9-cis Retinoic Acid Inhibits Growth of Breast Cancer Cells and Down-Regulates Estrogen Receptor RNA and Protein

Mark Rubin, Eyal Fenig, Angelika Rosenauer, Celia Menendez-Botet, Charles Achkar, Jacqueline M. Bentel, Joachim Yahalom, John Mendelsohn, and Wilson H. Miller, Jr.

ABSTRACT

All-trans retinoic acid (tRA) inhibits growth of estrogen receptor-positive (ER+) breast cancer cells in vitro, and a variety of retinoids inhibit development of breast cancer in animal models. 9-cis retinoic acid (9-cis RA) is a naturally occurring high affinity ligand for the retinoid X receptors, as well as the retinoic acid receptors (RARs). Whether 9-cis RA has a different spectrum of biological activity from tRA, which only binds RARs with high affinity, is largely unknown. We studied the effects of 9-cis RA on growth and gene expression in ER+ and ER− human breast cancer cells. 9-cis RA inhibited the growth in monolayer culture of several ER+, but not ER−, cell lines in a dose-dependent manner. Growth inhibition and morphological changes by 9-cis RA were similar to those of tRA, suggesting that the ability to bind both RAR and retinoid X receptors did not significantly augment growth inhibition or confer sensitivity to tRA-resistant lines. MCF-7 cells exposed to 9-cis RA showed a dose-dependent accumulation in G1. Northern analyses showed that RAR-α and RAR-β were not significantly regulated, while RAR-γ was up-regulated and retinoid X receptor α was down-regulated by 9-cis RA. Since interactions between tRA and ER-dependent transcription have recently been reported, we investigated whether these retinoids regulate expression of ER itself or estrogen-responsive genes. Both 9-cis RA and tRA induce down-regulation of ER mRNA and protein in MCF-7 cells. 9-cis RA down-regulates the estrogen-responsive genes PS2 and PR in MCF-7 cells as reported previously for tRA. In several ER-positive subclones, we found that the degree of ER expression and regulation, but not always estrogen-sensitivity, correlates with the growth-inhibitory effects of 9-cis RA. Further, in an ER−, retinoid-unresponsive breast cancer cell line, induced ER expression confers responsiveness to retinoid growth inhibition. These data, combined with reports of additive growth inhibition of tRA and tamoxifen in vivo, suggest that 9-cis RA might augment the ability of tamoxifen to inhibit growth of ER+ breast cancer cells in vivo.

INTRODUCTION

Retinoids are a group of natural and synthetic vitamin A derivatives that modulate growth and differentiation of many tissues and cell lines. Retinoidic acid derivatives have been investigated in several systems as potential agents of chemoprevention and treatment of human cancers (reviewed in Ref. 1). Retinoids bind to a group of nuclear receptors which form part of a larger superfamily of steroid/thyroid hormone nuclear receptors (2). The retinoid nuclear receptors are divided into RARs and RXRs subfamilies each composed of three subtypes (α, β, and γ). RAR has high affinity for both tRA and 9-cis RA, while RXR has high affinity for 9-cis RA only (3, 4). 9-cis RA is therefore a bifunctional ligand, capable of binding to both RAR and RXR. The potential for activation of RXR-selective pathways raises the possibility of biological consequences for 9-cis RA, in addition to those actions mediated through RAR. This study was initiated to examine the actions of 9-cis RA in breast cancer cell lines.

There is evidence that retinoids may induce differentiation and inhibit the growth of human breast cancer. Retrospective analyses have suggested a link between retinol consumption or serum levels of retinol and breast cancer incidence (5, 6). Mammary tumor formation in rodents induced by chemical carcinogens has been prevented by administration of retinoids (7–9). Many human breast cancer cell lines are growth inhibited by retinoids (10). Interestingly the response to tRA appears to correlate with expression of another nuclear hormone receptor, ER; ER+ cell lines are growth inhibited by retinoids and ER− cell lines are resistant to the effects of retinoids (10, 11). Retinoic acid nuclear receptors are expressed by both sensitive and resistant breast cancer cell lines, and there is some evidence that RAR expression may be higher in ER+ lines (12). There is also evidence that retinoids may act on estrogen pathways. The estrogen-stimulated expression of two genes, PS2 and PR, has been shown to be inhibited by tRA in ER+ cell lines (13, 14). In addition, transactivation assays show inhibition of estrogen response by RA (15), and expression of RXR inhibits estrogen gene activation (16). The down-regulation of target genes in the ER pathway suggests that retinoids may in part mediate their growth-inhibitory effects through interference of ER signal transduction. In this study, we report that 9-cis RA regulates the expression of both ER itself and downstream estrogen-induced genes. We further report that ER expression and regulation by RA correlates with the growth-inhibitory effects of 9-cis RA in several ER-positive subclones and that induction of ER expression in an ER− breast cancer line can confer RA responsiveness.

