The Targeted Disruption of Both Alleles of RARβ₂ in F9 Cells
Results in the Loss of Retinoic Acid-associated Growth Arrest*

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Teresa N. Faria†, Cathy Mendelsohn§, Pierre Chambon‡, and Lorraine J. Gudas†

From the †Department of Pharmacology, Weill Medical College of Cornell University, New York, New York 10021, the §Department of Urology, Columbia-Presbyterian Medical Center, New York, New York 10032, and the ‡Institut de Genetique et de Biologie Moleculaire et Cellulaire, College de France, BP 163, 67404 Illkirch Cedex, France

F9 teratocarcinoma cell lines, carrying one or two disrupted alleles of the RARβ₂ gene, were generated by homologous recombination to study the role of RARβ₂ in mediating the effects of retinoids on cell growth and differentiation. Retinoic acid (RA) does not induce growth arrest of the RARβ₂−/− cells, whereas the F9 WT and RARβ₂+/− heterozygote lines undergo RA-induced growth arrest. The RARβ₂+/− lines also exhibit a faster cell cycle transit time in the absence of RA. The RARβ₂−/− stem cells exhibit an altered morphology when compared with the F9 WT parent line, and after RA treatment, the RARβ₂−/− cells do not exhibit a fully differentiated cell morphology. As compared with F9 WT cells, the RARβ−/− cells exhibited a markedly lower induction of several early RA-responsive genes and no induction of laminin B1, a late response gene. The induction of RA metabolism in the F9 RARβ₂−/− cells following differentiation was not impaired. The research presented here, and prior research suggest that RARβ is required for RA-induced growth arrest in a variety of cell types and that RARβ also functions in mediating late responses to RA. These findings are significant in view of the reduced expression of RARβ transcripts in a number of different types of human carcinomas.

All-trans-retinoic acid (RA) is one of the most biologically active retinoids. RA exerts its effects, in part, by acting through two types of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (1–3), both of which are members of the nuclear receptor superfamily. Each of these RAR and RXR receptors has three isotypes (α, β, and γ), which are encoded by separate genes. In addition, for each RA iso-type, there are several isoforms, generated by differential promoter usage and alternative splicing (1, 3).

RARβ is one of the subtypes of the retinoic acid receptors. The RARβ gene has four isoforms: β₁, β₂, β₃, and β₄. β₂ is the most abundant RARβ isoform (3–6). There is a very high affinity retinoic acid-responsive element in the promoter of the RARβ₂ and RARβ₃ isoforms (4, 7–9), which is associated with the rapid transcriptional activation of RARβ₂ by RA in a variety of cells (4, 7, 8, 10–12). RARβ exhibits a restricted pattern of expression during development as well as in the mature organism (4, 6, 13, 14). This pattern of expression is different from those of the other RARs and suggests that RARβ performs specific functions distinct from those of RARα and RARγ.

F9 teratocarcinoma cells (15) express all known RA receptors, but RARβ mRNA is only present in high amounts after RA addition (10), consistent with the fact that RARβ₂ is the predominant RARβ isoform expressed in F9 cells (4, 6). F9 cells have recently been used in our laboratories to inactivate the RARα and RARγ genes by homologous recombination (16, 17). Both F9 RARα and RARγ null cell lines exhibit marked modulation of a variety of genes when compared with the F9 wild-type cells. This gene knockout approach has allowed the identification of a series of genes that are direct or indirect targets of RARα or RARγ, such as the “homeobox” genes of the Hoxb and Hoxa clusters, and the genes encoding the extracellular matrix proteins laminin and collagen IV(α1) (16, 17).

