Expression and Regulation of Retinoic Acid Receptors in Human Breast Cancer Cells

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ABSTRACT

Retinoic acid is known to inhibit mammary carcinogenesis in rodents and to inhibit proliferation and steroid hormone receptor gene expression in human breast cancer cells. Since these effects are likely to be mediated by nuclear retinoic acid receptors (RARs) the present study was initiated to determine the expression and regulation of RARs in human breast cancer cell lines. Differential cellular gene expression of the RARs was determined by Northern blot analysis of total RNA prepared from 5 ER* and 6 ER- cell lines. RARα was detected as mRNA species of 2.7 and 3.4 kilobases in all cell lines and the level of gene expression was greater in ER* cell lines (P < 0.001). RARγ mRNA (3.7 kilobases) was detected in seven of the eleven lines tested and was expressed most commonly in ER- cell lines. RARγ mRNA was expressed in all cell lines as a transcript of 3.4 kilobases at levels that were similar in both ER* and ER- cell lines. Retinoic acid failed to regulate the expression of the RARα and RARγ genes. The effect of steroid hormones on RARα and RARγ mRNA levels was also examined. In four PR* cell lines (T-47D, BT 474, MCF-7, and MDA-MB-361), progesterins markedly decreased RARα mRNA levels. The progestin effect on RARα mRNA levels in T-47D cells was detectable at concentrations of 0.05 μM and was maximal at 1 μM 16α-ethyl-21-hydroxy-19-nor-4-pregnen-3,20-dione ORG 2058, whereas dihydrotestosterone and dexamethasone were without effect. RARα and RARγ mRNA levels were rapidly decreased by progestin, and the effect was maximal 3–6 h after ORG 2058 treatment. However, the mRNA loss was transient, and recovery of RARα and RARγ mRNA levels was noted 12–24 h after retinoic acid treatment. Although RARγ mRNA returned to control levels by 24 h, the decrease in RARα mRNA was maintained at around 50% control until at least 48 h. In summary, RARα and RARγ were expressed in all human breast cancer cell lines and were regulated by progesterins in the PR* T-47D cell line. The previously reported ability of retinoic acid to down-regulate PR mRNA and the present demonstration that progesterins down-regulate RARα and RARγ mRNA suggest that mutual regulation may be a mechanism through which PR and the RARs interact in human breast cancer cells.

INTRODUCTION

The vitamin A-derived retinoids have antiproliferative and differentiating effects in a wide range of tissue and cell types (1–3) and have been shown to inhibit carcinogenesis. Low dietary or serum levels of β-carotene are associated with increased risk of a number of epithelial malignancies (1–3). Dietary or serum levels of β-carotene are associated with inhibition of a number of epithelial malignancies (1–3) and have been shown to inhibit carcinogenesis. Low dietary or serum levels of β-carotene are associated with increased risk of a number of epithelial malignancies (1–3). But dietary or serum β-carotene levels are associated with an increased risk of a number of epithelial malignancies (1–3). Antiestrogens, or selenium inhibit the initiation and promotion of mammary tumors induced in mice and rats by 7,12-dimethylbenz[a]anthracene or N-methyl-N-nitrosourea (4). A relationship between retinoid intake or serum levels of retinoids and human breast cancer incidence has been proposed but has been difficult to establish. Case-control and prospective studies have both supported (5, 6) and refuted (7–9) the hypothesis that low dietary or serum β-carotene levels are associated with an increased risk of breast cancer.

However, in addition to a possible involvement in the inhibition of mammary carcinogenesis, retinoids have an established role in the inhibition of growth of human breast cancer cells (10–15). Retinoids inhibit the growth of human breast cancer cells when administered alone (11, 13, 16, 17) or in combination with antiestrogens (10, 14) where synergism has been reported (15). Synergism has also been reported between RAα and interferon in the growth inhibition of human breast cancer cell lines (18). Furthermore, a phase 2 clinical trial of adjuvant therapy with combined retinyl acetate and the antiestrogen tamoxifen has been undertaken (19).

