Estradiol Induction of Retinoic Acid Receptors in Human Breast Cancer Cells

Shaun D. Roman, Christopher J. Ormandy, David L. Manning, Roger W. Blarney, Robert I. Nicholson, Robert L. Sutherland, and Christine L. Clarke

ABSTRACT

Retinoic acid inhibits proliferation and steroid receptor gene expression in human breast cancer cell lines. Retinoic acid receptors (RARα, -β, and -γ) are expressed in these cells and the expression of RARα is significantly greater in estrogen receptor (ER)-positive cells. This study was undertaken to determine whether the same relationship between RARα and ER gene expression was present in human breast cancers and to explore the possibility that the higher level of RARα in ER-positive cells was due to estrogen regulation of RARα gene expression. RARα and ER mRNA expression were determined by Northern blot analysis in 116 primary breast tumors; 94 (81%) tumors were ER-positive and of these 87 (93%) were also RARα-positive. The coexpression of ER and RARα was statistically significant (P = 0.0052 by χ² contingency analysis). There was also a positive correlation (by linear regression analysis) between the levels of expression of ER and RARα mRNA (r² = 0.251, P = 0.0001), which confirmed the relationship previously documented in breast cancer cell lines and suggested that RARα expression may be modulated in breast cancer in vivo by estrogens acting via the ER. The ability of estradiol to regulate RARα gene expression was examined in vitro using T-47D cells which had been rendered sensitive to estrogen by repeated passage in steroid-depleted medium. Estradiol increased RARα gene expression, but not that of RARβ or RARγ, in a concentration-dependent manner, with the effect being maximal at 10⁻¹⁰ M and less marked at higher concentrations. The effect was rapid, being detectable 1 h after and maximal 6 h after treatment with 10⁻¹⁰ M estradiol. Co-treatment of cells with estradiol and antiestrogens (tamoxifen or ICI 164384, 4 x 10⁻¹¹ M for 6 h) inhibited the estradiol induction of RARα gene expression, demonstrating that the effect was ER mediated. The estradiol sensitivity of the effect was underscored by the demonstration that addition of untreated serum to cells growing under steroid-depleted conditions was sufficient to induce maximal RARα gene expression. This effect was totally abolished by addition of ICI 164384. In summary, the demonstration that estradiol increased RARα mRNA levels in breast cancer cells supports the hypothesis that the correlation between RARα and ER gene expression in breast tumors and breast cancer cell lines is due to estradiol augmentation of RARα gene expression.

INTRODUCTION

The retinoid metabolites of vitamin A have been postulated to be both chemopreventative and chemotherapeutic cancer agents on the basis of human and animal studies. Low dietary or serum levels of β-carotene are associated with increased risk of a number of epithelial malignancies (1–3), and retinoids are known to act in combination with hormone deprivation in the prevention of mammary cancer in animals (1, 2, 4). Therefore, we investigated whether estradiol and antiestrogens (tamoxifen or ICI 164384, 4 x 10⁻¹¹ M for 6 h) inhibited the estradiol induction of RARα gene expression, because it is known that RARα mRNA is expressed in cells either that are programmed for cell death (32) or that become part of the nervous system (31). Murine RARγ mRNA is expressed predominantly in skin, cartilage, and bone (31, 33). We have previously characterized the expression of RAR γ mRNA in human breast cancer cell lines and found that ER-positive cell lines express greater levels of RARα than do ER-negative cell lines (34). RARβ expression was detected predominantly in ER-negative, epidermal growth factor receptor-positive cell lines. Because ER expression and epidermal growth factor receptor expression are markers of good and poor prognoses, respectively, in human breast cancer, RARα gene expression may be associated with good and RARβ gene expression with poor prognosis markers in breast cancer. Abnormalities in RARβ gene expression have been suggested to be associated with poor prognosis in lung carcinoma (35).

There is no published information on the expression of RARα in breast tumor biopsies, and one aim of this study was to determine whether RARα expression is greater in tumors which also expressed ER. Furthermore, we postulated that one potential explanation for higher levels of RARα gene expression in ER-positive human breast cancer cell lines was the presence of ER mediating the estradiol induction of RARα gene expression, because it is known that RARα gene expression can be regulated by retinoids and steroids (e.g., by RA) in a number of tissue types and by progesterins in breast cancer cells (34). Therefore, we investigated whether estradiol and antiestrogens regulated RARα gene expression in the ER-positive T-47D human breast cancer cell line.

