Evidence that Retinoic Acid Receptor β Induction by Retinoids is Important for Tumor Cell Growth Inhibition

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Running title: RARβ induction and growth inhibition.
Retinoic acid receptor β (RARβ) is thought to be involved in suppressing cell growth and tumorigenicity. Many premalignant and malignant cells exhibit a reduced RARβ expression. However, in some of these cells (e.g., H157 human squamous cell carcinoma cells), RARβ can be induced by retinoids (e.g., all-trans retinoic acid, ATRA) because its promoter contains a retinoic acid response element. To examine the hypothesis that RARβ induction is important for inhibition of cell proliferation by retinoids, we blocked ATRA-induced RARβ expression in H157 cells using a retroviral vector harboring multiple copies of antisense RARβ2 sequences. Antisense RARβ–transfected cells showed not only decreased expression of ATRA-induced RARβ protein but also reduced ATRA-induced RARE binding activity and transactivation. Importantly, all antisense RARβ transfectants of H157 cells were less responsive than vector transfected cells to the growth inhibitory effects of the retinoids ATRA and Ch55 in vitro. These results demonstrate that RARβ induction may play an important role in mediating growth inhibitory effects of retinoids in cancer cells.
Retinoids, a group of natural and synthetic vitamin A (retinol) derivatives, exert fundamental effects on the regulation of cell growth, differentiation, and development (1). Some retinoids are being evaluated as chemopreventive and chemotherapeutic agents for a variety of human cancers (2). Effective use of retinoids requires an understanding of their mechanism of action. Retinoids are thought to exert most of their effects by regulating gene expression primarily through two classes of nuclear receptors, RARs\(^1\) and RXRs (3, 4). Both types of receptor are members of the steroid hormone receptor gene superfamily of sequence-specific, ligand-activated transcriptional factors. The receptors are encoded by six distinct genes, RAR\(\alpha\), RAR\(\beta\), and RAR\(\gamma\), and RXR\(\alpha\), RXR\(\beta\), and RXR\(\gamma\). Each of these receptors includes several isotypes (e.g. \(\beta 1\), \(\beta 2\), \(\beta 4\)) formed by different splicing and usage of alternative promoters (3, 4). ATRA binds to and activates RARs, and 9-\textit{cis} retinoic acid bind to and activates both RARs and RXRs. RARs and RXRs modulate the expression of target genes by interacting as either homodimers or heterodimers with RAREs located in the promoter regions of target genes (3, 4). Some RAR genes, in particular the RAR\(\beta\) gene, contain RAREs in their promoters and can be induced by retinoids (5-8).

The RAR\(\beta\) RARE consists of two direct repeats of the core motif sequence (G/A)GTTCA seperated by five nucleotides (5-7). The level of RAR\(\beta\) transcript increases in many cell types in response to ATRA due to the presence of this RARE (5-8).

RAR\(\beta\) expression is suppressed in various types of malignant tumors including lung carcinoma (9-15). It has been suggested that the decrease in RAR\(\beta\) expression may lead to resistance to retinoids (16-21). Indeed, transfection of RAR\(\beta\) into RAR\(\beta\)-negative cervical, breast, and lung cancer cells increased cell responsiveness to growth inhibition and induction of apoptosis by retinoids (21-25) and restoration of RAR\(\beta\) expression in RAR\(\beta\)-negative lung cancer cell lines inhibited tumorigenicity in nude mice (26).
RARβ induction by retinoids has been demonstrated in vivo (16, 27, 28). However, it is not clear to what extent if any the induced RARβ contributes to the response to growth inhibitory effects of retinoids, or whether it plays no role in the overall response to retinoids.

To further understand the importance of RARβ induction, we constructed and transfected a retroviral expression vector harboring antisense RARβ2 into the H157 human lung squamous cell carcinoma cell line, which expresses RARβ only after ATRA treatment and found that blocking RARβ induction decreases cell sensitivity to retinoids.

EXPERIMENTAL PROCEDURES

Retinoids—ATRA and Ch55 were kindly provided by Dr. Werner Bollag (F. Hoffmann-La Roche, Basel, Switzerland) and Dr. Koichi Shudo (Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan), respectively. They were dissolved in dimethylsuloxide at a concentration of 10 mM under N₂ and stored in the dark at -80°C. Stock solutions were diluted to the desired concentrations with growth medium just prior to use.

Cell Culture—The H157 lung squamous cell carcinoma cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and grown in monolayer culture in a 1:1 (v/v) mixture of Dubecco's modified Eagle's medium and Ham's F12 medium containing 5% FCS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 37°C.

