The Role of Growth Factor Receptor Pathways in Human Breast Cancer Cells Adapted to Long-term Estrogen Deprivation

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Abstract

To study the long-term effects of estrogen deprivation on breast cancer, MCF-7Ca human estrogen receptor–positive breast cancer cells stably transfected with human aromatase gene were cultured in the steroid-depleted medium for 6 to 8 months until they had acquired the ability to grow. Proliferation of these cells (UMB-1Ca) was accompanied by increased expression of human epidermal growth factor receptor 2, increased activation of AKT through phosphorylation at Ser473 and Thr308, and increased invasion compared with parental MCF-7Ca cells. Estrogen receptor expression was also increased 5-fold. Although growth was inhibited by the antiestrogen fulvestrant, the IC50 was 100-fold higher than for parental MCF-7Ca cells. Aromatase inhibitor letrozole also inhibited growth at 10,000-fold higher concentration than required for MCF-7Ca cells, whereas anastrozole, exemestane, formestane, and tamoxifen were ineffective at 100 nmol/L. Growth of UMB-1Ca cells was inhibited by phosphatidylinositol 3-kinase inhibitor wortmannin (IC50 ~ 25 nmol/L) and epidermal growth factor receptor kinase inhibitor gefitinib (ZD 1839; IC50 ~ 10 μmol/L) whereas parental MCF-7Ca cells were insensitive to these agents. Concomitant treatment of UMB-1Ca cells with the signal transduction inhibitors and aromatase and tamoxifen restored their growth inhibitory effects. These studies show that estrogen deprivation results in up-regulation of growth factor signaling pathways, which leads to a more aggressive and hormone refractory phenotype. Cross-talk between ER and growth factor signaling was evident as inhibition of these pathways could restore estrogen responsiveness to these cells. (Cancer Res 2005; 65(9): 3903-10)

Introduction

The knowledge that steroids play a pivotal role in the development of breast cancer has been exploited clinically by the development of endocrine treatments, predominantly by estrogen withdrawal or antagonism of the actions of estrogen. The antiestrogen tamoxifen has had a major effect on the treatment of breast cancer (1). Aromatase inhibitors (2) are now proving to be effective and to increase survival (3–5). Nevertheless, despite the long-term benefits, some tumors eventually become resistant to therapy. The beneficial effects of treatment are limited by the ability of tumors to circumvent their need for steroid hormones although estrogen receptors (ER) are maintained in most cases (6, 7). The identification of mechanisms responsible for the development of these resistant conditions is therefore vital for the design of new diagnostics and therapeutic regimes. Estrogens are strongly associated with breast cancer development and tumor growth in both premenopausal and postmenopausal women. Estrogens stimulate the growth of breast cancer in about two thirds of patients whereas estrogen deprivation induces tumor regression. The effects of estrogens on tumor development are mediated by ERα, which induces gene transcription resulting in cell proliferation. The binding of estrogen to the ER induces a cascade of events leading to transcription of estrogen-responsive genes such as cyclin D1 and transforming growth factor α, which are known to stimulate mammary cell proliferation (8). Whereas estrogens stimulate both pre- and postmenopausal breast cancer, following menopause, breast tumors become more sensitive to estrogens as concentration of ER increases with age (9). Hence, first-line hormonal therapy for patients with ER-positive and hormone-sensitive breast cancer has been barrning estrogen from the in situ lesion with the antiestrogen tamoxifen. Whereas the ovary is no longer the main source of estrogen in older women, estrogen production is increased in peripheral sites, such as adipose tissue and breast tissue, and contributes to stimulation of breast cancer (10). Estrogen levels within the breast are equivalent to those of premenopausal women (11). Thus, the intratumoral conversion of androgens to estrogens by aromatase within the breast may be an important mechanism of autocrine stimulation in hormone-dependent breast cancer. Aromatase is an enzyme complex consisting of cytochrome P450arom, a 55 kDa protein of 503 amino acids, and NADPH cytochrome c reductase. Selective inhibition of aromatase for reduced estrogen production is a hypothesis (12) which is now proving useful in the clinic. Recently, aromatase inhibitors such as letrozole have been shown to have advantages over tamoxifen with respect to patient survival and tolerability in studies comparing aromatase inhibitors with tamoxifen as first-line and adjuvant treatments for postmenopausal patients with hormone-dependent advanced breast cancer (4, 13). Results to date indicate that aromatase inhibitors are more effective and better tolerated than tamoxifen. Although tamoxifen has proved to be a remarkably successful breast cancer therapy, patients eventually relapse with their tumors showing estrogen-independent growth and a more invasive cancer phenotype. Several mechanisms have been proposed that contribute to the development of this resistant phenotype. These comprise of activation of predominantly hormone-independent pathways, ligand-independent activation of the ER and ER-mediated transcription, and perturbation of the interactions between ER and coactivators and corepressors of transcription (14, 15). Several lines of evidence strongly suggest that enhanced signal transduction pathways may be one of the key adaptive changes accounting for tamoxifen-resistant growth in breast cancer.