The abbreviations used are: RA, retinoic acid; RAR, retinoid acid receptor; ER, estrogen receptor; PR, progesterone receptor; FCS, fetal calf serum; SFCS, dextran-coated charcoal-stripped fetal calf serum; 1S, RPMI 1640 medium containing 1% dextran-coated charcoal-stripped fetal calf serum; 5N, RPMI 1640 medium containing 5% fetal calf serum; T-47Dsd cells, T-47D cells grown in serum-depleted medium.

1 Supported by the National Health and Medical Research Council of Australia, the Australian Government's Cooperative Research Centre Program, and the Tenovus Organ.
MATERIALS AND METHODS

Materials. Materials were obtained from the sources listed previously (36). All three RAR probes contained the full-length coding region. The hRARα probe was a 1.9-kilobase EcoRI fragment (23), the hRARβ probe was a 1.4-kilobase EcoRI-BamHI fragment, and the hRARγ probe was a 1.5-kilobase EcoRI-BamHI fragment (25). The 18S rRNA probe used to control for RNA loading was a 30-base pair oligonucleotide complementary to rat 18S rRNA (37). The 2.1-kilobase ER complementary DNA clone OR8 has been described in detail previously (38, 39).

Patients and Tissue. Primary breast tumors were collected at surgery from patients undergoing simple or s.c. mastectomy and were frozen in liquid nitrogen within 15 min. Each tumor was histologically assessed and all contained significant numbers of malignant cells. Representative portions of the tissue were blocked for cryostat sectioning or were preserved at -70°C for RNA extraction and processed as described previously (40-42).

Cell Culture. The T-47D (breast carcinoma) and HS578T (breast carcinomasarcoma) cell lines were supplied by E. G. and G. Mason Research Institute (Worcester, MA) for the National Cancer Institute Breast Cancer Program Cell Culture Bank. Cells were maintained in phenol-red free RPMI 1640 medium supplemented with 6 mM glutamine, 14 mM sodium bicarbonate, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 20 μg/ml gentamycin, and 10 μg/ml porcine insulin, as described previously (43, 44), except that 5% FCS was used. Cells were passaged regularly to maintain almost continuous exponential growth. Cells were revived from frozen stocks at 3-month intervals and were shown to be free from Mycoplasma contamination using the Gen-Probe T.C. Rapid Detection System supplied by BioMediq (Doncaster, Australia).

T-47Dsd Cells. T-47D cells were grown as described above for 11 passages after thawing. Cells were then plated in phenol-red free RPMI 1640 medium supplemented as described above except for the use of 10% FSCS and 1 μg/ml insulin. After nine weekly passages in this medium, cells were maintained in medium with 5% FCS and 1 μg/ml insulin. Four passages later cells were frozen and stored in liquid nitrogen. The cells were viable upon thawing and maintained the same morphology and growth characteristics as observed prior to freezing. Cells were thawed in medium containing 10% FSCS and 1 μg/ml insulin. Doubling times of T-47Dsd cells were greater than those of the parent T-47D cells (average of approximately 90 h and 33 h, respectively), indicating that culture in medium containing charcoal-stripped serum had slowed cell growth.

Experimental Procedures. T-47Dsd cells were grown in RPMI 1640 medium supplemented as described above. Four days after plating the medium was replaced with medium containing 1% FSCS. Steroids were added to culture medium, from 1000-fold concentrated stock solutions in ethanol or dimethylsulfoxide, for the periods indicated in the legends to the figures.

