Tamoxifen-resistant Fibroblast Growth Factor-transfected MCF-7 Cells Are Cross-Resistant *in Vivo* to the Antiestrogen ICI 182,780 and Two Aromatase Inhibitors[1]

Sandra W. McLeskey, Lurong Zhang, Dorraya El-Ashry, Bruce J. Trock, Cecilia A. Lopez, Samir Kharbanda, Christopher A. Tobias, Lori A. Lorant, Rachel S. Hannum, Robert B. Dickson, and Francis G. Kern[2]

Lombardi Cancer Center [S. W. M., L. Z., B. J. T., C. A. L., S. K., C. A. T., R. S. H., D. E-A., R. B. D., F. G. K.], Departments of Biochemistry and Molecular Biology [D. E-A., F. G. K.], Cell Biology [R. B. D., L. Z.], Medicine [B. J. T.], and Pharmacology [S. W. M.], and the School of Nursing [S. W. M.], Georgetown University Medical Center, Washington, D. C. 20007

ABSTRACT

Although the antiestrogen tamoxifen has been the mainstay of therapy for estrogen receptor (ER)-positive breast cancer, successful treatment of responsive tumors is often followed by the acquisition of tamoxifen resistance. Subsequently, only 30–40% of patients have a positive response to second hormonal therapies. This lack of response might be explained by mechanisms for tamoxifen resistance that sensitize ER pathways to small amounts of estrogenic activity present in tamoxifen or that bypass ER pathways completely. To elucidate one possible mechanism of tamoxifen resistance, we treated ovariectomized tumor-bearing mice injected with fibroblast growth factor (FGF)-transfected MCF-7 breast carcinoma cells with the steroidal antiestrogen ICI 182,780 or one of two aromatase inhibitors, 4-OHA or letrozole. These treatments did not slow estrogen-independent growth or prevent metastasis of tumors produced by FGF-transfected MCF-7 cells in ovariectomized nude mice. FGF-transfected cells had diminished responses to ICI 182,780 *in vitro*, suggesting that autocrine activity of the transfected FGF may be replacing estrogen as a mitogenic stimulus for tumor growth. ER levels in FGF transfectants were not down-regulated, and basal levels of transcripts for estrogen-induced genes or of ER-mediated transcription of estrogen response element (ERE) luciferase reporter constructs in the FGF expressing cells were not higher than parental cells, implying that altered hormonal responses are not due to down-regulation of ER or to FGF-mediated activation of ER. These studies indicate that estrogen independence may be achieved through FGF signaling pathways independent of ER pathways. If so, therapies directed at the operative mechanism might produce a therapeutic response or allow a response to a second course of antiestrogen treatment.

INTRODUCTION

Because conventional therapy is not usually curative in clinical breast cancer, development of tamoxifen resistance, in which breast tumors previously growth-inhibited by tamoxifen become refractory, represents an important therapeutic dilemma. However, the development of tamoxifen resistance is not necessarily associated with progression to an ER[3]-negative phenotype. In many cases of clinical tamoxifen resistance, ER expression may be retained (1–4), implying that the resistance is due an alteration in activity of the tamoxifen/ER complex. Tamoxifen resistance in such a case could result from three possible mechanisms that, according to present knowledge, would not preclude successful treatment with an alternative hormonal therapy. First, alterations in the ER could arise, which might diminish or extinguish inhibitory responses to tamoxifen, leaving only its partial agonist effects to predominate (5–8). Second, tamoxifen resistance arising in the setting of an intact ER could be a result of altered intratumoral tamoxifen metabolism, which might produce more estrogenic metabolites locally (7, 9–11). Third, available tamoxifen could be sequestered by an increase in antiestrogen binding sites not associated with ERs (12). As mentioned, in each of these three instances, substitution of a hormonal therapy different from tamoxifen might result in a clinical response. Two such alternative therapies used in this report are steroidal estrogen antagonists, such as ICI 182,780, which lack the partial agonist activity of tamoxifen, and aromatase inhibitors, which inhibit endogenous estrogen production by all tissues, depriving the ER of its ligand.