As several aromatase inhibitors are now available in the clinic, we developed a model system to compare these agents and their...
effectiveness to antiestrogens (16–19). Our preclinical model system uses tumors from MCF-7 human breast cancer cell line stably transfected with human placental aromatase gene MCF-7Ca (20). This model has proved accurate in predicting outcomes of a number of clinical trials such as the Anastrozole Tamoxifen and the Combination trial (3, 17, 19). In the present study, we have used the MCF-7Ca cell line to investigate the mechanisms of resistance to estrogen deprivation. This cell line was grown in a steroid-depleted medium for a prolonged period of 8 months (18). The cell growth stopped initially but subsequently resumed. Our findings suggest that long-term estrogen deprivation leads to enhancement of both ER-mediated transcription as well as activation of growth factor receptor signaling pathways. Thus, these studies highlight the importance of the interaction between the ER and growth factor receptors in mediating proliferation in conditions of estrogen deprivation.

Materials and Methods

**Materials.** DMEM, penicillin/streptomycin solution (10,000 IU each), 0.25% trypsin-EDTA (1 mmol/L) solution, Dulbecco’s PBS, and genicin (G418) were obtained from Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT), and androstenedione, tamoxifen, DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-nylterrazolium bromide (MTT), and Tween 20 were obtained from Sigma Chemical Co. (St. Louis, MO). Mitogen-activated protein (MAP) kinase (MAPK) inhibitor PD98059, phosphatidylidyinositol-3-kinase (PI3K) inhibitor wortmannin, antibodies against phospho-AKT (Ser473 and Thr308), AKT, and MAPK, and in vitro kinase assay kits for AKT and MAPK were purchased from Cell Signaling Technology (Beverly, MA).

MCF-7 human breast cancer cells stably transfected with the human aromatase gene (MCF-7Ca) were provided by Dr. S. Chen (City of Hope, Duarte, CA; ref. 20). Letrozole (Femara, G20267) was provided by Dr. D. Evans (Novartis Pharma, Basel, Switzerland). Pure antiestrogen fulvestrant (Faslodex, ICI 182,780), aromatase inhibitor anastrozole, and epidermal growth factor receptor (EGFR) kinase inhibitor gefitinib (ZD 1839, Iressa) were supplied by Dr. A. Wakeling (AstraZeneca Pharmaceuticals, Macclesfield, United Kingdom). Enhanced chemiluminescence (ECL) kit and Hydroxy-ECL nitrocellulose membranes were purchased from Amersham Biosciences (Piscataway, NJ).

**Cell culture.** MCF-7Ca cells were routinely maintained in DMEM with 5% FBS, 1% penicillin/streptomycin solution, and 750 μg/mL G418. Selection of estrogen-deprived UMB-1Ca cells, early passage (passage 6) for MCF-7Ca cells were transferred into steroid-depleted medium, which consisted of phenol red–free improved MEM supplemented with 5% dextran–coated charcoal-treated serum, 1% penicillin/streptomycin, and 750 μg/mL G418. Cells were maintained in this medium for at least 8 months. After 8 months of estrogen deprivation, UMB-1Ca cells had acquired the ability to proliferate in this estrogen-depleted medium (18). Growth studies were done on synchronized cells by first transferring parental MCF-7Ca cells into steroid-free medium whereas UMB-1Ca cells were transferred to low-serum OptiMEM medium for 3 days before plating (1 × 10^6 cells/well) into 24-well plates. The next day, cells were washed with Dulbecco’s PBS and treated with steroid-free medium containing vehicle or the indicated concentrations of estrogens, androstenedione, antiestrogens, aromatase inhibitors, and PI3K inhibitor or MAPK inhibitor. 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 in serum-free medium, and cells were incubated for 2 hours. Medium was then removed and the formazan dye trapped in the living cells was dissolved in DMSO and absorbance was measured in a spectrophotometer at 560 nm. The results were expressed as a percentage of the cell number in the vehicle-treated control wells. IC_{50} values for inhibitors were calculated from the linear regression line of the plot of percent inhibition versus log inhibitor concentration.

