Rapid Development of Tamoxifen-stimulated Mutant p53 Breast Tumors (T47D) in Athymic Mice

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ABSTRACT

MCF-7 cells are used routinely to study tamoxifen-stimulated drug resistance in vivo. However, unlike MCF-7 cells, T47D cells express mutant p53 protein and lose the estrogen receptor (ER) during long-term estrogen deprivation in vitro [Pink et al., Br. J. Cancer, 74: 1227–1236, 1996 (erratum, Br. J. Cancer, 75: 1557, 1997)]. As a result, T47D tumors may respond differently from MCF-7 tumors to long-term tamoxifen treatment. Ovariectomized athymic mice were given injections bilaterally with T47D cells (5 × 10⁵) into the mammary fat pads. A rapidly growing estradiol responsive tumor (T47D:E2) was established and 0.5 mg of tamoxifen given daily blocked estrogen-stimulated growth. In subsequent experiments, low doses of tamoxifen (0.17 mg or 0.5 mg) did not produce tamoxifen-stimulated tumors at 14 weeks, whereas high-dose tamoxifen (1.5 mg) consistently produced tamoxifen-stimulated tumors (T47D:Tam; 17 tumors/20 sites) at 8 weeks. In contrast, 1.5 mg of tamoxifen produced tamoxifen-stimulated MCF-7 tumors (MCF-7:Tam2) at a slower rate (20 weeks) and less consistently (14 tumors/26 sites). When the T47D:Tam tumor was passaged, it grew maximally with either 1.5 mg of tamoxifen or a 1-cm estradiol (premenopausal levels) capsule, and similar results were obtained with MCF-7:Tam2 tumors. Interestingly, when T47D:Tam tumors were treated with the 0.5 mg of tamoxifen, tumors grew only to 50% maximum. All of the tumors originating from MCF-7 and T47D cells expressed ER at similar levels; therefore, tamoxifen did not select for an ER-negative tumor. In conclusion, we have shown that tamoxifen-stimulated T47D p53 mutant tumors can be developed rapidly with high-dose therapy (1.5 mg daily). The results from this model provide new opportunities to investigate the rapid development of drug resistance to adjuvant tamoxifen in patients with mutant p53 breast tumors.

INTRODUCTION

Tamoxifen is the endocrine treatment of choice for all stages of breast cancer (1). Up to 5 years of adjuvant treatment of patients with ER-positive disease confers a long-term survival benefit that can extend for at least 5 years after tamoxifen treatment is stopped (2). Although early studies with indefinite tamoxifen treatment illustrated the benefits of tamoxifen on preventing the appearance of primary mammary tumors in carcinogen-induced models (3), clonal selection of metastatic breast cancer in patients results in tamoxifen-stimulated disease. This is illustrated clinically by a withdrawal response when tamoxifen treatment is stopped (4–6). Tumors with acquired resistance to tamoxifen often retain the ER (7) and respond to a second line endocrine therapy such as a pure antiestrogen (ICI 182,780; Ref. 8), which has no estrogen-like properties, or an aromatase inhibitor (9), which blocks the synthesis of endogenous estradiol.

Only one laboratory model in vivo has been used routinely to study drug resistance to antiestrogens. MCF-7 human breast cancer cells are ER-positive and can grow into nonmetastatic solid tumors in athymic mice with estrogen supplementation (10, 11). Tamoxifen is able to block estrogen-stimulated growth (12). However, extended tamoxifen treatment for 8 months results in the appearance of ER-positive tamoxifen-stimulated tumors (13) that are transplantable (14–16). Although several hypotheses have been advanced to explain tamoxifen-stimulated growth, such as local metabolism of drug or mutant ERs (17–20), there is no unifying theory of tamoxifen-stimulated tumor growth or drug resistance. This is, however, not a surprise because all studies use tumors derived from one cell line that has a wild-type p53 protein (21). Because 50% of human breast cancer have mutant p53 (22, 23), we believed it was important to determine what effect a mutant p53 would have on acquired antiestrogen resistance.

