Anti-HER2 Antibody and Heregulin Suppress Growth of HER2-Overexpressing Human Breast Cancer Cells through Different Mechanisms

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ABSTRACT

Previous reports have shown that certain anti-HER2 antibodies and heregulin can inhibit clonogenic growth of breast and ovarian cancers that overexpress HER2. Anti-HER2 antibodies bind to HER2 directly, whereas heregulin does not bind to HER2 alone, but rather interacts with HER2 through the formation of heterodimers with HER3 or HER4. The purpose of the present study was to elucidate the mechanisms by which anti-HER2 antibody and heregulin inhibit tumor growth. The anti-HER2 monoclonal antibody (mAb) ID5 was found to block G1-S progression of the cell cycle, whereas heregulin inhibited passage through G2-M. Compatible with the effects on the cell cycle, treatment with mAb ID5 decreased levels of cyclin-dependent kinase (CDK) 2, cyclin E, and CDK6 proteins and reduced cyclin E-CDK2-associated kinase activity; mAb ID5-treated cells had increased p27Kip1 expression and an increased association of p27Kip1 with CDK2. In contrast, treatment with heregulin increased protein levels of CDK2, CDK6, CDC2, and cyclin B1. More Retinoblastoma protein was found in the hypophosphorylated state in the cells treated with mAb ID5, whereas more retinoblastoma protein was in the hyperphosphorylated state in hergulin-treated cells. Hergulin was able to induce cell differentiation as assessed by Oil Red O staining and apoptosis as assessed by sub-G1 peak on flow cytometry and the presence of DNA fragmentation in ApopTag histochemistry staining. Neither differentiation nor apoptosis was observed in the cells treated with mAb ID5. We conclude that anti-HER-2 mAb ID5 and heregulin exert growth inhibition through different mechanisms. In mammary cells overexpressing HER2, anti-HER2 mAb ID5 induces G1 arrest, whereas heregulin induces G2-M arrest, cell differentiation, and apoptosis.

INTRODUCTION

The human EGFR3 (or HER) family of tyrosine kinase receptors currently includes four members: HER1 (EGFR, c-erbB1), HER2 (neu, c-erbB2), HER3 (c-erbB3), and HER4 (c-erbB4; Refs. 1–4). The protein tyrosine kinase activity of HER2 can be induced by a number of growth factors, although no HER2-specific ligand has been identified. EGF, transforming growth factor α, and amphiregulin are all capable of stimulating HER2 activity by binding to HER1 and promoting its heterodimerization with HER2 (1–4). Similarly, heregulin binds to HER3 and HER4 and facilitates formation of heterodimers with HER2 (1–4).

Amplification of the HER2 gene and overexpression of HER2 protein are found in ~30% of breast and ovarian cancers. In many, but not all reports, HER2 overexpression has been associated with an unfavorable prognosis (1, 2, 5). Substantially greater expression of HER2 on cancer cells than on normal epithelial tissues permits selective targeting of malignant cells. HER2 is expressed on the cell surface where it can interact with ligands and antibodies (1, 2). A number of strategies have been evaluated for inhibiting the growth of cells that overexpress HER2, including the use of mAbs directed against the extracellular domain of the HER2 protein (6–9), recombinant single-chain antibodies specific for HER2 (10), anti-HER2 antibodies conjugated with immunotoxins, radioactive isotopes, or chemotherapeutics drugs (11–13), anti-HER2 antibody with CTL activity (14), intracellular antibodies (15, 16), antisense (17, 18), transcriptional repression of HER2 with adenovirus-5 E1A or mutant SV 40 large T antigen genes (19), and anti-HER- protein vaccination (20).