**Western blotting.** The cells were lysed in a buffer containing 0.1 mmol/L Tris, 0.5% Triton X-100, and protease inhibitor cocktail (Complete, Boehringer Mannheim, Germany). The protein concentration in each cell lysate was measured using the Bio-Rad method. Equal amounts of 50 μg of proteins were subjected to SDS-PAGE (3 hours at 60 V) and then transferred to a nitrocellulose membrane (1 hour at 90 V). The membrane was probed for specific primary antibody as specified in the protocol of the manufacturer. Immunoreactive bands were visualized using the enhanced chemiluminescence detection reagents according to the instructions of the manufacturer and quantitated by densitometry using Molecular Dynamics software (ImageQuant).

**In vitro protein kinase assay.** The AKT kinase assay was carried out according to the protocol of the manufacturer. Briefly, AKT protein in 200 μL cell lysate, which contains ~200 μg total protein, was immunoprecipitated with immobilized monoclonal antibody for AKT. The next day, the reaction was carried out in the presence of 50 μL 1× kinase buffer supplemented with 200 mmol/L ATP and 2 μg GSK3β fusion protein and incubated for 30 minutes at 30°C. The reaction is terminated by addition of 25 μL 3× SDS sample buffer. The samples are boiled for 5 minutes, vortexed, and then microcentrifuged for 2 minutes. The sample (30 μL) is loaded on to a SDS-PAGE gel and analyzed by Western blotting. The gel is probed with phospho antibody for the specific substrate of each kinase. The gel is quantitated using ImageQuant (Molecular Dynamics). Kinase assays for MAPK were done in a similar manner; Elk-1 was used as substrate for MAPK activity assay (21–23).

**In vitro chemoinvasion assay.** Cells were labeled with fluorescent dye (calcine AM, Molecular Probes, Inc.) and then 100,000 cells/well were placed in the upper chamber of 24-well plates separated by polycarbonate filter membrane of 5 μm pore size, coated with 1% fibrinogen and collagen. Cells were incubated overnight in these chambers with 1% FBS in the bottom chamber that served as chemotaxtractant. The next day, the number of cells that migrated to the lower chamber was counted using FITC index and compared with the number of cells plated. Results were expressed as percentage of cells that migrated to the lower chamber. The percentage of migration was compared with the noninvasive parental MCF-7Ca and the invasive cell line MDA-MB-231 (24).

**Statistical analysis.** All experiments were done twice in replicates of same size and the results are expressed as mean ± SE where applicable. The effects of treatment were compared with MCF-7Ca control cells using Student’s t test on Sigma Plot 2000 and P values of less than 0.001 (P < 0.05) were considered statistically significant.

**Results**

**Progression of hormone-dependent and noninvasive MCF-7Ca cells to estrogen independence and a more invasive phenotype after long-term estrogen deprivation.** MCF-7Ca cells were synchronized by transferring them to steroid-depleted medium for 3 days whereas UMB-1Ca cells were transferred to reduced-serum medium OptiMEM for 3 days. As shown previously, when cultured in estrogen-deprived medium, proliferation of MCF-7Ca cells slowed down but was increased in response to estradiol (E2; ref. 18). MCF-7Ca cells showed maximum growth stimulation in response to 1 nmol/L E2 and 25 nmol/L androstenedione (P < 0.005; ref. 18, 25). UMB-1Ca cells, which had acquired the ability to grow in an estrogen-deprived environment, did not respond to treatment with E2 or androstenedione (aromatizable estrogen precursor; Fig. 1A) indicating their growth was estrogen independent. Thus, after prolonged estrogen deprivation, UMB-1Ca cells have acquired the ability to grow in absence of estrogen and are not responsive to the mitogenic effects of E2.

