The Effects of Androgens and Antiandrogens on Hormone-responsive Human Breast Cancer in Long-Term Tissue Culture

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SUMMARY

We have examined five human breast cancer cell lines in continuous tissue culture for androgen responsiveness. One of these cell lines shows a 2- to 4-fold stimulation of thymidine incorporation into DNA, apparent as early as 10 hr following androgen addition to cells incubated in serum-free medium. This stimulation is accompanied by an acceleration in cell replication. Antiandrogens [cyproterone acetate (6-chloro-17α-acetate-1,2α-methylene-4,6-pregnadiene-3,20-dione) and R2956 (17β-hydroxy-2,2,17α-trimethoxyestradi-4,9,11-triene-1-one)] inhibit both protein and DNA synthesis below control levels and block androgen-mediated stimulation. Prolonged incubation (greater than 72 hr) in antiandrogen is lethal. The MCF-7 cell line contains high-affinity receptors for androgenic steroids demonstrable by sucrose density gradients and competitive protein binding analysis. By cross-competition studies, androgen receptors are distinguishable from estrogen receptors also found in this cell line. Concentrations of steroid that saturate androgen receptor sites in vitro are about 1000 times lower than concentrations that maximally stimulate the cells. Changes in quantity and affinity of androgen binding to intact cells at 37°C as compared with usual binding techniques using cytosol preparation at 0°C do not explain this difference between dissociation of binding and effect. However, this difference can be explained by conversion of [3H]-5α-dihydrotestosterone to 5α-androstenediol and more polar metabolites at 37°C. An examination of incubation media, cytoplasmic extracts, and crude nuclear pellets reveals probable conversion of [3H]testosterone to [3H]-5α-dihydrotestosterone. Our data provide compelling evidence that some human breast cancer, at least in vitro, may be androgen dependent.

INTRODUCTION

We have recently examined several cell lines of human breast cancer maintained in long-term tissue culture. The hormone responsiveness of some of these cell lines to estrogen (6, 7) and glucocorticoid (8) encouraged us to examine the effects of androgens and antiandrogens.

Information characterizing the interaction between androgens and breast cancer would be desirable for several reasons. First, androgens can affect the growth of breast cancer in animals. Pharmacological administration of androgens to rats bearing dimethylbenzanthracene-induced mammary carcinomas is associated with objective tumor regression (19, 22). Shionogi 115 cells, from a mouse mammary cancer in continuous tissue culture, have been shown to be stimulated by physiological concentrations of androgen (21), thus suggesting that some breast cancer might be androgen responsive in addition to being estrogen responsive.

Evidence also indicates that tumor growth in humans may be significantly altered by androgens. About 20% of patients with metastatic breast cancer will show objective tumor regressions when treated with androgens (1). A somewhat higher proportion of patients will respond to adrenalectomy (4). The efficacy of this latter therapy has been attributed to the removal of androgen precursors of adrenal origin, primarily dehydroepiandrosterone and Δ4-androstenedione. Clear-cut evidence that human breast cancer is androgen dependent has not, however, been previously available.

Finally, androgen receptor proteins have recently been demonstrated in some human breast cancer samples (3, 9, 12) and have been shown to be distinct from both estrogen receptor and sex steroid-binding globulin. It is clear that most if not all steroid hormone actions are mediated by an initial interaction of steroid hormone and receptor (13). Since estrogen receptor determination in human breast cancer appears to be of value in guiding therapeutic decisions in the management of metastatic breast cancer by predicting which patients will respond to hormonal manipulations (14), it is reasonable to hope that further delineation of the mechanism of interaction of androgens with breast cancer might also have therapeutic implications.

Study of these processes, however, has been hampered by the lack of a suitable model system in which the action of one hormone could be studied independently of the actions of other trophic hormones. In addition, in vivo model systems for androgen action are significantly perturbed by interactions of androgen with sex steroid-binding globulin as well as by effects of androgens on concentrations of many other hormones including gonadotrophins, prolactin, and estrogen (18).