Construction of the LNASβVI Retroviral Vector Harboring Multiple Copies of hRARβ2-specific cDNA Fragment Cloned in a Reverse Orientation—The retroviral vector LNSX, obtained
from Dr. D. Miller (Fred Hutchinson Cancer Research Center, Seattle, WA) (29), was used in this study. We introduced into LNSX vector multiple copies of hRARβ2 cDNA fragments corresponding to the initial site of RARβ2 translation in an antisense orientation by ligating 385-bp *Bam H I*/Sph I cDNA fragments released from pSG5-RARβ plasmid (30) into unique *Hind* III, *Cla* I, and *BamH I* sites through blunt end ligation with T4 DNA ligase, respectively. We obtained a series of vectors harboring different number of copies of RARβ2 cDNA inserts in an antisense orientation as identified by sequencing and enzymatic digestions. Vectors harboring 1, 2, 3, 4, 5, and 6 antisense RARβ2 inserts were designated as LNASβ, LNASβII, LNASβIII, LNASβIV, LNASβV and LNASβVI, respectively. The LNASβVI shown in Fig. 1 was used in all subsequent experiments.

*Retroviral Transduction*—LNASβVI or LNSX as a vector control were transfected directly into PA317 amphotropic packaging cell line by the calcium phosphahe precipitation method (31). The transfected cells were cultured in G418 (500 µg/ml), and individual clones of resistant cells were picked up using cloning cylinders after 14 days and expanded. The supernatants from these retroviral producers were titered on thymidine kinase-negative NIH3T3 target cells as described previously (31). Supernatant from the highest titer producer clone for either LNASβVI or LNSX (up to 5 x 10⁵/ml) was used in all subsequent experiments.

H157 cells were plated at 1:10 split in 6 cm diameter tissue culture dishes (Corning Incorporated, Corning, NY). On the second day, the medium was replaced with 2 ml of fresh medium containing different amounts of viral supernatant and 8 µg/ml of polybrene, and 2 h later, 2 ml of fresh medium was added. Cells were split at 1:10 into 10 cm diameter dishes and selected after 24 h using G418 (1000 µg/ml)-containing medium. Surviving clones were isolated after 12 to
15 days using cloning cylinders. The rest of the clones from different dishes were pooled as pool transfectants. Transfectants were expanded and maintained under continuous G418 selection at 500 µg/ml.

**RNA Purification and Northern Blotting**—Total cellular RNA purification and Northern blotting were performed as previously described (32). Twenty micrograms of RNA was loaded per lane. The 385-bp *BamH I*/Sph I fragment of RARβ from pSG5 expression vector harboring human RARβ2 cDNA (30), obtained from Dr. Pierre Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Illkirch, C.U. de Strasbourg, France), was used as a probe for Northern blotting. The 340-bp *EcoR I*/Xba I cDNA for GADPH, purchased from Ambion (Austin, TX), was used as a control of RNA loading.

**Protein Extraction and Western Blotting**—Nuclear extracts were prepared from H157 transfectants by a method described by Pollock et al (33). Eighty µg of protein was electrophoresed through a 10% polyacrylamide slab gel and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) by electroblotting. The blot was immersed in blocking solution (15% nonfat milk in PBS) at room temperature for 1 h and then incubated with a 1:50 dilution of rabbit anti RARβ polyclonal antiserum (generously provided by Dr. P. Chambon) in blocking solution overnight at 4°C with agitation. The blot was washed four times with blocking solution and incubated with 125I-labeled goat anti-rabbit IgG at a 1:2500 dilution in blocking solution at room temperature for 1 h. The blot was then washed four more times with blocking solution containing 0.1% Tween 20, and exposed to X-ray film at -80°C for 2 to 5 days.
Gel Mobility Shift and Supershift Assay—Nuclear extracts from H157 transfectants used in this assay were the same as those prepared for Western blotting. The synthetic oligonucleotides indicated below were labeled with $[^{32}\text{P}]$ ATP (4 000 Ci/mol; ICN Radiochemicals, Irvine, CA) using T4 polynucleotide kinase.

Wild-type (Wt) RARE-β2: 5'-TCGAGGGTAGGGTTACCGAAAGTTCACTCG-3'

Mutant (Mu) RARE: 5'-TCGAGGGTAGGGcTTACCGAAAGTTCA-3'

Nuclear extracts were preincubated with 2 µg of poly (dI-dC)poly (dI-dC) for 15 min on ice and then incubated with labeled RARE-β2 (approximately 10 000 cpm) for 15 min on ice in the presence of 10 mM Tris-HCl pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 5 mM MgCl$_2$, and 20% glycerol. For the gel supershift assay, receptor-specific monoclonal antibodies of RARs and RXRs (provided by Dr. P. Chambon) (0.3 µl) were added to the reaction mixture. The reaction mixtures (10 µl) were electrophoresed on a 5% polyacrylamide gel containing 25 mM Tris-HCl pH 8.5, 192 mM glycine, and 1 mM EDTA, dried, and exposed to X-ray film.