MATERIALS AND METHODS

Materials. FBS was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Propidium iodide was obtained from Sigma Chemical Co. (St. Louis, MO), and RNase A was purchased from United States Biochemical (Cleveland, OH). Phenol red and phenol red-free DME:F12 (1:1) high glucose medium, penicillin, streptomycin, and L-glutamine were obtained from Sloan-Kettering Institute Central Media facility. αMEM and phenol red-free αMEM were obtained from Gibco-BRL (Burlington, Ontario, Canada). tRA was purchased from Sigma, and 9-cis RA was kindly provided by Dr. Alex Wood (Hoffmann-La Roche, Nutley, NJ). The compounds were dissolved at a concentration of 10−2 M in DMSO, maintained in the dark at −70°C, and all manipulations were performed in a light-reduced environment. Final dilutions of all test compounds were made in culture medium. Final solvent concentrations during incubation were always <0.1% (v/v). These concentrations had no effect on cell growth.

Received 5/26/94; accepted 10/19/94.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by a Career Development Award from the American Cancer Society (W. M.), the Lederle Scholar in Clinical Oncology Fund (M. R.), the Memorial Sloan-Kettering Cancer Center, New York, New York 10021 [M. R., E. F., C. M-B., C. A., J. M. B., J. Y., J. M.], and Lady Davis Institute, Jewish General Hospital and McGill University, 3755 Cote-Sainte-Catherine, Montreal, Quebec H3T 1E2, Canada [A. R., W. H. M.].

2 To whom requests for reprints should be addressed.
Hybridization probes were isolated from plasmids containing: a 0.56-kilobase PstI restriction fragment of the human pS2 gene (ATCC, Rockville, MD); a 1.3-kilobase EcoRI restriction fragment of the human estrogen receptor gene (ATCC); a 0.6-kilobase PstI restriction fragment of the human RAR-α cDNA (18); a 1.4-kilobase SST1/HindIII restriction fragment of the human RAR-β cDNA (19); a 1.9-kilobase EcoRI restriction fragment of the human RAR-γ cDNA (20); a 1.8-kilobase EcoRI restriction fragment of the human RXR-α cDNA (21); a 2.3-kilobase EcoRI restriction fragment of the human RXR-β cDNA (a gift of R. M. Evans), a 700-base PCR-amplified cDNA fragment of human AR; a 700-base EcoRI fragment of hIGF-1R (ATCC); and a 1.2-kilobase restriction fragment of the chicken glyceraldehyde 3-phosphate dehydrogenase cDNA (22).

**Cell Lines.** Early passage MCF-7, T47-D, ZR75-1, BT-474, MDA-MB-231, and MDA-MB-468 were obtained from the ATCC. S30 cells were a generous gift from Dr. V. Craig Jordan (23). BAC2 is an MCF-7 line kindly provided by M. Osborne (New York Hospital, New York, NY) that was originally obtained from the Michigan Cancer Foundation. Karyotype analysis, performed by the cytogenetics laboratory at Memorial Sloan-Kettering Cancer Center, showed that the MCF-7 (ATCC) line and BAC-2 had very similar structural abnormalities and marker chromosomes, while they differed in average chromosome number.

