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Altered Expression of Estrogen-regulated Genes in a Tamoxifen-resistant and ICI 164,384 and ICI 182,780 Sensitive Human Breast Cancer Cell Line, MCF-7/TAM^R-1[1]

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ABSTRACT

A stable, tamoxifen-resistant subline, MCF-7/TAM^R-1, of the human breast cancer cell line MCF-7 has been established in tissue culture after long-term treatment with 10^{-6} M tamoxifen. The MCF-7/TAM^R-1 cell line grows equally well in the presence and absence of tamoxifen, whereas the steroidal antiestrogens ICI 164,384 and ICI 182,780 exert profound inhibitory activity on cell proliferation, although higher concentrations are required to inhibit these cells compared to the parent cells. The MCF-7/TAM^R-1 cells grown in tissue culture deviate from parent characteristics by the complete lack of expression of progesterone receptors even when grown with estradiol, by an altered tamoxifen regulation of M_r 52,000 cathepsin D synthesis and secretion, and by lack of tamoxifen stimulation of an estradiol down-regulated M_r 42,000 protein with presumed growth inhibitory function. MCF-7/TAM^R-1 cells are estrogen receptor positive. The estrogen receptors have wild-type characteristics with respect to (*a*) binding of estradiol, tamoxifen, and ICI 164,384; (*b*) estrogen and antiestrogen regulation of the estradiol-regulated proteins pS2, M_r 61,000 α_1-antitrypsin-like protein, M_r 66,000 α_1-antichymotrypsin-like protein, and corresponding mRNAs; and (*c*) estrogen and antiestrogen regulation of a transiently transfected estrogen responsive reporter gene. We suggest that the lack of tamoxifen up-regulation of the M_r 42,000 protein synthesis in MCF-7/TAM^R-1 cells may at least partly explain the resistance to tamoxifen treatment. The sensitivity to the growth inhibitory activity of ICI 164,384 and ICI 182,780 may be ascribed to the maintenance of the pure antagonistic effect of these steroidal antiestrogens on MCF-7/TAM^R-1 cells. Our results indicate that treatment with pure antiestrogens may be effective when patients become refractory to tamoxifen therapy.

INTRODUCTION

Treatment with the antiestrogen tamoxifen is first line endocrine therapy for most breast cancer patients with estrogen receptor-positive primary tumor, but almost all patients with advanced disease will eventually develop tamoxifen resistance. Fortunately, many of these patients respond to second and third line of hormone therapy (1). However, the mechanisms for antiestrogen resistance are not yet clarified; therefore, it is extremely important for a rational treatment strategy to be devised to obtain detailed knowledge of antiestrogen resistance. Recent results from studies on clinical material and with antiestrogen-resistant human breast cancer cell lines in tissue culture or in athymic nude mice have revealed that several different mechanisms may be underlying the development of resistance (2–4).

Presence of estrogen receptors is obligatory for response to antiestrogen treatment, and loss of expression of ER[3] is, therefore, the most obvious mechanism for development of antiestrogen resistance. The incidence of progression to ER negativity has been evaluated by a comparison of ER content in primary breast tumors and metastases from the same patient. Patients with uniformly ER-positive tumors usually have ER-positive metastases (93%), whereas ER heterogeneous tumors in most cases give rise to ER-negative metastases (5). In general, tumors classified as ER positive give rise to ER-positive metastases in about 70% of the cases (5, 6), and the observation of a response rate to endocrine therapy of about 60–70% in patients with advanced disease with ER-positive primary tumors could, at least for some of the patients, be explained by lack of ERs in the metastatic lesions. Relapse following successful antiestrogen therapy may also be due to progression of ER-negative tumor cells during treatment; however, clinical data show that many tumors that progress after first responding to tamoxifen therapy express ERs (7).

Expression of ERs with abnormal function could be a mechanism that leads to antiestrogen resistance in tumor cells classified as ER positive, and at present, papers have reported on the presence of ER proteins deviating from the wild-type protein (8), expression of variant mRNAs generated by alternative RNA splicing, and mutations in the ER gene (9–14). In a few cases, the function of such variant ERs has been studied, and two interesting abnormal functions have been disclosed: (*a*) one which is considered dominant positive by being transcriptionally active without binding hormone (15), and (*b*) one which is dominant negative by inhibiting the action of the normal receptor (12, 16). A review of papers describing the ER-positive antiestrogen-resistant cell lines established in tissue culture after long term treatment with antiestrogen reveals that most of the ER functions in these cells appear normal; however, each resistant cell line displays one or more features of abnormal ER function (17–22).

Antiestrogen resistance may not necessarily be associated with loss of ERs or abnormal function of ER. Tumors, which during the progression to hormone insensitivity up-regulate the synthesis of growth stimulatory factors and/or down-regulate the synthesis of growth inhibitory factors, may not, in spite of normal binding of antiestrogen and normal function of the ER machinery, exhibit a decrease in growth potential (23). ER-negative tumor cells or stromal cells may also support growth of ER-positive cells via paracrine mechanisms and thereby sustain growth of ER-positive tumor cells in spite of tamoxifen treatment. Finally, the observed tamoxifen resistance in transplanted tumors of MCF-7 cells in athymic nude mice seems to be due to a pharmacological change in the metabolism of tamoxifen giving rise to compounds with estrogenic activity (24). Similar indications of changed pharmacology of tamoxifen have also been found in human breast tumor biopsies (25).

In this paper, we describe a tamoxifen-resistant cell line established in our laboratory from MCF-7 cells via two series of long term treatment with tamoxifen in tissue culture. The antiestrogen resistant cell line MCF-7/TAM^R-1, originally called the AL-1 cell line (22, 26), is estrogen receptor-positive. Most of the ER-mediated functions are regulated normally, but with cells grown in tissue culture, we have

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[3] The abbreviations used are: ER, estrogen receptor; PgR, progesterone receptor; DME, Dulbecco's minimal essential medium; F12, Ham's nutrient mixture F-12; FCS, fetal calf serum; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SSPE, sodium chloride-sodium phosphate-EDTA buffer; SSC, standard sodium citrate; CAT, chloramphenicol acetyl transferase; IC_{50}, the concentration that inhibited growth by 50% relative to untreated controls; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; vit-tk-CAT, vitellogenin-thymidin kinase-chloramphenicol acetyl transferase.

observed a complete lack of PgR expression and estrogen inducibility and a lack of tamoxifen regulation of secreted proteins with growth regulatory function. This stable, tamoxifen-resistant cell line is sensitive to the steroidal antiestrogens ICI 164,384 and ICI 182,780, indicating that these new antiestrogens may be valuable compounds in the treatment of breast cancer patients with tumor progression while on tamoxifen therapy.

