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Retinoid signaling in immortalized and carcinoma-derived human uroepithelial cells

Przemyslaw Waliszewski [a], Miroslawa Waliszewska [a], Niquel Gordon [a], Robert E. Hurst [a,b,c,*], Doris M. Benbrook [b,d], Arindam Dhar [d], George P. Hemstreet III [a,c,e]

[a] *Department of Urology, University of Oklahoma Health Sciences Center, 920 S.L. Young Blvd., PO Box 26901, Oklahoma City, OK 73190, USA*
[b] *Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, 920 S.L. Young Blvd., PO Box 26901, Oklahoma City, OK 73190, USA*
[c] *Department of Environmental Health, University of Oklahoma Health Sciences Center, 920 S.L. Young Blvd., PO Box 26901, Oklahoma City, OK 73190, USA*
[d] *Department of Obstetrics and Gynecology, University of Oklahoma Health Sciences Center, 920 S.L. Young Blvd., PO Box 26901, Oklahoma City, OK 73190, USA*
[e] *Department of Pathology, University of Oklahoma Health Sciences Center, 920 S.L. Young Blvd., PO Box 26901, Oklahoma City, OK 73190, USA*

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Abstract

This paper investigates the presence and functionality of retinoid signaling pathways in human urinary bladder carcinoma and SV40-immortalized uroepithelial cell lines. Only two of eight cell lines were proliferation-inhibited by 10 μM of either all-*trans* or 13-*cis*-retinoic acid. Transactivation of the CAT gene under control of a retinoid-responsive element demonstrated functionality of the signaling pathway in both sensitive cell lines and four of six resistant cell lines. Relative RT-PCR analysis of a panel of retinoid-responsive and inducible genes demonstrated changes in expression levels of all the genes in response to-retinoic acid treatment together with numerous aberrations dysregulations. We conclude that retinoid signaling may be a target for inactivation during tumorigenesis by uncoupling gene expression, proliferation and differentiation. Therefore retinoids are more likely to be effective for chemoprevention than for treatment of bladder carcinomas.

Keywords: Retinoic acid; Retinoid; Bladder carcinoma (human); Chemoprevention; Uroepithelium

1. Introduction

Bladder carcinoma represents an ideal opportunity for pharmacologic chemoprevention because individuals at risk can be identified by epidemiologic risk factors (Ruder et al., 1990), clinical features (Heney et al., 1983) and biomarkers (Hemstreet et al., 1998, 1996a,b; Bonner et al., 1993) and the success of chemoprevention can be monitored by biomarkers in urinary cells (Hemstreet et al., 1998, 1996b; Decensi et al., 1994). Retinoids are among the more active chemopreventive agents under investigation (Lotan, 1996; Greenwald et al., 1995). However, clinical trials of retinoids for prevention of recurrent bladder cancer have yielded less impressive results (Decensi et al., 1994; Studer et al., 1984; Trump, 1994) than might be expected from strongly positive animal studies (Moon et al., 1992) and large effects on diverse cell types in vitro (Takatsuka et al., 1996).

Retinoids act through a complex signaling system. Retinoic acid or other derivatives bind to members of

* Corresponding author. Tel.: +1-405-2713930; fax: +1-405-2713118; e-mail: robert-hurst@ouhsc.edu.

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the RAR and RXR family of nuclear receptor/transcription factors. The ligand-bound complexes induce genes with retinoic acid response elements (RAREs), including inducing the receptors themselves through the ligand-bound receptor/transcription factor (Sporn and Roberts, 1983; Gudas, 1992; Evans, 1988; Pfahl et al., 1994; Chambon, 1994; Mangelsdorf et al., 1995). The ligand-bound receptors also can inhibit expression of genes by acting through specific negative response elements (Lee et al., 1998; Clark et al., 1995; Radoja et al., 1997; Chang and Wei, 1997; Kirfel et al., 1997). Retinoids also can act by inhibiting transcription factor AP-1 (Li et al., 1996; Salbert et al., 1993; Fanjul et al., 1994; Shao et al., 1995). However, the determinants of the retinoid-responsive phenotype have not been elucidated other than to show that expression of retinoid-responsive genes (Gudas, 1992) and various retinoid binding proteins and the metabolism of retinoids are important to the retinoid-sensitive phenotype (Takatsuka et al., 1996). What the effects of carcinogenesis with its attendant losses of critical regulatory functions might be upon retinoid signaling is unknown, but it is an important question highly relevant to the use of retinoids in chemoprevention.

