Paradoxical Action of Fulvestrant in Estradiol-Induced Regression of Tamoxifen-Stimulated Breast Cancer

Clodia Osipo, Csaba Gajdos, Hong Liu, Bin Chen, V. Craig Jordan

Background: Long-term tamoxifen treatment of breast cancer can result in tamoxifen-stimulated breast cancer, in which estrogen inhibits tumor growth after tamoxifen withdrawal. We investigated the molecular mechanism(s) of estradiol-induced tumor regression by using an in vivo model of tamoxifen-stimulated human breast cancer.

Methods: Growth of parental estradiol-stimulated MCF-7E2 and long-term tamoxifen-stimulated MCF-7TAMLT xenografts in athymic mice was measured during treatment with vehicle, estradiol, estradiol plus tamoxifen, tamoxifen alone, estradiol plus fulvestrant, or fulvestrant alone. Apoptosis was detected by the terminal deoxynucleotidyltransferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. Protein expression was assessed by western blot analysis. mRNA expression was assessed by real-time reverse transcription–polymerase chain reaction. All statistical tests were two-sided.

Results: MCF-7E2 tumor growth was stimulated by estradiol (cross-sectional area at week 13 = 1.06 cm², 95% confidence interval [CI] = 0.82 to 1.30 cm²; P < .001) compared with control (0.06 cm², 95% CI = −0.02 to 0.14 cm²), but tumor growth was inhibited by tamoxifen or fulvestrant. MCF-7TAMLT tumor growth was stimulated by tamoxifen) cross-sectional area at week 10 = 0.60 cm², 95% CI = 0.50 to 0.70 cm²; P < .001) compared with control (0.02 cm², 95% CI = 0.00 to 0.04 cm²). For MCF-7TAMLT tumors that were initially 0.35 cm², estradiol-induced regression to 0.18 cm² (95% CI = 0.15 to 0.21 cm²; P < .001), and tamoxifen or estradiol plus fulvestrant enhanced tumor growth to 1.00 cm² (95% CI = 0.88 to 1.22 cm²). Estradiol increased the number of apoptotic cells in tumors by 23% (95% CI = 20% to 26%; P < .001) compared with all other treatments, decreased estrogen receptor α (ERα) protein expression, increased the expression of Fas mRNA and protein, decreased the expression of HER2/neu mRNA and protein and nuclear factor κB (NF-κB) protein but did not affect Fas ligand protein expression compared with control. Paradoxically, fulvestrant reversed this effect and stimulated MCF-7TAMLT tumor growth apparently through ERα-mediated regulation of Fas, HER2/neu, and NF-κB. Conclusion: Physiologic levels of estradiol induced regression of tamoxifen-stimulated breast cancer tumors, apparently by inducing the death receptor Fas and suppressing the antiapoptotic/prosurvival factors NF-κB and HER2/neu. [J Natl Cancer Inst 2003;95:1597–1608]

Tamoxifen is currently used to treat all stages of estrogen receptor α (ERα)-positive breast cancer (1). Tamoxifen is also used as a chemopreventive agent for pre- and postmenopausal women at high risk for breast cancer (2,3). The benefits of tamoxifen as an adjuvant therapy continue for up to a decade after completion of 5 years of treatment (4). However, resistance to tamoxifen does occur by clonal selection of breast cancer cells that, paradoxically, grow in response to...
tamoxifen (5,6). These tumors are ERα-positive, and their growth continues to be regulated by ERα. Consequently, new endocrine therapies have now been established to inhibit growth of breast tumors before or after tamoxifen therapy has failed. These therapies include the use of aromatase inhibitors to inhibit 17β-estradiol (hereafter estradiol) synthesis (7–9) and the pure antiestrogen fulvestrant (Faslodex or ICI 182,780) (10,11) to target the ERα for ubiquitin-mediated degradation (12,13). The assumption made for using these second-line therapies is that if the formation of the estradiol–ERα complex is prevented, then breast tumors that have acquired resistance to tamoxifen will regress. These conclusions are based, in part, on laboratory models of early drug resistance to tamoxifen (1–2 years) that show that breast cancer cells in vivo grow in the presence of estrogen or tamoxifen (14–16). However, a more complex situation with estradiol has been documented for tumors after 5 years of tamoxifen treatment, that is, postmenopausal levels of estradiol inhibit the growth of tamoxifen-stimulated MCF-7 breast tumors in vivo (17,18) and estrogen-deprived MCF-7 cells in vitro (19). Thus, another hypothesis is emerging that the ERα mediates the dual actions of estradiol initially as a growth stimulator in breast cancer cells and then as a growth inhibitor in breast cancer cells after long-term therapy with tamoxifen or aromatase inhibitors. What is still unclear is the mechanism of estradiol-induced growth inhibition in breast cancer cells that are stimulated by tamoxifen or that grow spontaneously when deprived of estradiol.

Song et al. (19) showed that MCF-7 cells deprived of estrogen for up to 24 months in vitro (MCF-7LTED cells) express Fas, a member of the tumor necrosis factor α receptor family, in contrast to the parental MCF-7 cells, which do not express Fas. These authors also demonstrated that, although both parental and MCF-7LTED cells express Fas ligand (FasL), estradiol treatment increases the levels of FasL and induces apoptosis in Fas-expressing MCF-7LTED cells only.

The Fasl/Fas death receptor pathway is activated by cross-linking Fas (CD95/APO-1), a type I transmembrane protein expressed by a variety of nucleated cells, to FasL, a type II transmembrane protein expressed by activated T cells and or various other non–T cells. The cross-linking of FasL to Fas results in trimerization of Fas, activation of downstream caspases, and induction of apoptosis (20–24). The FasL gene promoter contains an estrogen response element and is regulated by estradiol (25). From these results, Song et al. (19) concluded that estrogen-induced tumor regression might result from the estrogen-mediated overexpression of FasL, which would induce Fas-mediated apoptosis in MCF-7LTED cells.

