Retinoid Refractoriness Occurs during Lung Carcinogenesis Despite Functional Retinoid Receptors

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ABSTRACT

Retinoids have demonstrated activity in the prevention of second primary tumors in patients with non-small cell lung cancer (NSCLC). They also contribute to the normal growth and differentiation of human bronchial epithelial (HBE) cells. Because retinoids mediate their actions through retinoid nuclear receptors (RARs and RXRs), aberrant signaling through retinoid receptors could contribute to lung carcinogenesis. Using a lung carcinogenesis model consisting of normal, premalignant, and malignant HBE cells, we examined all-trans retinoic acid (t-RA)-induced changes in cellular growth. These studies revealed that t-RA treatment inhibited the growth of normal HBE cells, but premalignant and malignant HBE cells were relatively resistant to t-RA. Coincident with the development of retinoid refractoriness, basal expression of the retinoic acid nuclear receptor β (RAR-β) increased. Analysis of receptor function by gel shift and transient transfection assays of normal, premalignant, and malignant HBE cells demonstrated that receptor-DNA binding and transcriptional activation properties were intact in the t-RA-refractory malignant HBE cells. To compare these findings to NSCLCs in patients, we investigated retinoid receptor expression in NSCLCs. A subset of the tumors expressed RAR-β, reflecting the RAR-β expression observed in the malignant HBE cells in culture. These findings demonstrate that retinoid receptor function was intact in the t-RA-refractory malignant HBE cell line, suggesting that the defect in retinoid signaling in this lung carcinogenesis model is not intrinsic to the retinoid receptors.

INTRODUCTION

Vitamin A and related "retinoids" are dietary components that have been the focus of cancer prevention studies. These studies have shown that retinoids are potent inhibitors of field carcinogenesis. In patients with oral leukoplakia, treatment with 13cRA reversed the premalignant lesions (1). In patients who had undergone resection of head and neck cancers, treatment with 13cRA prevented second primary tumors of the upper aerodigestive tract (2). Similarly, in patients who had undergone surgical resection of stage I NSCLC, retinyl palmitate treatment reduced the incidence of second primary lung tumors (3). The clinical utility of retinoids in the prevention of lung cancer is currently restricted by our limited insight into how retinoids prevent cancer. Laboratory studies have begun to examine mechanisms by which retinoids control HBE cellular growth and differentiation. Retinoids can suppress the growth and squamous differentiation of bronchial epithelial cells (4–8), but this effect of retinoids is lost during HBE neoplastic transformation (7).

Retinoid signaling occurs primarily through nuclear receptors. Retinoid receptors are members of the steroid receptor superfamily and include the RAR and RXR gene families, each containing three members (α, β, and γ; Refs. 9–13). These receptors bind retinoids, activating the receptors to function as transcription factors. Ligand binding is specific; both t-RA and 9cRA can bind to RARs, whereas only 9cRA binds RXRs (14, 15). In DNA binding and transcriptional activation by ligand, retinoid receptors function as heterodimers of RAR and RXR or as RXR homodimers. RAR/RXR heterodimers bind RAREs in target gene promoter regions that consist of DR2 and DR5 motifs [i.e., (AGGTCA), AGGTCA, where n = 2 or 5 nucleotides; Refs. 16–18]. RXRs form homodimers that bind specifically to target genes containing DR1 response elements (19, 20). Recent studies have shown that although RXR can bind to its ligand in the RXR homodimer (20), only RAR can bind the ligand in the RAR/RXR heterodimer (21).

Recent studies on patients with premalignant or malignant epithelial lesions have revealed a basis for investigations into the role of retinoid signaling in epithelial neoplasia. In patients with cancer of the head and neck, RNA in situ hybridization studies of mucosal biopsies revealed lower RAR-β expression in dysplastic and neoplastic aerodigestive epithelial cells than in adjacent normal regions (22). RAR-β expression was also lower in oral leukoplasia lesions than in adjacent normal mucosa (23). RAR-β was not expressed in some NSCLC cell lines (7, 24). Although regions of chromosome 3p adjacent to RAR-β are frequently deleted in lung cancer, investigations in lung cancer cell lines have shown that disruption of the RAR-β gene is infrequent (7).