Our group and others have reported that anti-HER2 antibodies can inhibit breast and ovarian cancer growth in vivo and in vitro (6–9, 12, 13, 21–23). Antibodies specific for HER2 promote formation of HER2 homodimers, but do not interact with HER3 or HER4 (24). Heregulin can also inhibit growth of breast and ovarian cancer cells that overexpress HER2 (21, 25–28). The hergulin family (also called neuregulin, neu differentiation factor, glial growth factors, and acetylcholine-receptor-inducing activity) includes a large group of secreted and

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The abbreviations used are: EGFR, epidermal growth factor receptor; HER, human epidermal growth factor receptor; CDK, cyclin-dependent kinase; mAb, monoclonal antibody; Rb, retinoblastoma; PI3-K, phosphatidylinositol 3-kinase.
membrane-attached growth factors, expressed as alternatively spliced isoforms from a single gene (29). Unlike anti-HER2 antibodies, members of the heregulin family cannot bind to HER-2 alone. Instead, the ligand interacts with homodimers of HER3 or HER4 or with heterodimers formed from HER2 with HER3 or HER4 (30, 31). Signaling through these receptors, heregulin can regulate a variety of responses in cultured cells, including proliferation, differentiation, and survival (29).

Although both anti-HER2 antibody and heregulin can inhibit growth of cancer cells that overexpress HER2, antibody and ligand (heregulin) interact with different configurations of receptors and might signal through different pathways. In this study, we provide evidence that anti-HER2 antibody and heregulin induced cell cycle arrest at different phases of the cell cycle in the human breast cancer cell line SKBr3. Heregulin induced cell differentiation and apoptosis, whereas anti-HER2 antibody primarily arrested cells at G1 phase with negligible induction of differentiation and apoptosis.

MATERIALS AND METHODS

Cell Culture. The human breast cancer cell line SKBr3, obtained from the American Type Culture Collection (Manassas, VA), was grown in complete medium, containing RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in humidified air with 5% CO2 at 37°C. For all experiments, cells were detached with 0.25% trypsin-0.02% EDTA. For cell culture, 2 × 10^5 exponentially growing cells were plated into 10-cm tissue culture dishes or 1 × 10^4 into 24-well plates in complete medium. After culture for 24 h in complete medium, cells were treated with antibodies (65 nM) or heregulin (0.3–1 nM) in medium with 2% dialyzed fetal bovine serum (Life Technologies, Inc.) at 37°C for the indicated time intervals. In case of pretreatment with PI3-K inhibitors, the cells were first incubated with LY294002 (10 μM) or wortmannin (2 μM) overnight in complete medium and then subjected to heregulin treatment.

Preparation and Purification of Anti-HER2 Antibodies. Anti-HER2 murine mAb ID5 was obtained from Applied Biotechnology/Oncogene Science (Cambridge, MA). Hybridoma cells specific for ID5 were used to produce ascites fluid as reported previously (21). mAb ID5 is of the IgG1/κ subclass. Murine mAb producing hybridoma MOPC21 (IgG1/κ) was obtained from the American Type Culture Collection and used as an isotype-matched control that did not bind to HER2.

Reagents. Antibodies reactive with p15 Ink4B , p16 Ink4A , p19 Ink4D , p21 Cip1 , p27 Kip1 , cyclin D1, and CDK2 were purchased from Oncogene Research Products (Cambridge, MA). Antibodies to p27 Kip1 , CDK2, CDK4, CDK6, and cyclin E were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to Rb and p53 were obtained from Upstate Biotechnology Incorporated (Lake Placid, NY). A mAb to actin was purchased from Sigma Chemical Co. Recombinant human heregulin β1 was obtained from NeoMarkers, Inc. (Fremont, CA).

