Modulation of Tamoxifen Sensitivity by Antisense *Bcl-2* and Trastuzumab in Breast Carcinoma Cells

Ryungsa Kim, M.D., Ph.D.[1]
Kazuaki Tanabe, M.D., Ph.D.[2]
Manabu Emi, M.D.[2]
Yoko Uchida, M.D.[2]
Tetsuya Toge, M.D., Ph.D.[2]

[1] International Radiation Information Center, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan.

[2] Department of Surgical Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan.

Address for reprints: Ryungsa Kim, M.D., Ph.D., International Radiation Information Center, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3 Kasumi Minamiku, Hiroshima 734-8553, Japan; Fax: (011) 81 822575877; E-mail: rkim@hiroshima-u.ac.jp

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BACKGROUND. Because the overexpression of HER-2 and Bcl-2 is associated with resistance to tamoxifen (TAM), the authors examined the effect of antisense (AS) *Bcl-2* on sensitivity to TAM compared with the effect of trastuzumab on sensitivity to TAM in breast carcinoma cell lines.

METHODS. Drug sensitivity was assessed in vitro using a [3-4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay with the breast carcinoma cell lines ZR-75-1, MDA-MB-453, and BT-474. AS *Bcl-2* 18-mer phosphorothioate oligonucleotide was applied. Apoptotic cell death was assessed with the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labeling method, and gene expression was evaluated with Western blot analysis.

RESULTS. The expression of Bcl-2 was identified in ZR-75-1 and BT-474 cells and, to a lesser extent, in MDA-MB-453 cells. Overexpression of HER-2 was identified in BT-474 cells, and moderate expression was identified in MDA-MB-453 and ZR-75-1 cells. Combination treatment with trastuzumab or AS *Bcl-2* enhanced TAM sensitivity in ZR-75-1 cells, which showed 50% inhibitory concentration (IC_{50}) values of 0.9 μM (7.2-fold increase) and 0.5 μM (13.0-fold), respectively. Combination treatment with trastuzumab or AS *Bcl-2* slightly enhanced TAM sensitivity of BT-474 cells, with IC_{50} values of 3.0 μM (1.3-fold) and 1.5 μM (2.6-fold), respectively. The sensitivity of MDA-MB-453 cells to TAM was not enhanced by combination with trastuzumab or AS *Bcl-2*. Modulation of TAM sensitivity by AS *Bcl-2* was superior to modulation by trastuzumab in HER-2-expressing and *Bcl-2*-expressing breast carcinoma cells. Enhanced sensitivity in combination with AS *Bcl-2* was associated with down-regulation of Bcl-2 and pAkt, which was correlated with the induction of Bax and caspase-3, leading to apoptosis.

CONCLUSIONS. AS *Bcl-2* appeared to be superior to trastuzumab with respect to regulating the signal-transduction pathways involved in breast carcinoma cells. ***Cancer*** **2005;103:2199–207.** *© 2005 American Cancer Society.*

KEYWORDS: antisense, Bcl-2, tamoxifen, trastuzumab, breast carcinoma.

Tamoxifen (TAM) is a promising agent for antiestrogen therapy in the adjuvant setting in patients with estrogen receptor (ER)-positive breast carcinoma. A survival benefit of TAM as adjuvant therapy has been shown even in the presence of axillary lymph node metastasis and regardless of menopausal status.[1,2] Although TAM is used as an antiestrogen treatment, failure occasionally occurs in the metastatic setting. Recent advances in antiestrogen therapy suggest that second-line therapy in this situation may include the use of aromatase inhibitors, which reportedly was effective against metastatic breast carcinoma in patients who were treated previously with TAM.[3–5] A survival benefit of anastrozole in the adjuvant setting also was reported recently.[6] Nevertheless, because TAM resistance is an inevitable phenomenon in situations of prolonged survival with met-

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astatic breast carcinoma, overcoming this resistance may provide increased survival.

The Bcl-2 oncoprotein is a critical regulator of apoptosis induced by both antitumor drugs and antiestrogens in breast carcinoma.[7,8] Apoptosis is important in the antitumor effect, and molecular mechanisms by which antitumor drugs and antiestrogens induce apoptosis have shown that mitochondrial dysfunction is involved.[9,10] The overexpression of Bcl-2 also is involved in the acquisition of resistance to antitumor drugs and antiestrogens, which involves inhibition of the signal-transduction pathways that lead to apoptosis.[11,12] The regulation of Bcl-2 expression is mediated by the promoter region of the ER-responsive element in breast carcinoma, and the prognosis for patients with Bcl-2-overexpressing tumors is better compared with the prognosis for patients with non-Bcl-2-overexpressing tumors.[13] However, the induction of Bcl-2 protein expression during chemoendocrine treatment occasionally causes resistance to antitumor drugs and antiestrogens. Thus, the induction of Bcl-2 expression is important in influencing TAM sensitivity in patients with breast carcinoma.