In this study a model system of eight uroepithelial cell lines was used to investigate retinoid signaling and the changes induced by malignant transformation in uroepithelial cells. These cell lines are representative of cells at the early stages of immortalization (HUC-BC and HUC-PC), as well as carcinomas, including transitional cell (T24, J-82, 5637, TCC-Sup), squamous cell (SCaBER) and papillomas (RT4). Transactivation of RARE-containing plasmid was used to assess retinoid signaling. Constitutive and retinoid-modulated expression of the nuclear receptor and retinoid-binding protein genes as well as of genes in several pathways linked to retinoid signaling, namely epidermal growth factor receptor (EGF-R), the angiogenic MK cytokine, ICAM-I and transglutaminase were assessed by relative RT-PCR. Since bladder carcinogenesis itself involves multiple genotypic combinations to produce the two, basic papillary and invasive phenotypes (Kallioniemi et al., 1995; Presti et al., 1991; Knowles, 1995; Reznikoff et al., 1996) and transformed cells lose their capability to respond to the normal hormonal constraints of proliferation, it is likely also that the hierarchy of retinoid-responsive and retinoid-modulated genes is uncoupled during the course of bladder tumorigenesis. If expression of the entire hierarchy of retinoid-responsive genes is triggered by the retinoid receptors, then we would expect to observe that the expression of the retinoid-reponsive genes is predictable from the expression of the retinoic acid receptor genes in different cell lines. On the other hand, if these genes form a system that interacts strongly and is modulated by other inputs that themselves may be modified by the carcinogenic process, then neither sensitivity to retinoids nor expression of individual genes will be predictable from receptor expression. Reports that these pathways are redundant (Chambon, 1994) and linked to other pathways through other response elements such as steroid-responsive elements in the promoters of retinoid-responsive genes and vice versa (Shibata et al., 1997; Waliszewski et al., 1997a), suggests this may well be the case. Our findings strongly support such a model and suggest that retinoid signaling is a common target of the carcinogenic process, but that no single gene seems to be a primary target.

2. Materials and methods

Human embryonal cell line FHS 738BL, human urinary bladder papilloma-derived cell line, RT4 and human urinary bladder carcinoma-derived cell lines HT 1376, J-82, SCaBER, T24, TCC-Sup and 5637 were obtained from the American Type Culture Collection. SV40-immortalized human uroepithelial cell lines HUC-BC and HUC-PC were obtained from Dr C. Reznikoff (Bookland et al., 1992).

2.1. Cell culture, retinoid stimulation and RNA isolation

Initially, we attempted to perform the studies in serum-free medium, but only J-82 cells grew well. Range-finding experiments with 10 and 100 nM, 1 and 10 μM were performed, which showed that inhibition was only significant at 10 μM. Hence, 2000 cells of each line were seeded into three 96-well plates with 10% fetal bovine serum-containing medium and allowed to attach for 24 h. Thirty wells in each plate were controls without added retinoid, two were blanks and the remaining 64 were treated with all-*trans*-retinoic or 13-*cis*-retinoic acid (Sigma, St. Louis, MO) with daily changes of medium to ensure a roughly constant concentration of retinoid. The media recommended by the American Type Culture Collection (ATCC) were used; MEM for TCC-Sup, J-82 and SCaBER, McCoy's 5A for T-24, RPMI for 5637 and Ham's F-10 for HUC-PC, HUC-BC and RT-4. The concentrations of all-*trans*-retinoic acid and vitamin A in the FBS used to supplement the medium were checked by HPLC (Boylan and Gudas, 1992) and were below the detection limit of 10 nM. Retinoids were kept at −20°C and dilutions were kept on ice in foil-wrapped tubes. To minimize photolysis, lights in the room and hood were switched off when retinoids were pipetted. After 6 days the number of cells was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Because the data were found to be normally distributed by the Shapiro–Wilk

Table 1
Sequences of primers made locally for amplification of specific retinoid-responsive genes

Gene	Sense primer (5′–3′)	Antisense primer	Size (bp)
hRARα	TTGTCTGTCAGGACAAGTCC	CCACTTCAAAGCACTTCTGC	188
hRARβ	CATCGAGACACAGAGTACCA	GCTCTCTGTGCATTCCTGCT	~500
hRARγ	ACCTCCTCGGGTCTATAAGC	CGCAAACTCCACAATCTTGA	~500
hRXRα	CAGGCAAGCACTATGGAGTGT	TGGCCAGGCACTTCTGGTA	165
hRXRβ	CTTTGTGCAATCTGCGGGGAC	TGGCCAGGCACTTCTGATATC	192
CRBP I	GTCGACTTCACTGGGTACTGGA	TTGAATACTTGCTTGCAGACCACA	441
CRABP I	CGGCACCTGGAAGATGCG CA	CCACGTCATCGGCGCCAAAC	370
CRABP II	CCCAACTTCTCTGGCAAC TGGA	CTCTCGGACGTAGACCCTGGT	410
MK	CTG CAA CTG GAA GAA GGA	TTC CCT TCC CTT TCT TGG	206
TG	GGG CGA ACC ACC TGA ACA AA	GGT CAC TAC CTA GCA TGT TGT	300
β-Actin	GCGCTCGTCGTCGACAACGGC	ATGGGGTAGCTCGTGCCGTAGCAGT	220
36B4	ATGTGAAGTCACTGTGCCAG	GTGTAATCCGTCTCCACAGA	~500

test, the means of growth relative to control were compared by the Student *t*-test.