MCF-7 and MCF-7Ca cells are weakly invasive. To examine whether UMB-1Ca cells progress to a more invasive phenotype, in vitro chemoinvasion assays were done (24, 26). MCF-7Ca cells showed 11.14 ± 0.44% migration. UMB-1Ca cells showed
Figure 1. A, effect of E2 and androstenedione on the growth of MCF-7Ca and UMB-1Ca human breast cancer cells in vitro. MCF-7Ca cells were cultured in steroid-free medium for 3 days before plating and UMB-1Ca cells were cultured in reduced-serum medium OptiMEM 3 days before plating. Triplicate wells were then treated with the indicated concentrations of E2 and androstenedione for 8 days and the media were refreshed every 3 days. Cell proliferation was measured on day 9 using the MTT assay as described in Materials and Methods. Cell viability is expressed as the percentage of the cells compared with the control wells (MCF-7Ca cells). Columns, mean of triplicate experiments; bars, SE. For MCF-7Ca cells, estradiol or androstenedione treatment significantly increased cell viability (P < 0.005). B, in vitro chemoinvasion assay. Cells labeled with fluorescent dye were plated (100,000 cells/well) and placed in the upper chamber of 24-well plates separated by a polycarbonate filter membrane of 5 μm pore size, coated with 1% fibronectin and collagen. 1% FBS was added to the bottom chamber to serve as a chemotactic attractant. Cells were incubated overnight in the chambers and the next day the number of cells in the bottom chamber was counted using FITC index and compared with the number of cells plated. Results are expressed as the percentage of cells that migrated to the bottom chamber. Percentage migration of UMB-1Ca cells was compared with the noninvasive parental MCF-7Ca and the invasive cell line MDA-MB-231 (**, P < 0.0005 compared with parental MCF-7Ca cells). C, in vitro antiproliferative effects of letrozole, exemestane, anastrozole, formestane, tamoxifen, and fulvestrant (100 nmol/L each) on UMB-1Ca human breast cancer cells. UMB-1Ca cells were cultured in reduced-serum medium OptiMEM for 3 days before plating. Triplicate wells were then treated with 100 nmol/L of aromatase inhibitors or antiestrogens for 8 days and the media were refreshed every 3 days. Cell proliferation was measured on day 9 using MTT assay as described in Materials and Methods. The percentage of the cells was compared with the control wells. Columns, mean of triplicate experiments; bars, SE. Letrozole- (*) and fulvestrant- (**) treated cells were statistically different from control (*, P < 0.005; **, P < 0.0005).
39.73 ± 2.23% migration, and MDA-MB-231 showed 85.19 ± 3.78% migration (Fig. 1B). Thus, UMB-1Ca cells showed a significant increase in their ability to migrate compared with parental MCF-7Ca cells (P < 0.0005). These results suggest that when hormone-dependent breast cancer cells progress to estrogen independence, they develop a more aggressive and invasive phenotype.

Next, we examined the effect of endocrine manipulations on UMB-1Ca cell proliferation. Cells were treated with different aromatase inhibitors anastrozole, formestane, exemestane, and letrozole, or antiestrogens tamoxifen and fulvestrant. UMB-1Ca cells showed significant growth inhibition in response to fulvestrant (100 nmol/L, P < 0.0005) and partial growth inhibition in response to letrozole (100 nmol/L, P < 0.005; Fig. 1C). Fulvestrant inhibited growth of both UMB-1Ca and MCF-7Ca in a dose-dependent manner. The IC50 value for MCF-7Ca was 5.37 ± 1.9 nmol/L whereas the IC50 for UMB-1Ca cells was 295.8 ± 6.8 nmol/L (data not shown). Thus, UMB-1Ca cells were 100-fold less sensitive to the antiestrogen fulvestrant than MCF-7Ca cells. UMB-1Ca cells were resistant to growth inhibitory effects of tamoxifen or aromatase inhibitors anastrozole, exemestane, and formestane. However, the cells were sensitive to a much large dose of letrozole with IC50 for growth inhibition of 1.25 ± 0.2 nmol/L for MCF-7Ca cells but 8.95 ± 3.4 μmol/L for UMB-1Ca cells (data not shown). Thus, UMB-1Ca cells are 10,000-fold less sensitive to letrozole than parental MCF-7Ca cells.

ERα expression in UMB-1Ca cells was increased by 4.5-fold compared with the levels in MCF-7Ca cells (Fig. 2). However, progesterone receptor (which is an estrogen inducible gene) expression was not significantly changed (Fig. 2), suggesting that ERα transcriptional activity was maintained. From these results it is clear that although UMB-1Ca cells show estrogen-independent growth, ERα is still functional and is involved in the growth of these cells.