There are a number of potential mediators of retinoid action. Nuclear receptor proteins have been characterized: RARα, β, and γ (20–25) and related receptors whose ligands have yet to be described (RxR; Ref. 26). The retinoic acid receptors belong to the gene superfamily of transcriptional modulators which includes receptors for the steroid hormones, thyroid hormone, and vitamin D (27). In addition to the nuclear RARs, ubiquitous cellular retinoic acid-binding proteins (I and II) and retinol-binding proteins (28, 29) have been described. Retinoic acid is believed to be the biologically active ligand for cellular retinoic acid-binding proteins and RARs (29), and since no evidence of cellular retinoic acid-binding proteins binding to DNA has been reported any effect of RA on transcription is likely to be mediated by the RARs.

The potential role of retinoids as chemopreventative or growth-inhibitory agents in breast cancer raises the question of whether breast cancers express RARs. There are currently no data on RAR gene expression in breast tumor biopsies. T-47D breast cancer cells have been shown to contain RARα, β, and γ (24), and we have previously shown in these cells (30) that RA decreases PR levels, leading to a decreased progestin response (31). However, neither the gene expression nor regulation of RARs has been examined in other breast cancer cells. Furthermore, despite the ability of retinoids and antiestrogens to inhibit breast cancer cell growth in combination, no information exists on the relative gene expression of RARs and ER in breast cancer. Accordingly, this study aimed to determine whether RAR gene expression is associated with expression of ER in these lines and is regulated by steroid hormones.

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7 The abbreviations used are: RA, retinoic acid; PR, progesterone receptor; RAR, retinoic acid receptor; ER, estrogen receptor; ORG 2058, 16α-ethyl-21-hydroxy-19-nor-4-pregnen-3,20-dione; poly(A) RNA, polyadenylated RNA.
MATERIALS AND METHODS

Materials. Materials were obtained from the sources listed previously (30). Two hRARα probes were used and gave the same results: a 1.9-kilobase EcoRI fragment (20) or a 2.9-kilobase EcoRI fragment (21), both containing the full-length coding region. The hRARβ probe was a 1.4-kilobase EcoRI-BamHI fragment containing the full-length coding region (22). Two hRARγ probes were used and gave the same results: a 1.5-kilobase EcoRI-BamHI fragment (24) or a 1.6-kilobase EcoRI fragment (25), both containing the full-length coding region. The 18S ribosomal RNA probe used to control for RNA loading was a 30-base pair oligonucleotide complementary to rat 18S ribosomal RNA (32).

Cell Culture. ER+ (T-47D, MCF-7M, MDA-MB-361, BT 474, and MDA-MB-134) and ER- (MDA-MB-231, MDA-MB-330, BT 20, and Hs0578T) 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. The T-47D cells used in these studies are growth stimulated by estradiol, and PR gene expression can be increased by approximately 50% by culture in the presence ofestradiol. MDA-MB-453 (ER+) cells were supplied by Dr. C. M. McGrath (Meyer L. Prentis Cancer Center, Detroit, MI). All cell lines were maintained in RPMI 1640 supplemented with 5 mM glutamine, 14 mM sodium bicarbonate, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 20 µg/ml gentamycin, 10 µg/ml porcine insulin, and 10% fetal calf serum as previously described (33, 34). Cell stocks were passed regularly to maintain almost continuous exponential growth. Cells were revived from frozen stocks at 3 monthly intervals and were shown to be free from Mycoplasma contamination using the Gen-Probe T.C. Rapid Detection System supplied by BioMediq (Doncaster, Australia). The identities of cell lines were confirmed by DNA fingerprint analysis using human α-globin 3′-HVR and mucin HVR probes.7

Experimental Procedures. Cells were grown in RPMI 1640 supplemented as described above, except that the serum concentration was 5%. Four days after plating the medium was replaced with medium containing 1% dextran-coated charcoal-stripped fetal calf serum. Steroids or retinoids were added to culture medium from lOOOx concentrated stock solutions in ethanol or dimethyl sulfoxide for the periods indicated in the figure.

