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Research Paper

9-cis Retinoic Acid Induces Insulin-like Growth Factor Binding Protein-3 through DR-8 Retinoic Acid Responsive Elements

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KEY WORDS

insulin-like growth factor, insulin-like growth factor binding protein-3, 9-cis retinoic acids, retinoic acid receptor-β, non-small cell lung cancer

ABBREVIATIONS

DR	direct repeat
IGF	insulin-like growth factor
IGFBP-3	insulin-like growth factor binding protein-3
NSCLC	non-small cell lung cancer
RARE	retinoic acid responsive element
RAR	retinoic acid receptor

ACKNOWLEDGEMENTS

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ABSTRACT

Retinoic acids, which have shown potential chemopreventive and therapeutic activities for several neoplastic diseases in vitro, modulate the growth-promoting and anti-apoptotic activities of insulin-like growth factors (IGFs), in part by influencing the expression of insulin-like growth factor binding protein-3 (IGFBP-3). This study sought to investigate the effect of 9-*cis* retinoic acid (9*c*RA) on the expression of IGFBP-3 and the underlying mechanisms involving retinoic acid receptor-β (RAR-β).The pharmacologic activity of 9*c*RA was characterized by monitoring target modulation as well as by evaluating the underlying mechanisms in NSCLC cells. Treatment of 9*c*RA inhibited proliferation of a part of NSCLC cell lines including H460 cells in clinically-achievable concentrations and induced IGFBP-3 expression in dose- and time-dependent manners. Transient transfection with a reporter constructs driven by the human IGFBP-3 gene promoter indicated that 9*c*RA induces gene expression via the -534 to -445 region (relative to translation start site) of the IGFBP-3 promoter. Unilateral deletion and site-directed mutagenesis identified a retinoic acid responsive element (RARE), a direct repeat of two GGGTCA-related hexanucleotides separated by just 8 bp (DR-8-type response element). A cotransfection assay with a RAR-β expression vector potentiated (and with *si*RNA for RAR-β, diminished) the effect of 9*c*RA on IGFBP-3 expression. IGFBP-3 gene expression by 9*c*RA is mediated by a distinct DR-8 RARE located in the proximal region of the IGFBP promoter and involves the RAR-β, a putative tumor suppressor in NSCLC.

INTRODUCTION

Retinoids are one of the most extensively studied agents in the secondary and tertiary chemoprevention of lung cancer. A common problem with these chemicals is pan-retinoid toxicity, yet numerous natural and synthetic retinoids—effective inhibitors of tumor cell growth in vitro and in vivo and selective for the different retinoid receptor isotopes—have been generated that circumvent pan-retinoid toxicity. The tumor-suppressive activity of selective retinoids and rexinoids have been established preclinically, and emerging clinical trials are supportive of the chemotherapeutic and chemopreventive potential of these compounds in multiple oncological indications, with reduced toxicity.[1]

One recent chemopreventive trial, which randomized former smokers to 13*c*RA plus α-tocopherol, 9*c*RA, or placebo in an attempt to reverse premalignant lesions with the desired biomarker, retinoic acid receptor-β (RAR-β), being upregulated in bronchial epithelium, showed that administration of 9*c*RA decreased the percentage of subjects with detectable squamous metaplasia in biopsy sites and increased RAR-β expression.[2] Although RAR-β is effectively upregulated by 9*c*RA in bronchial epithelium, data implicating maintenance of RAR-β intratumorly in NSCLC patients showed a poorer prognosis have cast RAR-β in a less favorable light.[1,3] Retinoid refractoriness, in terms of inducing RAR-β expression or apoptosis with treatment of retinoids, is attributable, at least partially, to aberrant promoter methylation and histone H3 acetylation. One study showed retinoid receptor function to be intact in the t-RA-refractory malignant HBE cell line, however, suggesting that the defect in retinoid signaling in this lung carcinogenesis model was not intrinsic to the retinoid receptors.[4] Such findings indicate the urgent need to discover a cellular mediator of chemoprevention of retinoic acids other than RAR-β.

Insulin-like growth factor (IGF) binding proteins (IGFBPs), composed of 216 to 289 amino acids and representing a major reservoir of circulating IGFs, mobilize IGFs to peripheral tissues and regulate their biologic activities.[5] IGFBP-1, -2 and -4 traverse the endothelial barrier and carry IGFs into peripheral tissues. Whereas IGFBP-3, the most abundant such binding protein in circulation, can reach 150 kD when bound to IGF-I, cannot penetrate endothelial cells, and has been implicated in regulating the content of

IGFs in circulation.[5,6] IGFBP-3 regulates IGF-1 bioactivity by sequestering IGF-1 away from its receptor in the extracellular milieu, thereby inhibiting the critical role of IGFs in cellular growth, differentiation, transformation, and apoptosis.[5,7,8] Besides its IGF-I-dependent function, IGFBP-3, like IGFBP-1 and IGFBP-5, has IGF-I-independent anti-proliferative and pro-apoptotic effects.[9]

The bioactivity of IGF system is modulated by retinoids, which not only decreases directly the level of IGF-1[10] but also increases IGFBP-3.[11,12] IGFBP-3, in turn, mediates a wide variety of growth suppression signals, including retinoic acid. Several findings further suggest that IGFBP-3 might be a good candidate as a surrogate marker of chemoprevention. These include a negative correlation between serum IGFBP-3 levels and various solid cancer risks;[13] an association between expression of IGFBP-3 and prognosis of stage I NSCLC patients;[14,15] and a protective role of IGFBP-3 against the effects of systemic IGF-I,[8] which has been implicated in the development of lung cancer. Thus, an urgent need exists for an effective modality inducing IGFBP-3 expression. The chemotherapeutic and chemopreventive potentials of retinoids and rexinoids might also originate, in part, from induction of IGFBP-3.[16] Yet the mechanism by which IGFBP-3 gene transcription is stimulated by retinoic acids has not been well-studied.

