Enhanced Sensitization to Taxol-induced Apoptosis by Herceptin Pretreatment in ErbB2-overexpressing Breast Cancer Cells

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ABSTRACT

The recombinant humanized anti-ErbB2/HER2 monoclonal antibody Herceptin (Trastuzumab) has been shown to significantly enhance the tumoral effects of antitumor drugs such as paclitaxel (Taxol) in patients with ErbB2-overexpressing breast cancers. Here, we investigated the molecular mechanisms by which Herceptin enhances the antitumor effects of Taxol. Because activation of p34Cdc2 is required for Taxol-induced apoptosis and because overexpression of ErbB2 blocks Taxol-induced apoptosis by inhibiting p34Cdc2 activation, we studied the effect of Herceptin treatment on p34Cdc2 kinase activation and apoptosis in Taxol-treated human breast carcinoma cell lines MDA-MB-435, SKBr3, MDA-MB-453, and 435.eB, which is an ErbB2 transfectant of MDA-MB-435. Herceptin treatment down-regulated ErbB2, reduced the inhibitory phosphorylation of Cdc2 on Tyr-15, and down-regulated the expression of p21\(^{Cip1}\), a Cdc2 inhibitor. Herceptin plus Taxol treatment led to higher levels of p34Cdc2 kinase activity and apoptosis in ErbB2-overexpressing breast cancer cells, which is likely attributable to inhibition of Cdc2-Tyr-15 phosphorylation and p21\(^{Cip1}\) expression. Because significant dephosphorylation of Cdc2-Tyr-15 and down-regulation of p21\(^{Cip1}\) occur at least 24 h after Herceptin treatment, we investigated whether 24 h Herceptin pretreatment would render ErbB2-overexpressing breast cancer cells more sensitive to Taxol-induced apoptosis compared with the simultaneous treatment of Herceptin plus Taxol. Indeed, Herceptin pretreatment increased Taxol-induced apoptosis and cytotoxicity in vitro and more effectively inhibited the growth of tumor xenografts with enhanced in vivo apoptosis. Thus, Herceptin treatment of ErbB2-overexpressing cells can inhibit ErbB2-mediated Cdc2-Tyr-15 phosphorylation and p21\(^{Cip1}\) up-regulation, which allows effective p34Cdc2 activation and induction of apoptosis upon Taxol treatment. Herceptin pretreatment renders ErbB2-overexpressing breast cancers more susceptible to Taxol-induced cell death, which may have important clinical therapeutic implications.

INTRODUCTION

The erbB2 gene (also known as HER2, neu) encodes a M\(_{r}\) 185,000 transmembrane glycoprotein (p185\(^{ErbB2}\)), which belongs to the epidermal growth factor receptor family of receptor tyrosine kinases (1, 2). The ErbB2 receptor tyrosine kinase can be involved in the regulation of a variety of vital functions controlled by any of the ErbB receptor family members, including cell growth, differentiation, and apoptosis (3). The ErbB2 receptor tyrosine kinase plays an important role in human malignancies. It is overexpressed in ~30% of human breast carcinomas (4, 5) and in many other types of human malignancies (6). Studies of individuals with ErbB2-overexpressing tumors have shown that they have a significantly lower overall survival rate and a shorter time to relapse than those patients whose tumors did not overexpress ErbB2. ErbB2 overexpression has been correlated positively with lymph node metastasis in node-positive patients (5, 7–9). We have also observed that ErbB2 overexpression can enhance the intrinsic metastatic potential of breast cancer cells (10).

In addition to conferring aggressive malignant behavior, ErbB2 overexpression has been observed to affect the sensitivity of breast cancer cells to several anticancer agents (3). Our previous studies demonstrated that ErbB2 overexpression in breast cancer cells confers increased resistance to the chemotherapeutic agent paclitaxel (Taxol; Refs. 11, 12). This is supported by another study in which Herceptin, a humanized anti-ErbB2 antibody, enhanced the antitumor activity of Taxol and doxorubicin against ErbB2-overexpressing human breast cancer xenografts (13). Furthermore, results of the Herceptin Phase III trial demonstrated that the Taxol response rate of patients with ErbB2-overexpressing breast cancers was significantly higher among patients receiving Taxol plus Herceptin than those receiving Taxol alone (14, 15).