Activation of growth factor receptor pathways. Numerous studies have shown the involvement of growth factor pathways in the proliferation of breast cancer cells after prolonged estrogen deprivation (6, 27). It is known that estrogens can stimulate growth factor production, whereas growth factors can regulate the process of ER-mediated transcription. Therefore, we examined the expression of the growth factor receptor erbB-2, an EGFR that is activated in a ligand-independent manner and is overexpressed in 20% of the hormone therapy refractory breast cancer patients. UMB-1Ca cells show a 4.1-fold increase in human EGFR-2 (HER-2) protein compared with parental MCF-7Ca cells (Fig. 3A). It has been shown that overexpression of HER-2 in MCF-7 breast cancer cells results in MAPK hyperactivity. MAPK hyperactivity promotes increased association of ER with coactivators and reduces association with corepressors, thus favoring estrogen inducible gene transcription (27, 28). However, UMB-1Ca cells did not show overexpression of phospho-MAPK (Fig. 3A) and MAPK was not found to be hyperactive (Fig. 3B). To confirm this finding, we examined the effect of MAP/extracellular signal-regulated kinase kinase 1 inhibitor PD98059 on growth of UMB-1Ca cells. Antiproliferative effects of this drug were not observed in UMB-1Ca cells (data not shown) although other cells that express hyperactive MAPK were inhibited by PD98059. These results suggest that MAPK pathway is not involved in growth adaptation of these cells.

Previous reports suggest that phosphorylation site Ser167 of ER is involved in the ability of HER2 to mediate tamoxifen resistance (6, 27). This is consistent with other reports that propose an active interplay between PI3K/AKT signaling and ER. ERα has also been shown to bind to the regulatory subunit p85 of PI3K in a ligand-dependent manner activating AKT and downstream survival pathways. AKT has been shown to induce phosphorylation of ERα at Ser167, activate ER-mediated transcription, and protect MCF-7 cells from tamoxifen-induced apoptosis (28–30). Therefore, we examined expression and activation of the AKT protein. Phosphorylation of AKT at Ser473 or Thr308 is responsible for activation of AKT. UMB-1Ca cells showed increased expression of phospho-AKT at Ser473 by 3.3-fold and at Thr308 by 2.7-fold (Fig. 4A). Also, the kinase activity of the protein in UMB-1Ca cells was found to be higher than in parental MCF-7Ca cells (Fig. 4B).

It is known that growth factor receptors can activate the PI3K/AKT signaling pathway. PI3K is an enzyme that causes activation of PI3K-dependent kinase 1 (PDK1), which in turn activates AKT. Once activated, AKT phosphorylates a number of proteins leading to regulation of metabolism (via GSK3β), translational control (via p70S6k), cell survival (via FKHR, BAD, and caspase-9), and cell cycle regulation through cyclin D1. To confirm our finding that the AKT pathway is activated in UMB-1Ca cells, we examined several of the proteins in this pathway, including phospho-PDK1 (Fig. 5A), which is upstream of AKT, along with downstream targets of AKT including Bcl-XL, phospho-Bad, phospho-GSK3β, and Forkhead receptor (Fig. 5A). Activation and up-regulation of all these proteins confirms that the PI3K/AKT pathway is indeed activated in UMB-1Ca cells. Furthermore, Pten, a protein encoded by tumor suppressor gene PTEN, which negatively regulates the PI3K/AKT pathway, was examined. There was no change in the expression of phospho-Pten confirming that Pten was turned off in both MCF-7Ca and UMB-1Ca cells (Fig. 5A).

Figure 2. Western immunoblotting analysis of whole cell lysates from MCF-7Ca and UMB-1Ca cells cultured in vitro for estrogen and progesterone receptor. Blot shows ERα protein expression at 66 kDa and progesterone receptor (PgR) protein expression at 98 kDa. Experimental protocol was as described in Materials and Methods. Lane 1, MCF-7Ca; lane 2, UMB-1Ca. Blots were stripped and probed for β-actin (bottom) to verify equal amount of protein loaded in each lane. Representative of three independent experiments.

1 Unpublished data.
AKT and ER Pathway Cross-Talk after Long-term Estrogen Deprivation

Figure 3. A. Western immunoblotting analysis of whole cell lysates from MCF-7Ca and UMB-1Ca cells cultured in vitro for HER2, MAPK, and phospho-MAPK. HER-2 protein expression at 185 kDa, phospho- and total MAPK protein expression at 42 and 44 kDa. Experimental protocol was as described in Materials and Methods. Lane 1, MCF-7Ca; lane 2, UMB1Ca. Blots were stripped and probed for β-actin to verify equal amount of protein loaded in each lane. Representative of three independent experiments. B, MAPK activity assay. Cells were cultured in steroid-free medium with indicated treatment. Cell lysates were prepared as described in Materials and Methods. Two-hundred micrograms of proteins were subjected to immunoprecipitation using specific MAPK antibody. The next day, after adding kinase reaction buffer and glutathione S-transferase (GST) fusion protein of Elk-1 (MAPK substrate), samples were incubated at 30°C for 30 minutes. The reaction was terminated by addition of 3× sample buffer and samples were analyzed using Western blotting as described in Materials and Methods. Representative of three independent experiments.

