Herceptin-induced Inhibition of Phosphatidylinositol-3 Kinase and Akt Is Required for Antibody-mediated Effects on p27, Cyclin D1, and Antitumor Action

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ABSTRACT

We have examined whether inhibition of phosphatidylinositol-3 kinase (PI3K) and its target, the serine/threonine kinase Akt, play a role in the antitumor effect of the HER2 antibody Herceptin. Herceptin inhibited colony formation, down-regulated cyclin D1, and increased p27 protein levels in the HER2 gene-amplified BT-474 and SKBR-3 human breast cancer cells. These effects were temporally associated with the inhibition of PI3K activity in vitro as well as Akt function as measured by steady-state levels of phospho-Ser473 Akt and kinase activity against glycogen synthase kinase (GSK)-3β. These responses were not observed in MDA-361 and MDA-453 cells, which do not exhibit HER2 gene amplification and are relatively resistant to Herceptin. Treatment of BT-474 cells with Herceptin inhibited the constitutive tyrosine phosphorylation of HER3 and disrupted the basal association of HER3 with HER2 and of HER3 with p85α potentially explaining the inhibition of PI3K. Treatment with either Herceptin or the PI3K inhibitor LY294002 increased the level of p27 in the nucleus>cytosol, thus increasing the ratio of p27:Cdk2 in the nucleus and inhibiting Cdk2 activity and cell proliferation. Antisense p27 oligonucleotides abrogated the increase in p27 induced by Herceptin and prevented the antibody-mediated reduction in S phase. Transduction of BT-474 cells with an adenovirus-encoding active (myristoylated) Akt (Myr-Akt), but not with a β-galactosidase control adenovirus, prevented the Herceptin- or LY294002-induced down-regulation of cyclin D1 and of phosphorylated GSK-3β and prevented the accumulation of p27 in the nucleus and cytostol. In addition, Myr-Akt prevented Herceptin-induced inhibition of the cell proliferation of BT-474 cells and Herceptin-induced apoptosis of SKBR-3 cells. These data suggest that (a) changes in cell cycle- and apoptosis-regulatory molecules after HER2 blockade with Herceptin result, at least in part, from the inhibition of Akt; and (b) disabling PI3K and Akt is required for the antitumor effect of HER2 inhibitors.

INTRODUCTION

The HER2/neu (erbB2) proto-oncogene product is a member of the HER (erbB) family of transmembrane receptor tyrosine kinases, which also includes the EGFR1 (HER1, erbB1), HER3 (erbB3), and HER4 (erbB4). Except for HER2, the binding of receptor-specific ligands to the ectodomain of EGFR, HER3, and HER4 results in the formation of homodimeric and heterodimeric kinase-active complexes.

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3 The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; PI3K, phosphatidylinositol-3 kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-triphosphate; MAPK, mitogen-activated protein kinase; IMEM, improved minimal essential medium; Tdt, terminal deoxynucleotidyl transferase; TUNEL, Tdt UTP nick end labeling; FACS, fluorescence-activated cell sorting; PMSF, phenylmethylsulfonyl fluoride; HH1, histone H1; TLC, thin layer chromatography; mAb, monoclonal antibody; Myr-Akt, myristoylated Akt; β-gal, β-galactosidase; MOI, multiplicity/multiplicities of infection; FISH, fluorescent in situ hybridization; Cdk, cyclin-dependent kinase; MM, mismatch; AS, antisense; P-Tyr, phosphorylated Tyr; P-GSK, phosphorylated GSK; P-Akt, phosphorylated Akt; P-MAPK, phosphorylated MAPK; GSK, glycogen synthase kinase.
dependent on the composition of the heterodimeric receptor complexes. Although kinase-deficient, HER3 is able to directly couple to PI3K (29, 30), a lipid kinase involved in the proliferation, survival, adhesion, and motility of tumor cells (31–33). Because of (a) the potent transforming ability of HER2/HER3 heterodimers (b) the co-expression and association of HER2 and HER3 in breast cancer cell lines as well as in mouse transgenic and human mammary tumors (20, 34–39), and (c) the ability of HER2 to activate PI3K and its downstream targets (36, 40–43), we have examined whether the inhibition of this signaling pathway is required for the antitumor effect of the HER2 blocking antibody Herceptin. The data presented below strongly suggest that the inhibition of PI3K and its target, the serine-threonine kinase Akt, is required for the antitumor action of Herceptin against HER2-overexpressing breast cancer cells.