The T47D human breast cancer cell line is ER/progesterone receptor-positive and is derived from a pleural effusion (24). These cells express a mutant p53 (25), and clones have been derived that are exquisitely sensitive to the stimulatory effects of...
estradiol (26, 27). The p53 protein comprises three functional domains: transactivating domain, sequence-specific zinc-binding domain, and tetramerization domain. Within the zinc-binding domain are two regions, L2 and L3 loops, also called zinc-binding domains (residues 163–195 and 236–251), that are important for DNA binding and protein stabilization (28). Missense mutations in the zinc-binding domain are the most frequently found and have been shown to predict poor outcome in patients (29, 30).

T47D cells contain only single copies of a missense mutation at residue 194 (within the zinc-binding domain, L2) which could explain why p53 is nonfunctional in these cells (25). If p53 cannot bind response elements in DNA, this may diminish or abolish its ability to regulate the cell cycle. Another study that analyzed the regulation of subcellular compartmentation of mutant versus wild-type p53 proteins as a function of the cell cycle showed that mutant p53 present in T47D cells was present in the nucleus at different times compared with the wild-type p53 expressed in MCF-7 cells (31). Interestingly, transfection of wild-type p53 into T47D cells is incompatible with cellular growth, whereas in MCF-7 cells, which express wild-type p53, transfection of wild-type p53 has no effect on cellular growth (21).

Early attempts to grow solid tumors from T47D cells were unsuccessful in athymic mice because, it was believed, T47D cells required prolactin in addition to estrogen supplementation for optimal growth. Interestingly, tumors would grow only if athymic mice were cotransplanted with the rat pituitary cell line GH3 (32). We have reexamined this observation and successfully developed estrogen-stimulated T47D tumors. Unlike MCF-7 cells, which retain the ER under estrogen-deprived conditions in vitro (33–35), T47D cells lose ER expression in vitro and become refractory to both estrogen and antiestrogens (36, 37). Naturally, we believed this phenomenon could be related to the difference in p53 status and encouraged us to pursue additional studies in vivo. Our initial hypothesis was that T47D cells would not form an antiestrogen-stimulated tumor because the drugs would cause selection pressure and encourage the growth of an ER-negative clone. However, this was not the case, because ER-positive T47D tumors rapidly become refractory and form ER-positive tamoxifen-stimulated tumors during high-dose tamoxifen therapy.

MATERIALS AND METHODS

The human breast cancer cell lines T47D and MCF-7 were originally obtained from American Type Culture Collection (Rockville, MD). These cells were karyotyped by Cellmark (Germantown, MD) and shown to be authentic T47D or MCF-7 cells (data not shown). DNA sequence analysis of the p53 gene in T47D cells was performed by OncorMed (Gaithersburg, MD) and revealed only homozygous mutation in exon 6 at nucleotide 580 of codon 194 changing leucine (CCT) to phenylalanine (TTT; data not shown). This mutation is reported in the Thierry Soussi database. Analysis of the exons 5–9 of the p53 gene in MCF-7 cells revealed only wild-type sequence.

Athymic Mouse Model. The T47D and MCF-7 tumors used in these parallel experiments originated from a bilateral inoculation of 5 × 10⁵ T47D or MCF-7 cells suspended in Hanks’ buffered saline solution into the mammary fat pads of ovariectomized BALB/c nu/nu mice supplemented with estrogen as described previously (38). Ovariectomized 4–5-week-old athymic mice (Harlan Sprague Dawley, Madison, WI) were subsequently bilaterally transplanted s.c. in the axillary mammary fat pads with 1-mm³ pieces of T47D or MCF-7 tumors using a trochar. The Animal Care and Use Committee of Northwestern University approved all of the procedures involving animals.

Hormone and Drug Treatments. Mice were divided into groups of 10 and were treated with E2 (Sigma, St. Louis, MO), tamoxifen (Sigma), or combinations. E2 pellets containing 1.7 mg of E2 (Innovative Research of America, Toledo, OH) were implanted s.c. in the back of the mouse on the same day as tumor transplantation. Silastic E2 capsules (0.3 cm or 1 cm in length) were made as described previously (39), implanted s.c., and replaced after 8–10 weeks of treatment. The 0.3-cm estradiol capsules produced a mean 83.8 pg/ml of serum E2, whereas 1.0 cm E2 capsules produced a mean 379.5 pg/ml serum E2 (40). Each was designed to represent the low or high E2 levels observed in post- or premenopausal women respectively.

