Tamoxifen-induced Apoptosis in Breast Cancer Cells Relates to Down-Regulation of bcl-2, but not bax and bcl-XL, without Alteration of p53 Protein Levels

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ABSTRACT

Tamoxifen (TAM) has been shown to induce apoptosis in breast cancer cells. bcl-2 family genes, which can interact with each other, have been shown to interfere with apoptosis after various stimuli. In this study, we investigated the effects of TAM on bcl-2 family gene products bcl-2, bax, and bcl-XL, and on p53 levels in estrogen receptor-positive MCF-7 breast cancer cells. We found that TAM induced time- and concentration-dependent down-regulation of bcl-2 at both the mRNA and protein level. Down-regulation of bcl-2 correlated with TAM-induced apoptosis. In addition, estradiol treatment significantly increased bcl-2 protein expression and blocked the reduction of bcl-2 by TAM. TAM did not, however, affect bax, bcl-XL, or p53 expression at the mRNA or protein level. Our results demonstrate that TAM can induce apoptosis in a time- and dose-dependent manner by modulating bcl-2 levels in breast cancer cells, and down-regulation of bcl-2 induced by TAM was not accompanied by alterations in p53 levels.

INTRODUCTION

TAM is commonly used for adjuvant therapy of breast cancer and is a key drug for chemoprevention of breast cancer in high-risk women (1–3). TAM is believed to inhibit the growth of breast cancer mainly through competing with estrogen for ER binding. The mechanism of action of TAM remains unclear, although its breast cancer antiproliferative effect may be via a receptor-mediated cytostatic activity, a nonspecific activity, or a receptor-mediated cytotoxic activity. Previous reports have suggested that TAM induced characteristic morphological changes consistent with apoptosis, including condensation of cytoplasm and convolution of nuclear and internucleosomal DNA fragmentation in breast cancer cells (4–6). This suggests that TAM inhibits the growth of breast cancer by inducing apoptosis.

The bcl-2 gene, which was isolated by its juxtaposition to the immunoglobulin heavy chain locus in follicular lymphoma, codes a M, 26,000 protein that inhibits apoptosis (7). Recently, several bcl-2 family genes have been identified by their homology with bcl-2 (8). Some bcl-2 family members are inhibitors of apoptosis, including bcl-2 (9), bcl-XL (10), BAG-1 (11), and mcl-1 (12), whereas others are inducers of cell death, including bax (13), bad (14), and bcl-XL (15). These members interact with each other via their BH3 domain in an antagonistic fashion to regulate apoptosis. Apoptosis is controlled by the ratio of various bcl-2 family members (16). When levels of apoptosis promoters (bax and bcl-XL) increase, apoptosis is accelerated, whereas when the inhibitors of apoptosis (bcl-2 and bcl-XL) increase, the cells are predisposed to be resistant to apoptosis in response to external stimuli. Previous reports have demonstrated that changes in the ratio of protoapoptotic to antiapoptotic proteins result in susceptibility to apoptosis, and a high bax: bcl-2 ratio has been found to correlate with etoposide-induced apoptosis (17). It has been suggested that estrogen enhances resistance to chemotherapy drugs such as Taxol and cisplatin by increasing the bcl-2:bax ratio (18, 19). Overexpression of HER2 in MCF-7 cells has been shown to suppress TAM-induced apoptosis by up-regulating bcl-2 and bcl-XL protein (20). However, whether TAM directly modulates bcl-2, bax, or bcl-XL expression is not known. On the other hand, p53 can modulate susceptibility to cytotoxic drugs by inducing apoptosis (17). Expression of p53 is up-regulated after DNA damage, such as by radiation or cytotoxic chemotherapy. In addition, wild-type p53 has been shown to induce apoptosis by increasing levels of bax and decreasing levels of bcl-2 (21). However, p53 is not present for all instances of apoptosis, and the effects of TAM on p53 have not been examined.

