Overexpression of HER2 Modulates Bcl-2, Bcl-X_L, and Tamoxifen-induced Apoptosis in Human MCF-7 Breast Cancer Cells[1]

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ABSTRACT

Overexpression of HER2 in estrogen receptor (ER)-positive human breast tumors has been associated with resistance to endocrine therapy. Here we investigated the effects of HER2 on expression of apoptotic pathways and modulation of tamoxifen-induced apoptosis in ER-positive MCF-7 breast cancer cells. We report that HER2 overexpression in MCF-7 cells is accompanied by up-regulation of antiapoptotic Bcl-2 and Bcl-X_L proteins and suppression of tamoxifen-induced apoptosis. In addition, human tumor cell lines that are both ER positive and overexpress HER2 also express enhanced levels of Bcl-2 compared to cells that are either ER positive or overexpress HER2 alone. Our findings suggest that possible deregulation of antiapoptotic Bcl-2 and Bcl-X_L may be associated with the enhanced survival of HER2-overexpressing and ER-positive breast cancer cells treated with antiestrogens.

INTRODUCTION

Breast cancer is the second leading cause of cancer death in women in the United States. A number of studies have demonstrated that about one-third of breast cancer patients respond to endocrine therapy, and this population is usually ER[3] positive (1). Despite the presence of ER, a significant proportion of patients do not respond to hormone therapy. One potential mechanism for development of resistance to antiestrogen therapy is the influence of growth factor pathways such as HER2 (also known as c-*erb*-B2 or c-*neu*). The HER2 encodes a M_r 185,000 transmembrane receptor tyrosine kinase glycoprotein (2) that has been shown to be overexpressed and/or amplified in breast cancer (3). *In vitro* studies have shown the development of tamoxifen resistance in ER-positive MCF-7 human breast cancer cells upon overexpression of the HER2 gene product (4). Clinical studies also demonstrated that the overexpression of HER2 in ER-positive breast tumors is associated with a decreased response to hormone treatment (5, 6). The biochemical mechanism leading to tamoxifen resistance of ER-positive breast cancer cells is not well understood.

Programmed cell death, or apoptosis, is a physiological mechanism of cell death that is dependent on both preexisting proteins and *de novo* protein synthesis (7, 8). Apoptosis plays an important role during development, metamorphosis, organ involution, and in many diseases including cancer (8). Apoptosis is characterized by nuclear condensation and fragmentation and degradation of DNA into oligonucleosome fragments (7, 8). Regulation of apoptosis is a complex process and involves a number of genes including *Bcl-2, Bcl-X, Bax,* and related family members (7–10). *Bcl-2* encodes a protein that protects cells against apoptosis (7). The *Bcl-X* gene gives rise to two distinct mRNAs by differential splicing that encode Bcl-X_L and Bcl-X_S proteins (9). Bcl-X_L is related to Bcl-2 in inhibiting apoptosis; in contrast, Bcl-X_S is a dominant-negative inhibitor of both Bcl-2 and Bcl-X_L (9). Bax is another protein with apoptosis-promoting function and forms homodimers and heterodimers with Bcl-2 (10). It is being proposed that the Bcl-2:Bax ratio may be important in regulating the nature of the apoptotic response; if Bax predominates, apoptosis is accelerated, and the antiapoptotic activity of Bcl-2 is antagonized (9, 10). The significance of expression of Bcl-2 and related family members in human breast cancer cells is not well defined, as yet. Bcl-2 may play a role in breast cancer development because this protein is overexpressed in 70% of breast cancers (11). Recent studies using immunocytochemical staining have shown an inverse relationship between the levels of Bcl-2 and HER2 expression (12, 13). Breast cancer MCF-7 cells express easily detectable levels of both Bcl-2 and Bcl-X_L (14, 15). Furthermore, up-regulation of Bcl-2 in MCF-7 breast cancer cells has been shown to be associated with resistance to tamoxifen (1–10 μM)-induced apoptosis (16).

Since overexpression of Bcl-2 and Bcl-X_L in cancer cells has been shown to result in suppression of apoptosis in response to a number of anticancer drugs (7, 8, 15, 17), these observations suggest that cancer cells may depend on Bcl-2 and/or Bcl-X_L or related members to prevent apoptosis, and that deregulation of apoptotic pathway(s) may modulate the sensitivity of breast cancer cells to therapeutic agents such as tamoxifen. In the studies presented here, we investigated the possible role of HER2 overexpression on apoptotic pathways and modulation of sensitivity of ER-positive breast cancer cells to tamoxifen-induced apoptosis using MCF-7 cells as a model system. We report that HER2 overexpression is accompanied by up-regulation of Bcl-2 and Bcl-X_L, as well as suppression of tamoxifen-induced apoptosis in MCF-7 cells.

