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Responses to Retinoic Acid of Tamoxifen-sensitive and -resistant Sublines of Human Breast Cancer Cell Line MCF-7[1]

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ABSTRACT

Growth of the human breast cancer cell line MCF-7 is known to be inhibited both by antiestrogens such as 4-hydroxytamoxifen (OHTAM) and by retinoic acid (RA). Uncloned MCF-7 cells (UNC) and two cloned sublines, one sensitive to antiestrogens (E-3) and the other resistant to them (RR), were used in this study. Growth of UNC and E-3 was inhibited by either OHTAM (10^{-7} M) or RA (10^{-6} M), and this inhibition could not be overcome by the simultaneous addition of estradiol. Subline RR, which was originally selected for resistance to tamoxifen, was resistant to both OHTAM and RA as measured by either growth in culture or colony forming ability. RR was resistant to RA at all concentrations tested between 10^{-9} M and 10^{-6} M. The inhibition of uncloned MCF-7 cells by RA was dose dependent between 10^{-9} M and 10^{-6} M. Subline E-3, however, exhibited a mixed response to RA. At 10^{-9} M and 10^{-8} M, growth was stimulated, but at 10^{-7} M and 10^{-6} M it was inhibited. The level of estrogen receptor was measured in the same experiment by using a whole cell assay. In the uncloned MCF-7 cultures and in both the RR and E-3 sublines the level of estrogen receptor was increased between 50 and 200% by RA.

The production of plasminogen activator by MCF-7 cells is stimulated by estrogen. RA had a dual effect on plasminogen activator production. In the absence of estrogen, RA inhibited production below the unstimulated level, but in cells stimulated by estrogen, RA increased plasminogen activator production. The results reported here support possible interactions between the mechanisms by which cells respond to estrogen, antiestrogens, and retinoids.

INTRODUCTION

Retinoids have been studied extensively in recent years as agents which affect cellular differentiation and proliferation and for their possible use in the prevention and treatment of cancer (1). Retinoic acid, in particular, inhibits the growth of a number of cell lines derived from human cancers, including several breast cancer cell lines (2–5). There is some evidence that only those lines which contain estrogen receptor are sensitive to inhibition by retinoic acid (5, 6). Both estrogen and retinoic acid act through receptors which are members of the steroid and thyroid hormone receptor superfamily (7). This group can be broken into two groups based on their structure and the consensus sequences of the response elements to which they bind, and both the estrogen and retinoic acid receptors are in the same group (8). This relationship between the structures and response elements of estrogen and retinoic acid receptors may form a basis for the observation, above, that only those breast cancer cell lines which contain estrogen receptor are sensitive to retinoic acid. The human breast cancer cell line MCF-7 contains many receptors including those for estrogen (9). Three RARs[3] have been described, RAR-α, RAR-β, and RAR-γ (10–14), and MCF-7 cells express at least two of them, RAR-α and RAR-γ (15, 16). The observation that retinoic acid can inhibit the estradiol-induced expression of the transforming growth factor α and *pS2* genes in MCF-7 cells (16) indicates some further link(s) between their actions on breast cancer cells. One of us (W. B. B.) has developed a series of sublines of MCF-7 which differ in tumorigenicity and other properties (17). These include E-3, a subline quite similar to the uncloned MCF-7 (UNC) and RR, a subline selected for its resistance to the antiestrogen tamoxifen (18, 19). The basis for the resistance of subline RR to antiestrogens has not been proved. It has a receptor which appears to be normal, as judged by its binding to estradiol and its ability, as a receptor-estrogen complex, to bind in an apparently normal way to chromatin. On the other hand, chromatin prepared from subline RR binds the receptor-estrogen complex normally, but shows an altered pattern of binding of the receptor-antiestrogen complex (19). Based on these observations, it is possible that the basis of subline RRs resistance to antiestrogens lies in alterations in the nuclear acceptor sites to which the estrogen receptor binds. The initial observation that subline RR is also resistant to the growth inhibitory effects of retinoic acid prompted us to do a more thorough study of the responses to both retinoic acid and tamoxifen of our uncloned MCF-7 and the two cloned sublines, E-3 and RR. Their varied responses to retinoic acid, reported here, should make them a useful system in which to further study the interactions of retinoic acid, estrogen, and antiestrogens.

