Expression of Constitutively Active Akt-3 in MCF-7 Breast Cancer Cells Reverses the Estrogen and Tamoxifen Responsivity of these Cells in Vivo

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Abstract

Purpose: Prior studies had suggested that Akt activity is elevated in a subset of breast cancers. In this study, to test the effect of active Akt-3 on estrogen receptor function, we have produced MCF-7 cells, which express active Akt-3 and examined the estrogen responsiveness of these cells in vivo and in vitro.

Experimental Design: MCF-7 cells expressing active Akt-3 were studied for estradiol (E2) responsiveness in vitro by both using an estrogen receptor element reporter construct as well as looking at induction of endogenous genes. These cells were also studied in vivo after injection into nude, ovariectomized mice by following tumor growth rates in the presence or absence of E2, tamoxifen, or the pure antiestrogen, ICI 182,780 (fulvestrant).

Results: Akt-3-expressing cells were found to produce tumors in mice in the absence of E2 that were approximately equivalent in size to control cells in mice given E2. Moreover, the formation of tumors by the Akt-3 cells was greatly suppressed by E2, stimulated by tamoxifen, and unaffected over, the formation of tumors by the Akt-3 cells was greatly suppressed by E2, stimulated by tamoxifen, and unaffected by ICI 182,780. In the in vitro assays for gene induction by E2, the Akt-3-expressing cells exhibited similar E2 and tamoxifen responsiveness as the control cells.

Conclusions: These results indicate that expression of active Akt-3 in MCF-7 cells results in E2-independent tumor growth. Moreover, the growth of these tumors is inhibited by E2 and enhanced by tamoxifen. Finally, these tumors are resistant to ICI 182,780. These findings suggest that the amount of active Akt present in breast cancers may be important in the relative efficacy of different treatments.

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3 The abbreviations used are: PIP3, phosphatidylinositol 3,4,5-phosphate; ER, estrogen receptor; ERE, estrogen-responsive element; E2, estradiol; DMEM:F12, DMEM:Ham’s F-12; PRF, phenol red free; FBS, fetal bovine serum.
endogenous Akt-3 and are estrogen responsive. The present work reports on the in vivo and in vitro effects of Akt-3 on the estrogen responsiveness of these cells. In particular, we have found that Akt-3 expression renders the MCF-7 cells estrogen-independent for tumor formation. Moreover, E2 inhibits the tumor formation by the Akt-3-expressing MCF-7 cells, whereas tamoxifen actually stimulates their growth. Finally, a pure estrogen antagonist (ICI 182,780 or fulvestrant; Ref. 17) had little effect on their growth. These results suggest that the amount of active Akt present in breast cancer may play a role in determining the efficacy of different treatments.

Materials and Methods

Cell Lines and Culture Conditions. MCF-7 cells (a kind gift of Dr. Daisy De Leon, Loma Linda University, Loma Linda, CA) were grown in DMEM:F12 (Life Technologies, Inc., Grand Island, NY) containing phenol red and supplemented with 10% FBS (Gemini, Calabasas, CA), streptomycin (Life Technologies, Inc.), and penicillin (Life Technologies, Inc.). Where indicated, the cells were transferred to PRF, serum-free DMEM:F12. The MCF-7 cells were transfected with either the pCDNA 3.1 Zeo (+) plasmid (Invitrogen, Carlsbad, CA) or the same plasmid encoding a constitutively active version of Akt-3 (18). Transfections were carried out with Lipofectamine (Invitrogen), and the cells were selected with Zeocin (650 μg/ml; Invitrogen) and cloned by limiting dilution. The resulting clones were screened with anti-p-Akt antibodies (Cell Signaling, Beverly, MA). PC-3 cells were cultured in RPMI 1640 supplemented with 5% FBS, penicillin, and streptomycin.