It is known that transcriptional activation by retinoids is mediated through two families of nuclear hormone receptors, the retinoic acid receptors, the RARs and the retinoid X receptors (RXRs). Three distinct RXR and RAR genes have been identified, and, of these nuclear hormone receptors, RAR-β is considered to be a tumor suppressor gene in NSCLC.[17-19] The retinoic acid receptors are ligand-dependent transcription factors that bind to *cis*-acting DNA sequences called retinoic acid responsive elements (RAREs) and retinoid X responsive elements (RXREs), located in the promoter region of their target genes.[20] In this study, we report that IGFBP-3 gene expression by 9cRA involves RAR-β and is mediated by a distinct DR-8 responsive element located in the proximal region of the IGFBP-3 promoter.

MATERIALS AND METHODS

Chemicals, cell culture and treatment. 9*c*RA, (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid (TTNPB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenbyl tetrazolium bromide (MTT), anti-β-actin antibody and other chemicals were obtained from the Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated. Anti-IGFBP-3, anti-RAR-β, and anti-RXR-β antibodies and *si*RNA for RAR-β were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Stealth™ RNAi Negative Control Duplexes, lipofectamine™ 2000 were purchased from Invitrogen (Carlsbad CA, USA). Human NSCLC cell lines used in this study (NCI-H596,- A549, -H460, -H1299 and COS7) were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS; Life Technologies, Inc., Gaithersburg, MD, USA). For treatment of each retinoic acid derivative, cells were maintained prior to harvest by splitting and incubation in RPMI-1640 medium supplemented with 2% FCS. To determine the effects of 9*c*RA on NSCLC cell proliferation, the NSCLC cell lines were plated at concentrations of 1×10^3 cells per well in 96-well plates. The next day, cells were treated with various titers of 9*c*RA and then incubated in 2% RPMI-1640 medium for the assay period. At the end of the assay period, the proliferation of treated cells was measured by using the MTT assay, as previously described elsewhere. Six replicate wells were used for each assay; data from replicate wells are presented as mean values with 95% confidence intervals (CIs). Three independent experiments were performed with similar results; representative results of one experiment are presented.

Soft agar colony formation assay. A colony formation assay was done using 24-well plates. Each well contained 1 mL of 0.5% agar in RPMI-1640 medium supplemented with 2% FCS as the bottom layer, 1 mL of 0.35% agar in 2% RPMI-1640 medium and 3,000 cells as the top layer with or without 500 nM 9*c*RA. Cultures were maintained under standard culture conditions. The number of colonies was determined with an inverted phase-contrast microscope at X 100 magnification; a group of >10 cells was counted as a colony. The data are means ± SE of four independent wells at optimum time of 14 days after the start of cell seeding.

Analysis of gene expression. Western blot analysis was performed by the standard procedure, employing antibodies raised against human RAR-β, human IGFBP-3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (Sigma). For detection of the intended protein, 30 to 50 μg of total protein was size fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and blocked with 5% nonfat dry milk in Tris-buffered saline-Tween (20 mM Tris HCl, pH 7.6; 150 mM NaCl; 0.1% Tween-20). Membranes were incubated with primary antibodies (Santa Cruz Biotechnology) overnight at 4°C and then with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 hr at room temperature. Immunoreactive proteins were detected using an enhanced chemiluminescence (ECL) kit and Hyperfilm ECL (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA). Relative amounts of the proteins of interest were determined using the ScionImage® software.

RNA preparation and semi-quantitative RT-PCR. Total cellular RNA, isolated from NSCLC cells using TRIzol (Invitrogen), was reverse transcribed and amplified using gene-specific primers and the Superscript™ One-Step RT-PCR System with Platinum™ TaqDNA Polymerase (Invitrogen). The following primer pairs were used: IGFBP-3 sense, 5'-ACC CCC TCC ATT CAA AGA TAA TCA-3', IGFBP-3 antisense, 5'-ACT TAT CCA CAC ACC AGC AGA AGC-3'; RAR-β sense, 5'-ATG ACT TTC TCA GAC GGC CTT ACC-3', RAR-β antisense, 5'-CCT TTA GCA CTG ATG CTA CGG AGA-3'; GAPDH sense, 5'-TGG GTG TGA ACC ATG AGA AGT ATG-3', GAPDH antisense, 5'-GTT TTT CTA GAC GGC AGG TCA GGT-3'. Cycling conditions were 1 cycle of 50°C for 30 min; 1 cycle of 94°C for 2 min; and 32 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min.

Plasmid preparation. A 1.9-kb IGFBP-3 promoter and its deletion mutants pGL2-Δ1600 and pGL2-Δ1675, subcloned to the luciferase reporter vector pGL2-basic (Promega Corp., Madison, WI, USA), were generous gifts from Dr. Oh Y (Oregon Health & Science University, Portland, OR, USA). Reporter vector nomenclature, which specifies the number of nucleotides removed from the 5'-end of pGL2-IGFBP-3, was adopted from Walker et al.[21] pGL2-Δ862 was prepared from an oligo corresponding to nucleotides -1064 to -64 (relative to the translation start site designated +1) of the IGFBP-3 promoter generated by PCR with the following primers: sense, 5'-GAG ATA AGC TTG AAT CCC AAC GC-3'; anti-sense, 5'-GCG AAG CTT TGG AAT CCA GGC A-3'. The same method was used to prepare pGL2-Δ1393 (-534 to -64; sense primer, 5-GTA TGC AAG CTT CCC CGA CAC C-3'; anti-sense primer, 5'-GCG AAG CTT TGG AAT CCA GGC A-3') and pGL2-Δ1482 (-445 to -64; sense primer, 5'-GGT GCA AGC TTG GCC AGG AGT-3'; anti-sense primer, 5'-GCG AAG CTT TGG AAT CCA GGC A-3'). Oligos were subcloned into the *Hind*III site of the pGL2-Basic vector.