Herceptin (or Trastuzumab) was developed from an anti-p185\(^{ErbB2}\) murine monoclonal antibody (4D5) that binds to the extracellular domain of ErbB2 and down-regulates the expression of cell surface ErbB2 proteins (16). Herceptin has demonstrated tumor inhibitory and chemosensitizing effects to Taxol and several chemotherapeutic agents in preclinical studies and in Phase II and Phase III clinical trials (13, 14, 17, 18). Currently, Herceptin is the only United States Food and Drug Administration-approved antibody therapeutic for treatment of cancers that overexpress ErbB2.

Taxol is a potent and highly effective antineoplastic agent in the treatment of advanced, drug-refractory, metastatic breast cancers (19, 20). Taxol induces tubulin polymerization and microtubule formation (21), blocks the cell cycle in mitosis, and induces programmed cell death (apoptosis; Ref. 22). Taxol may induce apoptosis through different mechanisms, depending on cell lines and culture conditions, which can involve c-Jun NH\(_{2}\)-terminal kinase activation, nuclear factor-\(\kappa\)B activation, p66 Shc phosphorylation, or mitogen-activated protein kinase pathways (extracellular signal-regulated kinase and p38) and can be p53 independent (23–28). Recently, we demonstrated that activation of p34Cdc2 is required for Taxol-induced apoptosis of breast cancer cells (12). Furthermore, we found that at least two mechanisms were involved in the ErbB2 inhibition of p34Cdc2 activation and Taxol-induced apoptosis: (a) ErbB2 overexpression can inhibit p34Cdc2 activation and apoptosis in Taxol-treated breast cancer cells by up-regulating p21\(^{Cip1}\), an inhibitor of cell cycle-dependent kinases (12); and (b) ErbB2 overexpression can inhibit p34Cdc2 activation and Taxol-induced apoptosis by increasing phosphorylation of Cdc2 on Tyr-15, a critical inhibitory phosphorylation site on p34Cdc2 (29).

On the basis of this understanding of the mechanisms of ErbB2-mediated Taxol resistance, we hypothesized that Herceptin may sensitize ErbB2-overexpressing breast cancer cells to Taxol-induced apoptosis by allowing efficient activation of p34Cdc2 kinase after Taxol treatment. In this study, we investigated whether Herceptin treatment of ErbB2-overexpressing breast cancer cells, by down-regulation of p34Cdc2...
ErbB2, could lead to inhibition of p21Cip1 expression and reduced Cdc2-Tyr-15 phosphorylation, which allows for efficient p34Cdc2 activation and apoptosis when cells are treated with Taxol. We also investigated whether Herceptin pretreatment, which renders ErbB2-overexpressing breast cancer cells more susceptible to p34Cdc2 activation and apoptosis upon Taxol treatment, more effectively sensitized these cells to Taxol-induced apoptosis.

MATERIALS AND METHODS

Cell Lines and Culture. Human breast cancer cell lines MDA-MB-435, the 435.eB transfectants, SKBr3, and MDA-MB-453 were obtained, established, and cultured as described previously (11).

Antibodies and Reagents. Herceptin was provided by Genentech, Inc. (South San Francisco, CA). rhu IgG1 was purchased from Calbiochem (San Diego, CA), and paclitaxel (Taxol) was from the Bristol Myers-Squibb Company (Princeton, NJ). Human p185ErbB2 monoclonal antibody (Ab-3) was purchased from Oncogene Science, Inc. (Cambridge, MA), and human p21Cip1 polyclonal and p34Cdc2 monoclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho Tyr15-p34Cdc2 polyclonal antibody was purchased from Cell Signaling Technology, Inc. (Beverly, MA).

Herceptin Treatment and Western Blot Analysis. Cells were cultured to ~80% confluence before Herceptin treatment. Cells were then incubated with either 10 or 20 nM Herceptin for 24, 36, or 48 h, depending on the cell line. Cells were washed with cold PBS and lysed with immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris at pH 7.4, 1 mM ethylene glycol-bis-tetraacetic acid, 0.2 mM sodium vanadate, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% NP40). One hundred μg of protein from each sample were separated by 8 or 12% SDS-PAGE, transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA), and probed by specific antibodies against p185ErbB2, phospho Tyr15-p34Cdc2, p21Cip1, and p34Cdc2. Protein signals were detected using the enhanced chemiluminescence detection system (Amersham Corp., Arlington Heights, IL).

