Estrogen Promotes Chemotherapeutic Drug Resistance by a Mechanism Involving Bcl-2 Proto-Oncogene Expression in Human Breast Cancer Cells

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ABSTRACT

Recent studies have shown that the Bcl-2 protein suppresses programmed cell death or apoptosis induced by a variety of stimuli including chemotherapeutic drugs. Because estrogen promotes the survival of estrogen-dependent breast cancer cells in vivo, we investigated whether estrogen might regulate levels of Bcl-2 gene expression in an estrogen-responsive human breast cancer cell line. Estrogen receptor-positive MCF-7 human breast cancer cells cultured in the presence of estrogen express the 8.5-kb Bcl-2 mRNA transcript. Depletion of estrogen from the medium results in loss of expression of the mRNA, whereas reexposure to estrogen markedly induces the Bcl-2 transcript. The changes in Bcl-2 mRNA are paralleled by changes in Bcl-2 protein levels. Estrogen-induced increases in Bcl-2 are significantly inhibited by inclusion of the pure antiestrogen ICI 164,384 in the medium. The Bax protein that heterodimerizes with Bcl-2 and promotes cell death is expressed in MCF-7 cells grown in the presence of estrogen and is unaffected by culture in estrogen-free medium. Estrogen depletion doubles the sensitivity of MCF-7 cells to the cytotoxic effects of Adriamycin compared with cells cultured in medium supplemented with estrogen, consistent with a decrease in the Bcl-2 levels. MCF-7 cells treated simultaneously with estrogen and ICI 164,384 exhibit markedly lower resistance to Adriamycin compared with cells treated with estrogen alone. In the absence of estrogen, MCF-7 cells transfected with Bcl-2 expression plasmids display a marked increase in resistance to Adriamycin. In the presence of estrogen, MCF-7 cells expressing Bcl-2 antisense transcripts are rendered twice as sensitive to acute Adriamycin cytotoxicity as a control clone. We conclude that estrogen can promote resistance of estrogen receptor-bearing human breast cancer cells to chemotherapeutic drugs through a mechanism that involves regulation of the Bcl-2 proto-oncogene.

INTRODUCTION

Approximately 65% of human breast tumors express ERs and of these about one-half are dependent on E2 for growth and tumorigenicity. E2-dependent mammary carcinomas regress after ovarietomy through a pathway involving programmed cell death or apoptosis, suggesting that E2 may also contribute to the survival of these cells. Whereas it is not completely understood how estrogen regulates the growth of these cells, it is known that hormonal induction of growth factors such as transforming growth factor α contributes to the proliferative actions of E2. E2 can up-regulate the receptors for epidermal growth factor and c-erbB2 (neu), the receptor for hergulin (6, 7). The c-myc gene is also rapidly induced in ER+ breast cancer cells after exposure to E2. In contrast, little is known about specific mechanisms by which E2 promotes the survival of E2-dependent breast cancer cells.

Recent studies indicate that the signals that ultimately cause cellular proliferation must interplay with mechanisms that control cell death. The Bcl-2 proto-oncogene plays an important role in this process because expression of the protein product prevents programmed cell death induced by a variety of stimuli and insults including growth factor depletion, stress, c-myc expression in the absence of growth factors, p53, and most chemotherapeutic agents.

We undertook this study to determine whether E2 treatment of ER-expressing breast cancer cells might alter the expression of proteins involved in the cellular control of apoptosis. Our results indicate that Bcl-2 expression in the ER+ MCF-7 human breast cancer cell line is dependent on E2, whereas Bax expression is independent of E2. In addition, we show that E2 promotes increased survival of MCF-7 cells exposed to the chemotherapeutic drug Adriamycin. Experimental alterations in the acute cytotoxicity of Adriamycin in transfected MCF-7 cells that constitutively express either sense or antisense Bcl-2 transcripts in the absence or presence of E2 suggest that E2-mediated drug resistance involves induction of the Bcl-2 proto-oncogene.