Although the mechanisms of tamoxifen resistance de-

Received 7/3/97; revised 11/26/97; accepted 12/10/97.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported by NIH Grants CA50376 (to F. G. K.), CA09218 (to F. G. K. and S. W. M.), CA53185 (to F. G. K. and R. B. D.), CA66154 (to S. W. M.), CA71465 (to D. E-A.), and Cancer Center Grant CA51008; American Cancer Society Grant IRG-193 (to S. W. M.); U.S. Army Medical Research and Material Command Grants DAMD 17-94-4172 (to D. E-A.) and DAMD 17-94-J-4173 (to S. W. M.); and a Susan Komen Foundation Fellowship (to L. Z.).
[2] To whom requests for reprints should be addressed, at Southern Research Institute, P. O. Box 55305, 2000 Ninth Avenue South, Birmingham, AL 35255-5305. Phone: (205) 581-2480; Fax: (205) 581-2877; E-mail: kern@sri.org.

[3] The abbreviations used are: ER, estrogen receptor; FGF, fibroblast growth factor; IMEM, improved minimal essential medium; X-gal, 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside; FBS, fetal bovine serum; 4-OHA, 4-hydroxyandrostenedione; NK, natural killer; CCS, charcoal-stripped calf serum; ERE, estrogen response element; CAT, chloramphenicol acetyltransferase; RT, reverse transcription.

scribed above should be amenable to alternative hormonal therapy, early results for small numbers of tamoxifen-resistant patients have shown that only about 30–40% of such patients have a positive response to subsequent ICI 182,780 or aromatase inhibitor therapy (13–20). These data imply alternative mechanisms for tamoxifen resistance. Constitutive production of autocrine growth factor(s) or growth factor receptors by tumor cells has been proposed as a mechanism for tamoxifen resistance that may or may not involve ER pathways. Evidence supporting this hypothesis is gained from the acquisition of estrogen-independent growth in tumor models, including the one used in this report, in which growth factors or growth factor receptors have been overexpressed in estrogen-dependent breast carcinoma cell lines (21–26). In addition, recent clinical data showing decreased efficacy of tamoxifen in treating tumors overexpressing *c-erbB2* (27) supports a role for growth factor signaling in clinical tamoxifen resistance. Because some growth factor signaling pathways, including the ERB-B pathway, have been shown to interact with ER signaling pathways (25, 28–32), increased growth factor signaling could be one mechanism by which cells could become sensitive to previously ineffective amounts of estrogenic stimulation produced by the partial agonist activity of tamoxifen itself or its estrogenic metabolites, above. In cases in which such interactions have been demonstrated, the growth factor and ER pathways may act collaboratively (25), making the final outcome susceptible to pharmacological manipulations of either pathway and implying that second line hormonal therapies might have an effect. However, increased autocrine or intracrine growth factor signaling might also bypass the need for ER-mediated growth stimulation in tumor cells or affect stromal components of the tumor, such as endothelial or immune cells (33–36), to alter the tumor environment in ways conducive to tumor growth. In either case, alternative hormonal therapies might not be effective.

Recently, cell-specific coactivators and corepressors have been identified for steroid hormone receptors, including the ER, which may influence steroid receptor-induced transcription positively or negatively (37, 38). Thus, the activity of tamoxifen in inhibiting or even stimulating tumor growth might depend on the relative expression of various stimulatory or inhibitory cofactors in a particular tumor (39, 40). However, transient transfection experiments suggest that tamoxifen-resistant tumors produced by such mechanisms should still be sensitive to pure antiestrogens (40).