Northern blot analysis was carried out as previously described (35). Briefly, total RNA was isolated by the guanidinium isothiocyanate/cesium chloride method (36). RNA (20 or 30 µg) was separated electrophoretically and transferred to Zeta-probe nylon membranes (Bio-Rad, Richmond, CA). Membranes were hybridized as described previously (30), except that the probes were labeled by random priming using the Amersham multiprime DNA labeling system (Amersham, North Ryde, New South Wales, Australia) to a specific activity of 1 × 10⁶ to 2 × 10⁷ dpm/µg complementary 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.

Densitometric data from four separate experiments, were normalized to generate Fig. 2, for RARα and RARβ to MDA-MB-231 cells and for RARγ to T-47D cells. The arbitrary units recorded by densitometry of the area specifically hybridizing to the probe were set at 1.0 for these cell lines. Where a probe failed to detect any specific binding the arbitrary units were given a value of 0. Statistical significance was tested using one-way analysis of variance. The statistics that are presented as significant for the means of all experiments were also significant when each experiment was analyzed individually.

RESULTS

Retinoic Acid Receptor Gene Expression in Human Breast Cancer Cell Lines. The gene expression of RARs was examined in a range of ER+ and ER- human breast cancer cell lines. Total RNA from 11 cell lines was analyzed for the expression of the RARα, β, and γ genes. RARα mRNA was detected as a pair of mRNA species of 2.7 and 3.4 kilobases in all cell lines (Fig. 1). A 3.7-kilobase RARβ mRNA was detected in 7 of the 11 cell lines and consistently in total RNA from 4 lines, notably the ER+ MDA-MB-231, MDA-MB-330, HBL100, and Hs0578T lines (Fig. 1). In MDA-MB-361, BT 474, and BT 20, RARβ mRNA was detected in 1 of 4, 2 of 4, and 1 of 3 experiments, respectively. When Northern blot analysis of poly(A)+ RNA from MDA-MB-231, MDA-MB-330, HBL100, MDA-MB-361, BT 474, and BT 20 was performed, RARβ mRNA was detected in all the lines (data not shown). In cell lines which were negative for RARβ even prolonged exposure of filters failed to detect specific bands. RARγ mRNA was expressed in all cell lines as a single transcript of 3.4 kilobases (Fig. 1).

RARα gene expression was significantly greater in ER+ cell lines (P < 0.001) (Fig. 2A). The mean RARα mRNA level for these lines was approximately 2-fold greater than in ER- cells. The level of RARβ gene expression varied among the cell lines, and there was no significant difference between the mean level of RARβ gene expression in ER+ and ER- cells (Fig. 2B), although RARβ was expressed more commonly in ER+ cell lines. Hs0578T, a breast carcinosarcoma cell line (37), expressed more than 4-fold more RARβ mRNA than any other cell line studied. The level of RARγ gene expression varied among the cell lines examined, but there was no significant difference between RARγ gene expression in ER+ and ER- cells (Fig. 2C).

Regulation of RARα and RARγ Gene Expression by Retinoic Acid. The ability of RA to regulate RARα and RARγ gene expression was examined in two ER+ (BT 474 and MCF-7M)
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Fig. 2. Relative levels of gene expression of RARs in human breast cancer cell lines. Data obtained by densitometric scanning of autoradiograms from four separate experiments were normalized to MDA-MB-231 (A and C) or T-47D cells (B) as described in "Materials and Methods" and presented as arbitrary units (mean ± SE). Bottom, ER status of the cells. A, RARα; B, RARβ; C, RARγ.

and two ER- (HBL100 and BT20) cell lines (Fig. 3). Either no effect or a modest decrease in RARα and RARγ mRNA levels was noted, except for BT20 cells, where a modest increase in RARγ was observed. Similarly, in T-47D cells, there was no effect of RA on RARα and RARγ mRNA (31), whereas RA treatment caused an increase in RARβ mRNA from barely detectable to low levels (not shown).