Immunocomplex-Kinase Assays. Fourteen h after cells were plated, the culture medium was replaced with medium containing 0.5% FBS with or without Herceptin (10 or 20 nM) for 24 h. Then cells were treated with or without Taxol (0.02 or 0.05 μM) for different time periods (3 or 8 h). Immunocomplex-kinase assays of p34Cdc2 kinase activity were performed as described previously (12).

Flow Cytometry Analyses of Apoptotic Cells. Cells were cultured in 0.5% FBS in the absence or presence (Herceptin pretreatment) of Herceptin (5, 10, and 20 nM) for 24 h. Cells were then treated with or without Taxol (0.02 and 0.05 μM) or Herceptin (0, 5, 10, and 20 nM) plus Taxol for 1 h, washed once, and incubated for another 24 h in the absence or presence of Herceptin. Cells were harvested by trypsinization, fixed in 1% formaldehyde on ice for 20 min, and washed once with 70% ethanol. For multiparameter flow cytometry analysis, the cells were incubated in terminal deoxynucleotidyl transferase solution (0.1M sodium cacodylate, 1 mM CoCl2, 0.1 mM DTT, 0.005 mg/ml BSA, 10 units of TdT, and 0.5 μM biotin-16-dUTP) at 37°C for 30 min, transferred to 100 μl of staining solution (4X SSC, 5% dry milk, 0.15% Triton X-100) and washed once with 70% ethanol. For multiparameter flow cytometry analysis, the cells were incubated in terminal deoxynucleotidyl transferase solution (0.1M sodium cacodylate, 1 mM CoCl2, 0.1 mM DTT, 0.005 mg/ml BSA, 10 units of TdT, and 0.5 μM biotin-16-dUTP) at 37°C for 30 min, transferred to 100 μl of staining solution (4X SSC, 5% dry milk, 0.15% Triton X-100) and washed once with 70% ethanol. For multiparameter flow cytometry analysis, the cells were incubated in terminal deoxynucleotidyl transferase solution (0.1M sodium cacodylate, 1 mM CoCl2, 0.1 mM DTT, 0.005 mg/ml BSA, 10 units of TdT, and 0.5 μM biotin-16-dUTP) at 37°C for 30 min, transferred to 100 μl of staining solution (4X SSC, 5% dry milk, 0.15% Triton X-100) and washed once with 70% ethanol. For multiparameter flow cytometry analysis, the cells were incubated in terminal deoxynucleotidyl transferase solution (0.1M sodium cacodylate, 1 mM CoCl2, 0.1 mM DTT, 0.005 mg/ml BSA, 10 units of TdT, and 0.5 μM biotin-16-dUTP) at 37°C for 30 min, transferred to 100 μl of staining solution (4X SSC, 5% dry milk, 0.15% Triton X-100) and washed once with 70% ethanol.

Fig. 1. Herceptin down-regulated ErbB2, reduced phosphorylation on Tyr-15 of p34Cdc2, and inhibited p21Cip1 expression in ErbB2-overexpressing breast cancer cells. MDA-MB-435 cells and 435.eB transfectants (A) and SKBr3 and MDA-MB-453 cells (B) were treated with incremental concentrations of Herceptin (20 nM in A and 10 nM in B) or IgG for various times (0, 24, or 36 h). C, MDA-MB-435 cells, 435.eB transfectants, and SKBr3 were untreated (−) or treated (+) with Taxol (0.02 mM for MDA-MB-435 and 435.eB; 0.05 μM for SKBr3) for 24 h. Western blot analysis was performed as described in “Materials and Methods.” All experiments described in this study have been repeated at least three times.
X-100, and 2.5 ng/μl Avidin–FITC), and incubated in the dark at room temperature for 30 min. Finally, the cells were stained in 500 μl of propidium iodide solution (0.5 μg/ml of propidium iodide, and 0.1% RNase A). Flow cytometry analyses were performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Cell cycle profiles were analyzed with Multicycle software (Phoenix Flow Systems, San Diego, CA), and FITC signals were analyzed with Epics Elite software (Coulter Corp., Miami, FL).