MATERIALS AND METHODS

Cells and Growth Experiments. The MCF-7 cell line was originally obtained from The Breast Cancer Task Force Cell Culture Bank, Mason Research Institute (Worcester, MA). The cell line has been gradually adapted to grow in medium with low serum concentration (27), and at the initiation of these experiments, the cells were propagated in DME/F12 medium (1:1) supplemented with 0.5% FCS, 2 mM glutamine, and 6 ng/ml insulin (Novo-Nordisk, Copenhagen, Denmark). The MCF-7/TAM^R-1 cell line, previously called AL-1, was established by two series of treatments with 10^{-6} M tamoxifen as described earlier (22). The MCF-7/TAM^R-1 cells were propagated continuously in the presence of 10^{-6} M tamoxifen from passage 372 (onset of the second tamoxifen treatment), which is considered as passage 1 of the resistant cell line. After the discovery of the weak estrogenic activity of phenol red (28), both MCF-7 and the MCF-7/TAM^R-1 cells were propagated in phenol red-free DME/F12 medium supplemented with 1% FCS, 2 mM glutamine, and 6 ng/ml insulin. The shift to growth medium without phenol red and with 1% FCS had neither influence on cell proliferation rate nor on the pattern of secreted proteins. Both MCF-7 and MCF-7/TAM^R-1 cells were passaged every week by trypsinization and were seeded with 5×10^3 cells/cm^2. Medium was changed every second or third day. The cell lines were regularly tested by the Hoechst staining method (29) and found to be free of *Mycoplasma*.

Cultures used for growth experiments were seeded in plastic T25 flasks or 2 cm^2 multiwell dishes (Nunc, Denmark) 2 days before the onset of the experiments. Estradiol (Collaborative Research), tamoxifen, ICI 164,384, and ICI 182,780 (ICI Pharmaceuticals) were dissolved in 96% ethanol in stock solutions 1000-fold higher than the concentration used and added to the experimental media at the time of change of medium, which was every second or third day during the experiment. Triplicate flasks or quadruplicate wells were used for determination of cell numbers, either after trypsinization and counting in a Bürker-Türch chamber or after cell lysis and counting of cell nuclei in an automatic cell counter (Analys Instrument AB/VDA; Ref. 26).

Tumorigenicity. Near confluent cultures were trypsinized, washed once with PBS, and resuspended in PBS. Approximately 10^7 cells were inoculated s.c. in female nude mice at the site of the fourth mammary gland. Estrogen was administered as estrone in the drinking water (0.5 μg/ml estrone; Sigma E9750). The animals were observed for tumor formation weekly for a minimum of 11 weeks and up to 6 months. Tumors were excised and frozen at −80°C until ER and PgR determination.

ER and PgR Determinations. Tumor tissue was homogenized in a Mikro-Dismembrator (B Braun) with precooled (−180°C) pulverization chambers. Cytosols were prepared according to the European Organization for Research and Treatment of Cancer guidelines (30). The ERs were extracted from the nuclear pellet with an extraction buffer consisting of 10 mM Tris (pH 8.5)-1 mM monothioglycerol-10% glycerol-600 mM KCl for 1 h at 0°C; the supernatant, after centrifugation at 100,000 × *g*, was used for determination of nuclear receptors. Tissue culture cells were harvested from near confluent cultures and homogenized in a glass-glass tightly fitting Potter-Elvehjem homogenizer; cytosol and nuclear extract were prepared as described previously (27). The content of free ER and free PgR in cytosol preparations was determined by ligand binding assay (dextran coated-charcoal assay) according to the recommendations of the European Organization for Research and Treatment of Cancer Receptor Group (30). ER contents in the nuclear extracts were determined with the Abbott ER-EIA monoclonal kit (Abbott Laboratories, North Chicago, IL), which is insensitive to KCl concentrations up to 800 mM and which measures both free and hormone-bound ERs (31).

Secreted Proteins. MCF-7/TAM^R-1 cells were propagated for 1 week without tamoxifen before onset of the experiments. MCF-7 and MCF-7/TAM^R-1 cells were seeded in 2 cm^2 multiwell dishes in control medium with 1% FCS at a density of 1.5×10^4 cells/well. Two days after seeding, experimental media containing the indicated concentrations of hormones and/or antihormones were added, and medium was renewed every second or third day. At day 6, the wells were washed with PBS and labeled for 6 h with [^{35}S]-methionine (NEG-009H; NEN Research Products; specific activity, ~1000 Ci/mmol) or a mixture of [^{35}S]methionine and [^{35}S]cysteine (NEG-072) in serum-free DME-medium with 1:10 the normal concentration of the amino acids used for labeling. Treatment of labeled, conditioned medium and SDS-polyacrylamide gel electrophoresis analysis on 15%, 15–20% gradient, and 20% polyacrylamide gels under denaturating conditions were performed as described previously (32). Kodak XAR-5 films were used for autoradiography, and scanning was performed by a LKB Ultroscan laser densitometer.

Northern Analysis. Cells were seeded in T-150 flasks, and after 2 days, the medium was changed to experimental medium containing either 10^{-9} M estradiol, 10^{-6} M tamoxifen, or 10^{-8} M ICI 164,384. The medium was renewed on days 3 and 5, and on day 6 the cells were harvested by trypsinization, pelleted, and frozen in liquid nitrogen. RNA preparation and Northern analysis were preformed as described previously (33). Briefly, the cell pellets were homogenized in 4 M guanidinium thiocyanate, and RNA was isolated by centrifugation over a CsCl cushion. To ensure the quality of RNA, 10 μg RNA from each preparation was denatured by dimethylsulfoxide/glyoxal, electrophoresed on a 1.0% agarose gel in 10 mM phosphate buffer (pH 7.0), and stained with acridine orange. Ten-μg RNA samples from both cell lines were electrophoresed as described above and transferred by Northern blotting to the same nylon membrane (NY 13N; Schleicher and Schuell, Dassel, Germany). RNA ladder, 0.24–9.5 kilobases, (BRL, New York) was used as molecular size markers.

Purified plasmid-derived complementary probes were labeled with [α-^{32}P]-dATP (Amersham, Buckinghamshire, England) using the Multiprime DNA labeling kit (Amersham) to a specific activity of $1–2 \times 10^9$ dpm/μg DNA, and Northern blots were hybridized as described before (33). Oligonucleotide probes were end-labeled with [gamma-^{32}P]ATP (Amersham) to a specific activity of $0.5–2 \times 10^7$ dpm/pmol using T4 polynucleotide kinase (BRL), and prehybridization of Northern blots was carried out at 55°C for at least 1 h in 6X SSPE (1X SSPE, 150 mM NaCl-10 mM NaH_2PO_4-1 mM EDTA), 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, and 0.5% SDS. For hybridization, the nylon membranes were then incubated 12 h at 55°C in 6X SSPE and 0.1% SDS containing 4×10^6 dpm/ml of ^{32}P-labeled oligonucleotide. Unbound probe was removed from the membranes by washing 1.5 h at 55°C with 2X SSC (1X SSC, 0.15 M NaCl-15 mM sodium citrate, pH 7.0,) and 0.1% SDS followed by a 10-min wash at 55°C with 6X SSC, 0.1% SDS, and a 3-min wash at 70°C in 6X SSPE and 0.1% SDS. The blots were exposed to Kodak XAR-5 or AGFA Structurix-D7pFW films at −80°C. Scanning of autoradiograms was performed by a LKB Ultroscan laser densitometer. All Northern analyses were repeated two times on independent RNA preparations, and the results were reproducible.

