Activation of Mitogen-Activated Protein Kinase in Xenografts and Cells during Prolonged Treatment with Aromatase Inhibitor Letrozole

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Abstract

Ovariectomized mice bearing tumor xenografts grown from aromatase-transfected estrogen receptor (ER)–positive human breast cancer cells (MCF-7Ca) were injected s.c. with 10 μg/d letrozole for up to 56 weeks. Western blot analysis of the tumors revealed that ERs (ERα) were increased at 4 weeks but decreased at weeks 28 and 56. Expression of erbB-2 and p-Shc increased throughout treatment, whereas growth factor receptor binding protein 2 (Grb2) increased only in tumors proliferating on letrozole (weeks 28 and 56). In cells isolated from tumors after 56 weeks and maintained as a cell line (LTLT-Ca) in 1 μmol/L letrozole, ERα was also decreased whereas erbB-2, adapter proteins (p-Shc and Grb2), and the signaling proteins in the mitogen-activated protein kinase (MAPK) cascade were increased compared with MCF-7Ca cells. Growth was inhibited in LTLT-Ca cells but not in MCF-7Ca cells treated with MAPK kinase 1/2 inhibitors U0126, and PD98059 (1 μmol/L). PD98059 (5 μmol/L) also reduced MAPK activity and increased ERα to the levels in MCF-7Ca cells. Epidermal growth factor receptor kinase inhibitor, gefitinib (ZD1839) inhibited growth of LTLT-Ca cells (IC50 ~ 25 μmol/L), PD98059 (5 μmol/L) also reduced MAPK activity and increased ERα to the levels in MCF-7Ca cells. Epidermal growth factor receptor kinase inhibitor, gefitinib (ZD1839) inhibited growth of LTLT-Ca cells (IC50 ~ 10 μmol/L) and restored their sensitivity to tamoxifen and anastrozole. In xenografts, combined treatment with ER down-regulator fulvestrant and letrozole, prevented increases in erbB-2 and activation of MAPK and was highly effective in inhibiting tumor growth throughout 29 weeks of treatment. These results indicate that blocking both ER- and growth factor–mediated transcription resulted in the most effective inhibition of growth of ER-positive breast cancer cells. (Cancer Res 2005; 65(12): 5380-9)

Introduction

Estrogens play an important role in the development and progression of breast cancers (1). Incidence of this malignancy increases as women age. In postmenopausal women, the ovaries are no longer the major source of estrogen synthesis and production of estrogens occurs mainly in peripheral tissue, such as adipose tissue (2). Endocrine therapy is the most important treatment for hormone-dependent postmenopausal breast cancer patients. Current treatment options for these patients include two strategies to reduce the effects of estrogens on tumor growth. One strategy is to block estrogen binding to estrogen receptor (ER) with antiestrogens, and the other is inhibition of estrogen synthesis with aromatase inhibitors. The antiestrogen tamoxifen has been used since the 1970s for the treatment of breast cancer (3) and has been shown to delay recurrences and contralateral breast cancer. However, tamoxifen exhibits both estrogen agonist and antagonist actions, depending on the tissue. Thus, tamoxifen acts like estrogen in the endometrium (4) and increases the risk of endometrial cancer. In addition, patients are at increased risk of strokes (5). To avoid agonist effects of antiestrogens and improve efficacy and safety, an alternate approach was taken by us in the early 1970s (6, 7). Aromatase inhibitors block the conversion of androgens to estrogens and do not have agonist effects. Two classes of aromatase inhibitors, steroidal (e.g., exemestane) and nonsteroidal (e.g., anastrozole and letrozole), are now available. To investigate the effectiveness of aromatase inhibitors, our laboratory developed a xenograft tumor model using human hormone-responsive (ER positive) breast cancer cells stably transfected with the human aromatase gene (MCF-7Ca). In this model, MCF-7Ca cells (8) are grown as tumors in the ovariectomized, immunosuppressed mice (9, 10). The tumors serve as autocrine sources of estrogen by aromatizing androgens. As the production of adrenal androgens is deficient in these mice (11), they are supplemented with androstenedione throughout the experiment. The resulting tumor xenografts are sensitive to both the antiproliferative effects of antiestrogens and aromatase inhibitors (9, 10, 12). Results from the recent clinical trials confirmed our studies and showed that aromatase inhibitors are more effective than tamoxifen as first-line therapy for postmenopausal patients with hormone-responsive breast cancer (13–15).

