A Novel Retinoic Acid Receptor β Isoform and Retinoid Resistance in Lung Carcinogenesis

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Background: We previously reported that all-trans-retinoic acid (RA) treatment can prevent in vitro transformation of immortalized human bronchial epithelial (HBE) cells. Methods: To determine whether methylation inhibits RARβ expression in HBE cells, we used sodium bisulfite sequencing to compare RARβ P2 promoter methylation patterns in RA-sensitive (BEAS-2B) and RA-resistant (BEAS-2B-R1) HBE cells. Immunoblotting was used to assess induction of the RARβ, placental transforming growth factor β (PTGF-β), Fos-related antigen 1 (Fra-1), and transglutaminase II (TGase II) proteins by RA following treatment with azacitidine, a DNA demethylating agent. The expression, transcriptional activity, and growth suppressive activity of RARβ1', a novel RAR isoform, were evaluated in lung cancer cells transfected with RARβ1', and expression was also studied in paired normal lung tissues and lung tumors. All statistical tests were twosided. Results: Hypermethylation was observed in the 3′ region of the RARβ P2 promoter of BEAS-2B-R1 but not BEAS-2B cells. Azacitidine treatment of BEAS-2B-R1 cells restored RA-inducible RARβ2 and PTGF-β expression but not that of RARβ1', Fra-1, or TGase II. RARβ1' expression was repressed in RA-resistant BEAS-2B-R1 cells and in lung cancers, compared with adjacent normal lung tissues. BEAS-2B-R1 cells transiently transfected with RARβ1' had increased RA-dependent activation of a retinoic acid receptor element (RARE)-containing reporter plasmid compared with vector control (mean = 3.2, 95% confidence interval [CI] = 3.1 to 3.3 versus mean = 1.4, 95% CI = 1.3 to 1.5; P<.001). In H358 lung cancer cells transiently transfected with RARβ1', RA treatment restored target gene expression compared with that in vector-transfected cells and suppressed cell growth compared with that in untreated cells (4 μM; treated mean = 0.49 versus untreated mean = 1.0, difference = 0.51, 95% CI = 0.35 to 0.67, P = .003; 8 μM: treated mean = 0.50 versus untreated mean = 1.0, difference = 0.50, 95% CI = 0.26 to 0.74, P = .015). Conclusion: Restoration of RARβ1' expression may overcome retinoid resistance in lung carcinogenesis. [J Natl Cancer Inst 2005;97:1645–51]

Vitamin A deficiency has been reported to cause preneoplastic changes in the bronchial epithelium and to be associated with an increased risk of lung cancer (1,2). Although results from initial clinical trials support a role for retinoids (natural and synthetic derivatives of vitamin A) in the chemoprevention of aerodigestive tract cancers (3,4), data from randomized trials suggest that carotenoids or retinoids are not effective for lung cancer chemoprevention in the general population, although a benefit may exist for nonsmokers (5–8). Clinical resistance to classical retinoids has been seen in lung cancer and is due, at least in part, to loss of retinoic acid receptors β (RARβ) expression during lung carcinogenesis (9–18). Loss of RARβ expression is often caused by genomic loss or epigenetic silencing due to genomic methylation or histone deacetylation (11,19). Hypermethylation of the RARβ promoter is an early event in lung carcinogenesis and is one of the most frequent methylation defects detected in the histopathologically normal bronchial epithelium of heavy smokers (20). However, whether loss of RARβ2 expression due to RARβ promoter methylation explains clinical resistance to RA is unknown.

We previously reported that all-trans-retinoic acid (RA) treatment can prevent in vitro transformation of immortalized human bronchial epithelial (HBE) cells and that this chemoprevention involves the proteasome-dependent degradation of G1 cyclins (21,22). In a later study, we derived an RA-resistant HBE cell line (BEAS-2B-R1) by selecting RA-sensitive (BEAS-2B) HBE cells with increasing concentrations of RA. Unlike parental BEAS-2B cells, BEAS-2B-R1 cells treated with RA do not degrade cyclin D1 or cyclin E or undergo G1 arrest (23). RA treatment does not induce RARβ or RA target gene expression in these cells (23,24).