Regulation of RARα Gene Expression by Steroid Hormones. The higher gene expression of RARα in ER+ cells raised the interesting and potentially important possibility that the gene expression of RARα was regulated by estrogens or other steroid hormones. To test this, T-47D cells, which contain appreciable levels of RARα, were treated (6 h, 10 nM) with the following steroids: the synthetic progestins 17α-dimethyl-19-norpregn-4,9-diene-3,20-dione (R 5020), 17α-acetoxy-6a-methyl-4-pregnene-3,20-dione (MPA), and ORG 2058; the glucocorticoid dexamethasone; 17β-estradiol; and the androgen dihydrotestosterone (DHT). RARα mRNA was decreased by the progestins (Fig. 4). However, neither dexamethasone nor dihydrotestosterone had any effect on RARα gene expression, whereas 17β-estradiol caused a modest increase in the gene expression of RARα (Fig. 4).

Since T-47D cells contain high levels of PR the regulation of RARα gene expression by the synthetic progestin ORG 2058 was examined in addition in other PR+ cell lines, namely BT 474, MCF-7M, and MDA-MB-361 (Fig. 5). In the three cell lines examined, progestin decreased RARα gene expression. The magnitude of the effect was greatest in BT 474, where levels were reduced to 65% of control. The effect was less extensive in MCF-7M and MDA-MB-361 (78% and 85% of control, respectively).

Fig. 3. Regulation of RARα and RARγ mRNA by retinoic acid. In A, Northern blot analysis was performed, as described in "Materials and Methods," on 20 μg of total RNA isolated from BT 474, MCF-7M, HBL100, and BT20 cells after treatment for 6 h with 1 μM retinoic acid or vehicle. Filters were probed for RARα and RARγ and autoradiograms exposed for 28 h and 7 days, respectively. In each case filters were stripped and reprobed for 18S rRNA and the result is shown in B. 18S filters were exposed for 17 h without intensifying screens. In B, the data obtained by video densitometric scanning of the autoradiograph, from a single experiment, are expressed as a percentage of the vehicle control. □, RARα mRNA; ●, RARγ mRNA.
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Fig. 4. Regulation of RARα mRNA by steroid hormones. In A, T-47D cells were treated for 6 h at 10 nM with vehicle (control) or the steroids indicated. Northern blot analysis was performed on 20 μg of total RNA as described in "Materials and Methods." RARα, filter probed for RARα mRNA; 18S, same filter probed for 18S rRNA. The filters were exposed for 2 days and 4 days, respectively, without intensifying screens. In B, the data obtained by video densitometric scanning of the autoradiograph, from a single experiment, are expressed as a percentage of the vehicle control.

Regulation of RARα and RARγ by the Synthetic Progestin ORG 2058. The concentration dependence of the ORG 2058 effect was determined in the T-47D line. Cells were treated with 0.05–100 nM ORG 2058 for 6 h, and Northern blot analysis was performed. There was a concentration-dependent loss of RARα mRNA which was maximal at 1 nM ORG 2058 (28% of control) (Fig. 6). The decline in RARα mRNA was detectable at concentrations as low as 0.05 nM ORG 2058 (79% of control) (Fig. 6).