Activation of RAR-β expression can restore normal growth and differentiation in premalignant and malignant epithelial cells. For example, 13cRA treatment activated RAR-β expression in oral leukoplasia lesions, and this induction was associated with partial or complete remission of this premalignant condition (23). Similarly, constitutive expression of RAR-β in the CALU-1 squamous cell lung cancer cell line through stable transfection inhibited CALU-1 tumorigenicity in nude mice (25). These studies have begun to reveal the molecular basis of retinoid biology and the contribution of the retinoid pathway to the control of aerodigestive epithelial cellular growth and differentiation.

This study further investigated the contribution of retinoid signaling to the control of HBE cellular growth and differentiation by examining whether retinoid receptor expression and function are altered during lung carcinogenesis. Using a lung carcinogenesis model consisting of normal, premalignant, and malignant HBE cells, we correlated t-RA-induced changes in cellular growth with retinoid receptor expression and function. These studies showed that retinoid receptor function was intact in malignant HBE cells that are refractory to the growth-inhibitory effects of retinoids, demonstrating that the defect in retinoid signaling in these cells is not intrinsic to the retinoid receptors.
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MATERIALS AND METHODS

Cell Lines and Growth Conditions. A lung carcinogenesis model that includes normal, premalignant, and malignant HBE cells was used in these studies. Normal HBE cells were grown from bronchial epithelium, which was harvested from fresh surgical specimens obtained from patients undergoing lobectomy procedures. The mucosal layer was steriley stripped from bronchial specimens, cut into small pieces, and placed on a plastic tissue culture plate containing a thin layer of medium (Serum-free Keratinocyte Medium; Gibco-BRL, Gaithersburg, MD). When HBE cells had grown from these tissues into a confluent monolayer population, they were expanded for use in experiments. For each experiment, normal HBE cells from a single patient were used.

For the purposes of this study, premalignant cell lines were defined as immortalized nonmutorigenic cells, and malignant cell lines as immortalized tumorigenic cells. The premalignant BEAS-2B HBE cell line was obtained from Dr. Klein-Szanto by growing BEAS-2B cells within the lumen of a rat trachea placed s.c. in a nude mouse. Within the rat trachea, the cells were exposed to CSC via a beeswax pellet, and tumors developed. After 6 months, the tracheas cell lines derived from immortalized BEAS-2B cells that were used in this study are listed in Table 1 and include: BEAS-2B, which is nonmutorigenic; 1198, which is nontumorigenic and was isolated following treatment with a beeswax pellet containing no CSC; 1198, which is nontumorigenic and was derived following treatment with CSC; and the CSC-derived tumorigenic cell line 11701. The normal HBE, BEAS-2B, and 1199 cells grow in defined serum-free medium (Serum-free Keratinocyte Medium; Gibco-BRL) containing EGF and bovine pituitary extracts (26), and 3% serum is required for the growth of premalignant 1199 and malignant 11701 cells (27). One patient was grown on standard plasticware (Falcon; Becton-Dickinson, Bedford, MA) at 37°C with pCO₂ of 5% (26). Tryptsin (PET) and soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) were used in the cell culture.

Assay of Retinoid-induced Changes in Growth. Normal, premalignant, and malignant HBE cells were seeded at 2 × 10⁶ cells/10 cm plate, and t-RA (10⁻⁶ M; Sigma) or medium alone was added 1 day later when the cells were in log-phase growth. During t-RA treatment, control and t-RA-treated cells were grown without EGF, because EGF abrogates the growth-inhibitory effect (8). Medium was changed every 2 days. After 5 days of treatment, the cells were trypsinized and counted by using a hemocytometer. The total number of viable cells (trypan blue-exclusive) was calculated. Results were expressed as the means and SDs of triplicate plates.

