Insulin-Like Growth Factor-I Receptor Signaling and Resistance to Trastuzumab (Herceptin)

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Background: Trastuzumab (Herceptin), an anti-HER2/neu receptor monoclonal antibody that inhibits growth of ErbB2-overexpressing breast cancer, is used to treat such cancers. Development of resistance to trastuzumab, however, is common. We investigated the mechanisms of resistance to trastuzumab, and IGF-IR signaling interferes with the growth-inhibitory action of trastuzumab.

Methods: MCF-7/HER2-18 and SKBR3 human breast cancer models were used to assess cell proliferation, colony formation in soft agar, and cell cycle parameters. Throughout, we used trastuzumab at a dose of 10 μg/mL and IGF-I at a dose of 40 ng/mL. All statistical tests were two-sided.

Results: Trastuzumab inhibited the growth of MCF-7/HER2-18 cells, which overexpress HER2/neu receptors and express IGF-I receptors (IGF-IRs), only when IGF-1R signaling was minimized. For example, in 1% fetal bovine serum (FBS), trastuzumab reduced cell proliferation by 42% (P = .002); however, in 10% FBS or IGF-I, trastuzumab had no effect on proliferation. In SKBR3 cells, which overexpress HER2/neu receptor but express few IGF-IRs, trastuzumab reduced proliferation by 42% (P = .008) regardless of IGF-1 concentration. When SKBR3 cells were genetically altered to overexpress IGF-IRs and cultured with IGF-I, trastuzumab had no effect on proliferation. However, the addition of IGF-binding protein-3, which decreased IGF-1R signaling, restored trastuzumab-induced growth inhibition. Conclusions: In breast cancer cell models that overexpress HER2/neu, an increased level of IGF-IR signaling appears to interfere with the action of trastuzumab. Thus, strategies that target IGF-IR signaling may prevent or delay development of resistance to trastuzumab. [J Natl Cancer Inst 2001; 93:1852–7]

The HER2/neu (ErbB2) proto-oncogene encodes a 185-kd transmembrane receptor protein with intrinsic tyrosine kinase activity. No soluble ErbB2 ligand has been identified, but ligand binding to the other members of the ErbB family induces receptor heterodimerization and activation of ErbB2 (1–5). Overexpression of ErbB2 was hypothesized to be associated with an aggressive phenotype, and this association was observed in clinical correlative studies (6,7).

Many methods have been used to therapeutically target HER2 in HER2-overexpressing cancers (8,9), including the use of anti-HER2/neu antibodies (10,11). Preclinical studies (11–13) demonstrated the antiproliferative activity of the murine 4D5 antibody and a humanized version of 4D5, named trastuzumab (Herceptin; Genentech, San Francisco, CA), against HER2/neu-overexpressing breast cancers. Clinical trials (14–17) have shown that trastuzumab has important activity against HER2-positive metastatic breast cancer.

Trastuzumab is often cited as a prototype for rationally designed antineoplastic drugs that target critical signal transduction pathways (8,17,18). However, not all HER2-overexpressing cancer cells respond to treatment with 4D5 or trastuzumab (19), and the clinical benefit of the drug is limited by the fact that most cancers become resistant to trastuzumab therapy in less than 12 months (16,17). The mechanisms of resistance to trastuzumab are poorly understood.

The association between insulin-like growth factor (IGF) physiology and neoplasia is supported by epidemiologic evidence that higher levels of circulating IGF-I are associated with increased risk of several cancers (20–26) and laboratory evidence that IGF signaling pathways are perturbed in neoplastic cells (27–29). IGF-I receptor (IGF-IR) activation stimulates signaling pathways involved in mitogenesis and cell survival (30,31). Therefore, we examined the possibility that IGF-IR signaling interferes with the antiproliferative actions of trastuzumab.

Materials and Methods

Cell Culture and Transfection

SKBR3 human breast cancer cells (from the American Type Culture Collection, Manassas, VA) and MCF-7/HER2-18 cells (32) (provided by Dr. M. Alaini-Jamali, McGill University, Montreal, PQ, Canada) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO2 and 95% air.

