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Reduced tamoxifen accumulation is not associated with stimulated growth in tamoxifen resistance

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Abstract To study tamoxifen resistance-stimulated growth, 30 female ovariectomized nude mice were implanted with tamoxifen-resistant tumors and treated with 10–1000 μg/day of tamoxifen citrate subcutaneously. Tamoxifen stimulated MCF-7 tumor growth in a dose-dependent manner, with tumoral tamoxifen concentrations increasing proportionally to the dose (1–13 nmol/g), as measured by high-performance liquid chromatography (HPLC). Flow-cytometric analysis revealed that tamoxifen-resistant tumors had a different DNA content as compared with wild-type MCF-7 cells. In contrast to earlier results, these data suggest that tamoxifen resistance-stimulated growth is associated with increasing rather than decreasing tumoral tamoxifen concentrations. Furthermore, the observed ploidy changes in the tamoxifen-resistant tumors imply that a genetic basis may exist for the development of tamoxifen resistance.

Key words Breast Cancer · Tamoxifen · Resistance

Introduction

Acquired tamoxifen resistance is a problem that will develop in virtually all breast cancer patients receiving tamoxifen [19]. Preclinical and clinical studies indicate that breast tumors not only become resistant to tamoxifen but are also stimulated to grow by the drug [3–5, 9–12, 14, 16, 17]. Potential mechanisms of acquired tamoxifen resistance include alterations in estrogen receptor (ER) status or levels of the receptor and enhanced biologic mechanisms for circumvention of tamoxifen cytotoxicity such as growth factors and antiestrogen-binding sites [19]. It has also been proposed that a reduced tumoral concentration of the parent drug and/or alterations in the relative amounts of metabolites would lead to acquired tamoxifen resistance and/or tamoxifen-stimulated growth [12].

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Materials and methods

Nude-mouse study

The tamoxifen-resistant MCF-7 tumors were maintained in nude mice given 500 μg tamoxifen citrate subcutaneously on a daily basis. The tamoxifen-resistant tumors were excised and serially transplanted as previously described [11, 12] into 30 female ovariectomized BALB-C/nu/nu nude mice purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, Ind.). The animals were divided into six groups of five mice each. Each mouse received only one implant, and all implants were placed in the right scapular area. The control group received the vehicle only, whereas the treatment groups received either 10, 100, 250, 500, or 1000 μg tamoxifen citrate. On day 1 after transplantation, subcutaneous treatment with tamoxifen citrate in peanut oil was instituted, and after 7 weeks of daily administration the animals were euthanized by cervical dislocation. The tumor sizes (length × width) were measured with a ruler on days 18, 25, 32, 40, and 48 after transplantation.

Tamoxifen concentration

In all, 22 of the MCF-7 tumors (5 each from the 10- and 1000-μg treatment groups and 4 each from the 100-, 250-, and 500-μg treatment groups) were analyzed for tamoxifen concentration using high-performance liquid chromatography (HPLC) as previously described [18].

Northern-blot analysis

Total cellular RNA was prepared from the MCF-7 wild-type cells and tamoxifen-resistant MCF-7 tumors as described by Chomczynski and Sacchi [2]. Agarose gel electrophoresis was used to separate aliquots of 10 μg/sample, which were then transferred to a reinforced nitrocellulose membrane. Hybridization with a [^{32}P]-deoxycytidine triphosphate-labeled *mdr1* cDNA probe (generously provided by Dr. M. Gottesman)

