Development of Tamoxifen-stimulated Growth of MCF-7 Tumors in Athymic Mice after Long-Term Antiestrogen Administration

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ABSTRACT

Long-term tamoxifen (TAM) therapy was examined in athymic mice bearing MCF-7 tumors of different sizes. Six months of TAM treatment caused no increase in tumor size (compared to placebo treatment) for animals treated initially following implantation of tumor pieces of approximately 1 mm³ or for animals with 0.2-cm² tumors (grown with 1 month of estradiol treatment). Tumors could be regrown with estradiol treatment in animals treated with either therapy and these tumors contained both estrogen and progesterone receptors. However, more tumors could be restimulated with estradiol following pretreatment with TAM than with placebo. A third group of animals had larger tumors (grown with 7 weeks of estrogen treatment to a ~0.6-cm³ area) before TAM or placebo treatment. These tumors partially regressed after 4 months of TAM or placebo therapy but began to regrow in both groups until the end of the experiment at 8 months. Tumors that grew in both groups were estrogen receptor positive and when retransplanted into athymic animals could grow with estradiol. However, the tumor that grew during TAM therapy, when retransplanted, could grow successfully only with further TAM treatment. Tumors growing with TAM contained double the estrogen receptor content of the estradiol-stimulated MCF-7 tumors that were not exposed to TAM [390 ± 37 (SE) fmol/mg protein versus 174 ± 14 (SE) fmol/mg protein]. These results may represent a form of TAM resistance, i.e., TAM dependence that may occur before hormone independecne is exhibited.

INTRODUCTION

Tamoxifen, a nonsteroidal antiestrogen (see Ref. 1 for a review), is the first-line endocrine therapy for the treatment of advanced breast cancer. Laboratory studies have demonstrated that TAM is a tumorstatic agent (2–7), and it is proving to be of value for long-term chemosuppressive adjuvant therapy in patients with Stage II breast cancer (8–11).

Current treatment strategies are aimed at maintaining control of hormone-dependent tumor growth; however, the therapy can be expected ultimately to fail with the development of tumor resistance.

The aim of this study was to provoke the failure of long-term tamoxifen therapy in a laboratory model in vivo. Antiestrogens can inhibit estradiol-stimulated growth of breast cancer cell lines transplanted into athymic mice (6, 7, 12, 13). However, Osborne et al. (14) have recently demonstrated that long-term tamoxifen therapy cannot continue to control MCF-7 tumor growth. The present study, conducted in parallel with Osborne’s study, compliments and extends his findings by an investigation of estradiol withdrawal and long-term tamoxifen therapy on the growth of different sizes of MCF-7 tumors in athymic mice.

MATERIALS AND METHODS

The breast cancer cell line MCF-7, was obtained from ATCC, Rockville, MD. These cells were karyotyped by Dr. Lorraine Meisner at the cytogenetics laboratory of the State of Wisconsin Hygiene Laboratory and shown to be authentic MCF-7 cells (data not shown). The MCF-7 tumor used in these experiments was derived from an inoculation of 10³ MCF-7 cells into estrogenized athymic mice as described previously (7). Ovariectomized BALB/c 4–5-week-old athymic mice were implanted s.c. in the axillary mammary fat pads with 1-mm³ pieces of MCF-7 tumor. This tumor had been passaged previously in vivo four times in estrogenized animals. Estrogen stimulation of this tumor was consistent over these passages in vivo and no growth was observed with placebo or TAM administration (data not shown).

Hormone Treatments. Estradiol and TAM pellets were custom made (Innovative Research of America; Toledo, OH) and implanted s.c. on the backs of animals via trocar on the same day of tumor transplantation. Estradiol pellets were replaced every 8 weeks and contained 1.7 mg of 17β-estradiol. TAM-free base pellets containing 5 mg of drug were replaced every 4 weeks in all experiments. TAM-free base was a gift from Stuart Pharmaceuticals, Wilmington, DE. Tamoxifen administered at this dose has been shown previously to inhibit estrogen-stimulated growth of MCF-7 tumors in athymic mice (7).

Tumor Measurements. Tumor measurements were performed weekly using Vernier calipers. Tumor area was calculated using the formula

\[
\frac{1}{2} \times w \times l
\]

Solid tumors were confirmed to be adenocarcinomas by histological examination.

Tumor Receptor Measurements. MCF-7 tumors were excised from animals and stored in liquid nitrogen until assay for hormone receptors. ER was measured by using a commercially available monoclonal antibody kit (ER-EIA) from Abbott Laboratories, North Chicago, IL. Procedures followed recommended kit protocol except that 0.4 mM KCl (Sigma; St. Louis, MO) was added to the homogenization buffer. The addition of KCl has previously been shown not to affect ER-EIA values measured by this kit (15).