Assays of Retinoid Receptor Expression. Retinoid receptor expression was examined in normal, premalignant, and malignant HBE cell lines by Northern analysis of total cellular RNA. Cells were seeded at 2 × 10⁵ cells/10 cm plate, and 1 day later, the medium was changed to contain t-RA (10⁻⁶ M) or medium alone. The medium was changed every 2 days. After 5 days of treatment, total cellular RNA was extracted by lysis of cells in 4.0 M guanidinium isothiocyanate, followed by ultracentrifugation over a 5.7 M cesium chloride cushion. For treatment with actinomycin D or cycloheximide, 2 days following the seeding of 11701 cells, the medium was changed to treat the cells with cycloheximide (10 μg/ml) or actinomycin D (1 μg/ml) for 6 h, and RNA was then extracted. For Northern analysis, 30 μg of total cellular RNA prepared from each sample were subjected to electrophoresis on a 1% agarose gel containing 2% formaldehyde, stained with ethidium bromide, photographed, transferred to a nylon membrane (Duralon UV; Stratagene, Inc., La Jolla, CA), and hybridized to an (α-³²P)-dCTP-labeled retinoid receptor cDNA probe obtained from Dr. Ronald Evans (Salk Institute, San Diego, CA; Refs. 9–13). Random priming was performed by using the Prime It kit (Stratagene). Identical membranes were used for hybridization to each of the receptor cDNA probes. Following high-stringency washing (0.1X SSC at 65°C), the membranes were exposed overnight to photographic film using an intensifying screen at ~80°C.

Because the baseline level of RAR-β expression was below the sensitivity of Northern analysis in normal and some premalignant HBE cells, RT-PCR techniques were performed as described elsewhere (7). Primers for RAR-β were (sense) 5'-CAGACATTCAGTCCAGGGAGATC-3' and (antisense) 5'-AA TTGTGCTCGATGTCAGACC-3'; these amplified a 329-bp PCR product. Primers for GAPDH were (sense) 5'-CGTCGCCCGCCAGCACTAC-GCTC-3' and (antisense) 5'-GGAGTCCACTGGCGTCTACCACC-3'; these amplified a 344-bp PCR product. Briefly, in two separate tubes, 1 μg of total cellular RNA underwent reverse transcription using equal volumes of a stock of reverse transcriptase reaction mixture [25 mM MgCl₂, 5 mM KMCL, 10 mM Tris-HCl (pH 8.3), 1 unit/μl RNase inhibitor, 2.5 units/μl Moloney murine leukemia virus reverse transcriptase, and 2.5 μM random hexamers]. PCR was then performed on the cDNA product in each tube, using primers to RAR-β, in one tube and primers to GAPDH in the other tube. The PCR reaction took place in 2 mM MgCl₂, 5 mM KCl, 10 mM Tris-HCL, 2.5 units AmpliTag DNA polymerase, and 0.2 μM primers. The reaction used a thermal cycler (Perkin Elmer-Cetus, Norwalk, CT) and consisted of an initial 5-min incubation at 95°C, followed by 30 amplification cycles (95°C for 1 min, 58°C for 1 min, and 72°C for 3 min) and a final extension step (72°C for 10 min). Equal volumes of the PCR product from each sample were subjected to electrophoresis on a 2% agarose gel stained with ethidium bromide and photographed. The PCR products were transferred to a nylon membrane and hybridized to the RAR-β cDNA probe (as described above) to confirm the identity of the visualized bands. Semiquantitative RT-PCR analysis was performed to quantify RAR-β and GAPDH in serial dilutions of the cDNAs, and the PCR products were electrophoresed, transferred, and hybridized to [³²P]dCTP-labeled RAR-β and GAPDH probes. Counts were quantitated from PCR products obtained in the linear range of the PCR assay using a Betascope 603 Blot Analyzer (Betagen, Inc.). Fold-induction of RAR-β expression by t-RA treatment was determined after correcting for cDNA loading based on GAPDH intensity.

Assays of Retinoid Receptor Function. To examine the effects of retinoid treatment on receptor function in HBE cells, gel shift and transient transfection assays were performed. The oligomers used in gel shift assays were commercially prepared (Oligos, Etc., Inc., Wilsonville, OR) from published DNA sequences (18). Oligomer sequences were as follows (response elements are underlined):

Wildtype DR5 RARE: TCGAGGTTAGGCTTCAAGGCAAAGTCCTCG AGGCCCCATCCAGGCAAGTTGACG
Mutated DR5 RARE: TCGAGGTTAGGCTTCAAGGCAAAGTCCTCG AGGCCCCATCCAGGCAAGTTGACG

