Characterization of a Receptor-negative, Hormone-nonresponsive Clone Derived from a T47D Human Breast Cancer Cell Line Kept under Estrogen-free Conditions

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ABSTRACT

We have established an estrogen receptor- and progesterone receptor-negative, hormone-nonresponsive breast cancer cell line from a receptor-positive, hormone-responsive line grown under estrogen-free conditions. T47D breast cancer cells were cultured under estrogenized conditions (in phenol red-containing medium supplemented with whole fetal bovine serum) and cloned to produce line T47D:A18. The parental T47D line was also estrogen deprived (in phenol red-free medium supplemented with dextran-coated charcoal-treated fetal bovine serum) for more than 1 year and subsequently clone T47D:C4 was established. T47D:A18 was estrogen receptor and progesterone receptor positive as determined by both ligand binding assay analysis and enzyme immunoassay analysis. T47D:C4 cells were estrogen receptor and progesterone receptor negative and mRNA for these receptors was not detected. Incubation of hormone-responsive T47D:A18 cells with 17β-estradiol caused a 3-fold increase in cell growth over 8 days when compared to control. This stimulation of growth was completely inhibited by the anti-estrogens 4-hydroxytamoxifen (0.1 μM) and ICI 164,384 (1.0 μM). Receptor-negative T47D:C4 cells were refractory to the effects of both 17β-estradiol and the antiestrogens.

T47D:A18 cells grown under both estrogen-containing and estrogen-free conditions expressed low levels of transforming growth factor (TGF)-α and epidermal growth factor receptor mRNA. In the presence of estrogen, high levels of TGF-β1 mRNA were detected in T47D:A18 cells. These levels decreased when T47D:A18 cells were grown in estrogen-free media. Conversely, TGF-β2 mRNA was not detected in T47D:A18 cells cultured under estrogenic conditions; however, message was detected after the cells were cultured under estrogen-free conditions. T47D:C4 cells expressed low levels of TGF-α, epidermal growth factor receptor, TGF-β1, and TGF-β2 mRNA. These studies characterize a novel hormone-nonresponsive cell line which has been established from a hormone-responsive cell line grown under estrogen-free and drug-free conditions. Further analysis of these lines should provide valuable information concerning the development of antiestrogen-resistant breast cancer.

INTRODUCTION

The antiestrogen TAM is an effective anticancer agent for the treatment of hormone receptor-positive breast cancer and is generally not effective against hormone receptor-negative disease (1). One potential mechanism of resistance to TAM is through the conversion of a tumor from a hormone-responsive, receptor-positive state to a hormone-nonresponsive, receptor-negative state. This type of resistance to TAM has been demonstrated in tumors from breast cancer patients. In retrospective clinical studies, Hull et al. (2) found that there was a 19% conversion of ER-positive tumors to ER-negative tumors when sequential biopsies were performed. These investigators also found a 44% conversion of initially PR-positive tumors to PR-negative tumors (3). This rate of conversion increased to 56% in patients who had received an endocrine therapy such as TAM. Patients whose tumors had lost PR and had become refractory to endocrine therapy had a significantly decreased chance of overall survival.

Various rodent model systems have been used to study mechanisms of resistance to hormonal treatment. Sluyser et al. (4) utilized a transplantable GR mouse mammary tumor model to study the loss of receptors in tumors over time. The conversion from receptor-positive, hormone-responsive tumors to receptor-negative, hormone-nonresponsive tumors was facilitated by growth in ovariectomized, estrogen-deprived animals. These studies illustrate the dynamics involved in the outgrowth of hormone-nonresponsive cell populations. However, the environment in vivo is extremely complex, so attempts are being made to study this phenomenon in vitro by using human breast cancer cell lines. These studies may provide clues to the cellular changes that occur, and uncover potential targets for future therapies.