MATERIALS AND METHODS

Cell Lines and Cultures. Human breast cancer MCF-7 cells and its clones, MCF-7/neo and MCF-7/HER2–18 cells

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[3] The abbreviations used are: ER, estrogen receptor; mAb, monoclonal antibody.

(kindly provided by Dr. C. K. Osborne; MCF-7/neo and MCF-7/HER2–18 cells have easily detectable levels of ER; Ref. 4), were maintained in DMEM-F12 (1:1) supplemented with 10% FCS. Other human breast cancer cells (T47-D, ZR-75-R, MDA-231, SKBR-3, MDA-468, MDA-453, MDA-361, SKOV-3, and OVCAR-3) were obtained from the American Type Culture Collection and were grown in the recommended culture media containing 10% serum. All experiments were performed with cells in logarithmic growth phase by controlling the plating density. When desired, subconfluent (about 50% or less confluent) cells were cultured in phenol red-free DMEM-F12 (1:1) supplemented with 5% charcoal-dextran-treated FCS for 24 h before tamoxifen treatment. Exponentially growing cells treated with the desired agents were used to prepare cell extracts as described (18, 19).

Western Immunoblotting. Cell lysates containing an equal amount of total protein (15–25 μg) were resolved on a SDS-PAGE, followed by transfer onto nitrocellulose (18, 19). Membranes were blocked in 1% BSA in TBS, followed by probing with either anti-Bcl-2 mAb (clone 124; Dako Corp.) or anti-Bcl-X mAb (20) or anti-Bax-polyclonal (Santa Cruz), and immune complexes were detected by using a secondary antibody-based alkaline phosphatase color reaction or ^{125}I-labeled protein A or the ECL method (for detection of Bax; Ref. 18). As an internal control, the blot was always cut into two pieces after transfer of proteins. The lower portion of the blot was probed with a specific antibody, and upper portion was probed with an unrelated actin antibody (Sigma Chemical Corp.). Low-molecular-mass colored markers (Amersham Corp.) were used as standards. For reprobing the blots, a nitrocellulose filter was stripped in 0.1 M glycine buffer (pH 2.5) for 1 h and neutralized in 1 M Tris-Cl, pH 8.0 (18). The quantitation of specific protein bands was performed by using a protein database scanner (Molecular Dynamics).

Quantitation of Apoptosis. To measure apoptotic cell death, we used a "cell death" ELISA (Boehringer Mannheim, Indianapolis, IN) that measures cytoplasmic DNA-histone complexes generated during apoptotic DNA fragmentation but not free histone or DNA that may be generated during nonapoptotic cell death (14–16). MCF-7 cells (2×10^4) were plated into each well of a 48-well plate. After the desired treatment, the cells were washed with PBS, and cytoplasmic extracts were made from both adherent and nonadherent cells, according to the manufacturer's protocol. Control and tamoxifen-treated cell extracts were equalized on the basis of equal cell number, as well as protein in the extracts. Briefly, cells were first coated with anti-histone antibody, loaded with cytoplasmic extracts, and followed by incubation with anti-DNA second antibody conjugated with peroxidase. ELISA was developed with peroxidase substrate, and the absorbance at 405 nm was measured using the Microplate autoreader; results are presented as the fold increase over control untreated cells.

RESULTS

Expression of Bcl-2 in Human Breast Cancer Cell Lines. To examine the possible relationship between HER2 and ER and apoptotic pathway(s) in human breast cancer cells, we determined the expression of Bcl-2 in a panel of breast cancer cell lines using a anti-Bcl-2 mAb in Western immunoblotting. All the cell lines we tested have easily detectable levels of Bcl-2 expression (Fig. 1 and Table 1). Results indicated that there was no significant difference in the levels of Bcl-2 between ER-positive cells, such as MCF-7 and T47-D, and HER2-overexpressing cells, such as SKBR-3 and MDA-453. In contrast, it was interesting to note that ER-positive cells with HER2 overexpression, such as ZR-75-R and BT-474, demonstrated a significantly higher (2–5-fold) levels of Bcl-2 protein. This pattern of Bcl-2 expression was also observed in human ovarian cancer cells as OVCAR-3 cells (ER positive and overexpressing HER2) express a higher levels of Bcl-2 than SKOV-3 cells (HER2 overexpressing).