pGL2-Δ1393 was used as a single-stranded DNA template for the production of mutant pGL2-Δ1393(R) with the primer AAG GGG TAA GGG CGG CGG AAA AAA GGA GAT CGG GGG TG and its reverse sequence. pGL2-Δ1393(F) was generated using CAC CCC GAG AGC GGA AGA AAA AAG GGC GGC GGG GTC AA-5' and its reverse sequence. pGL2-Δ1393(B) was generated using pGL2-Δ1393(R) and the same primer set as for pGL2-Δ1393(F). pcDNA™3.1D/V5-His-TOPO® was purchased from Invitrogen, and a full-length cDNA encoding RAR-β was inserted according to the manufacturer's instructions with the following primers: 5'-CAC CAT GGT TGA CTG TAT GGA T-3' and 5'-CTA GTT TCT GGT TGC ATG AAA TG-3'. Induction of RAR-β was confirmed by

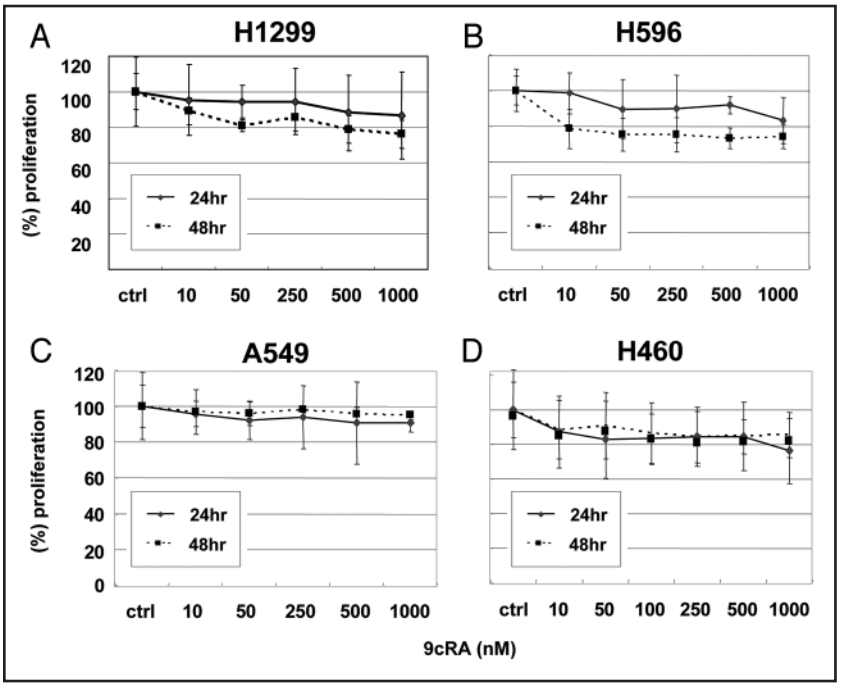

Figure 1. Effect of 9cRA on the proliferation of NSCLC cells. (A) NCI-H1299, (B) -H596, (C) -A549 and (D) -H460 NSCLC cells were treated with vehicle or indicated concentration of 9cRA and colorimetric MTT assays were then performed as described in "Materials and Methods." Results are expressed relative to the density of cells incubated in the vehicle alone. Each value is the mean (± SD) from eight identical wells. Each experiment was repeated three times. (Solid line, 24 hr treatment; dashed line, 48 hr).

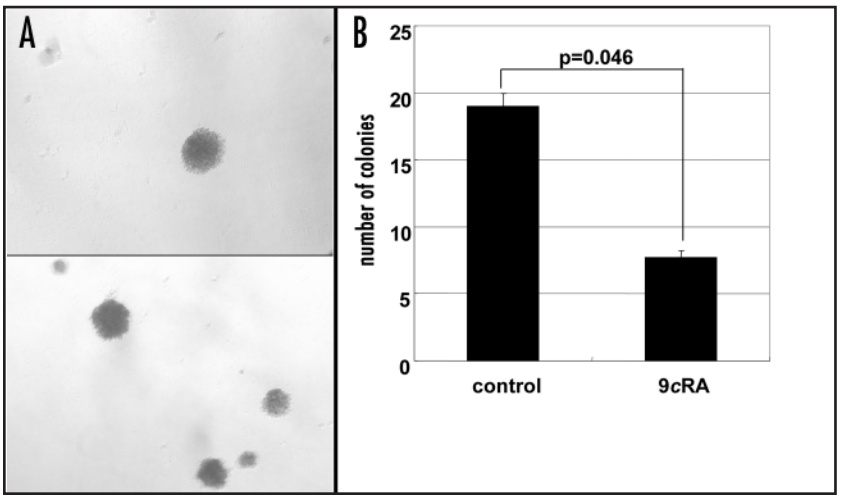

Figure 2. Soft agar colony formation assay in H460 cells treated with 500 nM 9cRA or control (vehicle only). (A) Representative photographs from three independent experiments taken under an inverted phase-contrast microscope at X100 magnification. (B) Quantitative analysis of soft agar colony formation of H460 cells treated with 9cRA or vehicle. Columns, mean of four independent wells in two weeks incubation from the start of cell seeding; bars, SE.

Western blot analysis of COS7 and H460 cells transfected with pcDNA™3.1D/V5-His- TOPO®-RAR-β.

Luciferase assay. H460 and COS7 cells were transiently transfected with lipofectamine-2000 reagent according to the manufacturer's instructions (Invitrogen). Cells from each line were seeded at a density of 5 x 10^4 cells/well in 24-well plates. At 70% confluence, cells were transfected with 200 ng/well of luciferase reporter plasmid, 30 ng/well of CMV-β-gal control vector (GibcoBRL), and either pcDNA™3.1D/V5-His-TOPO®-RAR-β or *si*RNA for RAR-β in serum-free RPMI-1640 medium. After 6 hr, the medium was changed to RPMI-1640 supplemented with 10% FCS. Cell lysates were collected for luciferase assay 48 hr after transfection. Transfections were performed in triplicate and experiments repeated at least three times. Luciferase activity was measured according to the manufacturer's instructions (Promega) and normalized against β-gal activity.