The development of antiestrogen-resistant, receptor-negative cell populations has not previously been observed in cell lines cultured in a drug-free environment. An antiestrogen-resistant T47D breast cancer cell line (T47D∞) was established in vitro without drug treatment. However, T47D∞ cells contain low levels of ER and high levels of PR. MCF-7 cells have been continuously treated with antiestrogens such as LY117018 and TAM to select for resistant cells in vitro (6–8). However, the resulting antiestrogen-resistant MCF-7 lines also retain steroid receptors and remain sensitive to stimulation by 17β-estradiol. The lack of emergence of receptor-negative, antiestrogen-resistant cells may be a result of the conditions used for breast cancer cell culture. Recently, it was reported that phenol red, a pH indicator present in most cell culture media, contains an estrogenic contaminant that stimulates breast cancer cell growth in vitro (9–12). Therefore, all breast cancer cell lines cultured in vitro have an increased basal growth rate and are refractory to further growth stimulation by 17β-estradiol (13–15). However, these cells retain ER and PR and their basal growth rate is inhibited by antiestrogens. Therefore, it appeared that receptor-negative, hormone-resistant cell populations may not readily emerge under estrogen-free, drug-free conditions.

We have studied the effects of long-term estrogen deprivation on hormone-responsive T47D breast cancer cells (16) in culture.
The initially hormone-responsive T47D (A line) became refractory to both estrogens and antiestrogens during long-term estrogen deprivation and the estrogen receptor (and its mRNA) became undetectable (C line). An ER-negative and PR-negative, hormone-nonresponsive clone was isolated from the T47D C line after these cells were cultured in estrogen-free conditions for approximately 1 year (17). Our overall goal is to determine the biochemical changes that occur in breast cancer cells that makes them refractory to antiestrogens. In this way we can establish a mechanism for the phenomenon and perhaps develop a strategy to prevent antiestrogen resistance from occurring or possibly determine whether the effect is reversible. As a first step, we characterized the T47D clone (C4) along with a receptor-positive, hormone-responsive clone (A18) which is representative of the parental T47D line (A line). The results are compared with the hormone-nonresponsive steroid receptor-negative breast cancer cell line MDA-MB-231.

MATERIALS AND METHODS

Cell Culture. The T47D cell line (16) used in these studies was originally obtained at passage 81 from the American Type Culture Collection (Rockville, MD) and was designated line T47D:A (17). Line T47D:C was established from line T47D:A at passage 119. Clone T47D:A18 was established from line T47D:A at passage 171 by dilution cloning. After approximately 1 year of culture in estrogen-free conditions, line T47D:C was cloned at passage 149 to produce clone T47D:C4. Cells were cloned by dilution in 96-well tissue culture dishes (0.5 cell/well) and only wells containing single colonies were used to establish the cell lines. T47D:A and its clone, T47D:A18, were routinely cultured in RPMI 1640 supplemented with 10% FBS (heat inactivated), 6 ng/ml bovine insulin (Sigma Chemical Co., St. Louis, MO), and penicillin (100 units/ml)/streptomycin (100 μg/ml). T47D:C and its clone, T47D:C4, were routinely cultured in RPMI 1640 without phenol red indicator supplemented with 10% dextran-coated charcoal-treated-FBS, bovine insulin (as above), and penicillin/streptomycin (as above). MDA-MB-231 cells (18) were cultured in phenol red-containing minimal essential medium supplemented with insulin (as above), penicillin/streptomycin (as above), 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, and 5% dextran-coated charcoal-coated-cell serum. All tissue culture reagents were obtained from Gibco Laboratoires (Grand Island, NY) unless otherwise stated.

All cell stocks were routinely cultured in T150 flasks (Corning, Park Ridge, IL) in a humidified atmosphere of 95% air/5% CO2 at 37°C with medium changes every 3 days. Cells were removed from flasks with 0.25% trypsin/EDTA solution for passage.

Growth Response Studies. To determine the growth rates of clones T47D:A18 and T47D:C4, cells were cultured as described above and plated into 24-well plates at 5 × 104 cells/0.5 ml/well on day 0. On day 1 and on day 4, fresh medium containing test compounds (0.5 ml/well) was added. Cells were harvested for DNA measurements on days 1–9 and assays for total DNA/well were performed.