In the present study, we used BEAS-2B-R1 RA-resistant HBE cells to investigate mechanisms responsible for RARβ silencing. We studied the methylation status of the RARβ P2 promoter, which is hypermethylated in certain lung cancer cells that lack RARβ2 expression (11). We then investigated whether treatment with the DNA demethylating agent azacitidine (also known as 5-azacytidine), alone or in combination with RA treatment, would restore RARβ expression and thereby induce the
expression of previously identified retinoid target genes in BEAS-2B-R1 cells. Also, we investigated the structure, expression, and function of RARβ1’, a previously unrecognized RARβ isoform, in lung cancer cells and in lung tumors and adjacent normal lung tissues.

**MATERIALS AND METHODS**

**Cell Culture**

BEAS-2B and BEAS-2B-R1 cells were cultured in serum-free LHC-9 media in the dark at 37 °C with 5% CO₂ in a humidified incubator as previously described (23, 24). H358, H441, and A549 lung cancer cell lines and COS African green monkey kidney fibroblast cells were obtained and passaged as recommended by American Type Culture Collection (Manassas, VA). H358, H441, and COS cell lines were cultured in RPMI media supplemented with 100 IU/mL of penicillin and 100 μg/mL of streptomycin. A549 cells were cultured in Ham’s F12 media containing l-glutamine and 10% fetal bovine serum (FBS) supplemented with 100 IU/mL of penicillin and 100 μg/mL of streptomycin.

**Compounds and Treatments**

RA and trichostatin A (Sigma Chemicals, St. Louis, MO) were prepared as 10 mM stock solutions in dimethyl sulfoxide (DMSO) and stored in liquid nitrogen. Azacitidine was obtained from the National Cancer Institute (Bethesda, MD) and dissolved in phosphate-buffered saline, pH 6.3. Trichostatin A treatment was performed at a dosage of 200 nM for 48 hours. Azacitidine treatment was performed at a dosage of 1 μM for 48 hours.

**Tumor Acquisition**

Non-small-cell lung tumors and paired normal lung tissues were acquired as part of a Dartmouth-Hitchcock Medical Center Institutional Review Board (IRB)–approved tumor acquisition protocol. The banked tissues do not carry patient identifiers and are exempt from IRB review under the Code of Federal Regulation Title 45, part 46.

**Genomic DNA Methylation Sequencing**

Genomic DNA was isolated from BEAS-2B and BEAS-2B-R1 cells using TRI Reagent (Molecular Research Center, Cincinnati, OH) per the manufacturer’s protocol and was modified with sodium bisulfite, which converts unmethylated cytosines to uracil and amplified by polymerase chain reaction (PCR) using established techniques and primers that are specific for bisulfite-modified DNA: 5'-AAGTGAGTTGTTAGGATGGAGGG-3' (sense) and 5'-CCTATAATATCCA AATAATCATTTACC-3' (antisense) (11). PCR products were then independently ligated into the TOPO-TA cloning vector (Invitrogen, Carlsbad, CA) using the manufacturer’s protocol, transfected into One Shot competent cells (Invitrogen) per the manufacturer’s protocol, selected on ampicillin-containing agarose plates, and individually expanded for analyses. Eight independent clones from each reaction were sequenced using established techniques (25) to assess the methylation frequency of each CG doublet in the RARβ P2 promoter, which has been identified as hypermethylated in lung cancer cells that lack RARβ2 expression. The genomic sequence of the region studied (nucleotides +106 to +250) is as follows: 5'-CGAAGACGGAGCATCCGAG CAGGTTTTGCTCG GCAACCGTGGGGTAGTCGGAAAGCATTGCCGAAGGGGTT TTTTGCAAGCTATTCTTGGAAAGAAGACCTGGATCTTT TCTGGGAAACCCCGCCCCGGCGTGGATGGCC-3'.

**Protein Extraction and Immunoblot Analysis**

Immunoblot analyses of extracts from the described experiments were performed using previously optimized techniques (23). In brief, protein extracts were obtained by lysing cells in radioimmunoprecipitation assay buffer (10 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1 mM NaF, and 1 mM sodium orthovanadate) followed by centrifugation at 10000g for 15 minutes at 4 °C. The protein concentration of the supernatant was assayed using the Bradford method (Bio-Rad, Hercules, CA). Proteins were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes for immunoblot analyses as previously described (23).