MATERIALS AND METHODS

Plasmid DNA Construction and Isolation. The Bcl-2 expression plasmid was constructed by subcloning an EcoRI restriction fragment from the human Bcl-2 cDNA into a vector that contains the cytomegalovirus promoter/enhancer and termination sequences from the SV40 small T intron. The sense and antisense orientation expression plasmids were verified by restriction enzyme analysis and DNA sequencing. The human Bcl-2 cDNA was obtained as a gift from Dr. Michael Cleary (Stanford University). Bax cDNA was the gift of Dr. Stanley Korsmeyer (Howard Hughes Medical Institute, St. Louis, MO), and glyceraldehyde phosphate dehydrogenase was obtained from Dr. Balwant Tuana (University of Ottawa). All plasmid DNAs were purified using Qiagen (Chatsworth, CA) column chromatography.

Cell Lines and Transfections. MCF-7 ER+ breast cancer cells were obtained from Dr. Leigh Murphy (University of Manitoba). MDA-MB-231 breast cancer cells do not express the ER and were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in α-MEM (GIBCO-BRL) containing phenol red free medium supplemented with nonessential amino acids, 0.3% glucose, 5% fetal bovine serum (GIBCO-BRL), and 2 μg/ml gentamicin sulphate (α-MEM). Cultures were maintained at 37°C in 5% CO2. For experiments requiring E2 depletion, cells were precultured for 7–10 days with several changes of medium in phenol red free α-MEM (GIBCO-BRL) containing 5% fetal bovine serum stripped of steroids by absorption to dextrancoated charcoal for 45 min at 45°C (DMEM). E2 (Sigma Chemical Co., St. Louis, MO) was added for indicated times from a 1-mm stock solution in ethanol. The pure antiestrogen ICI 164,384 (the gift of Dr. A. E. Wakeling, Zeneca Pharmaceuticals) was used at a concentration of 10^{-7} M from a 1-mm stock solution in ethanol. To generate stable cell lines expressing the sense and antisense Bcl-2 transcripts, 5 μg of the expression plasmid pT218 (control) was cotransfected with 5 μg of cytomegalovirus-Neo into MCF-7 cells using a CaPO4/DNA precipitate method (21). Cells were incubated with precipitate for 18 h, then washed with PBS and subjected to hypotonic shock for 3 min using aMEM containing 20% glycerol. Cells were selected for neomycin resistance in medium containing 50 μg/ml of G418 over a 12-day period with 3 changes of medium. The presence of neomycin resistance was confirmed by a 50 μg/ml of G418 over a 12-day period with 3 changes of medium. When no further cell death was evident, individual colonies were isolated using a sterile pipette tip and transferred to a multiwell plate, expanded, and assayed for expression of the transfected gene by North- ern analysis and for protein by Western blot analysis. Positive clones were passaged at least 10 times, and Bcl-2 protein was assessed a second time by Western blot analysis to ensure clonal integrity before use in cytotoxicity assays.

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3 The abbreviations used are: ER, estrogen receptor; E2, estrogen, ER+, ER positive, ER-, ER negative.

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Fig. 1. Northern blot analysis of Bcl-2 mRNA in human breast cancer cell lines. Twenty μg of total RNA were isolated and separated on a denaturing agarose gel and transferred to Hybond N for hybridization. All Bcl-2 blots were autoradiographed at -70°C for 7 days with an intensifying screen, followed by hybridization to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe as an internal control for RNA loading. A, E2 regulation of Bcl-2 gene expression. MDA-MB-231 ER− cells and MCF-7 ER+ cells were E2 depleted by culture for 7 days in DMEM before treatment for 48 h with vehicle or 10^-8 M E2. The blot was hybridized with a human Bcl-2 cDNA probe encompassing the coding region. Bars, 5.5- and 8.5-kb species of Bcl-2 mRNA.