**Reporter Plasmids, Transient Transfection and Luciferase Activity Assay** — The (RARE)$_3$-TK-Luc reporter plasmid, which contains three direct repeats of RARE from the P2 promoter region of the human RARβ2 gene (from –59 to –33) connected to herpes simplex virus thymidine kinase (TK) promoter, and TK-LUC control reporter plasmids were provided by Dr. R. A. Heyman (Ligand Pharmaceuticals, San Diego, CA). The AP1-TK-Luc reporter plasmid, which contains the luciferase gene controlled by a promoter fragment of the collagenase gene (-74 to –63) harboring a consensus AP-1 binding site (TGAGTC) connected to the TK promoter, was obtained from Dr. J. Kurie (The University of Texas M. D. Anderson Cancer Center, Houston, TX). pCH110 plasmid encoding β-galactosidase (β-gal) was purchased from Pharmacia Biotech (Piscataway, NJ).
plasmid purification, transfection, and luciferase activity assay procedures were the same as
described previously (34).

Cell Growth Assay—The effects of retinoids on the growth of different transfectants were
evaluated by the sulforhodamine B assay as previously described (32). Colony formation assay
was performed as follows: cells were plated at a density of 2 000 cells in 6 cm diameter tissue
culture dishes (Corning Incorporated) and treated on the next day with retinoids. The medium
was replaced with fresh medium containing retinoids every 3 days. After a 12-day treatment,
colonies were stained with 0.5% methylene blue in 70% ethanol and counted.

RESULTS

Establishment of H157 Cells Stably Expressing AntisenseRARβ by Retroviral-mediated
Transduction—H157 cells express undetectable levels of RARβ (by Northern blotting) but high
level of induced RARβ after treatment with ATRA or other retinoids. This cell line was infected
with the LNASβVI retroviral vector (Fig. 1), and individual G418-resistant clones were isolated.
These clones were designated H157-LNASβ. Vector only control clones were obtained by
infecting the same cell line with the retroviral vector LNSX, which does not contain the antisense
RARβ sequence (Fig. 1). These control clones were designated H157-LNSX.

Northern blotting was performed on transfectants to determine the expression level of
antisense RARβ mRNA. The 4.3 kb antisense mRNA controlled by the SV40 promoter and the
antisense mRNA larger than 5 kb driven by the LTR promoter were detected using a 385 bp BamH
I/Sph I RARβ2 cDNA fragment as a probe in all the LNASβVI-infected clones but not in the
LNSX-infected control cells (Fig. 2A).
Western blot analysis using an RARβ-specific polyclonal antiserum (35) revealed high levels of RARβ protein in ATRA-treated LNSX-P cells (vector only) (Fig. 2B). In contrast, ATRA induced less RARβ protein in most antisense RARβ-transfected clones, indicating that antisense RARβ expression was effective in the cells.

Effects of Antisense RARβ Transfection on ATRA-induced RARE Binding and Transactivation — To examine the effect of stable transfection of antisense RARβ on the formation of complexes between nuclear receptors and RARE, we performed gel shift and supershift assays, which are very sensitive methods for detecting functional RAR proteins. Fig. 3A shows that a weak signal of shifted band was observed with extract from untreated LNSX-P cells. This band was decreased in untreated LNASβ-9 cells. Antibodies against RARα and RARβ failed to supershift complexes in untreated LNSX-P or LNASβ-9 cell extracts. However, antibodies against RARγ and RXRs did supershift complexes in both cell types. Treatment with ATRA increased markedly the amount of shifted complexes in LNSX-P cells but only a very small increase in the amount of shifted complex was observed in ATRA-treated LNASβ-9 cells. Importantly, antibodies against RARβ supershifted a major complex in the ATRA-treated LNSX-P cells but not at all in the LNASβ-9 cells (Fig. 3A). These results clearly show that the antisense RARβ transfectants do not express functional RARβ even after ATRA treatment whereas the vector controls exhibit a large increase in functional RARβ. The band specificity was assured by the fact that they were competed out by a 100-fold molar excess of nonradioactive wild-type RARE, but not by same amount of nonradioactive mutated RARE (data not shown).

