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MCF7/LCC2: A 4-Hydroxytamoxifen Resistant Human Breast Cancer Variant That Retains Sensitivity to the Steroidal Antiestrogen ICI 182,780[1]

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Abstract

The development of resistance to the antiestrogen tamoxifen occurs in a high percentage of initially responsive patients. We have developed a new model in which to investigate acquired resistance to triphenylethylenes. A stepwise *in vitro* selection of the hormone-independent human breast cancer variant MCF-7/LCC1 against 4-hydroxytamoxifen produced a stable resistant population designated MCF7/LCC2. MCF7/LCC2 cells retain levels of estrogen receptor expression comparable to the parental MCF7/LCC1 and MCF-7 cells. Progesterone receptor expression remains estrogen inducible in MCF7/LCC2 cells, although to levels significantly lower than observed in MCF-7 and MCF7/LCC1 cells. MCF7/LCC2 cells form tumors in ovariectomized nude mice without estrogen supplementation, and these tumors are tamoxifen resistant but can be estrogen stimulated. Significantly, MCF7/LCC2 cells have retained sensitivity to the steroidal antiestrogen ICI 182,780. These data suggest that some breast cancer patients who acquire resistance to tamoxifen may not develop cross-resistance to treatment with steroidal antiestrogens.

Introduction

Although approximately one-third of all patients with metastatic breast cancer will initially benefit from endocrine therapy, the majority of these patients relapse. A subsequent temporary response to second line endocrine therapy is observed in about 50% of patients (1). Acquisition of an antiestrogen-resistant phenotype and subsequent development of a multihormone-resistant phenotype appear to be both critical and potentially inevitable steps in the malignant progression of hormone-responsive breast tumors (2, 3). There are few experimental models in which to study acquisition and reversal of antiestrogen resistance. Bronzert *et al.* (4) obtained a stable antiestrogen-resistant MCF-7 variant designated LY-2 following *in vitro* selection with the benzothiophene antiestrogen LY117018. However, LY-2 cells have lost their ability to form tumors when injected into ovariectomized or E2[4]-supplemented athymic nude mice and have acquired resistance to both triphenylethylene and steroidal antiestrogens (5). Other antiestrogen resistant models include FGF-4-transfected MCF-7 cells (6) and *in vivo*-selected variant MCF-7 cells (7, 8). However, these models produce TAM-stimulated tumors (6–8). To derive an antiestrogen-

resistant variant which retained tumorigenic potential, did not require E2 for tumor formation, and was not stimulated by TAM, we performed an *in vitro* selection of the hormone-independent MCF7/LCC1 cells (9, 10) against the potent triphenylethylene antiestrogen 4-OHTAM. MCF7/LCC1 cells retain the phenotype of their immediate predecessor (MCF7/MIII), being E2 independent *in vitro*, E2 responsive *in vivo*, and antiestrogen sensitive (10) and exhibiting increased metastatic potential (9). The present report describes the isolation and characterization of a 4-OHTAM-resistant MCF-7 variant (MCF7/LCC2) derived from MCF7/LCC1 cells. MCF7/LCC2 cells retain an E2-independent phenotype when growing either *in vitro* or *in vivo*, and express levels of ER equivalent to MCF-7 cells. Significantly, MCF7/LCC2 cells are resistant to the inhibitory effects of 4-OHTAM but remain sensitive to the pure steroidal antiestrogen ICI 182,780 *in vitro* (11).

Materials and Methods

Cell Lines. MCF-7 cells were obtained from Dr. Marvin Rich (Michigan Cancer Foundation). MCF-7 cells were routinely maintained in IMEM (Biofluids, Bethesda, MD) supplemented with 5% fetal calf serum. Establishment and characterization of the hormone-independent MCF-7/LCC-1 variant has been previously described (9, 10). MCF-7/LCC-1 and MCF-7/LCC-2 cells were serially passaged in CCS-IMEM, prepared as previously described (5). All cell lines were tested and found to be free from contamination with *Mycoplasma* species.