The time course of the regulation of RARα by ORG 2058 was characterized in T-47D cells, where RARγ gene expression was also examined. Cells were treated with 10 nM ORG 2058 for 3, 6, 12, 24, and 48 h, and a decrease in both RARα and RARγ mRNA levels was observed. The effect was rapid and was essentially maximal by 3 h, when RARα mRNA levels were 40% and RARγ mRNA levels were 60% of control. The down-regulation of both RARα and RARγ mRNA was transient, and a recovery was noted for both mRNAs beginning 6–12 h after treatment (Fig. 7). RARα mRNA recovered to between 60 and 80% of control levels by 24 h and then declined thereafter. By 12 h, RARγ mRNA levels had recovered and maintained control levels for up to 48 h.

DISCUSSION

Gene expression of RARs has been described previously in mammalian cells. RARα and RARβ mRNA have been detected in normal human tissues such as prostate, spinal cord, liver, and breast tissue (38). RARα gene expression is not developmentally regulated (39), whereas RARβ mRNA is expressed in cells that are either programmed for cell death (40) or that become part of the nervous system (39). Murine RARγ mRNA is expressed predominantly in skin, cartilage, and bone (39, 41).

There are no data on the expression of RAR genes in breast tumors, and with the exception of a previous report on RAR gene expression in T-47D cells (24), there are no data on RAR gene expression in breast cancer cell lines. Accordingly, the gene expression of RARs was examined in ER+ and ER- human breast cancer cell lines. RARα mRNA was detected in all cell lines, consistent with its presence in the normal breast (38), and its expression in ER+ cells was higher than in ER- cell lines, irrespective of whether individual experimental data or pooled mean data were analyzed. RARγ mRNA was detected in all human breast cancer cell lines, which was of interest although of unknown significance given the relatively restricted distribution of this receptor in normal tissues (39, 41). RARγ mRNA levels were similar in all lines and were unrelated to steroid hormone receptor status. RARβ mRNA was detected in seven cell lines, and in only four of these was it detected in all experiments; in MDA-MB-361, BT 474, and BT 20 cells RARβ mRNA was not consistently detected when Northern blot analysis of total RNA was performed. However, RARβ was expressed in these cells, as evidenced by the demonstration of its mRNA on analysis of poly(A)+ RNA (not shown); the failure to consistently detect it in total RNA may reflect differences in the
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**Fig. 6.** Concentration-dependent regulation of RARα mRNA by ORG 2058. In A, T-47D cells were treated for 6 h with 0.05–100 nM ORG 2058. Northern blot analysis was performed on 20 μg of RNA as described in “Materials and Methods.” RARα, filter probed for RARα mRNA; 18S, same filter probed for 18S rRNA. The RARα filter was exposed for 2 days and the 18S filter for 4 days without intensifying screens. In B, the data obtained by video densitometric scanning of the autoradiograph, from a single experiment, are expressed as a percentage of the vehicle control.

sensitivity of detection of low-abundance mRNAs in total versus poly(A)+ RNA. Interestingly, five of the seven cell lines which expressed RARβ mRNA [in either total or poly(A)+ RNA] were ER−.

RA treatment failed to markedly alter levels of RARα or RARγ mRNA in ER+ and ER− breast cancer cell lines. This was consistent with our demonstration that the mRNA levels of these receptors were unaffected by RA in T-47D cells (31) and was consistent in the main with the effect of RA on RARα and RARγ gene expression in other systems. RA had no effect on the expression of RARα and RARγ in hepatoma cells (38), hematopoietic cells (42–44), or murine F9 teratocarcinoma cells (45). In the retinol-deprived adult rat both RARα and RARγ mRNAs were unaffected by RA in the lung and liver (46). However, there is also some evidence that retinoids are able to increase RARα gene expression, e.g., in the testes of the retinol-deprived adult rat (46, 47) and in murine P19 and RAC65 teratocarcinoma cell lines (48). The differing effect of RA on RARα gene expression in different cell types suggests that the regulation may be cell type specific. There is further evidence in support of this theory; the binding of RARα to a RA response element was found to be increased by coinubcation with a nuclear extract, and the binding was altered, depending on the cell type of the extract (49). In addition to a possible cell-specific effect, there is evidence that RARα mRNA isoforms, resulting from exon splicing variations, are differentially regulated by RA; in murine P19 and F9 teratocarcinoma cell lines expression of the RARα2 mRNA isoform was increased by RA, whereas RARα1 expression was unaffected (50).

