Overexpression of HER2 Modulates Bcl-2, Bcl-X<sub>L</sub>, and Tamoxifen-induced Apoptosis in Human MCF-7 Breast Cancer Cells<sup>1</sup>

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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<sub>L</sub> 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<sub>L</sub> 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 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 Mr 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<sub>L</sub> and Bcl-X<sub>s</sub> proteins (9). Bcl-X<sub>L</sub> is related to Bcl-2 in inhibiting apoptosis; in contrast, Bcl-X<sub>s</sub> is a dominant-negative inhibitor of both Bcl-2 and Bcl-X<sub>L</sub> (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<sub>L</sub> (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<sub>L</sub> 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<sub>L</sub> 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<sub>L</sub>, 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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we determined the expression of Bcl-2 in a panel of breast cancer cell lines. To examine the possible relationship between HER2 overexpression 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) to express 45 times the increased HER2 levels; Ref. 4) or control mock neo-transfected cells (MCF-7/Neo cells; Ref. 4). Results in Fig. 2A show that HER2 overexpression in MCF-7 cells (Fig. 2A, 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. 2A, Lane 1) or mock/neo-transfected (Fig. 2A, Lane 2) MCF-7 cells. Data in Fig. 2, B and C, demonstrated that HER2 overexpression in MCF-7/HER2-18 cells (Fig. 2B) was associated with enhanced Bcl-2 expression. There was no effect of HER2 overexpression on the

**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 125I-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 x 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 an 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).

**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) to 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. 2A show that HER2 overexpression in MCF-7 cells (Fig. 2A, 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. 2A, Lane 1) or mock/neo-transfected (Fig. 2A, Lane 2) MCF-7 cells. Data in Fig. 2, B and C, demonstrated that HER2 overexpression in MCF-7/HER2-18 cells (Fig. 2B) was associated with enhanced Bcl-2 expression. There was no effect of HER2 overexpression on the
levels of an unrelated proteins such as actin (Fig. 2C) 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. 2C, 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-XL and Bcl-XS in MCF-2/Neo and MCF-7/HER2–18 cells using a well characterized anti-Bcl-X mAb that recognizes both Bcl-XL and Bcl-XS (20). Results in Fig. 3A indicated that HER2 overexpression in MCF-7 cells also lead to enhanced expression of Bcl-XL with no change in the levels of Bcl-XS. We also examined the expression of Bcl-XS in other breast cancer cell lines, and results indicated that the relative levels of Bcl-XS 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-XL 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. 3B indicated that there was no significant change in the expression of Bax between MCF-7/Neo (Fig. 3B, Lane 1) and MCF-7/HER2–18 cells (Fig. 3B, Lane 2).

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-XL, 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-XL 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...
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the potential mechanisms of resistance to tamoxifen, a phenom-
enon 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 overexpres-
sion of HER2 in MCF-7 cells results in increased expression
(2–5-fold) of Bcl-2 and Bcl-XL, and suppression of tamox-
ifen-induced apoptosis compared to cells transfected with
neo vector alone. Our observation of enhanced Bcl-2 expres-
sion in HER2 overexpressing (also ER-positive) breast can-
cer 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 dif-
ferences between the methods in those two studies (12, 13)
that used immunochemical method rather than physical de-
tection of the \( M_\text{c} \), 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 \( M_\text{c} \), 26,000 Bcl-2
in MCF-7/HER2–18 cells (Fig. 2C; 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 \( M_\text{c} \), 28,000 protein in
MCF-7 cells (22). The finding that HER2 overexpression did
not influence the levels of proapoptotic Bax and only en-
hanced the levels of Bcl-2 and Bcl-XL suggests that HER2
overexpression in MCF-7 cells may shift the Bcl-2:Bcl-XL
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-XL are antiapop-
totic 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-XL.

Our finding of enhanced expression of Bcl-2 and Bcl-XL 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-XL
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-overexpres-
sion 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-XL 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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