We now report the characterization of an androgen-responsive human breast cancer cell line grown in continuous...
tissue culture that obviates the above difficulties. Androgen effects are demonstrable in totally defined medium that contains no additional hormonal or serum factors. We are optimistic that such a model system may significantly aid in the study of mechanisms of androgen responsiveness.

**MATERIALS AND METHODS**

**Cells and Culture Techniques.** Methods for propagation of cells are as previously described (6, 7). All cell lines have been maintained for at least 1 year without changes in responsiveness. All lines have been tested for Mycoplasma (Flow Laboratories, Rockville, Md.) and were shown to be free of contamination. All cell lines used in the present study synthesize enzymatically active \( \alpha \)-lactalbumin. (M. E. Lippman and B. Vonderhaar, unpublished observations; \( \alpha \)-lactalbumin activity was detected using an enzymatic assay in which cell homogenates are incubated with \( [1^4C] \)UDP-galactose and formation of \( [1^4C] \)lactose is measured.)

**Sucrose Density Gradient Analysis and Binding Studies.** Analysis of androgen receptors on sucrose gradients and by competitive protein binding assay was as previously described (6, 8). Briefly, 100,000 \( \times \) g supernatants of cell homogenates were prepared and incubated with \( 5 \times 10^{-9} \) M \( [\text{H}] \)-5\( \alpha \)-DHT with or without competitor. After treatment with dextran-coated charcoal, these extracts were layered onto 5 to 20% sucrose gradients in 10% glycerol. The purity of \( [\text{H}] \)DHT (107 Ci/m mole; Amersham Searle Corp., Evanston, Ill.) was checked by thin-layer chromatography (10) and was shown to be more than 98% radiochemically pure. For studies designed to examine the specificity of binding, aliquots of cytoplasmic extract were pipetted into tubes containing various concentrations of unlabeled steroid as 1000-fold concentrates in ethanol. Radiolabeled steroid, either \( 10^{-9} \) M \( [\text{H}] \)-5\( \alpha \)-DHT as above or \( 17\beta \)-[\( \text{H} \)]estradiol (100 Ci/m mole; Amersham Searle), was added immediately thereafter. Labeled steroid was used at \( 10^{-9} \) M, which is only slightly above the dissociation constant for the receptor and therefore not saturating, in order to keep nonspecific binding below 10% of the total binding.

**Effect of Androgens on Cell Growth.** Cells growing logarithmically were harvested with sterile 0.02% EDTA and plated in 60-mm sterile plastic Petri dishes in MEM with serum. After 24 hr, the medium was changed to medium without serum. 5\( \alpha \)-DHT or R2956 (17\( \beta \)-hydroxy-2,17\( \alpha \)-tri-methylsterola-4,9,11-triene-3-one) at a concentration of \( 10^{-4} \) M in ethanol was added to one-third of the dishes, respectively, to give a final concentration of \( 10^{-7} \) M. Dishes in triplicate were harvested daily, and cells were either counted in a hemocytometer or assayed for total protein.

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**Whole-Cell Binding Experiments.** Cells growing in MEM plus 5% fetal calf serum were changed to MEM without serum on the day prior to the start of the experiment. Cells were harvested by suspension in trypsin-EDTA and were washed twice in PBS. The cells were then dispersed in MEM supplemented with 25 m\( \mu \)M N-tris(hydroxymethyl)methylglycine (pH 7.2) at a density of about 10\(^6/\)ml. Cells were then added to 12- x 75-mm glass tubes containing \( [\text{H}] \)-5\( \alpha \)-DHT at various concentrations with or without a 1000-fold excess of unlabeled 5\( \alpha \)-DHT. Tubes were shaken in a water bath at either 0° or 3 4° or 37° for 1 hr. After the incubation, the medium was diluted with 3 ml of ice-cold PBS, and the cells were collected by centrifugation for 30 sec in a Clay Adams serofuge. The cells were washed twice in ice-cold PBS, suspended in 0.5 ml of distilled water, and sonically dispersed for 4 sec at the lowest setting in a Branson sonicator; aliquots were taken for protein determination by the technique of Lowry et al. (11) or counted in Aquasol (New England Nuclear, Boston, Mass.) in a Packard liquid scintillation counter (efficiency for tritium, 80-40%). Results were analyzed by the method of Scatchard (20).