The HER-2 oncoprotein is a member of the epidermal growth factor family, which is involved in cell proliferation, angiogenesis, and metastasis in tumor cells.[14,15] Overexpression of HER-2 is observed in 20–30% of breast carcinomas and is associated with drug resistance and a poor prognosis.[16] Transfection of the HER-2 gene into breast tumor cells leads to resistance to antitumor drugs, such as paclitaxel, docetaxel, and TAM.[17,18] Overexpression of HER-2 leads to phosphorylation of Akt, which activates nuclear factor κB (NF-κB) to inhibit apoptosis[19] mediated by inhibitor-of-apoptosis proteins.[20] Furthermore, activation of pAkt is correlated with induction of Bcl-2 expression through cyclic AMP-responsive element-binding protein (CREB) and coupling to an antiapoptotic signaling network.[21] Thus, antiapoptotic signaling pathways that involve HER-2 and Bcl-2 play a pivotal role in the gain of resistance to apoptosis in response to antitumor drugs and antiestrogens in breast carcinoma cells. In the current study, we examined the enhancement of TAM sensitivity by antisense (AS) *Bcl-2* compared with trastuzumab in breast carcinoma cells to assess the therapeutic efficacy of AS strategies for overcoming resistance to endocrine therapy in receptor-positive breast carcinomas.

MATERIALS AND METHODS

Chemicals, Drugs, and Supplies

Chemicals and supplies were purchased from the following suppliers: RPMI 1640 and fetal bovine serum were from GIBCO BRL (Tokyo, Japan). AS *Bcl-2* phosphorothioate oligonucleotide (AS *Bcl-2* ODN) and mismatch AS *Bcl-2* (MM *Bcl-2*) 18-mer oligonucleotide were from Biologica Company, Ltd. (Tokyo, Japan). Transfectam was from Biosera (Marlborough, France). Anti-Bcl-2, anti-HER-2, anti-pAkt, anti-Bax, anti-caspase 3, and antiactin rabbit polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Peroxidase-conjugated antirabbit immunogloblin G (IgG) was from Upstate Biotechnology (Lake Placid, NY). The enhanced chemiluminescence (ECL) Western blot detection system was from Amersham Pharmacia Biotech, Ltd., (Buckinghamshire, United Kingdom). The in situ cell death detection kit was from Boehringer Mannheim (Indianapolis, IN). TAM was from Sigma Chemical Company (Tokyo, Japan). Trastuzumab kindly was supplied by Chugai Pharmaceutical Company, Ltd. (Tokyo, Japan).

Cell Lines

The human breast carcinoma cell lines ZR-75-1, MDA-MB-453, and BT-474 were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 supplemented with 10% heart-inactivated fetal bovine serum plus 1% penicillin/streptomycin and 1% glutamine. Cultures were maintained in a 5% CO_2, humidified incubator at 37 °C, and experiments were performed with exponentially growing cells.

The [3-4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium Bromide Assay

The [3-4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was performed according to a previously described method.[22] Briefly, cells (2×10^3) were treated with antitumor drugs for 48 hours. The tetrazolium agent was then added to each well. This was followed by a 4-hour incubation. Then, the culture medium supernatant was removed and dissolved mixed with dimethyl sulfoxide. After thorough formazan solubilization, the absorbance of each well was measured with a microculture plate reader at 540 nm. Growth inhibition was expressed as the ratio of the mean absorbance of treated cells to that of control cells. Each experiment was performed in triplicate, and the growth inhibition rate was calculated as a 50% inhibitory concentration (IC_{50}) value.

The Terminal Deoxynucleotidyl Transferase-Mediated Biotinylated UTP Nick-End Labeling Method

The terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated UTP nick-end labeling (TUNEL) method for assessment of apoptotic cell death was carried out according to the manufacturer's protocol, as described previously.[23] Briefly, the cells were

treated with drugs and then incubated with TdT and TdT buffer (biotin-16-dUTP), the reaction was made visible with nitroblue tetrazolium, and the cells were photographed. Apoptotic cell death was evaluated by the appearance of brightly labeled nuclei and apoptotic bodies. The induction of apoptosis was represented as the mean ± standard deviation from four random microscopic fields and was compared with that in untreated cells.

Treatment with AS *Bcl-2* Oligonucleotide

The AS *Bcl-2* phosphorothioate ODN sequence was as follows: 5′-TCT CCC AGC GTG CGC CAT-3′.[24] The sequence of the MM *Bcl-2* was as follows: 5′-TCT CCC AGC ATG TGC CAT-3′. AS and control ODNs were added to the cells in the form of complexes with cationic lipopolyamine according to the manufacturer's protocol, as described previously.[25] Briefly, the medium was removed, and serum-free RPMI was added to the cells. The ODN-lipopolyamine complex was prepared and added dropwise to the cells. After 12 hours of incubation, cells were washed with serum-free RPMI, and RPMI containing 10% fetal bovine serum was added to the cells. Cells were then treated with AS *Bcl-2* ODN twice for 12 hours per treatment. After the second treatment with AS *Bcl-2* ODN, cells were incubated with antitumor drugs for 48 hours, and cell viability was assessed with the MTT assay. Because the specificity and optimal concentration for AS *Bcl-2* have been reported in previous studies,[24,26] the appropriate concentration of AS ODNs used in this study was 1.0 μM. The treatment with 1.0 μM AS *Bcl-2* for at least 12 hours did not affect cell viability of breast carcinoma cells.