Fig. 1 Effect of anti-HER2 mAb ID5 and heregulin on anchorage-independent growth. SKBr3 cells in exponential growth were treated with mAb ID5 (65 nM), heregulin (0.3 nM), control mAb MOPC21 (65 nM), or diluent alone, as described in “Materials and Methods.” A, growth in soft agar assay was measured to assess anchorage-independent growth. Percent inhibition (%) was calculated as 100 times [1 – (colony numbers from antibody- or heregulin-treated samples / colony number from diluent-treated samples)]. Colony numbers were counted after a 14-day incubation in soft agar in the continuous presence of antibodies, ligand, or diluent. Data include mean ± SD. The asterisk indicates a statistically significant difference (P < 0.05) from controls (diluent or MOPC21). B, percent inhibition of clonogenic growth in soft agar after incubation with mAb ID5 for different intervals. The cells were incubated with mAb ID5 for different intervals, then washed, and subsequently incubated in medium with (add-back) or without mAb ID5. Colony numbers were counted after a 14-day incubation in soft agar. C, percent inhibition of clonogenic growth in soft agar after incubation with heregulin for different intervals. The cells were incubated with heregulin for different intervals, then washed, and subsequently incubated in medium with (add-back) or without heregulin. Colony numbers were counted after a 14-day incubation in soft agar.
Anchorage-dependent Growth. Cell growth was determined by a modified “Crystal Violet Mitogenic Assay” (32). Briefly, SKBr3 cells were plated in triplicate at a density of $1 \times 10^4$ cells in 24-well tissue culture plates. The cells were treated with antibodies (65 nM) or heregulin (0.3 nM) and incubated up to 4 days. At daily intervals, the cells were washed with PBS, fixed in 1% glutaraldehyde in PBS, and stained with 0.5% crystal violet (Sigma Chemical Co.) in methanol. The dye was eluted with Sorenson's buffer (0.9% sodium citrate, 0.02 N HCl, and 45% ethanol), and the eluted dye was measured by a microplater reader (V_max, Molecular Devices, Sunnyvale, CA) at lengthwave 560 nm.

Anchorage-independent Growth. To determine the anchorage-independent cell growth of SKBr3 cells, the soft agar colony-forming assay was used as reported in our previous studies (21).

Cell Cycle Analysis. Cell cycle distribution was analyzed by flow cytometry. Cells treated with antibodies or heregulin for 24 h were trypsinized, washed once with PBS, and fixed overnight in 70% ethanol. Fixed cells were centrifuged at 300 g for 10 min and washed with PBS. Cell pellets were resuspended in PBS containing 50 μg/ml RNase A and 50 μg/ml propidium iodide and incubated for 20 min at 37°C with gentle shaking. Stained cells were filtered through nylon mesh (41 μm) and analyzed on a Coulter flow cytometer XL-MCL (Coulter Corporation, Miami, FL) for relative DNA content. Doubllets and cell debris were excluded from the DNA histograms. The percentages of sub-G1 cell population were determined based on relative DNA content. The percentages of cells in different cell cycle compartments were determined using the MULTICYCLE software program (Phoenix Flow Systems, San Diego, CA).

Preparation of Total Cell Lysate and Western Immuno blot Analysis. The procedures for preparation of total protein and Western immunoblot analysis were performed as described previously (33).

Immunoprecipitation and Kinase Activity Assay. Aliquots of total cell lysates containing equal amounts of protein in lysis buffer [137 mM NaCl, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1 mM DTT, 1% NP40, 10% glycerol, and protease inhibitors] were precleared with 2 μg of normal mouse or rabbit IgG (Santa Cruz Biotechnology, Inc.) together with 20 μl of protein A/G agarose conjugate. CDK2, CDK4, CDK6, and cyclin E20 of protein A/G agarose conjugate. CDK2 and cyclin E-associated kinase activities were measured with a histone H1 kinase assay (34). CDK4, CDK6, and cyclin D1 associated kinase activities were measured using a glutathione S-transferase-Rb kinase assay (34). After washing four times with lysis buffer and twice with 1× kinase buffer [20 mM Tris-HCl (pH 7.4), 7.5 mM MgCl2, and 1 mM DTT], the agarose pellets were resuspended in 30 μl of kinase buffer containing 20 mM Tris-HCl (pH 7.4), 7.5 mM MgCl2, 1 mM DTT, 20 μM ATP, 6 μCi of [γ-32P] ATP (6000 Ci/mmol; Amersham Pharmacia Biotech, Arlington Heights, IL), protease inhibitors, and 3 μg of histone H1 (Boehringer Mannheim, Indianapolis, IN) or GST-Rb fusion protein (Santa Cruz Biotechnology). The mixture was incubated at 30°C for 30 min for the histone H1 kinase assay and at 30°C for 60 min for the GST-Rb kinase assay. The reaction was stopped by boiling the sample in Laemmli SDS loading buffer for 5 min, and samples were resolved on a 6–12% SDS-PAGE. The gel was then dried and subjected to autoradiography.