FGFs and their receptors have been shown to be present with high frequency in breast cancer specimens (41–50). Evidence for a possible role for FGF signaling in the estrogen-independent growth of breast tumors is gained from study of clonal and polyclonal FGF-transfected MCF-7 cell lines, which are capable of forming large, progressively growing tumors in ovariectomized or tamoxifen-treated nude mice. Moreover, the FGF-transfected cells are metastatic, forming micrometastases in lymph nodes, lungs, and other organs (21, 22, 51). The estrogen-independent and tamoxifen-resistant growth of FGF-transfected MCF-7 cells suggests an interaction between FGF signaling pathways and ER-activated pathways that could occur at the level of the ER itself or at the end point of both pathways, where they impinge on growth mechanisms. If FGF-mediated growth pathways bypass the ER pathway to affect growth directly, we would expect that growth would be unaffected by hormonal treatments devoid of agonist activity. We therefore sought to determine the sensitivity of the estrogen-independent tumor growth of FGF-transfected MCF-7 cells to ICI 182,780 or aromatase inhibitors. In contrast to what was seen with ERB-B signaling pathways, we report that FGF-mediated pathways appear to provide an alternative growth stimulatory signal that is not dependent on ER activation.

MATERIALS AND METHODS

Cell Lines. FGF-transfected MCF-7 cell lines have been described previously (21, 22, 51, 52). Briefly, the ML-20 clonal cell line is a MCF-7-derived cell line that is stably transfected with a *lacZ* expression vector. The *in vitro* and *in vivo* growth characteristics of ML-20 cells are indistinguishable from wild-type MCF-7 cells (51), and >90% of the cells routinely stain positive for β-galactosidase expression by X-gal staining (52). MKL-F (FGF-4-transfected; Ref. 52) and FGF-1 clone 18 (FGF-1-transfected) cells (22) resulted from the stable transfection of the ML-20 clonal cell line with expression vectors for FGF-4 (also known as hst-1/K-FGF) and FGF-1 (also known as acidic FGF or aFGF), respectively. Both cell lines continue to stably express β-galactosidase, allowing effects of FGF overexpression on metastatic capability to be assessed by X-gal staining of organs and tissues of tumor-bearing mice. The MKL-4 cell line was derived by transfecting wild-type MCF-7 cells (of similar passage number used for the ML-20 transfection) with an expression vector for FGF-4, which produced the clonal MKS-1 cells (21). These cells were then retransfected with an expression vector for *lacZ*, yielding MKL-4 cells (51). Cells were maintained in IMEM (Biofluids, Rockville, MD) supplemented with 5% FBS in a humidified, 37°C, 5% CO_2 incubator in routine culture until used for tumor cell injection.

Drugs. ICI 182,780 was kindly donated by Dr. Alan Wakeling of Zeneca Pharmaceuticals (Macclesfield, England), and was administered s.c. at a dose of 5 mg in 0.1 ml of vehicle every week. For the experiment depicted in Fig. 1, powdered drug was first dissolved in 100% ethanol and spiked into warmed peanut oil (Eastman Kodak, Rochester, NY) to give a final concentration of 50 mg/ml. For the experiments depicted in Fig. 1, *B* and *C*, 50 mg/ml preformulated drug in a vehicle of 10% ethanol, 15% benzyl benzoate, 10% benzyl alcohol, brought to volume with castor oil, was supplied by B. M. Vose (Zeneca Pharmaceuticals). 4-OHA was donated by Angela Brodie (University of Maryland, Baltimore, MD) and was administered s.c. at a dose of 1 mg/mouse/day 6 days of the week in a vehicle of 0.3% hydroxypropylcelluose. Letrozole was donated by Dr. Ajay Bhatnagar (Novartis, Ltd., Basil, Switzerland) and was administered via gavage at a dose of 1 mg/mouse/day 6 days of the week in a vehicle of 0.3% hydroxypropylcellulose. Sustained-release (60 day) pellets containing 5 mg of tamoxifen were obtained from Innovative Research of America (Sarasota, FL) and implanted s.c. in the interscapular area at the time of tumor cell injection.

