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 \( \alpha \) (ER\( \alpha \)) protein expression, increased the expression of Fas mRNA and protein, decreased the expression of HER2/neu mRNA and protein and nuclear factor \( \kappa B \) (NF-\( \kappa B \)) 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 ER\( \alpha \)-mediated regulation of Fas, HER2/neu, and NF-\( \kappa B \). 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-\( \kappa B \) and HER2/neu. [J Natl Cancer Inst 2003;95:1597–1608]

Tamoxifen is currently used to treat all stages of estrogen receptor \( \alpha \) (ER\( \alpha \))-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

Affiliations of authors: Robert H. Lurie Comprehensive Cancer Center, Feinberg School of Medicine, Northwestern University, Chicago, IL (CO, HL, BC, VCI); Department of Surgery, University of Alabama, Birmingham (CG).

Correspondence to: V. Craig Jordan, OBE, PhD, DSc, Robert H. Lurie Comprehensive Cancer Center, Feinberg School of Medicine, Northwestern University, 8258 Olson, 303 E. Chicago Ave., Chicago, IL 60611 (e-mail: vcjordan@northwestern.edu).

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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 H9251 ER second-line therapies is that if the formation of the estradiol resistance to tamoxifen (1 sions are based, in part, on laboratory models of early drug resistance to tamoxifen will regress. These conclu-
ingrowth of breast tumors before or after tamoxifen therapy has

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-7LTD 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-7LTD cells express Fas ligand (FasL), estradiol treatment increases the levels of FasL and induces apoptosis in Fas-expressing MCF-7LTD 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 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-7LTD 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 surveill-
ance 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 hu-
man MCF-7TAMLt breast cancers in vivo, a model generated by treating mice carrying tamoxifen-naive 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 ther-
apy 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 inves-
igated 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-
responsible 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 antifungal 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. Fulves-

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 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
fulvestrant 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 passaged 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
treated 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-
모xifen 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 deoxyuri-
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
triplet 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
mM/mL, leupeptin at 10 mg/mL, aprotinin at 10 mg/mL, pepsta-
in at 10 mg/mL, Na-p-tosyl-L-chloromethyl ketone hydro-
chloride [i.e., TLCK] at 10 mg/mL, N-p-tosyl-l-phenylalanine
chloromethyl ketone [i.e., TPCK] at 10 mg/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), phosphor-
ylated 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), NF-κB
p65 subunit (rabbit anti-human p65 antibody at 2 μg/mL;
Upstate 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 goat
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'-GCCGTTGAAACACACGATGTTGA-3' and
5'-'TTTCCAA-
GGACTGACTTGGAAG-3', respectively. The sequence for the
TGF-α probe was 5'-AGCCCTCATATAGCGCTCC-
TAGAAATG-3', Sequences for the forward and reverse
primers for human Fas were 5'-TGGTGAAGAGCCTCAGTGA-3' and
5'-CAGTCCCCATGGTCTTCCAC-3', respectively. The
sequence for the human Fas probe was 5'-CCAATTCCTGCGC-
AAGCCTGTCCTCC-3'. Sequences for the forward and re-
verse primers for human HER2/neu were 5′-ACTGCAGAGG-CTCGGGATT-3′ and 5′-ACGGCCAGGGCATAATTGT-3′, respectively. The sequence for the human HER2/neu probe was 5′-TGCGAGGCACCATCCTTGG-3′. For all real-time reverse transcription–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 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 ER pathway to inhibit the growth of MCF-7TAMLT tumors.

To investigate whether estradiol is acting as a 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 = .99), 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-7TAMLT Tumors

To determine whether ERα expression and function are altered in MCF-7TAMLT 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
endogenous human-specific estrogen-responsive gene (30,31). Estradiol 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-7LTE 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-7TAMLT tumors. To determine whether Fas plays a role in estradiol-induced apoptosis in MCF-7TAMLT tumors, we measured levels of Fas protein and mRNA in extracts of MCF-7E2 and MCF-7TAMLT tumors. Fas protein was expressed in parental MCF-7E2 tumors treated with estradiol plus tamoxifen and in MCF-7TAMLT 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

![Fig. 4. Expression of Fas ligand (FasL) protein, Fas protein, and mRNA in parental MCF-7E2 and tamoxifen (TAM)-stimulated MCF-7TAMLT tumors. A) Western blot analysis for human FasL protein (p38) and for human Fas protein (p45) in both parental MCF-7E2 and MCF-7TAMLT tumors at 10 weeks treated as previously described in Fig. 1, C. Blots shown are representative of three independent experiments, all with similar results. B) Human Fas mRNA copy number detected by real-time reverse transcription–polymerase chain reaction. Copy number was normalized as described in Fig. 3, B. * \( P < .001 \) compared with MCF-7E2 control group (one-factor analysis of variance). \( \dagger \) \( P < .005 \) compared with MCF-7TAMLT control group (one-factor analysis of variance). All statistical tests were two-sided.
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-7E2 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-7E2 (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 in 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-7E2 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-7E2 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
pressing the prosurvival/antiapoptotic factors HER2/neu and NF-κB in the progression of acquired resistance consists of at least three phases (15). During the treatment phase, breast tumors grow in the presence of estradiol and regress in response to 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-7E2 or to estradiol in MCF-7TAMLT 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-7TAMLT 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 could 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
estrogen-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 exacerbate the disease by stimulating growth, as illustrated in this study. Paradoxically, a strategy of treatment with estrogen alone, either exogenous estrogen or the woman’s endogenous estrogen may be sufficient to produce short-term control of disease progression. In the latter clinical scenario, it may be informative to document how long tamoxifen withdrawal responses occur in women with high levels of circulating estrogen after extended antihormonal therapy (i.e., tamoxifen or aromatase inhibitors). For the future, it may also be important to consider developing an estrogen challenge test or gene profile to distinguish between patients with phase I and those with phase II acquired resistance to tamoxifen or long-term estrogen deprivation.

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 antihormonal and hormonal therapies, may be possible by exploiting ERα further as a drug target.

REFERENCES

NOTES

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