MATERIALS AND METHODS

Cell Lines, Kinase Inhibitors, and Antibodies. The human breast cancer cell lines BT-474, SKBR-3, MDA-453, and MDA-361 were obtained from the American Type Tissue Culture Collection (Manassas, VA) and maintained in IMEM (Life Technologies, Inc. Rockville, MD) supplemented with 10% FCS. Herceptin was purchased from the Vanderbilt University Hospital Pharmacy (Nashville, TN). LY294002, a specific inhibitor of the p110 catalytic subunit of PI3K (44), was from BIOMOL (Plymouth Meeting, PA). For immunoprecipitations and/or immunoblot analysis, the following antibodies were used: GS-3B and p27 (Transduction Laboratories, Lexington, KY); HER2/neu and HER3 (Neomarkers, Freemont, CA); P-Tyr (Upstate Biotechnology, Lake Placid, NY); cyclin D1 (PharMingen, San Diego, CA); MAPK, P-GSK-3B, Akt and Ser473 P-Akt (New England Biolabs, Beverly, MA); P-MAPK (Promega, Madison, WI); p53, cdk2, and c-jun (Santa Cruz Biotechnology, Santa Cruz, CA). Protein content in cell lysates was measured by the BCA method (Pierce, Rockford, IL).

Monolayer Growth, Colony Formation, and TUNEL Assays. Cells were seeded in 6-well plates in IMEM/10% FCS at a density of 3 × 10^4 cells/well; Herceptin or LY294002 was added the next day. Medium and inhibitors were replaced with fresh medium and inhibitors every other day until cells were harvested by trypsinization and counted with a Zeiss Coulter Counter (Beckman Coulter, Miami, FL). Colony-forming assays were performed as described previously (45) in the presence or absence of Herceptin. Tumor cell colonies measured ≥50 μm were counted using an Omnicon 3800 Colony Counter at 4°C. Colony Analyzer V2.1A software (Imaging Products International, Inc.). To measure apoptosis, adherent cells in IMEM/10% FCS were treated with Herceptin for 72 h, harvested by scraping, and pooled with floating cells. TUNEL assay was performed using the APO-BrdU kit (Phoenix Flow Systems, San Diego, CA). Flow cytometric detection of FITC-positive cells was performed using the APO-BrdU kit (Phoenix Flow Systems, San Diego, CA). Flow cytometric analysis was performed as described previously (45) using the primary antibody at 4°C. TUNEL assay was performed using the APO-BrdU kit (Phoenix Flow Systems, San Diego, CA). Flow cytometric detection of FITC-positive cells was performed using the APO-BrdU kit (Phoenix Flow Systems, San Diego, CA). Flow cytometric analysis was performed as described previously (45) using the primary antibody at 4°C.

In Vitro Kinase Reactions. Cells were lysed in NP40 lysis buffer described above; 100–300 μg of protein from whole cell lysates or 50–100 μg of protein from cytoplasmic and nuclear fractions were precipitated overnight at 4°C with immobilized-Akt 1G1 monoclonal IgG2a (New England Biolabs) or a Cdk2 antibody, respectively. The immunoprecipitates were washed extensively with NP40 lysis buffer followed by washings in the respective kinase buffer. Cdk2 activity against HH1 was measured as described by Lenferink et al. (42). For Akt activity, reactions were performed at 37°C for 30 min in a total volume of 30 μl containing kinase buffer [50 mM Tris (pH 8.0), 10 mM MgCl2, 0.1 mM EGTA, 0.1 mM ATP, 1 mM DTT, 1 mM PMSF, 2 μg/ml leupeptin and aprotonin], 10 μg [γ-32P]ATP (specific activity, 3000 Ci/mmol; Amersham Pharmacia), and 0.04 μg GSK-3B (Sigma). All of the reactions were terminated with the addition of 6× Laemmli sample buffer and resolved using SDS-PAGE followed by autoradiography. For the determination of PI3K activity, cells were seeded at a density of 5 × 10^4 cells/100-mm dish 24 h prior to treatment with Herceptin or LY294002. After treatment, the cells were washed twice with 137 mM NaCl, 20 mM Tris (pH 7.5), 1 mM CaCl2, and 1 mM MgCl2 and were lysed in the wash buffer supplemented with 10% glycerol, 1% NP40, 1 mM PMSF, 10 μg/ml aprotonin, 10 μg/ml leupeptin, 10 mM NaF, and 200 mM Na3VO4. Cell extracts were precleared by centrifugation and then were precipitated overnight with a P-Tyr mAb (Upstate Biotechnology) and protein A-Sepharose. Immunoprecipitates were washed three times with 1% NP40 in PBS; 2 × with 100 mM Tris (pH 7.5) and 0.5 mM LiCl; twice with 10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA; and twice with kinase assay buffer [10 mM Tris (pH 7.5), 100 mM NaCl, 4 mM MgCl2, 1 mM EDTA, 0.5 mM EGTA]. All of the wash buffers contained 200 μM Na3VO4. The beads were suspended in 40 μl of kinase buffer followed by the addition of 10 μg [γ-32P]ATP and sonication in 0.2 mg/ml IP2 (Avanti). The kinase reaction proceeded for 10 min at room temperature and was terminated with stop buffer (1:1, methanol:HCl) followed by extraction with chloroform. The reaction products were separated by TLC on 1% oxalate-pretreated TLC with chloroform:methanol:acetic acid:water (60:20:18:11) and were detected by autoradiography.