Electrophoretic mobility shift assay. Three double-stranded 28-mer pairs (bp -488 to -461 from mRNA cap site), corresponding to wild- or mutant-type RARE, were synthesized by Genotech, Inc. (Daejun, Korea). The wild-type oligomer consisted of 5'-GAA GGG GTA AGG GCG GCG GGG TCA AGG A -3' and its reverse complement, while the mutant-type was 5'-GAA GAA AAA AGG GCG GCG AAA AAA AGG A-3' and its reverse complement. These oligomers were end-labeled with [α-^{32}P]-ATP with a T4 polynucleotide kinase (Promega) according to the manufacturer's instructions.

H460 crude nuclear extract preparation and EMSA were performed as previously described.[19] Briefly, 5 ug of purified nuclear protein extracts were incubated in 20-μl reaction mixtures (20 mM HEPES, pH 8.4; 100 mM KCl; 20% glycerol; 0.1 mM EDTA; 50 ng/ml poly dA-dT; 0.05% NP-40; 1 mg of bovine serum albumin, BSA) at room temperature for 10 min prior to addition of 0.2 ng of radiolabeled oligonucleotide probe. Doses of wild- or mutant-type RARE consensus oligomer (Promega) were added for competition assays. After 20 min of room temperature incubation, samples were separated on 5% acrylamide gels in 0.5X Tris-borate-EDTA buffer. For EMSA supershift studies, 1 ug of polyclonal RAR-β or RXR-β antibodies (Santa Cruz Biotechnology) or, as a control, polyclonal HA antibody (Santa Cruz Biotechnology) were added for 30 min of incubation following the standard binding reaction. Gels were transferred, dried, and exposed to Hyperfilm ECL (Amersham Pharmacia Biotech).

Statistical analysis. For colony formation assay, Mann-Whitney U test was used to examine the differences in colony numbers between treatment groups. Comparisons of relative levels of luciferase activity among the different transfections were conducted using Kruskal-Wallis H test, followed by Mann-Whitney U test for comparison of relative values between groups. All statistical tests were two sided. $p < 0.05$ was considered statistically significant.

RESULTS

9cRA increases IGFBP-3 and RAR-β expression. First, to investigate the effect of 9*c*RA on the anchorage dependent growth of NSCLC cells, we treated NSCLC cells with indicated dose of 9*c*RA for 24 hr to 48 hr and performed MTT assay. 9*c*RA did not influence on the proliferation of A549 cells and H1299 cells, whereas inhibited proliferation in H596 and H460 cells (representative data are shown in Fig. 1). Figures 2A and B showed that treatment of 500 nM 9*c*RA inhibited the anchorage-independent growth of H460 NSCLC cells. For controls, 19 ± 2.1 (mean ± SE of four wells) colonies per field were counted compared with 8 ± 0.6 colonies per field counted in 9*c*RA treated wells (Fig. 2B). Compared with control cells, treatment of 500 nM 9*c*RA results in 59.7% inhibition of colony formation (Fig. 2B; $p < 0.046$, Mann-Whitney U test).

To elucidate whether 9*c*RA is involved in the regulation of IGFBP-3 gene expression, we examined the effect of 9*c*RA on expression of the IGFBP-3 gene protein in vitro using H460 NSCLC cells cultured at various concentrations and times. As shown in Figure 3A, 9*c*RA increased IGFBP-3

expression in a dose-dependent manner until it reach 500 nM, with a maximum 3.4-fold induction reached at 48 h of exposure to 500 nM 9*c*RA. Reverse-transcribed mRNA was obtained from cells following the indicated conditions, and the results of subsequent PCR are shown in Figure 3B. High 9*c*RA concentration in the culture medium consistently induced an increase in IGFBP-3 mRNA. 9*c*RA also increased RAR-β protein and RAR-β mRNA levels at similar concentrations and times (Fig. 3A and B). The 9*c*RA-mediated induction suggests the involvement of RAR/RXR in IGFBP-3 gene activation, although analysis of the 9*c*RA effects does not allow discrimination between the relative involvement of RAR and RXR receptors in the activation of IGFBP-3 expression. To elucidate whether RARs play a role in IGFBP-3 gene expression, the synthetic RAR-specific agonist TTNPB was used. As shown in Figure 3C, TTNPB increased IGFBP-3 protein expression in a dose-dependent manner. At the lower concentration (0.1 to 10 nM), this effect was three orders of magnitude higher than those observed with 9*c*RA.

9cRA increases IGFBP-3 gene expression at the transcriptional level. To investigate the underlying mechanisms in which 9*c*RA increases IGFBP-3 gene expression, H1299 NSCLC cells were transiently transfected with a pGL2 luciferase reporter vector, driven by the wild-type IGFBP-3 promoter and its serial deletion mutants (Fig. 4A and B), and then treated with 9*c*RA. Selection for H1299 cells was based on a previous experiment that showed these cells to have lower levels of endogenous RAR-β and high transfection efficiency compared to other NSCLC cell lines.[20] As shown in Figure 5, pGL2-Δ862 exhibited a 2.5-fold induction of promoter activity by 9*c*RA. When a unilateral deletion construct carrying the -534 to -64 region of the IGFBP-3 promoter (pGL2-Δ1393) was introduced, the inductive effect of 9*c*RA was similar to that of pGL2-Δ862, whereas further deletion of the promoter region to -445 bp (pGL2- Δ1482) abolished the inductive effect of 9*c*RA. These results indicate that 9*c*RA acts on IGFBP-3 expression at the transcriptional level and further suggest the presence of a retinoid responsive region between -534 and -445 in the proximal IGFBP-3 promoter.