Despite the efficacy of these treatments, resistance ultimately occurs resulting in disease progression (16). In tamoxifen-resistant breast cancer cell lines, resistance is associated with up-regulation of growth factor signaling pathways (17, 18). It has been suggested that cross-talk between growth factor receptor signaling pathways and steroid receptors may play an important role in endocrine resistance (19–21). Many growth factor receptors on the cell membrane, such as epidermal growth factor receptor and erbB-2 can activate mitogen-activated protein kinase (MAPK) through a Shc-Grb2-mediated pathway (22). Growth factor receptor binding protein 2 (Grb2) and Shc adapter proteins play critical roles in coupling activated growth factor receptors to specific cellular signaling pathways. It has been shown that transgenic mice expressing high levels of either Shc or Grb2 have dramatic acceleration of mammary tumorigenesis (23). The role of these adaptor proteins is to link the activated tyrosine kinase receptors to a guanine nucleotide exchange factor (SOS) transducing the

Note: Preliminary data were reported at the Aromatase 2004 meeting.

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signal to Ras. This in turn activates the core of the cascade composed of Raf, MAPK kinase 1/2 (MEK1/2), and MAPK. Raf activation is stimulated following its translocation to the plasma membrane, a process that requires interaction with the membrane-localized GTPase, Ras-GTP. Activation of MAPK is critical for a large number of Ras-induced cellular responses. Activated MAPK can regulate targets in the cytosol (p90RSK) and also translocate to the nucleus where it phosphorylates a variety of transcription factors regulating gene expression. Activated MAPK can activate Elk, either directly by phosphorylation at Ser118 (24), or indirectly through p90RSK by phosphorylation at Ser167 (25). Transcriptional effects of the ER are modulated by interactions with coregulatory proteins that function as coactivators or corepressors (26, 27).

To determine the mechanism by which tumors adapt to growth on letrozole, we investigated expression of signaling proteins in tumors of letrozole-treated mice and also cells (LTLT-Ca) isolated from tumors treated with letrozole for 56 weeks.

Materials and Methods

Materials

MCF-7 human ER-positive breast cancer cells stably transfected with the human aromatase gene (MCF-7CA) were kindly provided by Dr. S. Chen (City of Hope, Duarte, CA; ref. 8). Dulbecco's PBS (DPBS), DMEM, penicillin/streptomycin solution, trypsin/EDTA solution, and geneticin (G418) were from Life Technologies, Inc. (Grand Island, NY). Fetal bovine serum (FBS) and charcoal/dextran-treated FBS (CSS) were from Hyclone (Logan, UT). Phenol red–free improved MEM (IMEM) and trypsin/verseen were from Biosource Biofluids Cell Culture Products (Rockville, MD). Matrigel Basement Membrane Matrix was obtained from BD Biosciences (Bedford, MA). Matrigel Basement Membrane Matrix was obtained from BD Biosciences (Bedford, MA). All tissue culture flasks and plates were from Costar (Cambridge, MA). MCF-7CA cells were inoculated into the mice as previously described (10, 28, 29). Subconfluent (80%) MCF-7CA cells were washed with DPBS and scrambled into DPBS. Cells were collected by centrifugation and resuspended in Matrigel (10 mg/mL). Each mouse received s.c. injections at two sites on each flank with 0.1 mL of cell suspension (2 × 10^7 cells/mL). As athymic mice are deficient in adrenal androgens (19), they were supplemented with daily injections of the aromatase substrate androstenedione (Δ4A, 100 μg/d) for the duration of the experiment.

