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Short communication

Tamoxifen stimulates in vivo growth of drug-resistant estrogen receptor-negative breast cancer

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Abstract. An estrogen receptor-negative, multidrug-resistant MDA-MB-A1 human breast cancer cell line was grown in culture with and without a noninhibitory concentration (0.5 μ*M*) of tamoxifen for 122 days. Tamoxifen-treated and control cells were inoculated into opposite flanks of nine nude mice, where they produced measurable tumors in every case. Six of the animals were treated with tamoxifen at 500 μg/day for 22 days. Although no inhibitory nor stimulatory effect of tamoxifen was seen in vitro, tamoxifen had a clear tumor-growth-stimulating effect in mice. The most pronounced stimulatory effects were observed in the cells that had been cultured with tamoxifen. Within 3 weeks of the start of tamoxifen therapy, the cells grown in the presence of tamoxifen produced tumors with a mean size of 380 mm^2, whereas the cells not pretreated with tamoxifen had tumors of 220 mm^2. In contrast, in mice not receiving tamoxifen, the sizes of the tumors were 190 and 140 mm^2, respectively. These preliminary results suggest that prolonged in vitro tamoxifen exposure induces cellular changes that result in tumors that are stimulated to grow faster in mice following tamoxifen treatment.

Introduction

Tamoxifen is a triphenylethylene antiestrogen that has widely been used in the hormonal treatment of breast cancer. In estrogen receptor (ER)-positive breast cancers, it produces an objective response rate of about 50% [17]. Even 10% of ER-negative breast cancers will respond to tamoxifen [9]. Unfortunately, virtually all tumors will ultimately develop resistance to tamoxifen [12]. Moreover, studies on rats have indicated that tamoxifen may in fact induce aggressive, hormone-independent mammary tumors to grow [7, 14, 18]. A variant of the human mammary cancer cell line MCF-7 has also been reported to stimulated by tamoxifen [10]. Tamoxifen has even b found to induce new primary cancers in the liver [15] a in the endometrium [1, 8, 11]. The aim of this pilot st was to investigate the effect of long-term tamoxifen ex sure on an ER-negative, multidrug-resistant (MDR) hun mammary cancer cell line transplanted into nude m treated with tamoxifen.

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Materials and methods

Cell lines. The human ER-negative MDR-positive MDA-MB-A1 br cancer cell line was obtained from the late Dr. William McGuire. MDA-MB-A1 cell line was developed by keeping the cells at continu doxorubicin pressure at a concentration of 2 μg/ml. The cells were gr in Improved Minimum Essential Medium (IMEM) supplemented 10% fetal bovine serum (FBS) and 10^{-9} *M* insulin in Corning T culture flasks and were maintained in 5% CO_2 and 95% air at 37°C. C were exposed for 122 days to 0.5 μ*M* tamoxifen. Control cells were grown and maintained in the same manner.

Tamoxifen accumulation. Tamoxifen was quantified in the cells u high-performance liquid chromatography (HPLC) as previously scribed [6]. The results were expressed as the area under the concen tion curve (AUC) in micromoles per 10^6 cells per day. The equipm used consisted of a Beckman model 320 gradient liquid chromatogr (Fullerton, Calif.), two model 110A pumps, a model 420 controller, a Spectraphysics 4100 integrator (Piscataway, N.J.).

Flow cytometry. Cells were stained with a modified Krishan techni [13], after which they were processed as previously described [2]. F cytometric measurements were performed on an EPICS 753 instrum (Coulter Cytometry, Hialeh, Fla.), and compartmental analysis of D histograms was accomplished with MODFIT software (Verity Softw House, Inc., Topsham, Me.).

Northern-blot analysis. Total cellular RNA was prepared from the tured cells and tumor xenografts by a single-step method descri previously [3]. Aliquots of 10 μg/sample were separated by agarose electrophoresis and transferred to a reinforced nitrocellulose membr Hybridization with a [^{32}P]-deoxycytidine triphosphate-labeled *m* cDNA probe (generously provided by Dr. M. Gottesman) was perfor under appropriate conditions. The *mdr1* probe was then removed f the membrane by boiling for 30 min in 1 mmol ethylen

Table 1. Doubling times and accumulation of tamoxifen (TAM) to the MDA-MB-A1 and MDA-MB-231 cell lines

Cell line	Doubling time (h)	Accumulation of TAM (AUC, μmol 10^{-6} cells day^{-1})			
		AUC	Passages	AUC	Passages
MDA-MB-A1:					
TAM 0.5 μ*M*	31.61±5.52	11.07	(11–17)	36.79	(11–26)
Control	25.73±5.72	–		–	
MDA-MB-231:					
TAM 0.5 μ*M*	23.17±2.13	17.56	(13–20)	50.50	(12–31)
Control	21.05±2.41	–		–	

aminetetraacetic acid (EDTA)/l and 1% sodium dodecyl sulfate (SDS). The membrane was then reprobed with a radiolabeled cDNA probe for beta-actin so as to correct for variability in RNA loading.