PR levels were determined from tumor tissues homogenized in non-KCl-containing ER-EIA buffer with 10% glycerol added. Specific ligand receptor binding was done using a Rainen PR assay kit (New England Nuclear, Boston, MA). This kit used [³H]-17α-methyl-R5020 (91 Ci/mmol) with and without a 100 times excess of unlabeled R5020 incubated with tumor cytosols overnight at 4°C. Free and bound ligand were separated using a dextran-coated charcoal separation method. PR values were quantitated using Scatchard analysis.

Single-point binding analysis of ER was done on some tumors using the same competitive ligand binding assays as for PR except that a saturating dose of 6 nM [³H]-17β-estradiol was used. Protein values were determined in cytosols using a modified Bradford protein assay (16) with a commercially prepared Coomassie blue protein dye reagent (Bio-Rad, Richmond, CA) against a goat IgG standard (Sigma).

Statistical Analysis. Differences in mean tumor area and receptor measurements between groups were measured using analysis of variance followed by unpaired Student's t test.
RESULTS

Tamoxifen Inhibition of Estrogen-stimulated MCF-7 Tumor Growth. Ovariectomized animals implanted with MCF-7 tumors showed an expected growth stimulation with estradiol pellet supplementation (Fig. 1). However, no visible growth was seen after 2 months of TAM therapy. Removal of the TAM pellets enabled the tumors to be stimulated to grow with estradiol, but an inhibition of estrogen-stimulated growth was apparent when TAM therapy was continued. Thus, TAM demonstrated antiestrogenic effects on short-term administration.

Effects of Long-Term Tamoxifen Therapy on Different MCF-7 Tumor Sizes. In these experiments, the effect of long-term TAM was evaluated for different sizes of MCF-7 tumors. Ovariectomized athymic animals were treated with estradiol pellets at the time of implantation for 0, 4, and 7 weeks. After the removal of estradiol pellets, animals were treated with TAM or placebo pellets for >6 months at (a) the initial implantation size, (b) =0.2-cm² or (c) =0.6-cm² tumor size.

No growth of MCF-7 tumors was achieved in animals treated immediately with TAM or placebo pellets for 6 months of therapy (Fig. 2). After 6 months of therapy, the addition of estradiol pellets to all animals caused regrowth of some tumors. No significant difference in tumor size was seen after 8 weeks of estradiol stimulation between tumors treated with placebo or TAM. However, only 3 of a possible 22 tumors regrew to a measurable size in placebo-treated animals compared to 12 of 14 possible tumors from animals pretreated with TAM.

A partial regression of the 0.2-cm² tumor size was observed in animals treated with TAM or placebo for 6 months (Fig. 3). There was no significant difference in average tumor size between groups after the 6 months of therapy. The addition of estradiol to all animals caused a regrowth of 11 of a possible 19 tumors in placebo-treated animals and 18 of 19 tumors in TAM-treated animals. Again, as with tumors regrown with estradiol (Fig. 2), no difference in tumor size was observed after 8 weeks of regrowth (Fig. 3). The tumors in both groups treated with TAM showed a larger percentage of regrowth with estradiol. Additionally, ER and PR were measured in these tumors after 8 weeks of estradiol stimulation (Table 1).

Although ER levels were higher in TAM-treated tumors than in placebo-treated tumors, these were not significantly different. PR levels in these tumors were significantly higher in TAM-treated tumors (P < 0.03) than in placebo-treated tumors. ER and PR levels were comparable to levels seen in MCF-7 tumors grown after 8 weeks with the same dose of estradiol (118 fmol/mg cytosol protein).

Interestingly, the uterine wet weights of all animals treated with TAM showed no dramatic increase in weight (compared to placebo-treated animals; P < 0.001) after all groups were treated with estradiol (Table 1). Prior treatment with TAM appeared to make the uterus refractory to further estradiol stimulation.

When MCF-7 tumors were grown to approximately 0.6-cm² after 7 weeks of estradiol and then treated with TAM or placebo, a reduction of tumor size was again seen as in the smaller =0.2-cm² tumors. Both TAM- and placebo-treated groups showed a 58 and 46% reduction in tumor size, respectively, after 4 months of therapy (Fig. 4). After 6 months of treatment, tumors began to regrow in both groups and tumors were significantly larger in TAM-treated groups compared to control (P < 0.05).