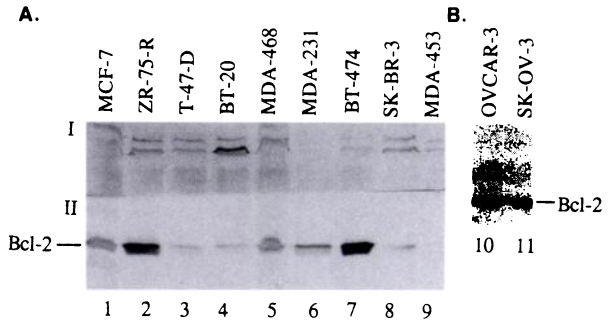

Fig. 1 Expression of Bcl-2 in human breast cancer cell lines. Exponentially growing cells were used to prepare cell extracts. Aliquots containing equal amounts of protein (50 μg) were resolved onto a 10% SDS-PAGE. Proteins were transferred to nitrocellulose, immunoblotted with an anti-Bcl-2 mAb, and visualized using an alkaline phosphatase color system. In *A*, the same blot was cut into two pieces, and *upper panel AI* was blotted with unrelated antibody to show the relative amount of nonspecific proteins in different lanes. In *B*, cell extracts from ovarian cell lines were immunoblotted with an anti-Bcl-2 mAb.

Table 1 Summary of Bcl-2 expression in breast cell lines

Bcl-2 expression[a]	Cell lines
++++	ZR-75-R, BT-474
++	MDA-231, MDA-468, MCF-7
+	T-47-D, BT-20, SK-BR-3
0	MDA-453

[a] Bcl-2 levels from immunoblot in Fig. 1, semiquantitative manner.

Effect of HER2 Overexpression on the Levels of Bcl-2 in MCF-7 Cells. The results in Fig. 1 suggest that the overexpression of HER2 in ER-positive breast cancer cells is associated with increased Bcl-2 expression. To further test this hypothesis, we used MCF-7 cells stably transfected with human HER2 (MCF-7/HER2–18 cells express 45 times the increased HER2 levels; Ref. 4) or control mock neo vector-transfected cells (MCF-7/Neo cells; Ref. 4). Results in Fig. 2*A* show that HER2 overexpression in MCF-7 cells (Fig. 2*A*, *Lane 3*) lead to up-regulation (2.5–6-fold; $n = 8$) in the levels of Bcl-2 compared to the levels in the parental (Fig. 2*A*, *Lane 1*) or mock/neo-transfected (Fig. 2*A*, *Lane 2*) MCF-7 cells. Data in Fig. 2, *B* and *C*, demonstrated that HER2 overexpression in MCF-7/HER2–18 cells (Fig. 2*B*) was associated with enhanced Bcl-2 expression. There was no effect of HER2 overexpression on the

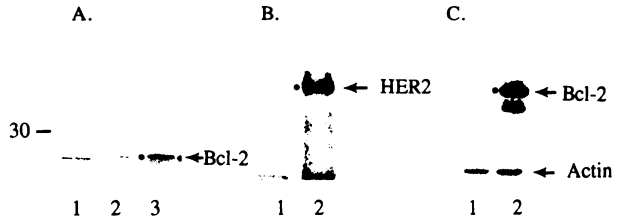

Fig. 2 Effect of HER2 on the levels of Bcl-2 in MCF-7 cells. *A*, exponentially growing cells were used to examine the Bcl-2 expression in different MCF-7 clones. Aliquots containing equal amounts of protein (20 μg) were resolved onto a 15% SDS-PAGE, immunoblotted with a anti-Bcl-2 mAb, and visualized using an alkaline phosphatase color system. *Lane 1*, control MCF-7 cells; *Lane 2*, MCF-7/Neo cells; *Lane 3*, MCF-7/HER2–18 cells. *B*, overexpression of HER2 in MCF-7 cells. Labeled cell lysates from MCF-7/Neo cells (*Lane 1*) and MCF-7/HER2–18 cells (*Lane 2*) were immunoprecipitated with an anti-HER2 mAb 9G6 and resolved on a 7% SDS-PAGE. *C*, cell extracts from MCF-7/Neo cells (*Lane 1*) and MCF-7/HER2–18 cells (*Lane 2*) were resolved on 12% SDS-PAGE, immunoblotted with an anti-Bcl-2 mAb (*upper panel*), and followed by sequential reprobing with anti-actin Ab (*lower panel*).