For gel shift assays, cells were seeded and treated with t-RA (10⁻⁶ M) or medium alone for 5 days as described above. Nuclear extracts were prepared as described elsewhere (28) from normal, premalignant, and malignant HBE cells following 5 days of treatment with t-RA (10⁻⁶ M) or medium alone by resuspending trypsinized cells in buffer A (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10% glycerol, 10 mM KCl, 10 mM monothioglycolic acid, 1 mM PMSF, 0.5 μg/ml leupeptin, and 0.5 μg/ml aprotinin). The cells were homogenized, and the nuclei were collected by centrifugation. Nuclear pellets were solubilized in buffer B (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10% glycerol, 600 mM KCl, 1 mM DTT, 10 mM monothioglycerol, 10 mM PMSF, 0.5 μg/ml leupeptin, and 0.5 μg/ml aprotinin). The extracts were centrifuged at 100,000 x g, and the supernatants were subjected to dialysis against buffer C (10 mM Tris-HCl (pH 7.5), 10 mM KCl, 1 mM EDTA, 20% glycerol, 1 mM DTT, 10 mM monothio-
glycerol, 1 mM PMSF, 0.5 μg/ml leupeptin, and 0.5 μg/ml aprotinin]. The resulting dialysate was nuclear protein.

For use in gel shift assays, DRS RARE oligomers were end-labeled with [γ-32P]ATP (4000 Ci/mmol) using T4 polynucleotide kinase (Boehringer-Mannheim, Indianapolis, IN) and purified on a 4% nondenaturing polyacrylamide gel. Nuclear extracts (10 μg) were preincubated with 2 μg of poly(deoxyribo)nuclease-deoxyxycytidylic acid) for 15 min at 4°C and then incubated with labeled DNA (6000 cpm) for 15 min at 4°C in the presence of 10 mM Tris-HCl (pH 7.5), 10 mM KCl, 1 mM EDTA, 20% glycerol, 1 mM DTT, and 5 mM MgCl2 (29). For competition experiments, 10-fold excess nonradioactive oligomers were added prior to the addition of radioactive oligomers. The reaction mixture was subjected to electrophoresis on a 5% nondenaturing polyacrylamide gel. The gel was then dried and autoradiographed using an amplifying screen at −80°C.

Retinoid receptor function in normal, premalignant, and malignant HBE cells was further examined by transient transfection assays using luciferase reporter plasmids containing DRS RAREs (RAR-TK-LUC and RAR-ΔM-LUC; Ref. 30). These plasmids contain either a thymidine kinase or a mutated mouse mammary tumor virus long terminal repeat (ΔM) heterologous promoter and were a gift from Dr. Ronald Evans. In addition, receptor transcriptional activity was examined by using reporter plasmids containing the endogenous RAR-β promoter from −1470 to +160 (−1470RAR-β-LUC) or from −60 to +160 (−60RAR-β-LUC) inserted into the promoterless P19 luciferase reporter plasmid; these were gifts from Dr. Frank Kruit (Huberecht Laboratory, the Netherlands); Ref. (31). Cells were seeded at a density of 2 × 105 cells/well on a 6-well plate. The next day, transfections were performed in the presence of lipofectamine (GIBCO-BRL) and 2 μg of reporter plasmid. The following day, the transfection mixture was removed, and fresh medium containing t-RA (10−8 M) or medium alone was added. Treatment continued for 24 h, and the cells were then harvested in lysis buffer [1% Triton X-100, 25 mM gly-gly (pH 7.8), 15 mM MgSO4, 4 mM EGTA, and 1 mM DTT]. Luciferase activity present in the cell lysate was determined with a Lumat B 9501 luminometer (Berthold, Mannheim).