In the present study, we investigated whether TAM-induced apoptosis is related to the expression of bcl-2 family genes and p53 protein. We tested TAM-induced apoptotic activity and examined the effects of TAM on bcl-2 family genes (bcl-2, bax, and bcl-XL) and p53 in MCF-7 breast cancer cells. We found that TAM-induced apoptosis is accompanied by down-regulation of bcl-2 levels without alterations in p53 in breast cancer cells.
MATERIALS AND METHODS

Cell Culture and Reagents. MCF-7 human breast cancer cells, which are ER positive, were obtained from the ATCC (Rockville, MD). The cells were maintained in DMEM with 10% FBS at 37°C in a humidified incubator and 5% CO₂. Cells were grown to confluence and passaged with the use of trypsin-EDTA. For apoptosis experiments, cells at approximately 2 × 10⁶ cells/75 cm² flask were placed in phenol red-free DMEM with 10% DCC-FBS to remove steroids (22). In this study, the concentration of estradiol in DCC-FBS was undetectable (<8 pg/ml) by using the estradiol detection kit (Diagnostic Products Corp., Los Angeles, CA), whereas FBS contained 29 pg/ml of estradiol. After 48 h incubation, the culture medium was removed, and a new medium containing TAM was added. TAM and E₂ were purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in ethanol as a 10⁻² stock solution.

Quantitation of Apoptotic Cells by ELISA. For quantitation of apoptosis induced by TAM, we used a cell death detection ELISAkit (Boehringer-Mannheim, Mannheim, Germany) to measure cytoplasmic DNA-histone complexes generated during apoptotic DNA fragmentation, as described previously (20). Each experiment was performed in triplicate. Absorbance at 405 nm was measured using a microplate reader, and the results are expressed as the enrichment factor relative to the untreated controls.

RT-PCR Analyses of bcl-2, bax, and bcl-X mRNA Levels. Semiquantitation of bcl-2, bax, and bcl-X mRNA levels were performed using RT-PCR. Total cellular RNA was isolated using the Trizol reagent according to the manufacturer’s instructions. (Life Technologies, Inc.). One μg of RNA was used for cDNA synthesis by using the First Strand cDNA Synthesis kit (Pharmacia Biotech, Uppsala, Sweden). A typical PCR consisted of 5 μl of cDNA, 0.2 mM deoxynucleotide triphosphate, 1 μM of each primer, 1× PCR buffer, and 2.5 units of Taq polymerase. The PCR profile was 95°C for 40 s, 65°C for 40 s, and 72°C for 2 min for 35 cycles, followed by extension for 7 min at 72°C. The primer pairs used in this study for PCR amplification were as follows: bcl-2 (459 bp), upstream 5′-GGTGCCACCTGTGGCCACCTG-3′ and downstream 5′-CTTCTACAATGAGCTGCG-3′; bax (538 bp), upstream 5′-CTTCACCTTGCGCCCAGATAAGG-3′; and downstream 5′-CAGCTCTGAGCAGATCATGAAGACA-3′; bcl-XL (780 bp), upstream 5′-TTGGGACATGGACTGGTGATGA-3′ and downstream 5′-TGTAATATGGTATGGTCAG-3′; and β-actin (838 bp), upstream 5′-ATCTGGCACCACAC-CTTCTACAAATGAGCTGGC-3′ and downstream 5′-CGTCATAACTCTCTGTTGATCCACATCTGC-3′. After PCR, aliquots of the reactions were analyzed on 2% agarose gels and visualized using ethidium bromide. The amount of mRNA was semiquantitated by comparing relative intensities of the amplified β-actin to an equal amount of cDNA.

Western Blot Analyses. Total proteins were extracted from approximately 5 × 10⁶ cells treated with TAM for up to 72 h. Briefly, cells were washed with ice-cold PBS and lysed in 50 mM Tris (pH 7.6), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 10 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.02% sodium azide for 30 min at 4°C. Insoluble material was removed by centrifugation at 12,000 × g for 15 min. Equal amounts of proteins were size fractionated under denaturing conditions on 12.5% SDS-polyacrylamide gels and transferred to Clearblot P membrane (Atto, Tokyo, Japan) as described previously (23) and were reprobed with anti-bcl-2 (clone 124; Dako, Glostrup, Denmark), anti-bax (MBL, Nagoya, Japan), anti-bcl-XL (Transduction Laboratories, Lexington, KY), or anti-p53 antibody (PAb1801; Oncogene Science, Cambridge, MA) as described previously (23) and were visualized using an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK). Proteins were size fractionated under denaturing conditions on 12.5% SDS-polyacrylamide gels and transferred to Clearblot P membrane (Atto, Tokyo, Japan) as described previously (23) and were reprobed with anti-bcl-2 (clone 124; Dako, Glostrup, Denmark), anti-bax (MBL, Nagoya, Japan), anti-bcl-XL (Transduction Laboratories, Lexington, KY), or anti-p53 antibody (PAb1801; Oncogene Science, Cambridge, MA) as described previously (23) and were visualized using an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK).