Probes. The probes used were cathepsin D, oligonucleotide 5′-TTAACG-TAGGTGCTGGACTTG-TCGCTGTTGTACTT-3′ (34); pS2, *Eco*RI linearized pS2 (35); α_1-antitrypsin, 1.4-kilobase *Eco*RI fragment from phAT85 (American Type Culture Collection No. 61595); α_1-antichymotrypsin, 1.0-kilobase *Pst*I fragment from phACT235 (American Type Culture Collection no. 61601); and rat GAPDH, 1.2-kilobase *Pst*I fragment from pRGAPDH-13 (36).

Transient Transfection Studies. Cationic liposome-mediated transfection with Lipofectin reagent (BRL) was performed according to the manufacturer's instructions. About 80% confluent cultures in 60-mm tissue culture dishes (Nunc, Denmark) were cotransfected with 1 μg vit-tk-CAT (37) and 10 μg pβGal plasmid (38) using 50 μl Lipofectin reagent. pBR322 (11 μg) was used for mock transfections, and 10 μg pRSV-CAT (39) were used as a positive control for transfection. Twenty-four h were used for transfection, followed by 48–72 h in experimental medium before harvest of the cells. The cells were washed twice with PBS and harvested with 1.5 ml 1 mM EDTA in PBS by scraping with a rubber policeman. Cells were collected by centrifugation at 10,000 × *g* for 5 min and resuspended in 100 μl 0.25 M Tris buffer, pH 7.8. The cells were lysed by freezing and thawing five times, and cell debris was removed by centrifugation at 10,000 × *g* for 15 min at 4°C. Equal amounts of supernatants (50 μl) were used for β-galactosidase assay for 4 h (40), and cell extracts (heated to 65°C for 10 min to remove deacylase activity) adjusted to equal β-galactosidase activity were analyzed for CAT activity (40), using [^{14}C]chloramphenicol (59.5 mCi/mmol; NEC-408; NEN Research Products). Thin layer chromatography on silica gel 60 F254 (0.2 mm; Merck) was

performed as described in Sambrook *et al.* (40). Kodac XAR-5 films were used for the autoradiograms.

RESULTS

Establishment of the Tamoxifen-resistant MCF-7/TAM^R-1 Cell Line and Growth Studies. The tamoxifen-resistant cell line was established from MCF-7 cells treated in two series with 10^{-6} M tamoxifen. During the first treatment, nearly all cells died, but a few grew up as isolated colonies in medium without tamoxifen. Tamoxifen retreatment of cells propagated from one of the surviving colonies gave rise to the MCF-7/TAM^R-1 cell line (22). Growth curves for the MCF-7 and the MCF-7/TAM^R-1 cell lines are shown in Fig. 1. The doubling time of MCF-7 cells is close to 24 h, and a similar doubling time is found for the tamoxifen-resistant cell line in both control medium (phenol red containing DME/F12 medium supplemented with 0.5% FCS, 2 mM glutamine, and 6 ng/ml insulin) and in medium with 10^{-6} M tamoxifen. Cell proliferation of the parent MCF-7 cell line is inhibited by tamoxifen, and after growing in medium with tamoxifen for 7 days, the majority of these cells will not survive a subcultivation in the presence of tamoxifen, whereas the MCF-7/TAM^R-1 cell line can be subcultured in the presence of tamoxifen with high plating efficiency.

To test the stability of the tamoxifen-resistant phenotype of the MCF-7/TAM^R-1 cell line, propagation was performed for 15 passages without tamoxifen and then a growth experiment similar to the one presented in Fig. 1 was performed. No difference in cell proliferation rate was observed in medium with and without 10^{-6} M tamoxifen (data not shown), and the MCF-7/TAM^R-1 cell line grown for 15 passages without tamoxifen could be transferred to tamoxifen-containing medium and passaged continuously in the presence of tamoxifen, indicating that this subline grown in tissue culture under the above mentioned conditions expresses a stable, tamoxifen-resistant phenotype with respect to growth characteristics.

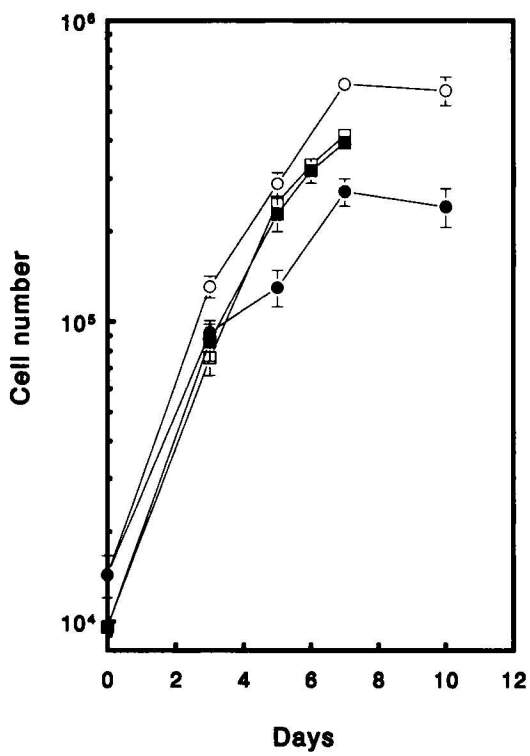

Fig. 1. Growth experiment with MCF-7 and MCF-7/TAM^R-1 cells. The MCF-7/TAM^R-1 cells were grown for 2 weeks without tamoxifen before onset of the experiment. Cells (1.25×10^5) were seeded per T25 flask in control medium (DME/F12 medium supplemented with 0.5% FCS, 2 mM glutamine, and 6 ng/ml insulin). Two days after seeding (day 0), the medium was changed to experimental medium, which could be either control medium or medium with 10^{-6} M tamoxifen. Medium was renewed every second or third day. On days 3, 5, 6, 7, and 10, triplicate cultures were trypsinized, and cell number was determined by counting in a Bürker-Türck chamber. Mean cell number and SD are shown by: ○, MCF-7 cells in control medium; ●, MCF-7 cells in control medium with 10^{-6} M tamoxifen; □, MCF-7/TAM^R-1 cells in control medium; ■, MCF-7/TAM^R-1 cells in control medium with 10^{-6} M tamoxifen.