To isolate total RNA for gene expression studies, cells were cultured with or without 10 μM all-*trans*-retinoic acid for 48 h in Phenol Red-free medium (Glover et al., 1988), washed three times with cold PBS and treated with 4M guanidinium thiocyanate according to Chomczynski and Sacchi (1987). The RNA quality was checked by electrophoresis.

2.2. Apoptosis assay

Cells were grown and treated in separate 75 cm^2 flasks with retinoic acid as described above. On the day of harvest, the supernatant was withdrawn and placed in a centrifuge tube. The cell layer was then treated with 0.05% trypsin to release the cells, which were then combined with the cells in the supernatant. The cells were centrifuged at 1000 rpm for 5 min and washed twice with buffered saline. The cells were brought to a final volume of 10 ml, placed on ice and 500 μl of 10% formaldehyde was added. After 15 min, the cells were centrifuged, washed, taken up in a final volume of 0.5 ml of buffered saline and added to ice-cold 70% ethanol. The cells were labeled with FITC-conjugated anti-BRdU antibody (Molecular Probes, Eugene, OR) and assayed as described using the TUNEL assay (Gorczyca et al., 1994).

2.3. Transactivation of CAT gene under RARE control and CAT assay

The pBLCAT2 reporter plasmid containing the chloramphenicol acetyl transferase (CAT) gene linked to a thymidine kinase (tk) promoter on the 5′ end to allow for a low basal activity and containing the SV40 polyadenylation signal attached to the 3′ end of the CAT gene to allow stabilization of the message was used as a control for the basal level of activity. The same plasmid containing the retinoic acid response element (RARE) from the RARβ gene placed upstream from the promoter (RAR-tk-CAT) was used to test for functionality of retinoic acid signaling in all the cell lines. The plasmids were transfected by the calcium phosphate method (Maniatis et al., 1990). CAT activity was measured with a CAT ELISA kit (Boehringer-Mannheim, Indianapolis, IN) after stimulation with 10 μM all-*trans*-retinoic acid for 24 h in comparison to the control.

2.4. Identification of mRNAs of expressed genes

Some 2 μg in total of RNA was digested with DNase I to eliminate artifacts from genomic sequences and processed to first strand cDNA by reverse transcription using random primers (Boehringer-Mannheim, Indianapolis, IN). Conditions for RT-PCR for each cDNA were optimized, but most conditions used 32 cycles of PCR at 96°C for 1 min, 52°C for 1 min and 72°C for 2.5 min. The primers concentration was 150 ng/μl. The PCR products (25 μl/lane) were separated on 1.5% agarose gel. Identities of the bands were confirmed by: (a) a single band of the expected size was observed; (b) restriction enzyme digestion to yield the expected size products; and (c) in the case of hRARα and hRARβ Southern blot analysis with independent probes. The *Eco*RI fragments of hRARα were prepared from the full-coding sequence cDNA, generously provided by Magnus Pfahl to one of us (DMB). The full-length cDNA sequences of hRARβ, hRARγ and β-actin were labeled with the Pharmacia random priming kit and used as probes. Primers were synthesized at the local Oklahoma Center for Molecular Medicine core facility. The sequences and the size of the expected products are listed in Table 1.

2.5. Relative RT-PCR

To compare constitutive levels of expression of retinoid-responsive genes with levels found subsequent

to stimulation and to compare the levels of expression of the cell lines among each other, the technique of Relative RT-PCR was used. This technique normalizes the amounts of cDNA to the concentration of a common housekeeping gene, in this case β-actin, which is not affected by retinoid treatment (Rao et al., 1997). The cDNA samples were amplified for β-actin using conditions optimized for its amplification. Amplification for 21 cycles produced bands that were within the linear range of the PCR procedure and under such conditions the fluorescence of the ethidium bromide-labeled bands were proportional to the relative concentrations of β-actin cDNA. The band densities were used to normalize the amount of cDNA in each sample. If eight cell lines were to be compared before and after stimulation with retinoic acid, this meant normalizing 16 cDNA samples. Once normalized, however, the concentrations of other cDNAs could be compared to each other as well as before and after stimulation. Eleven different retinoic acid-responsive genes were assayed in this way. The amplification of each cDNA was performed under conditions optimal for the particular cDNA. For example, hRARα yielded bands within the linear range of the ethidium bromide staining method after 33 amplifications. Each particular cDNA analysis was optimized in this manner to ensure that the midrange of expression was within the linear range of the PCR. All amplifications were performed in duplicate and a separate aliquot was always amplified for β-actin to ensure that the normalization was correct. A more sensitive Southern blot version of the assay using a >500 bp radioactive probe was used with hRARβ and hRARγ and normalization against 36B4 phosphoprotein using 20 cycles of amplification for the receptors and 18 for the 36B4 (Roy et al., 1995).

2.6. Sequencing of hRARα DNA-binding domain cDNA

The hRARα PCR products from all the cell lines were ligated into pCR vector using the TA-cloning kit (Invitrogen, San Diego, CA) and sequenced using the Sequanase kit (Amersham, Arlington Heights, IL) with Sp6 and T7 primers.