Increased activation of AKT is associated with growth of UMB-1Ca cells. To establish whether activated AKT is involved in the growth adaptation of UMB-1Ca cells to the low estrogen environment, the effect of PI3K inhibitor wortmannin on cell growth was determined in culture. Proliferation of UMB-1Ca cells was inhibited (IC50 = 17.54 ± 1.37 mmol/L) by wortmannin, whereas MCF-7Ca cells showed no effect of this agent on growth rate (data not shown). This suggests that AKT is involved in the growth of UMB-1Ca cells but does not have a role in MCF-7Ca. Our report provides evidence that growth factor pathways are activated in breast cancer cells after prolonged estrogen deprivation and interact with the ER.

Cross-talk between growth factor receptor and estrogen receptor α pathway. In addition to the role of AKT in survival, it is also documented that AKT can activate ERα through phosphorylation at Ser118 and Ser167. Interaction between AKT and ERα was evident when cell lysates of MCF-7Ca and UMB-1Ca cells were first immunoprecipitated with anti-AKT antibody and then immunoblotted with the antibody for ERα. Increased association between AKT and ERα was observed in UMB-1Ca cells (Fig. 5B), whereas when cells were treated with wortmannin and ER down-regulator fulvestrant, no association between ER and AKT was detected. Further evidence that ER and AKT interaction was gained from a set of experiments in which the growth factor receptor pathway activation in UMB-1Ca cells was obstructed by either PI3K inhibitor wortmannin or EGFR kinase inhibitor gefitinib (ZD 1839, Iressa). Gefitinib (ZD 1839) is also known to inhibit HER-2-mediated cell proliferation and is currently in clinical trials. Gefitinib (ZD 1839) had no effect on growth of MCF-7Ca cells but inhibited growth of UMB-1Ca cells (IC50 = 6.87 ± 2.68 μmol/L). The cells were also treated concomitantly with either tamoxifen or anastrozole. Both compounds were effective in MCF-7Ca cells (IC50 = 28 ± 1.27 mmol/L for anastrozole and 128 ± 0.2 mmol/L for tamoxifen) but were ineffective in inhibiting the growth of UMB-1Ca cells by themselves (Fig. 1C, dose response curve not shown). However, in combination with gefitinib (ZD 1839), the responsiveness of the cells to tamoxifen and anastrozole was restored (Fig. 6). Cell proliferation was inhibited 40% with gefitinib (ZD 1839) alone (58.48 ± 1.24% cell proliferation compared with control), 60% with combination of gefitinib (ZD 1839) and tamoxifen (34.96 ± 1.15% cell proliferation compared with control), and 65% with combination of gefitinib (ZD 1839) and anastrozole (26.46 ± 2.42% cell proliferation compared with control). Similar results were obtained when gefitinib (ZD 1839) was used in combination with anastrozole (20.81 ± 0.24% cell proliferation compared with control). Similar results were obtained with the combination of gefitinib (ZD 1839) and tamoxifen (17.24 ± 0.24% cell proliferation compared with control)

Figure 4. A. Western immunoblotting analysis of whole cell lysates from MCF-7Ca and UMB-1Ca cells cultured in vitro for AKT. Phospho-AKT (Ser473) and phospho-AKT (Thr308) protein expression at 60 kDa, and total AKT protein expression 60 kDa. Experimental protocol was as described in Materials and Methods. Lane 1, MCF-7Ca; lane 2, UMB-1Ca. Blots were stripped and probed for total AKT and for β-actin to verify equal amount of protein loaded in each lane. Representative of three independent experiments. B, AKT kinase activity assay. Cells were cultured in steroid-free medium with indicated treatment. Cell lysates were prepared as described in Materials and Methods. Two-hundred micrograms of proteins were subjected to immunoprecipitation using specific AKT antibody. The next day, after adding kinase reaction buffer and GST fusion protein of GSK3β (AKT kinase substrate), samples were incubated at 30°C for 30 minutes. The reaction was terminated by addition of 3× sample buffer and samples were analyzed using Western blotting as described in Materials and Methods. The results obtained were representative of three independent experiments.
were observed when PI3K inhibitor wortmannin was combined with tamoxifen or anastrozole. In addition, when wortmannin and fulvestrant were combined, an additive effect on growth inhibition was seen. Wortmannin and fulvestrant both showed 40% growth inhibition alone at 10 nmol/L (60.59 ± 9.29% cell proliferation compared with control in wortmannin-treated group and 60.54 ± 3.62% cell proliferation compared with control in fulvestrant-treated group). However, when combined, these drugs cause 80% growth inhibition (22.47 ± 1.97% cell proliferation compared with control).