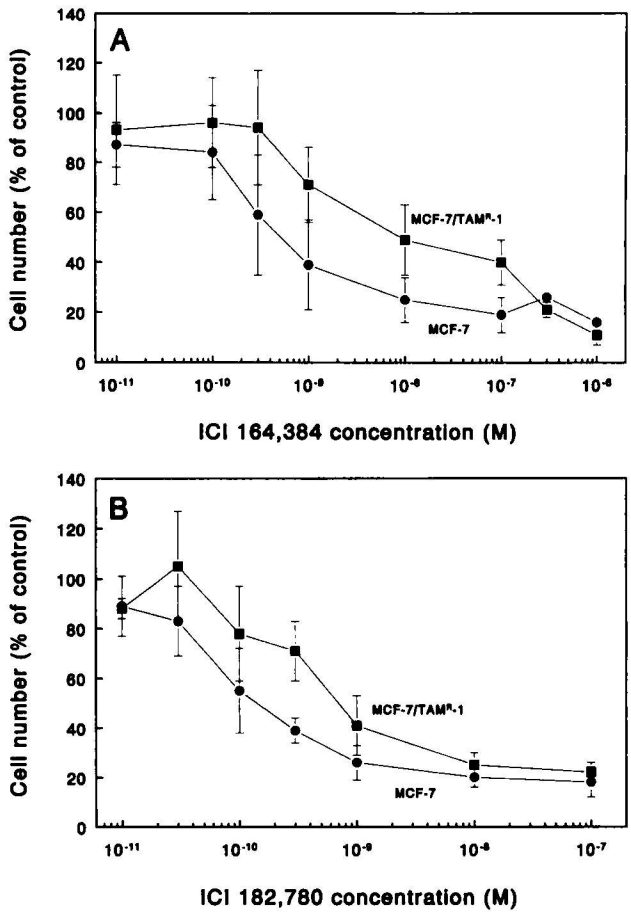

Fig. 2. Dose-response curves for effect of ICI 164,384 (*A*) and ICI 182,780 (*B*) on cell proliferation of MCF-7 cells and MCF-7/TAM^R-1 cells. Cells were seeded in multiwell dishes with 1.5×10^4 cells per well. Two days after seeding, medium was changed to experimental medium containing the indicated concentrations of the ICI compounds. Medium was renewed every second or third day. Cell numbers were determined after 6 days in experimental medium by counting of cell nuclei in an automatic cell counter. The mean values (expressed as a percentage of the corresponding control cultures) and SDs of four to six experiments are shown.

To elucidate whether resistance to tamoxifen results in cross-resistance to other antiestrogens, we have studied the effect of the new steroidal antiestrogens ICI 164,384 and ICI 182,780 on growth of MCF-7/TAM^R-1 cells. Fig. 2 clearly illustrates that the tamoxifen-resistant cell line is sensitive to both steroidal antiestrogens, although the sensitivity of the MCF-7/TAM^R-1 cells to the steroidal antiestrogens is reduced compared to parent MCF-7 cells. The IC_{50} for ICI 164,384 was 9×10^{-9} M for MCF-7/TAM^R-1 cells, which is 18-fold higher than the IC_{50} of 5×10^{-10} M for ICI 164,384 inhibition of parent MCF-7 cells. The IC_{50} for ICI 182,780 was 7×10^{-10} M for MCF-7/TAM^R-1 cells, which is 5-fold higher than the IC_{50} of 1.4×10^{-10} M for ICI 182,780 inhibition of MCF-7 cells. For both cell lines, the growth inhibitory effect of the steroidal antiestrogens in concentrations up to 10^{-7} M is fully estrogen reversible (results not shown).

Tumorigenicity in Nude Mice and ER and PgR Content. MCF-7 cells will form tumors in nude mice provided estrogen is administered, and most tumors are already palpable after 1 week (Table 1). The antiestrogen-resistant cell line also requires estrogen administration for tumor formation in nude mice, and except for one

tumor that appeared after 1 week, palpable tumors in the other five animals inoculated with MCF-7/TAM^R-1 cells appeared after 5 to 11 weeks (Table 1). Tumors originating from either inoculated MCF-7 or MCF-7/TAM^R-1 cells (excised from animals treated with estrogen) contain both free ERs and free PgRs. No difference in PgR level is observed between tumors arising from parent or tamoxifen-resistant cells. The ER level is significantly lower in tumors from animals injected with MCF-7/TAM^R-1 cells (Mann-Whitney U test; $P = 0.002$).

ER and PgR Content in MCF-7 Cells and in MCF-7/TAM^R-1 Cells Grown in Tissue Culture with Different Growth Media. Table 2 shows that the tamoxifen-resistant cell line contains free ER when grown in tissue culture in control medium with 1% FCS (and without tamoxifen for 15 days). The average level of free ER is lower than in the parent MCF-7 cell line grown under similar conditions, but due to the large SD values, the ER levels are not significantly different. The equilibrium dissociation constant for binding of estradiol to ER is the same for the parent and the resistant cell lines, about 10^{-10} M. There is no measurable content of PgRs in the tamoxifen-resistant cell line grown in control medium, whereas the parent cell line contains a significant level of PgR in control medium, which contains low levels of estrogen compounds supplied via the FCS content. In medium supplemented with optimal estradiol concentration (10^{-9} M), the MCF-7 cells contain very few free ERs and a high level of PgR, 2050 fmol/mg cytosol protein. The MCF-7/TAM^R-1 cells grown in estradiol-containing medium contain no free ERs and few, if any, free PgRs. MCF-7 cells grown in medium with 10^{-6} M tamoxifen contain few free ERs and a significantly reduced level of PgR compared to MCF-7 cells grown in control medium. The tamoxifen-resistant cell line contains neither free ERs nor free PgRs when grown with tamoxifen. After growth in the presence of 10^{-8} M ICI 164,384, no detectable level of free ER or free PgR is observed in the MCF-7 cells, whereas a low level of free ER and no free PgR are found in the MCF-7/TAM^R-1 cell line. Both cultures of MCF-7 and MCF-7/TAM^R-1 cells grown in control medium and in medium with estradiol or antiestrogens contain salt-extractable ERs in the nucleus (results not shown).

The complete lack of PgR expression and estrogen inducibility observed to occur in the MCF-7/TAM^R-1 cells grown in tissue culture is an interesting alteration, and we have investigated whether this characteristic is maintained after long term growth in medium without tamoxifen. The result of PgR determinations disclosed a small increase in estrogen-induced PgR level during the first 2–4 weeks without tamoxifen, and the level then stayed rather constant during the propagation for up to 15 passages without tamoxifen. The average estrogen-induced PgR level was 290 fmol/mg cytosol protein, which is only 14% of the PgR level in MCF-7 cells grown with estradiol.

Table 1 *Tumor takes in athymic nude mice. Estrogen and progesterone receptor content*

Near confluent cultures were harvested by trypsinization and about 10^7 cells were inoculated s.c. at the fourth mammary gland of female athymic nude mice. Some of the animals received estrogen supplementation. The animals were observed weekly for about 6 months. ER and PgR contents were determined by the dextran charcoal-coated assay on cytosols prepared from tumors originating from inoculated MCF-7 or MCF-7/TAM^R-1 cells (mean and SD). The numbers in parentheses are the number of tumors included in the analyses.