Methods

First-line treatment with letrozole and tamoxifen in vivo. When the tumors reached a measurable size (~300 mm^3), -4 weeks after MCF-7CA cell inoculation, animals were assigned to three groups (n = 20 per group) so that total tumor volume was similar in each group. The groups were injected with vehicle (control), or with tamoxifen (100 μg/d), or letrozole (10 μg/d). The doses of letrozole and tamoxifen used had been previously determined to be maximally effective in reducing tumor growth (10, 28, 29). Drugs were prepared as suspensions in 0.3% hydroxypropyl cellulose. Tumors were measured weekly with calipers and volumes were calculated using the formula 4/3π × r_1 × r_2 × r_2 (where r_1 < r_2). Two mice per group were sacrificed at 4, 28, and 56 weeks and their tumors collected, cleaned, weighed, and stored at -80°C for analysis (Fig. 1; 30).

Long-term letrozole-treated cells (LTLT-Ca). Tumor cells were isolated from the above mice treated with letrozole for 56 weeks. Tumors were cut into small pieces in IMEM containing 10% CSS and 2% penicillin/ streptomycin and further disrupted by repeatedly drawing into a pipette. The cells were incubated at 37°C, stirring overnight with collagenase type 1-A (1 mg/mL), hyaluronidase type 1-A (5 mg/mL), amphotericin B (2.5 μg/mL), and polymyxin B sulfate (50 units/mL). The following day, cells were washed thrice with IMEM, centrifuged at 4°C for 20 minutes each time, and filtered through a 70-μm filter, followed by a 40-μm filtration. Cells were suspended in phenol red–free IMEM with 10% CSS and 2% penicillin/ streptomycin and plated into Petri dishes. Next day, the dishes were washed thrice with DPBS and the medium was changed. After 1 week to remove fibroblasts, phenol red–free trypsin was added followed by washing with DPBS. Epithelial cells were cultured in the above medium with 750 μg/mL G418 and 1 μmol/L of letrozole. The medium was changed twice a week until colonies started to proliferate. Over the next 2 to 3 weeks, serum levels in the medium were reduced from 10% to 5% CSS and penicillin/ streptomycin level from 2% to 1%. Growing colonies were selected and each colony was added to one of a 6-well plate in phenol red–free IMEM supplemented with 5% CSS, 1% penicillin/streptomycin, 750 μg/mL G418, and 1 μmol/L of letrozole. LTLT-Ca xenografts. Cells were prepared for inoculation as described earlier. Subconfluent LTLT-Ca cells were washed with DPBS and scrambled into DPBS. Cells were collected by centrifugation and resuspended in Matrigel (10 mg/mL). Each mouse received s.c. injections at one site on each flank with 0.1 mL of cell suspension (2 × 10^7 cells/mL). Mice were divided into three groups (n = 5) and injected s.c. daily for 13 weeks with vehicle, androstenedione (Δ4A, 100 μg/d), or androstenedione plus letrozole (10 μg/d) from the day of inoculation.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. For cell growth studies, MCF-7Ca and LTLT-Ca cells were transferred into steroid-free medium for 3 days before plating (1 × 10^4 cells per well) into 24-well plates. The next day, cells were washed with DPBS and treated with steroid-free medium containing androstenedione (25 nmol/L) and the indicated concentrations of letrozole, anastrozole, tamoxifen, fulvestrant, gefitinib, U10126, or PD98059. The medium was changed every 3 days, and the cells were counted 9 days later using the MTT assay. MTT (500 μg) was added to each well, and cells were incubated for 2 hours. Medium was then removed and formazan dye trapped in the living cells was dissolved in DMSO and the absorbance was measured in a spectrophotometer at 560 nm. The results were expressed as a percentage of the cell number compared with the vehicle-treated wells (control).