Clonogenic Assay. Cells (1500–4000 per well) were seeded on 24-well plates. The culture medium was replaced 14 h later with medium containing 0.5% FBS with or without Herceptin. After incubation for 24 h, Taxol or Herceptin plus Taxol was added at Taxol concentrations ranging from 0 to 2.5 μM and incubated for 1 h. Cells were then washed with serum-free medium and incubated in the presence or absence of Herceptin. The culture medium was changed every 2 days until colonies were stained with crystal violet on day 8. Colonies containing >10 cells were counted under the microscope.

Tumorigenicity Assay. The 435.eB cells in log-phase growth were trypsinized, washed twice with PBS, and centrifuged at 250 × g. The viable cells were then counted. Viable cells (1 × 10⁶) in 0.15 ml of Matrigel (BD Biosciences, Bedford, MA) were injected into the mammary fat pad of 10-week-old female ICR-SCID mice (Harlan, Indianapolis, IN) under aseptic conditions. Nine or 10 mice were injected for each treatment group. Tumors were measured by calipers in two orthogonal diameters, and the volumes were calculated using the formula for a prolate ellipsoid: volume (mm³) = W × L²/2, where W and L are the major and minor diameters (in millimeters), respectively. Tumor volumes are presented as mean values.

Immunohistochemical Staining of ErbB2. Immunohistochemical staining was performed on 5-μm slices. After deparaffinization and rehydration, sections were subjected to heat-induced epitope retrieval by immersion in a 0.01 M citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked for 30 min in 3% hydrogen peroxide. The slides were then incubated for 30 min at room temperature in a humidified chamber with anti-ErbB2 antibody (A0485; DAKO). The immunoperoxidase staining was done using the LSAB2 peroxidase kit (DAKO). The antigen-antibody immunoreaction was visualized using 3-3′-diaminobenzidine as the chromogen. The slides were washed, counterstained with hematoxylin, dehydrated with alcohol, cleared in xylene, and mounted. Patient samples that were shown previously to express high levels of ErbB2 were used as positive controls. The negative control was performed by replacing the primary antibody with normal horse serum. Moderate to strong membrane staining in shown previously to express high levels of ErbB2 were used as positive controls. The negative control was performed by replacing the primary antibody with normal horse serum. Moderate to strong membrane staining in were the major and minor diameters (in millimeters), respectively. Tumor volumes are presented as mean values.

In Situ TUNEL Assay. The breaks of fragmented DNA in apoptotic cells were detected in situ by nick-end labeling with the TUNEL method using ApopTag Plus Peroxidase in situ apoptosis detection kit (Intergen, Purchase, NY). Briefly, slides were digested with 20 μg/ml protease K (Boehringer Mannheim) for 15 min at room temperature. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 5 min. Equilibration buffer was then applied directly to the slide for 30 min. Slides were incubated with digoxigenin-dUTP and TdT in a humidified chamber at 37°C for 1 h. The reaction was terminated with a 10-min incubation in stop/wash buffer. After washing in PBS, the sections were covered with anti-digoxigenin-peroxidase for 30 min at room temperature, followed by color development with 3,3′-diaminobenzidine. The slides were counterstained with hematoxylin. The positive control slides included in the kit were stained as described above as positive controls. The negative controls were done by substituting TdT with PBS.

Assessment of Apoptotic Cells. Apoptotic cells and bodies were counted from 10 HPFs with ×400. Cells were considered apoptotic if the whole nuclear area of the cell labeled positively. Apoptotic bodies were defined as small, positively labeled globular bodies in the cytoplasm of the tumor cells, which could be found either singly or as groups.