RNA Isolation and Analysis. Total cellular RNA was isolated using the LiCl-urea method (22). RNA samples (20 μg) were size fractionated in 1.1 M formaldehyde agarose gels (21) and transferred to Hybond N (Amersham) for hybridization with random-primed 32P-labeled cDNA probes. Densitometry was performed using a LKB Ultrascan densitometer.

Western Blot Analysis. Cultured cells were washed twice with PBS and then lysed in 400 μl/10^6 cells of 50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 10 μg/ml phenylmethylsulphonyl fluoride, 1 μg/ml aprotinin, and 0.02% sodium azide for 30 min at 0°C. Insoluble material was removed by centrifugation at 12,000 × g for 15 min. Protein concentrations were determined using a BioRad kit. Proteins (25 μg) were size fractionated under denaturing conditions on 12.5% SDS-polyacrylamide gels and transferred to polyscreen membranes (Dupont New England Nuclear). A mouse mAb, 4D7 (Pharmingen)(used in fig. 6 only), a rabbit polyclonal antiserum specific for human Bcl-2 (23), and a rabbit polyclonal antisemur specific for human Bax were used for Western blot analysis. Proteins were visualized after incubation with peroxidase-conjugated second antibody (Sigma) and chemiluminescent substrate (Dupont New England Nuclear). A rabbit antibody against α-actin (Sigma) was used to control for protein loading.

Cytotoxicity Assays. MCF-7 cells 1 × 10^5 in α-MEM or depleted of E2 by a 7—10-day culture in DMEM were cultured for 18 h in 24-well dishes and then treated with the indicated concentrations of Adriamycin (Adria Laboratories, Ontario) for 48 h. Cell viability was assessed by trypan blue dye exclusion, and the results were expressed as a percentage relative to the same cells cultured in the absence of drug (mean ± SD; n = 3). No differences in the doubling times between antisense and control-transfected cell lines were noticed.

RESULTS

Estrogen Promotes Bcl-2 mRNA Expression in ER+ MCF-7 Breast Cancer Cells. Because of the ability of E2 to prevent the regression of many ER+ mammary tumors by a process involving apoptosis, we have studied the effects of E2 on the expression of genes involved in the regulation of apoptosis in ER- and ER+ breast cancer cells. As shown in Fig. 1a, MCF-7 ER+ breast cancer cells that were cultured in E2-free medium for 7 days contained very low levels of Bcl-2 mRNA. Treatment of MCF-7 cells with 10^-8 M E2 for 48 h resulted in a 6-fold induction of the 8.5-kb Bcl-2 mRNA. Bcl-2 mRNA is undetectable in ER- MDA-MB-231 cells grown in E2-free medium for 7 days or in medium supplemented with 10^-8 M E2 for 48 h. A constitutive low level of hybridization with the Bcl-2 probe was seen coincident with the 28S ribosomal RNA, which may correspond to the 5.5-kb transcript described by Tsujimoto and Croce (24). Alternatively, it may be due to nonspecific hybridization as a result of the GC-rich nature of the Bcl-2 cDNA.

To study the kinetics of E2 regulation of Bcl-2 gene expression, a time course of the induction of Bcl-2 mRNA was performed as shown in Fig. 1b and elevations in Bcl-2 mRNA levels were detectable between 8 and 24 h after E2 treatment. The control lane in this figure contains RNA from MCF-7 cells that were maintained in medium containing unstripped fetal bovine serum and phenol red (α-MEM). These cells contain constitutively high levels of Bcl-2 transcript compared with E2-deprived cells.