Western Blot Analysis

Western blot analysis was performed according to a previously described method.[27] Briefly, whole-cell lysates were extracted with lysis buffer (10 mM Tris-HCl, pH 8.0; 0.15 M NaCl; 1 mM ethylenediamine tetraacetic acid; 10 mM 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate; 10 μg/mL aprotinin; and 0.02 mM phenylmethylsulfonyl fluoride), and the protein concentration was determined from 10 μg of each sample. Proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and were transferred to polyvinylidene difluoride membranes with a polyblot. Filters were blocked in 5% skim milk in phosphate buffered saline (PBS) for 1 hour. After incubation with primary antibody for 24–48 hours, filters were washed with PBS and then incubated with secondary antibody in 5% skim milk in PBS for 1 hour. After washing the membranes with PBS for 30 minutes, bands were visualized with the ECL Western blot detection system. Films were exposed for 15–30 minutes. The intensity of the bands was quantified by densitometric scanning with NIH Image software (version 1.56). For controls, the rabbit IgG serum was used.

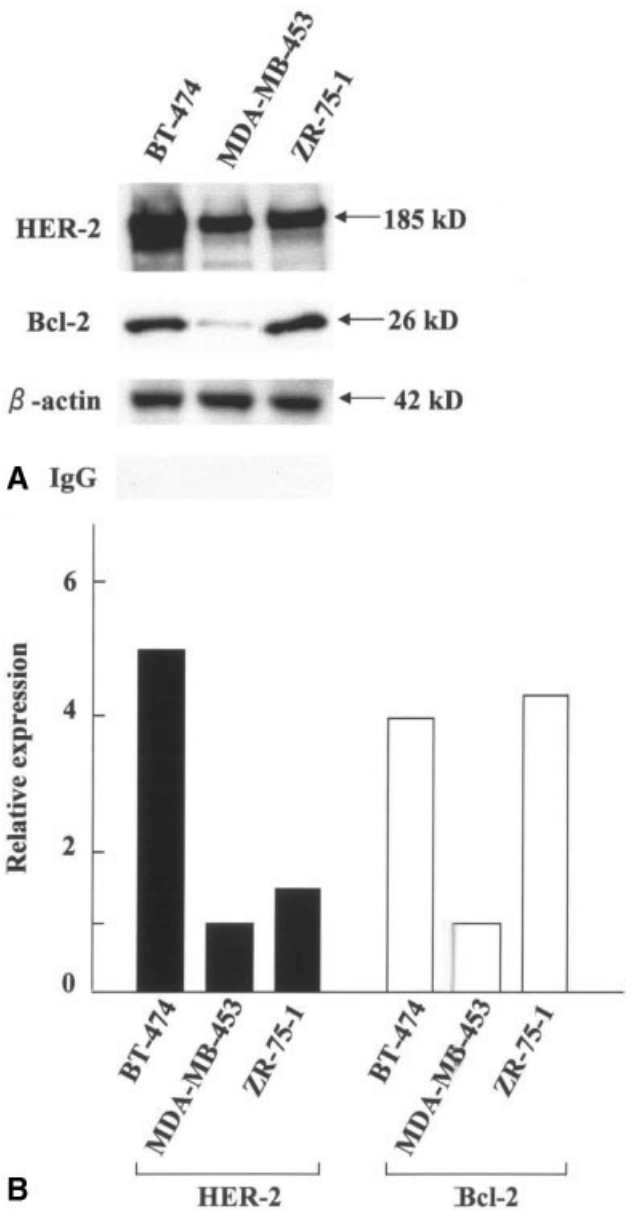

FIGURE 1. Expression levels of HER-2 and Bcl-2 proteins are illustrated in the BT-474, MDA-MB-453, and ZR-75-1 breast carcinoma cell lines. (A) Western blot analysis of HER-2 and Bcl-2 expression. (B) Quantification of HER-2 and Bcl-2 expression by densitometric analysis. The relative expression in the MDA-MB-453 cell line was compared with the expression in the BT-474 and ZR-75-1 cell lines (for details, see Materials and Methods). Results are shown from two representative, independent experiments.