Furthermore, we found that induction of RARE transcriptional activation by ATRA was markedly decreased in antisense RARβ transfected cells in comparison with LNSX-transfected
cells. As shown in Fig. 3B, RARE transactivation was increased 20-30 fold in both LNSX-transfected clones but only 8-fold in two LNASβ transfectants. One possible explanation for the residual transactivation capacity in LNASβ transfected cells is the presence of other RARs and RXRs. Fig. 3B shows that RARγ and at least one RXR protein and traces of RARβ can be supershifted from complexes with RARE in LNASβ-9 cells after ATRA treatment. It is plausible to suggest that these receptors are responsible for the activation of RARE-Luc reporter in the antisense RARβ transfectants.

The partial suppression of RARE transactivation in the LNASβ transfected cells appeared to be specific for RARE because the transactivation of another reporter construct mediated by AP-1 binding was not suppressed in these transfectants (Fig. 3C).

**Antisense RARβ Transfection Decreases Cell Responsiveness to Retinoid Treatment**—We next compared the responsiveness of LNASβVI-transfected clones to retinoid treatment with that of LNSX-transfected cells. Fig. 4A shows the effects of different retinoids on the population growth of LNASβVI-transfected and LNSX-transfected cells. Four LNASβVI-transfected clones exhibited much lower sensitivity to ATRA treatment than 3 LNSX-transfected cells did. ATRA at concentrations of 1 µM and 2.5 µM caused 20-40% growth inhibition in LNSX-transfected cells but less than 20% growth inhibition in all LNASβ VI-transfected clones (Fig. 4A). The synthetic retinoid Ch55 is a pan RAR-selective agonist and has better receptor binding affinities, especially to RARβ than ATRA but is much more active that ATRA in inhibiting the growth of H157 and other lung cancer cells (32). To better demonstrate the low responsiveness of antisense RARβ-transfected cells, we further examined the effects of Ch55 on the growth of different LNSX-
transfected cells and LNASβVI-transfected clones. As shown in Fig. 4A, Ch55 caused dose- and time-dependent growth inhibitory effects in all LNSX-transfected cells. However, the responsiveness of all LNASβVI-transfected cells to Ch55 was lower than that of LNSX-transfected cells: Ch55 at 1 µM and 2.5 µM inhibited cell growth by 40-60% and 65-80%, respectively, in LNSX-transfected cells but by less than 20% and 35%, respectively, in LNASβVI-transfected cells after 6-day treatment (Fig. 4A). In addition, we analyzed the effects of ATRA and Ch55 on the colony formation of different transfectants. Similar to the population growth inhibition results, all LNASβVI-transfected cells exhibited less sensitivity (about 50%) than LNSX-transfected cells to inhibition of colony formation by ATRA and Ch55 (Fig. 4B). These results clearly show that blockage of RARβ induction decreased the cell responsiveness to retinoids.

DISCUSSION

It has been suggested that RARβ may play a role as a tumor suppressor. This hypothesis was based on the observation that RARβ levels decreased in a variety of tumor cell lines including lung carcinomas (9-13) as well as in premalignant and malignant epithelial tissues in vivo (14-17, 27, 28). It has been shown that RARβ expression, which is suppressed at early stages of head and neck and lung carcinogenesis, can be induced by retinoid treatment (27, 28, 36). The induction of RARβ by retinoids is not surprising because the RARβ gene promoter contains a RARE (5-8). However, it was not clear whether this induction is important for the overall effect of ATRA on cell phenotype, namely whether RARβ increase plays a role in growth inhibition. Our study has addressed this question by blocking RARβ induction by ATRA using a retroviral vector harboring antisense RARβ. The results show clearly that the antisense vector was effective in decreasing the
level of the RARβ protein and that this was accompanied by a decrease in the response of the transfected cells to two RAR-selective retinoids (ATRA and Ch55). Thus, our data support the conclusion that the induction of RARβ by retinoids may be an early step in the cascade of events leading to growth inhibition.

We used the H157 lung carcinoma cells, which do not express RARβ constitutively but can be induced to express this receptor after ATRA treatment. These cells were, therefore, a good system to explore the role of RARβ induction in mediating the growth inhibitory effects of retinoids. We used for the first time antisense RARβ to block induction of RARβ by retinoids. This approach does not involve severe selective pressure as isolation of transfectants expressing sense RARβ would because the H157 cells do not express endogenous sense RARβ. Therefore, the expression of exogenous antisense RARβ should not affect them until they are treated with ATRA. Our present study provides the first direct evidence supporting the important role of RARβ induction in mediating growth inhibitory effects of retinoids in human cancer cells. Our in vitro finding that induction of RARβ by retinoids is important for growth inhibition may explain the correlation that we had established previously in vivo between the induction of RARβ and clinical response in patients with oral premalignant lesions (27) and patients with renal cell carcinoma (16).