9cRA activates IGFBP-3 promoter activity via RAR-β. In order to determine those transcription factors critical for 9*c*RA-induced IGFBP-3 gene expression, H460 cells were transfected with either *si*RNA for RAR-β or a RAR-β expression vector under the influence of 500 nM 9*c*RA or the vehicle for 48 hr. Treatment with *si*RNA for RAR-β decreased IGFBP-3 expression, while the RAR-β expression vector potentiated 9*c*RA inducement of IGFBP-3. Transfection with the RAR-β expression vector in the absence of 9*c*RA also induced IGFBP-3, although not at a level comparable to that for the combined treatment with 9*c*RA and the RAR-β expression vector (Fig. 6A).

To further elucidate RAR-β involvement in IGFBP-3 gene activation, pGL2-Δ1393 containing a putative RARE within the IGFBP-3 gene promoter (-534 to -445) was transfected into H1299 NSCLC cells. These cells were tested under the effects of *si*RNA for RAR-β and a RAR-β expression vector plus 9*c*RA (Fig. 6B). IGFBP-3 promoter activity was enhanced when cells were cotransfected with RAR-β and decreased with *si*RNA for RAR-β. In contrast, 9*c*RA in the presence of either *si*RNA resulted in marginal stimulation of IGFBP-3 promoter activity.

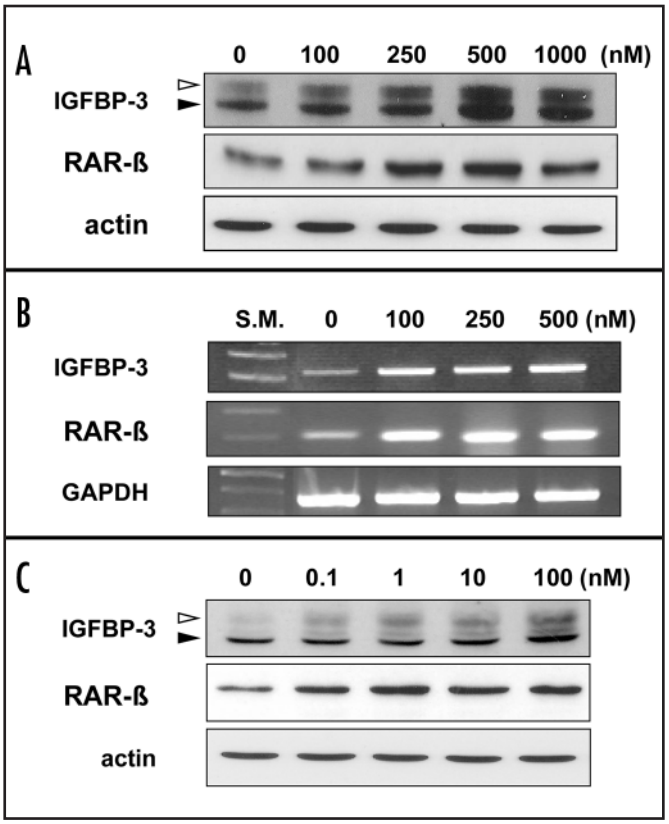

Figure 3. Induction of IGFBP-3 and RAR-β in response to 9*c*RA and TTNPB. H460 NSCLC cells were treated with the indicated doses of 9*c*RA or vehicle for 48 hr. Whole cell extracts were prepared and analyzed by immunoblot (A). mRNA expression was evaluated by semi-quantitative RT-PCR (B). Expression of IGFBP-3 and RAR-β after treatment with TTNPB was evaluated by Western blot (C). The experiment was repeated three times with identical results. Actin and GAPDH were used for equal dose control. S.M., DNA size marker; ▶, 42 kD IGFBP-3; ▷, glycosylated form of IGFPB-3.

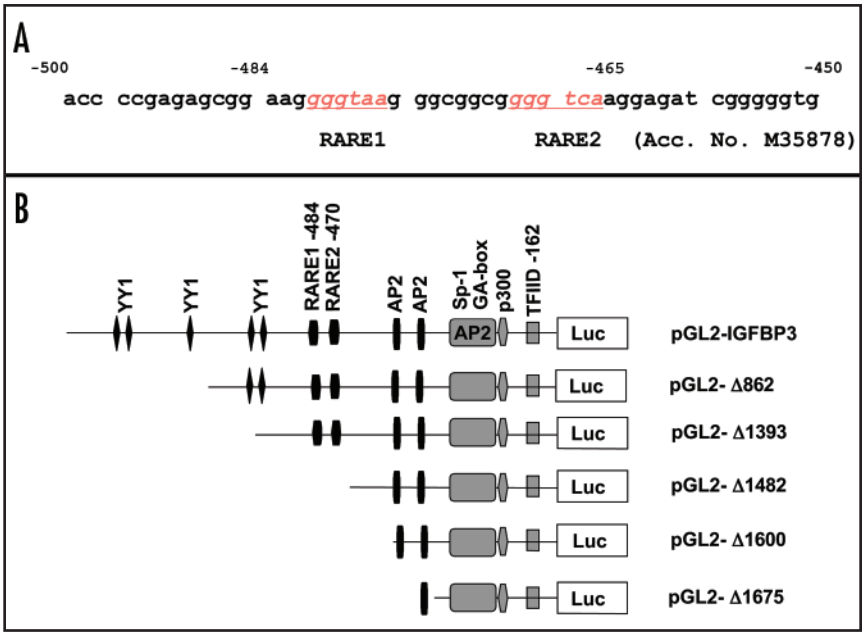

Figure 4. Sequence and schematic diagram depicting the human IGFBP-3 promoter used in this study. Sequence analysis of IGFBP-3 promoter shows a putative DR-8 RARE approximately 484 bp 5' to the translation start site (A). This approximately 1.9-kb portion of the human IGFBP-3 promoter also includes a cluster of 11 p53 sites, five Yin and Yang-1 (YY1) sites, a GC-rich/Sp-1 site, and two consensus AP2 sites. The DR-8 RARE is deleted in pGL2-Δ1482, as is the entire DR-8 RARE in pGL2-Δ1393 (B). Numbering of plasmid follows the designation of Walker et al.[21]