**Cell Culture.** MCF-7 cells were maintained in phenol red DME:F12 high glucose medium containing 5% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. T47-D, ZR75-1, BT-474, MDA-MB-231, and MDA-MB-468 were maintained in phenol red DME:F12 high glucose medium containing 10% FBS, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

**Cell Proliferation Studies.** MCF-7 and MDA-MB-231 were plated at 1.5 × 10^4 and 1.0 × 10^4 cells, respectively, in 12-well plates. T47-D, ZR75-1, BT-474, and MDA-MB-468 were plated at 3.5 × 10^4, 4.5 × 10^4, 5.0 × 10^4, and 5 × 10^4 cells/well, respectively, in 6-well plates. Media was changed 24 h later to control (DMSO, 0.1%) and experimental media containing indicated concentrations of retinoids. Cells were grown protected from light in a humidified incubator in 5% CO_2 at 37°C. Control and experimental media were renewed at the specified times. On the days indicated, triplicate wells were trypsinized and counted by Coulter Counter Model ZM. MDA-MB-231 cells and ER-expressing S30 transfectants were plated at 1 × 10^4 cells/well in 24-well plates in αMEM (GIBCO-BRL) with 5% FCS (MDA-MB-231) or phenol red-free αMEM with 5% FCS treated with dextran-coated charcoal as described (24). The following day, media was replaced with experimental media containing indicated concentrations of retinoids. Retinoid-containing medium was replaced on day 3 of treatment. Cell counts were obtained as above on quadruplicate wells at the indicated time points.

Trylitzed thyidine incorporation assays were performed on cells plated in 24-well plates at 1.6 × 10^3 cells/well in media containing 5% FCS. One μCi of [methyl-3H]thymidine (2 Ci/mmol, DuPont New England Nuclear) was added to each well on day 4 for 2 h. The cells were lysed in 0.1 N NaOH, extracted with trichloroacetic acid, and tritium incorporation was measured using a Beckman Instruments (Fullerton, CA) scintillation counter.

**Flow Cytometry.** Trypstinized cells were washed with DME:F12 medium containing 5% FBS followed by DME:F12 medium containing no serum and then DME:F12 medium containing 5% DMSO. Cells were resuspended at 10^6 cells/ml in 5 mM MgCl_2-10 mM Tris-Cl (pH 7.6)-0.5% NP40-50 μg/ml propidium iodide-100 μg/ml RNase A for 30 min at 37°C. Cell cycle analysis was performed on a Becton Dickinson FACScan Flow Cytometer using Cellfit software.

**HPLC.** Cells were plated at 1 × 10^6 in duplicate in 35-mm culture dishes. Media was changed after 24 h to contain 10^{-6} M tRA or 10^{-6} M 9-cis RA. The tRA used in these experiments contained less than 1% of the 9-cis isomer (data not shown). To explore whether interconversion of isomers tRA and 9-cis RA accounted for the similarity of responses to these agents, [3H]tRA metabolism in MCF-7 cells was analyzed by HPLC. tRA added to MCF-7 cells is rapidly metabolized, with the level of 9-cis RA never exceeding 1% of the total RA concentration throughout the course of the experiment (data not shown).

**Northern Analysis.** At the time points indicated, cells were washed twice with PBS and extracted with guanidine thiocyanate to prepare total cellular RNA (25). Ten μg of total RNA per lane from the indicated time points were loaded and electrophoresed on a 1% formaldehyde/agarose gel and blotted onto nitrocellulose filters. The filters were hybridized to probes radiolabeled by random priming, washed, and autoradiographed as described (26).

**ER and PR Protein Level Determination.** After the indicated times cells were washed twice with PBS, and all samples were kept on ice. Cells were scraped from plates and suspended in 400 mM sodium chloride-10 mM Tris-Cl (pH 7.4)-1.5 mM EDTA (pH 7.9)-1 μg/ml leupeptin-1 μg/ml aprotinin-1 mM ME-30 μM phenylmethylsulfonyl fluoride. After 1 h at 0°C, cells were centrifuged at 14,000 rpm in a microfuge and supernatant was collected for immunoassay of ER and PR using an EIA diagnostic kit (Abbott Diagnostics).