Tumor Cell Injection. The procedure for tumor cell injection has been described previously (21). Briefly, tumor cells were scraped into their normal growth medium, and viable cells were quantified using trypan blue exclusion. The cells were

resuspended in their normal growth medium at a density of 66.7×10^6 cells/ml, and 0.15 ml (containing 10 million cells) were used to inject ovariectomized mice (nude or *beige/nude/xid*) into the mammary fat pad. For the experiment involving MKL-4 cells and nude mice (Fig. 1*A*), each mouse was injected bilaterally into the thoracic mammary fat pads (two injections per mouse). There were seven mice in the vehicle group and five mice in each treatment group. For the experiments involving MKL-4 cells and *beige/nude/xid* mice (Fig. 2), four tumor cell injections were given, two on each side in the thoracic mammary fat pad and two in the inguinal mammary fat pad; treatment groups consisted of four mice. For the experiments involving MKL-F and FGF-1, clone 18 cells (Fig. 1, *B* and *C*), each mouse was injected once in the right thoracic mammary fat pad. There were seven mice in the each vehicle group, and treatment groups consisted of five or six mice each. Tumors resulting from the injections were measured twice weekly in three dimensions using calipers. Tumor volume is the product of the largest dimension, the orthogonal measurement, and the tumor depth, as described previously (21). Because the FGF-1-transfected clone 18 cell line produces tumors that in some cases are surrounded by a fluid-filled sac that confounds tumor measurements (22), these tumors were measured postmortem by weighing them.

Determination of Metastasis. Organs were harvested from tumor-bearing animals, fixed briefly, and stained with X-gal as reported previously (51) and viewed through a dissecting microscope (Olympus SZH). Clusters of blue-staining cells were identified as micrometastases. In accordance with previous results, no macrometastases were identified (21, 22, 51, 53).

Growth Assays. Anchorage-dependent and anchorage-independent growth assays were performed as described (21). Briefly, for anchorage-dependent growth, cells were plated in 24-well culture dishes at a density of 10,000 cells/well for the time course experiments (Fig. 4) and 20,000 or 30,000 cells/well for the concentration-response experiments (Fig. 5). For growth in FBS, following overnight attachment, treatments were added at the indicated concentrations, and cells were counted on the indicated days. For growth assays under estrogen-depleted conditions, cells were stripped of estrogens during a 24-h period the day following plating by changing the medium four times to phenol red-free IMEM supplemented with 5% CCS (21). We have found that this stripping procedure allows complete removal of estrogens without substantial proliferation of cells before treatments are added. Following the stripping procedure, on day 0, treatments were added, and counting of cells was done as above.

Doubling times were determined according to the following equation: doubling time $= t_2 - t_1/3.32\log(N_2/N_1)$, where N_2 and N_1 are the number of cells at times t_2 and t_1, respectively. N_1 and N_2 are the means of quadruplicate determinations.

Anchorage-independent assays in FBS-containing medium were done as described previously (21). For experiments using estrogen-depleted conditions, cells were stripped of estrogens over a 24-h period as described above before being plated in soft agar. Colonies greater than 60 μm were counted using an Omnicon 3600 Image Analysis system.

ER Assays. [^{3}H]Estradiol binding has been described previously (54, 55). Briefly, cells grown to 70% confluence were stripped with twice daily medium changes over 4 days with 5% CCS in phenol red-free IMEM. The prolonged stripping method allows ERs to become up-regulated to maximal levels. Cells were harvested, washed sequentially at 4°C with serum-free, phenol red-free IMEM followed by TEG (10 mM Tris, pH 7.4, 1 mM EDTA, 10% glycerol), and resuspended in 1 ml of TEG plus 1 mM DTT, 0.5 M NaCl and a cocktail of protease inhibitors (1 mg/ml leupeptin, 77 μg/ml aprotinin, 1 μg/ml pepstatin A). A whole-cell extract was prepared by homogenization with 40 strokes in a Teflon-glass Dounce homogenizer followed by centrifugation at $105{,}000 \times g$ for 30 min. Protein content of the supernatant was determined by the method of Bradford (56), and protein concentrations were adjusted to 2 mg/ml. Extracts were incubated with 10 nM [^{3}H]17β-estradiol with or without a 100-fold excess of unlabeled estradiol for 16 h at 4/C. Unbound ligand was removed by absorption with dextran-coated charcoal followed by centrifugation. Aliquots of the supernatant were counted in a Beckman liquid scintillation counter.