Western Blot Analyses. Cells and frozen tumor tissues were disrupted in lysis buffer [50 mM HEPES (pH 7.6), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na3VO4, 10 μM aprotinin, 10 μM leupeptin, 100 mM okadaic acid, 10 mM NaF, 2 mM NaPPi, 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulphonyl fluoride]. Lysates were stabilized by centrifugation for 15 min at 15,000 × g before immunoprecipitation or Western blotting. The protein concentrations in each sample were measured, and an equal amount of protein from each cell type was used for Western blotting or immunoprecipitation. Akt phosphorylation was determined by probing Western blots with p-Akt-Ser473 antibody (Cell Signaling). Akt-1 levels were measured by Western blotting with an antibody to Akt-1 (Transduction Laboratories, San Diego, CA). For immunoprecipitations of the ER, lysates were incubated for 2 h at 4°C with 10 μl of anti-ER-α antibody (Santa Cruz Biotechnology, Santa Cruz, CA) bound to 25 μl of protein A beads. The beads were washed twice with lysis buffer, twice with PBS, and analyzed by Western blotting with the anti-ER antibody or an anti-pSer167 ER antibody (Cell Signaling). Detection of bound antibody was carried out after a subsequent incubation with secondary antibody and using one of the following substrates: Western Lightning CDP Star (Perkin-Elmer, Boston, MA); West Pico Chemiluminescence (Pierce, Rockford, IL); or NBT/BCIP (Life Technologies, Inc.) Reagents.

Tumor Studies. MCF-7 cells were harvested by trypsinization, added to serum containing medium and washed twice with cold HBSS. The cells were resuspended in HBSS containing 0.04% DNase (Sigma, St. Louis, MO). Cells (4 × 106/mouse) were injected s.c. into the flank of ncr-nu female mice (Taconic, Germantown, NY). As indicated, mice were concurrently implanted with either E2 (SE-121, 0.72 mg, 60-day release; Innovative Research of America, Sarasota, FL) or tamoxifen (SE-361, 5 mg, 60-day release; Innovative Research of America) pellets. In some experiments, mice received a weekly s.c. injection of 100 μl of fulvestrant (50 mg/ml; AstraZeneca, Wilmington, DE). Tumors were removed after 28 days, weighted, and measured and either sectioned or lysed as described above.

ERE Reporter Assays. MCF-7 cells in 6-well plates were cotransfected with an ERE-luciferase (firefly) reporter plasmid, pVit ERE-Luc (Ref. 19; a gift of Dr. Ron Weigel, Jefferson University) and a control plasmid-encoding Renilla luciferase (pRL-CMV; Promega, Madison, WI) using FuGene 6 (Roche, Indianapolis, IN). Eighteen h after transfection, cells were washed twice with PBS and then incubated in PRF DMEM:F12 containing 10% charcoal-stripped FBS (Gemini) and the specified agents (either vehicle, E2, or tamoxifen). After an additional 18 h, firefly and Renilla luciferase activities were determined sequentially in the lysates according to the manufacturer’s protocol (Promega).

Induction of Endogenous Gene Transcription. After 5 days in PRF DMEM:F12 with 10% charcoal-stripped FBS, cells were washed twice with PBS and then incubated in PRF, serum-free DMEM:F12, and either vehicle, 1 mM 4-hydroxy-tamoxifen or 10 nM E2 as specified. Twenty-four h later, the cells were lysed, total RNA was extracted using Trizol (Life Technologies, Inc.), electrophoresed, and transferred to membranes. Membranes were prehybridized (Clontech ExpressHyb Hybridization Solution, Palo Alto, CA) for 2 h at 65°C and then incubated with 32P-labeled probes for pS2 (20) or GREB1 (Ref. 21; labeled by the random primer labeling kit from Invitrogen) overnight at 65°C, washed, and exposed for autoradiography. The rRNA was visualized by ethidium bromide staining.

Proliferation Assays. MCF-7 cells were washed twice with PBS and seeded into 96-well plates at 1000 cells/well in PRF, serum-free DMEM:F12 with penicillin, streptomycin, and 0.02% BSA. After the specified times, 20 μl/well MTS CellTiter Aquous One Solution (Promega) were added, and the absorbance at 490 nm was recorded after 45 min with an ELISA plate reader. Triplicate control wells containing the same media and the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium solution were used to determine background absorbance. A standard curve with various numbers of the different cell types tested was used to determine the absorbance/cell number.