The importance of the FasL/Fas death pathway as a mediator of apoptosis is clearly demonstrated in immune surveillance (22). In breast cancer, tumor escape from immunologic surveillance results from the induction of apoptosis of Fas-bearing, activated lymphocytes by FasL-bearing breast cancer cells (22). An earlier study (24) comparing normal human breast epithelium to breast carcinomas demonstrates that FasL mRNA and protein are overexpressed in carcinomas but that the expression of Fas mRNA and protein is almost completely undetected. Thus, by increasing the expression of FasL and simultaneously decreasing that of Fas, breast tumors evade the immune response and continue to grow.

In addition to dysregulation of apoptotic signals, such as those in the Fasl/Fas pathway, breast carcinomas overexpress the prosurvival factor HER2/neu, a member of the epidermal growth factor receptor family of receptor tyrosine kinases (26,27), and the antiapoptotic nuclear factor κB (NF-κB) (28,29). In breast carcinomas, constitutively active HER2/neu and/or NF-κB appear to be critical for tumor survival and growth by blocking apoptotic signals from death receptors (28,29).

The goal of this study was to investigate the mechanism of estradiol-induced tumor regression in tamoxifen-stimulated human MCF-7TAMLt breast cancers in vivo, a model generated by treating mice carrying tamoxifen-naïve MCF-7E2 tumors with tamoxifen for more than 5 years. This model is an example of breast tumors that are resistant to long-term tamoxifen therapy and is useful for examining the effects of various second-line treatments in tumors exposed to tamoxifen for 5 years. Thus, the model could mimic a clinical situation in which 5 years of adjuvant tamoxifen therapy may result in the evolution of a tamoxifen-sensitive breast cancer to a clinically undetected tamoxifen-stimulated micrometastatic breast cancer. We investigated whether estradiol causes tumor regression by inducing apoptosis. We examined ER function by measuring levels of transforming growth factor α (TGF-α) mRNA, an estrogen-responsive gene (30,31). Expression of Fasl protein, Fas mRNA and protein, HER2/neu mRNA and protein, and NF-κB and cyclooxygenase-2 proteins was measured to determine whether the Fasl/Fas death pathway and/or the HER2/neu/NF-κB prosurvival/antiapoptotic pathways were modulated by estradiol in MCF-7TAMLt tumors.

Materials and Methods

Cell Culture

The human breast cancer cell line MCF-7 was purchased from the American Type Culture Collection (Manassas, VA) and maintained at 37 °C in a 95% humidified air/5% CO2 atmosphere in phenol red–containing RPMI-1640 medium supplemented with 10% fetal bovine serum, insulin at 0.6 μg/mL, 1% L-glutamine, 1% nonessential amino acids, and a 1% solution of penicillin, streptomycin, and an antimycotic agent (Gibco-BRL, Life Technologies, Long Island, NY).

Drug Treatments

The following standard drug dosages were used throughout this study, unless otherwise stated. Estradiol was given subcutaneously in 0.30–cm silastic capsules (Baxter Healthcare, Mundelein, IL) to achieve postmenopausal serum levels of estradiol (83.8 pg/mL in serum) (32). Tamoxifen (Sigma-Aldrich, St. Louis, MO) at 1.5 mg/day was given orally by gavage. Fulvestrant (AstraZeneca, Cheshire, U.K.) was dissolved in 100% ethanol and diluted in peanut oil; 5 mg was then injected subcutaneously twice a week.

Growth of MCF-7E2 Tumors In Vivo

The mammary fat pads of 4- to 6-week-old ovariectomized BALB/c nu/nu athymic mice (Harlan Sprague Dawley, Madison, WI) were bilaterally inoculated with 0.10 mL containing 1 × 107 MCF-7 cells suspended in sterile phosphate-buffered saline. MCF-7 cells were inoculated into athymic mice, and...
mice were treated with estradiol to achieve estradiol-stimulated
tumor growth (MCF-7E2 tumors (15,17,32–34). Tumors were
maintained by serial passage of solid tumors into athymic mice
as described previously (17). Fifty mice were bi-transplanted
with parental MCF-7E2 tumors, and groups of 10 mice each
were randomly assigned to the following treatments: vehicle,
estradiol, estradiol plus tamoxifen, estradiol plus fulvestrant, orulvestrant alone.

**Growth of MCF-7TAMLT Tumors In Vivo**

Long-term tamoxifen-stimulated tumors (MCF-7TAMLT)
were developed by re-transplanting growing estradiol-
dependent MCF-7 tumors into new athymic mice and treating
the mice with tamoxifen (17,33,35). Selected tamoxifen-
stimulated MCF-7 tumors were then serially passed into
new athymic mice, and mice were treated with tamoxifen
for more than 5 years, as described (17). The mammary fat pads
of 50 ovariectomized BALB/c nu/nu athymic mice, 4–6
weeks old, were bi-transplanted with tumors, and groups of
10 mice were randomly assigned to the following treatments:
vehicle, tamoxifen alone, estradiol alone, fulvestrant alone, or
estradiol plus fulvestrant. In another experiment, 40 mice
were bi-transplanted with MCF-7TAMLT tumors and were
billed with tamoxifen. Another 10 mice (controls) were
bi-transplanted with MCF-7TAMLT tumors and were treated
with vehicle alone. When the mean tumor cross-sectional area
in tamoxifen-treated mice reached 0.35 cm², groups of 10
mice were randomly assigned to the following treatments:
tamoxifen alone, estradiol alone, estradiol plus fulvestrant, or
fulvestrant alone. The previous experiment was repeated as
described above except this time 30 mice were bi-
transplanted with MCF-7TAMLT tumors and treated with
tamoxifen. When the mean tumor cross-sectional area
in tamoxifen-treated mice reached 0.32 cm², mice were with-
drawn from tamoxifen treatment, and groups of 10 mice were
randomly assigned to the following treatments: continue ta-
moxfen alone, discontinue tamoxifen, or continue estradiol
alone. Tumors were measured weekly with vernier calipers.
The cross-sectional area was calculated by multiplying the
length (l) by the width (w) and by π and dividing the product
by 4 (i.e., lwπ/4).