RARβ mRNA expression has been reported to be greatly reduced in breast cancer cells (18, 19), oral and epidermal squamous cell carcinoma (cell lines and tissues) (20–24), and lung carcinoma lines and tissues (25–28). In breast cancer cell lines, RA is not able to induce RARβ mRNA expression, even though these cells can transcriptionally activate an exogenous RARβ RARE (retinoic acid response element) (19). When the expression of the RARβ gene is restored in breast cancer cell lines via an exogenous RARβ cDNA expression vector, the cells acquire sensitivity to RA-mediated apoptosis and growth arrest (29–31). Transfection of a human epidermoid lung cancer cell line with RARβ causes decreased tumorigenicity (32). In addition, overexpression of RARβ₂ in HeLa cells induces growth inhibition (33), while expression of RARβ antisense mRNA decreases RA sensitivity in responsive cell lines (31) and causes an increased frequency of carcinomas in transgenic mice (34). The loss of RARβ may be an essential step in neoplastic progression, since there is evidence of a progressive decrease in RARβ mRNA expression during breast carcinogenesis (35) and greatly reduced RARβ mRNA expression in morphologically normal tissue adjacent to breast carcinomas (36). Therefore, characterizing the role of RARβ in the control of RA-mediated differentiation and growth arrest may lead to a greater understanding of the mechanisms underlying the development and progression of cancer.

We have generated RARβ₂ knockout F9 cells by homologous recombination in order to study the role of RARβ₂ in mediating the effects of retinoids on cell growth and differentiation. RA is incapable of inducing growth arrest in these RARβ₂−/− cells, indicating that in F9 cells RARβ₂ is required for the growth inhibitory actions of RA. This finding is significant in light of the reduced expression of RARβ transcripts in a number of carcinomas, and it suggests that RARβ could regulate RA-induced growth arrest in a variety of cell types.
The RARβ2 disruption vector has been described in detail previously (37). F9 WT cells were cultured under standard conditions, and electroporation was performed as described previously (16, 17, 38). The cells were treated with 1 μM all-trans-RA and all-trans-ROL (Sigma), all-trans-4-oxoROL (Hoffman-LaRoche, Nutley, NJ), 4-HPR (fenretinide, R. W. Johnson Pharmaceutical Institute, Raritan, NJ), BMS 188,649 (Bristol-Myers Squibb Pharmaceutical Research Institute, Buffalo, NY) and 9-cis-RA (Hoffman-LaRoche, Nutley, NJ). [3H]RA was purchased from NEN Life Science Products. Southern and Northern analyses were performed as described (41). For Northern analyses, 10 μg of total RNA were loaded per lane and the signals were quantitated by phosphorimaging (Molecular Dynamics). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed under standard conditions as described (42) using the primers described previously (37) for 30 cycles. The probes for the Northern blots have been described previously (16, 17), except for the murine p450RAI (CYP26) probe, which was purchased from Genome Systems, St. Louis, MO (Expressed Sequence Tag Database accession no. AA 239785). Western blot analysis was performed as described (42, 43). Cleared lysates were analyzed for protein content by the Bradford method (Bio-Rad), and 150 μg of protein was loaded per lane. A rabbit polyclonal antibody (1:10 000) directed against RARβ (RPβF2; Ref. 44) or a mouse monoclonal antibody (1:1000) directed against RXRa (4BX3A2; Ref. 45) were used. Immune complexes were detected with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse serum (1:2000), respectively, and the SuperSignal Ultra Chemiluminescent Substrate (Pierce). Extraction of retinooids and high performance liquid chromatography (HPLC) were performed as described (39, 46).

RESULTS

Generation of Targeted Disruptions in the RARβ Gene—The RARβ2 isoform was inactivated with a disruption vector described previously (37). The neomycin-resistance gene was inserted into the exon 4 of the RARβ gene, which encodes the 5′-untranslated and the A2 region sequences of the RARβ isoform. The insertion of the neomycin resistance gene in this region disrupts only the RARβ2 isoform of the RARβ gene. F9 WT cells were electroporated and selected in G418. Resistant colonies were isolated and screened by Southern analysis for the expected change in size of the 6.5-kb wild-type (WT) KpnI genomic fragment (the mutated allele is 4.3 kb), using a probe derived from DNA sequence located 5′ of the sequence contained in the targeting construct. Two heterozygous lines were generated, F9 WT-β2–291 and F9 WT-β2–270, out of a total of 800 colonies screened (Fig. 1A). To target the second allele, the heterozygous lines were selected in high concentrations of GA18 for 21 days (16, 17, 38). One clone was isolated, out of 300 surviving colonies (F9 WT-β2–270–110), as a result of a second recombination event in which the second WT allele was disrupted (Fig. 1A). This clone was then subcloned, and eight subclone lines were generated that showed similar behavior (data not shown).

