive SK-RC-06 cells, whereas low levels of RAR-γ could be detected in 6 of 10 cell lines. We next examined the effects on RAR expression after exposure to cRA for 48 h. Levels of RAR-α mRNA in all 12 cell lines were not regulated by cRA (Fig. 2 shows data for Caki, SK-RC-02, -04, -17, and -45 cell lines; data for SK-RC-06, -07, -18, -35, -38, -39, and -49 cell lines are not shown). Similarly, transcripts for RAR-β were not induced by treatment with cRA in any retinoid-resistant cell line (Fig. 2), and expression of RAR-γ transcripts was not induced or up-regulated by cRA treatment (Fig. 3).

### RAR-β Expression in SK-RC-06 Cells

In contrast to other RCC cell lines, expression of RAR-β was up-regulated in cRA-sensitive SK-RC-06 cells, which had exhibited low levels of constitutive RAR-β expression (Fig. 4). Maximum RAR-β mRNA expression was achieved within 48–72 h of exposure to cRA (data not shown).

Expression of RAR-β is reported as undetectable by total cellular Northern analysis in other epithelial tumors, but transcripts have been detected in these cells by using more sensitive detection assays, such as the PCR amplification of cDNA. PCR amplifiers specific for human RAR-β₁ and -β₂ were designed, and amplified cDNA was derived from mRNA of all 12 RCC cell lines. RAR-β₁-specific transcripts could be detected in those RCC cells in which RAR-β transcripts were not detectable by Northern analysis (Fig. 5A). In contrast, RAR-β₂-specific transcripts were detected only in SK-RC-06 cells and not in the 11 other RCC cell lines or in cDNA derived from cultured normal human kidney cells (Fig. 5B).
Effects of Retinoic Acid on Renal Carcinomas

A critical area in the understanding of RA biology is the role played by retinoid receptors. It is currently viewed that retinoids can affect growth and differentiation pathways in tumor cells by stimulating specific retinoid receptors to activate or repress the growth-regulatory genes influencing proliferation or differentiation (3). Multiple isoforms of both the RAR and RXR subtypes have been described (10), and the formation of RAR-RXR heterodimers that can influence DNA-binding affinity and specificity illustrates the complexity of retinoid-mediated transcription (11). RA binds with high affinity to RARs but not RXRs (40, 41); therefore, we focused our analysis in this report on RA expression before and after cRA treatment. The data suggest that the antiproliferative action of cRA in RCC cells is mediated through RAR-β because: (a) basal levels of RAR-β were only detected in RA-sensitive SK-RC-06 cells; (b) induced RAR-β expression in these cells was concomitant with induced growth inhibition; and (c) no correlation between growth inhibition and expression of RAR-α or RAR-γ was observed (Table 1). It is possible that cRA induces growth inhibition in RCC cells by binding to an RAR-RXR heterodimer. However, the importance of RAR-β involvement in any retinoid receptor heterodimer is highlighted because 11 of 12 cRA-resistant RCCs lacked RAR-β expression at the level of total cellular RNA. Furthermore, analyses of selective retinoid-resistant and -sensitive RCCs did not find a correlation between growth inhibition and RXR-β or RXR-γ expression.5

RAR-β-specific transcripts were detected in RA-resistant RCC cells using a sensitive reverse transcription-PCR, indicating low levels of RAR-β expression in RCC cells. Lack of RAR-β expression is reported in numerous epithelial malignancies (13, 14, 42). RAR-β has been implicated as a tumor suppressor gene, the absence of which results in cell transformation and/or maintenance of the neoplastic phenotype (43). This hypothesis is based in part on the observations that RAR-β is located on chromosome 3p24, which is at or near a commonly

4 R. J. Motzer, unpublished data.

5 A. D. Hoffman and D. M. Nanus, unpublished data.
deleted region in a variety of malignancies, including RCC and lung cancer (44–46), and that introduction of a wild-type RAR-β into epidermoid lung cancer cells results in decreased tumorigenicity (43). We did not detect any deletions in the RAR-β gene in any cell line or in DNA extracted from 20 primary RCC specimens. Studies of lung cancer cells also did not detect deletions in the RAR-β gene (47) or mutations in the RAR-β promoter region (48) and suggest that other regulatory factors inhibit RAR-β transcription. The observations that RAR-β is not expressed at the cellular RNA level in most RCC cells and that up-regulated RAR-β expression induced by cRA inhibits growth indicate that RAR-β may induce or repress the transcription of another protein(s) that directly mediates the antitumor activity.

The implication of a role played by RAR-β in retinoid signaling in RCCs is in agreement with reports that link retinoid responsiveness in different cell types to expression of a specific retinoid receptor gene (12–15). Our prior work revealed that RA resistance in NT2/D1 cells is linked to repressed expression of RAR-γ but not RAR-α or RAR-β (15). Resistance to RA is overcome in these cells by overexpressing RAR-γ (16). In cultured human lung cells, inhibition of normal tracheobronchial epithelium is associated with an induction of RAR-β but not RAR-α, whereas RA-resistant lung cancer cells show no induction of RAR-β expression after RA treatment (13). A similar correlation also has been reported in breast cancer cells (14). In cultured cervical cancer cells, an additive increase in growth inhibition after combined treatment with synthetic retinoids is associated with a similar increase in transcriptional activation of the RAR-β response element, suggesting that retinoid-induced growth inhibition is mediated through RAR-β (49). Association of response to retinoid therapy with RAR expression has also been documented in vivo (50). Expression of RAR-β is lost in premalignant oral lesions and restored by treatment with isoretinoin, which significantly correlates with a clinical response (51).

Our analysis of RAR isoform expression (using a reverse transcription-PCR assay) shows that RAR-β-specific transcripts were present only in RA-sensitive SK-RC-06 cells. RAR-β1 is expressed in human fetal cells but not in adult kidney cells (38) or cultured adult human kidney cells. The RAR-β isoforms possess different functions as indicated by differing patterns of expression and differential phosphorylation in response to RA (52). Our data suggest that RAR-β1 is involved in cRA-induced growth arrest of RCC cells.

In conclusion, the correlation of sensitivity to cRA with the expression of RAR-β1 suggests that the antiproliferative effects of cRA in RCC cells are mediated through RAR-β1. Ongoing studies are examining the effects of the introduction of RAR-β1 into RA-resistant RCC cells. The fact that most RCCs are resistant to cRA indicates that the encouraging clinical results observed in patients treated with cRA and IFN-α are not simply a result of the antitumor effect of cRA. Our prior work indicates that the addition of cRA to IFN increases the antiproliferative effects of IFN in IFN-sensitive RCC cells but not in IFN-resistant RCC cells (20). Elucidating the mechanism by which cRA augments the effects of IFN is under study.

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Unpublished data.


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