**RESULTS**

The effect of 9-cis RA on the proliferation of MCF-7 cells in tissue culture is shown in Fig. 1. Continuous exposure of cells to 9-cis RA for 7 days led to concentration-dependent inhibition of cell growth with >90% inhibition achieved at the highest retinoid concentration (10^{-5} M) and 50% inhibition at 10^{-7} M. As shown in Fig. 1, tRA and 9-cis RA resulted in similar growth inhibition at all dose levels. Morphological changes in MCF-7 after exposure to retinoids have been described (27). Flatting and spreading of cells in tissue culture exposed to 6 days of 9-cis RA were similar to that induced by tRA (data not shown). Growth inhibition by 9-cis RA was compared to tRA in other ER+ cell lines as shown in Fig 2. 9-cis RA showed a similar concentration-dependent inhibition of cell growth. ER− cell lines are known to be relatively resistant to the growth-inhibitory effects of tRA and other retinoids known to activate RARs (10, 11). We examined whether the potential additional stimulation of RXR-specific pathways might broaden the spectrum of growth inhibition of breast cancer cells. Two ER− cell lines, MDA-MB-231 and MDA-MB-468, were studied. As shown in Fig. 2, MDA-MB-231 growth responded to neither retinoid, while MDA-MB-468 showed minimal
Fig. 2. Growth of additional ER+ and ER- breast cancer cell lines after continuous exposure to 10⁻³ to 10⁻⁶ M 9-cis RA or tRA. Media was changed on day 3. Triplicate wells were trypsinized and cell number calculated by Coulter Counter on day 5 (T47D, ZR75-1, MDA-MB-468). ●, tRA; ○, 9-cis RA; points, mean; bars, SEM.

inhibition without significant dose dependency or difference between retinoids.

Growth inhibition was further explored by studying the effects of 9-cis RA on the cell cycle. Since retinoids have been shown to result in an accumulation of some cells in G₁, we studied whether the observed growth-inhibitory effects of 9-cis RA were reflected in altered cell cycle kinetics. MCF-7 cells were grown for 3 days in the presence of 9-cis RA and flow cytometry analysis showed a dose-dependent accumulation of cells in G₁ (Fig. 3). We then looked for changes in the expression of genes associated with the action of retinoids. To explore whether 9-cis RA regulates the expression of retinoid receptors, MCF-7 cells were grown in the presence of either retinoid and were harvested at the indicated time points. Nuclear receptors RAR-α (Fig. 4A) and RXR-β (data not shown) were expressed but not significantly regulated by either retinoid (data not shown). RAR-β mRNA was neither detected nor induced with 9-cis RA at the level of total cellular Northern analysis. After exposure to either retinoid, RXR-α mRNA was down-regulated while RAR-γ mRNA was up-regulated (Fig. 4A). Quantification of gene regulation by densitometry showed a 50% decrease of RXR-α and a 60% increase in RAR-γ after 1 day of 9-cis RA (Fig. 5).

We next found that 9-cis RA affects the expression of other nuclear steroid hormone receptors in MCF-7 (Fig. 4B). Beginning at 6 h, both ER and AR mRNA were down-regulated. As shown in Fig. 5, down-regulation of ER mRNA as determined by densitometry was moderately greater for 9-cis RA (25% of control expression after 1 day) than for tRA (40% of control expression) and was maximal between days 1 and 3. As reported for tRA, 9-cis RA decreased expression of pS2, a target gene in the estrogen pathway (Figs. 4B and 5) (13). We examined whether these effects of 9-cis RA were dose dependent. Cells exposed to a lower concentration of retinoid (10⁻⁷ M) showed down-regulation of both ER and pS2 but to a lesser degree than that of 10⁻⁶ M retinoid (data not shown). To determine that this apparent regulation of ER mRNA was not a function of the state of confluence of the cultures, we measured ER mRNA levels in cultures of untreated MCF-7 cells varying in degree of confluence and found no difference (data not shown).

Since expression of IGFs has been associated with the development of estrogen independence (28), and since tRA has been reported to inhibit IGF-stimulated growth of MCF-7 cells (29), we examined the effects 9-cis RA on expression of the IGF-1R. Beginning at 6 h, total cellular mRNA for IGF-1R was down-regulated by 9-cis RA, with a maximum decrease of 75% at 3 days (Figs. 4B and 5). Thus, treatment with 9-cis RA reduced expression of two steroid hormone receptors.
Fig. 3. Flow cytometry analysis of MCF-7 cells grown continuously in the presence of DMSO or the indicated retinoid for 3 days. The percentage of cells in G1 or S phase is shown for each treatment. Points, mean of 3 separate flow cytometric analyses; bars, SEM.