In order to show that the RARβ2–/– line lacked detectable RARβ2 mRNA, RT-PCR analysis was performed (Fig. 1B). Whereas bands of the appropriate size for RARβ2 were detected in the F9 WT and RARβ2+/– 270 lines, no bands of that size were detected in the RARβ2–/– line. Thus, the RARβ2–/– cells do not express the RARβ2 transcripts which are expressed in the F9 WT cells. The expression of retinoic receptors was evaluated by Northern and Western analysis (Fig. 1C). As expected, there is no detectable induction of RARγ transcripts upon treatment of the F9 RARβ2–/– cells with 1 μM RA. In contrast, in the heterozygous lines and the F9 WT line, there was a large (∼20-fold) induction of the RARγ transcripts after RA treatment. The expression of RXRα and RXRβ mRNA was similar in all the lines. The expression of RXRβ mRNA increased slightly (approximately) 2-fold after RA treatment of the F9 RARβ2–/– cells, in contrast to the decrease in RXRγ mRNA observed in RA-treated F9 WT and RARβ2+/– cells. The levels of RXRα protein (Fig. 1C) were lower in the RARβ2+/– and RARβ2–/– than in the wild type cells. In summary, the RARβ2–/– line has a lower level of RXRα protein and (after RA treatment) a higher amount of RARγ mRNA. The other retinoid receptors are present at levels similar to those seen in F9 WT cells (Fig. 1C).

The levels of the RARβ protein in the F9 WT, F9 RARβ2+/–, and F9 RARβ2–/– cell lines were evaluated by Western analysis (Fig. 1D). After 48 h of RA treatment, the RARβ protein is induced in the F9 WT cells and is induced to a lesser extent in the F9 RARβ2+/– cells. No RARβ protein was detected in the F9 RARβ2–/– cells (Fig. 1D).
presence of 1 replicate wells at a density of 3000 cells/well in the absence or in the presence of 1 μM RA, and cells were counted on the indicated days. The results are plotted as cell number versus day of culture. Note the different y axis scales in each of the four graphs. Data points, means of trilplicate samples; bars, S.E. This experiment was repeated three times with similar results.

Analysis of the Growth of the F9 RARβ2−/− Cells—The responsiveness of the F9 RARβ2−/− line to RA-induced growth arrest was evaluated (Fig. 2). Unlike the F9 WT line and the heterozygote lines, the F9 RARβ2−/− line did not grow arrest in response to RA. Other retinoids and retinoid agonists were tested, including a RXR pan-agonist (BMS 188,649), and these were also not able to induce growth arrest in the F9 RARβ2−/− cell line (Fig. 3), although they were effective to varying degrees in arresting the growth of the parent F9 WT cell line. This suggests that RARβ2 is required for all of these compounds to exert their growth inhibitory effects in F9 cells.

Interestingly, both of the independently generated F9 RARβ2+/− heterozygote lines grow faster that the WT F9 cells in the absence of RA (Fig. 2, note the different y axis scales). The mean doubling time for the parent WT cell line was 22 h, whereas the F9 RARβ2+/− 270 heterozygote line had a mean doubling time of 17 h, indicating that reduced levels of RARβ2 protein are associated with a decrease in the cell cycle transit time.