A preliminary report of this work has been reported (20).

MATERIALS AND METHODS

Cell Culture. The human breast cancer cell line MCF-7 was obtained from Dr. H. D. Soule (21) and grown as described previously (17). The BT20 breast cancer cell line which lacks estrogen receptor was also obtained from Dr. Soule and was grown under similar conditions but with fetal calf serum. E-3 and RR are sublines of MCF-7 which were obtained by cloning MCF-7. The general properties of these sublines have been described previously (17).

Growth Curves and Colony-forming Assay. Cells were grown on the bottoms of sterile glass scintillation vials (5 cm^2) in 2 ml of the standard medium with Earle's base, 2% calf serum, and without insulin in a humidified atmosphere of 5% CO_2:95% air. Cells were plated at 2.5×10^4 cells/vial (5×10^3 cells/cm^2). Stock solutions of retinoic acid and 4-hydroxytamoxifen were prepared in ethanol and added to culture media so that the ethanol concentration did not exceed 0.1%. Drugs were added as indicated for each experiment. The media were changed, and cell counts were made every 2 or 3 days at the times indicated by the data points in the figures. The procedure used to count cells has been described (22). For the colony-forming assay, 400 cells were plated in 1 ml of the same medium containing 5% calf serum in 35-mm tissue culture dishes. Each dish also contained 1 ml of medium containing twice the final concentration of the drug being tested. After 2 weeks, the cells were fixed with methanol:acetic acid, 3:1, stained with methylene blue, and the colonies were counted.

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[3] The abbreviations used are: RAR, retinoic acid nuclear receptor; OHTAM, 4-hydroxytamoxifen; RA, all-*trans*-retinoic acid.

Measurement of Estrogen-binding Capacity. Cells were grown in 2% calf serum in glass scintillation vials as described above for growth curves. Estrogen-binding capacity was measured in intact cells following a 60-min incubation at 37°C with [^{3}H]estradiol as described previously (23, 24). Results have been normalized to cell number.

Plasminogen Activator Assay. The procedures used to assay plasminogen activator production by MCF-7 cells have been described previously (25, 26). Cells were plated in T-25 flasks (5×10^5 cells/flask) in 5% calf serum which had been stripped of endogenous steroids by treatment with dextran-coated charcoal (24). They were changed to fresh media 2 days later and 3 days after that were changed to fresh media containing the indicated concentrations of estradiol and/or retinoic acid. Two days later they were given fresh media and after 2 more days the media were collected, centrifuged to remove cells and cell debris, and assayed for plasminogen activator activity by using the ^{3}H-labeled fibrin assay described previously (25, 26). Cell counts were also made and the results are expressed as milliunits of plasminogen activator per million cells.

Statistical Analysis. Statistical significance of the experimental results was obtained by the 2-sample *t* test.

RESULTS

Effect of Retinoic Acid on MCF-7 Cell Growth. It has been reported previously that growth of the uncloned MCF-7 human breast cancer cell line is inhibited by both RA (2–4) and the antiestrogen OHTAM (27). The results shown in Fig. 1*A* show that this is true of our MCF-7 cells too. We have described a series of cloned sublines of MCF-7 (17), and tested two of them for their sensitivity to RA. Subline E-3 is very similar to the uncloned line in many respects and its growth is inhibited by both OHTAM and RA (Fig. 1*B*). The subline RR was selected for its resistance to tamoxifen. As shown in Fig. 1*C*, it is resistant to OHTAM and also to RA. We also measured the sensitivity of the human breast cancer cell line BT20 which lacks estrogen receptors. As shown in Fig. 1*D*, it too is resistant to both OHTAM and RA. The resistance of subline RR to both OHTAM and RA was also seen in a colony-forming assay, as shown in Fig. 2*A* for OHTAM and in Fig. 2*B* for RA. The results of a dose-response curve of uncloned MCF-7 and the two sublines, E-3 and RR, are shown in Fig. 3*A*. Here cells were grown for 10 days in the presence of various concentrations of RA. The results were different for each of the three cultures tested. The uncloned MCF-7 (UNC) was inhibited by RA in a simple dose-dependent manner and, as shown previously, subline RR, which was selected for resistance to tamoxifen, is resistant to RA at all concentrations tested. Subline E-3, however, gave a mixed response. Although its growth was inhibited by high concentrations of RA, it was actually stimulated at low concentrations.