Immunofluorescent Localization of p27kip1. BT-474 cells were seeded on coverslips in 6-well plates at a density of 4 × 10^4 cells/well. After an 8–24 h incubation with Herceptin or LY294002, the cells were washed with PBS, fixed in 4% paraformaldehyde/PBS for 10 min, washed, and stored overnight at 4°C. Cells were then permeabilized with 0.1% Triton X-100/PBS for 15 min, washed, and then incubated for 1 h with a p27 mAb (Transduction Laboratories) diluted 1:250 in 0.05% Triton X-100/PBS. After three washes with 0.05% Triton X-100/PBS, the cells were incubated for 45 min with antimouse Cy3 (Jackson Immunoresearch, West Grove, PA) diluted 1:500 in 0.05% Triton X-100/PBS. The cells were washed six times, stained with 1 mg/ml Hoechst, and mounted in Aquapoly Mount (PolySciences, Inc.). Cy3 immunofluorescence was recorded with a Princeton Instruments cooled digital CCD camera on a Zeiss Axioshot upright microscope.

Studies with AS p27 Oligonucleotides. The sequences of the 15-mer p27 and MM phosphorothioates (provided by M. Flanagan, Gilead Sciences, Foster City, CA) were reported previously (47). BT-474 cells in the presence or absence of Herceptin were treated with 30 nm oligonucleotides for 3 h as described by Lenferink et al. (42). To control for nonspecific effects of cefotaxim, cells treated with neither AS p27 nor MM oligonucleotides were, nevertheless, treated with 2 μg/ml cefotaxim (53815, Gilead Sciences). Forty-eight h after treatment with oligonucleotides, the cells were either trypsinized and subjected to cell cycle analysis by flow cytometry or were lysed and subjected to p27 and HER2 immunoblot procedures as indicated above.

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Myr-Akt and β-gal Adenoviral Infection. Adenoviral vectors were provided by Dr. W. Ogawa (Kobe University, Kobe, Japan) and have been described elsewhere (48). BT-474 cells were seeded at a density of 3 × 10^5 cells/60-mm dish 24 h before transduction with adenoviral vectors at 80 plaque-forming units/cell (MOI) as described by Sakaue et al. (49). More than 90% of BT-474 cells infected at a similar MOI with a β-gal adenovirus exhibited blue staining. Infections were conducted for 5 h in serum-free medium followed by a 48-h incubation in IMEM/10% FCS before the addition of antibodies or kinase inhibitors.

HER2/neu FISH. Adherent tumor cells were trypsinized, washed, and then swollen in (hypotonic) 0.075 M KCl and fixed in 3:1 methanol/acetic acid prior to seeding onto slides. After an overnight incubation at room temperature, the slides were denatured in 2× SSC/70% formamide at 73°C for 5 min. Denatured slides were dehydrated in 70, 85, and 100% ethanol for 1 min each. The slides were next dried and 10 μl of the Vysis LSI HER2 SpectrumOrange and CEP 17 SpectrumGreen probe mixture (Downers Grove, IL) were applied to them. Slides were sealed with rubber cement, incubated overnight in a humidified chamber at 37°C, washed in 0.4× SSC/0.3% NP40 for 2 min at 73°C, and then rinsed in 2× SSC/0.1% NP40 at room temperature. After adding DAPI II counterstain (10 μl) to each hybridization area on the slides, coverslips were applied onto them. Signal enumeration was performed at ×400 magnification in a fluorescence microscope equipped with a dual-pass filter (Chromatechnology, Brattleboro, VT) for the simultaneous detection of SpectrumOrange and DAPI and a single-pass filter for the detection of SpectrumAqua. Enumeration of HER2 and CEP 17 signals was performed on 25 consecutive cells. Images of representative cells were captured at ×630 with a single-bandpass filter for the detection of SpectrumOrange, SpectrumAqua, or DAPI using IP Labs imaging software package (Scanalytics, Inc., Fairfax VA).