RESULTS

Down-Regulation of p21Cip1 and Decreased Cdc2-Tyr-15 Phosphorylation in Herceptin-treated ErbB2-overexpressing Breast Cancer Cells. Our previous studies demonstrated that activation of p34Cdc2 kinase is required for Taxol-induced apoptosis and that overexpression of ErbB2 inhibits activation of p34Cdc2 and apoptosis of Taxol-treated cells by up-regulation of p21Cip1 and phosphorylation of Cdc2-Tyr15 (12, 29). To investigate whether Herceptin, which is known to down-regulate ErbB2 expression, will decrease ErbB2-mediated p21Cip1 up-regulation and Cdc2-Tyr15 phosphorylation in ErbB2-overexpressing breast cancer cells, MDA-MB-435, 435.eB, SKBr3, and MDA-MB-453 cells were treated with Herceptin or control human IgG for various time intervals. Western blot analyses demonstrated that 24- and 36-h Herceptin treatments down-regulated ErbB2 protein expression in cell lines overexpressing ErbB2 either naturally, SKBr3 and MDA-MB-453, or ectopically, 435.eB (Fig. 1). Notably, parallel to or after ErbB2 down-regulation, reduced phosphorylation on Tyr-15 of p34Cdc2 and decreased p21Cip1 protein levels were also detected in these cells after 24 or 36 h Herceptin treatment (Fig. 1). The control IgG had no significant effect on ErbB2 expression, Cdc2-Tyr-15 phosphorylation, or p21Cip1 expression. Additionally, ErbB2, p34Cdc2, or p21Cip1 expression was not significantly changed upon 24-h Taxol treatment (Fig. 1C). These data indicate that Herceptin effectively down-regulated ErbB2 expression, leading to inhibition of ErbB2-mediated p21Cip1 up-regulation and Cdc2-Tyr-15 phosphorylation in ErbB2-overexpressing breast cancer cells.
Herceptin Renders ErbB2-overexpressing Cells More Susceptible to p34\(^{\text{Cdc2}}\) Activation upon Taxol Treatment. It is known that dephosphorylation on Tyr-15 of p34\(^{\text{Cdc2}}\) can lead to activation of this cyclin-dependent kinase (30). Additionally, we have shown previously that p21\(^{\text{Cip1}}\) inhibits Cdc2 kinase activity, and reducing p21\(^{\text{Cip1}}\) expression by antisense p21\(^{\text{Cip1}}\) oligonucleotide allowed effective Cdc2 activation when cells were treated with Taxol (12). We therefore examined whether decreased Cdc2-Tyr-15 phosphorylation and reduced p21\(^{\text{Cip1}}\) expression by Herceptin treatment could result in activation of p34\(^{\text{Cdc2}}\) kinase in ErbB2-overexpressing breast cancer cells and whether better activation of p34\(^{\text{Cdc2}}\) will be observed in ErbB2-overexpressing breast cancer cells treated with Herceptin plus Taxol. The 435.eB transfectants were either untreated, cultured with human IgG control antibody, or treated with 0.02 \(\mu\)M Taxol, 20 nm Herceptin or Herceptin plus Taxol simultaneously (Fig. 2A, Lanes 1–5). In addition, because decreased Cdc2-Tyr15 phosphorylation and reduced p21\(^{\text{Cip1}}\) expression were detected at least 24 h after Herceptin treatment, cells were pretreated with Herceptin for 24 h, followed by the addition of Taxol (Fig. 2A, Lane 6). The kinase activity of p34\(^{\text{Cdc2}}\) immunoprecipitated with anti-p34\(^{\text{Cdc2}}\) antibody was measured using histone H1 as substrate. As expected, activation of Cdc2 kinase was readily detected in 435.eB cells treated with Taxol. Activation of p34\(^{\text{Cdc2}}\) kinase was indeed detected after 24 h of Herceptin treatment. Moreover, simultaneous treatment with Herceptin plus Taxol showed an additive effect on the activation of p34\(^{\text{Cdc2}}\) (Fig. 2A). The additive effect on p34\(^{\text{Cdc2}}\) activation is more apparent in cells pretreated with Herceptin for 24 h, followed by Taxol treatment (pretreatment, Fig. 2A). Similar effects on Cdc2 activation were also observed in SKBr3 cells upon Taxol treatment, Herceptin treatment, Herceptin plus Taxol simultaneous treatment, and 24 h of Herceptin pretreatment followed by Taxol (Fig. 2B). These results demonstrate more effective p34\(^{\text{Cdc2}}\) activation in ErbB2-overexpressing breast cancer cells treated with Herceptin plus Taxol.