Quantification of Cell Death and Apoptosis. MCF-7 cells were plated in 60-mm plates in PRF media containing 5% charcoal-stripped FBS and treated as indicated with vehicle (ethanol) or 1 μM 4-hydroxy-tamoxifen (Sigma). The media and treatments were changed every other day. After 7 days, triplicate plates were trypsinized, stained with trypan-blue, and counted. Apoptosis was measured after staining the cells with fluorescent annexin V-FITC (Roche) and fluorescence-activated cell sorting analysis as per the manufacturer’s recommendations. The cells were also stained with propidium iodide to identify necrotic cells, and these were gated out so as not to be included in the analyses.
Results

Transfection and Expression of Constitutively Active Akt-3 in MCF-7 Cells. MCF-7 cells were transfected with either empty plasmid or a plasmid encoding a constitutively active version of Akt-3. After drug selection, either the uncloned pool population of cells was used or, for the Akt-3-transfected cells, individual clones of Akt-3-expressing cells were isolated. In A, the levels of active, phosphorylated Akt in the parental MCF-7 cells, the plasmid transfected pool (Puncl.), the Akt-3-transfected pool (A3uncl.), and the two clones of Akt-3-expressing cells (A3B5 and A3G4) are shown as assessed by Western blotting (WB) with an antibody to the pSer473 site in Akt-1, which also cross-reacts with the functionally equivalent site pSer472 in Akt-3 (18). To verify that comparable amounts of cellular protein were analyzed, levels of an irrelevant protein (insulin-degrading enzyme, IDE) were assessed by immunoblotting (37). For comparison, the parental MCF-7 cells were also treated with insulin as indicated to show the levels of endogenous p-Akt (mostly Akt1) in these cells after stimulation. In addition, to determine whether the expression of Akt-3 affected the levels of Akt-1 in these cells, the total amounts of Akt-1 were assessed in the various cells lines by immunoblotting with an antibody to this protein. The levels of active phosphorylated Akt-3 expressed in the MCF-7 cells (A3G4) were also compared with the levels of active endogenous Akt in a PTEN-deficient prostate cell line (PC-3) cells (B).

Constitutively active Akt-3 did not materially change the endogenous levels of Akt1 in these cells (Fig. 1A). The levels of active phosphorylated Akt-3 in the transfected Akt-3 cells (A3G4) were comparable with the endogenous levels of phosphorylated Akt in a prostate cancer cell line (PC-3) that lacks PTEN (23) and contains constitutively active Akt (Fig. 1B).

Tumor Formation by the Akt-3-expressing MCF-7 Cells. The uncloned population of MCF-7 cells transfected with the plasmid (Puncl.) or Akt (A3uncl.) as well as a cloned population of Akt-3-expressing cells (A3B5) were injected into the hind flanks of nude female mice. After 28 days, the tumors were measured (Fig. 2A). As expected, E2 supplementation greatly increased the size of the tumors formed by the control MCF-7 cells. The Akt-3-expressing MCF-7 cell clone (A3B5) formed larger tumors than the control cells in the absence of E2. Most surprising, tumors formed by the Akt-3-expressing cells were smaller when E2 was given to the mice (Fig. 2A).

To eliminate the influence of endogenous E2 on these results, subsequent studies were performed with ovariec-tomized mice. In these mice, the Akt-3-expressing MCF-7 cells (A3B5) again formed tumors in the absence of E2 (Fig. 2B). The sizes of these tumors were comparable with those formed by the control MCF-7 cells when the mice with these cells were given E2, indicating that...
Akt-3 expression rendered the A3B5 cells estrogen independent. These results were not unique to the particular clone analyzed because similar results were obtained with the population of uncloned Akt-3-expressing cells (Fig. 2B).

As was observed in the nonovariectomized mice, E2 inhibited the growth of tumors from Akt-3-expressing cells in the ovariectomized mice (Fig. 2B). Tumor formation by the Akt-3-expressing MCF-7 cells was then measured in ovariectomized mice given either tamoxifen, a partial ER antagonist, or ICI 182,780 (fulvestrant), a pure ER antagonist (17). Tamoxifen caused a 3-fold increase in the size of the tumors formed with the Akt-3-expressing cells. In contrast, fulvestrant had no significant effect on tumor size (Fig. 3).