2.7. Scatchard analysis

Scatchard analysis was performed as described (Waliszewski et al., 1997b) using radio labeled all-*trans*-retinoic acid (New England Nuclear, Boston, MA) on proteins in nuclear and cytoplasmic fractions obtained by the method of Dignam et al. (1983). Protein concentrations were measured by the Bio-Rad Protein Assay (BioRad, Hercules, CA).

3. Results

3.1. Inhibition of proliferation of cell lines

As shown in Fig. 1, the HUC-BC and TCC-Sup cell lines gave a statistically significant decreased number of cells when treated with 10 μM of either all-*trans* or 13-*cis*-retinoic acid as compared to untreated controls. The Shapiro–Wilk test demonstrated the data were distributed normally and the Student *t*-test was significant at $P \leq 0.05$. The proliferation of the remaining cell lines was not affected significantly by either isomer. The HUC-BC line, which was found to be sensitive to both isomers, derives from the HUC-PC line, which is resistant to either isomer (Fig. 1). As determined by the morphology of the cells and by exclusion of Trypan Blue by over 95% of the cells, 10 μM of either retinoid was not toxic to the cells.

3.2. Apoptosis of cells treated with all-trans-retinoic acid

The percent of cells found to be in apoptosis after 48 h of treatment with 10 μM all-*trans*-retinoic acid is shown in Fig. 2. With the exception of the J-82 cell line, for which 25% were found to show an apoptotic response after 48 h, the other lines showed 10% or less apoptotic cells. Background apoptosis levels in the absence of added retinoid were all in the range of 2–5%. The J-82 cell line did not show a marked proliferation inhibition response (Fig. 1) in spite of having the highest apoptotic response.

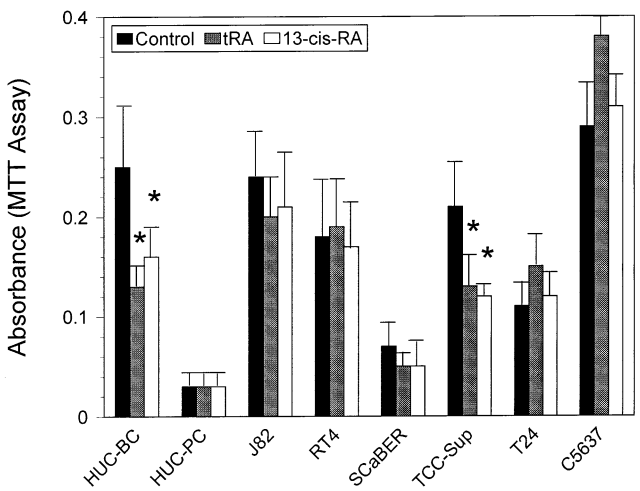

Fig. 1. Inhibition of proliferation by 10 μM all-*trans* and 13-*cis*-retinoic acids as assessed with the MTT assay. Results display the ratio of mean absorbances of control (without retinoid) ($n = 90$) and-retinoic acid-treated cultures ($n = 192$) in the MTT assay. Bars marked with an asterisk differed significantly ($P < 0.05$) from controls.

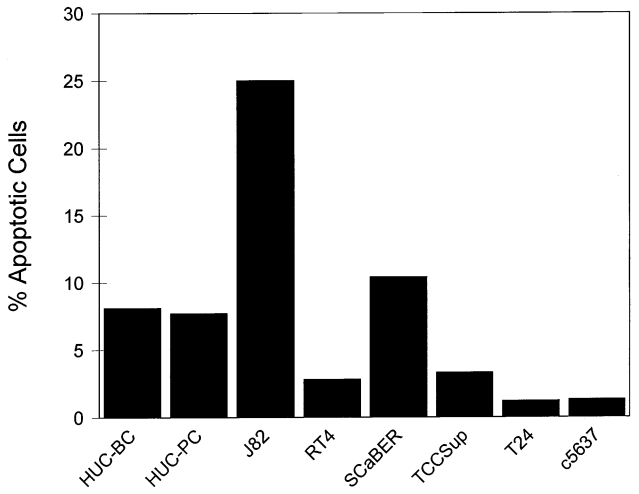

Fig. 2. Apoptotic response of cell lines grown for 48 h in 10 μM all-*trans*-retinoic acid. The percent of cells found to be apoptotic by the TUNEL assay are shown by cell type.

3.3. Binding assays in nuclear and cytoplasmic fractions

Scatchard analysis of the nuclear fractions of two of the cell lines (J-82 and TCC-Sup) yielded hyperbolic binding curves, indicative of the presence of more than a single binding protein. Because these data were not informative beyond this observation, they are not shown, nor were the other cell lines analyzed further.