The Animal Care and Use Committee of Northwestern Uni-
versity approved all of the procedures involving animals.

**Apoptosis Assay**

The following two-step method was used to detect apoptosis:
the terminal deoxynucleotidyltransferase–mediated deoxuri-
dine triphosphate nick-end labeling (TUNEL) assay followed by
tetramethylrhodamine deoxyuridine 5'-triphosphate (TMR)
staining. An in situ cell death assay with the TMR red detection
kit (Roche Diagnostic, Indianapolis, IN) was performed on tu-
mor sections from paraffin-embedded blocks, according to the
manufacturer’s instructions. The TUNEL assay was done in
triplicate and repeated four times with independent tumors from
each treatment group. The percentage of apoptosis was cal-
bulated by dividing the number of TMR-positive cells or TUNEL-
positive cells by the total number of epithelial cells (identified by
4',6-diamidino-2-phenylindole and hematoxylin–eosin staining)
and multiplying the result by 100.

**Western Blot Analysis**

Tumors were pulverized in liquid nitrogen and resuspended
in lysis buffer (1% Triton X-100, 1 mM EDTA, 150 mM NaCl,
50 mM Tris–HCl [pH 7.4], phenylmethylsulfonyl fluoride at 25
mg/mL, leupeptin at 10 µg/mL, aprotinin at 10 µg/mL, pepsta-
tin at 10 µg/mL, Na-p-tosyl-L-chloromethyl ketone hydro-
chloride [i.e., TLCK] at 10 µg/mL, N-p-tosyl-L-phenylalanine
chloromethyl ketone [i.e., TPCK] at 10 µg/mL, 100 mM NaF,
10 mM orthovanadate) (all supplied by Sigma-Aldrich). The
extract was subsequently sonicated at level 1 three times at
1-second intervals with a Microson sonicator (Misonix, Farm-
ington, NY) and then centrifuged for 5 minutes at 5000g at 4 °C.
The supernatant was collected, and protein concentration was
determined with the Bradford assay (Bio-Rad Laboratories, Her-
cules, CA). Equal amounts of protein (25–50 µg) were loaded
into lanes of a 7% gel for sodium dodecyl sulfate–polyacrylam-
ide gel electrophoresis. Proteins were transferred to a blot for
western blot analysis of ERα (1 : 200 dilution of rabbit anti-
human antibody; clone G20; Santa Cruz Biotechnology, Santa
Cruz, CA), β-actin (1 : 20 000 dilution of mouse anti-human
antibody; clone A15; Sigma-Aldrich), FasL (1 : 1000 dilution
of mouse anti-human antibody; clone 33; BD Transduction Labs,
San Diego, CA), Fas (1 : 500 dilution of rabbit anti-human
antibody; clone C-20; Santa Cruz Biotechnology), phosphory-
lated HER2/neu (rabbit anti-human phosphorylated residue
Y1248 antibody at 1 µg/mL; Upstate Biotechnology, Lake
Placid, NY), HER2/neu (1 : 200 dilution of mouse anti-human
HER2/neu antibody AB-11; Neomarkers, Fremont, CA), NFκB
p65 subunit (rabbit anti-human p65 antibody at 2 µg/mL; Up-
state Biotechnology), and cyclooxygenase-2 (COX-2) (rabbit
anti-human antibody at 1 µg/mL; Upstate Biotechnology). The
appropriate secondary antibody conjugated to horseradish
peroxidase was used to detect the primary antibody (either
gold anti-rabbit or goat anti-mouse immunoglobulin G (Santa Cruz
Biotechnology). The blot was developed with an enhanced
chemiluminescence (ECL) kit (Amersham, Arlington Heights,
IL). The membrane was exposed to Kodak X-Omat film for
10–30 seconds (Kodak, Rochester, NY).