TABLE 1
Modulation of Tamoxifen Sensitivity by Trastuzumab and Antisense *Bcl-2* in Breast Carcinoma Cell Lines

	IC_{50} value (μM)					
Cell line	TAM	Trastuzumab	AS *Bcl-2*	Trastuzumab and TAM	AS *Bcl-2* and TAM	MM *Bcl-2* and TAM
BT-474	4.0	3.9	3.6	3.0 (1.3)	1.5 (2.6)	3.8
MDA-MB-453	0.7	0.8	0.65	0.75	8.0	0.8
ZR-75-1	6.5	6.3	5.9	0.9 (7.2)	0.5 (13.0)	6.3

IC_{50}: 50% inhibitory concentration; TAM: tamoxifen; AS: antisense; MM: mismatch. Data were derived from triplicate cultures and are presented as mean values (n = 3 cultures; standard deviation < 5%. Values in parentheses indicate the x-fold decrease in the IC_{50} value.

Statistical Analysis

Statistical significance was determined with Student *t* tests or analysis of variance (ANOVA) with the Fisher least-squares difference (LSD) test for multiple comparisons. *P* values < 0.05 were considered significant.

RESULTS

Expression of Bcl-2 and HER-2 in Breast Carcinoma Cell Lines

To assess the expression of Bcl-2 and HER-2 in breast carcinoma cell lines, Western blot analysis was carried out. Figure 1 shows that Bcl-2 protein was expressed in BT-474 and ZR-75-1 cells, whereas BCl-2 expression in MDA-MB-453 cells was observed to a lesser extent. Expression of Bcl-2 in ZR-75-1 cells was somewhat higher than in BT-474 cells. These findings are consistent in part with the expression of ER and progesterone receptor in these cells; BT-474 and ZR-75-1 cells express both ER and progesterone receptor, whereas MDA-MB-453 cells are negative for both receptors (data not shown), in agreement with the previous reports.[28,29] These observations of Bcl-2 expression also are in agreement with previous reports. HER-2 was overexpressed in BT-474 cells and was expressed to a small extent in ZR-75-1 and MDA-MB-453 cells. These results are in agreement with a previous report due to mechanisms other than gene amplification, which lead to overexpression of the HER-2/*neu* protooncogene in carcinoma cells.[30]

Modulation of Sensitivity to TAM by Trastuzumab or AS *Bcl-2*

To assess sensitivity to TAM in breast carcinoma cell lines, IC_{50} values were determined by MTT assay. Table 1 shows that the mean IC_{50} values were 6.5 μM in ZR-75-1 cells, 0.7 μM in MDA-MB-453 cells, and 4.0 μM in BT-474 cells. These results indicate that MDA-MB-453 cells are relatively sensitive to TAM compared with the other cell lines, even though this cell line does not express hormone receptor. Treatment with trastuzumab increased the sensitivity to TAM in HER-2-overexpressing cells. The effect of AS *Bcl-2* on TAM sensitivity was then assessed. Figure 2 shows that the dose-response curve for TAM was enhanced by treatment with trastuzumab or AS *Bcl-2* in ZR-75-1 and BT-474 cells. Furthermore, TAM sensitivity was enhanced significantly to the greatest extent in combination with 0.5 μM TAM and AS *Bcl-2* in the treatment arm ($P < 0.05$; ANOVA with Fisher LSD test). In contrast, TAM sensitivity was not enhanced by either trastuzumab or AS *Bcl-2* in MDA-MB-453 cells; rather, it appeared that treatment with AS *Bcl-2* decreased sensitivity to TAM. The IC_{50} values are summarized in Table 1. Treatment with trastuzumab alone did not differ from TAM in any of the cell lines, whereas treatment with AS *Bcl-2* alone showed a slight increase in the IC_{50} value compared with TAM. Treatment with trastuzumab enhanced the sensitivity to TAM of ZR-75-1 cells, as shown by a 7.2-fold decrease in the IC_{50} value, whereas treatment with trastuzumab slightly increased the sensitivity to TAM of BT-474 cells, as shown by a 1.3-fold decrease in the IC_{50} value. Sensitivity to TAM of MDA-MB-453 cells was not altered by treatment with trastuzumab. Treatment with AS *Bcl-2* enhanced TAM sensitivity in ZR-75-1 cells by 13.0 fold and in BT-474 cells by 2.6 fold, whereas TAM sensitivity in MDA-MB-453 cells was not altered by treatment with AS *Bcl-2*. MM *Bcl-2* ODN had no effect. These results indicate that modulation of HER-2 or Bcl-2 by trastuzumab or AS can enhance sensitivity to TAM in receptor-positive breast carcinoma cells.