ER was measured in all tumors by two methods. Unoccupied ER was measured using a single saturating dose of [³H]estradiol with and without a 100-fold excess of cold ligand on tumor cytosol homogenized in 10 mM Tris buffer (see “Materials and Methods”). Total receptor was measured using a monoclonal antibody (ER-EIA) assay with cytosol from tumors homogenized in the Tris buffer containing 0.4 M KCl buffer.
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Table 1 Steroid receptor values (fmol/mg cytosol protein) of MCF-7 tumors treated with placebo or tamoxifen pellets for 6 months and then restimulated with estradiol pellets

<table>
<thead>
<tr>
<th>Tumor area size</th>
<th>Treatment group</th>
<th>Estrogen receptor by ER-EIA</th>
<th>Progesterone receptor</th>
<th>Uterine wet wt (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Implantation size</td>
<td>Placebo 6 mo → E₂ 8 wk</td>
<td>96 ± 8 (3)²</td>
<td>85 ± 12 (3)</td>
<td>146.9 ± 7.0</td>
</tr>
<tr>
<td>TAM 6 mo → E₂ 8 wk</td>
<td>132 ± 15 (12)</td>
<td>140 ± 19 (10)</td>
<td>29.4 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>Approximately 0.2 cm²</td>
<td>Placebo 6 mo → E₂ 8 wk</td>
<td>98 ± 18 (8)</td>
<td>61 ± 12 (8)</td>
<td>105.2 ± 4.1</td>
</tr>
<tr>
<td>TAM 6 mo → E₂ 8 wk</td>
<td>128 ± 10 (17)</td>
<td>158 ± 17 (17)</td>
<td>18.9 ± 2.2</td>
<td></td>
</tr>
</tbody>
</table>

* Progesterone receptor levels were significantly different in all TAM-treated groups from placebo-treated groups (P < 0.03).
* Uterine wet weights were significantly different from placebo-treated groups in all TAM-treated groups (P < 0.001).
* Mean ± SE.
* Numbers in parentheses, number of observations.

As expected in TAM-treated tumors, an average of less than 10 fmol/mg of cytosol protein of ER was measured by ligand-binding assays compared to 145 fmol/mg cytosol protein measured by ER-EIA (P < 0.001) (Table 2). Thus, it appears that the majority of receptors in TAM-treated tumors is occupied. By comparison, a larger amount of unoccupied ER is measured in placebo-treated tumors using the radioactive ligand binding assay (45 fmol/mg cytosol protein). The amount measured by ER-EIA was 91 fmol/mg cytosol protein. Therefore, despite regrowth during TAM and placebo therapy, these tumors still maintain their estrogen receptor content. PR was also measured in both tumor groups but there appeared to be no significant stimulation of receptor in TAM-treated tumors compared to control (Table 2). These levels were also much lower than those seen in MCF-7 tumors restimulated with estradiol after tamoxifen or placebo treatment.

Interestingly, TAM caused no increase in uterine wet weight after 7 months of therapy alone (Table 2). The average weights were also similar to the uterine weights of animals treated with TAM and refractory to estradiol stimulation.

Retransplantation of MCF-7 Tumors That Grew during Tamoxifen or Placebo Therapy. One tumor from each group which began to regrow during TAM or placebo therapy in the previous experiment (Fig. 4) was excised for retransplantation. The individual growth rates of these tumors were similar (Fig. 5). The TAM-treated tumor is designated T66, while the placebo-treated tumor is designated C72. Both tumors were transplanted into ovariectomized animals to investigate their growth characteristics with estradiol and TAM.

In the case of animals treated with estradiol, both C72 and T66 MCF-7 variants were stimulated to grow and were not significantly different in size after 8 weeks. Furthermore, TAM inhibited the estradiol-stimulated growth (Fig. 6A). However, when placebo or TAM alone was given to other animals (Fig. 6B), no growth was seen in C72 tumor implants. In contrast, sustained growth was eventually seen in TAM-treated animals bearing T66 tumor implants (10 of 12 possible) and slight growth was seen in placebo animals (3 of 12 possible). ER levels in C72 and T66 tumor cytosols were similar to each other in groups treated with estradiol pellets or estradiol + TAM pellets (Table 3).

However, in T66 tumors treated with TAM only, ER levels were 2-fold higher than estradiol-treated tumors. PR levels also

Fig. 4. Mean MCF-7 tumor growth (bars, SE) in animals treated with estradiol pellets for 7 weeks. Animals had estrogen removed (arrow) and were randomized into groups treated with placebo pellets (n = 10) (Δ) or TAM pellets (n = 12) (●) for 7 months.

Fig. 5. Growth of two individual MCF-7 tumors from Fig. 4. Long-term TAM-treated tumor T66, □; placebo-treated tumor C72, △.
were lower in this TAM-treated tumor compared to the estradiol-treated tumor (P < 0.04) (Table 3).

The T66 MCF-7 tumor was retransplanted again and growth was retained in TAM-treated animals. In animals treated with placebo, little or no growth was observed. Steroid receptor levels were also similar to those seen previously in this variant (data not shown).