**Conversion of 5\( \alpha \)-DHT to 5\( \alpha \)-Androstane-3\( \alpha \),17\( \beta \)-dilol.** T-75 flasks of confluent MCF-7 cells were incubated at 37° in 10 ml of serum-free MEM containing \( 10^{-7} \) M \( [\text{H}] \)-5\( \alpha \)-DHT (44 Ci/m mole) for 0, 1, 4, and 8 hr. The medium at each time point was collected and extracted with 2 volumes of methylene chloride. The organic phase was separated by centrifugation and evaporated to dryness under nitrogen. The residues were redissolved in 1 ml of methylene chloride containing 200 \( \mu \)g each of androstenediol, androstenedione, testosterone, and 5\( \alpha \)-DHT. Fifty \( \mu \)l were applied to duplicate thin-layer Silica Gel G plates (20 \( \times \) 20 cm) (Analytech, Inc., Newark, Del.), and the chromatograms were developed by ascending chromatography in chloroform:methanol (98:2, v/v). The steroid spots on each plate were visualized by streaking the ascending paths of each sample with H\( \text{SO}_4 \):methanol (1:1, v/v) and heating at 90° for 2 hr; the other plate was quantitated by counting 0.5-cm sections of the silica in Aquasol (New England Nuclear) in a Packard liquid scintillation counter.

**Conversion of Testosterone to 5\( \alpha \)-DHT.** The same procedure as above was followed utilizing \( 10^{-7} \) M \( [\text{H}] \)testosterone (82 Ci/m mole) as the steroid to be studied. Extractions were done after 1 and 4 hr of incubation. However, the plates were developed twice to ensure adequate separation of the 5\( \alpha \)-DHT from the testosterone. Recovery, monitored with \( [\text{H}] \)testosterone, is given in "Results."
RESULTS

The effects of $10^{-7}$ M 5α-DHT or the antiandrogen R2956 (2) on growth of the MCF-7 cell line are shown in Chart 1. 5α-DHT stimulates cell division above control levels. Inhibition by $10^{-7}$ M R2956 is apparent after 2 days in culture. A stimulatory effect of androgen and an inhibitory effect of antiandrogen on total protein per dish are also seen. The exact mechanism of the R2956 effect may not be via an interference with an androgen-stimulated pathway as discussed below, since androgens incompletely reverse the effects of antiandrogen treatment. In the experiment shown, the cells were in serum-free medium. We observe that the cells grow more slowly under serum-free conditions even when the medium is supplemented with hormones. This is not surprising considering the many potentially growth-promoting components in serum. Nonetheless, it is clear that androgen stimulates cell division above control.

The effects of various concentrations of 5α-DHT on [3H]thymidine and [14C]leucine incorporation into macromolecules in the MCF-7 human breast cancer cell line are shown in Chart 2. Incorporation of amino acid or nucleoside begins to increase over control levels at a 5α-DHT concentration of $5 \times 10^{-9}$ M. 5α-DHT at $10^{-6}$ M stimulates thymidine incorporation 300% and leucine incorporation 200% when incorporation rates are measured 48 hr after the addition of hormone. There is a rapid decrease in stimulation seen as 5α-DHT concentrations rise above $10^{-6}$ M. In the experiment shown, $10^{-5}$ M 5α-DHT reduces incorporation to control levels when measured at 48 hr. Frequently, values significantly below control are obtained at this steroid concentration. This variation may be due to metabolic effects discussed below. The stereoisomer 5β-DHT is nonstimulatory at any concentration tested (Table 1). If the cells are left in 5α-DHT at concentrations $>10^{-6}$ M, the cells round up from the surface of the dish, detach, and die after about 72 hr. This effect may be nonspecific since $10^{-6}$ M 5β-DHT also inhibits these cells. 5β-DHT, $10^{-4}$, is without effect. Cell lines that lack androgen receptor activity and are unstimulated by lower concentrations of 5α-DHT are also inhibited by 5β-DHT at $10^{-6}$ M. This result is similar to that seen with high molar concentrations of estrogen and human breast cancer cell lines as previously observed (7).