Primary antibodies to RARβ (rabbit polyclonal, 1:500), actin (goat polyclonal, 1:1000), placental transforming growth factor β (PTGF-β; goat polyclonal, 1:500), and Fos-related antigen 1 (Fra-1; rabbit polyclonal, 1:500) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The transglutaminase II ( TGase II; mouse monoclonal, 1:500) antibody was purchased from NeoMarkers (Fremont, CA). Species-appropriate mouse or rabbit (Amersham Biosciences Corporation, Piscataway, NJ) or goat (Santa Cruz Biotechnology) horseradish peroxidase–conjugated secondary antibodies were applied at a dilution of 1:3000 and binding was visualized using chemiluminescence (ECL Plus; Amersham Biosciences Corporation). Blocking peptide studies were performed for RARβ immunoblots of normal lung protein using a peptide specific to the C terminus of RARβ, per the manufacturer’s protocol (Santa Cruz Biotechnology). The specificity of the RARβ1’ band was confirmed by identifying the complete loss of the RARβ1’ band in an immunoblot probed with the blocking peptide with the presence of the band in an immunoblot probed without blocking peptide performed simultaneously under otherwise identical conditions.

**Reverse Transcription–PCR Amplification of RARβ Isoforms**

Total RNA was extracted from BEAS-2B cells, normal lung tissues, and malignant lung tissues using TRI Reagent (Molecular Research Center) per the manufacturer’s protocol. RARβ isoforms were PCR amplified with cDNA generated from 1 μg of total RNA from each sample using the First-Strand cDNA synthesis system (Invitrogen) per the manufacturer’s protocol. Annealing temperatures for the reactions were optimized for each primer pair. First-round amplification was performed for 35 cycles. Nested amplification was performed for 10 cycles using 5 μL of the first-round PCR product. Primers used to amplify RARβ isoforms were as follows: RARβ1, 5'-TGAAGCTCCAGGTGC TACTG-3' (sense) and 5'-TGTTGTTGACTGCAACTT-CA-3' (antisense); RARβ2 and RARβ4, 5'-AACGGAAGCGATCC GAGCAG-3' (sense) and 5'-ATTGGTCTGGCAGACAGAAG CA-3' (antisense); and RARβ1', 5'-ATGAGGAATGAAAGCTGA-3'.
GTAGA-3' (sense) and 5'-ATTTGTCTGAGCAGACGAA GCA-3' (antisense) (26).

5' Rapid Amplification of cDNA Ends PCR

RACE-Ready cDNA from human lung was purchased from Invitrogen. Two primers complementary to the fourth exon of RARβ were designed for nested 5' rapid amplification of cDNA ends (5'-RACE) PCR amplification of RARβ isoforms: first-round primer 5'-ACTTGGTGG CCAGTCACTGATTTTG-3' and second-round primer 5'-CTTCCGACACGAAGCAGGG TTTGTA-3'. RACE products were cloned using the TOPO-TA vector (Invitrogen) and were sequenced as described previously (25). One of the products identified was a novel RARβ isoform, designated RARβ1', which was registered as GenBank accession number DQ083391.

Luciferase Assay

The cloned products from the 5'-RACE PCR amplification were digested with EcoRI and XhoI restriction endonucleases (New England Biolabs, Beverly, MA) to release the exon 4 segment. An existing RARβ-pSG5 construct containing the full length RARβ cDNA in the pSG5 expression vector was also digested with appropriate restriction endonucleases. The size-fractionated fragments were gel purified and the 5' portion of RARβ' was ligated into a truncated 3' portion of the RARβ-pSG5 segment at exon 4 using T4 DNA ligase (Invitrogen) forming an RARβ'-pSG5 expression construct. Sequencing was performed on separate clones to confirm the presence of the desired construct. COS kidney fibroblast cells and BEAS-2B-R1 cells were both cotransfected with the RARβ1' expression plasmid, the RARβ2 expression plasmid, or empty vector, and with a retinoic acid response element–thymidine kinase (RARE-TK) fusion gene construct containing a firefly luciferase reporter gene, and a TK–Renilla luciferase reporter gene construct (as a control for transfection efficiency) using the FuGENE transfection system (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s protocol. All transfections were performed in triplicate. BEAS-2B-R1 cells were cultured without exogenous RA for 7 days before transfection. Cells were then treated with RA at various doses or with vehicle (DMSO) for 3 hours. Luciferase activity was determined using the Dual Luciferase Reporter System (Promega, Madison, WI). Firefly luciferase measurements were normalized to Renilla luciferase measurements as a control for transfection efficiency.