Effect of Estrogen on RA Inhibition. Although the lines tested showed similar patterns of resistance to RA and OHTAM, RA inhibition cannot be reversed by estrogen as inhibition by OHTAM can be (data not shown).

Effect of RA on Estrogen Receptor Level. To further explore possible relationships between the actions of retinoids and estrogens on MCF-7 cells, we measured the effect of RA on the level of estrogen receptor in MCF-7 cells by using the whole cell assay we have used before (23, 24). The results are shown in Fig. 3*B*, in which the level of estrogen receptor was measured in the same experiment shown in Fig. 3*A*. In all three cultures, RA caused an increase in the level of estrogen receptor, but the cultures differed in the degree of the response, with the greatest increase being observed in the uncloned MCF-7 cells. The decline in receptor level in uncloned MCF-7 and E-3 seen at the highest concentrations of RA used may be due to the fact that

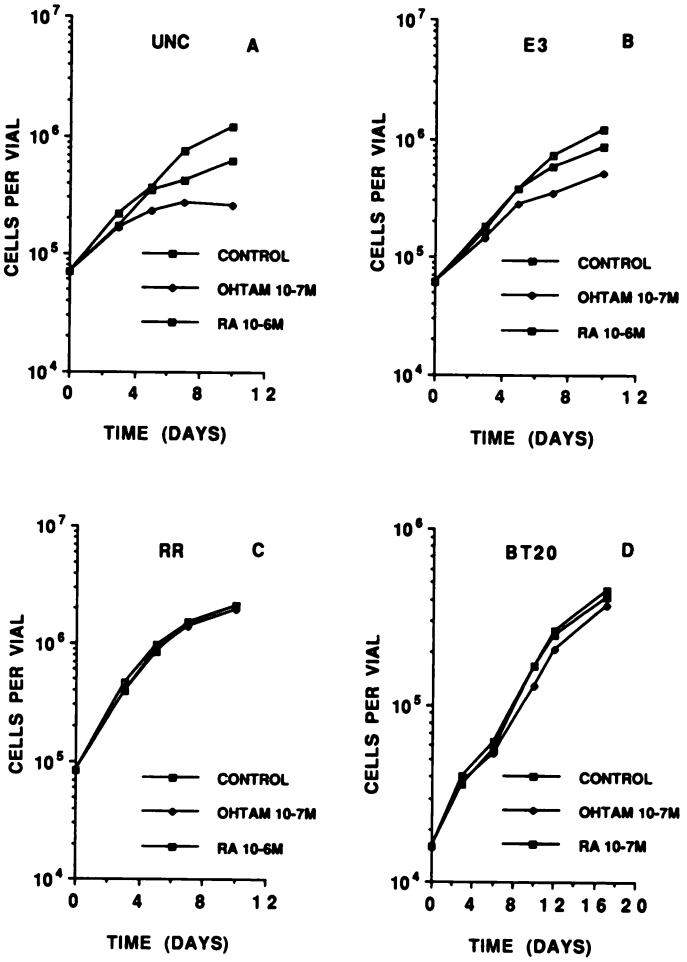

Fig. 1. Effect of RA and OHTAM on the growth of human breast cancer cells. Cells were plated as described in "Materials and Methods", and 3 days (UNC, E-3, and RR) or 1 day (BT20) later (day 0), the media were changed to media containing the indicated concentrations of RA or OHTAM. *A*, uncloned MCF-7 (*UNC*). *B*, E-3, a subline of MCF-7 resembling the uncloned culture in most ways. *C*, RR, a subline of MCF-7 resistant to antiestrogens. *D*, BT20, a human breast cancer cell line lacking estrogen receptors. *Points*, mean of duplicates; SD was smaller than the symbols.