Northern analysis was carried out on total RNA isolated by the guanidinium isothiocyanate/cesium chloride method (45) from breast cancer cell lines, as described previously (46). RNA (20 or 30 μg) was separated electrophoretically and transferred to Zeta-probe nylon membranes (Bio-Rad, Richmond, CA). Tumor tissue was homogenized in liquid nitrogen and resuspended in guanidinium isothiocyanate. Total RNA was isolated as described above, subjected to electrophoresis, and transferred to membranes as described previously (40). The integrity of RNA preparations from tumor samples was assed following ethidium bromide staining of denatured formaldehyde-agarose gels, and samples (approximately 2%) showing RNA degradation were discarded. Membranes were hybridized as described previously (36), except that the probes were labeled by random priming, using the Amersham Multiprime DNA labeling system (Amersham, North Ryde, Australia), to a specific activity of >10^8 dpm/μg DNA. After washing, the filters were exposed to Kodak X-Omat AR film with or without intensifying screens. Densitometry was performed on films exposed without intensifying screens, using a Bio-Rad 620 video densitometer and 1D Analyst program or a Pharmacia LKB laser densitometer and GelScan XL program. Breast tumor samples were classified as positive for ER or RARα mRNA expression if the integrated area, expressed in arbitrary densitometric units, exceeded 1.5.

RESULTS

Expression of RARα mRNA in Human Breast Carcinomas.

Expression of RARα and ER genes at the mRNA level was examined in 116 human breast tumor biopsies, and representative samples are shown in Fig. 1. RARα was predominantly detected as a single mRNA species, in contrast to the two species of similar intensity observed in breast cancer cells (34), whereas ER was detected as two distinct transcripts except where high ER levels were expressed and individual transcripts could not be distinguished (Fig. 1, first two lanes). Ninety-four (81%) tumors were ER-positive and 102 (88%) were RARα-positive (Table 1). Eighty-seven (93%) ER-positive tumors were also RARα-positive. However, RARα expression was noted in some ER-negative tumors, as evidenced by the observation that 15 of 22 ER-negative tumors were RARα-positive (Table 1). The data were analyzed by χ² contingency analysis and a significant correlation was found between ER and RARα status (P = 0.0052). In addition to the relationship between RARα and ER status there was a quantitative relationship between ER and RARα mRNA levels; a highly significant correlation (r² = 0.215; P = 0.0001) was found (Fig. 2) between the levels of expression of ER mRNA and RARα mRNA by linear regression analysis. These data agreed with the previously reported study of human breast cancer cell lines (34) and showed that both ER-positive and ER-negative cell lines and tumor biopsies expressed RARα but ER positivity was associated with higher levels of RARα mRNA.


The regulation of RARα gene expression was examined in T-47D cells grown under steroid-depleted conditions. Initially the concentration dependence of estradiol induction of RARα mRNA was examined (Fig. 3). Cells were treated for 6 h with estradiol concentrations ranging from 10^{-12} M to 10^{-8} M and were harvested, and 30 μg of total RNA were subjected to Northern blot analysis. Expression of RARα, -β, and -γ mRNA was examined. RARα mRNA levels were increased in an apparently biphasic manner (Fig. 3A). Maximal induction of almost 200% was observed at a concentration of 10^{-10} M estradiol. At concentrations greater than 10^{-10} M estradiol the effect was submaximal (Fig. 3B). RARγ was not induced by estradiol at any of the concentrations examined and was expressed at slightly lower

<p>| Table 1 ER and RARα status in human breast tumor biopsies |
|---------------------------------|-----------------|-----------------|
|                                 | ER-positive     | RARα-negative   |</p>
<table>
<thead>
<tr>
<th>No. of biopsies</th>
<th></th>
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<tbody>
<tr>
<td>ER-positive</td>
<td>87</td>
<td>7</td>
</tr>
<tr>
<td>ER-negative</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>102</td>
<td>14</td>
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than control levels after estradiol treatment. RARβ was not detectable in T-47D cells and estradiol did not induce it to detectable levels (data not shown).

The time course of estradiol induction of RARα gene expression was also examined. T-47D cells grown under steroid-depleted conditions were treated with $10^{-10}$ M estradiol for 1, 6, 12, 24, or 48 h. Total RNA was examined for RARα gene expression (Fig. 4). The timing of the induction of RARα gene expression was rapid, being maximal by 6 h after estradiol addition. There was a subsequent fall in the levels of RARα mRNA, which reached a plateau from 12 to 48 h of approximately 170% of control. It is interesting that the plateau level was approximately twice the initial level of RARα gene expression, because ER-positive human breast cancer cells express approximately twice the levels of RARα mRNA, compared with ER-negative cells (34).