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Western blotting. Protein extracts from tumor tissues were prepared by homogenizing the tissue in T-PER containing protease inhibitors. The cells were lysed in a lysis buffer containing 0.1 mmol/L Tris, 0.5% Triton X-100, and protease inhibitors. Equal amounts (60 μg) of protein from each sample was separated on a denaturing polyacrylamide gel and transferred to nitrocellulose membrane. The protein-bound membranes were then incubated for 1 hour at room temperature with 0.1% Tween 20 in PBS (PBS-T) and 5% nonfat dry milk to block nonspecific antibody binding. The membranes were incubated with respective primary antibodies as specified in manufacturer’s protocol, and specific binding was visualized by using species-specific immunoglobulin G followed by ECL detection and exposure to ECL X-ray film. Bands were quantitated by densitometry using Molecular Dynamics Software (ImageQuant).

In vitro kinase activity assay for mitogen-activated protein kinase. The MAPK kinase assay was carried out according to the manufacturer’s protocol. Briefly, 200 μL of cell lysate that contains ~200 μg of total proteins were immunoprecipitated with 15 μL of resuspended immobilized monoclonal antibody for p44/42 MAPK. The mixture was stirred with gentle rocking overnight at 4°C. The next day, the tubes were microcentrifuged for 30 seconds at 4°C and pellet washed twice with 500 μL of 1× lysis buffer at 4°C followed by two washings with 500 μL of 1× kinase buffer. The pellet was suspended in 50 μL of 1× kinase buffer, supplemented with 200 μmol/L ATP and 2 μg fusion protein of kinase substrate Elk-1 (GST-Elk-1), and incubated for 30 minutes at 30°C. The reaction was terminated by addition of 25 μL 3× SDS sample buffer. The samples were boiled for 5 minutes, vortexed, and microcentrifuged for 2 minutes. The sample (30 μL) was loaded on to a SDS-PAGE gel and analyzed by Western blotting. The membrane was probed with phospho-Elk-1 antibody.

Statistical Analysis
Linear mixed-effects models were fitted to estimate average tumor volume within each treatment group. Each experiment was analyzed separately. The mean effect of treatment in each group and random effects for each mouse within a group were estimated. Data on tumor volume were longitudinal and unbalanced. The duration of treatment varied across the treatment groups. It was not possible to measure the same number of multiple tumors per mouse at all time points. In addition, diagnostic plots suggested that models of exponential growth were appropriate to the tumor growth data. Therefore, linear mixed-effects models were fitted to the natural logarithm of tumor volume over time. The mixed-effect model is a powerful and flexible way to analyze unbalanced longitudinal data (31). Linear models that incorporate fixed effects (entire population) and random effects (individuals drawn at random from a population) are mixed effects models (31) and were used for our previously published studies (32, 33). This approach allows an exponential variable controlling the rate of growth to be estimated for each of these treatment groups, with the random effects being estimated for each subject in a group (31).

All hypothesis tests were two sided. Adjustments for multiple comparisons were made by using either Tukey’s or Dunnett’s procedure (34, 35). Results are presented as treatment mean ± SE and compared at the 0.05 level of significance.

Results
Activation of Growth Factor Receptor Pathways in Tumors Treated with Letrozole
Ovariectomized mice were divided into three groups (n = 20 per group) to receive vehicle, letrozole (10 μg/d), or tamoxifen (100 μg/d). All groups received androstenedione supplement (100 μg/d) throughout the experiment. Tumor volumes were measured weekly and expressed as percentage change relative to the initial tumor volume (Fig. 1). As we previously reported (30), both treatments were effective in controlling tumor growth compared with vehicle. However, letrozole was more effective and delayed tumor progression twice as long as tamoxifen. Tumors treated with letrozole initially regressed. After 18 weeks, they had regained their starting size and thereafter were clearly unresponsive to the drug (Fig. 1). Two mice from each group were sacrificed at 4 weeks when tumors were regressing and at weeks 28 and 56 when tumors were growing on letrozole as indicated in Fig. 1. Tumors were collected for analysis.

To determine the mechanisms of loss of tumor growth inhibition by letrozole, immunoblot analysis was done at 4 weeks when tumors were regressing and 28 and 56 weeks when they were growing in the presence of letrozole. The results were compared with vehicle-treated tumors collected at week 4 (Fig. 2A-B).
The expression of ERα was first examined in these tumors. After 4 weeks, whereas tumors were responsive to letrozole, ERα increased up to 1.7-fold compared with the vehicle-treated tumors. However, after prolonged letrozole treatment for 56 weeks, ERα expression was decreased in these growing tumors up to 50% compared with control tumors. Expression of PGR that is an estrogen-regulated gene was unchanged despite the decrease in ERα expression (Fig. 2A).