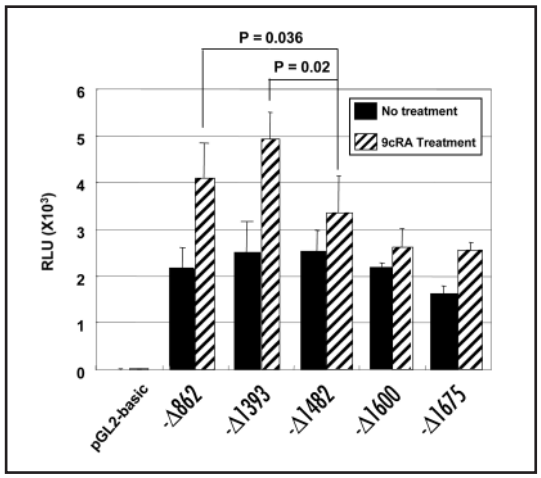

Figure 5. Luciferase assay of serial deletion mutants of IGFBP-3 promoter with or without treatment of 9*c*RA. 9*c*RA treatment induces IGFBP-3 promoter activity in the pGL2-Δ1393 reporter vector, which contains a putative RARE. pGL2-Δ 1482 and -Δ1600, in which the 5' element of the putative RARE and the entire putative RARE is deleted respectively, revealed a blunted response to 9*c*RA. Each experiment was repeated at least three times with identical results. RLU, relative luciferase activity.

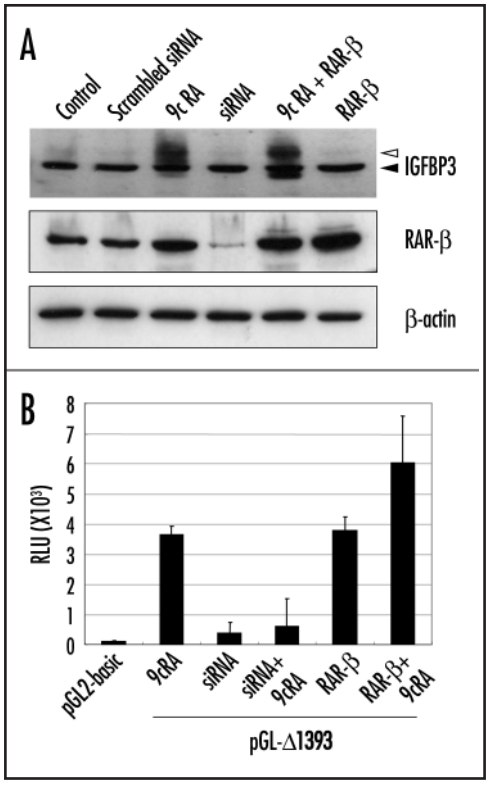

Figure 6. Treatment with *si*RNA for RAR-β decreased 9cRA induction of IGFBP-3; *si*RNA for RAR-β expression vector potentiated this induction. H460 NSCLC cells were treated with *si*RNA for RAR-β or a RAR-β expression vector under the influence of 500 nM 9*c*RA or the vehicle for 48 hr. Whole cell extracts were prepared and analyzed by immunoblot (A). Promoter activity of pGL2- Δ1393 was measured after treatment with *si*RNA for RAR-β or the RAR-β expression vector under the influence of 500 nM 9*c*RA or vehicle (B). Each experiment was repeated at least three times with identical results. RLU, relative luciferase activity.

Taken together, these data demonstrate the involvement of RAR-β in the 9*c*RA-induced transactivation of the IGFBP-3 gene.

Identification of retinoid responsive element in IGFBP-3 promoter. Sequence analysis of the IGFBP-3 promoter indicated a putative retinoid responsive element (RARE) composed of a DR-8 sequence (two directly-repeated TGACCT motifs separated by eight nucleotides; GGGTAA gggcggcgGGGTCA, nucleotides -484 to -465 upstream of the transcription start site) (Fig. 7A). Owing to data in the previous section suggesting involvement of RAR-β in IGFBP-3 gene transactivation, we suppose this putative responsive element to be a RARE. To determine whether this element mediates the retinoid response of the IGFBP-3 gene, three modified IGFBP-3 promoter plasmid constructs were transfected into H1299 NSCLC with 9*c*RA. The first construct corresponded to pGL2-Δ1393 of the IGFBP-3 promoter mutated to include the 5' GGGTCA motif of the putative RARE [pGL2-Δ1393(F)]. When compared to the wild-type construct (pGL2- Δ1393), mutation of this element in the RARE sequence led to a 12% reduction in the 9*c*RA inductive effect. In the second construct, mutation in the 3' motif of the putative RARE [pGL2-Δ1393(R)] of the promoter also decreased 9*c*RA-mediated induction (41.1%). In the third construct, mutations introduced in the GGGTCA-like motif [pGL2-Δ1393(B)] of the IGFBP-3 promoter further abolished 9*c*RA-mediated induction (70.5%; Fig. 7B). Our data suggest that the putative DR-8 RARE, located from -484 to -465 from the mRNA start site, participates in 9*c*RA-induced IGFBP-3 expression.

To further investigate the involvement of synthetic retinoic acids in the nuclear translocation of RAR-β and RXR-β, we performed Western blot analysis with a nuclear extract of H460 cells treated with the indicated doses of 9*c*RA, 13*c*RA, and TTNPB. Very low induction of RXR-β expression was observed in nuclear extracts from the synthetic retinoic acid derivatives treatment group, whereas RAR-β was strongly induced by 9*c*RA and 13*c*RA treatment (Fig. 8A).