RESULTS

Herceptin Inhibits HER2 Signaling in HER2 Gene-amplified Breast Cancer Cells. We initially examined the effect of the humanized IgG1 Herceptin against BT-474, SKBR-3, MDA-361, and MDA-453 human breast cancer cells, which are known to overexpress HER2 (20, 50). In a colony-forming assay, Herceptin inhibited BT-474 and SKBR-3 cells with an IC_{50} of 0.2 g/ml. However, doses as high as 20 g/ml did not inhibit MDA-361 and MDA-453 colony formation (Fig. 1A). The degree of gene amplification measured by DNA dot-blot analysis (45) as well as the levels of HER2 protein measured by HER2 antibody binding or immunohistochemical studies (51, 52) are reportedly different among these four cell lines. Thus, we next deter-

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**Fig. 1.** Herceptin inhibits colony survival of HER2-overexpressing breast tumor cells with HER2 gene amplification. In A, BT-474, SKBR-3, MDA-361, and MDA-453 cells (3 × 10^4 cells) were plated in 35-mm dishes in IMEM/10% FCS, 0.8% agarose, and 10 mM HEPES in the absence or presence of 0.2–20 μg/ml Herceptin. After 7 days, colonies measuring ≥50 μm were counted as indicated in “Materials and Methods.” Each bar, the mean ± SD of three dishes. In B, cell suspensions of all four cell lines were fixed and subjected to FISH analysis as described in “Materials and Methods.” In C, monolayers of the indicated cell lines were washed and solubilized in NP40 lysis buffer. The indicated amounts of protein were subjected to immunoblot analysis for HER2.
Fig. 2. Herceptin inhibits active Akt and MAPK and modulates cyclin D1 and p27 protein levels. In A and B, BT-474 and SKBR-3 breast cancer cells in IMEM/10% FCS were treated with 10 μg/ml Herceptin. At the indicated times, the cells were washed and harvested in lysis buffer. Seventy μg of protein from whole cell lysates were resolved by SDS-PAGE and subjected to the indicated immuno blot procedures. To the side of each panel, M, is in thousands. In C, dose-dependent effect of Herceptin on p27 levels. BT-474 cells were treated with 0.1-10 μg/ml Herceptin for 24 h. At this time, cells were washed, lysed, and tested for p27 protein content by immunoblot analysis. In D, exponentially growing MDA-361 and MDA-453 cells in IMEM/10% FCS were incubated with 10 μg/ml Herceptin. After 6-48 h, the cells were lysed and tested for the content of P-Akt, total Akt, P-MAPK, and total MAPK by immunoblot analysis as described in “Materials and Methods.” Each lane, 70 μg of protein from whole cell lysates.

The Akt kinase activity, as measured by the ability of Akt precipitates from Herceptin-treated BT-474 cells to phosphorylate a GSK-3β substrate in vitro, was severely reduced compared with that present in untreated cells (Fig. 3B). The inhibitory effect of Herceptin against PI3K and Akt kinases was similar to that induced in intact BT-474 by the PI3K inhibitor LY294002 (Fig. 3, A and B). At the same concentrations in which PI3K and Akt were inhibited, both Herceptin and LY294002 inhibited the growth of BT-474 cells (Fig. 3C), which suggested a link between the inhibition of PI3K-Akt and the antiproliferative effect of Herceptin.

In lysates from exponentially growing cells, HER3 antibodies were able to coprecipitate p85 and HER2 (Fig. 4A, middle and right panels), and p85 antibodies coprecipitated both HER3 and HER2 (Fig. 4A, left and right panels). In addition, a M, ~180,000 P-Tyr immunoreactive band was detectable in the HER3 precipitates (Fig. 4B, Lane 1), which indicated that, in BT-474 cells, HER3 is constitutively phosphorylated on Tyr and is associated with both HER2 and p85. Treatment with Herceptin induced a transient increase in HER3 phosphorylation at 1 h without disrupting the association with p85 (Fig. 4B, Lane 2). However, consistent with the inhibition of PI3K and Akt shown in Fig. 3, treatment with Herceptin for 24 h eliminated the basal phosphorylation of HER3 and the constitutive association of HER3 with p85 (Fig. 4B).