E2 Regulation of p26Bcl-2a in ER+ and ER- Breast Cancer Cell Lines. To confirm that E2-induced changes in Bcl-2 mRNA are reflected in corresponding alterations in the levels of p26Bcl-2a protein, we performed Western blot analysis on extracts from MCF-7 cells that had been cultured in the absence of E2 for 10 days and then treated with or without 10^-8 M E2 for 1—4 days. The results in Fig. 2a indicate that Bcl-2 protein levels are almost undetectable in E2-depleted cells, whereas E2 treatment results in a marked increase in p26-Bcl-2a within 24 h. These levels continued to rise over the next 4 days. To assess the participation of the ER in E2 effects on Bcl-2a expression, we tested the effects of the pure antiestrogen, ICI 164,384, on the E2-induced increases in p26Bcl-2a. The Western blot in Fig. 2b shows that ICI 164,384 almost completely prevented the E2-mediated increase in Bcl-2 protein levels. To compare Bcl-2 protein levels in ER+ protein levels in ER+ and ER- breast cancer cell lines, extracts from MDA-MB-231 cells and MCF-7 cells that had been cultured in complete α-MEM were subjected to Western blot analysis. As shown in Fig. 2c, p26 Bcl-2a was present in MDA-MB-231 cells at levels approximately 6-fold lower than those in MCF-7 cells (data normalized to the level of α-actin) as assessed by densitometry. We consistently detected a second immunoreactive protein of approximately M, 28,000 in MCF-7 cells and to a lesser extent in MDA-MB-231 cells that was not regulated by E2. This band was detected by both the monoclonal and polyclonal Bcl-2 antibodies, although the reactivity was weaker with the monoclonal. Additional studies are required to determine whether this represents a posttranslational modification of Bcl-2 or cross-reactivity with another protein.

Expression of Bax Protein Is Independent of E2. Because the Bcl-2: Bax ratio in the cell determines the relative sensitivity of cells to apoptotic stimuli such as growth factor withdrawal or exposure to cytotoxic drugs, we examined the regulation of Bax protein expres-
Estrogen Treatment of MCF-7 Cells Confers Resistance to Acute Adriamycin Cytotoxicity. Because E2 induces elevations in Bcl-2 mRNA and protein levels in MCF-7 cells, we sought to determine whether E2 depletion would alter the sensitivity of these cells to the induction of apoptosis by the anticancer drug Adriamycin, a drug commonly used in the treatment of breast cancer. Fig. 4 shows the effects of a 48-h exposure to Adriamycin on MCF-7 cell viability. MCF-7 cells cultured in the absence of E2 displayed approximately a 50% decrease in viability when cultured in 10^{-7} M Adriamycin compared to the same cells precultured with E2 for 2 days before treatment with Adriamycin. E2-mediated increases in drug resistance were also seen at higher concentrations of Adriamycin compared to E2-deprived cells.

To determine whether Bcl-2 expression alone could recapitulate the E2-mediated increase in resistance to Adriamycin, we generated clones of MCF-7 cells expressing high levels of either Bcl-2 antisense [MCF-7(Bcl-2(-))] or sense transcript [MCF-7(Bcl-2(+))]. Three positive clones expressing Bcl-2(+) and three clones expressing Bcl-2(-) were obtained. The relative level of p26Bcl-2a in MCF-7(Bcl-2(+)) clones S3, S4, and S5 compared with pooled control clones trans-

Fig. 2. Western blot analysis of Bcl-2 protein in human breast cancer cell lines. Cell extracts prepared as described in "Materials and Methods" were electrophoresed under denaturing conditions in 12.5% SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose. A. induction of p26Bcl-2a by E2 in MCF-7 cells. MCF-7 cells were cultured for 10 days in DMEM to deplete E2 and then treated with vehicle (con) or 10^{-8} M E2 for 1-4 days before Western blot analysis with Bcl-2 antibody. Proteins were visualized by reaction with peroxidase-conjugated second antibody and chemiluminescence as described in "Materials and Methods." kDa, molecular weight in thousands. B. effect of the pure antiestrogen ICI 164,384 on E2 induction of p26Bcl-2a. MCF-7 cells were cultured for 7 days in DMEM to deplete E2 and then treated with vehicle for 72 h (C), 10^{-8} M E2, or 10^{-8} M E2 with 10^{-7} M ICI 164,384 for the indicated times. Western blot analysis was performed as in A. C. comparison of Bcl-2 protein in MDA-MB-231 cells and MCF-7 cells. Approximately 100 μg of extract from MDA-MB-231 cells and 50 μg of MCF-7 cell extract from cells cultured in complete α-MEM were subjected to Western blot analysis with Bcl-2 antibody. Reactivity with α-actin antibody was used as a control for protein loading on both blots.