at these concentrations growth of both cultures was significantly inhibited. Although RA increased the level of estrogen receptor, an experiment using the uncloned MCF-7 showed that it had no effect on the K_d of the receptor. Untreated cells had 67,000 estrogen-binding sites/cell, and cells treated for 10 days with 10^{-8} M RA had 116,000 sites/cell, an increase of 73%. The K_d for both cultures was the same, 1.6×10^{-9} M.

Effect of RA on Plasminogen Activator Production. One of the effects of estrogen on MCF-7 cells is to increase the level of plasminogen activator produced and secreted into the medium (25). The stimulation observed is primarily due to the effect on the tissue type plasminogen activator (28). This stimulation is inhibited by OHTAM (25), which for plasminogen activator, unlike for progesterone receptor, acts as a pure estrogen antagonist (29). We therefore wanted to determine what effect RA had on plasminogen activator production by MCF-7 cells. As shown in Fig. 4, the effect of RA on plasminogen activator production by MCF-7 cells is dependent on estrogen. In the absence of estrogen, RA inhibits the basal level of plasminogen activator production by E-3 and RR cells. The results are the opposite in the presence of estrogen, however. Here, cells in which plasminogen activator production has been maximally stimulated by estrogen can be stimulated further by RA. This is especially evident in E-3.

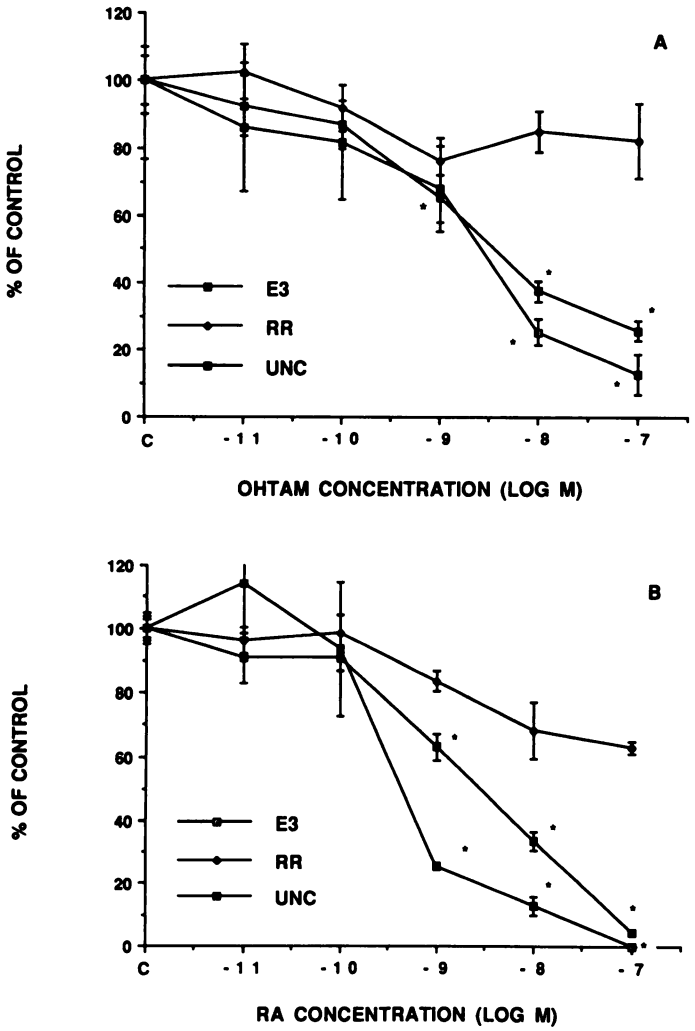

Fig. 2. Effect of various concentrations of RA and OHTAM on the colony-forming ability of MCF-7 cells. All cultures received 400 cells and media containing the concentrations of RA or OHTAM indicated. *A*, OHTAM. *B*, RA. *Points*, mean of triplicates; *bars*, SD. *, $P < 0.01$ *versus* untreated control (*C*).