Tamoxifen was first dissolved in ethanol and suspended in a solution of 90% carboxymethylcellulose (1% carboxymethyl-cellulose in double distilled water) and 10% polyethylene glycol 400/Tween 80 (99.5% polyethylene glycol and 0.5% Tween 80). Ethanol was evaporated under nitrogen before use. Tamoxifen was administered p.o. by gavage at various doses: 0.17, 0.5, or 1.5 mg per mouse per day 5 days a week. Tamoxifen at 0.5 mg resulted in serum levels of tamoxifen of 58 ± 7 ng/ml and at 1.5 mg 203 ± 100 ng/ml (mean ± SD; 40).

Tumor Measurements. Tumor measurements were performed weekly using Vernier calipers. The cross-sectional area was calculated using the formula: length × width/4 × π.

Statistical Analysis. Comparisons in mean tumor between the animal groups were analyzed by ANOVA each week and were followed by unpaired Student’s t test. The two-tailed P of the last previous week of each experiment was reported using StatMost 2.5 (Datamost Corp., Salt Lake, UT). Analysis of covariance was used to compare slopes of the rate of development of T47D and MCF-7 tamoxifen-stimulated tumors.

Western Blot Analysis. Cells were seeded at various concentrations into T-75 cm² tissue culture flasks and treated with hormone for 24 h. Tumors were homogenized by grinding in liquid nitrogen. The cell or tumor cell pellet was resuspended in protein extraction buffer [0.5% NP40, 2% Glycerol, 1 mM DTT, 1 mM EDTA, 150 mM NaCl, 50 mM Tris (pH 7.4), 1 mM EGTA, 3 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 9 μg/ml aprotonin, 25 μg/ml trypsin inhibitor, 25 μg/ml t-chymotrypsin]. Samples were incubated on ice with intermittent vortexing for 30 min and then pelleted. Supernatant was then collected and stored at −80°C. Protein concentration was measured using the Bio-Rad Protein Assay kit, and equal amounts of protein were run in a standard Western blot protocol. The ER primary antibody used was AER311 (Neomarkers, Fremont, CA) and β-actin antibody AC-15 (Sigma, St. Louis, MO) was used to standardize loading. The appropriate secondary antibody conjugated with horseradish peroxidase kit (Amersham Corp.) was used to visualize bands using an ECL visualization kit (Amersham Corp., Arlington Heights, IL). The
RESULTS

Ovariectomized athymic mice were injected bilaterally with \(5 \times 10^5\) T47D human breast cancer cells and supplemented with E2 pellets (14, 38) to create T47D:E2 tumors. Initial tumor take was 5 tumors/8 sites after 8 weeks. When T47D:E2 tumors reached 0.8 cm\(^2\) (data not shown), a single tumor was passaged into 40 mice by retransplanting 1 mm\(^3\) tumor pieces and treating 10 mice each with premenopausal estradiol levels delivered by a 1-cm E2 capsule (379.5 pg/ml), p.o. tamoxifen (0.5 mg), or a combination (Fig. 1). The final group served as the untreated control. After 12 weeks of treatment, tumors from the estradiol-treated animals grew rapidly and tamoxifen inhibited estradiol-stimulated growth, which demonstrated that tamoxifen was acting as an antiestrogen in this model. In this experiment, 0.5 mg tamoxifen alone stimulated the growth of only a single tumor which, when retransplanted and treated with 0.5 mg of tamoxifen, did not grow after 15 weeks of tamoxifen treatment (data not shown).

To determine whether tamoxifen inhibited the tumor take or tumor growth, we implanted 30 mice with E2 pellets (14), which deliver high-dose estradiol (1044 pg/ml), to establish growing tumors. After 5 weeks, animals were randomized into groups to continue E2 and stop or receive 0.17 mg or 1.5 mg tamoxifen daily (Fig. 4B). The E2 group grew rapidly during the first 13 weeks. The experiment was extended to 31 weeks until tamoxifen-stimulated tumors appeared. The majority of tamoxifen-stimulated tumors (14 tumors/26 sites) occurred in the 1.5-mg-tamoxifen group, as they had in the T47D model. How-
ever, the MCF-7:Tam2 tumors were retransplanted, and only one of four attempts produced viable tamoxifen-stimulated tumors. These data support the theory that selection pressure from high doses of tamoxifen will facilitate tamoxifen-stimulated tumor growth compared with the effect of lower doses. In addition, the data show that the T47D tumor line was more prone to tamoxifen failure compared with the MCF-7 tumor line. Using analysis of covariance to compare the slopes of the 1.5-mg-tamoxifen treatments of T47D and MCF-7 tumors, we determined that the T47D tumors produced tamoxifen-stimulated tumors at a faster rate and to a significantly greater extent compared with MCF-7 tumors (P < 0.001).