Selection Procedure. MCF-7/LCC1 cells in their 18th passage were selected stepwise against increasing concentrations of 4-OHTAM. Selection was begun against a concentration of 10^{-9} M 4-OHTAM, with the concentration increasing by half a decade after three successive passages of cells at each dose. The final concentration used was 10^{-6} M 4-OHTAM. Cells proliferating in 10^{-6} M 4OH-TAM were designated MCF7/LCC2, and their dose response to E2, 4OH-TAM and ICI 182,780 determined. MCF7/LCC2 cells were maintained for a further 10 passages in the presence of 10^{-6} M 4OH-TAM, followed by 10 passages in 5% CCS without the drug, cells being refed with fresh growth medium every 3 days. Subsequently, the response of MCF7/LCC2 cells to 4OH-TAM and ICI 182,780 reexamined. The MCF-7 origin of MCF-7/LCC-2 was determined by both karyotype and isozyme analyses as previously described (12). For karyotype analyses, 100 metaphases were examined.

Steroid Hormone Receptor Analyses. ER and PGR were determined using whole cell competitive binding assay as described previously (13, 14). Briefly, cells were grown in 24-well dishes (Costar, Cambridge, MA) and incubated for 60 min at 37°C with increasing concentrations of radiolabeled steroid in the absence or presence of a 200-fold excess of unlabeled competitor. For ER determinations cells were treated with 0.2–0.6 nM [3H]17β-estradiol (specific activity, 95 Ci/mmol; Amersham). PGR were estimated using 0.1–2.5 nM [3H]-ORG 2058 (specific activity 50.6 Ci/mmol; Amersham). To reduce progestin binding to glucocorticoid receptors, cells were incubated at 37°C with 100 nM hydrocortisone for 30 min prior to determining PGR. Radioactivity was extracted into ethanol and measured in a liquid scintillation spectrometer. Data was analyzed using the LIGAND receptor binding software (15).

Cell Growth *in Vitro*. The proliferative capacity of the cell lines was studied by determining the anchorage-dependent growth of cells growing in

CCS-IMEM. All 3 cell lines were grown for 5 or more days in 5% CCS-IMEM and then plated in 96-well Costar tissue culture dishes in either 5% CCS-IMEM, 5% CCS-IMEM containing 10^{-12} M to 10^{-6} M ICI 182,780, 5% CCS-IMEM containing 10^{-12} M to 10^{-6} M 4OH-TAM (Imperial Chemical Industries, United Kingdom), or 5% CCS-IMEM containing 10^{-12} M to 10^{-6} M E2. The cells were refed the appropriate medium every fourth day. Cell growth was determined using a crystal violet dye uptake assay, where the degree of dye uptake is directly related to cell number (16, 17). Briefly, cells were stained with the crystal violet stain by incubation with staining solution (0.5%; w/v) crystal violet in 25% (v/v; methanol) for 5 min at 25°C and rinsed gently twice with distilled H_2O. Cells were allowed to dry, and the dye was extracted by adding 0.1 M sodium citrate in 50% (v/v) ethanol and incubating at room temperature for 10–15 min. Absorbance was read at 540 nm using a Dynatech MR700 enzyme-linked immunosorbent assay reader (Dynatech, Chantilly, VA). The data are presented as the mean and SD of four determinations and represent the absorbance expressed as a percentage of untreated cell populations.

Cell Growth in Vivo. To determine tumorigenicity, 2×10^6 MCF7/LCC2 cells were inoculated s.c. into each flank of 6–8-week-old untreated ovariectomized female NMRI nude mice (Bomholtgaard, Ry, Denmark). Estrogen supplementation was provided by the s.c. implantation of a 0.72-mg E2 pellet (Innovative Research, Toledo OH). TAM treatment was administered by daily i.p. injection of 0.1 mg TAM. This treatment significantly inhibits growth of the parental MCF-7 tumors (18). Tumor area was recorded three times weekly, and tumor doubling time estimated by application of the Gompertz function (19).