Fig. 2 Effect of mAb ID5 and heregulin on cell cycle distribution in SKBr3 cells. Subconfluent cultures of SKBr3 cells were incubated with mAb ID5 (65 nM), heregulin (0.3 nM), diluent, or control mAb MOPC21 (65 nM) for 24 h. Cell cycle phases were determined by flow cytometry of cellular DNA content. A, a representative cell cycle distribution. B, the mean ± SD of cell cycle distributions obtained from five experiments.
**Materials and Methods.**

Used for Western blotting and immunoprecipitation, as described in kinase activities. B protein expression.

NY). Formed as described by the manufacturer (Intergen, Purchase, MOPC21 (65 nM), or diluent for 24 h. Total cell lysate was prepared and incubated with mAb ID5 (65 nM), heregulin (0.3 nM), control mAb and only 8.3% were in G2-M phase. In clear contrast, heregulin treatment promoted the G1 to S phase transition in SKBr3 cells (Fig. 3). Oil Red O Staining. A modified “Oil Red O in propylene glycol” method was used to visualize neutral lipids, as reported previously (26). Briefly, SKBr3 cells were cytospin to slides and fixed in 6% paraformaldehyde in PBS. After dehydrating in absolute propylene glycol for 5 min, the slides were stained with 0.5% Oil Red O in propylene glycol for 1 h. The cells were then differentiated in 85% propylene glycol for 2 min, rinsed in distilled water, counterstained with Harris hematoxylin for 2 min, washed in PBS, and mounted in glycerin.

Detection of Apoptosis. Monolayers of SKBr3 cells were treated with antibodies or heregulin for 72 h, trypsinized, pooled with detached cells, and pelleted at 300 × g for 10 min at 4°C. Two methods were used to assess possible apoptosis: one dependent on cell cycle analysis (see above for detail), and the other measure DNA fragmentation by histochemical staining. After cytospinning the SKBr3 cells to slides, the ApopTag peroxidase in situ apoptosis assay (catalogue #S7100) was performed as described by the manufacturer (Intergen, Purchase, NY).

**RESULTS**

Rapid Suppression of Anchorage-independent Growth of SKBr3 Breast Cancer Cells with mAb ID5 and Heregulin. In previous studies, we have shown that the anti-HER2 mAb ID5 inhibited anchorage-independent growth in vitro (12, 21). In the present study, we further confirmed that both mAb ID5 and heregulin (Fig. 1) inhibited anchorage-independent growth of SKBr3 cells. Moreover, incubation of SKBr3 cells with mAb ID5 for as little as 6 h inhibited anchorage-independent growth in soft agar culture (Fig. 1B). Incubation with mAb ID5 for 30 min failed to inhibit growth. If cells were washed after 30 min and returned to medium with mAb ID5, significant growth inhibition was observed (Fig. 1B). Comparable inhibition was observed if SKBr3 cells were incubated for 6 h and returned to medium without mAb ID5 (Fig. 1B).

Heregulin, a ligand that can bind to homodimers of HER3 or HER4 and to heterodimers of HER2 with HER3 or HER4 (30, 31), inhibited anchorage-independent growth of SKBr3 cells (Fig. 1). Growth inhibition was, however, less marked with heregulin than with mAb ID5 (Fig. 1). Incubation with heregulin for different intervals indicated that growth inhibition required 24 h, a longer interval than was required with mAb ID5 (Fig. 1C). These results suggest that both mAb ID5 and heregulin can suppress the anchorage-independent growth of SKBr3 breast cancer cells and that inhibition is determined within 6–24 h of treatment.