Characterization of the ER and E2 Responsiveness of the Akt-3-expressing MCF-7 Cells in Vitro. Levels of the ER in control and Akt-3-expressing cells were determined by immunoblotting. An approximate 70% decrease in ER levels was observed both in the uncloned population of Akt-3-expressing cells as well as the clone (A3B5) of Akt-3-expressing cells (Fig. 4A). The amount of ER was also lower in the tumors formed by the Akt-3-expressing cells compared with control cells (Fig. 4C). The amount of ER phosphorylated on Ser167 in the two cell types was also determined by immunoblotting. In contrast to the decreased total levels of ER in the Akt-3-expressing cells, the levels of Ser167-phosphorylated ER were greater in the Akt-3-expressing cells and tumors compared with control cells (Fig. 4, B and C).

To test the ability of the ER in the Akt-3-expressing cells to mediate an estrogen response, we transiently transfected the cells with an ERE-reporter construct, treated the cells with either E2 or tamoxifen, and then assayed the amount of luciferase activity generated. As expected, E2 stimulated the amount of transcriptional activity of this reporter construct in the control cells whereas tamoxifen did not (Fig. 5A). In the Akt-3-expressing clone, the basal levels of the ERE-reporter activity were lower, consistent with the lower ER levels in these cells. However, E2 still stimulated a response in these cells and the fold stimulation was comparable with that observed in the control cells (Fig. 5A). Similar results were obtained in the Akt-3-expressing pool population of cells (Fig. 5A). In the Akt-3-expressing cells such as the control cells, tamoxifen did not stimulate a response (Fig. 5A). The slight inhibition observed with tamoxifen may be attributable to residual levels of estrogenic compounds in the media.

The various cell lines were also tested for the ability of E2 to stimulate the transcription of two endogenous genes, pS2 and GREB1, which are known to be E2 responsive (20, 21). Cells were treated with E2 and then the amount of pS2 and GREB1 mRNA was determined by Northern blotting. E2 comparably induced the mRNA for both genes in the control plasmid-transfected cells, the Akt-3-expressing clone A3B5 as well as the Akt-3-expressing uncloned population (A3uncl.; Fig. 5B). In addition, the basal levels of these two mRNAs were comparable in the Akt-3-expressing cells and the parental MCF-7 cells, indicating that the ER in the Akt-3-expressing cells was not constitutively active. As with the reporter construct, tamoxifen did not stimulate the transcription of either endogenous gene in any of the cells but instead appeared to lower the basal level of transcription of these two genes.

Additional Characterization of theAkt-3-expressing MCF-7 Cells. To examine possible effects of active Akt-3 on the MCF-7 cells, we compared the growth characteristics of
Akt-3 and control plasmid-transfected cells. In media containing serum and the estrogenic material present in the media (including phenol red), no difference in the growth of these cells was observed (data not shown). However, in serum-free, PRF media, the Akt-3-expressing A3B5 cells were observed to continue to grow for as long as 9 days after placement into such media (Fig. 6). In contrast, the control cells stopped growing after 2 days in this media.

Because Akt can also affect the ability of various agents to induce cell death/apoptosis in cells, we examined the ability of tamoxifen to induce these responses in the Akt-3-expressing cells. The MCF-7 cells expressing Akt-3 (both A3G4 and A3B5) were found to be resistant to tamoxifen-induced cell death as assessed by trypan blue exclusion (Fig. 7A) and apoptosis as assessed by fluorescent annexin V binding (Fig. 7B) in comparison to the parental MCF-7 cells and the cells transfected with the empty plasmid (Puncl).

**Discussion**

Increased levels of active Akt have been identified in various tumors, including breast, glioblastomas, colon, and prostate (5–10). This increase in active Akt can result in these tumors from either increased levels of the Akt activator, PIP3, decreased levels of the PIP3 phosphatase PTEN, increased levels of growth factor receptors such as Her2, autocrine secretion of growth factors, increased levels of Akt, or a combination of these factors. In some cases, the increase in active Akt levels has been correlated with a poorer prognosis than in the case of tumors lacking the stimulated Akt (6, 7). In the present studies, we have generated MCF-7 cells expressing an increased level of active Akt-3 and determined the ability of these cells to form tumors in mice with or without the administration of E2, tamoxifen, and the pure estrogen antagonist ICI 182,780 (also called fulvestrant or Faslodex).