Cell line	Tumor takes − estrogen	Tumor takes + estrogen	Palpable tumors (wk)	Free ER[a] (*n*)	Free PgR[a] (*n*)
MCF-7	1[b]/11	6/8	1–6	316 ± 206 (8)	252 ± 196 (8)
MCF-7/TAM^R-1	0/11	6/9	1–11	26 ± 33 (5)	226 ± 88 (5)

[a] fmol/mg, cytosol protein.
[b] One small nonprogressively growing tumor.

Table 2 *ER and PgR content in MCF-7 and in MCF-7/TAM^R-1 cells grown in tissue culture in control medium, medium with estradiol, and medium with the antiestrogens tamoxifen or ICI 164,384*

MCF-7/TAM^R-1 cells were grown for 9 to 10 days without tamoxifen before onset of the experiment. Estradiol and antiestrogens were added to cultures in the early exponential growth phase, and cells harvested after 6 days. Mean and SD for ER and PgR content determined by dextran-coated charcoal assay on cytosol preparations are shown, and the number of experiments are indicated in the parentheses.

	MCF-7		MCF-7/TAM^R-1	
	Free ER[a] (*n*)	Free PgR[a] (*n*)	Free ER[a] (*n*)	Free PgR[a] (*n*)
Control medium 1% FCS	442 ± 272 (8)	361 ± 83 (8)	271 ± 138 (8)	<10 (8)
Medium with 10^{-9} M estradiol	14 ± 2 (4)	2050 ± 504 (4)	<10 (4)	17 ± 17 (4)
Medium with 10^{-6} M tamoxifen	18 ± 24 (3)	70 ± 64 (3)	<10 (4)	<10 (4)
Medium with 10^{-8} M ICI 164,384	<10 (3)	<10 (3)	42 ± 52 (4)	<10 (4)

[a] fmol/mg, cytosol protein.

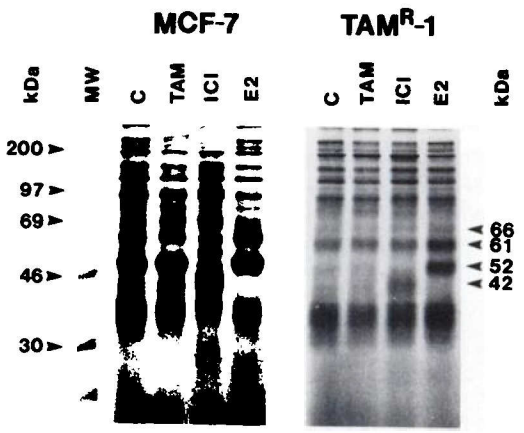

Fig. 3. Autoradiogram of [^{35}S]methionine-labeled proteins secreted from MCF-7 and MCF-7/TAM^R-1 cells analyzed by SDS-polyacrylamide gel electrophoresis. The cells were grown as described in Fig. 2. The concentrations of hormones and antihormones in the experimental media were 10^{-6} M tamoxifen (*TAM*), 10^{-8} M ICI 164,384 (*ICI*), and 10^{-9} M estradiol (*E2*). [^{35}S]Methionine in serum-free medium was added to the cultures at day 6, and conditioned media were collected after 6 h. Equal amounts of TCA-precipitable cpm were applied on each lane. *C*, lanes with conditioned medium from control cultures; *MW*, lane with molecular weight markers. *Arrows* to the *right*, positions for the estradiol-regulated proteins M_r 66,000 α_1-antichymotrypsin, M_r 61,000 α_1-antitrypsin, M_r 52,000 cathepsin D, and M_r 42,000 protein.

This result shows that the MCF-7/TAM^R-1 cells do not regain parent capacity to induce PgR synthesis when grown in tissue culture without tamoxifen.

Effect of Estradiol and Antiestrogens on the Synthesis of Secreted Proteins in MCF-7 and MCF-7/TAM^R-1 Cells. The observation of lack of synthesis and estrogen inducibility of the PgR protein encouraged a further analysis of hormone regulation of estrogen-regulated proteins in MCF-7 cells. Fig. 3 shows [^{35}S]methionine-labeled secreted proteins from MCF-7 and MCF-7/TAM^R-1 cells grown in control medium, medium with 10^{-6} M tamoxifen, medium with 10^{-8} M ICI 164,384, and medium with 10^{-9} M estradiol. The patterns of secreted proteins in control cultures of MCF-7 and MCF-7/TAM^R-1 are different in that MCF-7 cells secrete substantially more M_r 52,000 cathepsin D (5-fold; densitometric scanning) than MCF-7/TAM^R-1 cells. The MCF-7/TAM^R-1 cells secrete about twice as much M_r 61,000 α_1-antitrypsin as MCF-7 cells. A striking difference in the effect of tamoxifen on secreted proteins is noticed because tamoxifen has no effect on secreted proteins in MCF-7/TAM^R-1 cells, whereas tamoxifen stimulates the secretion of the M_r 52,000 cathepsin D protein (3-fold) and the M_r 42,000 protein (3-fold) in the parent MCF-7 cells. The steroidal antiestrogen ICI 164,384 inhibits M_r 52,000 cathepsin D secretion and stimulates M_r 42,000 protein syn-

thesis in both cell lines. Estradiol stimulates secretion of M_r 52,000 cathepsin D and M_r 61,000 α_1-antitrypsin and inhibits the secretion of the M_r 42,000 protein in both cell lines. The M_r 66,000 α_1-antichymotrypsin protein is secreted at a rather low level in control cultures of both cell lines, and no significant estrogen or antiestrogen regulation was found in these experiments.

In both cell lines, estradiol significantly stimulates the secretion of a [^{35}S]cysteine-labeled protein with M_r 7000–8000, presumed to be the pS2 protein (41), and cultures of both cell lines grown with either tamoxifen or ICI 164,384 do not secrete detectable levels of this protein (results not shown).

Effects of Estradiol and Antiestrogens on the Synthesis of Specific mRNAs Encoding Estrogen-regulated Proteins. To investigate whether the observed changes between the MCF-7/TAM^R-1 and the parent cell line in secreted estrogen-regulated proteins could be reflected also at the mRNA level, we studied the mRNA expression of cathepsin D, α_1-antitrypsin, α_1-antichymotrypsin, and pS2. Densitometric scannings of the Northern blots (adjusted for difference in loading by measuring the level of the housekeeping gene GAPDH) reveal that the level of cathepsin D, α_1-antitrypsin, and α_1-antichymotrypsin mRNA in MCF-7/TAM^R-1 cells grown in both control medium and in medium with estradiol, tamoxifen, or ICI 164,384 are considerably lower than in the corresponding parent cultures. Fig. 4*A* shows that the 2.2-kilobase cathepsin D mRNA expression in MCF-7 is significantly stimulated by both estradiol and tamoxifen, whereas only estradiol exerts a marked stimulation in MCF-7/TAM^R-1 cells. The level of pS2 mRNA in control cultures of MCF-7/TAM^R-1 cells is significantly higher than in parent MCF-7 cells, and estradiol stimulates pS2 mRNA level to the same extent in both cell lines. Tamoxifen inhibits pS2 mRNA in both cell lines (Fig. 4*B*). The α_1-antitrypsin mRNA expression is stimulated by estradiol and to a smaller extent by tamoxifen in both cell lines (Fig. 4*C*), whereas the α_1-antichymotrypsin mRNA synthesis in both MCF-7 and MCF-7/TAM^R-1 cells is stimulated by estradiol and is unresponsive to tamoxifen (Fig. 4*D*). ICI 164,384 inhibits the mRNA synthesis of all four genes investigated.