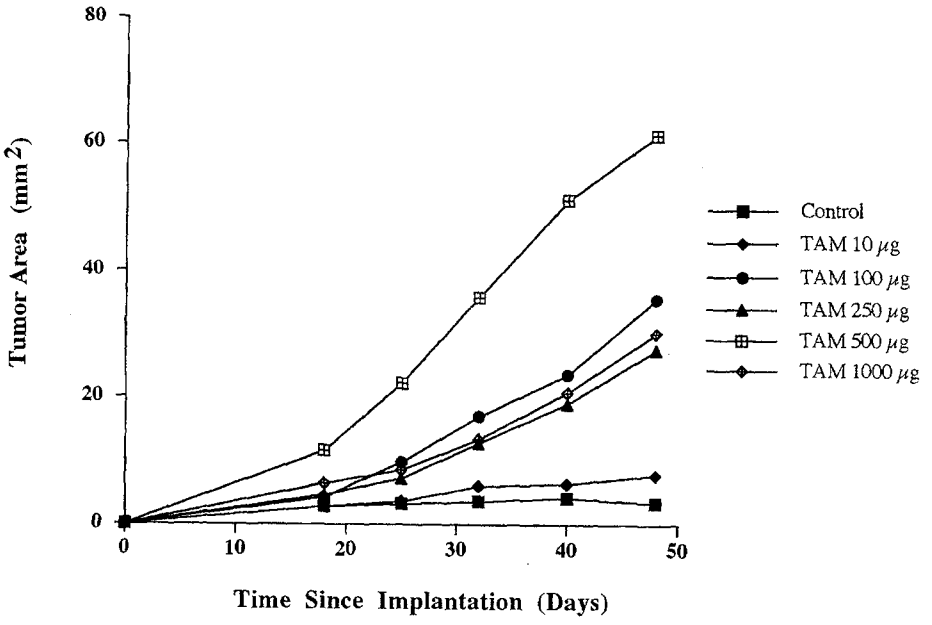


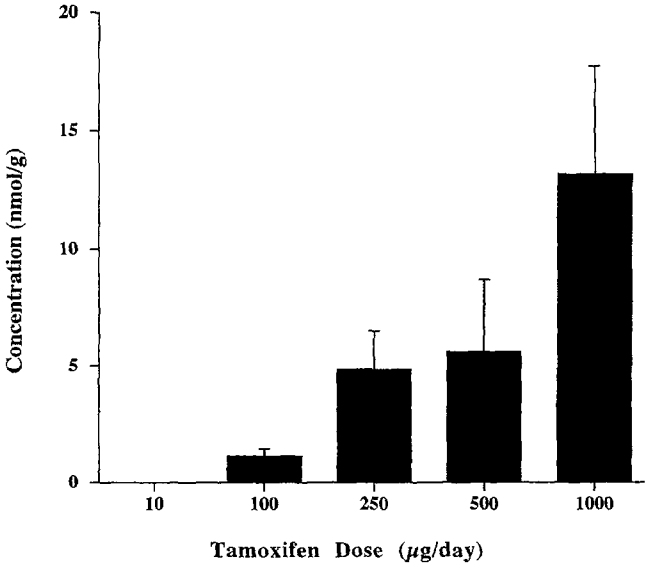
Fig. 1 Dose-dependent growth of MCF-7 tamoxifen-resistant transplant tumors in 30 female nude mice following daily administration of tamoxifen citrate for 7 weeks. Control vs 10 μg, $P<0.25$; vs 100 μg, $P<0.01$; vs 250 μg, $P<0.01$; vs 500 μg, $P<0.0005$; vs 1000 μg, $P<0.005$ (Student's one-tailed *t*-test)

was performed under appropriate conditions. The blots were washed and exposed to Kodak X-OMAT AR film at −70° C. Loading of mRNA was assessed by staining with ethidium bromide.

Flow cytometry

Cells were stained using a modified Krishan technique [8] and processed as described previously [1]. An EPICS 753 instrument (Coulter Cytometry, Hialeah, Fla.) was used to perform the flow-cytometric measurements, and compartmental analysis of DNA histograms was accomplished with MODFIT software (Verity Software House, Inc., Topsham, Me.). The flow cytometry was performed on tamoxifen-resistant MCF-7 tumors and on passage 35 of cells cloned from a tamoxifen-resistant MCF-7 tumor two transplant generations earlier. The cloned cells had been kept under tamoxifen pressure (2 μ*M*) until passage 32. The DNA content of tamoxifen-resistant tumors was compared with that of MCF-7 wild-type cells of passage 216.