For dose-response assays, cells were plated into 24-well plates as above with fresh medium containing test compounds added on days 0 and 4. All cells were harvested on day 7 of treatment and assays for total DNA/well were performed. 17β-Estradiol was purchased from Sigma Chemical Co. 4-OHT and ICI 164,384 (19) were obtained from ICI Pharmaceuticals (Macclesfield, England). All compounds were prepared in a concentrated form in 100% ethanol and diluted in cell culture medium. Final ethanol concentrations of the media never exceeded 0.2%.

DNA Assays. Cells were sonicated in the well for 12 s with a Micro Ultrasonic Cell Disrupter (Kontes, Vineland, NJ). Samples (50–100 μl) were taken for DNA determinations. Assays for DNA were performed by incubating samples with Hoechst 33258 dye (Calbiochem-Behring Corp., La Jolla, CA) according to a method by LaBarca and Paigen (20). Samples were analyzed on a SLM-Aminco Fluoro-Colorimeter III. Corrections were made for background medium fluorescence. All points for each DNA measurement represent a mean of three sampled wells.

Steroid Receptor Assays. The levels of ER and PR in T47D:A18 and T47D:C4 were determined by using a ligand-binding assay with Scatchard analysis (21) and by enzyme immunoassay analysis. Both of these techniques have been described previously (17).

mRNA Analysis. Cell stocks were grown in T150 flasks and were harvested for mRNA preparations by using the guanidinium isothiocyanate method (22). Briefly, cells were removed from flasks with trypsin/EDTA solution and rinsed twice with chilled phosphate-buffered saline. Guanidinium isothiocyanate (7 ml/6 × 105 cells) was added to the cell pellet and the resulting cell lysate was mixed vigorously for 2 min. The lysate was drawn through a 20-gauge needle and 0.5 g cesium chloride was added per ml of lysate. The cell solution was layered over a 5.7 M cesium chloride cushion and samples were spun at 30,000 × g for 20 h in a Beckman L565 Ultracentrifuge (SW50.1 rotor). The supernatant was removed and the RNA pellet was resuspended in a solution containing 5% phenol, 10 mM Tris (pH 7.5), and 1% SDS. This solution was extracted twice with phenol/chloroform/isooamyl alcohol (25:24:1) and twice with chloroform/isoamyl alcohol (24:1). The RNA was precipitated, resuspended in Diethylpyrocarbonate-treated water, and quantitated by spectrophotometry. Polyadenylated RNA was purified by polyuridylylate-Sepharose 4B chromatography (Sigma Chemical Co) by using the established protocol (23) and samples were requantitated. Recovery was consistently 5–8% of total RNA loaded.

Northern Blotting and Autoradiography. Northern blotting was performed as described previously (24). Briefly, samples of Polyadenylated RNA were denatured in formaldehyde/formamide and fractionated by electrophoresis on a 1.2% agarose/formamide gel (24). Prior to blotting, gels were treated with 50 mM sodium hydroxide/10 mM sodium chloride, neutralized with 100 mM Tris/HCl (pH 7.5), and equilibrated with 20× SSC. Transfer to Hybond N (Amersham, Arlington Heights, IL) was carried out using 20× SSC. Blots were then air dried and UV fixed. The blots were stained with methylene blue to determine the quality of transfer. Blots were then prehybridized in buffer (5× SSC; 10% sodium dextran sulfate; 0.1% Ficoll; 0.1% bovine serum albumin; 0.2% polyvinyl pyrrolidone; 20 mM sodium phosphate (pH 6.5); 0.2% SDS, 200 μg/ml salmon sperm DNA; and 50% deionized formamide) at 42°C overnight. Radio-labeled probe was added to the buffer (1–2 × 106 dpm/ml) and blots were hybridized at 42°C overnight. Blots were then washed in 2× SSC/0.2% SDS at room temperature 2 times for 1 h each, and then in 0.1× SSC/0.2% SDS at 65°C for 20 min. Autoradiography was performed using two Quanta III Dupont Cronex intensifying screens (Sigma Chemical Co.) at −70°C.