Statistical Analysis. Densitometry was performed using NIH Image 1.59 software (National Cancer Institute, Bethesda, MD). The intensities of the protein or mRNA bands were normalized to the actin bands and quantified by comparing with those of control cells. Statistical differences were evaluated by Student’s paired t test. P < 0.05 was considered as statistically significant.

RESULTS

TAM-induced Apoptosis Is Time- and Concentration-dependent. Cells grown on plastic flasks in DMEM with 10% DCC-FBS displayed typical epithelial patterns: a cluster of cells growing in a polygonal shape (Fig. 1A). Within 48 h of treatment with 10⁻³ TAM, MCF-7 cells showed a marked change in morphological pattern (Fig. 1B). The changes included membrane blebbing, cytoplasmic condensation, and DNA fragmentation. The percentage of apoptotic cells was determined using a cell death detection ELISAkit (Boehringer-Mannheim, Mannheim, Germany) to measure cytoplasmic DNA-histone complexes generated during apoptotic DNA fragmentation, as described previously (20). Each experiment was performed in triplicate. Absorbance at 405 nm was measured using a microplate reader, and the results are expressed as the enrichment factor relative to the untreated controls.

Western Blot Analyses. Total proteins were extracted from approximately 5 × 10⁶ cells treated with TAM for up to 72 h. Briefly, cells were washed with ice-cold PBS and lysed in 50 mM Tris (pH 7.6), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 10 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.02% sodium azide for 30 min at 4°C. Insoluble material was removed by centrifugation at 12,000 × g for 15 min. Equal amounts of proteins were size fractionated under denaturing conditions on 12.5% SDS-polyacrylamide gels and transferred to Clearblot P membrane (Atto, Tokyo, Japan). The membranes were probed with anti-bcl-2 (clone 124; Dako, Glostrup, Denmark), anti-bax (MBL, Nagoya, Japan), anti-bcl-XL (Transduction Laboratories, Lexington, KY), or anti-p53 antibody (PAb1801; Oncogene Science, Cambridge, MA) as described previously (23) and were visualized using an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK). Proteins were size fractionated under denaturing conditions on 12.5% SDS-polyacrylamide gels and transferred to Clearblot P membrane (Atto, Tokyo, Japan) as described previously (23) and were reprobed with anti-bcl-2 (clone 124; Dako, Glostrup, Denmark), anti-bax (MBL, Nagoya, Japan), anti-bcl-XL (Transduction Laboratories, Lexington, KY), or anti-p53 antibody (PAb1801; Oncogene Science, Cambridge, MA) as described previously (23) and were visualized using an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK).
morphology, including shrinkage, irregular shape and condensed chromatin in the nuclei (Fig. 1B). ELISA revealed that more than $10^{-8}$ M TAM had apoptotic effects on breast cancer cells (Fig. 2). Furthermore, TAM induced apoptosis in a time- and concentration-dependent manner. When treated with $10^{-8}$ M TAM for 72 h, the cells exhibited an ~8-fold higher apoptosis rate than untreated cells ($P < 0.0001$).

Effects of TAM on Expression of bcl-2, bax, and bcl-X<sub>L</sub> mRNA. To determine the effects of TAM on the expression of bcl-2, bax, and bcl-X<sub>L</sub> mRNA in MCF-7 cells, we performed RT-PCR. TAM ($10^{-8}$ M or more) decreased the bcl-2 mRNA levels in MCF-7 cells after 48 h exposure, and the decrease of bcl-2 mRNA was dose dependent (Fig. 3A). In addition, TAM treatment down-regulated bcl-2 mRNA expression in a time-dependent manner; the reduction of bcl-2 was by ~70% after 72 h exposure to $10^{-5}$ M TAM (Fig. 3B). There was no significant difference in either bax or bcl-X<sub>L</sub> mRNA levels before and after TAM treatment (data not shown).

Effects of TAM on Expression of bcl-2, bax, and bcl-X<sub>L</sub> Proteins. To determine the effects of TAM on the expression of bcl-2, bax, and bcl-X<sub>L</sub> proteins in MCF-7 cells, we performed Western blotting analysis. After 48 h of treatment, $10^{-10}$ TAM caused a slight but not significant increase in bcl-2 protein. TAM ($10^{-8}$ M or more) caused reduction in bcl-2 protein expression, $10^{-5}$ M TAM caused a 70% reduction compared with nontreatment cells (Fig. 4A). TAM had a concentration-dependent effect on bcl-2 protein (Fig. 4B). We failed to observe a phosphorylated isoform of bcl-2 protein in the Western blots. In addition, exposure to TAM did not alter expression of bax or bcl-X<sub>L</sub> proteins in MCF-7 cells (data not shown).