RARβ1' Transfection and Proliferation Assays

To investigate the function of RARβ1', we cotransfected H358 lung cancer cells with a puromycin resistance expression vector (p-Pur, BD Biosciences, Mountain View, CA) and with either the RARβ1' expression vector or an insertless vector (pSG5). Cells were then treated with 2 μg/mL of puromycin for 24 hours to select against untransfected H358 cells. After 7 days, cells were plated in 96-well plates at a density of 3 × 10^3 cells per well for proliferation assays. Cellular proliferation was measured using the CellTiter-Glo assay kit (Promega). Assays were performed in triplicate. Basal luminescence activity and an ATP standard curve were each determined. Cells were then treated with either 4 μM of RA or with DMSO (vehicle) for 24 hours. Luminescence activity and an ATP standard curve were determined each day that the assay was performed. Growth rates were calculated by subtracting the basal ATP content from ATP content measured after RA or vehicle treatments, as described previously (25). Growth rates were normalized to that of vehicle-treated cells.

Statistical Methods

Differences between groups of continuous variables were assessed using the two-sample t test. P<.05 (two-sided) was considered statistically significant.

RESULTS

RARβ P2 Promoter Methylation and RA-Induced Expression of Target Genes

To determine the methylation status of the RARβ P2 promoter was different in RA-sensitive (BEAS-2B) and RA-resistant (BEAS-2B-R1) HBE cells, we assayed the CG-methylation pattern of the P2 promoter in each cell line using bisulfite modification and PCR sequencing. Independent determinations of the RARβ P2 promoter methylation pattern in BEAS-2B and BEAS-2B-R1 cells revealed that most CG doublets were methylated (in ≥50% of samples) in both cell lines. The CG doublets at the 3' region of this promoter were more frequently methylated in BEAS-2B-R1 than in BEAS-2B cells (Fig. 1).

We previously showed that BEAS-2B-R1 cells do not express RARβ after RA treatment, whereas BEAS-2B cells do.

Fig. 1. DNA methylation patterns of the RARβ P2 promoter in BEAS-2B and BEAS-2B-R1 cells. Genomic DNA purified from BEAS-2B and BEAS-2B-R1 cells was subjected to sodium bisulfite modification and polymerase chain reaction (PCR) amplification. PCR products were cloned and eight individual clones from each reaction were sequenced. A) Relative positions in the promoter of methylated and unmethylated CpG islands in each cell line. The genomic DNA sequence of this region is listed in “Materials and Methods.” Numbers listed indicate nucleotide positions relative to the initiation of RARβ2 transcription (solid circles, ≥50% cytosine methylation; open circles, <50% cytosine methylation). B) Percentages of clones (y-axis) that demonstrated methylated cytosines at the nucleotide sites specified (x-axis). Most P2 promoter residues were methylated in both BEAS-2B (gray bars) and BEAS-2B R1 (black bars) cells. By contrast, CpG islands at the 3' region of the P2 promoter were more often methylated in BEAS-2B-R1 than in BEAS-2B cells.
To determine if RA-inducible RARβ expression could be restored to BEAS-2B-R1 cells by demethylation, BEAS-2B-R1 cells were treated with azacitidine, a demethylating agent, or with trichostatin A, a histone deacetylase inhibitor. Pretreatment with 1 μM azacitidine for 48 hours restored RA-inducible RARβ expression (Fig. 2), whereas pretreatment with 200 nM trichostatin A for 48 hours did not (data not shown). A second RARβ immunoreactive protein that migrated at 47 kDa was identified in BEAS-2B cells but was repressed in BEAS-2B-R1 cells (Fig. 2). The expression of this protein was not induced by RA treatment, azacitidine treatment, or combined RA and azacitidine treatments. The expression of the other RARβ and RXR isoforms was similar in BEAS-2B-R1 and BEAS-2B cells (data not shown).