Radio-labeled Probe Preparation. Human ER cDNA (AOR8; 2.1-kilobase insert in pBR322) (25) was obtained from Dr. Pierre Chambon (Strasbourg Cedex, France) and subcloned into the EcoRI site of pGEM3Z (Promega, Madison, WI). The human PR cDNA probe (entire coding region) (human PR in pGEM4) (26) was obtained from Dr. Edwin Milgrom (Le Kremlin-Bicetre Cedex, France). The cDNA probes for TGF-α (sp65C17N3 in sp65) (27) and TGF-β, [pXas (XBC-III Dupont Cronex intensifying screens (Sigma Chemical Co.) at −70°C.

DNA Fingerprinting. DNA fingerprinting (32) was performed by

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Cellmark Diagnostics (Germantown, MD), using Jeffreys' multilocus probes (33,15) (33, 34).

RESULTS

We studied the effects of different culture conditions on the growth rate of clones T47D:A18 and T47D:C4 (Fig. 1). T47D:A18 cultured under estrogenized conditions (in phenol red-containing medium with whole FBS) had approximately a 2-day doubling time (Fig. 1a). The addition of 17β-estradiol (1.0 nm) did not increase the growth rate of T47D:A18 cells above control levels. The potent TAM metabolite 4-OHT (0.1 μM) alone and in the presence of 1.0 nm 17β-estradiol decreased the growth rate of these cells. The pure steroidal antiestrogen ICI 164,384 (1.0 μM) (19) also inhibited the growth rate of T47D:A18 cells.

To evaluate the direct effect of estrogens and antiestrogens on cell growth, T47D:A18 cells were estrogen deprived for 14 days and then treated with test compounds (Fig. 1b). Under these conditions, the basal growth rate of control cells was decreased but 17β-estradiol (1.0 nm) was able to increase the cell growth rate approximately 3-fold during an 8-day treatment period. Addition of 4-OHT or ICI 164,384 alone did not decrease growth below control levels, but these compounds did completely inhibit 17β-estradiol-stimulated growth.

The long-term estrogen-deprived clone T47D:C4 exhibited a slow growth rate with approximately a 2.5- to 3.0-day doubling time (Fig. 1c). The addition of 17β-estradiol (1.0 nm), 4-OHT (0.1 μM), and ICI 164,384 (1.0 μM) alone or in combination did not alter the growth rate of these cells.

Dose-response studies were performed to determine if the T47D:C4 clone was completely refractory to hormonal treatment at any concentration (Figs. 2 and 3). After short-term estrogen deprivation, the growth of T47D:A18 cells was stimulated by increasing concentrations of 17β-estradiol (Fig. 2a). Increasing concentrations of 4-OHT and ICI 164,384 alone had no effect on cell replication (Fig. 2a), but did effectively inhibit 17β-estradiol (0.1 nm)-stimulated growth (Fig. 2b). Over the same concentration range, these compounds did not alter the growth of T47D:C4 cells (Fig. 3). T47D:C4 cells were refractory to any growth stimulation by 17β-estradiol and to any growth inhibition by the antiestrogens.

Since T47D:C4 cells were refractory to both 17β-estradiol and the antiestrogens, we investigated whether any changes had occurred in their expression of ER and PR protein. The ER and PR contents of T47D:A18 and T47D:C4 cells were determined by both ligand-binding assay and enzyme immunoassay analysis (17). T47D:A18 cells contained approximately 82 fmol/mg protein of ER and 1530 fmol/mg protein of PR as determined by ligand-binding assay analysis. After 14 days of estrogen deprivation, the levels of ER remained at approximately 69 fmol/mg protein, while the levels of PR dropped to approximately 116 fmol/mg protein. Similar results were obtained with enzyme immunoassay analysis (data not shown). Both ER and PR protein were undetectable in T47D:C4 cells.