Fig. 3. Effect of E2 on Bax expression. MCF-7 cells were cultured in α-MEM (con) or in E2-free DMEM for 8 days, followed by treatment with 10^{-7} M E2 for 0-48 hours before isolation of cell extracts as described in "Materials and Methods." Equal amounts of protein from each sample were electrophoresed and subjected to Western blot analysis as described above with an antibody specific for human p21Bax. Equivalency of loading was confirmed by reaction with anti-α-actin antibody.

Fig. 4. Estrogen promotes resistance to acute cytotoxicity of Adriamycin. MCF-7 cells were plated at a density of 10^5 cells/well in a 24-well plate. After 24 h, the cells were treated with concentrations of 10^{-7}, 10^{-6}, or 10^{-5} M Adriamycin for 48 h. Cell viability was assessed by trypan blue exclusion, and values represent percentages of duplicate control wells without drug. Points, mean of triplicate determinations; bars, SD. Three independent clones of MCF-7 cells transfected with the sense orientation of cytomegalovirus-neo were depleted of E2 by culture in E2-free DMEM before treatment with Adriamycin. The graph shows the average cytotoxicity obtained in pooled cultures of the three independent clones (Δ) and in clone S4 alone (□). Pooled MCF-7 clones transfected with cytomegalovirus-neo only were similarly rendered E2-free by 10 days of culture in DMEM and treated for 48 h with E2 (○) or vehicle (X) before addition of Adriamycin.

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Fig. 5. Western blot analysis of MCF-7 (Bcl-2) sense and antisense clones. A. 25 μg of protein derived from pooled control MCF-7 clones (C) and three independent Bcl-2(+) clones (S3, S4, and S5) maintained in estrogenic α-MEM were used for Western blot analysis of p26Bcl-2α as described in "Materials and Methods." B. Comparison of p26Bcl-2α protein levels in MCF-7(Bcl-2(+)) and control MCF-7 clones cultured in E2-free DMEM. C. Comparison of p26Bcl-2α protein levels in a MCF-7(Bcl-2(-)) and a control MCF-7 clone cultured in estrogenic α-MEM.

Because the inclusion of ICI 164,384 repressed the induction of p26Bcl-2α, we reasoned that ICI 164,384 would prevent the E2-mediated resistance from Adriamycin-induced cytotoxicity. For these experiments, depicted in Fig. 7, MCF-7 cells were treated simultaneously with E2 and ICI 164,384 before exposure to Adriamycin. In agreement with the results in Fig. 4, MCF-7 cells cultured in E2-free DMEM showed greater sensitivity to Adriamycin at the two lower concentrations compared with the same cells treated with E2. In the same experiment, cells treated simultaneously with both antiestrogen and estrogen were rendered markedly more sensitive to all concentrations of Adriamycin than cells cultured with or without E2. The additional cytotoxicity in ICI 164,384-treated cells may reflect antagonism of residual E2 within the cells or in the stripped serum.

DISCUSSION

Recent studies of the Bcl-2 oncogene have demonstrated its role in the prevention of apoptosis induced by a wide variety of stimuli and conditions (10–18). The mechanism by which Bcl-2 exerts its antiapoptotic effects is not fully resolved, although it has been speculated
to act as either a regulator of an antioxidant pathway that prevents oxidative damage such as lipid peroxidation caused by the generation of free radicals (25, 26) or as a regulator of intracellular Ca\(^{2+}\) compartmentalization (27). The antiapoptotic activity of Bcl-2 correlates with its intracellular ratio to another recently described protein called Bax (28). High levels of Bax have been shown to favor apoptosis in cells subjected to growth factor deprivation, whereas high levels of Bcl-2 prolong cell survival under the same conditions (28). The ability of Bcl-2 to oppose the activity of Bax is dependent on its ability to heterodimerize with Bax (28, 29). Our finding that E2 depletion results in a marked decrease in Bcl-2 expression but does not alter Bax gene expression indicates that, in the absence of E2, the Bax:Bcl-2 ratio is increased. Such a situation is consistent with the clinical and experimental observation that ER+ breast cancer tumors that are E2-dependent undergo apoptosis after E2 ablation (2).