A 4.7-kilobase, full-length human IGF-IR complementary DNA was obtained from the PEC/IGF-IR plasmid (33) by EcoRI digestion and was inserted into the pcDNA3.1(+) expression vector (Invitrogen Inc., Carlsbad, CA). SKBR3 cells stably transfected with pcDNA3.1(+)/IGF-IR were generated by calcium phosphate transfection and selected after 3 weeks in G418 at 800 μg/mL. (Life Technologies, Inc. [ Gibco BRL], Burlington, ON, Canada).

Cell Proliferation Assay

We used a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay after confirming high correlation of this end point with a cell number end point in preliminary work. We plated 105 cells in six-well plates in medium containing 10% FBS. After 24 hours, the medium was changed to test medium specific for each experiment. After 72 hours, the MTT assay was done in triplicate: MTT (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 1 mg/mL, the reaction mixture was incubated for 3 hours at 37 °C, and the absorbance was measured at 570 nm. The 95% confidence interval (CI) never exceeded 81% to 119% of the mean for each observation.

Trastuzumab was purchased from the Oncology Pharmacy of the Jewish General Hospital, Montreal, and was used at 10 μg/mL, unless otherwise specified; recombinant human IGF-binding protein-3 (IGFBP-3) and IGF-I were from Progine Incorporated, Mountain View, CA, and were used at 1 μg/mL and 40 ng/mL, respectively, unless otherwise specified. α-IR3, a blocking antibody against the IGF-IR, was from Oncogene Research Products (Boston, MA).

Soft-Agar Assay

Anchorage-independent cell growth was measured in six-well plates. A 1-mL layer of 0.8% agar (Sigma Chemical Co.) in tissue culture medium was
solidified in the bottom of each well. Cells to be assayed were suspended at 37°C in 1 mL of 0.35% agar in tissue culture medium, and then 3 x 10^5 MCF-7/HER2-18 cells, 5 x 10^5 SKBR3 cells, or 5 x 10^5 SKBR3/IGF-IR cells were added per well. Trastuzumab, IGF-I, and IGFBP-3 were then added in 2 mL of medium to the top of the agar, and the medium was changed every 3 days. After about 25 days, all of the colonies were counted under a dissection microscope. Colonies were defined as clusters of 30 or more cells. The 95% CI never exceeded 76% to 124% of the mean for each observation.

Flow Cytometry

The cells were plated in 100-mm dishes at 30% confluence. After 24 hours, the medium was changed to the test medium. After 72 hours, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and fixed in 70% ethanol at −20°C overnight. The cells were washed twice with ice-cold PBS and resuspended in propidium iodide buffer (i.e., PBS [pH 7.4], 0.1% Triton X-100, 0.1 mM EDTA, ribonuclease A [0.05 mg/mL], and propidium iodide [50 µg/mL]). After 30 minutes at room temperature, the cell cycle distribution was determined by flow cytometry with a FACSscan (Beckman-Coulter, Fullerton, CA). Duplicate experiments yielded similar results.

Western Blots

After each treatment, the cells were lysed in RIPA buffer (0.1 mM diethylamine phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and aprotinin at 0.2 µg/mL). Protein from clarified lysates (20–60 µg) was resolved electrophoretically on de-naturing SDS–polyacrylamide gels (8%–12%), transferred to nitrocellulose membranes, and probed with the following primary antibodies: anti-Cdk2 (where Cdk is cyclin-dependent kinase), anti- Cdk6, anti-cyclin E, and anti-IGF-IR (Santa Cruz Biotechnology [Santa Cruz, CA] and anti-p21^{Cip1}, anti-Cdk4, and anti-p27^{Kip1} from Neomarkers, Inc. [Fremont, CA]). The position of proteins was visualized with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies.