The overall stimulation of precursor incorporation into protein or nucleic acid may exceed the net increment in cell division shown in Chart 1 because of the growth-limiting, serum-free conditions used which probably enhance catabolism of cellular macromolecules.

If the cells are responsive to 5α-DHT, one would predict that they would contain specific cytoplasmic androgen receptors. Five to 20% sucrose density gradients in 10% glyceral of cytoplasmic extracts incubated with radiolabeled 5α-DHT are shown in Chart 3 for the androgen-responsive MCF-7 cell line. [3H]-5α-DHT ($5 \times 10^{-9}$ M) is bound to a molecule that sediments at about 8 S. This peak of binding is totally competed by the addition of a 100-fold excess of unlabeled 5α-DHT on a 1000-fold excess of the antiandrogen cyproterone acetate (6-chloro-17α-acetate-1,2α-methylene-4,6-pregnadiene-3,20-dione). A 1000-fold excess of unlabeled tamoxifen (ICI 46474; trans isomer of 1-[4-(2-dimethylaminoethoxy)phenyl]-1,2-phenyl-1-but-1-ene citrate), an antiestrogen, competes with less than 10% of the [3H]-5α-DHT bound to receptor, suggesting that the [3H]-5α-DHT is binding to an androgen receptor site. We were curious as to whether tamoxifen might compete with DHT for androgen receptor sites, since an appreciable response to antiestrogen therapy has been reported in women with metastatic breast cancer even if tumor samples lack estrogen receptor (14).

Binding of [3H]-5α-DHT to cytoplasmic extracts is shown in Chart 4. Using a dextran-coated charcoal assay, a high-affinity receptor site is demonstrable. The straight line obtained by Scatchard analysis (20) of the binding data (Chart 4, inset) suggests that [3H]-5α-DHT is binding to a class of receptors of uniform affinity ($K_d = 8.7 \times 10^{-10}$ M, $r = 0.982$). There are 45 fmoles of [3H]DHT bound per mg of cytoplas-
Our previous analysis of estradiol receptor in this same cell line revealed 83 fmoles of [3H]estradiol binding per mg of cytoplasmic protein with a similar Kd of $6.8 \times 10^{-10}$ ($r = 0.986$) (7). We invariably observe nearly twice the number of estrogen-binding sites in paired cytosol preparations. These differences in number of binding sites do not allow one to differentiate between estrogen and androgen receptors in these cells.

We therefore examined the receptors for differences in specificity of binding in the cross-competition studies shown in Charts 5 and 6. In Chart 5 are shown results of incubating 17β-[3H]estradiol with cytoplasmic extracts from MCF-7 human breast cancer in the presence of various concentrations of unlabeled competing steroids. Unlabeled 17β-estradiol and diethylstilbestrol compete with [3H]estradiol for receptor sites. The failure to observe the exactly predicted competition by unlabeled 17β-estradiol may be due to the sequence of addition of unlabeled steroid to cytosol followed by labeled steroid. The antiestrogens nafoxidine, tamoxifen, and Cl 628 (triphenylatedenylene derivatives) all competed at least 80% of the specifically bound [3H]estradiol from receptor when present in 2000-fold molar excess. At 2000-fold molar excess, 5α-DHT, testosterone, Δ4-androstenedione, and the antiandrogen R2956 all fail to compete significantly with [3H]estradiol for binding sites. This strongly suggests that [3H]estradiol is bound to a receptor with a restricted binding affinity for estrogens and antiestrogens. Results of a similar experiment are shown in Chart 6, but [3H]-5α-DHT was used as the trace. The ability of various steroids to compete with [3H]-5α-DHT for receptor sites was examined. 5α-DHT and testosterone as well as antiandrogens R2956 and cyproterone acetate completely prevent [3H]-5α-DHT from binding to specific binding sites. The slightly excessive competition of a 2-fold molar excess of unlabeled 5α-DHT probably is due to the order of addition used. Unlabeled steroid is added to cytoplasmic extract followed immediately by labeled ligand. The use of some-