Results

Origin of MCF7/LCC2 Cells. The isoenzyme phenotype of MCF-7/LCC-2 (not shown) is identical to both MCF-7 and MCF7/LCC1 (10, 12). The presence of the human/5 lactate dehydrogenase isozyme and the absence of the mitochondrial malic isoenzyme-2 (EC 1.1.1.40) are distinguishing characteristics of MCF-7 cells. Karyotype analyses revealed an aneuploid human female karyotype (Xq + 0), with chromosome counts in the triploid range. The presence of marker chromosomes M8 and M18 and the loss of marker chromosomes M21 and M25 in MCF-7/LCC-2 represent the only significant differences in the pattern of marker chromosome expression from that observed in MCF7/LCC1 cells. The distribution and probable origins of marker chromosomes have been previously reported (10, 20).

Steroid Hormone Receptor Expression. Steroid hormone receptor analyses demonstrated the presence of similar levels of high affinity ER in both MCF7/LCC1 and MCF7/LCC2 cells. These levels are similar to those present in MCF-7 cells and other related MCF-7 variants (12, 21). However, we observed a significant reduction in the levels of PGR expression in MCF7/LCC2 cells growing in the absence of E2, when compared with MCF7/LCC1 cells. The baseline levels of PGR expression in MCF7/LCC2 are comparable to those observed in MCF-7 cells (12, 21). MCF7/LCC2 cells retain an E2-inducible PGR expression similar to MCF7/LCC1 cells. However, the level of expression in E2-treated cells is significantly lower than that present in E2-treated MCF7/LCC1 cells (Table 1).

Growth Response of MCF7/LCC2 Cells to Estrogens and Antiestrogens in Vitro. MCF-7/LCC-1 cells respond poorly (approximately 125% of control; Fig. 1a) or not at all to E2 (10). However, MCF7/LCC-2 cells can be significantly stimulated by E2 in vitro (approximately 200% of control; Fig. 1a), but the degree of stimula-

Table 1 *ER and PGR expression in parental hormone-dependent MCF-7, hormone-independent MCF-7/LCC-1, and hormone-independent and antiestrogen-resistant MCF-7/LCC-2 cells.*

Data represent the number of receptor sites/cell and are expressed as the mean ± SD.

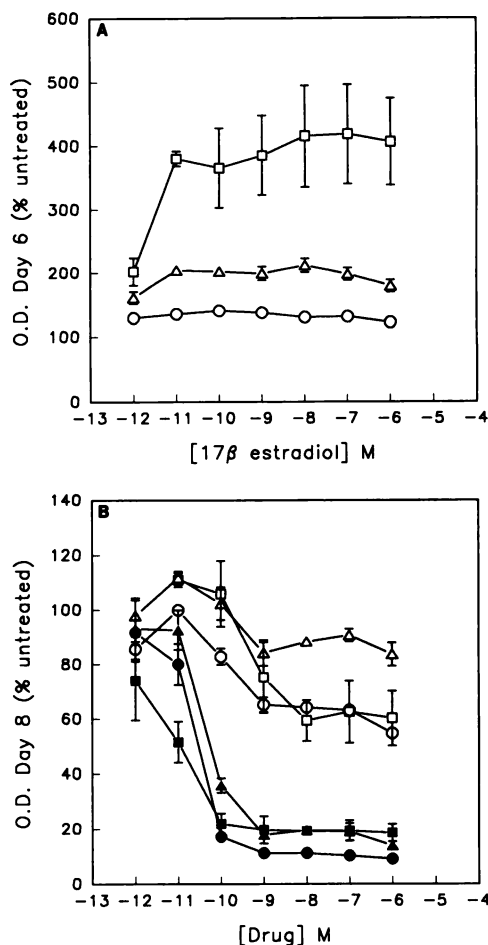| Cell line	ER	PGR −E2	PGR +E2
MCF-7	120,536 ± 20,003	9,841 ± 4,683	47,522 ± 12,005
MCF7/LCC1	116,576 ± 15,951	29,313 ± 7,293	74,909 ± 20,564
MCF7/LCC2	91,286 ± 1,331	6,418 ± 1,127	16,744 ± 1,723