Discussion

Our results indicate that after prolonged estrogen deprivation, MCF-7Ca cells are no longer responsive to growth effects of estrogen. This is in contrast to wild-type MCF-7 cells, which, after long-term estrogen deprivation, show hypersensitivity to estradiol (6, 7). However, UMB-1Ca cells retained some sensitivity to the inhibitory effects of the ER down-regulator fulvestrant, but at a 10-fold higher dose than needed to inhibit the growth of MCF-7Ca cells, indicating that ER is still functional in growth regulation of these cells (6).

Figure 5. A, Western immunoblotting analysis of whole cell lysates from MCF-7Ca and UMB-1Ca cells cultured in vitro for proteins in the PI3K/AKT pathway. Cells were cultured in steroid-free medium with indicated treatment. Cell lysates were prepared as described in Materials and Methods. Two-hundred micrograms of proteins were subjected to immunoprecipitation using antibody specific for phospho-PDK1 (Ser241) at 58 to 68 kDa, Bcl-XL protein expression at 30 kDa, phospho-BAD (Ser136) at 23 kDa, phospho-GSK3β (Ser9) protein expression at 46 kDa, phospho-FKHR (Ser256) protein expression at 75 kDa, and phospho-Pten protein expression at 54 kDa. Experimental protocol was as described in Materials and Methods. Lane 1, MCF-7Ca; lane 2, UMB-1Ca. Blots were stripped and probed for β-actin to verify equal amount of protein loaded in each lane. Representative of three independent experiments. B, communoprecipitation of whole cell lysates from MCF-7Ca and UMB-1Ca cells cultured in vitro for ERα protein association with AKT. The experimental protocol was as described in Materials and Methods. Two-hundred micrograms of cell lysate were subjected to immunoprecipitation with anti-AKT antibody and then were subjected to Western immunoblotting using ERα antibody. Lane 1, MCF-7Ca; lane 2, UMB-1Ca; lane 3, UMB-1Ca cells treated with wortmannin (10 nmol/L); lane 4, UMB-1Ca cells treated with Fulvestrant (10 nmol/L); lane 5, UMB-1Ca cells treated with wortmannin and fulvestrant (10 nmol/L each). Representative of three independent experiments.

Figure 6. In vitro antiproliferative effect of combination of signal transduction inhibitors gefitinib (ZD 1839; 5 μmol/L) and wortmannin (10 nmol/L) with tamoxifen and anastrozole on UMB-1Ca cells. Each set of columns shows the effect of signal transduction inhibitor alone, either tamoxifen or anastrozole alone, and combination at concentrations indicated. Starting from left, first bar is control, then gefitinib at 5 μmol/L, third tamoxifen at 1 μmol/L, then combination of gefitinib 5 μmol/L and tamoxifen 1 μmol/L. Combination of signal transduction inhibitor (STI) with antiestrogen (AE) tamoxifen or aromatase inhibitor (AI) anastrozole had a significantly greater effect on inhibition of UMB-1Ca cell proliferation than either drug alone (*, P < 0.05).
Evidence exists for the activation of PI3K and AKT by growth factor receptors leading to activation of Erα. Several reports have shown phosphorylation and transcriptional activation of Erα by AKT (30, 31). This report provides further indications that signaling pathways such as ER and growth factors are coupled. Thus, when UMB-1Ca and MCF-7Ca cell lysates were subjected to immunoprecipitation with anti-AKT antibody and probed with anti-Erα antibody, Erα-AKT complex was evident in UMB-1Ca cell lysates (Fig. 5B).

The ability of these pathways to affect Erα function suggests that Erα may be a point of convergence of the PI3K/AKT pathway. Although it is unclear whether these pathways act independently, cross-talk, or act together resulting in estrogen-independent growth of cells and loss of response to hormone therapy, involvement of these pathways seems to be clearly responsible. Thus, when AKT pathway was inhibited by wortmannin, UMB-1Ca cells were able to respond to aromatase inhibitors and antiestrogens. In another experiment, gefitinib (ZD 1839; EGFR kinase inhibitor) was used to inhibit HER-2 mediated cell proliferation. This restored the sensitivity of UMB-1Ca cells to tamoxifen- and anastrozole-mediated growth inhibition. These results further implicate the role of ER together with growth factor receptor pathways in the growth response of UMB-1Ca cells.