Blockade of G1-S Progression by mAb ID5 and of G2-M Progression by Heregulin. Cell cycle analysis was used to explore the possible mechanisms of mAb ID5- and heregulin-induced growth inhibition. SKBr3 cells, at low cell density, were allowed to grow in complete medium for 24 h before treatment for an additional 24 h with antibodies (mAb ID5 or MOPC21), heregulin, or diluent as described in “Material and Methods.” Among these treatments, anti-HER2 mAb ID5 increased the percentage of SKBr3 cells in the G1 phase of the cell cycle and heregulin increased the percentage of cells at the G2-M interface (Fig. 2, A and B). After mAb ID5 treatment, 74.4% of cells were in G1, 17.4% were in S phase, and only 8.3% were in G2-M phase. In clear contrast, heregulin treatment promoted the G1 to S phase transition in SKBr3 cells with 42.9% of cells in G1, 31.8% in S phase, and 25.4% in G2-M. Under the same culture conditions, neither diluent nor MOPC21-treated cells displayed G1 or G2-M arrest (Fig. 2B). The sub-G1 fractions in diluent-, MOPC21-, mAb ID5-, and heregulin-treated cells were 1.7%, 2.0%, 2.1%, and 3.2%, respectively, consistent with little apoptosis at this early interval.

**Oil Red O Staining.** A modified “Oil Red O in propylene glycol” method was used to visualize neutral lipids, as reported previously (26). Briefly, SKBr3 cells were cytospin to slides and fixed in 6% paraformaldehyde in PBS. After dehydrating in absolute propylene glycol for 5 min, the slides were stained with 0.5% Oil Red O in propylene glycol for 1 h. The cells were then differentiated in 85% propylene glycol for 2 min, rinsed in distilled water, counterstained with Harris hematoxylin for 2 min, washed in PBS, and mounted in glycerin.

**Detection of Apoptosis.** Monolayers of SKBr3 cells were treated with antibodies or heregulin for 72 h, trypsinized, pooled with detached cells, and pelleted at 300 × g for 10 min at 4°C. Two methods were used to assess possible apoptosis: one dependent on cell cycle analysis (see above for detail), and the other measure DNA fragmentation by histochemical staining. After cytospinning the SKBr3 cells to slides, the ApopTag peroxidase in situ apoptosis assay (catalogue #S7100) was performed as described by the manufacturer (Intergen, Purchase, NY).
cyclin B1-CDC2 (35). Using Western blot analysis, we examined the effect of mAb ID5 and heregulin on the expression of these cell cycle-related proteins in subconfluent cultures. The protein expression of CDK2, CDK6, cyclin B1, cyclin E, and CDC2 was found to decline after a 24-h incubation with mAb ID5 treatment (Fig. 3A). In contrast, expression of all of these proteins increased in heregulin-treated cells. The protein expression of CDK4 (Fig. 3A) and cyclin D1 (data not shown) did not change appreciably after treatment with antibody or heregulin.

We next examined the CDK activities in extracts of SKBr3 cells using histone H1 and GST-Rb kinase assays. Levels of histone H1 kinase activities associated with immunoprecipitable cyclin E and CDK2 (Fig. 3B) were lower in mAb ID5-treated cells. In agreement with levels of protein expression, CDK4 (data not shown) and cyclin D1 (Fig. 3B) activities assessed by GST-Rb kinase activities were unchanged. Thus, a decrease in protein expression of CDK2 and CDK6 and the reduction of cyclin E-CDK2-associated kinase activities may contribute to the G1 arrest produced by mAb ID5 in SKBr3 cells.

**Effect of mAb ID5 and Heregulin on the Rb and CDK Inhibitory Proteins.** Several proteins can regulate CDK activity. Two families of mammalian CDK inhibitors have been identified. One includes p21Cip1, p27Kip1, and p57Kip2, which can inhibit G1 cyclin/CDK activity. The other family of CDK inhibitors includes the INK4 CDK inhibitors p15Ink4B, p16Ink4A, p18Ink4C, and p19Ink4D, that inhibit CDK4 and CDK6 kinase, but do not inhibit CDK2 activity (34, 35). As shown in Fig. 4, A and B, the protein expression of p27Kip1 increased in the cells treated with mAb ID5, but did not increase in cells treated with heregulin or MOPC21. The time course data showed that mAb ID5-induced p27Kip1 increase occurred as early as 8 h after antibody treatment (Fig. 4B). To determine whether increased p27Kip1 expression contributed to the decreased kinase activity of CDK2 observed after treatment with mAb ID5, the physical association of p27Kip1 with CDK complex was analyzed by immunoprecipitation with an anti-p27Kip1 antibody. As shown in Fig. 4D, a significantly increased amount of p27Kip1 associated with CDK2 was observed in mAb ID5-treated cells. However, the amount of p27Kip1 that was associated with CDK4 or CDK6 did not change dramatically (Fig. 4D). Western blotting could not detect p21Cip1 in SKBr3 cells (data not shown). This result was consistent with the fact that this cell line has a mutant p53 gene (36). Expression of p16Ink4A changed little after treatment with mAb ID5 or heregulin (data not shown). The p15Ink4B and p19Ink4D CDK inhibitors were undetectable in SKBr3 cells (data not shown).