To investigate whether the effects of TAM on bcl-2 levels are time dependent, we added TAM to MCF-7 cells at a final concentration of $10^{-5}$ M for up to 72 h. After 48-72 h treatment, an ~80% reduction in bcl-2 protein was detected (Fig. 5A). TAM at a concentration of $10^{-5}$ M down-regulated bcl-2 protein levels in a time-dependent manner (Fig. 5B). Furthermore, this down-regulation in bcl-2 protein correlated with apoptosis detected by ELISA (Fig. 2). However, no significant changes in either bax or bcl-X<sub>L</sub> expression were detected (Fig. 6).

We also tested the effects of E<sub>2</sub> on TAM-induced apoptosis by adding $10^{-8}$ M E<sub>2</sub> with TAM. We found that addition of E<sub>2</sub>
blocked TAM induced apoptosis (Fig. 7A). E2 significantly increased bcl-2 protein levels with 10^{-8} M E2, causing 4.3-fold increase in bcl-2 protein levels (Fig. 7B). Moreover, E2 blocked the reduction of bcl-2 induced by TAM.

**Effects of TAM on p53 Expression.** We determined the effects of TAM on p53 protein expression by Western blotting analysis. As shown in Fig. 8, no differences in p53 protein levels were found between untreated cells and those treated with 10^{-2} to 10^{-6} M TAM for up to 72 h. Also, 10^{-10} to 10^{-6} M TAM did not affect wild-type p53 levels (data not shown).

**DISCUSSION**

In this study, we demonstrated that TAM, an antiestrogen, induces apoptosis in MCF-7 human breast cells in a time- and concentration-dependent manner, which is consistent with previous reports (5). bcl-2 plays an important role in the inhibition of apoptosis stimulated by various factors, such as irradiation, chemotherapeutic drugs, and withdrawal of growth factors (24, 25). Our results demonstrate that expression of bcl-2 is down-regulated in MCF-7 cells after culture with more than 10^{-8} M TAM at both the mRNA and protein levels. In contrast to our data, a previous report did not find an effect of 10^{-9} to 10^{-7} M TAM on bcl-2 protein levels in MCF-7 cells. Very recently, Burrow et al. (26) have reported that three different MCF-7 cells from different laboratories show different sensitivities to tumor necrosis factor-α-induced apoptosis. In their study, MCF-7 N (from ATCC) demonstrated a higher sensitivity than the other two variants of MCF-7 and showed internucleosomal DNA fragmentation. Thus, the discrepancy between Huang’s and our report might be attributable to the difference between the characters of these two MCF-7 cell lines, although both of them were obtained from ATCC. Moreover, the down-regulation of bcl-2 caused by TAM is time and concentration dependent and correlated with TAM-induced apoptosis. These observations strongly suggest that down-regulation of bcl-2 plays a critical
role in deregulation of apoptosis inhibition and also triggers apoptosis by TAM.

Whether cells enter apoptosis is determined by the ratio of proapoptotic:antiapoptotic factors. We did not find any alterations in bax and bcl-X\textsubscript{L} expression after TAM treatment. This conflicts with a report that ionizing radiation induces down-regulation of bcl-X\textsubscript{L} mRNA expression (27) and suggests that alteration of bcl-2 protein alone is sufficient to alter the bax:bcl-2 ratio or decrease the antiapoptotic levels and alter susceptibility to apoptosis. However, it is also possible that other members of the bcl-2 family, e.g., BAG-1 (11) and bad (14), may interfere with TAM-induced apoptosis rather than bax or bcl-X\textsubscript{L}.

Taxol, which activates a mitogen-stimulated protein serine/threonine kinase (i.e., c-Raf-1; Ref. 28), has been shown to induce apoptosis via phosphorylating bcl-2 protein in breast and prostate cancers (23, 29). TAM could not phosphorylate bcl-2 protein in MCF-7 cells. Thus, apoptosis induced by TAM is not due to a functional inactivation by phosphorylation of bcl-2 protein.