RESULTS

**Effects of t-RA on HBE Cell Number.** Cells used in these experiments are listed in Table 1. For the purposes of this study, premalignant cell lines are defined as immortalized, non-tumorigenic cells, and malignant cell lines are defined as immortalized, tumorigenic cells. Normal HBE cells are primary cultures derived from bronchial mucosal biopsies and are senescent. Normal, premalignant, and malignant HBE cells were treated with t-RA (10−6 M) or with medium.
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receptor expression during the malignant progression of HBE cells. Northern analysis of total cellular RNA was performed. Northern analysis revealed detectable RXR-α, -β, and -γ in untreated normal, premalignant, and malignant HBE cells, demonstrating no major change in RXR gene expression during lung carcinogenesis (Fig. 2). While t-RA treatment did not appreciably alter expression of RXR-α or -β, RXR-γ expression increased following t-RA treatment in the CSC-exposed cells, including premalignant 1198 cells and malignant 11701 cells. RXR-α and -γ were expressed at detectable levels in all cells examined (Fig. 2). In contrast, RAR-β was detected in untreated CSC-exposed cells (premalignant 1198 and malignant 11701) but not in untreated normal HBE cells or the premalignant cell lines BEAS-2B and 1799. These results demonstrate an increase in baseline RAR-β expression during the malignant progression of HBE cells.

Fig. 2. A, Northern analysis was performed on total cellular RNA extracted from the indicated cells following 5 days of treatment with medium alone (Lane 1) or with t-RA (10^{-8} M) (Lane 2). Identical membranes were hybridized to each of the indicated probes following random-priming with [α-32P]dCTP, washed, and autoradiographed for 48–72 h using an intensifying screen at —80°C. B2B, BEAS-2B cell line. B, a photograph of a representative ethidium bromide-stained gel demonstrates RNA loading per well.

Fig. 3. A, Northern analysis was performed on total cellular RNA extracted from the indicated cells following 5 days of treatment with medium alone (Lane 1) or with t-RA (10^{-8} M) (Lane 2). Identical membranes were hybridized to each of the indicated random-primed cDNA probes, washed, and autoradiographed for 48–72 h using an intensifying screen at —80°C. B2B, BEAS-2B cell line. B, a photograph of a representative ethidium bromide-stained gel demonstrates RNA loading per well.

RAR-β Expression Increases during Lung Carcinogenesis. In light of the central role of retinoid receptors in mediating retinoid actions, aberrant receptor expression could contribute to the retinoid refractoriness observed in lung carcinoma cells. In experiments designed to reveal alterations in baseline or t-RA-induced retinoid

alone for 5 days, and the total numbers of viable cells were counted. Treatment with t-RA reduced the number of viable normal HBE cells and premalignant 1799 cells to 30% and 70% of controls, respectively (Fig. 1). t-RA had no discernible effect on the number of CSC-exposed cells, which included the premalignant 1198 and malignant 11701 cell lines. These results demonstrate that, relative to normal HBE cells, premalignant and malignant HBE cells are refractory to the growth-inhibitory effects of t-RA.

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Fig. 4. Semiquantitative RT-PCR analysis of RAR-β expression was performed on total cellular RNA extracted from cells following 5 days of treatment with t-RA (10^{-8} M) or media alone. Serial dilutions of the cDNAs revealed that, for all of the samples, PCR analysis was within the linear range for RAR-β and GAPDH using cDNAs diluted 1:4 and 1:256, respectively. Following PCR performed at these dilutions, the products were electrophoresed, transferred to a nylon membrane, hybridized to [α-32P]dCTP-labeled RAR-β and GAPDH cDNAs, and autoradiographed for 90 min at —80°C. Included are control and t-RA-treated normal HBE cells (Lanes 1 and 2), control and t-RA-treated BEAS-2B cells (Lanes 3 and 4), control and t-RA-treated 1799 cells (Lanes 5 and 6), and 11701 cells as a positive control (Lane 7).

Fig. 5. Northern analysis of RAR-β expression was performed on total cellular RNA extracted from malignant 11701 cells following 6 h of treatment with medium alone (Lane 1), t-RA (10^{-8} M) (Lane 2), cycloheximide (10 μg/ml, Lane 3), or actinomycin D (1 μg/ml, Lane 4). Following hybridization and washing, the membrane was autoradiographed for 48 h using an intensifying screen at —80°C. Small arrows, the positions of the 28S and 18S ribosomal RNA bands; large arrow, RAR-β-specific bands. B, a photograph of the ethidium bromide-stained gel demonstrates RNA loading per well.

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Treatment with t-RA (10^{-6} M) increased expression of RAR-\(\alpha\), \(\beta\), and \(\gamma\) in malignant 11701 cells.