Next tamoxifen-stimulated MCF-7:Tam2 and T47D:Tam tumors were harvested and retransplanted to compare the growth characteristics of these two tumor models under similar conditions. Thirty athymic mice implanted with the MCF-7:Tam2 tumor were treated with 0.3-cm E2 capsule, 1.5 mg tamoxifen, or no drug (control). E2 resulted in very rapid growth of these tumors, which reached an average tumor area of 1 cm² by 8 weeks (Fig. 5). The same experiment was performed using the T47D:Tam tumors implanted into 30 athymic mice and treated with the same regimens. Low-dose E2 (0.3 cm capsule) resulted in growth of T47D:Tam to 1 cm² after 14 weeks (Fig. 6) and tamoxifen produced an almost identical growth response. Clearly, both tumors respond to estrogen and tamoxifen for growth.

In the final growth study, we confirmed the response of T47D:Tam tumors to premenopausal E2 (1-cm capsule) and, in addition, established the dose-response relationship of tamoxifen-stimulated growth. T47D:Tam tumors were retransplanted into 40 mice that were treated with 1 cm E2 capsule, 0.5 mg tamoxifen, 1.5 mg tamoxifen, or no drug (control; Fig. 7). Both the E2- and high-dose-tamoxifen-treated tumors grew immediately and rapidly, whereas control tumors did not grow. Interestingly, the 0.5 mg-tamoxifen group grew at a slower rate and to only one-half the maximal size attained by tumors stimulated by either E2 or 1.5 mg of tamoxifen. This experiment demonstrates that tamoxifen-stimulated tumors are able to grow with either E2 or tamoxifen, as previously described for MCF-7 tumors resistant to tamoxifen (14).

Lastly, we measured the levels of ER expression by Western blot in the T47D:E2 and T47D:Tam tumors and then compared the results.
pared these in vitro with T47D cells treated with E2 or the active metabolite of tamoxifen, 4-OHT (Fig. 8). We compared these findings with ER expression levels of MCF-7:E2 and MCF-7: Tam2 tumors and then compared these with MCF-7 cells in vitro treated with estradiol or 4-OHT. We found no obvious differences in ER expression in T47D or MCF-7, tumors whereas the in vitro results illustrated the two models of ER regulation published previously (35). Most importantly, the ER was not lost in T47D:Tam tumors.

DISCUSSION

Although tamoxifen is the endocrine treatment of choice for all stages of breast cancer (1, 2), approximately one-half of the patients with ER-positive breast cancer either do not respond to tamoxifen or rapidly fail tamoxifen treatment (2). A study of drug resistance to tamoxifen is, therefore, a priority in breast cancer either to predict who will not benefit from therapy or to counter the process and extend the antitumor actions of an effective treatment.

Laboratory investigations of drug resistance to tamoxifen have, almost exclusively, used the MCF-7 breast cancer cell line (13, 14, 20, 42). However, it is obvious that studies with a single cell line from a single patient (10, 11) cannot adequately describe all of the possible mechanisms involving drug resistance to tamoxifen that occur spontaneously in the clinic. Breast cancer growth is heterogeneous and multifaceted with numerous genetic alterations such as HER2 amplification (43–45), BCAR-1 amplification (46, 47), and p53 mutation (48, 49) modifying the response to tamoxifen through the ER signal transduction pathway.

MCF-7 and T47D cells are both ER-positive and sensitive to the stimulatory effects of E2 and the inhibitory effects of antiestrogens in cell culture (27, 50, 51). However, unlike the MCF-7 cell line (37), T47D cells can lose the ER during long-term estrogen deprivation (36, 52). The mechanism is unknown but the finding that p53 is mutated in T47D cells (53) and that MCF-7 and T47D cells have different control mechanisms for ER regulation (35, 54) raised the possibility that T47D cells would lose sensitivity to tamoxifen in vivo and would develop ER-negative hormone-independent tumors. In fact, our hypothesis was incorrect.