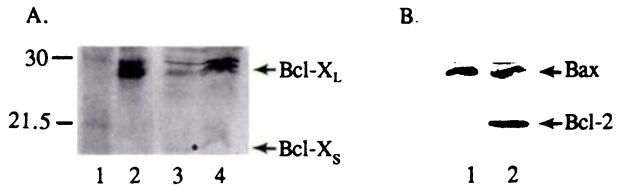

Fig. 3 Effect of HER2 on the levels of Bcl-X and Bax in MCF-7 cells. *A*, cell extracts (15 μg) from MCF-7/Neo cells (*Lanes 1* and *3*) and MCF-7/HER2–18 cells (*Lanes 2* and *4*) were resolved on a 12% SDS-PAGE and immunoblotted with an anti-Bcl-X mAb. *Lanes 1* and *2* and *Lanes 3* and *4* are from two different experiments. *B*, cell extracts from MCF-7/Neo (*Lane 1*) and MCF-7/HER2–18 (*Lane 2*) were immunoblotted with anti-Bax Ab (*upper panel*), followed by sequential reprobing with anti-Bcl-mAb.

levels of an unrelated proteins such as actin (Fig. 2*C*) and 2–5(A)synthetases (data not shown). In addition to Bcl-2 protein, the anti-Bcl-2 mAb used here cross-reacted with an additional protein in MCF-7/HER2–18 cells (Fig. 2*C*, *Lane 2*, see *band* under the Bcl-2) and will be investigated as a part of another study.

Effect of HER2 Overexpression on the Levels of Bcl-X and Bax in MCF-7 Cells. Since apoptosis in mammalian cells is also regulated by Bcl-X gene products, we examined expression of Bcl-X_L and Bcl-X_S in MCF-2/Neo and MCF-7/HER2–18 cells using a well characterized anti-Bcl-X mAb that recognizes both Bcl-X_L and Bcl-X_S (20). Results in Fig. 3*A* indicated that HER2 overexpression in MCF-7 cells also lead to enhanced expression of Bcl-X_L with no change in the levels of Bcl-X_S. We also examined the expression of Bcl-X_L in other breast cancer cell lines, and results indicated that the relative levels of Bcl-X_L were 3-fold higher in BT-474 cells (HER2-overexpressing, ER-positive cells) compared to SK-BR-3 (HER2-overexpressing cells) or MCF-7 (ER-positive cells; data not shown).

Since it has been proposed that the ratio of Bax to Bcl-2 or Bcl-X_L may be important in regulating apoptotic response (10) and the fact that reduced Bax expression has been correlated with the poor response to chemotherapy (21), we examined the effect of HER2 overexpression on the levels of Bax. Results in Fig. 3*B* indicated that there was no significant change in the expression of Bax between MCF-7/Neo (Fig. 3*B*, *Lane 1*) and MCF-7/HER2–18 cells (Fig. 3*B*, *Lane 2*).

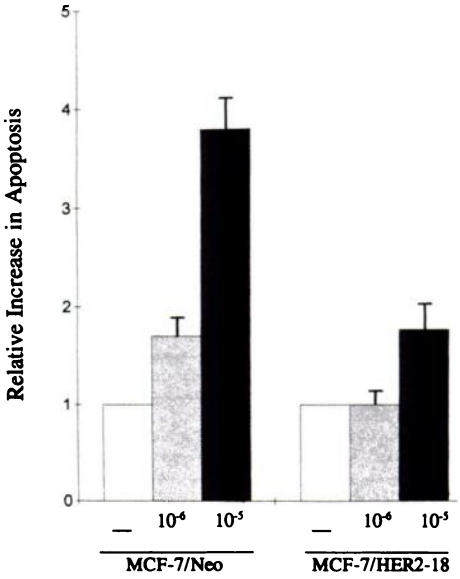

Fig. 4 Effect of HER2 on tamoxifen-induced apoptosis in MCF-7 cells. Expression of Bcl-X_L in breast cancer cells. Cells were grown in estrogen-free medium for 24 h and then treated with or without tamoxifen (10^{-6} or 10^{-5} M) for 24 h. Cell extracts were made, and induction of apoptosis was determined by quantitative ELISA. *Columns*, mean; *bars*, SE (n = 3).