Fig. 4. Northern blot analysis of retinoid-induced changes in gene expression in MCF-7 cells. Total cellular RNA was isolated from MCF-7 celis grown in media containing 10^{-6} M 9-cis RA or tRA for 6, 24, 72, or 120 h. After electrophoresis and transfer, membranes were hybridized to 32P-labeled cDNA probes: RAR-a, RAR-y, RXR-a, and glyceraldehyde 3-phosphate dehydrogenase (A); and ER, AR, pS2, IGF-1R, and glyceraldehyde 3-phosphate dehydrogenase (B). Ethidium bromide staining of the agarose gel is shown as an additional control for the amounts of RNA loaded in each lane (10 μg).

(ER and AR), an estrogen regulated gene (pS2), and the receptors RXR-α and IGF-1R, while the expression of the RARs was unchanged (RAR-α, RAR-β) or modestly increased (RAR-γ).

We next sought to confirm that the regulation of ER seen at the level of RNA expression extends to significant differences in protein expression. As shown in Fig. 6, ER protein levels were down-regulated after 1, 3, and 5 days of exposure to 9-cis RA. This down-regulation of ER protein, although seen with both 9-cis RA and tRA, was consistently greater with 9-cis RA. Similarly, down-regulation of PR protein levels occurred with either retinoid (Fig. 6b). Both retinoids at 10^{-7} and 10^{-8} M caused smaller decreases in both ER and PR protein levels (data not shown).

As discussed above, ER+ cell lines generally are not growth inhibited by tRA. We have shown that two ER+ cell lines are similarly not growth inhibited by 9-cis RA. We asked whether, in ER+ cell lines, 9-cis RA also fails to regulate expression of genes regulated in the ER+ MCF-7 line. Since MDA-MB-231 cells do not express either ER or pS2 mRNA, we examined IGF-1R and RXR-α. Both were expressed but not regulated by either retinoid (data not shown).

RA has been reported previously to have no effect on or to increase ER expression in MCF-7 cells (13, 30), but MCF-7 cells are known to vary in biological properties and karyotype (31). We thus compared the MCF-7 line used in the above experiments with an MCF-7 line of different origin (designated BAC-2) that differed in its response to 9-cis RA. The BAC-2 line was less growth inhibited by either tRA or 9-cis RA than the ATCC MCF-7 line, as assayed by tritiated thymidine after 4 days (see Fig. 7a) or total cell number after 7 days of treatment (data not shown). We correlated these differences in growth inhibition with gene regulation. As shown above, 9-cis RA decreased expression of both ER and pS2 mRNA and ER and PR protein in ATCC MCF-7 cells. In contrast, BAC-2 showed no regulation of ER mRNA or protein expression by 9-cis RA (Fig. 7b and data not shown). Of note, ATCC MCF-7 cells were less dependent on estrogen for growth than were BAC-2 cells. Thus, a highly estrogen-responsive MCF-7 subclone in which 9-cis RA does not regulate ER expression is relatively resistant to retinoid growth inhibition. We then compared the effects of retinoids on a subclone of MCF-7 derived to express IGF-II with its control subclone. We have found that the IGF-II-expressing subclone, clone 9, is resistant to growth inhibition by either tRA5 or 9-cis RA (data not shown), while the control subclone, clone 1, is inhibited by both retinoids. While both retinoids decrease ER expression in clone 1, neither retinoid regulates ER in the retinoid-resistant clone 9 (data not shown).

To further explore the relationship between ER expression and

---

4 E. Fenig, unpublished data.

5 J. M. B., submitted for publication.
growth inhibition by retinoids, we obtained a subclone of the ER− MDA-MB-231 cell line, S30, transfected to constitutively express ER protein. Growth of S30 cells was significantly inhibited by 6 days exposure to $10^{-6} \text{ M}$ 9-cis or tRA, while the parental cell line was unaffected (see Fig. 8). An even more profound growth inhibition was seen at $10^{-5} \text{ M}$ 9-cis or tRA, with minimal effects on the parental cells. Thus, the partial reconstitution of the ER pathway provided by the introduction of ER into an ER− cell line permits the cells to respond to retinoids.