We then determined the levels of mRNA for the ER and PR to establish if the loss of receptor protein in T47D:C4 cells was occurring at the level of transcription. Consistent with the level of ER protein, ER mRNA was detected in T47D:A18 cells under both estrogenized and estrogen-free conditions (Fig. 4a, lanes 1 and 2). Message for the ER was not detected in the T47D:C4 line or in the ER-negative MDA-MB-231 breast cancer cell line (Fig. 4a, Lanes 3 and 4). mRNA for the PR was

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Fig. 1. Growth curve of clones T47D:A18 and T47D:C4. Cells were plated into 24-well dishes at 5 × 10^4 cells/well and treated with compounds for 8 days. a, growth response of T47D:A18 cells cultured under estrogenized conditions; b, growth response of T47D:A18 cells cultured under estrogen-free conditions; c, growth response of T47D:C4 cells. control: •, 17β-estradiol (1 nm); O, 4-OHT (0.1 μM); ◊, ICI 164,384 (1 μM); ▲, 17β-estradiol (1 nm) plus 4-OHT (0.1 μM); △, 17β-estradiol (1 nm) plus ICI 164,384 (1 μM).
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Fig. 2. Dose response of T47D:A18 cells to increasing concentrations of 17\beta-estradiol, 4-OHT, and ICI 164,384. Cells were cultured under estrogen-free conditions for 14 days and during incubation with test compounds. a, □, 17\beta-estradiol; ●, 4-OHT; ▲, ICI 164,384; ■, control; ◊, 4-OHT plus 17\beta-estradiol (0.1 nM); △, ICI 164,384 plus 17\beta-estradiol (0.1 nM); □, control; ◊, 17\beta-estradiol (0.1 nM).

Fig. 3. Dose response of T47D:C4 cells to increasing concentrations of 17\beta-estradiol, 4-OHT, and ICI 164,384. a, □, 17\beta-estradiol; ●, 4-OHT; ▲, ICI 164,384; ■, control; ◊, 4-OHT plus 17\beta-estradiol (0.1 nM); △, ICI 164,384 plus 17\beta-estradiol (0.1 nM); □, control; ◊, 17\beta-estradiol (0.1 nM).

Factors in the development of hormone-independent breast cancer cell growth (35, 36). It has been postulated that hormone-nonresponsive cells may circumvent their hormone dependence by either constitutively producing growth factors or by increasing the levels of growth factor receptors which serves to amplify the signals from their respective ligands. We measured the levels of mRNA for selected growth factors to determine if T47D:C4 cells overexpressed their messages. T47D:A18 cells contained mRNA for TGF-\alpha and its receptor, EGFR (37), under both estrogenized and estrogen-free conditions (Fig. 6). T47D:C4 cells expressed low levels of message for this growth factor and its receptor. The levels of mRNA expression for EGFR and TGF-\alpha in hormone-independent MDA-MB-231 cells were much higher than in T47D:C4 cells.

The levels of mRNA for TGF-\beta_{1} and TGF-\beta_{2} were also determined in these cell lines (Fig. 7). T47D:A18 cells cultured under estrogenized conditions expressed high levels of TGF-\beta_{1}

expressed at high levels in T47D:A18 cells under estrogenized conditions, and these levels decreased dramatically after cells were deprived of estrogen for 14 days (Fig. 4b, Lanes 1 and 2). Again, mRNA for the PR (Fig. 4b, Lanes 3 and 4) was undetectable in receptor-negative lines T47D:C4 and MDA-MB-231.

To ensure that the T47D:C4 line was not a contaminant from the MDA-MB-231 line, DNA fingerprinting was performed (Fig. 5). The parental lines, T47D:A and T47D:C (Fig. 5, Lanes 1 and 3), and their clones, T47D:A18 and T47D:C4 (Fig. 5, Lanes 2 and 4), all displayed a distinct DNA banding pattern which was different from that of MDA-MB-231 cells (Fig. 5, Lane 5). This proved that T47D:C4 was of the T47D lineage and was not a receptor-negative contaminant from another cell line.

Recently, much attention has focused on the role of growth
mRNA, and unexpectedly, these levels decreased significantly after 14 days of estrogen deprivation (Fig. 7a, Lanes 1 and 2). The expression of TGF-β1 mRNA was low in T47D:C4 cells, but MDA-MB-231 cells had a high level of TGF-β1 mRNA (Fig. 7a, Lanes 3 and 4). Interestingly, the expression of mRNA for TGF-β2 in T47D:A18 cells appeared to be inversely regulated by estrogen as compared to the expression of TGF-β1 mRNA (Fig. 7b). Message for TGF-β2 could not be detected in T47D:A18 cells under estrogenized conditions, but message was detected in these cells after they were deprived of estrogen for 14 days (Fig. 7b, Lanes 1 and 2). T47D:C4 cells also expressed mRNA for TGF-β2 (Fig. 7b, Lane 3), and MDA-MB-231 cells expressed a high level of TGF-β2 mRNA (Fig. 7b, Lane 4).