Whereas the E2 inducibility of Bcl-2 of 2 provides a mechanism for chemotherapeutic resistance in ER+ cells, the low levels of Bcl-2 found in the ER- MDA-MB-231 cells suggests that these cells may rely on other mechanisms to modulate drug resistance. In this regard, the level of Bax mRNA in these cells is also low (data not shown). Our findings using breast cancer cell lines support the recent report that Bcl-2 expression is associated with ER+ tumors and that ER- breast cancer biopsies tend to lack this protein (30).

The mechanism by which E2 regulates Bcl-2 may involve direct transcriptional induction through an E2 response element in the Bcl-2 gene or an indirect pathway. There are no perfect consensus sequences for an E2 response element in the published Bcl-2 gene sequence (31); however, degenerate E2 response motifs interacting with Sp1 sites (32) and TA-rich binding protein binding sequences (33) have been shown to confer E2 responsiveness. In this regard, the Bcl-2 gene promoter contains several Sp1-binding sites (31).

Positive regulation of Bcl-2 protein levels have been reported in neuroblastoma cells induced to differentiate with phorbol esters and high doses of retinoic acid (19). There is also recent evidence that the tumor suppressor p53 is a negative regulator of Bcl-2 expression (34–36). The ability of p53 to modulate cellular proliferation has been shown to be altered not only by mutation but also by nuclear exclusion in breast cancer cells (37). In breast biopsies containing wild-type p53 (37), as well as MCF-7 cells which are homozygous for wild-type p53 (38), the proliferative state is associated with cytoplasmic sequestration of p53. Thus, because E2 induces proliferation of MCF-7 cells, potential nuclear exclusion of p53 might also contribute to the strong induction of Bcl-2 by E2.

A number of studies have shown that constitutive expression of the Bcl-2 proto-oncogene in a variety of cells results in a heightened resistance to chemotherapeutic agents that function by inducing programmed cell death (19, 20, 39, 40). The present studies indicate that this is also the case of ER+ breast cancer cells grown in the presence of E2 because of E2 significantly protected MCF-7 cells from Adriamycin-induced apoptosis compared with cells deprived of E2. By expressing the sense and antisense transcripts of Bcl-2 in MCF-7 cells, we have been able to exclude other effects of E2 on the cells that might contribute to survival in the presence of Adriamycin. E2-deprived MCF-7 cells with gene transfer-mediated elevations in Bcl-2 protein levels exhibited a similar degree of resistance to Adriamycin as E2-treated control MCF-7 cells. Consistent with the participation of Bcl-2 in mediating E2-induced drug resistance, MCF-7 cells expressing the antisense Bcl-2 transcript were markedly more sensitive to the cytotoxic effects of Adriamycin than were control cells, even though both were cultured in E2-containing medium.

The observation that the pure antiestrogen ICI 164,384 significantly inhibits E2 induction of p26Bcl-2a is consistent with the participation of the ER in induction of the Bcl-2 gene because antiestrogens have been shown to act through the ER to prevent ligand-induced transactivation (41). A number of studies have shown that antiestrogens alone or in combination with chemotherapeutic drugs are of significant therapeutic benefit in ER+ breast cancer over systemic chemotherapy alone (42, 43). Our findings support the notion that at least some antiestrogens can increase the sensitivity of breast cancer cells to Adriamycin by decreasing the cellular levels of Bcl-2a.

Taken together, our findings demonstrate a novel mechanism of action for E2 in that it confers chemotherapeutic drug resistance through regulation of Bcl-2 expression. Future studies will address the role of Bcl-2 in hormone ablation-induced programmed cell death in ER+ breast tumors.

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