Antisense RARβ2 has been used previously to generate transgenic mice with reduced levels of RARβ2. Interestingly, mice expressing the antisense RARβ2 transgene developed lung carcinomas, demonstrating that partial inactivation of RARβ2 predisposes the mice to lung cancer (37). The precise function of RARβ2 is still unknown, however, recent studies with F9 mouse teratocarcinoma cells rendered RARβ2 null by homologous recombination have found that the cells become resistant to growth arrest by ATRA. In addition, the cells were somewhat
compromised in their ability to undergo differentiation by ATRA and showed a markedly lower induction of several ATRA-responsive genes (38). These results suggest that RARβ is required for ATRA-induced growth arrest in F9 cells.

Severe defects in RARβ expression have been observed in cells where ATRA treatment cannot induce RARβ expression (39-41). Previous studies have demonstrated that transfection of RARβ sense expression vectors into cells deficient in this receptor resulted in restoration of growth inhibition, apoptosis, and decrease tumorigenicity by retinoids (21-25). Thus, both induction of endogenous RARβ by retinoids (this study) as well as expression of exogenous RARβ (studies by others) may lead to a similar result, namely growth inhibition by retinoids. However, the role of induction of endogenous RARβ by retinoids may be more relevant to explain ongoing clinical chemoprevention trials with retinoids.
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The abbreviations used are: RAR, retinoic acid receptor; RXR, retinoic X receptor; ATRA, all trans retinoic acid; RARE, retinoic acid responsive element; Ch55, (E)-4-[3-(3,5-di-tert-butylphenyl)]-3-oxo-1-propenylbenzoic acid.
Fig. 1. Structure of the retroviral control vector LNSX and the vector LNASβVI containing six antisense RARβ sequences.

Fig. 2. Detection of antisense RARβ mRNA (A) and RARβ protein induction (B) in H157 cells transfected with retroviral vectors. A, Total RNA was prepared from different H157 transfectants (LNSX, vector only; LNASβ, anti-sense RARβ transfectants) and subjected to Northern blotting as described under “Experimental Procedures”. B, Cells were culture without (-) or with 1 µM ATRA (+) for 5 days and refed fresh medium containing ATRA every 2 days. Nuclear extracts were prepared and subjected to Western blot analysis 24 h after the last feeding as described under “Experimental Procedures”. NS, non-specific band.

Fig. 3. Suppression of ATRA-induced RARE binding activity (A) and RARE transactivation (B) but not AP-1 transactivation (C) in antisense RARβ transfected H157 cells. A, Nuclear proteins used in this assay were the same as those used in Western blotting (Fig. 2) and gel supershift assay was performed as described under “Experimental Procedures”. B and C, Cells were grown for 24 h in six-well plates and then co-transfected with a reporter plasmid (RARE)₃-TK-Luc (B) or AP-1-TK-Luc (C) and β-gal enzyme-encoding plasmid using lipofectamine reagent for 6 h. After treatment with 10⁻⁶ M ATRA for 24 h, the cells were subjected to luciferase activity assay and β-gal activity assay as described under “Experimental Procedures”. Column, means of triplicate determinations; bars, SD. The numbers on top of the bars (B) represent the fold induction of RARE transactivation by ATRA over DMSO control.
Fig. 4. Decreased cell responsiveness to retinoid treatment in antisense RARβ transfected H157 cells in comparison with vector only transfected H157 cells. Growth inhibitory effects of ATRA and Ch55 were analyzed by estimating total cell population size (A) or number of colonies formed on culture dishes surface (B) as described under “Experimental Procedures”. For the colony formation assay (B), cells were continuously treated with ATRA (2.5 µM) and Ch55 (1 µM) for 12 days and refed fresh medium every 3 days. Points (A) or columns (B), means of quadruplicate (A) or triplicate (B) determinations; bars, SD.
Figure 1

[Diagram showing LNSX and LNASSβVI structures with LTR, NEO, SV, ASβ, and pA markers]
Figure 2

A

Antisense RARβ

GAPDH

28S

B

ATRA: - + - + - + - + - +

RARβ

NS
Figure 3

[Image of a gel electrophoresis diagram with bands labeled as 'supershifted bands', 'shifted bands', and 'free probe'.]
Evidence that retinoic acid receptor β induction by retinoids is important for tumor cell growth inhibition
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