To more sensitively examine the expression of RAR-\(\beta\) in normal HBE cells and premalignant BEAS-2B and 1799 cells, RT-PCR analysis of RAR-\(\beta_2\) expression was performed. The RAR-\(\beta_2\) isoform, along with \(\beta_4\) and \(\beta_5\), is normally present in lung tissue (35). Following transfer to a nylon membrane, the RT-PCR products hybridized to a radiolabeled RAR-\(\beta\) cDNA (Fig. 4) but not to RAR-\(\gamma\) (data not shown), confirming that the visualized bands were RAR-\(\beta\). Semi-quantitative analysis of RAR-\(\beta_2\) and GAPDH expression was performed to determine fold-induction of RAR-\(\beta_2\) expression following t-RA treatment. Serial dilutions of the cDNAs revealed that, for all of the samples, PCR analysis was within the linear range for RAR-\(\beta_2\) and GAPDH using cDNAs diluted 1:4 and 1:256, respectively. After correcting for differences in cDNA loading based on GAPDH intensity, these studies showed that t-RA treatment increased RAR-\(\beta_2\) expression in normal HBE and premalignant BEAS-2B cells 8.9- and 7.1-fold, respectively (Fig. 4). t-RA treatment did not appreciably change RAR-\(\beta_2\) expression in premalignant 1799 cells.

The change in basal RAR-\(\beta\) expression observed during HBE carcinogenesis suggests that transcriptional or posttranscriptional controls of RAR-\(\beta\) expression are altered in this carcinogenesis model. To examine this possibility, malignant 11701 cells were treated with either cycloheximide or actinomycin D for 6 h, and RAR-\(\beta\) expression was determined by Northern analysis (Fig. 5). Treatment with actinomycin D reduced RAR-\(\beta\) expression to undetectable levels, suggesting that transcriptional mechanisms contribute to the increased RAR-\(\beta\)-levels in untreated malignant 11701 cells. To further examine this question, transient transfections were performed on normal, premalignant, and malignant HBE cells using reporter plasmids containing segments of the RAR-\(\beta\) gene promoter. -60 RAR-\(\beta\)-LUC contains the promoter region from -60 to +160, which includes the DR5 RARE from -59 to -33 (31). -1470 RAR-\(\beta\)-LUC contains additional 5' promoter regions from -1470 to +160. These experiments showed that baseline reporter plasmid transcriptional activity was higher in untreated malignant 11701 cells than in untreated normal HBE or premalignant 1799 cells (Fig. 6). In untreated malignant 11701 cells, -1470 RAR-\(\beta\)-LUC activity was higher than that of -60 RAR-\(\beta\)-LUC, suggesting that RAR-\(\beta\) promoter sequences 5' of the DR5 RARE contribute to RAR-\(\beta\) expression in these cells. Furthermore, in malignant 11701 cells, t-RA treatment increased luciferase activity from either reporter plasmid (Fig. 6) demonstrating that transcriptional mechanisms contribute to t-RA-induced RAR-\(\beta\) expression in these cells. These results support the hypothesis that transcriptional mechanisms contribute to the increases in baseline and t-RA-induced levels of RAR-\(\beta\)-expression observed in malignant 11701 cells.

Retinoid Receptor Function Remains Intact. Prior studies have shown that t-RA binds RARs and activates the transcriptional properties of RAR:RXR heterodimers (21). Following ligand binding, RAR:RXR heterodimers activate DR2 and DR5 RAREs within gene promoters (16–18). We investigated whether these functional properties of retinoid receptors are altered in this carcinogenesis model, possibly contributing to the t-RA refractoriness observed in the malignant 11701 cells. Gel shift and transient transfection assays were performed to examine the ability of these cells to bind a DR5 RARE and activate transcription of a luciferase reporter gene under the control of a DR5 RARE oligomer containing the DR5 RARE from -59 to -33, along with -1 and -33, -5 normally present in lung tissue (35). Following transfection, the cells were treated with or without t-RA (10^{-6} M) for 24 h and harvested for luciferase assays. Results represent the means and SDs of results from five identical transfections. Variations in luciferase activities attributable to differences in transfection efficiencies between cell lines were corrected by comparing \(\beta\)-galactosidase activities of cells transfected with a \(\beta\)-galactosidase expression vector. NHBE, normal HBE cells.