Inhibition of PI3K and Modulation of p27. Blockade of HER2 with Herceptin or HER kinase inhibitors has been reported to redirect the Cdk inhibitor to p27 to Cdk2, thus leading to the growth arrest of HER-overexpressing cells (39, 42). Therefore, we examined whether the inhibition of PI3K with either Herceptin or LY294002 induced similar modulation of p27 in BT-474 cells. By immunofluorescence microscopy, we observed that most of p27 was present in the cytosol of proliferating BT-474 cells. Treatment with growth-inhibitory concentrations of Herceptin or LY294002 for 20 h resulted in an almost complete translocation of immunofluorescent p27 from cytosol to cell nuclei (Fig. 5A). This effect was evident as early as 8 h after a
treatment with LY294002 but required >16 h with the saturating dose of Herceptin. By immunoblot of cell fractions, we observed that most of p27 was in the cytosol of the proliferating cells. Treatment with Herceptin or LY294002 increased the p27 protein levels in both nucleus and cytosol but more so in the nucleus (Fig. 5B). In proliferating untreated cells, a low level of nuclear p27 precipitated with Cdk2 antibodies. However, in cells that were treated for 20 h with Herceptin or LY294002, higher levels of p27 associated with nuclear Cdk2, which resulted in the suppression of the ability of nuclear Cdk2 precipitates to phosphorylate HH1 in vitro (Fig. 5C). Similar results were obtained when using GST-Rb as a Cdk2 kinase substrate (data not shown).

To determine whether p27 played a role in the cell cycle arrest on blockade of HER2 with Herceptin, we used p27 AS oligonucleotides. In cells treated with cytofectin alone (control) or with MM oligonucleotides, but not in cells treated with AS p27 phosphorothioates, Herceptin up-regulated p27 levels (Fig. 6B). In cells treated with cytofectin alone or with MM oligonucleotides, treatment with Herceptin reduced the proportion of cells in S phase [11 versus 5% (P = 0.01) and 5.2% (P = 0.03), respectively] and increased the G1 cell fraction [70 versus 85% (P = 0.001) and 83% (P = 0.002), respectively]. In cells in which p27 was down-regulated by AS p27 oligonucleotides, Herceptin failed to statistically reduce the proportion of cells in S phase compared with controls [11 versus 8% (P = 0.28)]. Although the AS oligonucleotides did not eliminate p27 content completely (Fig. 6B), it is likely that they prevented a threshold level of p27 required for redirection to and inhibition of Cdk2. In cells preincubated with AS p27 oligonucleotides, Herceptin still induced an increase in the G1 cell fraction. However, this increase was less significant than that induced in cells treated with MM oligonucleotides [70 versus 76% (P = 0.03); Fig. 6A], which, all together, suggested that the modulation of p27 levels and/or its localization play a partial role in the growth arrest that follows HER2 blockade with Herceptin.

Constitutively Active Akt Abrogates the Antitumor Effect of Herceptin. We next examined whether forced expression of active Akt prevented Herceptin action. For this purpose, we used Myr-Akt, which consists of Akt1 ligated to a myristoylation sequence, resulting in an enzyme ~10-fold more active than the wild-type enzyme (48). In cells infected with a control β-gal adenovirus, both Herceptin and LY294002 inhibited PI3K as indicated by a marked reduction of
P-Ser473 Akt, P-GSK-3β, and cyclin D1 protein levels (Fig. 7A). However, in cells infected with Myr-Akt, neither inhibitor reduced the P-Akt levels. This unchanged P-Akt band likely represents the membrane-associated Myr-Akt protein phosphorylated in Ser-473. Similarly, P-GSK-3β and cyclin D1 levels were not reduced by Herceptin or LY294002 in cells expressing Myr-Akt (Fig. 7A), which supports the conclusion that Myr-Akt was indeed functional. In fractions from BT-474 cells that were infected with β-gal virus, and consistent with results with uninfected cells (Figs. 5, A and B), the majority of p27 was present in the cytosol. Myr-Akt eliminated the low basal nuclear levels of p27 but did not alter the basal cytosolic content of p27 (Fig. 7B). In cells infected with β-gal adenovirus, both inhibitors up-regulated p27 in the cytosol and in the nucleus. However, this accumulation of p27 in both compartments was markedly reduced in cells infected with a similar MOI of Myr-Akt adenovirus (Fig. 7B), which suggested that the accumulation of p27 that follows the blockade of HER2 and PI3K results from the inhibition of Akt.

Finally, we examined whether active Akt was able to counteract the antiproliferative effect of Herceptin in BT-474 cells, which in culture are highly sensitive to Herceptin-mediated growth arrest (39) but not to apoptosis (54). Treatment with Herceptin or LY294002 over a period of 6 days reduced cell proliferation >50% compared with untreated controls. However, infection with Myr-Akt almost completely prevented the antiproliferative effect of each inhibitor (Fig. 8A). The ability of active Akt to counteract the pro-apoptotic effect of Herceptin was studied in cultured SKBR-3 cells. Herceptin does not induce SKBR-3 cell cycle arrest as markedly as in BT-474 cells (39, 55) but has recently been shown to induce apoptosis of these cells (54). Treatment with either Herceptin or LY294002 for 72 h induced a 3-fold increase in the basal level of SKBR-3 cell apoptosis that was completely prevented by infection with Myr-Akt (Fig. 8B).