Effect of Estradiol and Antiestrogens on the CAT Activity from vit-tk-CAT-transfected MCF-7 and MCF-7/TAM^R-1 Cells. In order to study the ability of endogenous ERs in MCF-7 and MCF-7/TAM^R-1 cells to regulate the synthesis of an estrogen-regulated reporter gene (the vit-tk-CAT construct containing the estrogen response element from *Xenopus* vitellogenin A2 gene linked to the promoter of the herpes simplex virus 1 thymidine kinase gene and to the bacterial *CAT* gene; Ref. 37), we have made transient transfections of both cell lines with this construct and determined the CAT activity in cells grown with estradiol and antiestrogens. Both transfected cell lines showed increased CAT activity when grown in medium with estradiol and decreased CAT activity when grown in medium with tamoxifen or ICI 164,384. No differences in regulation of the synthesis of this estrogen-responsive reporter gene are thus observed between the cell lines.

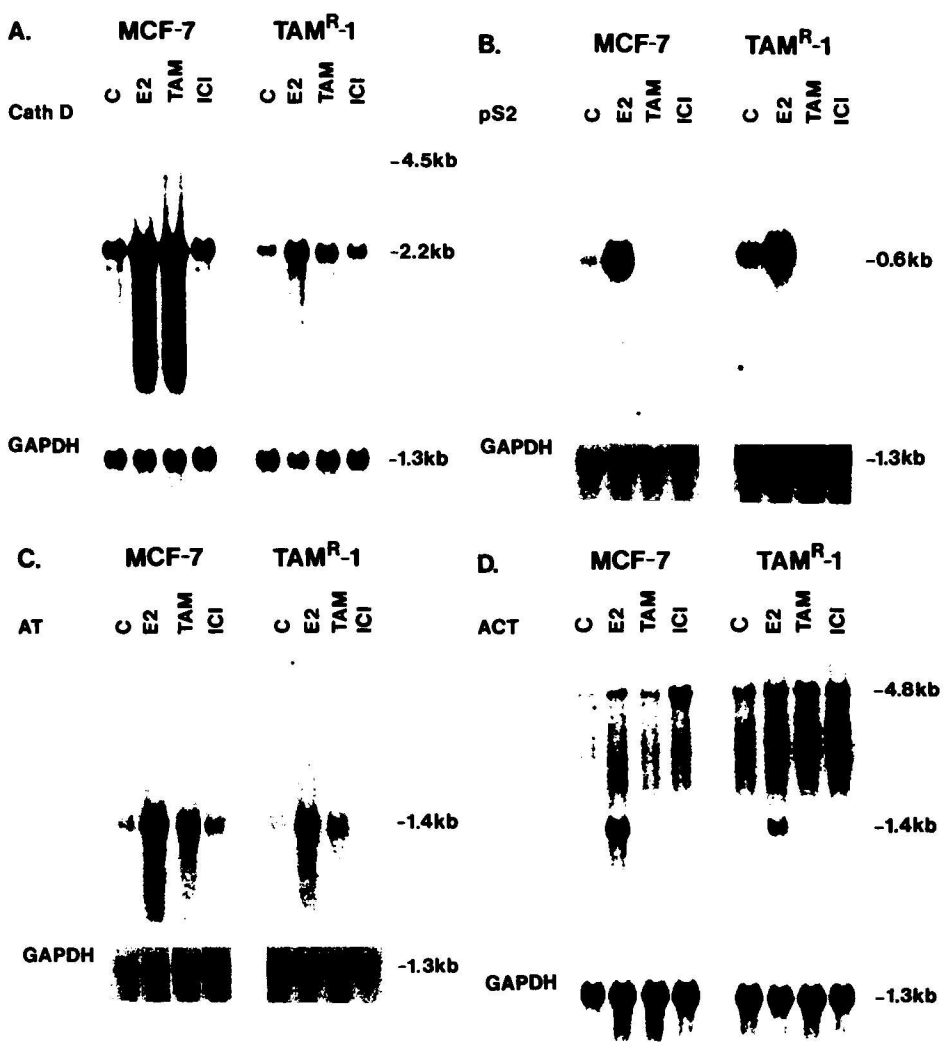

Fig. 4. Autoradiogram of Northern blots hybridized with ^{32}P-labeled probes for *A*, cathepsin D (*cath D*); *B*, pS2; *C*, α_1-antitrypsin (*AT*); and *D*, α_1-antichymotrypsin (*ACT*). Ten μg total RNA from MCF-7 and MCF-7/TAM^R-1 cells were analyzed by hybridization of the same Northern blot with specific probes against cathepsin D, pS2, α_1-antitrypsin, and α_1-antichymotrypsin. After hybridization, the probe was removed, and the blot was rehybridized with rat GAPDH as control for differences in RNA loading. *Kb*, kilobase.

DISCUSSION

The nonsteroidal antiestrogen tamoxifen has been used with success in the treatment of breast cancer patients. However, progression of tamoxifen-resistant tumors will eventually occur in most patients, and this raises the question of what treatments should be applied in these patients. In order to mimic the clinical situation to explore this question in a model system, we have established a tamoxifen-resistant cell line derived from MCF-7 cells after long term exposure to tamoxifen. This cell line, MCF-7/TAM^R-1, is estrogen receptor positive and sensitive to the inhibitory activity of the steroidal antiestrogens ICI 164,384 and ICI 182,780, indicating that treatment with steroidal antiestrogens may be beneficial for patients relapsing on tamoxifen therapy. Our investigations of the mechanisms underlying the development of tamoxifen resistance and maintenance of sensitivity to steroidal antiestrogens have revealed that the MCF-7/TAM^R-1 cell line has lost normal estrogen regulation of at least one gene (the PgR gene). The significance of this changed characteristic is at present unknown. Altered tamoxifen regulation of gene expression has been detected in MCF-7/TAM^R-1 cells, and this may contribute to tamoxifen resistance. The observed pure estrogen antagonistic effect of ICI 164,384 on gene expression in MCF-7/TAM^R-1 cells as well as in parent MCF-7 cells may explain the sensitivity to the inhibitory activity of the steroidal antiestrogens.