Northern Blots. Cells were grown to 50% confluence in IMEM supplemented with 5% FBS and then stripped of estrogens as described for the growth assays, above. Treatments of 0.1% ethanol (vehicle) or 10^{-8} M 17β-estradiol in the same medium were added. Cultures were harvested after 3 days of treatment, and RNA was extracted using RNAzol B (Tel-Test, Inc.) according to the manufacturer's directions. Thirty μg of each RNA were subjected to electrophoresis in a 1.2% formaldehyde/agarose gel and transferred to nylon (Hybond-N, Amersham Corp., Arlington Heights, IL) by capillarity. ^{32}P-labeled antisense riboprobes for pS-2, GAPDH, and cathepsin D were prepared and sequentially hybridized to the membrane overnight at 65°C [hybridization buffer was 50% formamide, 50 mM Na_2HPO_4, 0.8 M NaCl, 10 mM EDTA, 2.5 × Denhardt's solution (1× Denhardt's = 0.02% polyvinylpyrrolidone, 0.02% BSA), 0.2% SDS, 400 μg/ml yeast tRNA, and 400 μg/ml sonicated salmon sperm DNA with 10^6 DPM/ml of the appropriate probe]. The membrane was washed three times in 0.1% SDS/0.1× SSC at 80°C for the PS-2 and cathepsin D probes, and 75°C for the GAPDH probe. Autoradiograms and PhosphorImager (Molecular Dynamics Model 445SI) quantitation of individual hybridization signals were obtained between the sequential hybridizations. For the results depicted in Fig. 7, *A* and *B*, PhosphorImager values obtained for PS-2 or cathepsin were normalized to those obtained for GAPDH.

Progesterone Receptor mRNA Determination by RT-PCR. The primers for human progesterone receptor that produce a 205-bp PCR product have been described previously (57). The human GAPDH primers that produce a 437-bp PCR product are as follows: 5′-AAG GTC GGT GTG AAC GGA TTT G-3′ (sense) and 5′-TGG TGC AGG ATG CAT TGC TG-3′ (antisense). RT-PCR was performed with 0.1 μg of test RNAs, except T47D cells, where 0.02 μg was used, using the GeneAmp RNA PCR kit (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions with the following modifications: the RT reaction was primed with 0.0625 μM random hexamers in a volume of 40 μl, with 2 μl each of ^{35}S-labeled UTP and ^{35}S-labeled ATP (each 3000 Ci/mmol, 10 μCi/μl, Amersham Corp.) substituted for water in the reaction. Then, 20 μl of each RT reaction were transferred into two tubes for separate GAPDH and progesterone receptor PCR reactions.

Cycle analyses using RNA from ML-20, estradiol-treated cells (the highest expressors of progesterone receptor) revealed that amplification remained logarithmic at 35 cycles for the GAPDH reaction and 40 cycles for the progesterone receptor reaction, making these assays semiquantitative. The GAPDH PCR reaction was performed using standard reagent conditions recommended by the manufacturer and cycles of 95°C for 45 s and 50°C for 45 s for 35 cycles. For the progesterone receptor PCR reaction, final $MgCl_2$ concentrations were adjusted to 1.25 mM, and 0.25 M acetamide was included. Cycles were of 95°C for 45 s and 50°C for 45 s for 40 cycles. GAPDH and progesterone receptor reaction products were first visualized by ethidium bromide staining following electrophoresis in a 2% agarose gel. Products were then electrophoresed on a 4–20% acrylamide gel that was subjected to both autoradiography and PhosphorImager quantitation as described above.