Gel shift analysis was performed, showing the RARE to be an essential region for 9*c*RA-induced IGFBP-3 promoter activity in H460 NSCLC cells. For this study, we designed a 28-mer oligonucleotide (-488 to -461; WT) spanning the putative RARE in the IGFBP-3 promoter. When the nuclear extract from H460 cells was used, a DNA:protein complex was formed (Fig. 8B). Specificity of binding was verified by competition assays; in which each complex was successfully out-competed by increasing concentrations of unlabeled wild-type RARE consensus oligonucleotides (S.C.). Complexing was not abolished by treatment with unlabeled mutant RARE consensus oligonucleotides (N.C.) (Fig. 8B). We then used polyclonal antibodies raised against RAR-β or RXR-β to demonstrate that complexes indeed contained RAR-β. RAR-β antibody (Fig. 8C, lanes 2), but not a RXR-β and a control (anti-HA) antibodies (Fig. 8C, lane 3 and 4), led to disappearance of the complex.

DISCUSSION

With the advent of novel targeted agents such as COX-2 inhibitors, farnesyl transferase inhibitors, and epidermal growth factor receptor tyrosine kinase inhibitors, there has been a push to consider molecular targeted approaches to NSCLC and the development of appropriate surrogate markers for chemoprevention. Despite substantial advances in chemoprevention of breast, colon, bladder, and head/neck cancers, the prevention of lung cancer is still a distant goal. This is in part due to a lack of appropriate surrogate markers suitable for long-term follow-up care. RAR-β, regarded as a tumor suppressor in upper aerodigestive tract cancer, is one of the frequently-used surrogate markers in chemoprevention trials. One recent large-scale chemopreventive trial sought to reverse detectable squamous metaplasia in the bronchial epithelium of former heavy smokers with 9*c*RA or 13*c*RA plus α-tocopherol. The study showed that administration of 9*c*RA reduced the percentage of subjects with detectable squamous metaplasia and led to a statistically significant increase in RAR-β

expression in the biopsy site. Although RAR-β was effectively upregulated by 9*c*RA in the bronchial epithelium of former smokers, data implicating maintenance of RAR-β intratumoraly in stage I NSCLC in individuals with a poorer prognosis[3] has shed some doubt on these results.[1] Consecutive reports that elevated IGF-1 and decreased IGFBP-3 in circulation are related to the development of various solid cancers, however, not only bolster IGFBP-3 as a good candidate for surrogate marker but also urge the development of effective ways to up-regulate IGFBP-3.

Here, we demonstrated that the expression of IGFBP-3 in NSCLC cells was induced by the pan-retinoic acid receptor agonist, 9*c*RA, and that administration of this agent decreased frequency of metaplasia in the bronchial epithelium of former heavy smokers.[2] Induction of IGFBP-3 expression by 9*c*RA occurs at the transcriptional level, as measured by semi-quantitative RT-PCR. Concurrence of semi-quantitative RT-PCR and Western blot in the expression of IGFBP-3 and RAR-β indicated that 9*c*RA activates IGFBP-3 gene transcription, supporting the previous findings that a major consensus sequence for retinoic acid may be present in the promoter region of the IGFBP-3 gene.[24]

By using a RAR-selective agonist (TTNPB), we demonstrated that RAR-β expression was induced by 9*c*RA through a RAR-dependent signaling pathway and that RAR-β induction was correlated with IGFBP-3 induction. Further, expression of IGFBP-3 is mediated by RAR-β, as blocking of RAR-β expression by *si*RNA for RAR-β diminished expression of IGFBP-3. When H1299 cells were transfected with cDNA encoding RAR-β, the promoter activity of IGFBP-3 was increased; it was further augmented in the presence of 9*c*RA. Transfection of H460 cells with siRNA for RAR-β not only inhibited 9*c*RA-induced IGFBP-3 expression, but also IGFBP-3 promoter activity, indicating that RAR-β is directly involved in the mediation of IGFBP-3 induction by 9*c*RA. A connection between IGFBP-3 and RAR-β defines the signal intersection between the retinoid and IGF systems in cell growth regulation and suggests why the loss of RAR-β might be critical in NSCLC carcinogenesis and progression.

Several regulatory elements—a cluster of 11 p53 consensus binding sites; five consensus Yin and Yang-1 (YY1) sites; a sequence cluster comprised of a GC-rich/Sp1 site, AP-2 site overlapping a GA-box, and p300 DNA binding site—are found in the promoter of IGFBP-3.[21] Earlier work using rat IGFBP-3 promoter identified the major consensus sequences that could be activated by retinoic acid as an AP-2 and an Oct site, which locate from -78 to -71 and at -612, respectively, relative to the transcription start site. There are, however, minor differences between human and rat IGFBP-3 promoter sequences. Between the human and rat IGFBP-3 promoters, there is 65% sequence conservation within 1.2 kb of the promoter, and the rat IGFBP-3 promoter lacks a putative RARE in the 5'-untranslated flanking region.[24] Computer-based simulation using the transcription element search system (TESS; http://www.cbil.upenn.edu/tess) algorithm and our experiment using serial deletion mutants cloned into the pGL2-reporter vector showed that a RARE consisting of two half-sites arranged as a direct repeat with an 8-bp spacer (DR-8) is located from -484 to -465 relative to the translation start site.