![Fig. 6. Transient transfection assays were performed on the indicated cell lines using luciferase reporter plasmids containing portions of the RAR-\(\beta\) gene promoter from -1470 to +160 (-1470RAR-\(\beta\)-LUC) or from -60 to +160 (-60RAR-\(\beta\)-LUC), which contain the DR5 RARE from -59 to -33. Following transfection, the cells were treated with or without t-RA (10^{-6} M) for 24 h and harvested for luciferase assays. Results represent the means and SDs of results from five identical transfections. Variations in luciferase activities attributable to differences in transfection efficiencies between cell lines were corrected by comparing \(\beta\)-galactosidase activities of cells transfected with a \(\beta\)-galactosidase expression vector. NHBE, normal HBE cells.](image-url)
control of a DR5 RARE. Gel shift studies revealed that, in nuclear lysates from normal HBE and premalignant 1799 cells, there was minimal DR5 binding activity (Fig. 7). In contrast, DR5-binding activity was detected in nuclear lysates from both untreated and t-RA-treated malignant 11701 cells. Binding to the radioactive probe by nuclear lysates from malignant 11701 cells could be competed by prior incubation with a nonradioactive wild-type DR5 RARE but not by a nonradioactive mutated DR5 RARE, demonstrating that binding was both protein and DNA sequence specific. These studies show that, in this carcinogenesis model, DR5 RARE binding activity increased during HBE carcinogenesis.

To examine the transcriptional activation properties of retinoid receptors in these cells, transient transfection assays were performed. Reporter plasmids were used that contain a DR5 response element under the control of either a TK (RAR-TK-LUC) or ΔMMTV (RAR-ΔM-LUC) heterologous promoter. Compared to normal and premalignant HBE cells, untreated malignant 11701 cells contained increased basal receptor transcriptional activity (Fig. 8). Treatment with t-RA activated DR5 transcriptional activity in normal, premalignant 1799, and malignant 11701 cells (Fig. 8). Together with the above-mentioned gel shift assays and transient transfections with reporter plasmids containing portions of the RAR-β promoter, these studies demonstrate that retinoid receptor activity increased during malignant transformation of HBE cells in this lung carcinogenesis model.

**NSCLCs Are Heterogeneous in RAR-β Expression.** These studies demonstrate that, in this model, lung carcinogenesis was associated with an increase in RAR-β expression. To examine whether RAR-β expression in this carcinogenesis model represents a subset of NSCLCs in patients, we investigated RAR-β expression in 10 NSCLC biopsies. RNA in situ hybridization was performed using digoxigenin-labeled anti-sense retinoid receptor cRNA probes. Sense probes were used as a negative control to confirm that binding is specific to RAR-β. Of the 10 tumors, 5 were positive for RAR-β expression and 5 were negative. Tumors positive and negative for RAR-β expression are shown in Fig. 9, a and b, respectively. RNA in situ hybridization to RXR-α was positive in all 10 tumors (data not shown), confirming that the RNA was intact in all samples examined. These studies reveal that RAR-β is expressed in a subset of NSCLCs in patients, which reflects the RAR-β expression observed in the carcinogenesis model.