Recently, it has been suggested that growth factors can regulate ERα-mediated transcription (19). Therefore, the above tumor lysates were subjected to immunoblot analysis for erbB-2 expression. In the presence of letrozole, erbB-2 expression was increased about 2-fold throughout treatment (weeks 4, 28, and 56). Expression of adapter protein p-Shc was also increased 2-fold in letrozole-treated tumors at week 28 and 56 suggesting that ligand-independent activation of ER may occur in tumors proliferating during treatment with letrozole (Fig. 2B).

Mechanisms involved in loss of letrozole sensitivity in long-term letrozole-treated cells (LTLT-Ca). A cell line was developed from tumors of letrozole-treated mice to determine the mechanism of resistance to letrozole and investigate additional treatment possibilities. Cells (LTLT-Ca) were isolated from tumors of mice treated with letrozole for 56 weeks and were kept in the presence of 1 μmol/L of letrozole after isolation. To confirm that the cells had lost sensitivity to letrozole, we did in vitro and in vivo growth studies. As shown in Fig. 3A, LTLT-Ca cells were insensitive to the effect of letrozole in vitro, whereas proliferation of the parental MCF-7Ca cells was markedly inhibited. Furthermore, when LTLT-Ca cells were inoculated into groups of ovariectomized nude mice injected sc daily with either vehicle, androstenedione (100 μg/d), or androstenedione plus letrozole (10 μg/d), tumors grew equally well in the presence and absence of androstenedione (Fig. 3B). This finding suggests that the tumors no longer required estrogens for growth and is consistent with our previous report that tumors treated with letrozole for 40 weeks and transplanted into new animals did not require the aromatase substrate for proliferation (33). In addition, LTLT-Ca xenografts also grew equally well with and without letrozole, indicating development of letrozole insensitivity (Fig. 3B) in these tumors.

To confirm our in vivo data that tamoxifen and fulvestrant were not effective as second-line therapies after letrozole treatment (30), we cultured LTLT-Ca cells with these antiestrogens. Tamoxifen inhibited growth of MCF-7Ca as expected but was not effective in LTLT-Ca cells in vitro. A similar result was obtained with fulvestrant (Fig. 3C). ERα expression was decreased in LTLT-Ca cells at week 28 and 56 suggesting that ligand-independent activation of ER may occur in tumors proliferating during treatment with letrozole (Fig. 2B).
Figure 3. A, effect of letrozole on the growth of MCF-7Ca and LTLT-Ca cells. MCF-7Ca and LTLT-Ca cells were transferred into steroid-free medium for 3 days before plating (1 x 10^4 cells per well) into 24-well plates. The next day, cells were washed with DPBS and treated with steroid-free medium containing androstenedione (25 nmol/L) and the indicated concentrations of letrozole. The medium was changed every 3 days, and the cells were counted 9 days later using the MTT assay. The results were expressed as a percentage of the cell number compared with the vehicle-treated wells (control).

B, effect of letrozole on the growth of LTLT-Ca xenografts. Each mouse received s.c. injections at one site on each flank with 0.1 mL of suspension of LTLT-Ca cells (2 x 10^7 cells/mL). Mice were divided into three groups (n = 5 per group) and injected s.c. daily for 13 weeks with vehicle, androstenedione (Δ4A, 100 μg/d), or androstenedione plus letrozole (10 μg/d) from the day of inoculation. Tumor volumes were measured weekly and were expressed as the percent change relative to the initial tumor volume. C, effect of fulvestrant on the growth of MCF-7Ca and LTLT-Ca cells. MCF-7Ca and LTLT-Ca cells were transferred into steroid-free medium for 3 days before plating (1 x 10^4 cells per well) into 24-well plates. The next day, cells were washed with DPBS and treated with steroid-free medium containing androstenedione (25 nmol/L) and the indicated concentrations of fulvestrant. The medium was changed every 3 days, and the cells were counted 9 days later using the MTT assay. The results were expressed as a percentage of the cell number compared with the vehicle-treated wells (control).
cells (Fig. 4A) compared with the parental MCF-7Ca cells. PGR expression was unchanged in LTLT-Ca cells when compared with MCF-7Ca, indicating that transcriptional activity of ER is maintained despite low levels of ERα protein. These results are all consistent with our findings in the tumor xenografts treated with letrozole for 56 weeks.