Enhancement of Apoptotic Cell Death by Trastuzumab or AS *Bcl-2*

To assess the induction of apoptotic cell death by TAM in combination with trastuzumab or AS *Bcl-2*, TUNEL assays were carried out (Fig. 3). The apoptotic indices after treatment with 1.0 μM TAM, 0.1 μM trastuzumab, and 1.0 μM AS *Bcl-2* alone in ZR-75-1 cells were 1.2%, 2.6% $\pm$ 0.9%, and 3.1% $\pm$ 1.4%, respec-

tively; whereas cotreatment with TAM and 0.1 μM trastuzumab enhanced apoptotic cell death up to 18.0% ± 1.5%. Cotreatment with TAM and 1.0 μM AS *Bcl-2* enhanced apoptotic cell death to 24.0% ± 4.5%, whereas cotreatment with MM *Bcl-2* did not enhance apoptotic cell death in 3.0% ± 1.6%. In BT-474 cells, induction of apoptotic cell death by TAM also was enhanced in combination with AS *Bcl-2*. The apoptotic indices in response to treatment with TAM, trastuzumab, AS *Bcl-2*, trastuzumab plus TAM, AS *Bcl-2* plus TAM, and MM *Bcl-2* plus TAM were 2.5%, 3.5% ± 1.5%, 5.1% ± 1.6%, 11.4% ± 1.5%, 19.2% ± 2.1%, and 2.7% ± 2.2%, respectively. These findings indicate that, although combination treatment with trastuzumab or AS *Bcl-2* enhanced sensitivity to TAM in receptor-positive breast carcinoma cells, the enhancement of TAM sensitivity by AS *Bcl-2* was greater than the enhancement by trastuzumab in ZR-75-1 and BT-474 cells, which are Bcl-2-expressing breast carcinoma cell lines.

Mechanisms by which Treatment with Trastuzumab or AS *Bcl-2* Enhances TAM Sensitivity

To assess the mechanisms by which combination treatment with trastuzumab or AS *Bcl-2* enhances sensitivity to TAM, the expression of apoptosis-related proteins was analyzed. The expression of Bcl-2 protein and pAkt after treatment with 1.0 μM AS *Bcl-2* plus 1.0 μM TAM was down-regulated, and the expression of Bax and caspase-3 was increased after the treatment for apoptosis in ZR-75-1 cells, whereas cotreatment with MM *Bcl-2* did not increase the expression of these proteins (Fig. 4). Although trastuzumab treatment down-regulated pAkt activity to a lesser extent compared with AS *Bcl-2* treatment, which was connected with a slight induction of Bax and caspase-3 in BT-474 cells, trastuzumab did not down-regulate Bcl-2 expression. These results indicate that down-regulation of Bcl-2 by AS *Bcl-2* or down-regulation of HER-2 by trastuzumab activates apoptotic signal-transduction pathways in breast carcinoma cells and that the susceptibility to apoptotic cell death in response to AS *Bcl-2* may be superior to the response to trastuzumab in terms of dual inhibition of Bcl-2-mediated and Akt-mediated antiapoptotic signaling pathways.

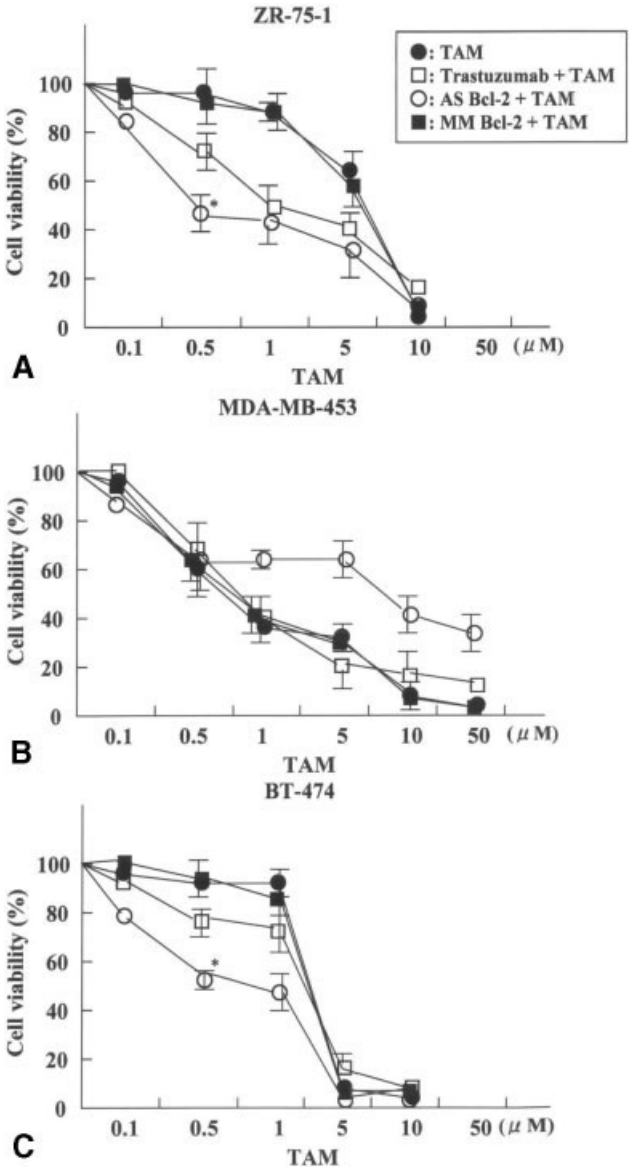

FIGURE 2. Dose-response curves for the modulation of sensitivity to tamoxifen (TAM) in combination with trastuzumab or antisense (AS) *Bcl-2* in the ZR-75-1 (A), MDA-MB-453 (B), and BT-474 (C) breast carcinoma cell lines (trastuzumab, 0.1 μM; AS and mismatch [MM] *Bcl-2*, 1.0 μM; for details, see Materials and Methods). Results are shown from three representative, independent experiments. Error bars indicate the mean ± standard deviation; asterisks indicate P values < 0.05 (analysis of variance with Fisher least-squares difference test).