Morphological Characterization of the F9 RARβ2−/− Cells—The morphology of the RARβ2−/− cells is markedly different from that of the parent F9 WT cells (Fig. 4). In the absence of RA, there are noticeable differences between the F9 WT cells and the F9 RARβ2−/− cells; the latter clump together in cell aggregates, which do not exhibit the irregular borders characteristic of F9 WT cells. Following RA treatment, F9 WT cells form long cellular processes that are absent in the RARβ2−/− cells, which look like undifferentiated cells (Fig. 4). F9 WT cells exhibit even more dramatic morphological alterations after the addition of cAMP and theophylline (47), unlike the RARβ2−/− cells. It should be noted that it is possible to observe a few morphological changes occurring in the F9 RARβ2−/− cells after treatment with RA; the RA-treated F9 RARβ2−/− cells become more flattened and exhibit more vacuoles. The F9 RARβ2+/− cells exhibit a similar morphology to F9 WT cells in both the absence and presence of RA (data not shown), indicating that both alleles of RARβ2 must be inactivated in order to observe alterations in the morphological phenotype.

Effects of RA on Gene Expression in F9 RARβ2+/− and F9 RARβ2−/− Lines—The F9 WT, F9 RARβ2+/−, and F9 RARβ2−/− cell lines were cultured in the presence of 1 μM all-trans-RA for the indicated times, and the expression of several genes which are transcriptionally activated by RA was evaluated (Fig. 5, A and B). In the heterozygous F9 RARβ2+/− lines, the induction of laminin B1, CRABP II, and P450RAI (CYP26) by RA was similar to or slightly lower than that in the F9 WT cells. The expression of the REX-1 gene, which in F9 WT cells is reduced similarly in the RARβ2−/− cells was that Hoxa-1 gene was always induced to a greater extent at earlier time points in the heterozygote lines than in the F9 WT cells. The expression of the REX-1 gene, which in F9 WT cells was reduced by ~2-fold after 48 h of RA treatment, was reduced similarly in the RARβ2−/− cells (2.4-fold). The greatest difference between F9 WT and F9 RARβ2−/− cells was that the Hoxa-1 gene was always induced to a greater extent at earlier time points in the heterozygote lines than in the F9 WT cells. In the experiment shown, there was an 18-fold induction of Hoxa-1 mRNA in the heterozygote lines cultured in the presence of RA for 48 h versus a 4.7-fold induction in F9 WT cells cultured under the same conditions (Fig. 5, A and B). The increased expression of Hoxa-1 in the heterozygote lines was transient, and after 96 h of RA treatment, the levels of Hoxa-1
mRNA were comparable in the F9 WT and the RARβ2+/− lines (7.3- and 6.5-fold induction). In the F9 RARβ2−/− line, the expression of early response genes such as p450RAI and CRABP II was RA-responsive, although the magnitude of the induction of these mRNAs was much lower in the F9 RARβ2−/− cells than in the F9 WT cells.

In contrast, the induction of the Hoxa-1 message after 48 h of RA treatment was only 2-fold lower in the RARβ2−/− cells than in the F9 WT cells. The reduction in the levels of REX-1 message after 24 h of RA treatment was comparable in the RARβ2−/− cells and the F9 WT cells (Fig. 5, A and B). Interestingly, the reduced REX-1 mRNA levels were not maintained in the RARβ2−/− cells, and by 96 h REX-1 levels were the same as those in the untreated cells (see Fig. 5B for normalized data). Laminin B1, a late response gene, was not induced in the RARβ2−/− cells (Fig. 5, A and B). In summary, the F9 RARβ2−/− cells exhibited a reduction in the magnitude of the induction of a number of RA-responsive genes following RA treatment.