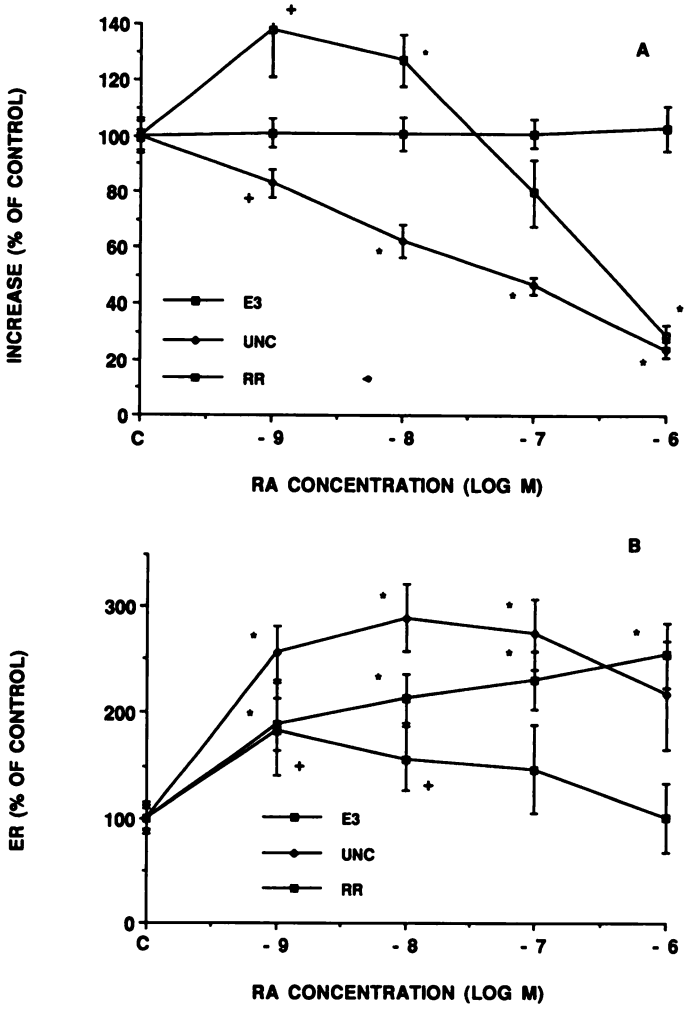

Fig. 3. Effect of various concentrations of RA on the growth and estrogen receptor level of MCF-7 cells. Cells were plated and 1 day later duplicate vials were taken for cell counts and the media in the rest were changed to the concentrations of RA indicated (day 0). Cultures were given fresh media containing RA on days 4 and 7. On day 10 three vials were used for cell counts and six vials were used for measuring the level of estrogen receptor. *A*, increase in cell number over 10 days. Controls increased 23-fold (*UNC*), 19-fold (*E-3*) and 42-fold (*RR*). *B*, estrogen receptor level. *Points*, mean of triplicates; *bars*, SD. *, $P < 0.01$ *versus* untreated control (*C*); +, $P < 0.025$ *versus* control (*C*).

DISCUSSION

The results presented here clearly indicate that there are interactions between the effects of estrogens, antiestrogens, and retinoic acid on MCF-7 cells. (*a*) In all MCF-7 sublines tested (Fig. 3*B*) retinoic acid increased the level of estrogen binding. RA has recently been shown to produce a 2-fold increase in estrogen receptor mRNA (16). (*b*) Plasminogen activator production, which is stimulated by estrogen in MCF-7 cells (25), was inhibited by retinoic acid in the absence of estrogen, but stimulated further by retinoic acid added simultaneously with a fully inducing dose of estradiol (Fig. 4). (*c*) A subline of MCF-7, RR, which was selected for its resistance to the antiestrogen tamoxifen proved to also be resistant to the growth inhibitory effects of retinoic acid (Figs. 1–3). In addition, as shown in Fig. 3, retinoic acid can inhibit or stimulate the growth of these MCF-7 sublines, depending on the concentration of RA used and the MCF-7 subline tested. A similar stimulation of MCF-7 cells by low concentrations of RA has been reported previously (3).