Herceptin Pretreatment Further Increases Taxol-induced Apoptosis. We reported previously that p34\(^{\text{Cdc2}}\) activation is required for Taxol-induced apoptosis in ErbB2-overexpressing breast cancer...
cells (12). The data described above demonstrate that p34Cdc2 kinase activity was increased by Herceptin treatment in ErbB2-overexpressing breast cancer cells. Thus, we next examined whether Herceptin treatment could enhance Taxol-induced apoptosis and whether Herceptin pretreatment was more effective in sensitizing ErbB2-overexpressing breast cancer cells to Taxol-induced apoptosis than simultaneous Herceptin plus Taxol treatment. The 435.eB transfectant and SKBr3 cells were treated as in Fig. 2, i.e., cultured in regular medium with human IgG control antibody, with Herceptin, with Taxol, with Herceptin plus Taxol added simultaneously, or with pretreatment of Herceptin for 24 h before the addition of Taxol (Fig. 3). After cells were collected, cell cycle stages and apoptosis were simultaneously determined by measuring DNA content with propidium iodide and by labeling DNA strand breaks with biotinylated dUTP shown as FITC-positive cells (31). Very low levels of apoptosis were detected in cells cultured in regular medium, with human IgG control antibody, or with Herceptin, whereas Taxol induced apoptotic cell death in both the 435.eB transfectant and SKBr3 cells (Fig. 3). In 435.eB transfectants simultaneously treated with Herceptin plus Taxol, Herceptin treatment slightly increased Taxol-induced apoptosis (Fig. 3A). Notably, in 435.eB transfectants pretreated with Herceptin for 24 h followed by Taxol treatment, Taxol-induced apoptosis was significantly (P = 0.001) increased by Herceptin pretreatment (Fig. 3A). In SKBr3 cells, Herceptin pretreatment showed increases in Taxol-induced apoptosis in a concentration-dependent manner (Fig. 3C). Thus, data from both the 435.eB transfectant and SKBr3 cells demonstrate improved sensitization to Taxol-induced apoptosis by Herceptin pretreatment than by simultaneous treatment with Herceptin plus Taxol.

**Enhanced Sensitization to Taxol Killing in ErbB2-overexpressing Breast Cancer Cells by Herceptin Pretreatment.** To examine whether Herceptin pretreatment further enhances the cytotoxic effect of Taxol over the Herceptin-Taxol simultaneous treatment, clonogenic assays were performed using 435.eB transfectants and SKBr3 cells. Cells were treated with various concentrations of Taxol plus IgG, simultaneously with Taxol plus Herceptin, or pretreated with Herceptin for 24 h followed by Taxol. The number of surviving colonies after each treatment was counted 8 days after treatment. As expected, Taxol reduced colony formation in a Taxol concentration-dependent manner (Fig. 4). Simultaneous treatment of Herceptin plus Taxol moderately increased the cytotoxic effect of Taxol on these two cell lines. Notably, Herceptin pretreatment significantly enhanced the cytotoxicity of Taxol. Specifically, in 435.eB cells, ~19% of the colonies survived after Herceptin pretreatment followed with near half the IC50 concentration of Taxol treatment (0.01 μM), whereas ~55% of the colonies survived after the simultaneous Herceptin-Taxol treatment with the same concentration of Taxol (Fig. 4A). Better sensitization to Taxol killing by Herceptin pretreatment was also observed in SKBr3 cells (Fig. 4B). These data indicate that Herceptin pretreatment sensitizes the ErbB2-overexpressing breast cancer cells to Taxol killing better than the simultaneous treatment.