**Real-Time Reverse Transcription–Polymerase Chain
Reaction for Human TGF-α, Fas, and HER2/neu mRNAs
in Tumors**

Total RNA, extracted from the tumors with the RNeasy mini
kit (Qiagen, Valencia, CA) according to the manufacturer’s
instructions, was reverse transcribed with TaqMan reverse
transcription reagents (Applied Biosystems, Hayward, CA) and
random hexamers as the primers, according to the manufacturer’s
instructions. Primers and probes for human TGF-α and Fas were
designed with Primer Express TM1.5 software (Applied Biosys-
tems) set at default parameters to select the most optimized
primer and probe sets for this system. The sequences for the
forward and reverse primers for human TGF-α were
5'-GCTGTGAAACACATGCACTGA-3' and 5'-TTTCCAAG-
GGACTGACTTGGAAG-3', respectively. The sequence for the
TGF-α probe was 5'-AGGCTCTACATATCGGCTCCC-
TAGAAGTG-3'. Sequences for the forward and reverse primers
for human Fas were 5'-TGGAAAGCTGTCATGTA-3' and
5'-CAGTCCCTAGTTTCTTTACCC-3', respectively. The
sequence for the human Fas probe was 5'-CCAATCCGTGCA-
AACGCCGTGCTCC-3'. Sequences for the forward and re-
verse primers for human HER2/neu were 5’-ACTGCAGAGG-
CTCCGATT-3’ and 5’-ACGGCCAGGGCATTTGT-3’, respectively. The sequence for the human HER2/neu probe was 5’-TGGCAAGCCACCCAGCTCTTGA-3’. For all real-time reverse transcript–polymerase chain reactions (RT–PCRs), 6-carboxyfluorescein was the reporter, and QSY7 was the quencher (MegaBases, Chicago, IL). The quantity of human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was also measured in each total cDNA sample for normalization. The probe and primers for GAPDH were purchased from Applied Biosystems. The PCR portion of the reaction was performed with the TaqMan PCR core reagent kit (Applied Biosystems). The 25-mL PCR mixture contained 50 ng of total cDNA, 100 nM probe, and 200 nM primers. Real-time PCR was performed with the ABI-Prism 7700 sequence detection system (Applied Biosystems). PCR conditions were 50 °C for 2 minutes and 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute.

Statistical Analysis

Tumor growth curves were analyzed longitudinally with a two-factor analysis of variance (ANOVA) to compare cross-sectional areas of all tumors for all treatment groups in a time-dependent manner. Tumors in athymic mice were assumed to be homogenous across individual mice. Tumors within and between mice were assumed to be independent of each other. Tumor growth curves represent means and 95% confidence intervals (CIs) of tumor cross-sectional areas. One-factor ANOVA was used to analyze differences in the percentage of apoptosis between the various treatment groups and the control group, as measured by the TUNEL assay. One-factor ANOVA was used to analyze differences in mRNA levels as detected by real-time PCR between the treatment groups and the control group. The error bars for the measurement of the percentage of apoptosis and mRNA copy number are 95% confidence intervals as calculated with StatMost 2.5 (DataMost, Salt Lake City, UT). All statistical tests were two-sided.

RESULTS

Growth of MCF-7E2 and MCF-7TAMLT Tumors In Vivo

The effects of estradiol, tamoxifen, and fulvestrant on the growth of tamoxifen-naïve MCF-7E2 and tamoxifen-stimulated MCF-7TAMLT tumors in athymic mice were investigated. The growth of parental MCF-7E2 tumors was stimulated more by estradiol than by estradiol plus tamoxifen, estradiol plus fulvestrant, or control (Fig. 1, A). At 13 weeks, the cross-sectional area of estradiol-treated tumors was 1.06 cm² (95% CI = 0.82 to 1.30 cm²) compared with that of control (0.06 cm², 95% CI = −0.02 to 0.14 cm²), estradiol plus tamoxifen (0.30 cm², 95% CI = 0.26 to 0.34 cm²), estradiol plus fulvestrant (0.01 cm², 95% CI = 0.00 to 0.02 cm²), or fulvestrant alone (0.01 cm², 95% CI = 0.00 to 0.02 cm²). In contrast, tamoxifen-stimulated MCF-7TAMLT tumors grew faster in mice treated with tamoxifen than in mice treated with estradiol, fulvestrant, or control vehicle (P<.001 for all) (Fig. 1, B). At 9 weeks, the cross-sectional area of MCF-7TAMLT tumors treated with tamoxifen (0.47 cm², 95% CI = 0.35 to 0.59 cm²; P<.001) was greater than that of tumors treated with control (0.02 cm², 95% CI = 0.00 to 0.04 cm²), fulvestrant alone, or estradiol alone. Surprisingly, the growth of MCF-7TAMLT tumors treated with a combination of estradiol plus fulvestrant was essentially the same as that of tumors treated with tamoxifen alone (Fig. 1, B). At 9 weeks, the cross-sectional area of tumors treated with estradiol plus fulvestrant was 0.47 cm² (95% CI = 0.32 to 0.62 cm²). In addition, when we increased the weekly dose of fulvestrant to 20 mg in combination with estradiol, tumors continued to grow (data not shown). These results suggest that estradiol may act through the ERα pathway to inhibit the growth of MCF-7TAMLT tumors.

To investigate whether estradiol is acting as a tumorstatic or tumoricidal agent, mice bi-transplanted with MCF-7TAMLT tumors were treated with tamoxifen until the cross-sectional area of tumors reached approximately 0.35 cm². At this point, tamoxifen was withdrawn, and mice were randomly assigned to treatment groups as described above. In estradiol-treated mice, MCF-7TAMLT tumors continued to grow for 2 weeks and then regressed rapidly, in contrast to tumors in mice treated with tamoxifen, estradiol plus fulvestrant, or fulvestrant alone (all P<.001; Fig. 1, C). At 10 weeks, the mean cross-sectional area of tumors in mice treated with estradiol was 0.18 cm² (95% CI = 0.15 to 0.21 cm²) compared with fulvestrant alone (0.44 cm², 95% CI = 0.37 to 0.51 cm²), tamoxifen (0.60 cm², 95% CI = 0.50 to 0.70 cm²), or estradiol plus fulvestrant (1.00 cm², 95% CI = 0.88 to 1.12 cm²). Thus, at physiologic levels, estradiol appears to act as a potent tumoricidal agent for MCF-7TAMLT tumors. Remarkably, tumors treated with estradiol plus fulvestrant grew robustly (Fig. 1, C).