Transient Transfection, Luciferase, and CAT Reporter Assays. ML-20 and clone 18 cells were plated in 6-well plates, allowed to attach overnight, and stripped of estrogens in a procedure similar to that for the growth assays (see above). Following stripping, cells were transfected by the calcium phosphate, low-CO_2 method (58). The luciferase plasmids pGLB-MERE or pGLB-MNON were obtained by inserting an approximately 1.48-kb fragment containing a glucocorticoid response element-deleted mouse mammary tumor virus promoter with either a substituted double consensus ERE (MERE) or the same sequence with the ERE palindromes scrambled (MNON) (59) into the *Hin*dIII site of pGLB (Promega, Madison, WI). Each dish received 2.5 μg of either pGLB-MERE or pGLB-MNON and 1.0 μg pCMV-CAT, which directs constitutive expression of CAT, cotransfected as a control for transfection efficiency. Following transfection, each well was washed twice with PBS and incubated for 48 h in medium containing vehicle (0.01% ethanol), 10^{-9} M estradiol, 10^{-7} M ICI 182,780, a combination of E_2 and ICI, 10 ng/ml FGF-1 plus 10 μg/ml heparin, or a combination of FGF, heparin, and ICI 182,780. (Duplicate samples of each treatment were used.) Cells were lysed and assayed for luciferase activity using the Luciferase Reporter Gene Assay (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. Luciferase values, expressed as relative light units, for each sample were corrected for background by subtracting the value of lysates of untransfected cells prepared in parallel. CAT expression was assayed using the CAT ELISA (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. Protein content of the lysates was determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL). Luciferase and CAT values, normalized for protein, were used to calculate mean specific relative light units/ng CAT.

Statistical Analyses. Statistical methods used for tumor growth have been described previously (53, 60). For Figs. 1 and 2, only mice surviving at the end of the experiment were included in the analysis. When no tumor developed from a particular injection, tumor volume was recorded as zero. The repeated measures ANOVA (60) was used to compare tumor volumes among the treatment groups using measurements taken over the entire time course of the experiment. In addition, final tumor volumes (or weights in the case of clone 18) were compared among treatment groups at the end of each experiment using ANOVA. For analysis of metastasis in Table 1, for each transfectant, analysis of covariance was used to compare the effects of treatment on total metastases, total distant metastases (lung metastases plus other metastases), lymph node metastases, lung metastases, and other metastases. The analyses were all conducted with final tumor volume (or weight for the clone 18 cells) included in the model as a covariate. The analyses considered the effects of all treatments simultaneously, as well as the effects of individual treatment comparisons (which were adjusted for multiple comparisons using Dunnett's method). For each transfectant, the effect of final tumor volume (or weight for clone 18) on the number of metastases was evaluated using linear regression (for each of the categories of metastasis described above). In Fig. 3, paired *t* tests were performed comparing control and transfected cells under different conditions of treatment. For the anchorage-dependent growth assays depicted in Fig. 4, we examined the effect of treatment on the rate of cell growth, using linear regression with an interaction between time and treatment. To compare cell growth rates and doubling times among the cell lines under specific treatment conditions, nested linear regression models were used. For Fig. 6, ANOVA was used to determine significant differences in ER binding among cell lines.