**Greater Inhibition of Tumor Growth by Herceptin Pretreatment.** To further determine whether Herceptin pretreatment may provide an advantage for tumor growth inhibition in vivo, female ICR-SCID mice were inoculated with 435.eB transfectants into the mammary fat pad to allow for the growth of tumor xenografts. Seven days after injection, mice were divided into five groups with at least nine animals in each group. The first group began treatment starting on day 7 with the rhu IgG monoclonal antibody (0.3 mg/kg) twice a week for another 4 weeks. The fourth group was given Taxol (10 mg/kg) i.p. on days 12 and 15, along with Herceptin (0.3 mg/kg). Treatment continued with Herceptin twice a week for 4 weeks. The fifth group was first treated with Herceptin (0.3 mg/kg) on days 7, 9, and 11. Then, mice were given Taxol (10 mg/kg) i.p. on days 12 and 15 along with Herceptin (0.3 mg/kg). Herceptin treatment continued twice a week for 4 weeks. A low dose of Herceptin (0.3 mg/kg) was selected to detect possible differences between the simultaneous treatment group (group 4) and the pretreatment group (group 5). Compared with the control group (group 1), treatment with either Herceptin (group 2) or Taxol (group 3) delayed tumor growth, whereas simultaneous treatment with Herceptin plus Taxol (group 4) showed slightly better tumor inhibition (Fig. 5A). Notably, Herceptin pretreatment followed by Taxol (group 5) inhibited tumor growth to a greater extent (P = 0.017) than the simultaneous treatment (group 4). These data indicate that Herceptin pretreatment can improve its sensitization effect on tumor growth inhibition by Taxol in ErbB2-overexpressing breast cancers.

To investigate whether down-regulation of ErbB2 and better induction of apoptosis are the underlying mechanisms for the enhanced tumor growth inhibition by Herceptin pretreatment, tumor samples were collected from each group and examined for ErbB2 expression and *in vivo* apoptosis. Overexpression of ErbB2 was found by immunohistochemical staining of tumor slides with anti-ErbB2 antibody from the IgG control-treated (group 1) and Taxol-treated (group 3) mice. However, ErbB2 expression levels were significantly reduced in Herceptin-containing treatment groups (groups 2, 4, and 5). *In situ* TUNEL assays detected fragmented DNAs within the nuclei of the...
tumor cells in each group, and intense TUNEL signals were occasionally observed in nuclear fragments or apoptotic bodies (Fig. 5B).

Compared with IgG control tumors (11 apoptotic cells/10 HPFs), Herceptin-treated or Taxol-treated tumors showed a slight increase in apoptosis (18 apoptotic cells and 22 apoptotic cells, respectively, per 10 HPFs), whereas Herceptin plus Taxol-treated tumors demonstrated a dramatic increase of in vivo apoptosis (Fig. 5C, group 4, 36/10 HPFs). Notably, an even higher incidence of apoptosis was detected in Herceptin pretreated tumors (group 5, 62/10 HPFs) than in tumors treated simultaneously with Herceptin and Taxol. These data indicate that Herceptin pretreatment primed the ErbB2-overexpressing breast tumors for efficient apoptosis induction in response to Taxol treatment, which paralleled the enhanced sensitization to tumor inhibition by Taxol.

DISCUSSION

In this study, we demonstrated that Herceptin treatment of ErbB2-overexpressing human breast cancer cells down-regulated ErbB2 expression and decreased the inhibitory phosphorylation of Cdc2 on Tyr-15 and the expression of p21Cip1, thus allowing efficient p34Cdc2 activation and apoptosis induction upon Taxol treatment. We also provide evidence that Herceptin pretreatment led to improved sensitization of ErbB2-overexpressing breast cancer cells to Taxol-induced apoptosis, cytotoxicity, and tumor growth inhibition than Herceptin and Taxol simultaneous treatment strategy.

Herceptin down-regulated the ErbB2 protein in the 435.eB transfectant, MDA-MB-453 cells, and SKBr3 cells. Herceptin-mediated ErbB2 down-regulation is known to result from multiple steps such as receptor internalization, protein degradation, and receptor recycling (32). However, the efficiencies of these processes may vary among different cell lines. Additionally, different cell lines may have divergent downstream elements in their signal transduction pathways that are responsible for their responses to ErbB2 down-regulation by Herceptin (33). Thus, it is not surprising that variation in response to Herceptin-mediated ErbB2 down-regulation was seen among ErbB2-overexpressing breast cancer cells (Fig. 1). This is also consistent with clinical trial data that breast cancers with ErbB2 overexpression have a Herceptin response rate of only ~15% (34).