In addition to increased activation of AKT, which leads to phosphorylation and ligand-independent activation of Erα, AKT confers additional survival advantage through activation of antiapoptotic proteins such as Bcl-xl, BAD, and FKHR. It is well documented that AKT acts as an important mediator of cell survival by directly inhibiting different proapoptotic signals, such as Bad and the forkhead family of transcription factors (32–34). Bad is a proapoptotic member of Bcl-2 family that can displace Bax from its binding to Bcl-2 and Bcl-xl, resulting in cell death. Survival factors such as AKT can inhibit the apoptotic activity of Bad by phosphorylating at Ser136. This phosphorylation event results in the binding of Bad to 14-3-3 proteins and inhibition of binding Bad to Bcl-2 or Bcl-xl. This cytosolic sequestration of Bad results in mitochondrial membrane stabilization and inhibition of both cytochrome c release and apoptosis (25). These proteins were all expressed at increased levels in UMB-1Ca cells as compared with MCF-7Ca cells. In addition to its role in survival, AKT is involved in regulation of cell cycle by preventing GSK3β-mediated phosphorylation and degradation of cyclin D1 and by negatively regulating the cyclin-dependent kinase inhibitor such as p27kip1.

In conclusion, our studies indicate that MCF-7Ca cells deprived of estrogen lose their ability to respond to the mitogenic effects of estrogen and become resistant to tamoxifen and several aromatase inhibitors. Only partial resistance was observed to aromatase inhibitor letrozole and the ER down-regulator fulvestrant, suggesting that these cells have not completely adapted to estrogen independence. These changes were accompanied by increased expression and activity of erbB-2 tyrosine kinase receptor and proteins in the PI3K/AKT signaling pathway. Inhibition of this pathway with gefitinib (ZD 1839) or wortmannin restored the sensitivity of the cells to antiproliferative effects of tamoxifen and anastrozole. Thus, targeting these pathways may be a useful approach for treating patients with tumors resistant to hormone therapy. In addition, as aromatase inhibitors may now become first choice of treatment for postmenopausal ER-positive breast cancer (3–5, 15), development of resistance to these drugs is a concern. In patients where AKT overexpression leads to loss of responsiveness to endocrine therapy, AKT might constitute a new prognostic marker as well as a target for therapeutic intervention.

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References
swelling-induced translocation of rat liver Na(+)/
taurocholate cotransport polypeptide is mediated via
the phosphoinositide 3-kinase signaling pathway. J Biol
22. Deregibus MC, Buttiglieri S, Russo S, Bussolati B,
Camussi G. CD40-dependent activation of phosphati-
dylinositol 3-kinase/Akt pathway mediates endothelial
cell survival and in vitro angiogenesis. J Biol Chem
induces the Akt-dependent activation of endothelial
nitric-oxide synthase in vascular endothelial cells. J Biol
“chemoinvasion assay”: a tool to study tumor and
endothelial cell invasion of basement membranes. Int J
25. Thiantanawat A, Long BJ, Brodie AM. Signaling
pathways of apoptosis activated by aromatase inhib-
EW. Epidermal growth factor promotes MDA-MB-231
breast cancer cell migration through a phosphatidyli-
nositol 3'-kinase and phospholipase C-dependent
Adaptive hypersensitivity following long-term estrogen
depivation: involvement of multiple signal pathways.
28. Kurokawa H, Arteaga CL. ErbB (HER) receptors can
abrogate antiestrogen action in human breast cancer by
multiple signaling mechanisms. Clin Cancer Res 2003;9:
511–58.
29. Nicholson BL, Gee JM. Oestrogen and growth factor
cross-talk and endocrine insensitivity and acquired
30. Campbell RA, Bhat-Nakshatri P, Patel NM,
Constantinidou D, Ali S, Nakshatri H. Phosphatidyli-
nositol 3-kinase/AKT-mediated activation of estrogen
receptor α: a new model for anti-estrogen resistance.
31. Sun M, Paciga JE, Feldman BL, et al. Phosphatidyli-
nositol-3-OH Kinase (PI3K)/AKT2, activated in breast
cancer, regulates and is induced by estrogen receptor α
(ERα) via interaction between ERα and PI3K. Cancer
32. Fry MJ. Phosphoinositide 3-kinase signalling in
breast cancer: how big a role might it play? Breast
33. Roynons D, Slegers H. Phosphatidylinositol 3-kinases
34. Hill MM, Hemmings BA. Inhibition of protein kinase
B/Akt. implications for cancer therapy. Pharmacol Ther
The Role of Growth Factor Receptor Pathways in Human Breast Cancer Cells Adapted to Long-term Estrogen Deprivation


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