**DISCUSSION**

The experiments reported here establish that 9-cis RA inhibits the *in vitro* growth of ER+ human breast cancer cells. Changes in growth inhibition, G1 accumulation, and morphology induced by 9-cis RA are equal or greater than those induced by equimolar concentrations of tRA. HPLC analysis indicates that this similarity did not occur because of interconversion of tRA to 9-cis RA (see "Materials and Methods"). We found that 9-cis RA does not broaden the action of tRA to include ER− cells. These results suggest that if selective activation of RXR pathways by 9-cis RA occurs, it does not contribute substantially to the phenotypic responses examined in this study. Although 9-cis RA may be significantly more active than tRA in some cell systems (32), another recent study finding similar responses to 9-cis RA and tRA suggests that selective activation of RXR pathways does not result in alterations of the phenotype (33). The modestly greater effects of 9-cis RA on gene expression in the absence of new phenotypic changes or an expanded range of sensitive cells may be due to a different rate of metabolism of 9-cis RA than tRA in these cells. Although no *in vitro* studies have been reported, we have evidence that multiple dosing of 9-cis RA does not cause the same pharmacokinetic changes as tRA in mice (17).

To begin to gain insight into mechanisms by which 9-cis RA and other retinoids inhibit breast cancer cell growth, we examined the regulation of genes known to be important in the response to retinoids in other cells or in the estrogen-dependent growth of breast cancer cells. We found that 9-cis RA inhibits RNA expression of the two nuclear receptors ER and AR in a dose- and time-dependent fashion. Significant decreases in ER protein were confirmed by immunoassay of extracts of retinoid-treated cells. We have also found that tRA down-regulates AR RNA and protein in AR+ prostate cancer cells.6 Further, as reported for tRA in MCF-7 and other ER+ cells (13, 14), we found that both 9-cis RA and tRA decreased expression of the genes pS2 and PR. That these estrogen-stimulated genes are also down-regulated by retinoids suggests that the decreased ER expression may be physiologically meaningful. These findings are consistent with recent reports of interactions between retinoid receptors and other steroid hormone receptors (15, 16, 34).

We also examined the effects of 9-cis RA on expression of RAR and RXR family members. Consistent with a prior report of RAR

---

6 J. M. B., unpublished data.
expression and regulation by tRA in breast cancer cells (12). RAR-α and RAR-β are unregulated and unexpressed, respectively. However, 9-cis RA induces a moderate increase in RAR-γ and a decrease in RXR-α expression. Retinoid-induced increases in RAR-γ mRNA levels may be explained by a retinoid response element known to exist in the promoter of the RAR-γ2 isoform (35). Although the human RXR-α promoter has not been defined and expression and regulation of RXR-α has not been reported in breast cancer cells, an inhibition of RXR-α expression has been seen in acute promyelocytic leukemia cells (36).

It has been reported that ER expression in MCF-7 cells is not decreased after 6–10 days of tRA treatment (13). In contrast, we found ER RNA expression most down-regulated between 6 h and 3 days (Fig. 4B). One possible explanation of this difference is our finding that ER RNA expression returns toward the control level after 5 days of treatment (Fig. 4B), especially with tRA. An additional possible explanation for these different results lies in the variability of MCF-7 lines (31). RA-induced gene expression appears to vary considerably among cell lines. Our data show that not all ER+ breast cancer cells, even variously derived MCF-7 cells, show ER regulation by 9-cis RA. While we found that AR mRNA was decreased by RA treatment in MCF-7 cells, one recent report found AR down-regulation in the ER+ T47-D breast cancer cell line but not an MCF-7 subclone designated MCF-7M (37). Similarly, RAR-γ was not up-regulated in MCF-7M as it was in our MCF-7 line.

These differences raise the question of the biological significance of the expression and regulation of other nuclear hormone receptors by retinoids. We examined different subclones of MCF-7 and found that ER down-regulation by retinoids correlates with growth inhibition by the retinoids. An MCF-7 subclone transfected with a growth factor and showing partial estrogen independence (28) did not show regulation of ER or significant growth inhibition by retinoids. In the MCF-7 cell subclone designated here as BAC-2, which showed a decreased response to the growth inhibitory actions of 9-cis RA and tRA, neither retinoid decreased the expression of ER RNA or protein.