In contrast with the evidence that RA either increases or does not affect RARα mRNA levels, there is abundant evidence that RA exposure increases the levels of RARβ mRNA (38, 45, 48, 51–56). RARβ mRNA was inducible in T-47D cells to low levels by RA (not shown). Expression of all three RAR mRNAs is induced by RA in melanoma cell lines (57). RA-mediated increases in RARα and RARβ mRNA are believed to be mediated by RA-binding RA response elements which have been found in one of the promoters of the genes encoding RARα and RARβ message species (58–63). A decrease in RARγ mRNA levels by RA has been reported (51, 64).

Having documented the expression of RAR genes in breast cancer cells and demonstrated differential expression of the RARα gene in ER+ and ER− cells, it was considered important to examine the hypothesis that expression of this gene was regulated by estrogen or other steroid hormones. This study has shown that RAR gene expression can also be regulated by steroid hormones in breast cancer cells. Short-term treatment of T-47D cells with steroid hormones resulted in a marked progestin-induced decrease in the concentration of RARα mRNA. Both the 3.4-kilobase and 2.7-kilobase RARα mRNA species were decreased by the three progestins tested. The effect of the synthetic progestin ORG 2058 on RARα mRNA was

**Fig. 7.** Time course of RARα and RARγ mRNA regulation by ORG 2058. In A, T-47D cells were treated for 3–48 h with 10 nM ORG 2058. Controls were treated for 24 h with vehicle. Northern blot analysis was performed on 20 μg of total RNA as described in “Materials and Methods.” RARα, filter probed for RARα mRNA; RARγ, same filter probed for RARγ mRNA; 18S, same filter probed for 18S rRNA. The RARα filter was exposed for 7 days, the RARγ filter for 4 days, and the 18S for 20 min, all without intensifying screens. In B, the data obtained by video densitometric scanning of the autoradiograph, from a single experiment, are expressed as a percentage of the vehicle-treated control. □, RARγ mRNA; ■, RARα mRNA.
examined in three PR+ cell lines in addition to T-47D cells. A decrease in RARα gene expression was found in all cells; however, the magnitude of the effect was not as great as in T-47D cells. One explanation for this decreased effectiveness of ORG 2058 could be that these cells contain lower levels of PR than T-47D cells (65). The other steroids were ineffective in modulating RARs mRNA levels in T-47D cells at the time and concentrations tested, and further experiments have shown that longer exposure of T-47D cells to these agents had no effect on RARα mRNA levels (not shown). There was a modest increase in RARα mRNA concentration with 17β-estradiol; the significance of this observation is unclear and requires further confirmation.

Reduction of RARα mRNA by the synthetic progestin ORG 2058 was detectable at subsaturating ligand concentrations and was maximal at concentrations approximating the $K_a$. The progestin effect was rapid but transient, however, with a recovery of both mRNAs being noted. The mechanism of the progestin-mediated decrease in RAR mRNA concentrations is unknown, and it is unclear whether these changes in mRNA concentrations are reflected in changes in the cellular RAR protein levels. These questions require further investigation and are of critical importance in understanding how progestin- and retinoid-mediated events interact and are controlled in breast cancer cells.

The present study has shown that RARs are expressed in human breast cancer cells, and modulation of RARα and γ gene expression by progestins has been documented. There is also evidence that RARβ may be preferentially expressed in ERα and ERβ in ERα cells. Thus like some other members of the steroid hormone/thyroid hormone receptor family, RARs may be markers of endocrine responsiveness and prognosis in human breast cancer. Further investigations are urgently required to define the roles of the various RARs in tumor responsiveness to retinoids and the relationship, if any, to known prognostic factors in breast cancer.

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