It is difficult to establish tamoxifen-stimulated tumors in athymic mice using estrogen-stimulated MCF-7 tumors. This is not surprising inasmuch as it has been difficult to demonstrate tamoxifen-stimulated breast cancer clinically (4–6), and the phenotype is only inferred by a second-line response to either a pure antiestrogen (8) or an aromatase inhibitor (9). In contrast, the p53 mutant T47D tumors produced tamoxifen-stimulated tumors with p.o. 1.5 mg tamoxifen faster and to a significantly greater extent compared with MCF-7 tumors. We believe that this is an important observation that could provide clues to the causes of tamoxifen failure.

Mice rapidly excrete tamoxifen, and high daily doses are required to replicate the circulatory levels observed in patients (55). In patients, tamoxifen accumulates to reach steady state within the first 4 weeks (56), and blood levels of tamoxifen are around 100–200 ng/ml using a 20-mg/day treatment regimen (57). The initial daily dose selected in our study (0.5 mg per day p.o.) was based on previous experience by Osborne’s group who used 0.35 mg per day via i.p. injection (12, 13). We demonstrate that 0.5 mg tamoxifen per day is effective as an antitumor agent when premenopausal levels of E2 are used to stimulate tumor growth (Fig. 1). However, 1.5 mg tamoxifen per day caused the development of tamoxifen-stimulated tumors with both MCF-7 and T47D breast cancers.

In an earlier study, we examined the circulating levels of tamoxifen and found that a dose of 0.5 mg of tamoxifen produced serum levels of 58 ± 7 ng/ml (mean ± SD) and at a dose of 1.5 mg, 203 ± 100 ng/ml serum tamoxifen was produced (40). It is difficult to relate the dose per mouse realistically to the
clinical use of tamoxifen because, unlike in humans, tamoxifen is metabolized and excreted rapidly in mice. Furthermore, the optimal therapeutic dose that is used in humans is controversial. The initial selection of a dose to test for the treatment of breast cancer was only estimated (58, 59) and subsequently established based on the observation that the drug was effective with no significant side effects. Depending on the country, tamoxifen is recommended at 20 mg per day (United States), 20 or 40 mg per day (United Kingdom), or 30 mg per day (Canada and Germany). There are suggestions that the doses used clinically are too high (60, 61), and a dose of 10 mg every other day is being considered for chemoprevention in well women (61).

The observations that both the T47D and MCF-7 tamoxifen-stimulated tumors remain ER-positive and will grow with either estradiol or tamoxifen is consistent with clinical observation (7). The mechanism for the alteration of tamoxifen from an antiestrogen to an estrogen appears to be consistent for the cell lines despite differences in ER regulation (35, 54). However, the observation that the development of tamoxifen-stimulated tumors occurs more rapidly with T47D cells is consistent with the fact that removal of effective cell cycle regulation by a mutant p53 could enhance the expression of the estrogen-like actions of tamoxifen during the development of resistance.

We have performed preliminary studies on the expression of VEGF, an angiogenesis promoter, in MCF-7 tamoxifen-stimulated tumors. In these tamoxifen-stimulated tumors, there is an increased expression of VEGF compared with that in MCF-7 estrogen-stimulated tumors, which supports the fact that tamoxifen-stimulated growth may be mediated by increased VEGF expression (62). We are currently determining VEGF expression in both estrogen- and tamoxifen-stimulated T47D breast tumors.

Although T47D and MCF-7 cells have different regulatory mechanisms that control the translation of the ERα gene (35, 54), both types of tamoxifen-stimulated tumors contain measurable amounts of ERα by Western blot (14). A model of cell selection could occur that exploits the estrogen-like actions of tamoxifen at ERα (63, 64). Recent studies have illustrated the promiscuous nature of the ERα-tamoxifen complex at complex gene targets based on the actual shape of the complex (65). Other antiestrogens, however, present different external surfaces (66), so that there is an opportunity to design new antiestrogens that do not exhibit early drug resistance and are not cross-resistant with tamoxifen. In conclusion, the MCF-7:Tam2 and T47D:Tam tumors will be valuable not only in evaluating new antiestrogens for potential clinical use but also in understanding the molecular mechanism of drug resistance in vivo.

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REFERENCES


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