In LTLT-Ca cells, growth factor receptor erbB-2 was upregulated, as well as adapter proteins Grb2 and p-Shc, suggesting that the MAPK signaling pathway may be activated in these cells. We further examined the MAPK signaling proteins p-Raf, p-MEK1/2, and p-MAPK and found that all of these phosphorylated proteins were increased in LTLT-Ca cells (Fig. 4B). Proteins downstream of MAPK, p-p90RSK and p-Elk, were also increased in LTLT-Ca cells compared with the parental MCF-7Ca cells (Fig. 4B).

We also did in vitro kinase activity assays for MAPK in MCF-7Ca and LTLT-Ca cells by measuring Elk-1 phosphorylation. MAPK activity was dramatically increased in LTLT-Ca cells compared with the MCF-7Ca cells (Fig. 4B). When LTLT-Ca cells were treated with 5 μmol/L of PD98059, MAPK activity returned to the level similar to that of MCF-7Ca cells.

Our studies indicate that growth factor receptors and the MAPK pathway are activated in LTLT-Ca cells. Therefore, we explored the effects of the growth factor receptor inhibitor Gefitinib and MEK1/2 inhibitors U0126 and PD98059 on cells insensitive to letrozole. None of these compounds were effective in inhibiting the growth of MCF-7Ca cells, whereas all of them inhibited growth of LTLT-Ca cells with IC50 of 10 μmol/L for gefitinib and IC50 of 25 μmol/L for U0126 and PD98059 (Fig. 5A-C). These data suggest that the growth factor receptor inhibitor and inhibitors of the MAPK cascade may be useful in blocking pathways activated by long-term letrozole treatment. Interestingly, LTLT-Ca cells treated with 5 μmol/L of PD98059 dramatically up-regulate expression of ERα compared with untreated LTLT-Ca cells, returning levels to those of the parental MCF-7Ca (Fig. 4A). Based on this finding, we hypothesized

**Figure 4.** A, expression of steroidal receptors ER and PGR, growth factor receptor erbB-2, and adapter proteins Grb2 and p-Shc in MCF-7Ca and LTLT-Ca cells. B, up-regulation of signaling proteins p-Raf, p-MEK1/2, p-MAPK, p-p90RSK, and p-Elk in LTLT-Ca cells when compared with the parental MCF-7Ca cells. Cell lysates were prepared as described in Methods. Equal amounts of protein (60 μg) were separated on a denaturing polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking nonspecific binding with 5% nonfat milk in PBS-T, the membranes were incubated with the respective primary antibodies, and specific binding was visualized by using species-specific immunoglobulin G followed by ECL detection (ECL kit) and exposure to ECL X-ray film. After exposure to X-ray film, the membranes were stripped and probed for β-actin to confirm that equal amount of proteins were loaded in each lane. Numbers below the blots represent fold change in protein expression compared with the control obtained by densitometric analysis. For MAPK activity, assay cell lysates were prepared as described in Methods. Proteins (200 μg) were subjected to immunoprecipitation using specific MAPK antibody. Next day, after adding kinase reaction buffer and GST fusion protein of Elk-1 (MAPK substrate), samples were incubated at 30°C for 30 minutes. Samples were analyzed by Western blotting using phospho-Elk-1 antibody.