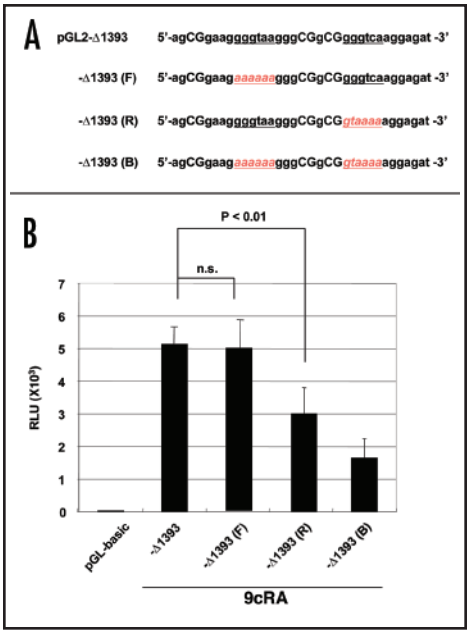

Figure 7. Site-directed mutagenesis introduced in either element of DR-8 RARE decreased the promoter activity of pGL2-Δ1393. Portion of sequence of pGL2-Δ1393, and those of its mutants pGL2-Δ1393 (A). Promoter activity of pGL2-Δ1393 and its mutants was measured under the influence of 500 nM 9cRA (B). Each experiment was repeated at least three times with identical results. RLU, relative luciferase activity.

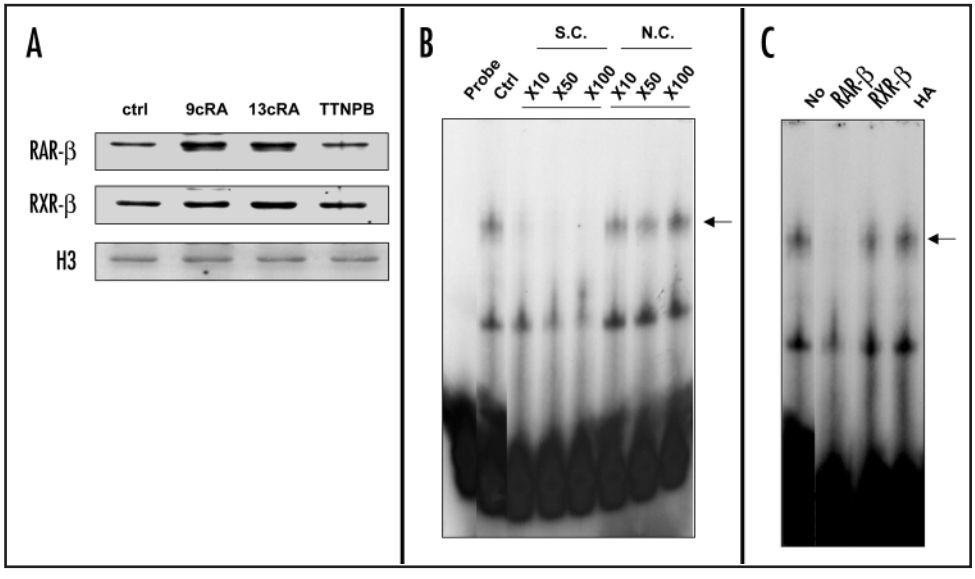

Figure 8. Treatment of synthetic retinoic acids increased nuclear expression and specific binding of RAR-β on the putative RARE. Immunoblot of nuclear extract of H460 cells treated by either 500 nM 9cRA, 1 μM 13cRA, or 1 nM TTNPB showed increased nuclear translocation of RAR-β (A). EMSA revealed specific binding of RAR-β to the putative DR-8 RARE. Incubation with anti-RAR-β antibody and an unlabelled RARE competitor led to disappearance of the DNA:protein complex signal; treatment with anti-RXR and mutated RARE did not (B and C).

This DR-8 in the human IGFBP-3 promoter is different from the canonical RARE, represented by DR-1 to DR-5.[25] The classical DR-5 elements are found in the promoters of the RAR-β itself[26] and several Hox and HNF genes.[27,28] DR-8 motifs previously identified in the promoter lesion of human H1 histone and the RAR-β gene interact with thyroid hormone receptors (TRs), RARs and RXR, COUP-TF, and v-erbA in a ligand-dependent manner.[29,30] In a study using the DR-8 sequence in the human H1 promoter, COUP-TF, which usually represses RAREs, enhanced transcriptional activation through the DR-8. In constrast, v-erbA completely repressed TR-RXR induction of the H1 gene.[29] The pattern of site selectivity is based on the spacing of DRs(1-5 rule) and polarity of RAR-RXR complexes.[31,32] Among the canonical RAREs, RAR-RXR complex have preference for DR-1, DR-2, and DR-5 RARE.[33] The facts that expression of RXR-β is suppressed in a large percentage of NSCLC,[34] its expression is relatively lower than that of other type of RXRs,[35] and as shown in (Fig. 8C), nuclear translocation of RXR-β is not influenced by 9*c*RA might be implicated in failure of supershift by RXR-β antibody. Further studies may be needed to identify appropriate partners of RAR-β in heterodimeric complex formation for binding of DR-8 RARE in IGFBP-3 promoter.

Our study demonstrates that reporter constructs containing DR-8 allowed a several-fold induction of IGFBP-3 promoter activity by 9*c*RA in the presence of RAR-β. Also, RAR-β binding to the DR-8 was enhanced in the presence of 9*c*RA. Several signaling pathways that play important roles during differentiation and cell proliferation thus influence the rate of IGFBP-3 transcription through a DR-8 response element in its promoter. Mutational analysis suggests that this site is functionally a DR-8 element for retinoic acid derivatives. We further demonstrated binding of RAR-β to the DR-8 element in electrophoretic mobility shift and competition assays between the RAR consensus sequence and another sequence bound by RAR-β.

Our experiments show that RAR-β is involved in the growth inhibitory activity of 9*c*RA via mediation of the induction of IGFBP-3 expression. Because both IGFBP-3 and RAR-β expression levels inversely correlate with cell proliferation and are regarded as putative tumor suppressor genes in NSCLC, our data suggest that retinoic acids such as 9*c*RA can influence cell proliferation via regulation of the DR-8 element. By elucidating the linkage mechanism between IGFBP-3 and RAR-β, our experiments have pinpointed an intersection between the retinoid and IGF systems. This information also expands knowledge of the downstream effectors of RAR-β and suggests how RAR-β might act as a tumor suppressor.

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