**DISCUSSION**

We have previously reported the generation of F9 teratocarcinoma cell lines carrying targeted disruptions of the RARα and RARγ genes (16, 17). We now continue the characterization of the roles of the RARs in mediating RA-induced F9 cell differentiation by generating cell lines carrying one or two disrupted alleles of the RARβ2 gene. The RARβ2 disruption completely abrogates the growth arrest that is one of the hallmarks of RA action in a variety of cell types. The F9 RARβ2−/− cells did not growth arrest in response to RA or a variety of other retinoids (Figs. 2 and 3). Thus, the data presented in this report, together with prior data from various F9 RAR and RXR knockout lines, strongly argue for a critical role for RARβ2 in...
mediating growth arrest in response to RA in F9 cells (Figs. 2 and 3). Whereas in the absence of RARβ2 no growth arrest in response to RA is observed in the F9 RARβ2−/− cells (Fig. 2), the RARα−/− and RARγ−/− lines grow arrest like WT cells, and the RXRα−/− and RXRγ−/− RARβ2−/− lines partially growth arrest after RA addition (16, 17, 48–50). Only the RXRα−/− RARγ−/− line shows no growth arrest in response to RA. Although in this line RARβ2 mRNA induction is decreased by 2–3-fold (49), this relatively small impairment of RARβ2 expression probably does not contribute to the observed lack of growth arrest of the RXRα−/− RARγ−/− cells (50). Since the RARβ2−/− line has a lower level of RXRα protein than F9 WT cells (Fig. 1C), it is possible that in these RARβ2−/− cells the RXRα:RARβ heterodimer is involved in mediating the RA-induced growth arrest.

The mechanism by which RA induces growth arrest is not known, although several reports have linked RARβ to growth regulation and apoptosis (see Introduction). Interestingly, the two independent F9 RARβ2+/− heterozygote lines, which arrested their growth normally in response to RA, showed a faster cell cycle transit time in the absence of RA (Fig. 2). At present we do not have an explanation for this observation. The finding that two independently derived RARβ−/− lines grow faster than the F9 WT cells in the absence of RA (Fig. 2) is particularly intriguing in light of the greatly reduced expression of RARβ in a variety of human cancers and preneoplastic cells (see Introduction). Our data support the hypothesis that reduced levels of RARβ confer a selective growth advantage to preneoplastic cells and, therefore, that the loss of one allele of RARβ may be a key step in neoplastic progression.

The RARβ2 disruption alters the morphology of these cells as compared with the F9 WT parent line (Fig. 4). In the absence of RA, this difference in morphology may result from the presence of low levels of RARβ2 transcripts in the F9 WT cells and in the RARβ2+/− heterozygote cells, but not in the RARβ2−/− line. After RA treatment, the F9 RARβ2−/− cells do not exhibit a typically differentiated morphology. A partial or complete lack of morphological differentiation following RA treatment has been previously observed in the F9 RARγ−/− lines (16), the F9 RXRα−/− lines (48), and in the double mutant lines F9 RXRα−/−:RARγ−/− and F9 RXRα−/−:RARβ−/− (50).

There was a markedly lower induction of several RA-responsive genes in the F9 RARβ2−/− cells when compared with F9 WT cells (Fig. 5). In contrast, in the F9 RARα−/− and RARγ−/− lines, the inactivation of RARα and RARγ respectively, caused a severely impaired induction of particular RA-responsive genes, whereas the induction of other RA-responsive genes was like that in F9 WT cells (16, 17). The modulation of gene expression in response to RA in the RARβ2−/− cells was actually strongest at early time points when compared with the F9 WT cells. This RA induction of gene expression diminished at later times, and laminin B1, a late response gene, was not induced in the F9 RARβ2−/− cells. Our data suggests that in F9 WT cells the initial response to RA is mediated via the RARα and RARγ receptors, but that the large increase in RARβ2 receptors after RA treatment (which occurs after ~16–24 h) is required for the maximal expression of RA-responsive genes at later times after RA addition. Alternatively, the lack of growth arrest in response to RA observed in the RARβ2−/− cells may contribute to a reduction in the magnitude of the differentiation response in these cells. That the initial RA response with respect to transcriptional activation of RA-responsive genes such as Hoxa1 is present in the F9 RARβ2−/− cells is consistent with prior data indicating that RXRα and either RARα or RARγ are the essential receptor pairs for these early responses during the RA-induced conver-

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