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Figure 5. Effect of gefitinib (A), U0126 (B), and PD98059 (C) on the growth of MCF-7Ca and LTLT-Ca cells. MCF-7Ca and LTLT-Ca cells were transferred into steroid-free medium for 3 days before plating (1 x 10⁶ cells per well) into 24-well plates. The next day, cells were washed with DPBS and treated with steroid-free medium containing androstenedione (25 nmol/L) and the indicated concentrations of gefitinib, U0126, and PD 98059. The medium was changed every 3 days, and the cells were counted 9 days later using the MTT assay. Percentage of the cell number compared with the vehicle-treated cells (control).
that the combination of treatment with gefitinib and an antiestrogen or aromatase inhibitor to block both pathways should inhibit cell growth more effectively than gefitinib alone. Tamoxifen alone did not have any effect on the growth of LTLT-Ca cells, as one would expect considering the fact that erbB-2 and downstream signaling proteins are up-regulated in these cells. However, when we combined tamoxifen with gefitinib, cell growth was inhibited to a statistically significantly greater extent than with gefitinib alone (P < 0.0001; Fig. 6A). The combination of gefitinib and anastrozole also showed better effects than either gefitinib or anastrozole alone (P < 0.0001; Fig. 6B).

Discussion

As aromatase inhibitors may become first-line therapy for postmenopausal hormone-dependent breast cancer patients, understanding the mechanisms of resistance to their growth controlling effects could provide clues to improving treatment. For this purpose, we have used a model system in which human breast cancer cells expressing the ER and stably transfected with the aromatase gene are grown as tumors in ovariectomized, immunosuppressed mice (30). These tumors are sensitive to the effects of estrogen, antiestrogens, and aromatase inhibitors (30, 32, 33, 36–39). However, overall tumor growth rate may vary between experiments. In this study, the tumors had initially regressed during treatment with letrozole (30) but grew back more slowly than in other reported investigations (32, 36) and had doubled after 32 weeks of treatment. Several mice were sacrificed during the course of the treatment for tumor analysis on weeks 4, 28, and 56. These were animals with the largest tumors, whereas mice with smaller tumors were continued on treatment. The data analysis and Fig. 1 show only the latter tumors which would be expected to have longer doubling times than typically seen in this model. Thus, no comparison of absolute treatment response time can be made between experiments.

Although letrozole caused marked inhibition of tumor growth in this mouse model for an extended period (33) and reduced estrogen levels by 90% in these tumors, the tumors eventually acquired the ability to grow in the presence of letrozole and were
refractory to second line therapy with antiestrogens and letrozole (100 μmol/L/d). Our results indicate that growth occurs by activating alternate signaling pathways. In this study, we present evidence that letrozole treatment by inhibiting estrogen synthesis, initially prevents tumor growth, up-regulates ERα, and induces activation of erbB-2 and MAPK. A similar finding of up-regulated AKT was noted in response to acute androgen deprivation that induced cell cycle arrest in prostate cancer cells (40). When tumors became insensitive to letrozole and were actively growing, ERα expression was decreased and Grb2 was up-regulated. Because Grb2 is known to amplify MAPK signaling, increase in expression of Grb2 could lead to a further increase in activated MAPK. However, additional studies to inhibit or block Grb2 would be necessary to determine this cause and effect relationship.

The above findings were confirmed by our studies in vitro with LTLT-Ca cells isolated from these tumors. The LTLT-Ca cells also grew in the presence of increasing concentrations of letrozole (0.1-10,000 nmol/L) and were refractory to hormonal therapies in vitro. LTLT-Ca cells showed increases in erbB2, Grb2, p-Shc, p-Raf, p-MEK1/2, p-MAPK, p-p90RSK, and p-Erk. Activation of these signaling proteins could result in phosphorylation of the ER despite its decreased level and independent of its binding to the ligand resulting in transcription and cell proliferation. We also found that LTLT-Ca cells treated with 5 μmol/L of PD98059 dramatically up-regulate expression of ERα compared with untreated LTLT-Ca cells, returning levels to those of the parental MCF-7Ca (Fig. 4A). This suggests that abrogation of MAPK leads to up-regulation of ERα and activation of ER-dependent transcription. Similar findings have been reported by Oh et al. (41) who showed that when MCF-7 cells were transfected with Raf-1, ER levels were decreased whereas treatment of these cells with PD98059 resulted in reexpression of ER. These findings imply that inhibition of MAPK could restore sensitivity of LTLT-Ca cells to tamoxifen and anastrozole. Thus, when cells were treated with gefitinib targeting growth factor receptors and resulting in inhibition of MAPK, sensitivity to both tamoxifen and anastrozole returned. Our findings suggest that better control over tumor growth may be achieved by blocking both growth factor receptor pathways as well as ER-mediated signaling. Recent data from Song et al. (42) suggest that ER can coopt growth factor pathways such as the EGF receptor pathway to stimulate the MAPK pathway. Thus, we do not exclude the possibility that other growth factor receptors besides ErbB-2 may be involved in the activation of downstream proteins.