To determine whether the estradiol-induced tumor regression was a consequence of the tamoxifen withdrawal, mice bi-transplanted with MCF-7TAMLT tumors were treated with tamoxifen until tumors had a cross-sectional area of 0.32 cm². At this point, tamoxifen was withdrawn, and mice were randomly assigned to the following treatment groups: continue tamoxifen treatment, discontinue tamoxifen treatment, or discontinue tamoxifen treatment and begin estradiol treatment (Fig. 1, D). In the estradiol-treated group, MCF-7TAMLT tumors regressed, with a mean cross-sectional area at 10 weeks of 0.10 cm² (95% CI = 0.04 to 0.16 cm²; P = .001), whereas tumors in the other groups continued to grow, with cross-sectional areas of 0.60 cm² (95% CI = 0.47 to 0.73 cm²) in the tamoxifen-treated group and 0.40 cm² (95% CI = 0.33 to 0.70 cm²) in the group for which tamoxifen was withdrawn. However, the growth rate was lower for tumors withdrawn from tamoxifen than for tumors treated with tamoxifen (P = .038).

Effects of Estradiol on Apoptosis

To determine whether apoptosis was involved in estradiol-induced regression of MCF-7TAMLT tumors, we used the TUNEL assay to quantitate apoptosis in tumor sections from all treatment groups at week 9 (Fig. 1, C) and week 10 (Fig. 1, D). MCF-7TAMLT tumors treated with estradiol had 23% (95% CI = 20% to 26%; P<.001) apoptotic cells compared with tumors treated with vehicle control (1.2%, 95% CI = 1.98% to 2.02%), tamoxifen (1.7%, 95% CI = 1.69% to 1.71%), fulvestrant (4.8%, 95% CI = 2.1% to 7.5%), or estradiol plus fulvestrant.
Expression and Function of ERα in MCF-7E2 and MCF-7TAML Tumors

To determine whether ERα expression and function are altered in MCF-7TAML tumors by long-term exposure to tamoxifen, we used western blot analysis to measure the level of ERα protein and real-time RT–PCR to measure ERα activity as reflected by the expression of TGF-α mRNA, an

(1.7%, 95% CI = 0.7% to 2.7%) (Fig. 2, A) or 20% (95% CI = 7% to 33%; P = .038) apoptotic cells compared with tumors withdrawn from tamoxifen (Fig. 2, B). More importantly, apoptosis was completely blocked (2.5%, 95% CI = 1.5% to 3.5%) in tumors treated with the combination of estradiol plus fulvestrant (Fig. 2, A), indicating that estradiol-induced MCF-7TAML tumor regression is the result of apoptosis mediated through the ERα pathway.

Expression and Function of ERα in MCF-7E2 and MCF-7TAML Tumors

Fig. 1. Growth of MCF-7E2 and MCF-7TAML tumors in athymic mice. Estradiol (E2) was given as a 0.30-cm silastic capsule subcutaneously, tamoxifen (TAM) was given orally by gavage at 1.5 mg/day, and 10 mg of fulvestrant was given subcutaneously as a 5-mg dose twice a week. For all panels, tumor areas were measured weekly with vernier calipers. Cross-sectional areas were calculated by multiplying the length (l) by the width (w) and by π and dividing the product by 4 (i.e., lwπ/4). A) MCF-7E2 tumors. Fifty ovariectomized athymic mice were bi-transplanted with tamoxifen-naive MCF-7E2 tumors, and groups of 10 mice were randomly assigned to the following treatments: vehicle (control), estradiol alone, estradiol plus tamoxifen, estradiol plus fulvestrant, or fulvestrant alone. *Cross-sectional area at 13 weeks of estradiol-treated tumors was statistically significantly different from that of tumors treated with control vehicle, estradiol plus tamoxifen, fulvestrant alone, or estradiol plus fulvestrant. **Cross-sectional area of tumors at 13 weeks treated with estradiol plus tamoxifen was statistically significantly different from that of tumors treated with vehicle control, fulvestrant alone, or estradiol plus fulvestrant. B) MCF-7TAML tumors. Another 50 athymic mice were bi-transplanted with MCF-7TAML tumors, and groups of 10 mice were randomly assigned to the following treatments: vehicle control, tamoxifen alone, estradiol alone, estradiol plus fulvestrant, or fulvestrant alone. C) Tamoxifen-treated MCF-7TAML tumors. Fifty mice were bi-transplanted with MCF-7TAML tumors, of which 40 were treated with tamoxifen until tumors had an area of 0.35 cm², and 10 were given vehicle from the beginning (control). Tamoxifen was withdrawn from the tamoxifen-treated mice, and groups of 10 mice were randomly assigned to the following treatments: tamoxifen alone, estradiol alone, fulvestrant alone, or estradiol plus fulvestrant. *Cross-sectional area at 10 weeks of tumors treated with estradiol was statistically significantly different than that of tumors treated with tamoxifen alone, fulvestrant alone, or estradiol plus fulvestrant. D) Tamoxifen withdrawal. Thirty mice were bi-transplanted with MCF-7TAML tumors and treated with tamoxifen until tumors had an area of 0.32 cm². Tamoxifen was withdrawn, and groups of 10 mice were randomly assigned to the following treatments: tamoxifen, estradiol, or no treatment. *Cross-sectional area at 10 weeks of tumors treated with estradiol or withdrawn from tamoxifen was statistically significantly different from that of tumors treated with tamoxifen alone. At the conclusion of the experiment, one tumor from each mouse was fixed in 10% buffered formalin and sectioned for analysis and the other was snap-frozen in liquid nitrogen and maintained at −80 °C for future use. Data are from a representative experiment; results of other experiments are similar. Each experiment was repeated at least three times, with 10 mice per group. Error bars = 95% confidence intervals. All statistical tests were two-sided.
endogenous human-specific estrogen-responsive gene (30,31). Estriadiol treatment reduced the level of ERα protein in both parental estradiol-dependent MCF-7 tumors and MCF-7TAMLT tumors compared with that in corresponding control tumors (Fig. 3, A). Tamoxifen treatment of MCF-7E2 or MCF-7TAMLT tumors almost restored levels of ERα protein to that detected in corresponding control tumors. ERα activity in MCF-7E2 and MCF-7TAMLT tumors followed a similar trend; estradiol treatment increased the expression of TGF-α in MCF-7E2 and MCF-7TAMLT tumors (both \( P<.001 \)) compared with that in control tumors (Fig. 3, B). The estradiol-induced expression of TGF-α mRNA was inhibited by tamoxifen in both MCF-7E2 and MCF-7TAMLT tumors and by fulvestrant in MCF-7TAMLT tumors. Because fulvestrant-treated MCF-7E2 tumors did not grow, they were not available for further analysis. In both MCF-7E2 and MCF-7TAMLT tumors, as measured by real-time RT–PCR, the level of ERβ mRNA was much lower (\(<1\%\); \( P<.001 \)) than that of ERα mRNA (data not shown), indicating that ERβ plays little, if any, role in estradiol-induced apoptosis in MCF-7TAMLT tumors. Thus, the ERα in MCF-7TAMLT tumors is apparently not dysregulated, and the paradoxical increase in the estradiol-induced apoptosis observed in such tumors may be mediated through the ERα pathway.