Our growth analyses have revealed that the cell proliferation rate of the MCF-7/TAM^R-1 cell line is the same in the presence and absence of tamoxifen and as high as observed for the parent cell line grown in control medium (Fig. 1). Even after growth for 15 passages without tamoxifen, the cell proliferation rate of the MCF-7/TAM^R-1 cells is not significantly affected by addition of 10^{-6} M tamoxifen. The new steroidal antiestrogens ICI 164,384 and ICI 182,780, which are much more potent growth inhibitors of MCF-7 cells than tamoxifen (26, 42), are able to inhibit growth of the tamoxifen-resistant MCF-7/TAM^R-1 cells. However, higher concentrations are required to obtain the same degree of inhibition as seen with the parent MCF-7 cells (Fig. 2). These results demonstrate that there is no cross-resistance between the nonsteroidal antiestrogen tamoxifen and the two new steroidal antiestrogens in this tamoxifen-resistant cell line, indicating that steroidal antiestrogens may be valuable as a new treatment of breast cancer patients who have become refractory to tamoxifen therapy. A similar lack of cross-resistance between tamoxifen and the steroidal antiestrogen ICI 182,780 is described for the tamoxifen-resistant MCF7/LCC2 cell line (43), whereas cross-resistance to antiestrogens of widely different structures (LY 117018, tamoxifen, 4-OH-tamoxifen, and steroidal antiestrogens) is observed in the antiestrogen-resistant LY2 cell line (17, 21).

The stability of tamoxifen resistance in human breast cancer cell lines seems to vary. The LY2, RTx6, and MCF-7/LCC-2 cell lines have been reported to have a stable, antiestrogen-resistant phenotype (17, 20, 43), whereas the R3 and R27 sublines revert to their normal (antiestrogen sensitive) charactistics once tamoxifen is removed from the growth medium (17, 21). The stability of the tamoxifen-resistant ZR-75–9a1 cells has been studied very carefully. It was found that the ER- and PgR-negative ZR-75–9a1 cells revert to ER- and PgR-positive, antiestrogen-sensitive cells if grown in tamoxifen-free complete medium for 4 weeks and remain ER- and PgR-negative and tamoxifen-resistant if propagated in estrogen-free medium or tamoxifen-containing medium. This rather rapid reversion to parent phenotype when tamoxifen is withdrawn argues against the fact that the ZR-75–9a1 could have arisen from a selective loss of ER-positive cells within a parent cell line heterogeneous with respect to ER content (44). Our MCF-7/TAM^R-1 cell line maintains the tamoxifen-resistant phenotype if grown in tissue culture in medium with tamoxifen and also if grown in tamoxifen-free control medium (phenol red free DME/F12 medium with 1% FCS) for 15 passages. The control medium cannot be considered a complete estrogen-free medium because small amounts of estrogen compounds are supplied via the serum content (26). The presence of PgR in parent MCF-7 cells grown in control medium (Table 2) actually verifies that this is the case since the PgR level is undetectable in parent cells grown with newborn calf serum (22), which contains lower levels of estrogen compounds compared to FCS. Our observation of a partial reversal of the ability of the MCF-7/TAM^R-1 cells to induce PgR (to 14% of the PgR level in MCF-7 cells) when the cells are propagated in tissue culture in tamoxifen-free control medium may therefore be ascribed to the presence of estrogen compounds in the control medium.

Tumors in athymic nude mice arising from inoculated MCF-7/TAM^R-1 cells contain PgR, require a significantly longer time to form palpable tumors than parent MCF-7 cells, and are only found in animals treated with estrogen supplementation (Table 1). These experiments clearly show that tumors arising in animals injected with MCF-7/TAM^R-1 cells have developed characteristics distinct from the MCF-7/TAM^R-1 cells growing in tissue culture. The estrogen dependence and the expression of PgR in the tumors indicate emergence of parent-like phenotype in these cells. We assume that the selection pressure in the nude mice is due to the generation of these tumors with changed characteristics compared to the inoculated cells. Whether these tumors arise from clonal expansion of subpopulations already existing in the MCF-7/TAM^R-1 cell line or whether the genetic instability and the selection pressure have provided a growth advantage for altered cells with parent-like phenotype are at present unknown. An interesting clinical observation reflecting such changes in characteristics dependent on the different selection pressures is the observation of breast cancer patients, who, after initial response to tamoxifen therapy, develop resistance and then after an appropiate time interval (at least 10 weeks) respond to a tamoxifen rechallenge (45).

The determination of ER and PgR content in MCF-7 and MCF-7/TAM^R-1 cells grown in tissue culture disclosed one major difference between the cell lines. The MCF-7/TAM^R-1 cells lack PgR expression in control growth medium, and no induction of PgR synthesis occurs in medium with optimal estradiol concentration. As demonstrated several years ago and also found here, a high concentration of tamoxifen inhibits PgR synthesis in MCF-7 cells (46), and the MCF-7/TAM^R-1 cells which are normally propagated in medium with 10^{-6} M tamoxifen have therefore been withdrawn 10 days from tamoxifen before onset of estrogen treatment. This excludes that lack of PgR induction in MCF-7/TAM^R-1 cells can be due to remaining tamoxifen and points toward an impaired function in estrogen induction of PgR synthesis. Whether this is due to a modification in the ER protein, which in several other aspects acts normally, or whether other transcription factors involved in regulation of PgR transcription may be altered, awaits further investigations. The lack of PgR expression and estrogen inducibility may be an early event in the development of antiestrogen resistance since the antiestrogen-resistant LY2 cell line displays similar characteristics (17), and we have recently found that four new ICI 164,384-resistant MCF-7 sublines established in our laboratory also lack PgR expression and estrogen inducibility.[4]

Changed uptake or changed ER binding of tamoxifen could be mechanisms rendering ER-positive breast cancer cells tamoxifen resistant. Tamoxifen treated MCF-7 and MCF-7/TAM^R-1 cells contain ERs in the nuclear salt extract and no free ERs in the cytosol preparations. This indicates that the ERs in both cell lines treated with tamoxifen are present as tamoxifen-bound, chromatin-associated re-

[4] Unpublished results.

ceptors. Changed uptake or binding of tamoxifen, therefore, does not seem to be involved in the tamoxifen resistance of this cell line.