In related studies, expression of AIB-1 was found to be markedly increased in LTLT-Ca cells (39). Intracellular kinases are known to activate not only the ER but also its coactivator protein AIB1 (43). Overexpression of AIB1 has been reported to be sufficient to activate ER despite low ER levels and cause its transcriptional activity (27). Although ERα expression was decreased in LTLT-Ca cells compared with the parental MCF-7Ca, transcription activity was maintained as indicated by the presence of PGR. However, PGR is an estrogen-regulated gene primarily involved in differentiation and may not be indicative of cell proliferation (44). Further studies of ER-mediated genes involved in proliferation are needed to determine whether growth of tumors on letrozole is due to enhanced ligand-dependent or ligand-independent transcription of ER.

It has been reported that when MCF-7 cells were grown in estrogen-depleted medium for a prolonged period (LTLD cells), the MAPK cascade is activated as well as the AKT pathway (37). We also found that the AKT pathway was activated when MCF-7Ca cells were deprived of estrogen in culture (UMB-1Ca; ref. 38). However, in our long-term letrozole treated MCF-7Ca cells (LTLT-Ca), the AKT pathway was not activated as determined by assay of AKT activity and Western immunoblotting for p-AKT (Ser473) and p-AKT (Thr308; data not shown). It is possible that these long-term letrozole-treated cells and tumors have adapted to more extreme conditions of estrogen deprivation making the cells insensitive to hormone therapy.

In the previous study, treatment of the mice was changed from letrozole to antiestrogen treatment when tumors had doubled in volume on letrozole (30). These tumors did not respond to second-line therapy. In another study, letrozole-refractory tumors transplanted into new animals not maintained on letrozole grew equally well with or without androstenedione supplement (33). Treatment with antiestrogens started when transplanted tumors reached a measurable size delayed tumor growth. This experiment was repeated and compared with mice maintained on letrozole (45) from the time of transplantation. These tumors grew slower initially, they did not respond to tamoxifen but did respond to fulvestrant and a higher dose of letrozole. Similarly, tumors in situ that had regressed on letrozole and later switched to a higher dose when tumors were growing but still small responded to this treatment (33). Expression of MAPK and erbB-2 in the transplanted tumors was not increased relative to MCF-7Ca tumors. However, ER expression was greatly increased (45). This suggests plasticity of the tumor cells as they regained some sensitivity to anti-hormone therapy. In contrast, the tumors used in the current study remained in situ and treatment was switched from letrozole to antiestrogens as would occur in the treatment of breast cancer patients. Although most patients are refractory to faslodex as second line treatment after an aromatase inhibitor, a small number of responses have occurred suggesting the possibility that not all patients reach the same degree of estrogen deprivation as represented by this model.

In conclusion, our studies show that the erbB-2 and MAPK signaling pathways are up-regulated in tumors and cells after long-term treatment with letrozole. Inhibition of the MAPK pathway by MAPK inhibitor or growth factor receptor inhibitor, gefitinib, restores tumor sensitivity to antiestrogen therapy. These findings may have useful application in improving treatment for breast cancer patients who relapse during aromatase inhibitor treatment.

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Activation of Mitogen-Activated Protein Kinase in Xenografts and Cells during Prolonged Treatment with Aromatase Inhibitor Letrozole

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