Protein expression of several RA target genes that were previously identified as being induced by RA in BEAS-2B cells but not in BEAS-2B-R1 cells were assessed after treatment with various combinations of RA and azacitidine or with vehicle. Azacitidine treatment restored the RA induction of PTGFβ expression to BEAS-2B-R1 cells but not RA-inducible expression of Tgase II or Fra-1 (Fig. 3).

**Identification of a Novel RARβ Isoform**

To identify the second RARβ protein repressed in BEAS-2B-R1 cells, we performed PCR analyses of RARβ1, RARβ2, and RARβ4 using published techniques (26). However, these RARβ isoforms were not amplified from BEAS-2B cDNA. To investigate the possible existence of a novel RARβ isoform, we performed 5′ RACE–PCR analysis using RNA derived from normal human lung tissue. The first round of PCR amplified several dominant species (Fig. 4, A), of which the largest species represented splice variants of RARβ2 and the smallest represented a novel isoform of RARβ present in normal human lung. Reverse transcription–PCR analysis confirmed the expression of this isoform in BEAS-2B cells (data not shown). This isoform contains an upstream exon spliced into exon 3 of RARβ with an in-frame start codon. This previously unrecognized RARβ isoform was designated as RARβ1′. The genomic structure of this isoform is shown in Fig. 4, B.

**Transient Transfection of BEAS-2B-R1 cells with RARβ1′**

Translation of RARβ4 or RARβ5 arising from a downstream start codon produces a low-molecular-weight RARβ protein that inhibits RA-induced transactivation of an RARE-containing reporter construct (27,28). To determine whether the small novel RARβ1′ also inhibits RA-inducible transactivation of RARE, we cotransfected BEAS-2B-R1 and COS cells with RARβ1′, RARβ2, or empty vector and with a RARE-containing reporter gene construct. We then treated transfected cells with RA or DMSO (vehicle control). Cells transfected with RARβ1′ or RARβ2 had higher RA-induced activation of the RARE reporter construct transfected cells (mean = 3.2, 95% confidence interval [CI] = 3.1 to 3.3, P <.001, and mean = 2.8, 95% CI = 2.6 to 3.0, P <.001, respectively) than vector-transfected cells (mean = 1.4, 95% CI = 1.3 to 1.5) (Fig. 5). A similar pattern of induction was observed in COS cells, although the basal reporter gene activity was not increased relative to vector transfectants in these cells, which

![Fig. 2](image1.png)  
**Fig. 2.** Immunoblot analysis of RARβ protein expression in BEAS-2B and BEAS-2B-R1 cells following azacitidine (5-AZA) and/or all-trans-retinoic acid (RA) treatments. BEAS-2B and BEAS-2B-R1 cells were treated with 1 μM of azacitidine for 48 hours followed by 4 μM of RA for 24 hours or treated with 5-AZA or RA alone. A novel RARβ immunoreactive protein, RARβ1′, with a molecular mass of 47 kDa was detected in BEAS-2B cells. Molecular mass markers are shown at right. RARβ2 is approximately 55 kDa (doublet). Actin was used as a control for protein loading and transfer.

![Fig. 3](image2.png)  
**Fig. 3.** Immunoblot analysis of all-trans-retinoic acid (RA)-target gene expression before and after RA, azacitidine (5-AZA), or combined azacitidine and RA treatments of BEAS-2B-R1 cells. BEAS-2B-R1 cells were treated with 1 μM of azacitidine or vehicle (dimethyl sulfoxide) for 48 hours followed by treatment with 4 μM RA or vehicle for 24 hours. Target genes previously shown to be transcriptionally repressed in BEAS-2B-R1 cells were assessed by immunoblot analyses. Analysis of RARβ2, placental transforming growth factor β (PTGFβ), Fos-related antigen 1 (Fra-1), and transglutaminase II (Tgase II) were performed in three independent experiments. Actin was used as a control for protein loading and transfer.