**DISCUSSION**

We have examined whether the blockade of HER2 with the humanized IgG1 Herceptin inhibits tumor cell growth by inhibiting PI3K function. Herceptin inhibited colony formation, P-MAPK, and P-Ser473 Akt, P-GSK-3β, and cyclin D1 protein levels (Fig. 7A). However, in cells infected with Myr-Akt, neither inhibitor reduced the P-Akt levels. This unchanged P-Akt band likely represents the membrane-associated Myr-Akt protein phosphorylated in Ser-473. Similarly, P-GSK-3β and cyclin D1 levels were not reduced by Herceptin or LY294002 in cells expressing Myr-Akt (Fig. 7A), which supports the conclusion that Myr-Akt was indeed functional. In fractions from BT-474 cells that were infected with β-gal virus, and consistent with results with uninfected cells (Figs. 5, A and B), the majority of p27 was present in the cytosol. Myr-Akt eliminated the low basal nuclear levels of p27 but did not alter the basal cytosolic content of p27 (Fig. 7B). In cells infected with β-gal adenovirus, both inhibitors up-regulated p27 in the cytosol and in the nucleus. However, this accumulation of p27 in both compartments was markedly reduced in cells infected with a similar MOI of Myr-Akt adenovirus (Fig. 7B), which suggested that the accumulation of p27 that follows the blockade of HER2 and PI3K results from the inhibition of Akt.

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induced phosphorylation of HER2 that was not blocked by mAb coreceptors to HER2. In OVCA420 ovarian cancer cells, EGF may not be effective in interrupting lateral signaling from HER2. The latter result also implies that at levels of HER2 present at high density, whereas, at low concentrations, the presence of a ligand was required for receptor multimerization and activation (22). The inability of Herceptin to block Akt activation; and (b) in HER2 gene-amplified cells in which Herceptin-bound HER2 can potentially still receive signaling input from co-overexpressed HER family members.

Treatment with Herceptin inhibited the PI3K target Akt in antibody-sensitive but not in antibody-resistant breast tumor cell lines. Both HER3-associated PI3K activity and HER2/HER3 heterodimers have been previously shown in MDA-361 and MDA-453 cells (20). The inability of Herceptin to block PI3K activity suggests that, in these cells, HER3-associated PI3K depends on signals other than HER2. The inhibition of PI3K in BT-474 cells was associated with the uncoupling of phosphorylated HER3 from HER2 and from p85. The kinetics of these effects were slow and probably reflect the reported ability of mAb 4D5 to remove HER2 from the plasma membrane (60, 4D5, the corresponding mouse hybridoma of Herceptin (58). In a recent report, tumor growth factor-α-induced proliferation of BT-474 cells was inhibited by ZD1839, a low-molecular-weight EGFR Tyr kinase inhibitor, but not by Herceptin (54). Finally, Herceptin did not inhibit the growth of MKN gastric cancer cells, which exhibit HER2 gene amplification (59) but which also overexpress highly phosphorylated EGFR (39). These data imply that Herceptin may not be effective: (a) in HER2-overexpressing cells with a single copy of the HER2 gene, like MDA-361 and MDA-453, in which HER2 may rely on lateral signals from HER coreceptors for activation; and (b) in HER2 gene-amplified cells in which Herceptin-bound HER2 can potentially still receive signaling input from co-overexpressed HER family members.