Fig. 1. Dose-response effects of 17β-estradiol, 4-hydroxytamoxifen, and ICI 182,780 on the anchorage-dependent growth of MCF-7, MCF7/LCC1, and MCF-7 LCC2 cells. (*A*) Effect of 17β-estradiol: MCF-7 (□); MCF7/LCC1 (○); and MCF7/LCC2 (△) cells growing *in vitro*. (*B*) Effect of 4-OHTAM (*open symbols*) and ICI 182,780 (*closed symbols*). Data represents the mean ± SD of 4 determinations.

tion is significantly less than that observed in E2-treated MCF-7 cells (approximately 400% of control; Fig. 1a).

While 10^{-6} M 4OH-TAM significantly inhibits MCF7/LCC1 and MCF-7 cell proliferation, the proliferation of MCF7/LCC-2 is reduced by ≤ 15% (Fig. 1b). However, MCF7/LCC2 cells retain sensitivity to the inhibitory effects of the steroidal antiestrogen ICI 182,780 similar to MCF-7 and MCF7/LCC1 cells (Fig. 1b). MCF7/LCC2 cells maintained for 10 passages in 5% CCS without TAM exhibit a similar pattern of responsivity. MCF-7 cells often produce a weak partial agonist (estrogenic) response to 4OH-TAM when growing in CCS-IMEM (5, 22). We did not observe any significant partial agonism in this study.

Growth of MCF7/LCC2 Cells in Vivo. Both MCF7/LCC2 (Fig. 2) and MCF-7/LCC-1 cells (10) are tumorigenic in untreated ovariectomized female nude mice, whereas wild-type MCF-7 cells do not produce tumors in these animals (5, 12, 21). The data in Fig. 2 show the effects of E2 and TAM on the growth of MCF7/LCC2 cells in vivo. MCF7/LCC2 cells retain the E2 responsivity of the parental MCF-7 cells, the MCF7/MIII cells from which MCF7/LCC1 cells were derived, and their immediate predecessor MCF7/LCC1 cells (10, 12). TAM treatment which significantly inhibits MCF-7 growth (18) does not influence the growth of MCF7/LCC2 tumors (Fig. 2). This contrasts with other potential in vivo models of TAM resistance in which TAM stimulates tumor growth.

Tumor doubling times were estimated from the data in Fig. 2 as described by Rygaard and Spang-Thomsen (19). Analyses of variance
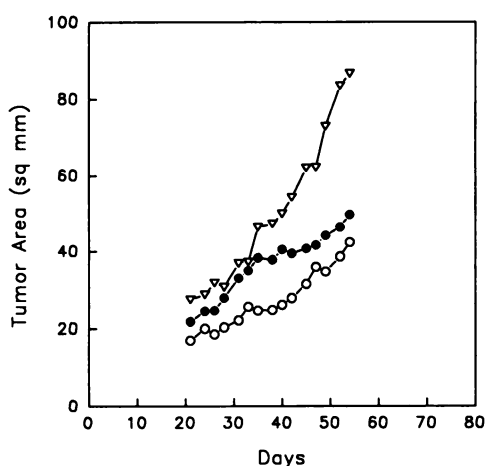
Fig. 2. Effect of E2 and TAM on the growth of MCF7/LCC2 cells in ovariectomized athymic nude mice: nonsupplemented (O); TAM (●); E2 supplemented (▽). Data represent mean tumor area. Tumor doubling times estimated from these growth curves are: untreated, 32.53 days; TAM treated, 31.31 days; E2 treated, 18.31 days. Analyses of variance indicate that the tumor doubling times for tumors in the TAM-treated and untreated are equivalent ($P > 0.05$). E2-treated tumors grow significantly more rapidly than both untreated and TAM-treated (analysis of variance, $P < 0.01$).