The sensitivity of human breast tumors to tamoxifen therapy is due to the estrogen-responsive or estrogen-dependent cell proliferation of the tumor cells. Complex interactions involving estrogen-regulated gene products are probably responsible for the estrogen-stimulated cell proliferation. Changes in estrogen or antiestrogen regulation of these gene products may accordingly lead to development of tamoxifen resistance. In order to elucidate whether such changes have occurred in the MCF-7/TAM^R-1 cell line, we have chosen to study estrogen and antiestrogen regulation of five estrogen regulated proteins, which are secreted from the cells and probably involved in growth regulation. These proteins are the estrogen-inducible pS2 and M_r 52,000 cathepsin D proteins, which have been studied in detail in other laboratories (41, 47). The function of the pS2 protein is unknown, and the M_r 52,000 cathepsin D has been described to exert mitogenic activity on estrogen-deprived MCF-7 cells (48). The three other proteins are two antiproteases, the M_r 66,000 α_1-antichymotrypsin and the M_r 61,000 α_1-antitrypsin which are stimulated by estrogen, and a M_r 42,000 protein which is down-regulated by estrogen and up-regulated by tamoxifen. The α_1-antichymotrypsin has been reported to be an estrogen-stimulated protein from two laboratories besides ours (49–52), and we have found that addition of polyclonal antibodies to α_1-antichymotrypsin to estrogen-treated MCF-7 cells reduced the estrogen stimulation by about 30% (52). We have been the first to describe estrogen stimulation of α_1-antitrypsin synthesis and secretion in MCF-7 cells (32, 51), and this observation has recently been confirmed in another laboratory (50). Estrogen stimulation of MCF-7 cells can be reduced by addition of polyclonal antibodies to α_1-antitrypsin, indicating that also this antiprotease may be involved in the mechanisms leading to estrogen stimulation (53). The estrogen down-regulated secreted M_r 42,000 protein has not yet been identified. As described previously (32), this protein is propably the same protein as the M_r 39,000 and the 37,000 proteins described by two other groups to be estrogen down-regulated and tamoxifen up-regulated in MCF-7 cells (54, 55). These two groups have independently suggested that this protein may have a growth inhibitory function. We have been able to partially purify the M_r 42,000 protein from conditioned medium from growth inhibited MCF-7 cells, and the fractions containing M_r 42,000 protein exert growth inhibitory activity towards MCF-7 cells (53). We assume that the M_r 42,000 protein is a negative growth regulatory factor, and experiments are in progress to purify the protein to homogeneity. In control growth medium, secretion of the above described proteins is not identical in the two cell lines (Fig. 3). The MCF-7 cells secrete substantially more M_r 52,000 protein and less M_r 61,000 α_1-antitrypsin than the MCF-7/TAM^R-1 cells. This difference does not affect the cell proliferation rate which in control growth medium is the same for both cell lines (Fig. 1). No difference appears in estradiol regulation of the secreted proteins or on cell proliferation. Tamoxifen has no effect on secreted proteins or on the cell proliferation rate in MCF-7/TAM^R-1 cells, whereas M_r 52,000 and M_r 42,000 protein secretion is significantly stimulated in MCF-7 cells, which are growth inhibited by tamoxifen. If these two secreted proteins are involved in growth regulation, it is likely to suggest that the mitogenic effect of the M_r 52,000 protein is of minor importance, as found previously in the MCF-7 cell variants, I13 and LY2 (56). However, the M_r 42,000 protein may have a growth inhibitory function because we find stimulation of M_r 42,000 protein synthesis associated with growth inhibition by tamoxifen and lack of tamoxifen stimulation of M_r 42,000 protein synthesis and secretion associated with resistance to the growth inhibitory effect of tamoxifen. The steroidal antiestrogen ICI 164,384 has a pure estrogen antagonistic effect on estrogen-regulated secreted proteins in both cell lines, and they are both growth inhibited by the ICI 164,384 compound and also by the related ICI 182,780 compound. Up-regulation of M_r 42,000 protein synthesis is also associated here with inhibition of cell proliferation.

Estrogen regulation often occurs at the mRNA level, and our Northern analyses have confirmed that the steady state levels of mRNA for pS2 protein, M_r 52,000 cathepsin D, M_r 61,000 α_1-antitrypsin, and M_r 66,000 α_1-antichymotrypsin are stimulated by estradiol in MCF-7 cells (35, 50, 57). In essence, the estrogen and antiestrogen regulation of the specific mRNAs correlate with the effects observed on the corresponding proteins. Apart from the pS2 mRNA, the overall levels of the estrogen-regulated genes are lower in the resistant cell line. The pS2 mRNA level is significantly higher in control cultures of MCF-7/TAM^R-1 cells compared to parent MCF-7 cells. An increased constitutive synthesis of pS2 mRNA has been found in a hormone-insensitive MCF-7 subline, and estradiol does not induce but rather suppress the pS2 mRNA level in this subline (58). A similar lack of estradiol stimulation of neither pS2 mRNA level nor pS2 protein synthesis and secretion is not found in MCF-7/TAM^R-1 cells. The tamoxifen-resistant cells also differ from parent cells in showing only a minor tamoxifen stimulation of the 2.2-kilobase cathepsin D mRNA level compared to the immense stimulation in MCF-7 cells. It should be emphasized that our parent MCF-7 cell line differs from the MCF-7 cell line in the laboratory of Westley and Rochefort (47) since tamoxifen in our cell line acts as a complete estrogen agonist on cathepsin D mRNA synthesis and cathepsin D secretion. Results similar to ours with tamoxifen stimulation of M_r 52,000 mRNA synthesis and M_r 52,000 protein synthesis have recently been published (59), and the discrepancy may be accounted for by differences in growth conditions or alternatively by selection of different MCF-7 sublines in different laboratories.

Transient transfection with the reporter plasmid, vit-tk-CAT, containing the vitellogenin estrogen response element upstream of the reporter gene (37) has been used to study the ability of endogenous ERs to regulate the synthesis of an estrogen-regulated exogenous gene. No differences in estradiol and antiestrogen regulation of CAT expression in transfected parent and antiestrogen resistant cells were observed. The ERs in the tamoxifen-resistant cell line must therefore be considered to function normally in this particular assay system, which may not mimic perfectly the endogenous gene systems (60).

In conclusion, we believe that long term treatment with tamoxifen may allow clonal expansion of antiestrogen-resistant cells, either already present in the parent cell line or developed by genetic instability during the selection period. It has been suggested that tamoxifen-resistant breast cancer cells may "read" tamoxifen as an agonist (61), and studies indicate that tamoxifen-resistant human breast tumors in nude mice and tumors from breast cancer patients relapsing on tamoxifen treatment may be growth-stimulated by a tamoxifen metabolite with estrogenic activity (24, 25), although a recent report does not find support for this hypothesis (62). We have found no indications of increased agonistic effects of tamoxifen in MCF-7/TAM^R-1 cells, and several of the observed alterations can be ascribed to a reduction in or lack of tamoxifen effect. This lack of tamoxifen effect does not seem to occur from lack of tamoxifen binding to ERs in the tamoxifen-resistant cells. We assume that the lack of tamoxifen up-regulation of the synthesis of the M_r 42,000 protein with presumed growth inhibitory function may be one of the changes involved in tamoxifen resistance, particularly because the ICI 164,384 compound exerts normal up-regulation of this protein in the MCF-7/TAM^R-1 cell and also inhibits growth. The lack of cross-resistance between tamoxifen and the steroidal antiestrogens ICI 164,384 and ICI 182,780 indicates that steroidal antiestrogens may be useful as a treatment at choice when patients develop resistance to tamoxifen therapy.

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