RESULTS

Estrogen-independent Growth of Tumors Produced by FGF-transfected MCF-7 Cells Is Not Inhibited by Treatment with a Pure Antiestrogen or with Aromatase Inhibitors. We have previously shown that both FGF-1- and FGF-4-transfected MCF-7 cells form progressively growing tumors in ovariectomized nude mice, as well as in similar mice treated with tamoxifen (21, 22, 53). Although ovariectomized mice could be expected to have substantially lower levels of estrogenic compounds than reproductively intact mice, some estrogens are synthesized at extraovarian sites, such as adrenal gland, liver, fat, or possibly the tumor itself. The transfected cells evidently still possess ERs, because they respond to estrogen and tamoxifen administered to the mice, as well as to these compounds used in tissue culture (21, 22). To test the hypothesis that growth of the FGF-transfected cells in ovariectomized or tamoxifen-treated nude mice is due to increased sensitivity to the small amounts of estrogens still present in ovariectomized nude mice, we tested the ability of a pure antiestrogen, ICI 182,780, and two aromatase inhibitors, 4-OHA and letrozole, to inhibit the estrogen-independent tumor growth produced by these FGF-transfected cell lines.

In a first experiment to test the above hypothesis, FGF-4-transfected MKL-4 cells were injected as before, and the mice were treated with vehicle, tamoxifen, or ICI 182,780. There were no significant differences in tumor volume among the treatment groups considered over the entire time course of the experiment ($P = 0.72$) or at the final time point (Fig. 1*A*; $P = 0.72$). Treatment with ICI 182,780 did not inhibit tumor growth below that achieved in vehicle-treated mice ($P = 0.675$). Thus, the failure of ICI 182,780 to inhibit the estrogen-independent growth exhibited by this cell line supports the hypothesis that such growth does not result from small amounts of estrogenic