The differential sensitivity among the four cell lines that we used in this study is in agreement with preliminary data showing that the clinical responses to Herceptin were limited to breast cancers with HER2 gene amplification and/or with the highest level of receptor protein expression (57). Because Herceptin is specific to HER2, and considering that only HER2 gene-amplified tumors are inhibited by the antibody, we speculate that only HER2 gene-amplified breast cancers are dependent on activated HER2. Along those lines, a recent study reported that breast tumors that stained positively with a mAb specific for Tyr-1248 phospho-HER2 were those with the highest level of total HER2 expression (21). This is also consistent with the ability of p185- neu, the rat homologue of HER2, to spontaneously oligomerize and activate its kinase when present at high density, whereas, at low concentrations, the presence of a ligand was required for receptor multimerization and activation (22). The latter result also implies that at levels of HER2 below those seen in HER2 gene-amplified cells, activation of the receptor may depend on ligand-activated coreceptors. Several studies suggest that blocking the HER2 ectodomain with Herceptin may not be effective in interrupting lateral signaling from HER coreceptors to HER2. In OVCA420 ovarian cancer cells, EGF induced phosphorylation of HER2 that was not blocked by mAb P-Akt in BT-474 and SKBR-3 cells, which exhibit HER2 gene amplification, but not in MDA-361 and MDA-453 cells, both with a single copy of the HER2/neu gene. This result is consistent with a previous report in which the latter two cell lines exhibited a modest reduction in [3H]thymidine incorporation but no reduction in the S-phase fraction when treated with 10 μg/ml Herceptin (56). Although MDA-361 and MDA-453 cells exhibit robust levels of constitutively phosphorylated HER2 (20, 54), quantitation of HER2 gene copy number as measured by DNA dot-blot analysis was only 2-fold, relative to normal human DNA (50). It is likely that this last method overestimated the gene copy number as suggested by the results with the more controlled FISH analysis (Fig. 1).

Fig. 7. Dominant active Akt rescues BT-474 cells from Herceptin action. BT-474 cells (3 × 10⁵ cells/60-mm dish) were infected with either β-gal or Myr-Akt adenoviruses for 48 h as indicated in “Materials and Methods” followed by treatment with either 10 μg/ml Herceptin or 40 μg/LY294002 for 24 h. Whole cell lysates were prepared and 70 μg of protein resolved by SDS-PAGE followed by the indicated immunoblot procedures. On the left of each panel, M r in thousands. In B, nuclear and cytoplasmic fractions were prepared as described in “Materials and Methods” from identically treated cells. Fractions were next analyzed for p27 or α-tubulin levels by immunoblot; α-tubulin was detected only in the cytosolic fractions.

Fig. 8. Dominant-active Akt rescues from the antiproliferative and apoptotic effect of Herceptin. In A, BT-474 cells (3 × 10⁵/well in 6-well plates) were infected with Myr-Akt or β-gal adenoviruses (48 h) followed by treatment with Herceptin (10 μg/ml) or LY294002 (40 μg). Fresh IMEM/10% FCS ± inhibitors was added on days 3 and 5. Cells were trypsinized and counted in a Coulter counter on day 6. Each data point, the mean ± SD of triplicate wells. Results were confirmed in two independent experiments. In B, 10⁵ SKBR-3 cells were infected with Myr-Akt or β-gal (Control) adenoviruses treated with the same concentrations of Herceptin or LY294002 for 72 h. Adherent and floating cells were harvested and assayed for evidence of apoptosis by Apo-BrdU analysis in the presence of Tdt. In parentheses on top of each panel, the percentage of FACS-positive apoptotic cells in the R1 gated areas, quantitated by flow cytometry.
and/or induce its homodimerization.\textsuperscript{4} Down-modulation of receptor-ligand complexes is a major attenuation mechanism of receptor-induced signaling. Therefore, antibody-induced HER2 down-modulation from the cell surface and/or HER2 dimerization with itself should result in less receptor available for heterodimerization with other HER family members and thus impair growth signals in HER2-dependent tumor cells. Interestingly, in MKN gastric cancer cells, Herceptin did not down-regulate PI3K signaling nor inhibit growth (39). The low-to-undetectable levels of HER3 in these cells led to the suggestion that the collaboration of HER2 with HER3 and its disruption by Herceptin are markers of HER2 dependence and Herceptin sensitivity, respectively (39). This logical speculation requires further investigation in human tumors but is also supported by the data presented above.