Thus, ER expression and regulation by RA correlates with the growth-inhibitory effects of 9-cis RA on several cell lines. This correlation suggests that the effects of retinoids on ER expression may have mechanistic significance. One possibility that must be considered is that retinoids may act as weak estrogens or as agonist/antagonists to the ER. We have performed experiments showing that the retinoid-induced inhibition of cell growth in ER-positive cells (Fig. 1) does not depend on the presence of estrogen in the media. MCF-7 cell growth is similarly inhibited by 10^{-9}–10^{-5} m RA in a dose-dependent manner when experiments are performed in chemically defined medium (data not shown). The recent literature provides additional compelling evidence to suggest that retinoids do not act as estrogens or antiestrogens in breast cancer cell lines. Additive effects of retinoids and antiestrogens (including 4-hydroxytamoxifen, 4-hydroxycyclophosphene, and LY117018) in breast cancer cell lines have been reported (38). Two recent studies show that RA inhibition of cell growth and estrogen-induced gene expression cannot be reversed by estrogens, as can tamoxifen-induced growth inhibition (15, 39). Three reports conclude from dose-response studies that there is no direct competition between retinoids and estrogens or progesterones for their respective receptors (16). It is our conclusion that retinoids inhibit ER activity in MCF-7 cells by actions downstream of ER.

However, these various cell lines clearly harbor many more differences than the expression or regulation of ER. We thus examined the effects of the induced expression of ER in a completely ER-independent ER+ cell line, MDA-MB-231. ER-expressing clones of this cell line have been constructed and shown to become growth-inhibited by estrogen by two groups, suggesting only a partial reconstitution of the ER-dependent transcription pathways present in naturally ER+ cells (23, 40). Our data show that even without an intact growth-stimulatory response to estrogen, the expression of ER in the S30-transfected clone renders the cells responsive to the growth-inhibitory effects of both retinoids. This supports the hypothesis that the expression of ER is a critical factor in the response to breast cancer cells to retinoids. Further studies may show which functional domains of ER are required for conferring retinoid response, the mechanism of retinoid regulation of ER and estrogen responsive genes, and whether other steroid hormone receptors could also confer retinoid sensitivity.

We have shown that 9-cis RA induces cell cycle arrest and growth inhibition in human breast cancer cell lines expressing ER. We found
that 9-cis RA regulates two retinoid receptor RNAs and consistently decreases expression of ER RNA and protein and of known estrogen-responsive genes in some but not all subclones of the human breast cancer cell line MCF-7. Although the mechanism of action of retinoids in these and other cell types remains unclear, we have presented evidence that expression and retinoid-regulation of ER are important to retinoid action. Considerable interest exists among clinical oncologists in using retinoids in chemoprevention and even selected metastatic cancers (41). Breast cancer has been suggested as a possible target for retinoids. These data support prior studies that suggest that most effective use of retinoids would be in tumors that retain ER. Further, since there is no evidence that retinoids can bind ER, the mechanisms of action of retinoids and traditional ER inhibitors such as tamoxifen are most likely different. tRA and tamoxifen have already been reported to produce additive growth inhibition in breast cancer cells (38), and the combination has been entered into Phase I breast cancer clinical trials (42). Since we found that retinoid sensitivity does not always correspond to estrogen sensitivity (in MCF-7 lines BAC-1 and BAC-2) and that a cell with a clearly abnormal response (MDA-231 transfected with ER) is inhibited by retinoids, it may be that response to retinoids will not always correlate with response to tamoxifen. Thus, combined retinoid-tamoxifen therapy might target subclones in a human tumor that would not respond to tamoxifen alone. Our data suggest that 9-cis RA, currently in Phase I clinical trials, is at least as effective as tRA in vitro. Studies of the metabolism of 9-cis RA in humans will show whether, as suggested in mice (17) and monkeys (43), this compound may not share with tRA the up-regulation of its own metabolism in humans.

ACKNOWLEDGMENTS

We thank A. DeBlasio for technical assistance.

REFERENCES


Downloaded from cancerres.aacrjournals.org on April 29, 2017. © 1994 American Association for Cancer Research.
9-cis Retinoic Acid Inhibits Growth of Breast Cancer Cells and Down-Regulates Estrogen Receptor RNA and Protein

Mark Rubin, Eyal Fenig, Angelika Rosenauer, et al.


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/54/24/6549

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.