One of the critical substrates of G1-related cyclins and CDKs is the Rb protein (35). When Rb protein is phosphorylated by the major cyclin-CDK complexes formed in G1, such as cyclin D-CDK4 or CDK6 and cyclin E-CDK2, transcription factors such as E2F are released that promote transition from G1 to S phase (35). Conversely, when Rb protein is not phosphorylated, transcription factors remain tightly bound and cell growth is arrested in the G1 phase. As shown in Fig. 4, A and C, more Rb protein became in the hypophosphorylated state in SKBr3 cells treated with mAb ID5 or heregulin. These observations are consistent with the ability of mAb ID5 to arrest SKBr3 cells in the G1 phase.
Phosphorylation in herregulin-treated cells is compatible with increased entry into S phase.

**Induction of Cell Differentiation by Treatment with Herregulin.** To determine whether growth inhibition by herregulin or mAb ID5 was associated with cell differentiation, we assessed synthesis and secretion of neutral lipids defined by Oil Red O staining. SKBr3 cells were treated with antibodies, diluent, or herregulin for 72 h, and Oil Red O staining was performed as described in “Materials and Methods.” A, representative staining of Oil Red O is shown. B, the percentage of positively stained cells came from five different experiments. Data indicate means ± SD. The asterisk indicates a statistically significant difference from controls (diluent or MOPC21).

**Fig. 5** Induction of cell differentiation by herregulin. SKBr3 cells were treated with antibodies (65 nM) and herregulin (1 nM) for 3 days. Cytoslines were prepared and stained with Oil Red O to detect lipid droplets, as described in “Materials and Methods.” A, representative staining of Oil Red O is shown. B, the percentage of positively stained cells came from five different experiments. Data indicate means ± SD. The asterisk indicates a statistically significant difference from controls (diluent or MOPC21).

**Induction of Apoptosis by Treatment with Herregulin.** Two different assays were used to determine whether growth inhibition produced by mAb ID5 or herregulin involved programmed cell death. SKBr3 cells were treated with antibodies, herregulin, or diluent for 72 h. After trypsinization, a portion of cells was analyzed by flow cytometry to determine the sub-G1 population. Another portion was cytospinned on slides and stained with ApopTag. Flow cytometric analysis (Fig. 6A) showed that...
Anti-HER2 antibody and heregulin inhibit growth of SKBr3 cells through different mechanisms

Fig. 6 Induction of apoptosis (sub-G1 fraction) by heregulin. Subconfluent cultures of SKBr3 cells were incubated with mAb ID5 (65 nm), heregulin (1 nm), control mAb MOPC21 (65 nm), or diluent for 72 h. Both floating and attached cells were harvested and subjected to two different assays, as described in “Materials and Methods.” A, sub-G1 fractions representing apoptotic cell population were determined by flow cytometric analysis. A representative histogram is shown. The number shown above bar came from five different experiments. B, apoptotic cells were determined by ApopTag assay. Data are means ± SD (from five experiments); the asterisk indicates a statistically significant difference from controls (diluent or MOPC21).