Effect of Antiestrogens on Estradiol Induction of RARα mRNA. Having shown that estradiol increased RARα mRNA levels, it was of interest to determine the role of ER in the effect. Because ER-positive human breast cancer cell lines express higher levels of RARα mRNA than do ER-negative cell lines, it was postulated that the estradiol induction of RARα gene expression was mediated by the ER and should be abrogated by antiestrogens. Therefore, cells were treated for 6 h with $10^{-10}$ M estradiol in the presence or absence of $4 \times 10^{-7}$ M concentrations of the antiestrogens tamoxifen and ICI 164384. Tamoxifen and ICI 164384 alone had no effect on RARα gene expression (Fig. 5). Estradiol induction of RARα mRNA was marked after 6 h with $10^{-10}$ M estradiol (Fig. 5), and addition of either tamoxifen or ICI 164384 totally abrogated the effect, supporting the conclusion that the increased RARα mRNA levels upon estradiol treatment were ER mediated.

Evidence That the Estrogens Present in Serum Are Sufficient to Induce RARα Gene Expression. Having shown that estradiol acting via the ER could be responsible for the high levels of RARα mRNA found in ER-positive human breast cancer cell lines, it was important to examine whether estrogens present in serum were sufficient to induce RARα. T-47D cells were grown as described in “Materials and Methods” and after 24 h in 1S the medium was changed again, either to fresh 1S or to 5N. Along with a change in medium, cells from either the 1S or 5N group were also treated with $10^{-11}$ M to $10^{-9}$ M estradiol, 100 nM ICI 164384, or vehicle. Addition of estradiol to cells growing in 1S increased RARα mRNA levels to 250% of control, as shown in Figs. 3–5. However, cells growing in 5N had elevated RARα mRNA levels that were 300% of those of control cells growing in 1S (Fig. 6A). Addition of estradiol to cells growing in 5N failed to elicit a further elevation of RARα mRNA levels, suggesting that the induction due to the serum was nearly maximal. If anything, addition of estradiol in the presence of 5N slightly reduced the induction of RARα gene expression (Fig. 6D). Furthermore, the induction due to serum was abrogated completely by the pure antiestrogen ICI 164384.
suggested that estrogens present in serum were solely responsible for the induction of RARα gene expression (Fig. 6D). Neither the medium change nor the addition of estradiol induced detectable levels of RARβ mRNA, as indicated by failure to detect RARβ in any sample, compared with Hs0578T, an ER-negative human breast cancer cell line used as a positive control (Fig. 6B). Likewise, RARγ mRNA levels remained unchanged under all of these experimental conditions (Fig. 6C).

**DISCUSSION**

We have previously documented the expression of RARα mRNA in human breast cancer cell lines (34) and to our knowledge this study represents the first documentation of the expression of RARα mRNA in human breast tumors. The majority of tumors expressed both ER and RARα mRNAs, and there was a significant relationship between ER status and RARα expression. Furthermore, there was a quantitative relationship between levels of ER and RARα mRNAs, which agreed with the observation previously made in breast cancer cell lines that ER-positive cells contain higher levels of RARα mRNA than do ER-negative cells. The results of this study demonstrate that, for the cohort of samples studied, RARα mRNA expression correlated with ER mRNA expression and by inference with other markers of good prognosis in breast cancer (47), suggesting that RARα may be one of a suite of genes reflecting the good prognosis phenotype.

Thus, in breast cancer RARα may be an important mediator of RA effects in ER-positive cells, and the effectiveness of retinoids as potential therapeutic agents may be related to ER/PR status. However, this does not preclude retinoid treatment being potentially beneficial in ER-negative tumors; if the higher expression of RARβ in ER-negative breast cancer cell lines (34) is also true for tumor biopsies, it is possible that RARβ may mediate retinoid effects in ER-negative tumors. However, little is yet known about the expression of RARβ or the RXRs in breast tumors, and detailed models for the action of retinoids in breast cancer await such information.