Effect of HER2 Overexpression on Apoptosis in MCF-7 Cells. Since HER2 overexpression in MCF-7/HER2–18 cells increases antiapoptotic Bcl-2 and Bcl-X_L, we examined whether overexpression of HER2 will modulate apoptosis. To test this possibility, we examined the sensitivity of MCF-7 cells with or without HER2 overexpression to undergo apoptosis in response to tamoxifen. Results in Fig. 4 show that HER2 overexpression is associated with significant suppression in the tamoxifen-induced stimulation of apoptosis because there was up to 1.8 ± 0.4 (n = 3) fold increase in apoptosis in MCF-7/HER2–18 cells compared to 3.8 ± 0.63 (n = 3) fold in MCF-7/Neo cells by 24-h tamoxifen (10^{-5} M) treatment.

DISCUSSION

It is generally recognized that the expression of HER2 in ER-positive breast cancer cells leads to development of resistance to endocrine therapy (4–6). In recent years, apoptosis has emerged as a physiological mechanism of cell death in response to a variety of chemotherapy drugs and hormones, including tamoxifen (16, 17). Overexpression of antiapoptotic proteins such as Bcl-2 and Bcl-X_L has been shown to result in the reduced sensitivity of a number of human cell lines to cytotoxic/inhibitory effects of chemotherapy drugs (7, 8, 13, 17). Therefore, deregulation of apoptotic pathways could constitute one of

the potential mechanisms of resistance to tamoxifen, a phenomenon generally associated with HER2 overexpression in ER-positive cells (4–6). To explore this hypothesis, we investigated the effect of HER2 overexpression on apoptotic pathways in MCF-7 cells.

The results presented here demonstrate that overexpression of HER2 in MCF-7 cells results in increased expression (2–5-fold) of Bcl-2 and Bcl-X_L and suppression of tamoxifen-induced apoptosis compared to cells transfected with neo vector alone. Our observation of enhanced Bcl-2 expression in HER2 overexpressing (also ER-positive) breast cancer cells is apparently not consistent with the reports from the literature showing an inverse correlation between HER2 and Bcl-2 expression (12 13). In this regard, it is important to note that those studies (12, 13) have correlated the levels of Bcl-2 with the individual expression of HER2 or ER and not with the co-overexpression of HER2 in ER-positive breast cancer cells. In addition, there may be other important differences between the methods in those two studies (12, 13) that used immunochemical method rather than physical detection of the M_r 26,000 Bcl-2 protein used in our study. In this context, it is worth noticing that the anti-Bcl-2 mAb (clone 124; Dako) used in immunocytochemical studies (12, 13) cross-reacts with proteins other than the M_r 26,000 Bcl-2 in MCF-7/HER2–18 cells (Fig. 2*C*, see *band* under Bcl-2) and also in human colorectal carcinoma DiFi cells (data not shown). Moreover, Teixeira *et al.* (22) have recently reported the cross-reactivity of both polyclonal and monoclonal anti-Bcl-2 antibodies, with an additional M_r 28,000 protein in MCF-7 cells (22). The finding that HER2 overexpression did not influence the levels of proapoptotic Bax and only enhanced the levels of Bcl-2 and Bcl-X_L suggests that HER2 overexpression in MCF-7 cells may shift the Bcl-2:Bcl-X_L ratio to Bax toward suppression of apoptosis as demonstrated in this study. In this context, it is important to note that, recently, enhancement in the levels of Bcl-2 has been shown to confer resistance to tamoxifen-induced apoptosis in MCF-7 cells (16). Because Bcl-2 and Bcl-X_L are antiapoptotic proteins, the observed resistance of tamoxifen-induced apoptosis in HER2-overexpressing MCF-7 cells may have a close relationship with enhanced expression of Bcl-2 and Bcl-X_L.

Our finding of enhanced expression of Bcl-2 and Bcl-X_L in MCF-7/HER2–18 cells have raised a number of new issues regarding the mechanism(s) of regulation of antiapoptotic gene products in ER-positive breast cancer cells and its modulation by HER2. Why is enhanced expression of Bcl-2 and Bcl-X_L restricted to HER2-overexpressing, ER-positive breast cancer cells? Is there any contribution of regulatory interaction(s) between HER2 and ER? The mechanism by which co-overexpression of HER2 in ER-positive breast cancer cells influences the levels of antiapoptotic proteins remains to be determined. This may occur at the transcriptional level and/or posttranscriptional level and may also involve enhanced mRNA stability, leading to an increase in protein expression. Our findings support the hypothesis of possible deregulation of apoptotic pathways and up-regulation of antiapoptotic Bcl-2 and Bcl-X_L in breast cancer cells; these biochemical changes may lead to enhanced survival of HER2-overexpressing, ER-positive cells upon endocrine therapy and thus could play a role(s) in breast cancer progression.

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