We have found previously that activation of p34Cdc2 is required for Taxol-induced apoptosis, but overexpression of ErbB2 inhibits Cdc2
activation upon Taxol treatment, leading to inhibition of apoptosis (12). In this study, we showed that Herceptin treatment permits efficient p34Cdc2 activation upon Taxol treatment of ErbB2-overexpressed human breast cancer cells (Fig. 2). The Herceptin-mediated efficient p34Cdc2 activation upon Taxol treatment is likely the result of reversals of two known ErbB2-mediated Cdc2 inhibitory mechanisms: (a) Herceptin decreased the ErbB2-mediated inhibitory phosphorylation of Cdc2 on Tyr15; and (b) Herceptin blocked the ErbB2-mediated up-regulation of p21Cip1 (Fig. 1). We propose that the reversals of these two known ErbB2-mediated Cdc2 inhibitory mechanisms are important molecular mechanisms contributing to Herceptin-mediated sensitization of ErbB2-overexpressing cells to Taxol-induced apoptosis (Fig. 3), which requires Cdc2 activation.

Other mechanisms or multiple signaling pathways may be involved in Herceptin-mediated Taxol sensitization in addition to the ones demonstrated here. For example, although Herceptin pretreatment only led to slightly better activation of p34Cdc2 than simultaneous treatment (Fig. 2), Herceptin pretreatment resulted in significant sensitization to Taxol-induced apoptosis, cell killing, and tumor inhibition (Figs. 3–5). This suggests that p34Cdc2 activation is only one of those multiple mechanisms or signaling pathways induced by Herceptin leading to Taxol sensitization. Consistent with this notion, it has been reported recently that anti-ErbB2 monoclonal antibody 4D5, from which Herceptin was developed, resulted in rapid dephosphorylation of ErbB2, accumulation of the cyclin-dependent kinase inhibitor p27kip1, and inactivation of cyclin-Cdk2 complexes (35). Notably, we have shown that Cdk2 kinase activities were dramatically inhibited when MDA-MB-435 cells were treated with Taxol (12). Another group showed recently that Taxol treatment significantly reduced the level of Cdk2 protein in MCF7 cells (28). Thus, it is possible that Cdk2 inhibition may also contribute to Herceptin-mediated sensitization of ErbB2-overexpressing breast cancer cells to Taxol. In addition, Taxol-mediated down-regulation of IκB-α, through the up-regulation of IκB kinase β subunit (26) and Taxol-mediated serine phosphorylation of the M_6,60,000 She isofrom may also be signaling events contributing to cell death (27). We are currently investigating the involvement of these mechanisms in Herceptin-mediated Taxol sensitization.

It has been reported that Herceptin enhances the antitumor activity of Taxol and doxorubicin against ErbB2-overexpressing human breast cancer xenografts (13). The report is consistent with data from the Herceptin Phase III clinical trial, in which Herceptin and Taxol were administered simultaneously (36, 37). Here, we report the observation that Herceptin pretreatment sensitizes ErbB2-overexpressing cells to Taxol-induced apoptosis, cell killing, and tumor inhibition to greater extents than simultaneous treatment with Herceptin plus Taxol (Figs. 3–5). This improved Taxol sensitization may be attributed, at least in part, to reduced Cdc2 inhibitory phosphorylation on Tyr-15 and down-regulation of p21^Cip1, which are achieved at least 24 h after Herceptin treatment in cultured cells (Fig. 1), allowing more efficient activation of Cdc2 and better induction of apoptosis (Figs. 2 and 3). In our in vivo tumorigenicity assays, we pretreated mice with Herceptin 5 days before giving Taxol. This pretreatment consisted of three Herceptin injections once every other day to allow enough time for inhibition of both Cdc2-Tyr-15 phosphorylation and p21^Cip1 expression. Although we were unable to determine Cdc2 kinase activities in tumor samples, in situ TUNEL assays demonstrated that Herceptin-pretreated tumors are more susceptible to Taxol-induced apoptosis than the simultaneous treatment group (Fig. 5, B and C). More importantly, the enhanced apoptosis in tumor samples correlated with improved inhibition of tumor xenografts in mice. Despite the need to investigate other possible mechanisms underlying the improved tumor inhibition by Herceptin pretreatment, our findings that Herceptin pretreatment better sensitizes ErbB2-overexpressing breast cancer cells to Taxol than the simultaneous treatment could have important therapeutic implications. It will be very important to determine whether Herceptin pretreatment may provide further improved clinical responses to Taxol in breast cancer patients compared with the practice of the simultaneous administration of Herceptin and Taxol.

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