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**Chart 2.** The effects of 5α-DHT on the incorporation of [3H]thymidine or [14C]leucine in acid-precipitable material. Values are means of quadruplicate determinations ± S.D.

**Table 1**

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Concentration</th>
<th>Thymidine incorporation (dpm $\times 10^{-15}$ μg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>32.8 ± 3.8*</td>
</tr>
<tr>
<td>5α-DHT</td>
<td>$10^{-7}$</td>
<td>51.7 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>$10^{-6}$</td>
<td>63.4 ± 0.95</td>
</tr>
<tr>
<td>5β-DHT</td>
<td>$10^{-7}$</td>
<td>33.5 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>$10^{-6}$</td>
<td>35.0 ± 1.0</td>
</tr>
</tbody>
</table>

* Means of triplicate determinations ± S.D.
what less than saturating concentrations of labeled ligand will also contribute to the excessive apparent competition by unlabeled 5α-DHT. While 17β-estradiol competes with [3H]-5α-DHT for receptor sites, it is about 100 times less effective as a competitor than are the 2 androgens. The antiestrogens nafinoxidina and tamoxifen are less effective competitors than either antiandrogen at equivalent molar ratios. The reduced C-19 metabolite, etiocholanolone, does not compete with [3H]-5α-DHT for receptor sites. Although the results are not shown in this chart, we were unable to demonstrate significant competition for the receptor site by 5β-DHT even when it was present in 2000-fold molar excess. We conclude that these cells contain 2 receptors: an androgen receptor with a much lower binding affinity for estrogen, and an estrogen receptor with virtually no affinity for androgens. 17β-Estradiol and the antiandrogen cyproterone acetate appear to have similar affinities for the androgen receptor.

Because of the ability of unlabeled testosterone to compete with [3H]-5α-DHT for androgen receptor sites, we examined the binding of [3H]testosterone to receptor directly. Since there is evidence that testosterone and not 5α-DHT is the "active" androgen in some tissues (13), we hoped that large differences in the affinity of testosterone and 5α-DHT for receptor might suggest which was the biologically relevant hormone. The binding data are shown in Chart 7. The binding data are replotted in the inset according to the Scatchard technique (20). The straight line that is obtained (r = 0.939) suggests that testosterone is binding to a single class of receptors of uniform affinity. The dissociation constant for testosterone, 1.3 × 10⁻⁹ M, is about 2-fold greater than that obtained for 5α-DHT. This small and possibly insignificant difference in binding affinity does not allow conclusion as to whether 5α-DHT and/or testosterone is the active androgen. For this particular cytosol preparation, we found total 5α-DHT- and testosterone-binding capacity to
be within 5% of each other. The good agreement of these numbers with the results shown in Chart 6 suggests that testosterone and 5a-DHT are binding to the same androgen receptor site.