DISCUSSION

This study indicates that both the anti-HER2 antibody mAb ID5 and the ligand heregulin inhibited growth of SKBr3 cells that overexpress HER2. The antibody and heregulin appeared, however, to exert their growth inhibitory action through different mechanisms. The mAb ID5 blocked the transition from G1 phase to S phase. The mAb ID5 induced G1 arrest that was associated with: (a) a reduction in the expression of CDK2, cyclin E, and CDK6; (b) a reduction of cyclin E- and CDK2-associated kinase activity; (c) an increase in the expression of p27Kip1; (d) an increased association of p27Kip1 with CDK2; and (e) hypophosphorylation of Rb protein. Heregulin, in contrast, was capable of promoting progression from G1 to S, with subsequent increase in cells at G2-M associated with hyperphosphorylation of the Rb protein. Possibly of greater importance, heregulin induced both apoptosis and differentiation in SKBr3 cells, whereas mAb ID5 did not.

At present, precise mechanisms by which anti-HER2 antibodies affect tumor growth are poorly understood. Not all anti-HER2 antibodies inhibit tumor growth, as different antibodies may signal through different pathways (8). Several mechanisms for antibody-induced growth inhibition have been proposed. Inhibition of tumor growth may be associated with an intrinsic ability of anti-HER2 antibodies to induce endocytosis (38, 39). Blocking signaling by preventing the heterodimerization between HER family members induced by growth factors such as heregulin or EGF might also contribute to growth inhibition by anti-HER2 antibody (39). Other studies have correlated the tumor inhibitory potential of anti-HER2 antibodies with their capacity to induce cellular differentiation (40, 41). Bacus et al. (41) reported that the anti-HER2 antibodies with tumor-inhibitory specificity induced growth arrest at late S or early G2 phase of the cell cycle and phenotypic cellular differentiation. In this study, we showed that anti-HER2 antibody mAb ID5 primarily affected the G1 phase of cell cycle and caused G1 arrest without induction of differentiation and apoptosis, apparently differing from the observations by Bacus et al. (41). The cause for this discrepancy is unclear at present. Different anti-HER2 antibodies that recognize different epitopes on the extracellular domain of HER2 molecule may exert different biological effects. Our data did support the notion that anti-HER2 antibodies may exert growth inhibition through cytosstatic effects. Data from the anti-HER2 antibody mAb 4D5 and its humanized 4D5 antibody (Herceptin) indicate that effects of 4D5 antibody are cytosstatic, not cytoidal because tumor growth resumed on termination of antibody treatment (22).

Several studies have shown that certain anti-HER2 antibodies exhibit apoptotic activity (42–45). Interestingly, anti-HER2 antibodies that bind near the transmembrane region of the HER2 extracellular domain (such as 4D5) have potent cytosstatic properties and induce little apoptosis, whereas the antibodies that bind the NH2 terminus of HER2 induce rapid apoptosis (43). However, prolonged treatment with 4D5 could induce a small amount of apoptosis (43). In our experiments, treatment with mAb ID5 up to 72 h still failed to induce a measurable amount of apoptosis.

4 X-F. Le and R. C. Bast, unpublished data.
**Fig. 7**  Effect of PI3-K inhibitors on heregulin-induced G₂-M arrest and apoptosis (sub-G₁ fraction). Aliquots of 2 × 10⁵ of SKBr3 cells were plated in 10-cm culture dishes and cultured for 24 h in complete medium. After pretreatment with the PI3-K inhibitors LY294002 or wortmannin overnight, cells were incubated with heregulin or diluent for 24–72 h. Both floating and attached cells were harvested and subjected to cell cycle analysis, as described in “Materials and Methods.” A, G₂-M fractions at 24 h after heregulin treatment were determined by flow cytometric analysis. A representative histogram is shown. The number shown is the mean percentage ± SD from three replicate experiments. B, sub-G₁ fractions representing apoptotic cell population at 72 h after heregulin treatment were determined by flow cytometric analysis. A representative histogram is shown. The number shown is the mean percentage ± SD from three replicate experiments.
increase in apoptosis (Fig. 6). Again, different properties of anti-HER2 antibodies may be responsible for these discrepancies.