![Fig. 4](image3.png)  
**Fig. 4.** Expression of RARβ1′ in RNA derived form normal human lung tissue. A) 5′ rapid amplification of cDNA ends-labeled cDNA was subjected to polymerase chain reaction (PCR) amplification using primers complementary to exon 4 of human RARβ. DNA sequence analyses of separate clones determined that the indicated largest species represent splice variants of RARβ2 and the smallest species (680 bp) revealed a novel isoform containing an in-frame AUG start codon. The 100-bp size ladder is shown at right. B) Genomic structure of the 5′ regions of RARβ2, RARβ4, and the novel isoform, RARβ1′. Arrows mark the initiation of transcription.
were cultured without exogenous RA (data not shown), unlike BEAS-2B-R1 cells, which were grown in media supplemented with RA (23).

### RARβ1′ Expression in Lung Cancer Cell Lines and in Paired Normal and Malignant Lung Tissues

The relative expression of RARβ1′ in lung cancers was sought using lung cancer cell lines as well as paired normal and malignant lung tissues. The RA response of several lung cancer cell lines was assessed by determining growth response following 4 μM RA treatment for 72 hours. The patterns of RARβ1′ and RARβ2 expression in these cells, as determined by immunoblot analyses, were compared with those in RA-responsive BEAS-2B HBE cells. RARβ2 protein was abundantly expressed in A549 cells and barely detected in untreated H358 and H441 cells (Fig. 6, A). By contrast, RARβ1′ protein expression was substantially lower in all three lung cancer cell lines than in BEAS-2B HBE cells (Fig. 6, A). Furthermore, when three sets of paired malignant and normal tissues were compared, RARβ1′ protein expression in de novo lung cancers was consistently lower than that of normal lung tissues (Fig. 6, B). The identity of this protein was confirmed with a blocking peptide specific for the C terminus of RARβ (data not shown). Another set of paired malignant and normal lung tissue with available RNA was subjected to reverse transcription–PCR analysis, from which RARβ1′ RNA was detected in the normal lung tissue but not in the paired malignant tissue (data not shown).

#### Growth Suppression in RA-resistant Lung Cancer Cells Transfected with RARβ1′

We then studied the functional consequences of restoring RARβ1′ expression in lung cancer cells. RA treatment slightly increased expression of RARβ2 and strongly increased that of the RA target gene PTGF-β in H358 cells (Fig. 7, A). To determine whether exogenous RARβ1′ expression in RA-resistant
cells would restore the growth-suppressive effects of RA treatment, we stably transfected H358 cells with either RARβ1’-pSG5 or an empty expression vector (pSG5) as a control. Transfected cells were then treated with RA or vehicle for 24 hours. RA treatment at 4 and 8 μM dosages had no effect on the growth of control cells (Fig. 7, B). In RARβ1’ transfected cells, by contrast, RA treatment suppressed growth (4 μM; treated mean = 0.49 versus untreated mean = 1.0, difference = 0.51, 95% CI on the difference = 0.35 to 0.67, P = .003; 8 μM: treated mean = 0.50 versus untreated mean = 1.0, difference = 0.50, 95% CI on the difference = 0.26 to 0.74, P = .015; Fig. 7, B). Engineered expression of RARβ1’ in H358 cells was also associated with restored RA induction of the RA target gene, TGase II (Fig. 7, C). By image quantification, TGase II expression was 2.4-fold greater in RA-treated RARβ1’-transfected cells than in RA-treated control vector-transfected cells.

**Discussion**

By investigating an RA-resistant HBE cell line BEAS-2B-R1 (derived by treating with increasing concentrations of RA), we have identified the existence of a novel RARβ isoform, RARβ1’. This isoform was expressed in normal lung and in RA-sensitive BEAS-2B cells but not in RA-resistant BEAS-2B-R1 cells, lung cancer cell lines, or clinical lung cancers. Transfection of this isoform into a RA-resistant lung cancer cell line, H358, that expresses low levels of RARβ2 conferred RA target gene induction and growth suppression following RA treatment. Thus, RARβ1’ has distinct biologic properties compared with RARβ2 and appears to function as a tumor suppressor in lung carcinogenesis.

Repression of RARβ is often observed in lung carcinogenesis and likely confers resistance to the growth-suppressive effects of vitamin A and its derivatives (9–20). In this study, a specific domain of the RARβ P2 promoter was found to be hypermethylated in RA-resistant HBE cells that in turn led to RARβ silencing. This region of the P2 promoter appears to serve a key role in methylation-mediated silencing of RARβ in the lung. Consistent with this hypothesis, a murine model of tobacco-induced lung carcinogenesis highlighted a similar region of promoter methylation that conferred RARβ silencing (16).