indicate that TAM-treated (31.38 days) and untreated tumors (32.53 days) have equivalent tumor doubling times ($P > 0.05$). E2-treated MCF7/LCC2 tumors (18.31 days) grow significantly ($P < 0.01$) more rapidly than both untreated and TAM-treated tumors. The growth rate of MCF-7/LCC-2 in the ovariectomized nude mice is similar to that of MCF-7/LCC-1 tumors (10).

Discussion

The process of malignant progression by breast cancer cells is frequently characterized by the progression of initially endocrine/ cytotoxic responsive tumors to a multidrug-resistant and multihormone-resistant phenotype (16). While acquired resistance to endocrine manipulation is a major clinical problem, there are only a few antiestrogen resistant models with which to study this process (for a recent review see Ref. 2). The most widely used LY-2 variant was derived from MCF-7 cells selected in vitro against the benzothiophene antiestrogen LY 117018 (4). LY-2 cells retain functional ER at levels significantly less than in the parental MCF-7 cells (4). However, LY-2 cells have lost their ability to form tumors when growing in ovariectomized or E2-supplemented athymic nude mice (5). In vivo selection of MCF-7 cells against TAM, or transfection with FGF-4 (6), produces tumors which are stimulated by TAM (7, 8). The in vivo selected TAM-stimulated tumors are dependent upon E2 or TAM for growth in vivo (7, 8). The clinical relevance of the LY-2 or TAM-stimulated phenotypes remains to be established (2, 5, 23).

We have derived a series of variants from the hormone-dependent, antiestrogen-sensitive, and poorly invasive MCF-7 human breast cancer cell population. Following one selection of MCF-7 cells in ovariectomized nude mice we isolated a population designated MIII (12). MIII cells do not require E2 for growth in vitro or in vivo (5, 12) and are inhibited by each of the major structural classes of antiestrogens in vitro (5) and by LHRH analogues when growing in intact female mice (3). A further in vivo selection produced the MCF7/LCC1 variant, which has a significantly reduced lag to the appearance of tumors in ovariectomized mice (10) and tends to express a more malignant phenotype than MIII cells (9). MCF7/LCC1 cells retain the hormone-independent and antiestrogen-sensitive phenotype of MIII cells (10).

To obtain an antiestrogen-resistant variant which retained tumorigenic potential but did not require E2 for tumor formation, we chose to select the hormone-independent MCF7/LCC1 cells against

4-OHTAM in vitro. The resultant cells designated MCF7/LCC2 have a number of critical characteristics which clearly separate them from other antiestrogen-resistant variants. For example, unlike the nontumorigenic LY-2 cells (5), E2-dependent and TAM-dependent/ stimulated in vivo selected MCF-7 cells (8), and E2-dependent MCF-7 RR cells (24), MCF7/LCC2 cells can form proliferating tumors in ovariectomized nude mice without E2 supplementation. Whereas LY-2 cells have lower ER expression and lost expression of PGR (4), MCF7/LCC2 cells retain levels of ER similar to MCF-7 cells. Although PGR remains E2 inducible, PGR levels in MCF7/LCC2 cells appear to be lower than their parental MCF7/LCC1 cells.

While MCF7/LCC2 cells are resistant to 4-OHTAM, they have not acquired cross-resistance to the steroidal antiestrogen ICI 182,780. This is in marked contrast to the LY-2 cells, which are resistant to all the major structural classes of antiestrogens (5). Another TAM-resistant MCF-7 variant (AL-1) retains partial sensitivity to the steroidal ICI 164,384 (25). If the development of a non-cross-resistance MCF7/ LCC2 phenotype occurs in human tumors, this would have considerable significance in the design of combined antiestrogen therapies using both triphenylethylenes and steroidal antiestrogens. For example, it might be expected that a subset of patients who respond initially but subsequently progress on TAM therapy may respond to a second line of endocrine treatment with a steroidal antiestrogen (e.g., ICI 182,780).