Roles of FasL and Fas in Estradiol-Induced Apoptosis

Song et al. (19) suggested that estradiol treatment of MCF-7TAMLT cells promoted apoptosis by increasing the expression of FasL, an estrogen-responsive gene belonging to the tumor necrosis factor α family, and thus activating the Fas death pathway. We detected similar levels of FasL protein in both MCF-7E2 and MCF-7TAMLT tumors, regardless of treatment (Fig. 4, A), a result indicating that FasL is not a likely target for estradiol-
induced apoptosis in MCF-7TAML T tumors. To determine whether Fas plays a role in estradiol-induced apoptosis in MCF-7TAML T tumors, we measured levels of Fas protein and mRNA in extracts of MCF-7E2 and MCF-7TAML T tumors. Fas protein was expressed in parental MCF-7E2 tumors treated with estradiol plus tamoxifen (Fig. 4, A). The expression of human Fas mRNA was increased by 2.5-fold (95% CI = 2.3- to 2.7-fold; P < .001) in estradiol-dependent parental MCF-7E2 tumors treated with estradiol plus tamoxifen compared with that in control tumors, and
Fas mRNA expression was increased 3.3-fold (95% CI = 2.6- to 4.0-fold; \( P = .005 \)) in tamoxifen-dependent MCF-7TAMLT tumors treated with estradiol compared with that in control tumors (Fig. 4, B). Thus, the expression of the Fas gene appears to be associated with estradiol-induced tumor regression of tamoxifen-stimulated breast cancer.

**Expression and Function of HER2/neu in MCF-7E_2 and MCF-7TAMLT Tumors**

The ER\(\alpha\) pathway regulates expression of the oncogene HER2/neu (36), and overexpression and/or amplification of HER2/neu is sufficient to promote resistance to tamoxifen in vitro and in vivo (37). To investigate whether the ER\(\alpha\) pathway modulates estradiol-induced regression of MCF-7TAMLT tumors by regulating HER2/neu, we measured HER2/neu mRNA with real-time RT–PCR and measured phosphorylated and total HER2/neu protein with western blot analyses in tumor extracts. Levels of HER2/neu mRNA were lower in MCF-7E_2 (decrease of 9.2-fold, 95% CI = 9.1- to 9.3-fold; \( P = .005 \)) and MCF-7TAMLT tumors (decrease of 5.1-fold, 95% CI = 2.3- to 7.9-fold; \( P = .045 \)) treated with estradiol than in their respective control tumors (Fig. 5, A). Remarkably, treatment with the combination of estradiol plus fulvestrant resulted in an increase of 5.5-fold (95% CI = 3.7- to 8.3-fold; \( P < .001 \)) of HER2/neu mRNA in MCF-7TAMLT tumors compared with that in vehicle-treated control tumors (Fig. 5, A). Levels of phosphorylated and total HER2/neu protein were decreased in regressing MCF-7E_2 tumors treated with estradiol plus tamoxifen compared with vehicle-treated control tumors and barely detected in estradiol-treated MCF-7TAMLT tumors (Fig. 5, B). MCF-7TAMLT tumors treated with fulvestrant plus estradiol expressed approximately fivefold more phosphorylated and total HER2/neu protein than control tumors (Fig. 5, B). Interestingly, mRNA levels do not appear to be consistent with steady-state protein levels; thus, this observation merits further analysis.