The mechanism whereby TAM down-regulates bcl-2 expression remains unknown. In breast carcinomas, bcl-2 has been shown to associate with ER status (30). Thus, bcl-2 may be regulated through the interaction of estrogen with ER (18). Furthermore, in MCF-7 cells, estrogen has been shown to inhibit cytotoxic drug-induced apoptosis through up-regulating bcl-2 levels (31). A previous study demonstrated that MCF-7 cells cultured in a medium containing 10% FBS express significantly higher bcl-2 levels than those cells exposed to estrogen-free medium for 14 days (18). This result suggests that estrogen contained in 10% FBS medium has a role in up-regulating bcl-2 levels. To eliminate the effect of endogenous estrogen, a medium with 10% DCC-FBS was used to investigate the effect of E\textsubscript{2} and TAM on bcl-2 levels. In our present study, 10\textsuperscript{-9} M E\textsubscript{2} significantly increased bcl-2 protein and blocked TAM-induced apoptosis. These results are consistent with a previous report that >10\textsuperscript{-9} M E\textsubscript{2} up-regulated bcl-2 levels in MCF-7 cells exposed to an estrogen-free medium for 4–14 days, and TAM reduced the rate of increase of bcl-2 levels (18). We have demonstrated that down-regulation of bcl-2 is associated with the apoptotic action of TAM. These data, along with previous reports, strongly suggest that down-regulation of bcl-2 is necessary for TAM-induced apoptosis and appears to be mediated by interaction between TAM and ER. The decrease in both mRNA and protein levels induced by TAM suggest that TAM acts via a transcriptional mechanism. It appears that the E\textsubscript{2}-ER complex can directly bind to the estrogen response element of the bcl-2 promoter. However, no consensus sequence (GGTCA-\textsubscript{nnn}-TGACC) for the estrogen response element has been clarified in the bcl-2 gene sequence (32). Dubik and Shiu (33) have localized a 116-bp region overlapping the P2 promoter in the human c-myc gene, which lacks the conventional estrogen response element sequence. They have also shown that estrogen activates c-myc expression via the interaction of E2-ER complex with the 116-bp regions. Thus, further study is needed to clarify whether an alternative sequence conferring estrogen responsiveness exists in the bcl-2 gene as in the c-myc oncogene.

bcl-2 expression may be down-regulated by p53 through binding to the silencer of bcl-2 (34), and p53 plays a critical role in apoptosis after treatment with cytotoxic agents or irradiation. To discover whether bcl-2 down-regulation is caused via a p53-mediated pathway, we examined the effects of TAM on p53 protein in MCF-7 cells. We did not find increases in p53 protein expression. Although in testicular tumors and other tumors p53 has been found to increase severalfold after treatment with cytotoxic agents, this is not the case for all of cell lines or all types of DNA damage (35, 36). Our results demonstrate that
TAM-induced apoptosis is probably not mediated by altering p53 levels. Similarly, in MCF-7 breast cancer cells exposed to hypoxia, no changes in p53 protein were found compared with cells under aerobic conditions, and only 50% of cells undergoing apoptosis were shown to be p53 positive (37). p53 is both a transactivator and a repressor of transcription, but it remains unclear whether the activities of p53 are required for apoptosis. Previous investigation indicated that p53-mediated apoptosis initiated by DNA damage can occur in the presence of actinomycin D or cycloheximide (36), which blocks RNA or protein synthesis. Taken together with our observations, this suggests that functional p53 might play a role in the regulation of apoptosis by TAM rather than through an elevation in p53 levels, and p53 may initiate apoptosis by a p53 activity not requiring transcription in breast cancer cells.

In breast cancers, bcl-2 expression has been shown to be a favorable prognostic factor and predictor of response to endocrine therapy (38–40). This improved response to endocrine therapy by bcl-2-positive tumors might be the result of down-regulation of bcl-2 by antiestrogen agents, with the resultant susceptibility to apoptosis. Our results provide a possible explanation for these clinical observations, showing that TAM can trigger apoptosis by down-regulating bcl-2 levels, control tumor progression, and improve responses of bcl-2-positive tumors. Thus, treatment modulating apoptosis may benefit breast cancer patients. Recently, several studies in vitro have shown that antisense oligodeoxynucleotide targeting the bcl-2 coding sequence can effectively reduce the viability of malignant cells by down-regulating bcl-2 levels in both small lung cancer and hematological malignancies (41, 42). Antisense therapy to reduce bcl-2 expression may provide a novel therapeutic strategy (43) and enhance the antitumor effects of TAM in bcl-2-positive breast cancers.

In conclusion, our results demonstrate that TAM can induce apoptosis in a time- and dose-dependent manner by modulating bcl-2 levels in breast cancer cells. The reduction of bcl-2 induced by TAM is likely regulated at the transcriptional level and is not accompanied by alterations in p53 levels. Further studies should be performed to investigate whether TAM effects on apoptosis and bcl-2 expression in MCF-7 cells can be extended to other antiestrogens or whether TAM can induce apoptosis and down-regulate bcl-2 levels in other ER-positive breast cancer cell lines.

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