Fig. 2 Dose-dependent cellular accumulation of tamoxifen in MCF-7 tamoxifen-resistant transplant tumors following daily administration for 7 weeks



Results

Tamoxifen stimulated the MCF-7 tamoxifen-resistant tumors to grow in a dose-dependent fashion up to 500 μg a day (Fig. 1). The lowest dose (10 μg/day) had no noticeable effect on tumor growth, whereas the 100-μg dose produced a stimulation that was statistically significant ($P<0.01$) as compared with the control value. The stimulatory effect began to taper off at the highest dose (1000 μg), indicating that an inhibitory concentration of tamoxifen was being approached.

The concentration of tamoxifen (ranging from 1 to 13 nmol/g) detected in the tumor tissue increased in direct relation to the dose (Fig. 2). Serum concentrations of tamoxifen ranged from 0 to 0.31 μ*M* at the 10–1000-μg/day dose levels. The mean serum concentration for the 500-μg/day dose that produced the largest tumors was 0.09 μ*M*. The mean (± SD) ratio of *cis*/*trans*-4-hydroxytamoxifen was 1.21 ± 0.07, 1.11 ± 0.03, and 0.74 ± 0.09 in the groups receiving 250, 500, and 1000 μg tamoxifen citrate, respectively. The concentration of *cis*- and *trans*-4-hydroxytamoxifen measured in the groups ranged from undetectable to 0.003 μ*M*.

Tamoxifen-resistant tumors and cells had a different DNA content as compared with wild-type MCF-7 cells (Fig. 3). It appears that the new clones selected by tamoxifen are not stable. These apparent genetic changes may be associated with the development of acquired tamoxifen resistance. Accordingly, the Northern-blot analysis showed that tamoxifen-resistant tumors, like wild-type MCF-7 cells, were negative for *mdr1* gene expression.

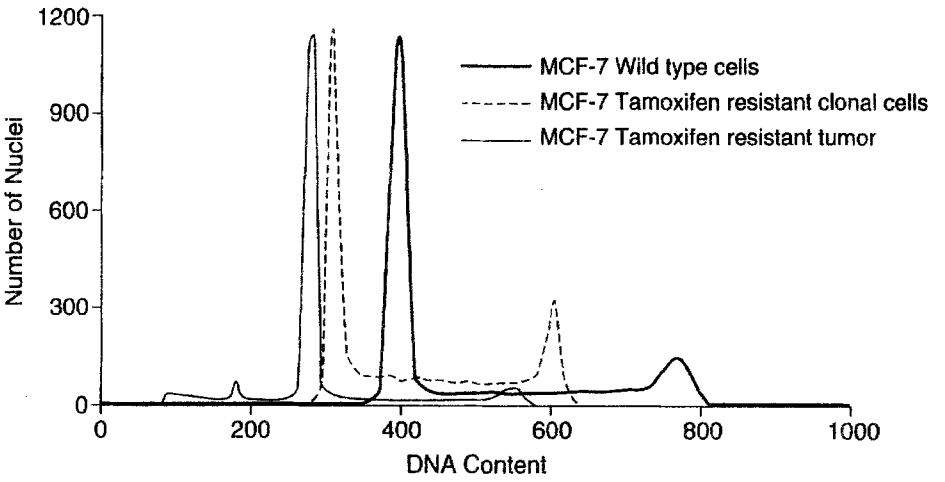


Fig. 3 Superimposed DNA histograms of MCF-7 wild-type cells, MCF-7 tamoxifen-resistant tumor cells, and MCF-7 tamoxifen-resistant cells derived from a MCF-7 tumor two transplant generations previously. The small peak to the *left* of the tumor cells is a reflection of mouse cells surrounding the tumor. The numbers on the *x*- and *y*-axis represent the relative amount of DNA (190 = diploid) and number of nuclei, respectively