3.4. Activity of RARE signaling

Fig. 3 shows the results of testing for retinoid signaling activity using transient transfection with a RARE-containing plasmid. All of the cell lines transiently transfected with the RARE-driven CAT construct showed a positive response (i.e. ratio of CAT activity in the presence of retinoic acid to that in its absence was

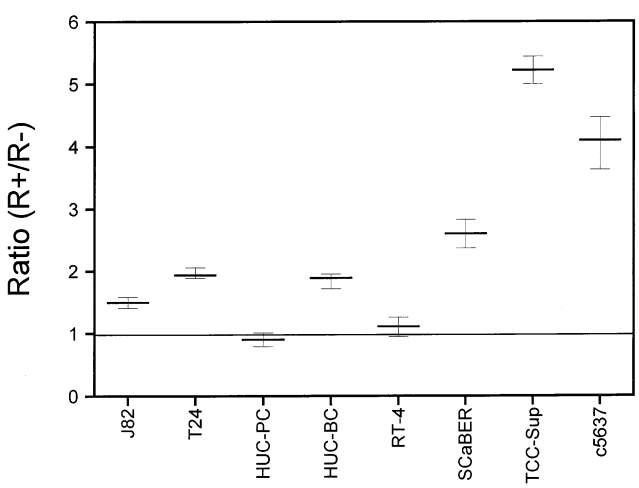

Fig. 3. Functionality of retinoid signaling pathways as tested with cell lines transfected with CAT constructs in the presence (R+) and absence (R−) of retinoic acid.

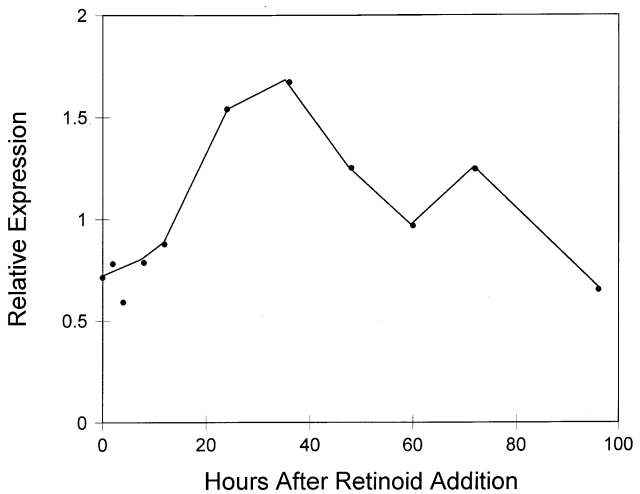

Fig. 4. Time course of expression of hRARα in TCCSup cells treated with 10 μM all-*trans*-retinoic acid. The cDNAs prepared from mRNA isolated from cells treated for different intervals were normalized according to the β-actin cDNA and analyzed by PCR. The mean density of the actin to which the data were normalized was 9.1 ± 0.6 arbitrary units.

greater than unity) to all-*trans*-retinoic acid except for RT-4 and HUC-PC. The level of stimulation for these two cell lines was within experimental error of unity and indicated signaling dependent upon RARE sequences was inactive. The level of RARE transactivation did not correlate with inhibition of proliferation seen in Fig. 1.

3.5. Time course of stimulation of hRARα expression

Fig. 4 illustrates the time course of the level of hRARα mRNA in TCCSup cells in response to treatment with 10 μM all-*trans*-retinoic acid. Expression increases significantly at 8 h, is maximal from between 36–48 h and begins to resolve thereafter as the system saturates. The level of mRNA reaches baseline after 96 h.

3.6. Expression of mRNAs of retinoid-responsive genes

Fig. 5 illustrates Relative RT-PCR analyses of hRARα, hRXRα, CRABP I and EGF-R. These are illustrative of the data summarized in Table 2, which compares the relative expression of all the genes studied. The list comprises genes encoding the receptors, including both hRAR and hRXR families; elements in other signaling pathways such as EGF-R, the angiogenic MK cytokine and ICAM-I; transglutaminase, a gene involved with apoptosis and differentiation of a number of epithelial cell types and CRBP I, CRABP I and II, three proteins that bind retinol and-retinoic acid respectively. All the cell lines expressed hRARα and hRXRα constitutively. The hRARβ receptor was not

Table 2
Constitutive and stimulated expression of mRNAs of selected retinoic acid receptor genes and binding proteins as determined by relative RT-PCR after 48 h stimulation with 10 μM all-*trans*-retinoic acid[a]

	HUC-BC		TCC-Sup		HUC-PC		J-82		SCa-BER		T-24		5637		RT-4	
	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C	T
hRARα	++	++	++	+++	++	+++	+++	+++	+++	+	+++	+	++	++	+++	+
hRARβ	U	U	U	V	U	++	U	U	U	U	U	+	U	U	U	V
hRARγ	+	++	V	+	+	++	U	V	+	+++	+	+++	+++	+++	V	+
hRXRα	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
hRXRβ	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
CRBP I	U	U	U	U	+++	+	U	U	+	+	U	U	+++	+	U	U
CRABP I	++	+	++	+++	++	+	U	U	+	+	U	U	U	U	U	U
CRABP II	+	+++	++	+++	++	+	+	+	+++	+++	+	+	+++	+++	+++	+++
TG	+++	+	+	+++	+	++	+	+++	+	+++	+++	+++	++	++	U	U
EGF-R	+++	+++	+++	+	+++	+	+++	+	+++	+++	++	+	++	+	++	+
ICAM-I	++	+++	++	+	+++	++	++	+++	++	+++	+++	++	+++	+	U	U
MK cytokine	+	+	+++	+++	++	+	++	+++	++	+++	+++	+	+++	++	U	U

[a] +++ high; ++ moderate; + low; V, very low (detectable only by radioactivity and 4 days film exposure); and U, undetectable.