**Expression and Function of NF-\(\kappa\)B in MCF-7E_2 and MCF-7TAMLT Tumors**

The ER\(\alpha\) (38) and HER2/neu (39,40) pathways regulate expression and function of NF-\(\kappa\)B, an antiapoptotic transcription factor.
factor (29), and the Fas gene promoter contains κB-binding sites (28,29,41). To investigate whether ERα acts through NF-κB to mediate the induction of Fas and thus estradiol-induced apoptosis in MCF-7TAML7 tumors, we determined the levels of NF-κB protein and measured its activity in tumors. The level of the NF-κB p65 subunit protein was almost undetectable by western blot analysis in estradiol-treated MCF-7TAML7 tumors in contrast to that in tumors treated with vehicle control, tamoxifen, fulvestrant, or estradiol plus fulvestrant (Fig. 5, C), suggesting that the estradiol–ERα complex has an as yet undetermined role in the regulation of NF-κB p65 protein levels in MCF-7TAML7 tumors. Thus, a mechanism other than simple hormonal regulation appears to mediate the expression of NF-κB in estradiol-treated MCF-7TAML7 tumors. To assess activity of NF-κB protein in tumors after various treatments, we examined the expression of COX-2, an NF-κB responsive gene (42,43). The level of cyclooxygenase 2 protein was lower in estradiol-treated MCF-7TAML7 tumors than in untreated MCF-7TAML7 tumors, whereas the level of COX-2 protein was lower in tamoxifen-treated MCF-7EΔ tumors than in untreated MCF-7EΔ tumors (Fig. 5, C). These data indicate a novel mechanism in which ERα appears to mediate estradiol-induced apoptosis and tumor regression of MCF-7TAML7 tumors by suppressing the prosurvival/antiapoptotic factors HER2/neu and NF-κB and inducing the death receptor Fas.

**DISCUSSION**

We demonstrated, to our knowledge for the first time, that the regression of tamoxifen-stimulated human breast cancer tumors in vivo induced by physiologic levels of estradiol appears to be mediated by increased apoptosis through suppression of the prosurvival/antiapoptotic factors HER2/neu and NF-κB and induction of the death receptor Fas. In such tumors, the pure antiestrogen fulvestrant completely blocked the growth inhibitory effect of estradiol and paradoxically stimulated growth when given in combination with estradiol. These results strongly suggest that regulation of Fas, HER2/neu, and NF-κB in response to treatment with estradiol is mediated by the ERα pathway.

Currently, postmenopausal women with ERα-positive breast cancer are treated with tamoxifen, aromatase inhibitors, or fulvestrant. Tamoxifen is recommended as a 5-year adjuvant therapy for women with ERα-positive, lymph node–positive and/or negative breast cancer and as a chemopreventive agent to women at high risk for breast cancer. During long-term adjuvant therapy, tumors can acquire resistance to tamoxifen. The evolution of acquired resistance consists of at least three phases (44). During the treatment phase, breast tumors grow in the presence of estradiol and regress in the presence of tamoxifen or an aromatase inhibitor (estrogen withdrawal) (45). In phase I resistance, acquired resistance to tamoxifen occurs when breast tumors grow in response to either tamoxifen or estradiol (45) and regress when either an aromatase inhibitor or fulvestrant is given after tamoxifen fails to inhibit growth (16). Phase II resistance is observed after 5 years of therapy with tamoxifen (long-term treatment phase). Our current in vivo model used in this article, in which MCF-7 breast tumors that have been exposed to tamoxifen for more than 5 years and have become resistant to it, represents an example of phase II drug resistance. The growth of these breast tumors is dependent on tamoxifen, and tumors rapidly regress in the presence of physiologic levels of estradiol (17). Phase II drug resistance is also observed in MCF-7 cells treated with raloxifene for more than 1 year in vitro, as described by Liu et al. (46). Thus, phase II drug resistance is not limited to tamoxifen or to development of tamoxifen-stimulated MCF-7 tumors in vivo but might result from a long-term exposure of breast cancer cells to tamoxifen or raloxifene. Our data (Fig. 1, B and C) suggest that the clinical use of fulvestrant to treat ER-positive tumors might not be beneficial during phase II resistance because the growth of such tumors is stimulated by treatment with estradiol plus fulvestrant. Furthermore, the use of aromatase inhibitors to block estradiol synthesis might stabilize disease rather than cause regression.

Before the use of tamoxifen (47–49) or first-generation aromatase inhibitors (50), postmenopausal women with breast cancer were treated with high-dose estrogens, such as diethylstilbestrol or ethinyl estradiol (49,51,52). Results from our study suggest that low levels of estradiol (83.8 pg/mL in serum) (32) are sufficient to induce apoptosis and the regression of tamoxifen-stimulated breast tumors. A study by Lonning et al. (53) showed that standard high-dose estrogen (5 mg of diethylstilbestrol, given three times per day) has a substantial antitumor effect in postmenopausal breast cancer patients who have been exposed to multiple endocrine therapies. Four of the 32 patients in that study achieved complete remission, six had partial remission, five had objective responses to high-dose diethylstilbestrol, and three had stable disease lasting from 6 months to more than 1 year. Clearly, there could be a profound advantage for patients if an apoptotic regimen targeting ER-positive tumors could be integrated into the overall treatment plan, especially because a recent laboratory study (17) shows that tamoxifen is again effective after such estrogen therapy.

Song et al. (19) suggested that overexpression of FasL is important for estradiol-induced apoptosis of Fas-expressing breast cancer cells after long-term estrogen deprivation in vitro. In contrast, we demonstrate in this article that FasL protein is expressed at essentially the same level in both tamoxifen-naive and tamoxifen-stimulated MCF-7 tumors (Fig. 4, A), regardless of the treatment, and thus would not be a limiting factor. Moreover, we show that both Fas mRNA and protein are induced in regressing tumors in response to tamoxifen in parental MCF-7EΔ or to estradiol in MCF-7TAML7 tumors (Fig. 4, A) and could regulate apoptosis. Thus, apoptosis—induced initially by tamoxifen and later by estradiol—appears to be mediated by a common pathway. In estradiol-treated MCF-7TAML7 tumors, fulvestrant treatment completely blocked estradiol-induced regression, enhanced tumor growth (Fig. 1, B and C), and, most importantly, blocked the estradiol-induced expression of Fas (Fig. 4, A). Thus, both apoptosis and Fas expression appear to be mediated by the estradiol–ERα complex.