Comparison of Charts 2 and 4 reveals that there is about a 100- to 1000-fold discrepancy between concentrations of DHT that half-maximally saturate androgen receptor and concentrations that half-maximally stimulate either leucine or thymidine incorporation. It would appear that every receptor site would have to be occupied by androgen before any induced response is seen. One possible explanation is that binding of [3H]DHT to cytosol preparations at 0° is of much higher affinity than that seen in intact cells at physiological temperatures or that androgen cannot enter intact cells. To explore this possibility intact cell binding experiments were performed at 0° and 37°. Scatchard analyses of the results are shown in Chart 8. At both 0° and 37° essentially the same number of binding sites are identified. The affinity of the cells for [3H]DHT appeared to be 3 times higher at 37° than at 0°, and both dissociation constants are within a factor of 10 of that seen on cytosol preparation at 0° (Chart 4). While the somewhat higher affinity of binding at 37° is surprising, this result may be due to temperature-dependent transformation of receptor and nuclear translocation that decreases the apparent Kd. It is certainly possible that a transformed receptor or one bound to chromatin sites might have an altered affinity for steroid. Whether or not this increase in binding affinity at 37° is significant, we must conclude that at least it is not strikingly reduced, the major point of the experiment. Furthermore, androgen is clearly able to enter and bind to high-affinity sites in these cells. Thus, alterations in either binding affinity or androgen uptake in intact cells at 37° do not appear to explain the discrepancy between androgen binding and cell stimulation. The straight lines obtained by Scatchard analysis at 0° and 37° (r = 0.956 and 0.994, respectively) suggest that,
active androgen available to the cells. In results not shown 5α-androstanediol was detectable in cell homogenates as early as 1 min following addition of [3H]-5α-DHT to the medium.

Because of the similar dissociation constant of the androgen receptor in these cells for [3H]-5α-DHT as compared to testosterone, one might surmise that 5α-DHT is also an "effector" androgen in these cells. For this to be true, one would expect the cells to contain 5α-reductase activity to convert circulating testosterone to 5α-DHT. This question was examined directly by incubating the cells with [3H]-testosterone for various times. At 10 min, 1 hr, and 4 hr the media, crude cytosol, and crude nuclear pellet were examined for radioactivity migrating on thin-layer chromatography plates in the position of 5α-DHT. At 1 hr 5.9% of the counts extracted from the nuclear pellet migrated in the positions of androstanediol and 5α-DHT. At 4 hr, of the counts that could be recovered in the nuclear fraction 13.9% migrated in the position of 5α-DHT and androstanediol. Obviously, the sum of both of these steroids represents a minimal estimate of 5α-reductase activity. At 1 hr 6.4% of the radioactivity found in the supernatant fraction after the nuclei were sedimented migrated in the positions of androstanediol and 5α-DHT. This was reduced to 5.16% at 4 hr. Less than 0.2% of the radioactivity in the media migrated with androstanediol or 5α-DHT at any time studied. Recovery monitored by adding a trace of [14C]-testosterone to the methylene chloride cell extracts was 75.6% for the 0 con-

even in intact cells, high-affinity binding of [3H]DHT is to a single class of receptor sites.

An alternative potential explanation for the discrepancy between binding affinities and optimal inducing concentrations of 5α-DHT is that the cell line is capable of transforming 5α-DHT to an inactive metabolite. Metabolism of androgens was examined directly by incubating the cells in serum-free medium containing [3H]5α-DHT and examining radioactive steroid products at various time intervals by thin-layer chromatography (Chart 9). Analysis of the incubation medium reveals that there is very significant conversion of [3H]-5α-DHT to a steroid migrating with an RF similar to that of 5α-androstanediol. After 1 hr of incubation almost one-half of the 5α-DHT has been converted to androstanediol.

As the time of incubation increases, there is further conversion of DHT to androstanediol as well as the appearance of a new peak of radioactivity near the origin of the plate and a large increase in counts not extractable in the methylene chloride phase, suggesting even further metabolism or conjugation of androgen to more polar products. The extent of conversion is nearly 70% by 8 hr. Thus, it appears likely that the discrepancy between optimal inducing concentrations of androgen and concentrations of androgen that bind to receptor is a function of metabolism and resultant inactivation of 5α-DHT to androstanediol or other metabolites. Also it is apparent that small changes in incubation conditions or cell density could drastically alter amounts of biologically active androgen available to the cells. In results not shown 5α-androstanediol was detectable in cell homogenates as early as 1 min following addition of [3H]-5α-DHT to the medium.