Regulation of RAR gene expression has been examined in a number of in vitro systems, and in particular the effects of retinoids have been studied in some detail. Gene expression of all three RAR mRNAs is induced by RA in melanoma cell lines (48). The level of RARα mRNA (49, 50) is increased by RA in murine embryonal carcinoma cells, whereas in leukemia cells RARα is not regulated by RA. Furthermore, it has been reported that RARα and RARγ are not regulated by RA in embryonal carcinoma cells without the addition of cyclic AMP analogues (51). RA causes an increase in all RARβ mRNA isoforms (52), which, in the case of RARβ2, is believed to be mediated by RAR binding to a RA response element in the promoter region of the RARβ2 gene (53–57). RARγ mRNA has been shown to be decreased by RA in murine embryonal carcinoma cells (58, 59). RA
does not regulate the expression of RARα and RARγ genes in T-47D human breast cancer cells (60).

There is a paucity of information on the regulation of the expression of RARs by ligands for the steroid hormone receptor members of the nuclear receptor gene superfamily. We have previously shown in T-47D human breast cancer cells that prostegins inhibit the expression of both RARα and RARγ genes in a time- and concentration-dependent manner (34). We now show that estradiol regulates RARα, but not RARγ, gene expression in a time- and concentration-dependent manner. Maximal induction of RARα was maintained over a narrow concentration range and at higher concentrations there was a decline in the level of induction. A similar biphasic effect of estradiol on cell growth has been previously reported in T-47D cells grown under steroid-depleted conditions (61). Whereas maximal growth stimulation occurred at 10^{-9} M estradiol in that study, RARα gene induction by estradiol in this study was an order of magnitude more sensitive. The different estradiol concentrations required for maximal effects on growth and RARα mRNA levels may be due to the methodological differences between the two studies; the earlier experiments were done in the presence of phenol red, a known estrogenic factor, whereas in the present study phenol red was omitted, which may explain the lower levels of estradiol required to elicit maximal stimulation of RARα mRNA.

Induction of RARα mRNA by estradiol was maximal 6 h after treatment and mRNA levels subsequently declined to a new steady state, which was still well above control. Interestingly, the timing of the induction by estradiol mirrors that of the previously reported depletion of RARα mRNA by progestins (34). In both cases the maximal effect was seen at 6 h and was followed by a recovery at 24 and 48 h towards but not reaching control levels. The decrease in the magnitude of the estradiol or progesterin effect on RARα mRNA beyond 6 h may be due to the well documented down-regulation of ER or PR by their homologous ligands (62). The observation that RARα mRNA was expressed in T-47Dsd cells, albeit at low levels, even in the absence of estradiol indicates that factors other than estradiol are likely to control the basal expression of this gene in breast cancer cells.

The inhibition of the estradiol effect by antiestrogens, such as ICI 164384 and tamoxifen, is evidence that the estradiol effect was ER mediated, and the inhibition of serum induction of RARα gene expression by ICI 164384 indicated that serum estrogens were responsible for the effect. The failure of estradiol to further induce RARα gene expression suggests that the magnitude of the increase in RARα gene expression upon serum addition was maximal.

The functional significance of the ability of estradiol to increase RARα mRNA levels is unclear at present. However, RA has been postulated to act as an antiestrogen under some experimental conditions (63). RA has been shown to inhibit estradiol-induced transcription of a number of transfected constructs; the estrogen-responsive promoters of PR, vitellogenin A2, pS2, and oxytocin all showed reduced estradiol-induced transcription upon RA treatment (63-65). It is attractive to speculate that the increased expression of RARα mRNA by estradiol, if matched by a corresponding increase in RARα protein concentration, may result in an antiestrogenic effect in the presence of RA and this may be one potential mechanism to limit the duration or magnitude of estradiol effects.

In summary, the present study has shown that estradiol is able to increase RARα gene expression and therefore that the previously reported (34) greater level of RARα mRNA expression in ER-positive breast cancer cell lines may be due to the stimulatory effect of serum estrogens. Estrogen regulation of RARs may be one explanation for the positive correlation between ER and RARα gene expression in breast tumor biopsies, suggesting that RARs may form part of a suite of genes determining the good prognosis phenotype in breast cancer.

ACKNOWLEDGMENTS

The authors are grateful to Professor P. Chambon (Strasbourg, France) for the hRAR complementary DNA clones, A. Wakeling for the ICI 164384, Y-E. Chiew and R. Hall for the 18S data on the tumor samples and the Department of Haematology, Westmead Hospital, for their support.

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