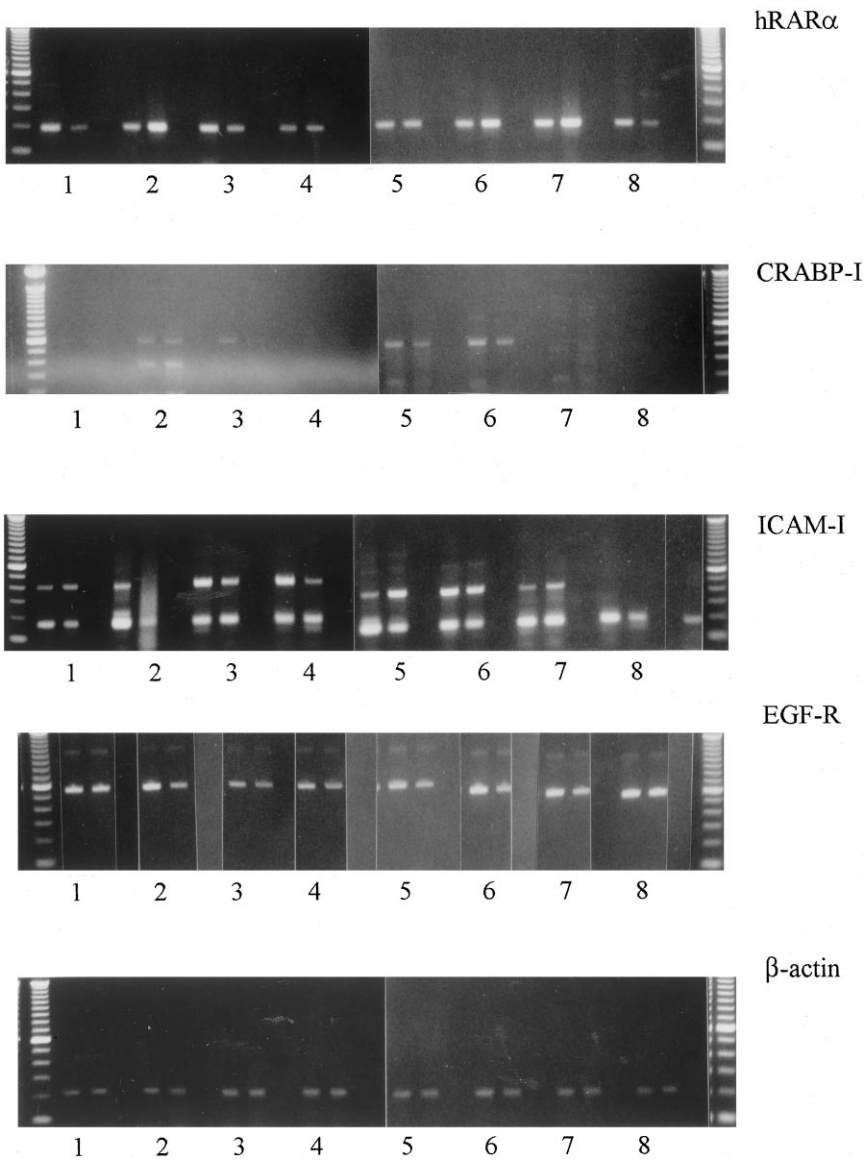

Fig. 5. Relative RT-PCR analysis with Ethidium Bromide labeling for hRARα, CRABP I, ICAM-I, EGF-R and the normalized β-actin comparing constitutive and all-*trans*-retinoic acid-stimulated expression. Lane 1, FHS; Lane 2, HUC-BC; Lane 3, HUC-PC; Lane 4, HT 1376; Lane 5, J-82; Lane 6, RT4; Lane 7, SCaBER; Lane 8, TCC-Sup; Lane 9, T24; and Lane 10, C5637. A 100 bp DNA ladder was used as a marker of molecular size. In each pair, the constitutive expression is on the left. In case of multiple bands, e.g. CRABP I, quantitation was with respect to the band corresponding to the band associated with the authentic cDNA.

expressed constitutively in any cell line in amounts detectable by Northern analysis or RT-PCR. The expected receptor product was detectable subsequent to retinoic acid treatment only in T24 and HUC-PC with the ethidium bromide assay, but with the more sensitive radioactivity assay, faint bands also could be discerned in the RT-4 and TCC-Sup cells after 4 days of exposure of the film as compared to 1 h needed to demonstrate hRARγ, which all the cell lines expressed subsequent to retinoic acid treatment and all but J-82 expressed constitutively.