We have found that expression of active Akt-3 at levels comparable with that present in a PTEN-deficient prostate cancer cell line was sufficient to render the tumor formation by these MCF-7 cells estrogen independent. This effect was not because of the particular clone of cells isolated because tumor formation from the uncloned population of Akt-3-transfected cells was similarly estrogen independent. In vitro growth of the Akt-3-expressing MCF-7 cells was also independent of E2 and serum.

What was most surprising was the finding that E2 potently inhibited the growth of the tumors formed by the Akt-3-expressing cells, whereas tamoxifen actually increased the size of the tumors formed by these cells. In contrast, in in vitro studies, E2 stimulated similarly an estrogen-responsive reporter construct as well as the induction of two estrogen-responsive genes in the control MCF-7 cells and the cells expressing active Akt-3. In addition, tamoxifen did not stimulate either of these responses in these in vitro assays in the cells expressing active Akt-3. As previously observed in one study of MCF-7 cells overexpressing...
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Results shown are representative of three independent experiments.

An increase in apoptosis was observed. Whether this is attributable to the increased levels of phosphorylation of Ser167 in the ER or attributable to a general decreased ability of various agents to induce apoptosis in cells expressing Akt is not clear from the present work.

The finding that E2 can inhibit the tumor formation by the Akt-3-expressing MCF-7 cells is quite similar to the prior observation that E2 has an antitumor effect on another breast cancer cell line, the T47D cells, when these cells overexpress protein kinase Cα (26). In addition, E2 was found to either inhibit growth or induce apoptosis in human breast cancer cells that were selected for their ability to grow in the absence of E2 (27, 28) or to grow in the presence of tamoxifen in vivo (29).

The mechanism(s) for these growth inhibitory effects of E2 are not known, although several genes have been identified which are regulated by E2 after acclimation of these cells to growth in the absence of E2 (30). It is possible that the increase in phosphorylation of the ER in the Akt-3-expressing cells may induce the expression or the degradation of these cofactors in the cells. In either case, this could modulate E2 and/or tamoxifen actions in these cells (31–33). In contrast, because ICI 182,780 is a pure ER antagonist that can induce a rapid down-regulation of the ER (34), this compound may be unable to induce the increase in tumor size observed with tamoxifen. Such a hypothesis is consistent with the observation that ICI 182,780 does not exhibit the partial agonist effects of tamoxifen in vivo.

Regardless of the mechanism, the observation that E2 inhibits and tamoxifen stimulates the formation of tumors by the Akt-3-expressing cells may have therapeutic implications. It is possible that an increase in Akt activity may contribute to the development of tamoxifen resistance in some patients. This is consistent with the observation that patients whose breast tumors had active Akt had a poorer prognosis than those who did not (6, 7). These findings are also consistent with the study that the nonsteroidal estrogen diethylstilbestrol was effective in some patients whose breast cancer was no longer responsive to tamoxifen (35, 36). The present finding that E2 could inhibit tumor growth by the Akt-3-expressing cells even when the pure antiestrogen ICI 182,780 (fulvestrant) could not suggests that the use of estrogenic compounds such as diethylstilbestrol should be additionally investigated in patients who are no longer responsive to tamoxifen. In addition, monitoring the Akt status of tumors could be useful in predicting whether or not a particular course of therapy (such as tamoxifen) should be used.

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Fig. 7  Effect of Akt-3 expression on tamoxifen induced cell death/apoptosis. Cells (parental MCF-7, plasmid transfected population called PuncI, and the Akt-3-expressing clones A3G4 and A3B5) were treated with with either 1 μM 4-hydroxy-tamoxifen (4-OH-Tam) or vehicle for 7 days. The number of viable cells was then determined by trypan blue exclusion, and the results are expressed for each cell type as the survival percentage of the tamoxifen versus the control vehicle-treated cells of the same type ± SD (A). Similarly tamoxifen-treated (dashed curves) and vehicle-treated cells (nondashed curves) were analyzed for apoptosis by FITC-annexin V binding (B). A shift to the right (increased fluorescence, FL1-H) on the fluorescence-activated cell sorting scan represents an increase in apoptotic cells. An overall increase in fluorescence/cell was observed in the Akt-3-expressing cells compared with the control cells in the absence of tamoxifen because these cells were larger. Results shown are representative of three independent experiments.

References


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