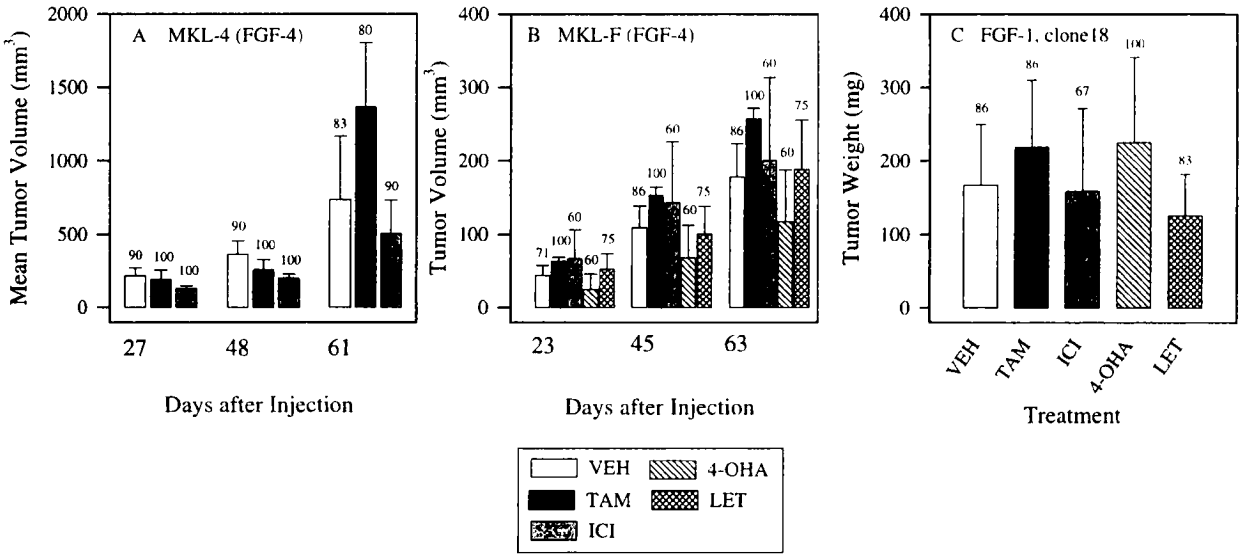

Fig. 1 Growth of FGF-transfected MCF-7 cells in ovariectomized nude mice is not inhibited by treatment with ICI 182,780, 4-OHA, or letrozole. Ten million cells from the indicated cell lines were injected into the mammary fat pads of ovariectomized nude mice treated with vehicle (*VEH*); a 5-mg, 60-day-release tamoxifen pellet (*TAM*); ICI 182,780, 5 mg s.c. every week (*ICI*); 1 mg of 4-OHA s.c. per day 6 days of the week (*4-OHA*); or 1 mg of letrozole per day via gavage 6 days of the week (*LET*). *Columns*, group mean; *bars* SE. *Numbers* above each column are the percentages of injections resulting in measurable tumors at that time point. *A*, volumes of tumors produced by one clonal FGF-4-transfected MCF-7 cell line, MKL-4, at the indicated number of days following tumor cell injection. *B*, volumes of tumors produced by a second clonal FGF-4-transfected MCF-7 cell line, MKL-F, at the indicated number of days following tumor cell injection. *C*, weights of tumors produced by a clonal FGF-1-transfected MCF-7 cell line, FGF-1, clone 18, weighed after sacrifice of the animals 28 days after tumor cell injection. (Because the FGF-1 producing MCF-7 cells may form fluid-filled sacs around the tumor, confounding tumor measurements before sacrifice, only postmortem weights are presented here.)

growth stimulation achieved by extraovarian estrogen production.

We wished to assess the effect of ICI 182,780 on metastasis as well as on tumor growth. In spite of its retention of the transfected *lacZ* expression plasmid, the MKL-4 cell line becomes heterogeneous over time with respect to β-galactosidase expression, such that a few cells have high expression, but most are negative (52). We therefore used a second clonal FGF-4-transfected MCF-7 cell line, MKL-F, the β-galactosidase expression of which is stable, for a subsequent experiment involving FGF-4-transfected MCF-7 cells. Because FGF-1 has also been shown to produce estrogen-independent *in vivo* growth when transfected into MCF-7 cells (22), we also included a clone of FGF-1-transfected cells designated clone 18, the β-galactosidase expression of which is also stable. For these experiments, two aromatase inhibitors, 4-OHA (61, 62) and letrozole (63), were also used to inhibit extraovarian synthesis of estrogens.

In agreement with the experiment using MKL-4 cells depicted in Fig. 1*A*, when the FGF-4-transfected MKL-F cells were used, there were no differences in tumor volume among treatment groups over all time points ($P = 0.382$), and ICI 182,780 did not decrease tumor growth below that obtained in vehicle-treated animals (Fig. 1*B*; $P = 0.837$ for the last time point). In addition, neither 4-OHA nor letrozole decreased tumor growth below vehicle-treated levels ($P = 0.571$ and 0.931 for the last time point, respectively).

FGF-1-transfected clone 18 cells form tumors that are sometimes surrounded by a fluid-filled sac (22, 53), preventing accurate tumor volume measurements during the course of the experiment. Consequently, when these cells were used (Fig. 1*C*), only terminal tumor weights were analyzed with ANOVA. As with the MKL-4 and MKL-F cells, ICI 182,780 did not inhibit estrogen-independent tumor growth in the clone 18 cells ($P = 0.977$). Administration of ICI 182,780 to animals injected with ML-20 cells, a clonal line of β-galactosidase-transfected wild-type MCF-7 cells (51), also produced no effect when compared with vehicle-treated animals [*i.e.*, no progressively growing tumors were obtained in either case (data not shown)]. In other, separate experiments, a polyclonal population of control vector-transfected ML-20 cells that forms progressively growing tumors in estrogen-supplemented mice (22) did not form tumors in either untreated or ICI 182,780-treated animals.[4] Thus, the continued progressive *in vivo* growth of FGF-transfected cells in ovariectomized animals treated with either a pure antiestrogen or aromatase inhibitors demonstrates that the estrogen-independent growth of these cells in untreated ovariectomized nude mice is not due to estrogenic activity produced at extraovarian sites.

Because ICI 182,780, 4-OHA, and letrozole were without effect in the experiments described above, we injected reproductively intact female mice for 2 weeks with these compounds at the same doses used in the above experiments to observe for activity in preventing effects of endogenous estrogens on the

[4] Unpublished results.

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.