The finding that the pure antiestrogen fulvestrant switch an estradiol-induced apoptotic signal to an estradiol-induced growth signal is intriguing. Fulvestrant is a steroidal compound with a 7α-alkylamide hydrocarbon side chain that reduces the level of cellular ERs (54) by disrupting dimerization of the ER; the protein monomer subsequently is targeted for ubiquitin-mediated degradation by the proteosome (13). We have observed a novel action of fulvestrant—that the combined treatment of fulvestrant with estradiol stimulates the growth of breast tumors exposed to tamoxifen treatment for 5 years (Fig. 1, B and C). Unexpectedly, fulvestrant partially blocked the
estradiol-induced decrease in the ERα protein level (Fig. 3, A). Thus, long-term exposure of breast tumors to tamoxifen leads to a situation in which the combined treatment of fulvestrant and estradiol does not efficiently lead to ERα degradation and actually promotes growth. Because the combined treatment with estradiol plus fulvestrant enhanced tumor growth but did not induce the expression of TGF-α mRNA (Fig. 3, B), non-genomic actions of ERα (i.e., actions not dependent on ERα–DNA interaction), such as extranuclear ERα to activate the MAPK/ERK1/2 pathway, or ERα-mediated activation of other transcription factors may contribute to cell survival and proliferation stimulated by such treatment. We have initiated studies to address whether ERα is sequestered and redistributed in MCF-7TAMLT tumors.

The exact role played by the ERα in the estradiol-induced expression of Fas and subsequent apoptosis of MCF-7TAMLT tumors remains unclear. However, transcription of the Fas gene is inactivated in many types of cancers by epigenetic events, such as hypermethylation of the promoter at specific κB sites (41). Our results demonstrate that estradiol decreases the expression and activity of NF-κB, an antiapoptotic factor, that has been shown to induce expression of apoptotic inhibitors such as Bcl-x(L) (55) and inhibitors of apoptosis, a group of proteins that inhibit caspases (56), in MCF-7TAMLT tumors (Fig. 5) and that fulvestrant inhibits this effect. These results suggest that the estradiol–ERα complex might act by decreasing NF-κB protein levels and thus allowing apoptosis mediated by the FasL/Fas death pathway to proceed.

Our data suggest that long-term exposure of MCF-7 breast tumors to tamoxifen results in supersensitivity to estradiol, so that perhaps only physiologic levels of estradiol are required to induce tumor regression. Similar observations were reported in a raloxifene-resistant MCF-7 tumor model in vitro and in vivo (46). These laboratory results have important clinical implications, particularly for the use of aromatase inhibitors and the pure antiestrogen fulvestrant as second-line therapies after tamoxifen. For patients with a recurrence of ER-positive, tamoxifen-dependent breast cancer after 5 years of adjuvant therapy (phase II resistance), second-line treatment with fulvestrant or aromatase inhibitors may produce tumor stasis or growth. More important, the use of fulvestrant in the patient with sufficient levels of circulating estrogen may produce tumor stasis or growth. More important, the use of fulvestrant in the patient with sufficient levels of circulating estrogen may produce tumor stasis or growth. More important, the use of fulvestrant in the patient with sufficient levels of circulating estrogen may produce tumor stasis or growth. More important, the use of fulvestrant in the patient with sufficient levels of circulating estrogen may produce tumor stasis or growth. More important, the use of fulvestrant in the patient with sufficient levels of circulating estrogen may produce tumor stasis or growth. More important, the use of fulvestrant in the patient with sufficient levels of circulating estrogen may produce tumor stasis or growth. More important, the use of fulvestrant in the patient with sufficient levels of circulating estrogen may produce tumor stasis or growth.

In conclusion, the use of a tamoxifen-stimulated human breast cancer model in vivo demonstrates that long-term tamoxifen treatment results in supersensitivity to estradiol in which apoptosis and tumor regression are induced by physiologic levels of estradiol. Moreover, we show that combined treatment with estradiol plus fulvestrant promotes the growth of MCF-7TAMLT tumors, strongly implicating the ERα pathway and suggesting that estradiol is required to induce tumor regression in tamoxifen-stimulated breast cancer. Furthermore, our study suggests a novel mechanism in which estradiol induces apoptosis of MCF-7TAMLT tumors by inducing the death receptor Fas and suppressing the prosurvival/antiapoptotic factors HER2/neu and NF-κB. Future studies to integrate these novel results into clinical practice require a focus on enhancing estrogen-induced apoptosis with chemotherapy or radiation therapy and blocking tumor cell survival pathways with antibodies or tyrosine kinase inhibitors to HER2/neu and/or epidermal growth factor receptor. We believe that if these concepts can be tested in the clinic, a new strategy of extended adjuvant therapy, through progressive schedules of aromatase and hormonal therapies, may be possible by exploiting ERα further as a drug target.

**References**


Wolf DM, Jordan VC. A laboratory model to explain the survival advantage observed in patients taking adjuvant tamoxifen therapy. Recent Results Cancer Res 1993;127:23–33.


NOTES

C. Osipo and C. Gajdos contributed equally to the studies.

Supported by the Avon Foundation, Specialized Programs of Research Excellence (SPORE) grant CA89018-03 in breast cancer research (to V. C. Jordan) from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, and the Lynn Sage Breast Cancer Research Foundation. C. Osipo is funded by the Judy Dlugie Memorial Fund Fellowship for Breast Cancer Research.

We thank Dr. Alfred Rademaker for his assistance in determining which statistical tests were necessary for our studies, Dr. Ruth O’Regan for her helpful discussion during the writing of this article, and Kathy Meeke for her assistance in performing the TUNEL assay.

Manuscript received February 10, 2003; revised August 21, 2003; accepted August 29, 2003.