DISCUSSION

Although improvement in patient survival has been observed in long-term TAM clinical breast trials, there is still a population of patients who eventually fail therapy (8–11). In this paper we have attempted to develop a possible laboratory model for this type of endocrine therapy failure using the MCF-7 breast cancer cell line grown in athymic mice. TAM-stimulated growth has been reported previously in a hormone-sensitive human endometrial tumor (EnCa101) by Clarke and Satyaswaroop (17). This EnCa101 tumor can be stimulated to grow with TAM alone in athymic mice. Paradoxically, endometrial carcinoma, like breast cancer, is treated successfully in some instances using TAM therapy (18–20).

One possibility for this phenomenon may be the weak estrogenic properties of TAM. Indeed work done in vitro by Berthois et al. (21) has shown that MCF-7 cells grown without the presence of the weak estrogen, phenol red, can be stimulated to grow with low concentrations of antiestrogens. Osborne et al. (6), in an earlier report, has also demonstrated some growth of MCF-7 cells in athymic mice with TAM or LY 156758 (an antiestrogen with high affinity for ER) administration alone. Clinically, some breast cancer patients experience tumor flare at the start of antiestrogen therapy (22–24). Furthermore, another report has observed tumor regression after TAM withdrawal in a patient failing therapy (25). These data alone with the partial successes seen with second-line endocrine therapies after TAM provide additional evidence of some form of TAM resistance that retains endocrine responsiveness (26–28).

In these experiments, we have observed that the inability of TAM therapy to prevent tumor growth in athymic mice may be a function of the tumor burden. Only in the larger (~0.6 cm³) MCF-7 tumors was a sustained growth seen in the presence of TAM therapy (Fig. 4). Our results have been confirmed again in a subsequent experiment (data not shown) and are in agreement with another report of MCF-7 TAM-stimulated growth (14). Although we have not observed sustained growth in smaller tumors with TAM, this may be a function of the variability seen with wild-type MCF-7 cells. Variability between ER content and tumorigenicity has been seen in MCF-7 cells from different laboratories (29).

Interestingly, a greater percentage of MCF-7 tumors could be restimulated to grow with estradiol treatment after pretreatment with TAM rather than placebo. This increased tumor viability may be a function of the estrogenic effects of TAM. Indeed TAM has been shown, like estrogen, to decrease some immune functions in mice after long-term administration (30).

Tumors treated with long-term TAM have ER levels which are similar to those treated with placebo therapy. Tumors restimulated with estradiol supplementation after either therapy also have similar ER levels. However, PR levels were elevated in those tumors treated with estradiol compared to those treated with TAM or placebo alone. It is curious, however, that although the ER receptor seems to be occupied when comparing ER-EIA levels to ER levels measured by the traditional ligand-binding assay there is no stimulation of PR in tamoxifen-treated animals.

In contrast to the estrogenic effects on TAM-treated tumors, mice treated with TAM for 7 months had uteri which were unstimulated compared to placebo-treated animals. TAM has been traditionally thought to act as an estrogen in the mouse uterus (31). An estrogenic effect on the uterus has been observed in our laboratory after 2 months of treatment with the dose of TAM used in this experiment (31). However, treating the mouse uterus for 6 months with TAM caused it to be refractory to estradiol stimulation (Table 1). Thus the estrogenic effects of TAM on the mouse uterus are not apparent after long-term administration.

Retransplantation of tumors that grew during TAM or placebo therapy constituted the last set of experiments. A tumor growing during TAM therapy (T66) was retransplanted into athymic mice and showed that it still required estradiol supplementation for growth. Also TAM could inhibit this estradiol-
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stimulated growth as previously noted in the literature (6, 32). A tumor growing during placebo treatment (C72) also behaved in a similar manner (Fig. 5). However, in tumors treated with TAM alone, only the T66 MCF-7 variant showed continued growth during placebo treatment (C72) also behaved as previously noted in the literature (6, 32).

In placebo-treated animals, there was some slight growth in 3 of 12 tumors, but this growth was not sustained (Fig. 6).

ER levels in these tumors treated with estradiol did not vary markedly with other estradiol-stimulated MCF-7 tumors; however, in the TAM-stimulated MCF-7 tumors, ER levels were doubled (Table 3).

We intend to define this MCF-7 variant further in vivo and to determine if other less estrogenic antiestrogens can inhibit TAM-stimulated growth. Experiments have been initiated to culture these tumor cells and to investigate whether their characteristics have changed in vitro from the parent cell line.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Lorraine Meisner of the State Hygiene Lab of Wisconsin for her cytogenetic analysis of our MCF-7 cells and Dr. Kennedy Gilchrist for undertaking a pathological examination of MCF-7 tumors. Thanks are due to Kathryn Edge for her expert technical typing.

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