DISCUSSION

In the current study, we showed that sensitivity to TAM was modulated by AS *Bcl-2* or trastuzumab in receptor-positive breast carcinoma cells. The enhancement of TAM sensitivity by AS *Bcl-2* was greater compared with enhancement by trastuzumab. This may be due to a difference in the Bcl-2-mediated antiapoptotic signaling pathway, which is correlated with the inactivation of pAkt. Down-regulation of HER-2 after treatment with trastuzumab does not necessarily decrease Bcl-2 expression, even in the presence of down-regulated pAkt. In contrast, treatment with AS *Bcl-2* down-regulated Bcl-2 protein as well as pAkt, suggesting that AS *Bcl-2* may exert its inhibitory action through both Bcl-2 and pAkt antiapoptotic

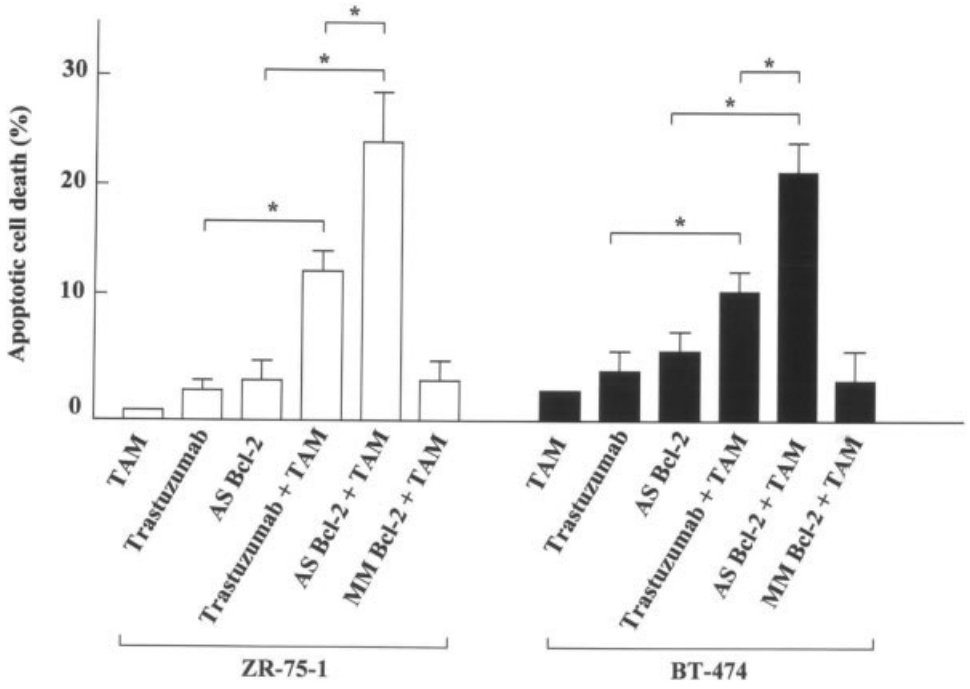

FIGURE 3. Apoptotic cell death was assessed using the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labeling method after treatment with tamoxifen (TAM) in combination with trastuzumab or antisense (AS) *Bcl-2* in the ZR-75-1 and BT-474 breast carcinoma cell lines. The apoptotic index was calculated compared with that in control cells (TAM, 0.5 μM; trastuzumab, 0.1 μM; AS *Bcl-2*, 1.0 μM; for details, see Materials and Methods). Results are shown from two representative, independent experiments. Error bars indicate the mean $\pm$ standard deviation; asterisks indicate *P* values < 0.05 (Student *t* test). MM *Bcl-2*: mismatch *Bcl-2*.

pathways in some breast carcinoma cells. Thus, it may be more effective to use AS *Bcl-2* than trastuzumab to enhance TAM sensitivity in some receptor-positive breast carcinomas.