Animal study. Nine female 4- to 5-week-old BALB-C/nu/nu athymic nude mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, Ind.). The MDA-MB-A1 cells were resuspended in sterile saline at a density of 3 million (200 μl). The cells (3×10^6) were injected subcutaneously on opposite sides of the same animal using a 20-gauge needle (in the left scapular area, control cells cultured without tamoxifen; in the right side, cells pretreated with tamoxifen). The tumors were allowed to grow for 3 weeks. Six mice were treated with 500 μg tamoxifen s.c. on a daily basis, whereas the remaining three animals were not treated. The tumor sizes (length × width) were measured with a ruler on days 7, 11, 18, and 22 after the institution of tamoxifen therapy and were expressed in square millimeters. Tamoxifen treatment was stopped at day 22, and the animals were sacrificed 2 weeks later.

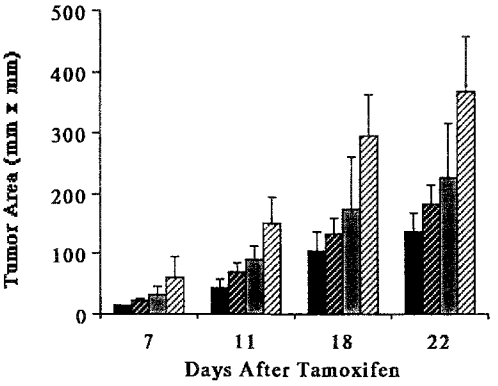

Fig. 1. Growth of the tumors produced by inoculation of MDA-MB-A1 cells into 9 nude mice. Tamoxifen-pretreated cells and nonpretreated cells were inoculated into the opposite flanks of each animal. *Black bars*, No tamoxifen in culture, no tamoxifen to mice ($n = 3$); *dark-striped bars*, tamoxifen in culture, no tamoxifen to mice ($n = 3$); *light bars*, no tamoxifen in culture, mice treated with tamoxifen ($n = 6$); *light-striped bars*, tamoxifen in culture, mice treated with tamoxifen ($n = 6$).

Results

The cellular accumulation of tamoxifen in MDA-MB-A1 cells and their doubling times are given in Table 1. For comparison, the wild-type MDA-MB-231 cell line was exposed to tamoxifen under the same conditions and the results are also shown in Table 1. The cellular accumulation of tamoxifen was about 30% lower in the MDA-MB-A1 cell line, and the MDA-MB-A1 cells appeared to grow at a slightly slower rate than the wild-type cells.

Cell-cycle measurements showed no effect of tamoxifen on the kinetics of the MDA-MB-A1 cell line as measured on days 65, 86, and 111 in culture. No blockade of the MDA-MB-A1 cells in the G_0G_1 phase or any reduction in the S-phase fraction was observed in the cells treated with tamoxifen as compared with the untreated control cells. Northern-blot analysis confirmed the expression of the *mdr1* gene at the start of the experiment. The mouse tumors, however, were found to be undetectable for the gene, indicating a loss of the MDR phenotype during culture without doxorubicin pressure.

In nude mice, the MDA-MB-A1 cells began to grow and produced tumors on both sides of every animal. Although no inhibitory or stimulatory effect of tamoxifen was seen in vitro, tamoxifen had a clear tumor-growth-stimulating effect in mice. The most pronounced growth observed following tamoxifen treatment occurred in the cells that had also been cultured with tamoxifen (Fig. 1). By 3 weeks after the start of tamoxifen therapy, the cells kept under tamoxifen in culture had produced tumors with a mean size of 380 (SD, 88.2) mm^2 and the cells not pretreated with tamoxifen had developed mean tumor areas of 220 (SD, 89.0) mm^2. In contrast, in the mice not treated with tamoxifen, the sizes of the tumors were 190 (SD, 31.5) and 140 (SD, 32.6) mm^2, respectively. After the mice had been sacrificed, the tumors were confirmed to be ER-negative by ligand binding assay.

Discussion

The results of the present study are in agreement with previous findings on the ability of tamoxifen to induce aggressive, hormone-independent tumors to grow [7, 14, 18]. The same phenomenon might theoretically happen during tamoxifen therapy of an ER-negative cytotoxic drug-resistant breast cancer. Because the tumors stimulated have been ER-negative, the induction is unlikely to be linked to a partial estrogen agonist action of tamoxifen, but rather to the oncogene/suppressor gene system.

We confirmed that the MDA-MB-A1 cell line was MDR-positive at the start of the experiment. A probable explanation for the observation that the mouse tumors did not express MDR is that the doxorubicin pressure was discontinued and the cells were devoid of the drug for 122 days prior to transplantation. It is interesting that tamoxifen accumulation was lower in MDA-MB-A1 cells than in the wild-type cells in vitro. One possible explanation would be that the MDA-MB-A1 cells express the MDR phenotype and tamoxifen is a substrate for P-glycoprotein [16]. Although Clarke et al. [4] showed no cross-resistance of ER-positive MCF-7 cells to tamoxifen after transduction with *mdr1* complementary DNA, such a link might exist in ER-negative tumor cells inherently resistant to tamoxifen.

Our observation that tamoxifen stimulates tumors derived from a MDR-positive cell line to grow raises an important consideration: should tamoxifen be given to

patients who have failed chemotherapy, particularly since MDR is more common than previously thought in breast cancer [19]? Future work is necessary to elucidate fully why tamoxifen stimulates drug-resistant and ER-negative cells to grow [5].

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