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trol, 100% at 1 hr, and 92% at 4 hr for the nuclear pellets. Values for the supernatants were 92 and 94% at 1 and 4 hr. Recoveries from media were 92 and 100% at 1 and 4 hr. We cannot exclude that in vivo, both testosterone and 5α-DHT are biologically active.

The effects of the antiandrogen R2956 (2) were investigated. As previously shown in Chart 1, 10⁻⁷ M R2956 inhibits cell division after 3 days in culture. Additional results are shown in Table 2. Thymidine incorporation is stimulated 120% by 10⁻⁶ M 5α-DHT when measured at 48 hr. R2956 at a concentration of 10⁻⁶ M inhibits thymidine incorporation to 63% of control values. 5α-DHT, 10⁻⁶ M, slightly reverses inhibition by 10⁻⁶ M R2956. Although R2956 can compete with [3H]-5α-DHT for receptor (Chart 6), the exact mechanism of action of R2956 cannot be assumed to be competitive for the receptor alone since the process is largely reversible by addition of 5α-DHT (Table 2). This may reflect a mechanism of action in addition to simple competition for cytoplasmic receptor. Preliminary results suggest that R2956 may inhibit thymidine incorporation but not leucine incorporation in other cell lines that appear to lack androgen receptor and are not stimulated by physiological androgens. Thus, while the partial reversal of antiandrogen effects by 5α-DHT and its known physiological actions (2) suggest that R2956 is an antiandrogen, its mechanism of action in tissue culture will require further study. We are particularly hampered by the rapid metabolism of 5α-DHT previously described. This may be a sufficient explanation for our inability to reverse antiandrogen effects.

The effects of another antiandrogen, cyproterone acetate, are shown in Table 3. Cyproterone acetate by itself has little effect on thymidine incorporation at concentrations less than 10⁻⁶ M. There is some inhibition seen at 10⁻⁶ and 10⁻⁵ M. 5α-DHT at a concentration of 10⁻⁶ M nearly doubles thymidine incorporation in 2 experiments. Combination of 10⁻⁶ M cyproterone acetate and 10⁻⁸ M 5α-DHT results in thymidine incorporation significantly above control but less than that seen with 5α-DHT alone. Thus cyproterone acetate effects appear to be reversible by androgen. These experiments would be easier to interpret if it were possible to use molar ratios of cyproterone acetate and 5α-DHT of about 200:1 since, as shown in Chart 6, cyproterone acetate competes less effectively than 5α-DHT for androgen receptor sites. Unfortunately, concentrations of 5α-DHT greater than 10⁻⁷ M are required for significant stimulation of the cells. Thus, it is difficult to achieve concentrations of cyproterone acetate that would not be nonspecifically lethal to the cells. Differences between Experiments 1 and 2 shown in Table 3 are not due to differing incubation times. In Experiments 1 and 2, cells were incubated in hormones for 36 hr before addition of labeled precursors. However, small differences in total cell number plated could easily alter the amount of 5α-DHT available during the incubation period. We presume that 5α-DHT metabolism as described above is likely to be a linear function of cell number, and 2-fold variations between experiments in number of cells plated are common.

The effects of androgens on 4 other human breast cell lines are shown in Table 4. The androgen-responsive MCF-7 cell line is shown for comparison. The other 4 lines all fail to show stimulation by 5α-DHT at 10⁻⁶ M concentration. Lower concentrations (10⁻⁷ and 10⁻⁸ M) were also tested and failed to stimulate these cell lines. For comparative purposes the quantity of androgen receptor is shown in Table 4. The G-11, MCF-7, and Evans E cell lines have detectable receptor activity. Despite this, the Evans E and G-11 lines do not respond to androgen under conditions we used.

**DISCUSSION**

Our studies strongly support the notion that some human breast cancers, at least in vitro, may be stimulated by androgens. In this work, we demonstrate that androgen receptor may be distinguished from estrogen receptor present in the same cell line. Furthermore, stimulation of macromolecular synthesis by androgen is probably mediated by interaction with this receptor since 5α-DHT, unlike testosterone, is not a substrate for aromatization (5) and shows virtually no ability to displace [3H]estradiol from estrogen receptor.