The clinical acquisition of resistance to one endocrine modality does not necessarily result in acquired resistance to other modalities. Approximately 50% of patients who demonstrate an objective response to first line endocrine therapy will upon relapse benefit from a second line endocrine therapy (1). Resistance to (or independence from) E2 in the MCF7/MIII variant does not produce cross-resistance to either antiestrogens (5, 12) or LHRH analogues (3). Acquired resistance to a triphenylethylene antiestrogen does not always induce resistance to steroidal antiestrogens, as indicated by the MCF7/LCC2 phenotype. Thus, the MCF-7 variants (MCF7/MIII, MCF7/LCC1, and MCF7/LCC2) provide the opportunity to investigate responsivity to antiestrogens and the development of cross-resistance both in vivo and in vitro, without the confounding necessity to provide E2 supplementation. Furthermore, these variants may lead to the identification of novel combined hormonal manipulative regimens using both triphenylethylene and steroidal antiestrogens, LHRH analogues, and possibly other endocrine treatments (3, 23).

The factors responsible for the acquisition of a triphenylethylene-resistant phenotype by MCF7/LCC2 cells remain unclear. Intervention with either cytotoxic chemotherapy or hormonal manipulation may contribute to progression by either inducing phenotypic changes and/or selecting for a resistant phenotype. We have previously suggested that the altered chromosomal number ranges of the variants may reflect a process of clonal selection (23, 26). Thus, we cannot exclude the possibility that antiestrogen-resistant MCF7/LCC2 cells were already present in the MCF7/LCC1 population.

Although selection or adaptation may facilitate emergence of the resistant phenotype, the mechanistic changes which confer the altered phenotype are unknown. We have recently discussed in detail the potential mechanisms which may produce antiestrogen resistance (2). However, there are a number of specific changes which could result in the phenotype observed in MCF7/LCC2 cells. For example, mutant ER are clearly present in some breast tumors (27), and these can produce different responses when occupied with triphenylethylene versus steroidal compounds (28). Elevated levels of antiestrogen binding sites could produce the non-cross-resistant MCF7/LCC2 phenotype, since these sites have a high affinity for triphenylethylene but not steroidal molecules (29). These sites most probably function as an intracellular "sink," rather than specific functional "antiestrogen re-

ceptors" (2). Osborne *et al.* (30) have demonstrated the ability of some cells to metabolize 4OH-TAM to its less potent *cis* isomer. Since it seems unlikely that the factor(s) reponsible for this activity would also metabolize steroidal antiestrogens, altered cellular metabolism of 4OH-TAM could contribute to the MCF7/LCC2 phenotype.

There is increasing evidence suggesting that triphenylethylenes and steroidal antiestrogens interact differently with the ER transcription complex. The steroidal ICI 164,384 has been reported to impair ER dimerization (31) and increase cellular ER turnover (32). In contrast, TAM does not prevent dimerization, and the resulting TAM-activated ER transcription complex alters the transcription of a number of specific genes (2, 31). While both triphenylethylene and steroidal antiestrogens alter the function of the ER transcription complex, this can occur through different mechanisms. Consequently, it is perhaps not surprising that some TAM-resistant cells do not acquire cross-resistance to steroidal ligands.

By continuous sequential selection of MCF-7 human breast cancer cells, we have demonstrated that this model can develop endocrine resistance in a stepwise manner. This observation closely reflects the clinical experience with the use of second line endocrine therapy and further substantiates the biological relevance of these models (23). We are currently using these models in an attempt to identify the molecular changes responsible for conferring increased metastatic potential (9), hormone-independence, and antiestrogen resistance in the presence of functional ER.

Acknowledgments

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