**DISCUSSION**

This study involved the examination of a carcinogenesis model in which the premalignant and malignant cells evolved from the same precursor cell. This precursor cell, BEAS-2B, underwent a series of steps required to proceed from an immortalized HBE cell to a fully malignant cell. These steps include passage through the xenograft model (1799), exposure to CSC (1198), and transformation from an...
immortalized, premalignant state to a fully malignant state (1170L). Although viral immortalization does not reproduce the same molecular events that occur in premalignant, dysplastic HBE cells in patients, T antigen-induced immortalization induces loss of normal growth controls, including cellular senescence, which mimics the growth advantage observed in dysplastic cells. The studies performed here demonstrate that the growth-inhibitory effects of t-RA decreased as the HBE cells became progressively more malignant. Other studies have shown that treatment of primate tracheobronchial epithelial cells with retinol (8) and of HBE cells with t-RA (7) induced growth inhibition. Similar to our results, prior studies have shown that Northern analysis of normal HBE cells and BEAS-2B cells did not detect RAR-β mRNA (24, 36), and RT-PCR analysis detected increased RAR-β mRNA in normal HBE cells following t-RA treatment (7). Corroborating our observations in NSCLC biopsy specimens, baseline RAR-β was detected in some NSCLC cell lines but not in others (7, 36–38). Treatment with t-RA increased RAR-β expression in some NSCLC cell lines (as we found in malignant 1170L cells) but not in others (37, 38). The reasons for this variability in baseline and t-RA-induced RAR-β expression in NSCLC cells are not known. Dysfunctional retinoid receptors that cannot activate the DR5 RARE in the RAR-β gene promoter are one possibility. Defects in retinoid receptor function were found in t-RA-refractory HL-60 leukemia cells and NT2/D1 teratocarcinoma cells that expressed aberrant RAR-α and RAR-γ, respectively (39, 40). However, this was not the case in t-RA-refractory malignant 1170L cells that activated DR5 RAREs in transient transfection assays, demonstrating that RAR:RXR heterodimers are functional in these cells. Prior work suggests that cofactors necessary for RAR-β expression other than RAR:RXR heterodimers are missing in some lung cancer cell lines (38, 41).

There was no evidence for missing cofactors in malignant 1170L cells, possibly reflecting the heterogeneous population of cells in primary HBE cultures.

Similar to our results, prior studies have shown that Northern analysis of normal HBE cells and BEAS-2B cells did not detect RAR-β mRNA (24, 36), and RT-PCR analysis detected increased RAR-β mRNA in normal HBE cells following t-RA treatment (7). Corroborating our observations in NSCLC biopsy specimens, baseline RAR-β was detected in some NSCLC cell lines but not in others (7, 36–38). Treatment with t-RA increased RAR-β expression in some NSCLC cell lines (as we found in malignant 1170L cells) but not in others (37, 38). The reasons for this variability in baseline and t-RA-induced RAR-β expression in NSCLC cells are not known. Dysfunctional retinoid receptors that cannot activate the DR5 RARE in the RAR-β gene promoter are one possibility. Defects in retinoid receptor function were found in t-RA-refractory HL-60 leukemia cells and NT2/D1 teratocarcinoma cells that expressed aberrant RAR-α and RAR-γ, respectively (39, 40). However, this was not the case in t-RA-refractory malignant 1170L cells that activated DR5 RAREs in transient transfection assays, demonstrating that RAR:RXR heterodimers are functional in these cells. Prior work suggests that cofactors necessary for RAR-β expression other than RAR:RXR heterodimers are missing in some lung cancer cell lines (38, 41). There was no evidence for missing cofactors in malignant 1170L cells, which expressed baseline RAR-β and increased RAR-β expression following t-RA treatment. The elevated basal DR5 RARE transcriptional activity in malignant 1170L cells may reflect the presence of a stimulatory cofactor that is not present in normal or premalignant cells.

Previously, retinoid receptor activation inhibited the growth of HL-60 cells (42). Despite their intact function, retinoid receptor activation induced by t-RA treatment in malignant 1170L cells did not suppress cell growth. These findings demonstrate a block in retinoid signaling. This block may be due to SV40 T antigen. T antigen binds and inactivates important growth-inhibitory proteins such as p53 and retinoblastoma protein (43). Growth-inhibitory pathways known to be activated by retinoid treatment include transforming growth factor β (44–46), which induces growth arrest at the G1-S cell cycle boundary through activation of cyclin-dependent kinase inhibitors (47–50). The role of these pathways in the growth-inhibitory effects of retinoids on normal HBE cells and of altered signaling through these pathways in lung carcinogenesis will be the focus of future studies.

REFERENCES


Fig. 9. RNA in situ hybridization was performed by using digoxigenin-labeled anti-sense cRNA probes to examine RAR-β expression in tumor biopsy specimens. The staining represents the detection of RAR-β mRNA by an anti-digoxigenin antibody. Tumors positive (A) and negative (B) for RAR-β expression are illustrated. Specificity for RAR-β was confirmed by showing that tumors staining positively for RAR-β did not hybridize to the digoxigenin-labeled sense RAR-β probe.


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