It is believed that antiapoptotic signaling pathways involve Bcl-2 and pAkt.[9,10] Overexpression of Bcl-2 has been found in 60–80% of breast carcinomas, and overexpression of Akt also has been reported in several human malignancies.[31,32] The molecular mechanisms by which overexpression of Bcl-2 or Akt produces resistance to apoptosis may involve the following: 1) inhibition of cytochrome c release from mitochondria and inhibition of homodimerization with Bax by Bcl-2, which blocks caspase cascades[33]; 2) phosphorylation of Bad and inhibition of caspase-9 activation by Akt[34,35]; 3) induction of Bcl-2 expression by Akt through CREB[36]; and 4) activation of NF-κB by Akt and activation of inhibitor-of-apoptosis proteins.[37] Thus, Bcl-2 and Akt are involved in multiple antiapoptotic signaling pathways. With respect to the mechanism of TAM resistance, it has been reported that breast carcinoma cells can undergo hormone-independent growth and, in turn, growth factor-dependent growth activated by the transcription factor AP-1.[38] Overexpression of HER-2 is observed in TAM-resistant cells,[39] and treatment with trastuzumab overcomes this resistance.[40] Moreover, TAM resistance also is induced by overexpression of Akt and Bcl-2.[41] Despite the fact that overall survival is better in patients with Bcl-2-overexpressing breast carcinoma than in patients with non-Bcl-2-overexpressing breast carcinoma, increased Bcl-2 expression causes resistance not only to antitumor drugs but also to TAM in breast carcinoma cells. pAkt-positive breast carcinomas are more prone to recur with distant metastases, which is an independent prognostic factor.[42] These findings indicate that modulation of HER-2 and Bcl-2 with AS and antibody to increase TAM sensitivity is a good strategy for the treatment of hormone-independent growth in patients with breast carcinoma who have failed TAM treatment. The clinical use of AS *Bcl-2* may be better than trastuzumab in terms of TAM modulation in receptor-positive breast carcinoma cells.

Trastuzumab, which is a chimeric, humanized anti-HER-2 antibody, provides a promising treatment for patients with metastatic breast carcinoma. Therapeutic efficacy in terms of response rate, response duration, and median survival in combination with doxorubicin and paclitaxel has been shown compared with monotherapy using chemotherapeutic agents.[43] In addition, a therapeutic benefit of combined vinorelbine and trastuzumab has been reported in patients who failed treatment with taxanes.[44] Possible mechanisms by which trastuzumab enhances sensitivity to antitumor drugs are as follows[45]: 1) down-regulation of the HER-2 receptor, 2) prevention of HER-2/HER-3 and HER-2/HER-4 heterodimer formation, 3) induction of G_1 arrest, and 4) induction of antibody-dependent cellular cytotoxicity. Treatment with trastuzumab overcomes resistance to TAM in breast carcinoma cells in vitro, and growth factor-dependent growth of

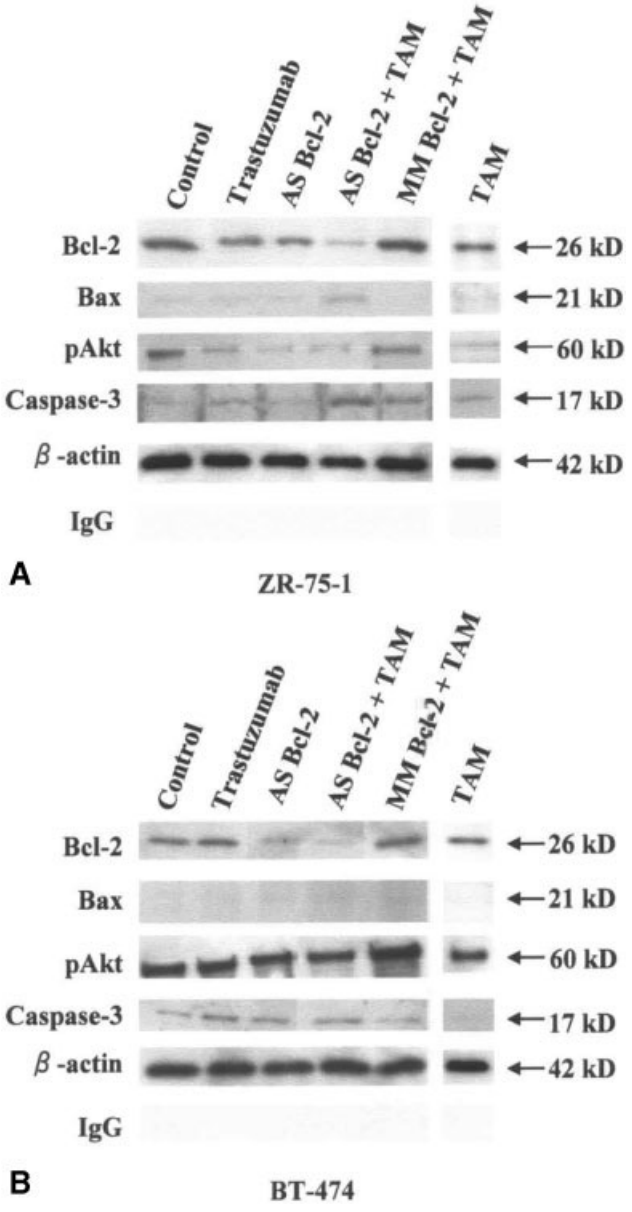

FIGURE 4. Changes in the expression of apoptosis-related proteins are illustrated after treatment with tamoxifen in (TAM) combination with antisense (AS) *Bcl-2* in the ZR-75-1 (A) and BT-474 (B) breast carcinoma cell lines (TAM, 1.0 μM; trastuzumab, 0.1 μM; AS *Bcl-2*, 1.0 μM; for details, see Materials and Methods). Results are shown from two representative, independent experiments. MM *Bcl-2*: mismatch *Bcl-2*; IgG: immunoglobulin G.