DISCUSSION

The major problem in breast cancer treatment is the development of resistance to anticancer therapeutic agents. The antiestrogen TAM is an effective agent in controlling the growth of ER-positive breast cancers (1). However, hormone-responsive breast cancer cells can become refractory to TAM treatment through the loss or alteration of their estrogen receptors. The goal of our program is to identify possible mechanisms involved in the development of resistance to antihormonal agents. As a first step, we have established a model system in vitro to compare a receptor-positive, hormone-responsive cell population to a receptor-negative, hormone-nonresponsive population derived from the same cell line (17).

Hormone-independent breast cancer cells may be able to survive and grow through utilization of paracrine and autocrine growth factors in the host environment (35, 36). There appear to be differences between hormone-responsive and hormone-nonresponsive cell lines in their expression of, and sensitivity to, certain growth factors. In a recent study, hormone-dependent MCF-7 breast cancer cells were transfected with the oncoprotein v-Ha-Ras. In contrast to the parental line, the transfectants...
Fig. 6. Northern blot analysis of TGF-α and EGFR mRNA. 

a. blot was hybridized with 32P-labeled probe for TGF-α mRNA; b, blot was hybridized with 32P-labeled probe for EGFR mRNA. Lane I, T47D:A18 cells grown under estrogenized conditions (in phenol red-containing RPMI medium supplemented with whole FBS). Lane 2, T47D:A18 cells grown under estrogen-free conditions (in phenol red-free RPMI medium supplemented with dextran-coated charcoal-treated-FBS) for 14 days. Lane 3, T47D:C4 cells; Lane 4, MDA-MB-231 cells. ACTIN, pattern of hybridization obtained with Drosophila ß-actin used as a control for gel loading. All lanes were loaded with 15 µg of polyadenylated RNA per lane; kb, kilobase.

Fig. 7. Northern blot analysis of TGF-β1 and TGF-β2 mRNA. a, blot was hybridized with 32P-labeled probe for TGF-β1 mRNA; b, blot was hybridized with 32P-labeled probe for TGF-β2 mRNA. Lane I, T47D:A18 cells grown under estrogenized conditions (in phenol red-containing RPMI medium supplemented with whole FBS). Lane 2, T47D:A18 cells grown under estrogen-free conditions (in phenol red-free RPMI medium supplemented with dextran-coated charcoal-treated-FBS) for 14 days. Lane 3, T47D:C4 cells; Lane 4, MDA-MB-231 cells. ACTIN, pattern of hybridization obtained with Drosophila ß-actin used as a control for gel loading. All lanes were loaded with 15 µg poly A*RNA per lane; kb, kilobase.

Hormone-nonresponsive breast cancer cells did not respond to exogenous estrogen in culture, and these cells were tumorigenic in nude mice without the need for estrogen supplementation (38). Transfectants had an increased expression of the growth factors TGF-α, TGF-β, and insulin-like growth factor-I (39). In addition, conditioned medium from the transfectants replaced estrogen in stimulating and sustaining hormone-dependent MCF-7 parental cell growth in vitro and in vivo (40). These data support the theory that growth factor secretion may be involved in promoting hormone-independent cell growth.

There is evidence that the stimulatory growth factor TGF-α, which produces its effects through the EGFR (37), is involved in breast cancer cell stimulation (41, 42). Receptor-positive cell lines have a low basal level of expression of TGF-α and EGFR (41, 43), and TGF-α expression increases in hormone-responsive cells in response to 17β-estradiol treatment. In contrast, hormone-independent lines have a very high, constitutive level of TGF-α and EGFR mRNA expression (43). High levels of EGFR expression have also been correlated with ER-negative, hormone-independent disease in patients (44). Hormone-responsive T47D:A18 cells express lower levels of TGF-α and EGFR mRNA than MDA-MB-231 cells. However, hormone-nonresponsive T47D:C4 cells do not have an increased expression of the mRNAs for these stimulatory growth factors as might be expected.