Each cell line showed a different expression of the binding proteins. The two HUC cell lines showed a single band in the position expected from CRABP I cDNA. TCC-Sup also showed fainter bands above and below the expected product while T24 and SCaBER yielded only the additional bands. The presence of these bands was reproducible with independent cDNA samples and therefore is not an artifact. The bands were not further identified. While all the cell lines expressed CRABP II at some level, several did not express CRABP I and only three expressed CRBP I.

In general, the cell lines showed remarkable differences in gene expression. The papilloma-derived RT-4 line was particularly aberrant in comparison to the other cell lines and failed to express many of the genes

that were expressed by the other lines. Overall, no correlation could be found between modulation of hRARα mRNA and expression of the other retinoid-responsive genes studied, between expression of the entire hierarchy of retinoid-responsive genes including hRARα and the phenotypic response of cells and between retinoid signal transduction as demonstrated by CAT assay and modification of expression of retinoid-responsive genes.

3.7. Sequencing of hRARα amplimers

The PCR products encoding the hRARα DNA-binding domains were all sequenced and the receptor from HUC-PC cells was found to contain a mutation, a G→T point mutation at position 48 of the cloned sequence, equivalent to position 606 of the hRARα. This mutation would substitute tyrosine for asparagine in the highly conserved DNA-binding domain. The mutation also was found in a second, independently-produced amplimer.

4. Discussion

In order to better understand and predict the response of bladder carcinoma and premalignant bladder lesions to retinoic acid and its derivatives, we investigated the sensitivity of SV-40-immortalized uroepithelial cells and urinary bladder carcinoma-derived cell lines to the antiproliferative and apoptosis-inducing action of all-*trans* and 13-*cis*-retinoic acids. We sought to identify any consistent links between these phenotypic responses and expression of the family of RAR and RXR receptors, as well as to the expression of a number of other genes known to be modulated by retinoic acid to determine if response could be predicted from expression of key retinoid signaling pathway genes.

Only two of eight cell lines showed inhibition of proliferation in response to treatment with 10 μM-retinoic acids; HUC-BC, one of the SV-40-immortalized cell lines and TCC-Sup, one of the carcinoma lines. No significant differences were observed between the two-retinoic acid isomers, suggesting they function similarly. The lack of correspondence with apoptosis indicates that the ability to be proliferation-inhibited or induced to apoptose represent independent phenotypes.

The functional studies indicated that a complex pattern of inactivation or dysregulation of retinoid signaling has occurred in some, if not most, cell lines. In two of the eight cell lines, transactivation of a CAT construct driven by RARE was not observed, thereby demonstrating that the system of receptors activated by retinoic acid is not functional in those cell lines. In one of these, the HUC-PC line, a mutation was observed in the hRARα gene. Assuming this mutation in the highly conserved DNA-binding domain would inactivate RARE transcriptional activation in general, the resistance of this line to all-*trans*-retinoic acid is explicable. Inactivation of retinoid signaling in the RT-4 line must be due to another mechanism. Scatchard analysis was not informative because of the complex nature of the binding curves, other than demonstrating that the cells contain more than single cytoplasmic and nuclear proteins capable of binding radiolabeled all-*trans*-retinoic acid.

In spite of inactivation of signaling acting through the particular RARE studied, expression of a number of genes was modulated in the HUC-PC cell line in response to-retinoic acid treatment, which demonstrates the presence of other, RARE-independent pathways by which gene expression is modulated by-retinoic acid (Shao et al., 1995). Transrepression of the AP-1 transcription factor, which some suggest is responsible for the anti-proliferation effect (Li et al., 1996; Fanjul et al., 1994), is but one example that has been identified to date.

The extensive dysregulation suggests that retinoid signaling is a target of carcinogenesis in the bladder. All of the retinoid receptors and retinoid-binding proteins would be expected to be unregulated by treatment with all-*trans*-retinoic acid because these contain a RARE sequence in the promoter, as does transglutaminase (Yan et al., 1996). The situation for the angiogenic cytokine MK is not so clear. The promoter contains a RARE, but the gene normally is expressed only in proximal tubule cells and was overexpressed in a variety of genitourinary tumor cell lines (Kitamura et al., 1993). The effect on EGF-R should be inhibitory or stimulatory, depending upon the cell line (Nutting and Chowaniec, 1992; Zheng et al., 1992). Recently an increasing awareness that mutations or other alterations in retinoid signaling pathways may be involved in the process of tumorigenesis itself has emerged (Arranz et al., 1994; Xu et al., 1994; Lotan et al., 1995). The mechanisms by which retinoid signaling are abolished are obscure, however. As has been observed for lung, cervical and other cancer cell lines, inactivation of hRARβ expression seems to be common (Xu et al., 1997a,b). None of the bladder cancer cell lines showed constitutive expression of this receptor at levels detectable by RT-PCR and only four showed any expression after stimulation with all-*trans*-retinoic acid. For two of those lines, the amount expressed was very low and was undetectable by ethidium bromide staining. One of the two lines that responded by upregulating hRARβ expression was the HUC-PC line. Given that hRARβ responds to hRARα, either the mutation of the hRARα gene in the HUC-PC cells does not inactivate upregulation of the hRARβ gene, or it occurs by a mechanism independent of hRARα.