Our studies also suggest an interesting mechanism whereby tumors may escape stimulation by low levels of androgen. We have shown that the MCF-7 cell line can rapidly metabolize 5α-DHT to androstenediol and to as yet unidentified metabolites or possibly conjugates. Previous workers (15, 16) have documented the ability of breast tumor samples to synthesize active androgen and estrogen from androgen precursors such as dehydroepiandrosterone. The metabolic potential of a tumor to either synthesize

| Table 2 Effects of 5α-DHT and R2956 on [³H]Thymidine incorporation in MCF-7 human breast cancer |
|---|---|
| Addition (M) | Thymidine incorporation (dpm x 10⁻²/μg protein/hr) |
| None | 25.4 ± 2.3a |
| R2956, 10⁻⁷ | 23.6 ± 1.2 |
| R2956, 10⁻⁸ | 16.2 ± 1.5 |
| 5α-DHT, 10⁻⁷ | 44.3 ± 3.0 |
| 5α-DHT, 10⁻⁸ | 57.3 ± 9.2 |
| 5α-DHT, 10⁻⁴ + R2956, 10⁻⁸ | 17.4 ± 0.45 |
| 5α-DHT, 10⁻⁴ + R2956, 10⁻⁶ | 23.2 ± 3.2 |

*a Means of triplicate determinations ± S.D.
The usefulness of estrogen receptor in guiding clinical androgen action. We are currently attempting to develop a human cell line in long-term tissue culture. Although a lion with an evaluation of receptors as well as steroid metabolism, we have shown to be distinct from estrogen receptor. Analysis of the mechanism of androgen action. System may provide palpable advantages for the study of androgen receptor in fresh human breast cancer specimens which we have shown to be distinct from estrogen receptor. The usefulness of estrogen receptor in guiding clinical decisions involving hormonal manipulations in human breast cancer is already established (14). Conceivably, analysis of tumor material for androgen receptors might similarly be of value.

Possibly the most significant aspect of the present work concerns the availability of a markedly androgen-responsive human cell line in long-term tissue culture. Although studies on the Shionogi carcinoma (21) and a recently characterized Syrian hamster tumor of the ductus deferens (17) have yielded important data, these cell lines show rather limited androgen responsiveness and have not been reported to be killed by antiandrogens. Thus, the current system may provide palpable advantages for the study of androgen action. We are currently attempting to develop cell lines resistant to the effects of antiandrogens in the hope that such cells will offer interesting reagents for the analysis of the mechanism of androgen action.

REFERENCES


Table 4

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Control*</th>
<th>5α-DHT, 10⁻⁴ M</th>
<th>5β-DHT, 10⁻⁴ M</th>
<th>Quantity androgen receptor*</th>
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<tbody>
<tr>
<td>MCF-7</td>
<td>32.8 ± 3.5*</td>
<td>63.4 ± 0.95</td>
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<td>G-11</td>
<td>32.6 ± 8.6</td>
<td>32.3 ± 2.1</td>
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<td>HT-39</td>
<td>24.9 ± 3.4</td>
<td>20.7 ± 5.6</td>
<td>27.6 ± 7.0</td>
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<td>MDA-231</td>
<td>49.5 ± 0.38</td>
<td>45.4 ± 1.1</td>
<td>46.7 ± 4.0</td>
<td>0</td>
</tr>
<tr>
<td>Eves E</td>
<td>31.0 ± 0.78</td>
<td>35.8 ± 2.8</td>
<td>28.0 ± 6.8</td>
<td>15.3</td>
</tr>
</tbody>
</table>

* fmol [3H]-5α-DHT bound per mg cytoplasmic protein.

Values are means of triplicate determinations ± S.D.
The Effects of Androgens and Antiandrogens on Hormone-responsive Human Breast Cancer in Long-Term Tissue Culture

Marc Lippman, Gall Bolan and Karen Huff


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