Discussion

In a previous study, we reported that MCF-7 tamoxifen-resistant tumors retain functional ER and are stimulated to grow with daily 500-μg doses of tamoxifen citrate for 4–6 months [12]. We speculated that tamoxifen-stimulated growth in this model is associated with reduced cellular accumulation of tamoxifen and with a shift of cellular metabolism of tamoxifen toward *cis* isomers that are less antiestrogenic than the *trans* isomers [12]. However, the present study shows that stimulation of growth in tamoxifen-resistant tumors is dose-dependent. Furthermore, the increasing tumoral tamoxifen concentrations seen with increasing doses produced larger tumors. The *cis/trans*-4-hydroxytamoxifen ratios either appeared to be unaffected or showed a diminishing trend despite increasing doses and tumor growth, thus potentially ruling out their direct role in tamoxifen-stimulated growth in tamoxifen-resistant tumors.

In the previous study, tamoxifen tumoral concentrations between sensitive and resistant tumors were compared [12]. The seemingly higher concentrations observed in the sensitive tumors may have been an artifact of the study design. In fact, the present study suggests that the serum concentrations were high for tamoxifen-resistant and -sensitive groups. We have no explanation for this difference. However, the comparison in the previous study between treatment groups was made following 2 weeks (sensitive stage) and 4–6 months (resistant stage) of tamoxifen therapy. The nude-mouse model utilized in this study required daily 50-μl subcutaneous peanut oil injections 5 days a week for 4–6 months. Thus, the total amount of peanut oil injected subcutaneously into each animal is 4–6 ml, causing tissue irritation, scarring, and incomplete absorption as evidenced by HPLC analysis of peanut oil reservoirs (data not shown).

Recently, Johnston et al. [7] published data in favor of the role of reduced accumulation. Patients with acquired tamoxifen resistance had lower tumoral tamoxifen concentrations than did patients with de novo resistance. However, the comparison was made between two forms of tamoxifen resistance, such that conclusions regarding the development of acquired tamoxifen resistance cannot be made. Moreover, the well-known withdrawal responses seen in some patients with acquired tamoxifen resistance [14, 17] are difficult to explain on the basis of reduced tamoxifen tumoral levels. In agreement with the present results, Jordan's group [5] has shown a dose-dependent stimulatory effect of increasing doses of tamoxifen in another tamoxifen-resistant MCF-7 model. Moreover, these investigators recently published data implying that altered metabolism of tamoxifen is not the cause of tamoxifen-stimulated growth [20].

The increased accumulation of tamoxifen seen in the present study is in accordance with the observation that tamoxifen-resistant cells were negative for the *mdr1* gene. The *mdr1* gene codes for P-glycoprotein, a cell-membrane protein responsible for the most well-known form of drug resistance, multidrug resistance (MDR) [13]. P-glycoprotein functions as an active efflux pump, and tamoxifen is one of the substrates of P-glycoprotein [15]. We have previously shown a reduced accumulation of tamoxifen in ER-negative MDA-MB-231-A1 human breast-cancer cells that are multidrug-resistant and express the *mdr1* gene [9].

Clonal selection by tamoxifen has previously been shown in breast-cancer cell lines [6, 16], which has resulted in aggressive tumor growth when these cells are implanted in nude mice [10, 16]. In the present study, the significance of the observed ploidy change is unknown; however, our data suggest that tamoxifen resistance and/or tamoxifen-stimulated growth may be associated with genetic changes following tamoxifen therapy.

In conclusion, tamoxifen stimulates tamoxifen-resistant MCF-7 tumor growth in a dose-dependent manner, seemingly by a nonhormonal mechanism. In contrast to the previous study [12], we now believe that tamoxifen-stimulated growth is related to increasing rather than decreasing tamoxifen concentrations in the MCF-7 nude-mouse models of tamoxifen resistance.

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