In this study, an increased amount of the p27kip1 CDK inhibitor was found in cells treated with anti-HER2 antibody mAb ID5. This finding is similar to previous reports that treatment with antibody against EGFR (HER1) stimulated the expression of the cell cycle inhibitor p27kip1, but not p21WAF1 (46, 47). p27kip1 protein expression is ordinarily constant throughout the cell cycle in many cell types (48). Recently, the anti-HER2 antibody mAb 4D5 has been shown to induce p27kip1 in an ovarian cancer cell line OVCA420 (49). Mimosine, a drug that induces G1 arrest by a different mechanism, was unable to induce p27kip1 (46). Also recently, Hynes et al. have presented similar observations with the anti-HER2 antibody 4D5 in SKBr3 cells. Treatment with mAb 4D5 induced G1 arrest, increased p27kip1 protein expression, decreased cyclin E/CDK2 kinase activity, and resulted in hypophosphorylated Rb expression (50). It seems that blockade of receptor tyrosine kinases of the HER family either by antibodies to HER1 (52) or HER2 (Refs. 49 and 50 and this study) or a tyrosine kinase inhibitor, herbigycin (46), can elicit increases in p27kip1 expression. The SKBr3 cell line used in this study expresses mutated p53 (36), and Western blot analysis revealed a lack of induction of p53 (data not shown) or p21Cip1 protein following mAb ID5 treatment. These data suggest that mAb ID5-induced G1 arrest is independent of p53-regulated mechanisms.

Heregulin has been reported to stimulate the growth of human normal mammary epithelial cells (51, 52) and breast tumor cell lines expressing low levels of HER2 (53). However, the biological effects of heregulin on breast cancer cell lines expressing high levels of HER2 are controversial. Both growth-stimulatory (53, 54) and growth-inhibitory effects (21, 25–28) have been reported. Our data demonstrate that heregulin B1 can suppress the anchorage-dependent and anchorage-independent growth of SKBr3 breast cancer cells that overexpress HER2 by inducing G2-M arrest, followed by apoptosis and cell differentiation. Bacs et al. (26) reported that heregulin was able to induce G2-M arrest and cell differentiation in human breast cancer AU-565 cells that overexpress HER2, but did not study apoptosis. Daly et al. (27) reported that heregulin could induce G2-M arrest and apoptosis, but cell differentiation was not studied. Recently, Weinstein et al. (28) also reported that heregulin was capable of mediating apoptosis in human and murine mammary tumor cell lines and murine tumors, but effects on the cell cycle were not determined. Daly et al. proposed that the apoptotic cells in heregulin-treated cells arise from the G2-M population of cells (27). Our data suggest that this is not the case with SKBr3 cells. Pretreatment of the cells with PI3-K inhibitors LY294002 and wortmannin can abolish heregulin-induced G2-M blockade at 24 h after treatment, but cannot abolish heregulin-induced apoptosis at 72 h (Fig. 7). Additional experiments need to address this issue. Most recently, Daly et al. (37) have reported that heregulin induces G2 progression with an increase in cyclin A, cyclin B, and Cdk1, and can also cause a G2 block from day 1 and apoptosis from days 2–3.

The mechanisms by which mAb ID5 and heregulin inhibited the growth of SKBr3 cells were quite different. These differences presumably reflect interaction with different HER family receptors and the subsequent alterations in signaling pathways. Anti-HER2 antibodies specifically bind to HER2 and produce HER2 homodimers (32). In contrast to antibodies that interact with HER2 homodimers, heregulin does not bind to HER2 alone. Instead, HER3 and HER4 function as the direct receptors for heregulin (30, 31). Via heterodimerization with HER3 or HER4 and transphosphorylation, HER2 can be tyrosine phosphorylated and activated by heregulin (31, 55). Several down-stream signaling pathways can be activated depending on whether cells are activated with specific ligands or antibodies (1–4). In our own preliminary observations, heregulin activated the PI3-K and JNK pathways, whereas mAb ID5 did not.4

In summary, this study demonstrates that anti-HER2 antibody mAb ID5-induced G2 arrest is associated with increased p27kip1 expression, decreased CDK2 and cyclin E expression, and declined CDK2- and cyclin E-associated kinase activities. No significant apoptosis and cell differentiation was observed during mAb ID5-induced growth inhibition. In contrast, heregulin induced G2-M arrest, followed by apoptosis and cell differentiation. Thus, antibody mAb ID5 and heregulin exert their growth inhibition of human breast cancer cells that overexpress HER2 through different mechanisms.

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