Other mechanisms by which cells can become resistant to-retinoic acid include elevated metabolism of the retinoids by cytochrome P-450 (Roberts et al., 1992) or elevated levels of retinoid-binding proteins in the cytoplasm that could sequester free-retinoic acid, thereby inhibiting liganded hetero or homodimer formation. In support of the first mechanism is the finding that the HUC-BC cell line, which expresses low levels of certain cytochrome P-450 enzymes (Bookland et al., 1992), is sensitive to all-*trans*-retinoic acid. Examination of Table 2 does not support the contention that elevated expression of any of the three retinoid-binding protein genes alone produces resistance. However, more detailed studies of protein levels will be required to confirm this finding. Refractoriness in the presence of functional receptors has been reported (Kim et al., 1995).

We speculate this resistance could be responsible for the failure of retinoids to show a higher activity in vivo than is typically observed in vitro or in animal studies (Decensi et al., 1994; Studer et al., 1984). Generally clinical trials of retinoids have sought to prevent recurrences rather than primary tumors. The presence of field disease is indicative of a very high risk for bladder carcinoma (Foresman and Messing, 1997) and many such patients may well be resistant to retinoids because of alterations in the retinoid pathway. In animal studies, however, the retinoid is administered before cancers develop and is therefore present during earlier stages of tumorigenesis. Previous studies have shown that premalignant oral mucosa cells show suppression of hRARβ just as do malignant cells, but unlike the malignant cells, show upregulation of the receptor by all-*trans*-retinoic acid (Lotan et al., 1995). Successful chemoprevention may therefore require identification of a subset of sensitive patients by the absence of biomarkers indicative of late field disease (Hemstreet et al., 1998, 1996a; Bonner et al., 1996), or administration to subjects identified as being at risk from epidemiological or biochemical factors well before late-stage premalignant cells emerge.

Examining several cell lines displays a fuller range of the effects of carcinogenic transformation than does examination of a single cell type in depth. The wide range of behavior seen in Table 2 demonstrates clearly that the system responds nonlinearly. In other words, knowledge of the expression of a variety of retinoic acid-responsive genes that supposedly are intimately involved in producing the response of cells to all-*trans*-retinoic acid provides little ability to predict the sensitivity of the cell line to all-*trans*-retinoic acid. While one could argue that we simply have not identified the proper set of genes, another explanation should be considered as well. It is distinctly possible that retinoid action, like the weather, represents a complex system. The role of complexity in biological systems is underappreciated, probably because we lack effective tools and theories for studying complex systems.

When a system is complex, components of the system cannot be isolated sufficiently to manipulate each variable individually and the system is inherently nonlinear, thereby making it difficult to assess causality in the conventional sense. In the case of retinoid signaling, the complexity is inherent because retinoid signaling is linked hierarchically to other signaling pathways, including steroid receptors (Shibata et al., 1997) and others (Leid et al., 1992). This complexity may not be apparent when only a single cell type is studied, nor is it restricted to bladder-derived cells. The entire hRXR and hRAR homo- and heterodimer system displays both specificity and redundancy (Forman et al., 1995; Zhang and Pfahl, 1993; Lufkin et al., 1993). However, the ability of retinoids tailored to activate specific receptor systems has very little to do with the overall response of lung cancer cells to retinoids (Sun et al., 1997). Thus, the phenotypic response will not be readily predictable from a knowledge of gene expression.

This situation actually is more common than perhaps has been recognized. Retinoid signaling represents a key and central pathway involving regulation of proliferation, differentiation and eventual cell death by apoptosis. The process of tumorigenesis involves escape from these normal controls and mutagenic inactivation of one single gene, the p53 gene, seems to represent a common means of escape, perhaps because this one gene plays such a common role in all the intersecting pathways involved. Nonetheless, rarely does the aberrant p53 genotype represent over 60% of the means by which a given type of cancer cell can escape apoptotic control (Shackney and Shankey, 1997). A number of investigators have suggested that the cell actually represents a complex set of interacting pathways in which mutation or change in expression of a single gene can have wide-ranging consequences on the expression of other genes (Lee et al., 1991). Multiple genotypes can lead to a common phenotype (Shackney and Shankey, 1997) and can therefore lead to a situation in which phenotype is not predictable with any accuracy from a knowledge of genotype. Retinoid signaling appears to be a frequent and early casualty of the carcinogenic process in bladder carcinogenesis, but there are obviously numerous means by which these controls can be subverted or inactivated. The possibility that the success of chemoprevention in animal models in which the agent is administered prior to emergence of the malignant phenotype is due to gene deletions and mutations of critical genes in this system of pathways must be considered, as must secondary alterations resulting from the highly linked nature of the system.

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