There is conflicting evidence regarding the expression of the inhibitory growth factor TGF-β in human breast cancer cells. Early studies on TGF-β did not differentiate between the various species of TGF-β that are now known to exist. The cDNAs for these species have recently been cloned, thus allowing investigators to differentially detect the specific RNAs. However, the corresponding TGF-β proteins and their activities are still indistinguishable by the majority of bioassays used. It appears from our results that at least two species of TGF-β mRNA are differentially regulated in breast cancer cell lines (28, 30). Knabbe et al. (45) reported that hormone-responsive MCF-7 cells contain TGF-β receptors and express TGF-β activity. The secretion of TGF-β was induced 8- to 27-fold when MCF-7 cells (cultured in the presence of phenol red) were treated with antiestrogens, but TGF-β mRNA levels were unaffected. Subsequent studies showed that TGF-β2 mRNA and TGF-β activity were regulated by antiestrogens (46).
In contrast to the above studies, other investigators have reported that hormone-responsive MCF-7 and T47D cells do not contain any TGF-β receptors and express low levels of TGF-β, mRNA (47) and TGF-β activity (48). These cells were not inhibited by this growth factor. Interestingly, Arteaga et al. (48) found that four ER-negative cell lines did express TGF-β receptors and activity, and were exquisitely sensitive to growth inhibition by this factor. In hormone-responsive T47D: A18 cells, expression of TGF-β, and TGF-β, mRNA appears to be inversely regulated by estrogen. However, T47D:C4 cells do not express high levels of the mRNA for either of these growth factors. Interestingly, and in contrast to other hormone-independent cell lines (18), T47D:C4 hormone-nonresponsive cells have a very slow growth rate. Furthermore, other biochemical characteristics of these cells do not appear to be consistent with those of a "classical" hormone-independent cell line, such as MDA-MB-231. Further study utilizing this defined culture system may help to define the precise role of growth factors in the regulation of breast cancer cell growth.

Recently, new pure steroidal antiestrogens, such as ICI 164,384, have been identified and are targeted for clinical development (19). It has been proposed that these new pure antagonists may be more effective antitumor agents against receptor-positive breast cancer cells because, unlike TAM, these agents lack any agonist activity. Pure antiestrogens may have a role in therapeutics to prevent the growth of tumors, such as endometrial carcinomas, that are sensitive to the estrogen-like properties of TAM (49, 50). However, the studies presented here have shown that an antiestrogen-sensitive breast cancer cell population can become resistant to antiestrogen treatment when cells are kept under estrogen-free conditions. Pure antagonists may have the same effect on breast cancer cell growth, to produce an antiestrogen-resistant cell population which is more difficult to treat. Nevertheless, if the cells grew more slowly and became quiescent, as was found in our study in culture, a therapeutic advantage may be obtained.

A variety of cell lines of differing origin have been used to study the factors involved in the development of hormone-independent disease. These cell types may not have any relation to each other in the process of the development of hormone-independent disease. In addition, the majority of human breast cancer cell lines were obtained from pleural effusions of patients who received large doses of mutagenic chemotherapeutic agents. Therefore, hormone resistance in lines such as MDA-MB-231 may have resulted from mutations induced by drug therapy. Similarly, cell lines that have been treated with mutagenic drugs in vitro, such as the MCF-7/Adr line (51), may also have become resistant through an accumulation of drug-induced mutations.

We have established a system to study the role of various factors in the establishment and promotion of hormone-independent disease within one cell line grown under drug-free conditions. Further study utilizing these T47D cell lines may help to define mechanisms which allow estrogen-dependent disease to become refractory to hormonal treatment. Most importantly, we are determining whether the T47D C4 clone can be "reeducated" to respond to estrogen by continuous culture in an estrogen-containing environment. If these breast cancer cells are only quiescent and can be encouraged to reownize their steroid receptor systems, then this reversible model system may be an intermediate stage before the development of true hormone-independent growth. These studies will ultimately aid in the development of new effective therapeutics for the treatment of breast cancer.

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