We next studied whether or not the inhibition of the PI3K target Akt is required for the antitumor effect of Herceptin. The NH\textsubscript{2}-terminal pleckstrin homology domain of Akt binds PI3K-induced PIP\textsubscript{3} in the plasma membrane, in which Akt is activated by 3-phosphoinositide-dependent kinase 1 (PDK1)-mediated phosphorylation. Once active, Akt phosphorylates an increasing number of substrates involved in apoptosis, cell cycle regulation, protein synthesis, and glycogen metabolism (Refs. 62–65 and Refs. therein). The substrates involved in regulation of cell death that are disabled by Akt include the Bcl-2 family member Bad, Forkhead transcription factors, 14-3-3 kinase, caspase-9, the cyclic AMP response-element binding protein (CREB; Refs. 62–65), and, more recently, p53 via MDM2-mediated phosphorylation and ubiquitination (66, 67). Other targets of Akt could potentially regulate cell cycle progression. Akt phosphorylates and inactivates GSK-3\textbeta, thus stabilizing nuclear \beta-catenin and increasing cyclin D1 transcription (68). By inactivating GSK-3\textbeta, Akt represses GSK-3\textbeta-mediated phosphorylation and the proteolytic turnover of cyclin D1, hence increasing cyclin D1 levels in the nucleus (69). Phosphorylation of the Cdk inhibitor p21\textsuperscript{WAF1} by Akt causes its cytoplasmic retention, preventing it from exerting its antiproliferative action in the nucleus (41). Akt has been shown to induce E2F activity (70) and the transcription of c-Myc (71). In addition, Akt may contribute to the induction of cell cycle progression by regulating the Cdk inhibitor p27. By phosphorylating Forkhead transcription factors, it can inhibit AFX-mediated transcription of p27 (72). Akt can also inhibit p27 protein levels (73). Interestingly, ectopic expression of the 3-phosphoinositide-specific phosphatase PTEN (74) results in the inhibition of Akt, an increase in p27 levels, and growth arrest (75, 76). Conversely, loss of PTEN function, a frequent event in human cancers, leads to derepression of Akt activity, down-regulation of p27, and cellular transformation (77). Finally, recent work has revealed that Akt can phosphorylate p27 directly in Thr-157, which results in its cytoplasmic retention and the loss of its growth-inhibitory effects (personal communications\textsuperscript{5} and Ref. 78).

Consistent with its ability to inhibit Akt function, Herceptin inhibited GSK-3\textbeta phosphorylation and increased p27 levels. As shown recently in BT-474 cells treated with a HER kinase inhibitor (42), the loss of phosphorylation in GSK-3\textbeta activates its catalytic activity against cyclin D1 in vitro, thus potentially explaining the reduction in cyclin D1 induced by Herceptin. All of these effects were abrogated by forced expression of active Akt (Fig. 7A). Treatment with Herceptin also resulted in higher nuclear and cytosolic levels of p27. The increase of p27 in the nucleus would increase the amount of p27 available for binding to and inhibiting Cdk2 and inducing cell cycle arrest (Fig. 5C). These responses were also prevented by Myr-Akt (Fig. 7B). Although the molecular determinants of a direct interaction between Akt and p27 require further investigation, these data suggest that the increase in the nuclear levels of p27 after treatment with Herceptin is attributable to the inactivation of Akt.

Finally, up-regulation of Akt activity by transfection of Myr-Akt prevented Herceptin-induced cytostasis of BT-474 cells and the apoptosis of SKBR-3 cells. LY294002 exhibited inhibitory effects that were similar to those induced by Herceptin, and these effects were also rescued by Myr-Akt (Fig. 8), supporting an important role for PI3K-Akt signaling in the HER2 dependence of these cells. BT-474 tumors in nude mice undergo complete regressions when treated with Herceptin (54, 79), which suggests that they are sensitive to Herceptin-induced apoptosis. However, Herceptin does not induce apoptosis of these cells in culture, impeding our ability to demonstrate a blocking effect of active Akt on Herceptin-induced BT-474 cell death. Nonetheless, the data presented suggest that the inhibition of PI3K-Akt may be an obligated step for the cell cycle arrest and/or apoptosis induced by the HER2 IgG1. These conclusions have important clinical implications for patients treated with Herceptin or other inhibitors of the HER network. Several studies suggest the presence of aberrant PI3K signaling in a cohort of breast carcinomas (reviewed in Ref. 80). Gershtein et al. (81) reported increased levels of p85 and PI3K catalytic activity in paired breast tumor over adjacent nontumor tissues in 79% of the specimens examined. Although mutations of PTEN occur in \textlt;5\% of breast cancers, a recent report suggests that the complete lack of PTEN protein in breast cancers with hemizygous deletions of the PTEN gene is not uncommon (82). Akt kinase activity is often increased in breast cancers with a poor prognosis (83). Akt3 mRNA is up-regulated in estrogen receptor-negative breast tumors (84), linking Akt3 with a more rapidly progressive, hormone-independent breast cancer phenotype. The cytoplasmic protein tyrosine kinases Src and BRK are overexpressed in a high percentage of breast cancers, and both of these kinases up-regulate signaling via PI3K and Akt (85, 86). It is then likely that many HER2-overexpressing tumors will also harbor genetic alterations in the PI3K-Akt signaling pathway and exhibit very high levels of constitutive Akt activity. These tumors may not respond to therapy with HER2 inhibitors alone. This hypothesis can be now be tested prospectively in present clinical trials with inhibitors of the HER signaling network.

\textbf{REFERENCES}


Herceptin-induced Inhibition of Phosphatidylinositol-3 Kinase and Akt Is Required for Antibody-mediated Effects on p27, Cyclin D1, and Antitumor Action

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