In this study, we observed not only that RARβ2 and RARβ1’ have distinct functions but also that their expression is regulated differentially. Although neither RARβ2 nor RARβ1’ was expressed in RA-resistant BEAS-2B-R1 HBE cells, pretreatment with azacitidine restored only RA-dependent RARβ2 expression but not expression of RARβ1’. This increased RARβ2 expression was accompanied by increased expression of only one of the other RA target genes assayed, PTGF-β. Thus, we hypothesized that RARβ1’ is required for the efficient induction of those RA target genes that were not restored by azacitidine treatment alone or by combined RA and azacitidine treatments. In support of this view, cotransfection of BEAS-2B-R1 cells with RARβ1’ and an RA-responsive reporter gene enhanced retinoid trans-activation of the RARE reporter construct. Also, exogenous expression of RARβ1’ in the H358 RA-resistant lung cancer cell line, which only slightly increases expression of RARβ2 following RA treatment, led to RA-dependent growth suppression and RA target gene induction, whereas no growth suppression was observed in H358 cells after RA treatment. These results strongly suggest that RARβ1’ has a critical role in retinoid signaling in lung carcinogenesis. Furthermore, the observation that the expression of RARβ1’ was repressed in BEAS-2B-R1 HBE and lung cancer cell lines and in lung cancer samples (but not in paired normal lung tissue) suggests that deregulation of this previously unrecognized RARβ isoform is important for conferring RA resistance. Restored expression of RARβ1’ may only partially restore retinoid response. It is also possible that loss of RARβ1’ expression will be necessary but not sufficient for conferring retinoid resistance. Further understanding of the role of RARβ1’ in response to retinoids would be gained by selective knockout of RARβ1’.

Azacitidine treatment of RA-resistant HBE cells conferred RA-dependent induction of RARβ2 expression and the activation of specific RA target genes. The results indicate that combining retinoids with a DNA demethylating agent might restore RARβ2 expression to lung cancer cells (11, 29–31). However, for an epigenetic targeting strategy to restore the full induction of RARβ target genes, the basis for RARβ1’ repression needs to be determined. RARβ1’ transcription is likely initiated by a distinct promoter near the first exon of this isoform. We did not find a classical RARE sequence in the promoter (data not shown), which is consistent with the inability of RA to induce RARβ1’ expression (Fig. 2). Characterization of the RARβ1’ promoter, regions of genomic DNA methylation, and histone acetylation changes within this region are the subject of future work. Combined treatment with a DNA demethylating agent and a histone deacetylase inhibitor might prove useful for restoring the expression and function of RARβ1’. For the success of this and other targeted approaches in lung cancer therapy, proof-of-principle studies are needed that confirm whether pharmacologic mechanisms identified in vitro are also engaged in the clinical setting (25). In this regard, studies to investigate the benefits of combining retinoids with chromatin-modifying drugs should evaluate changes in specific retinoid receptor isoforms as well as their target genes that would directly confer retinoid biologic effects (24).

This study is limited in part due to the number of cell lines studied. Future work will seek to extend our findings by investigating the effects of RARβ1’ expression in more cancer cell lines. Restored expression of RARβ1’ may restore retinoid sensitivity only in certain cell contexts. Furthermore, selective knockdown of RARβ1’ will be necessary to determine whether loss of this species is sufficient to confer retinoid resistance.

Taken together, the data presented here directly implicate a critical role for RARβ1’ in mediating retinoid biologic effects in the lung and perhaps other organ sites. RARβ1’ itself could serve as a novel molecular pharmacologic target. The frequent repression of RARβ1’ in lung carcinogenesis underscores its likely important biologic or clinical role. RARβ1’ repression, despite RA treatment, offers a mechanistic explanation for clinical retinoid resistance that has been reported previously (7, 8). Identification of pharmacologic approaches that restore RARβ1’ expression would provide a basis for future retinoid-based combination strategies for lung cancer therapy or chemoprevention.

**References**


Notes

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