carcinoma cells is modulated by restoring TAM sensitivity. In the current study, treatment with trastuzumab enhanced sensitivity to TAM in ZR-75-1 cells, whereas sensitivity to TAM in BT-474 cells was increased only slightly, even though BT-474 cells showed more receptor overexpression than ZR-75-1 cells. The difference in modulation of TAM sensitivity by trastuzumab and AS *Bcl-2* in ZR-75-1 and BT-474 cells may be due to activation of downstream antiapoptotic signal-transduction pathways. Treatment with trastuzumab down-regulates HER-2 expression at a low concentration of 10 nM. Down-regulation of Bcl-2 may occur through a different pathway. Thus, a possible explanation for the differential effect of trastuzumab in the enhancement of drug sensitivity may be its effect on the Bcl-2 signaling pathway in breast carcinoma. This implies that dual modulation of antiapoptotic signals mediated by Bcl-2 and pAkt will be more effective for enhancing TAM sensitivity than the modulation of each signal alone. The potential interaction between Bcl-2 and Akt for drug sensitivity has been implicated in previous reports. The enforced overexpression of Bcl-2 prevented daunorubicin-induced apoptosis through inhibition of Akt degradation in U937 leukemic cells.[46] The inhibition of Akt produced a decreased protein level of Bcl-2 in pancreatic carcinoma cells in association with the decreased function of NF-κB, which is capable of transcriptional activation of Bcl-2.[47] The integrity and function of a Akt/NF-κB/Bcl-2 signaling pathway may be dependent on various factors, including the cell type and genetic background of carcinoma cells.

AS strategies for the modulation of specific proteins involved in cell proliferation and apoptosis have been developed for protein kinase Cα,[48] c-Raf,[49] and Bcl-2.[50] The therapeutic efficacy of AS *Bcl-2* (G3139, Genasense, oblimersen sodium; Genta Inc.) combined with antitumor drugs has been examined in Phase III trials for chronic lymphocytic leukemia, multiple myeloma, and malignant melanoma. Although the recent results of Phase III trial of G3139/dacarbazine versus dacarbazine alone in patients with advanced malignant melanoma did not show a significant increase in overall survival by the addition of G3139, combination treatment with G3139 and dacarbazine showed a significant increase in progression-free survival and response rates compared with dacarbazine alone.[51] The clinical efficacy of G3139 for the treatment of solid tumors, including breast carcinoma, colon carcinoma, gastric carcinoma, small cell lung carcinoma, and bladder carcinoma, also is under investigation. We have shown that treatment with AS *Bcl-2* enhanced drug sensitivity to mitomycin C and paclitaxel in MDA-MB-231 cells by modulating the signal-transduction pathways involving Bax, cytochrome c, and caspase-3.[26] Furthermore, down-regulation of Bcl-2 by AS activated caspase-8 through both death receptor-dependent and receptor-independent pathways. Although the mechanism by which TAM exerts its cytotoxic effects occurs through competitive inhibition of estradiol binding to the ER-responsive element, treatment with TAM actually induces apoptotic cell

death in breast carcinoma cells and is associated with the induction of Bax, Fas, caspase-8, cytochrome c, and caspase-3 and with the activation of caspase cascades. In addition, treatment with TAM induces G0/G1 cell-cycle arrest in association with apoptosis.[52] These findings suggest that growth arrest by TAM triggers the activation of signal-transduction pathways leading to apoptosis. Assuming that signal-transduction pathways for apoptosis are activated after TAM treatment, it appears that the signaling pathways are mediated by death receptor-dependent and receptor-independent pathways and by activation of caspase cascades. Thus, because mitochondrial dysfunction by release of cytochrome c is a critical event in promoting apoptosis, it is conceivable that AS therapies for the modulation of Bcl-2-mediated antiapoptotic pathways provide a good strategy for the enhancement of TAM sensitivity in breast carcinoma cells.

In conclusion, the use of *Bcl-2* AS oligonucleotide is effective for the modulation of TAM sensitivity in HER-2 and Bcl-2-overexpressing breast carcinoma cells. Treatment with AS *Bcl-2* may be more effective than trastuzumab in terms of modulating antiapoptotic signaling pathways that involve Bcl-2 and Akt in hormone-independent growth in TAM-resistant breast carcinoma cells.

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