The results presented here do not provide evidence for any specific model which would explain these interactions. Enough is known about the mechanisms by which estrogens and retinoids act on cells, however, to make it possible to suggest probable explanations for these results. The receptors for estrogen

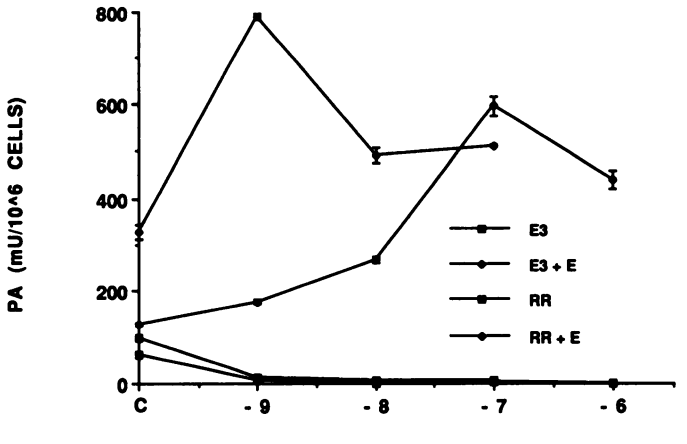

Fig. 4. Effect of estradiol and RA on plasminogen activator production by MCF-7 cells. Cells were treated for 4 days with the indicated concentrations of RA in the presence or absence of 10^{-8} M estradiol, and the media were assayed for plasminogen activator. *Points*, mean of triplicates; *bars*, SD. *C*, is untreated control.

and retinoic acid belong to the same superfamily of receptors which includes receptors for steroids and thyroid hormone (7). The most highly conserved portion of these receptors is the DNA-binding domain through which they bind to regions of DNA called hormone response elements. As has been shown for the mouse mammary tumor virus promoter, transcription may be regulated by more than one steroid hormone response element (30). These hormone response elements may be closely related (31) and in some cases one response element may mediate the response to more than one hormone (32). One of us (J. A. F.) has reported that RA can inhibit the estradiol-induced expression of the transforming growth factor α and *pS2* genes in MCF-7 (16). These observations raise the possibility that RARs and estrogen receptors may act directly to regulate the same genes. The results reported here are compatible with such a model. The antiestrogen-resistant subline RR has a normal estrogen receptor but may be resistant because it lacks a specific class of chromatin acceptor sites (sites on the chromatin which bind receptors). This class of sites binds estrogen receptors which have antiestrogens bound to them but does not bind estrogen receptors which have estrogens bound to them (19). If these are sites through which the retinoic acid receptor also acts to inhibit growth, then their loss would explain the resistance of RR cells to both antiestrogens and retinoic acid. There are no reports in which MCF-7 sublines resistant to either RA or antiestrogens have been tested for cross-resistance to the other. The effect of retinoic acid on plasminogen activator production could also be explained if the plasminogen activator gene is regulated by response elements for both estrogen and retinoic acid. We have shown earlier that both progesterone and estrogen stimulate production of plasminogen activator (25) and retinoic acid appears to regulate it too, although in opposite directions, depending on whether or not estrogen is present.

There is evidently a range of links between the effects of retinoic acid, estrogens, and antiestrogens on the MCF-7 breast cancer cell line. Further study of this system, including study of some of our other sublines and other responses to estrogen, antiestrogens, and RA should provide additional insights into the types of regulation possible in this system which is evidently responsive to more than one member of the family of receptors which includes those for retinoic acid and estrogen. In addition, a more detailed study of the specific RARs present in sublines exhibiting different responses to RA may help clarify the roles of these receptors in mediating the responses of breast cancer cells to RA.

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