Immunoprecipitation

To assess the effects of IGF-I and recombinant human IGFBP-3 on IGF-IR activation in SKBR3 and SKBR3/IGF-IR cells, we washed cultures that were 70%–80% confluent twice and then added serum-free medium for 24 hours. Fifteen minutes before harvesting, the cultures were treated with saline vehicle, IGFBP-3 at 1 µg/mL, and/or IGF-I at 40 ng/mL at 37°C. Monolayers were quickly washed twice with ice-cold PBS and lysed with 0.4 mL of lysis buffer (i.e., 10 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA [i.e., ethylene glycol bis[aminohexyl]aminoethyl] N,N',N''-tetraacetic acid], 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, and aprotinin at 0.2 µg/mL). Phosphotyrosine levels in IGF-IR were measured in protein extracts (300 µg) by use of anti-IGF-IR and a specific anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY).

For Cdk2-p27^{Kip1} and Cdk2-p21^{Cip1} immuno-precipitation experiments, clarified protein lysates (200 µg/mL) were precleared with 25 µL of protein A-Sepharose (Santa Cruz Biotechnology) and precipitated with 2 µg of anti-Cdk2 antibody (Santa Cruz Biotechnology) and 25 µL of protein A-Sepharose overnight at 4°C. Cdk2-p27^{Kip1} and Cdk2-p21^{Cip1} complexes were detected by immunoblotting.

Statistical Analysis

All of the data are shown as means and 95% CIs, unless otherwise specified. To assess the statistical significance of observed differences, we used Student’s t test. All statistical tests were two-sided, and P values less than .05 were considered to be statistically significant.

RESULTS

Effects of IGF-I and Trastuzumab on MCF-7/HER2-18 Cells

MCF-7/HER2-18 cells are MCF-7 human breast cancer cells that overexpress HER2/neu (32). We confirmed the observation (32) that the growth of neither MCF-7 nor MCF-7/HER2-18 cells in 10% FBS is inhibited by trastuzumab (data not shown). The growth of MCF-7/HER2-18 cells, however, was inhibited by trastuzumab when the FBS concentration was reduced (Fig. 1, A), suggesting that a component of FBS blocks the antiproliferative effect of trastuzumab. IGF-I (40 ng/mL) reduced trastuzumab-induced growth inhibition to the same extent as 10% FBS (Fig. 1, B).

MCF-7 cells express IGF-IRs, and their growth is inhibited by the anti-IGF-IR antibody α-IR3 or the IGF-binding protein IGFBP-3, both of which interfere with ligand–receptor interactions (34, 35). MCF-7/HER2-18 cells were responsive to IGF-I, and the modest trastuzumab-induced growth inhibition observed with 5% FBS was enhanced by α-IR3 or IGFBP-3 (Fig. 1, C). In 5% FBS, trastuzumab inhibited proliferation to 83% of control (P = .04), whereas trastuzumab plus α-IR3 at 0.5 µg/mL or trastuzumab plus IGFBP-3 at 1 µg/mL inhibited proliferation to 50% and 47% of control, respectively (P < .001 in each case). Dose–response data for IGFBP-3 effects on proliferation were shown in Fig. 1, D. When anchorage-independent MCF-7/HTR2-18 cell growth was assayed in soft agar with 10% FBS, trastuzumab alone did not inhibit colony formation, IGFBP-3 (1 µg/mL) alone reduced colony formation by 29%, and the combination reduced colony formation by 82%, which was statistically significantly greater (P < .001) than growth inhibition with either agent alone (data not shown).

Overexpression of IGF-IR and Trastuzumab-Induced Growth Inhibition of SKBR3 Cells

SKBR3 human breast cancer cells overexpress HER2/neu but have less than 10% the number of IGF-IRs in MCF-7/HER2-18 cells. The IGF-IR-transfected SKBR3 clone SKBR3/IGF-IR has about sevenfold more IGF-IRs than MCF-7/HER2-18 cells. Mock-transfected SKBR3 neo cells and SKBR3 cells have approximately the same number of IGF-IRs, as estimated by densitometric scanning of western blots (data not shown).

In contrast to the findings with MCF-7/HER2-18 cells, trastuzumab inhibits SKBR3/neo cell proliferation in the presence or absence of FBS (11). Maximal trastuzumab-induced growth inhibition of SKBR3/neo cells was 42% (Fig. 2, A). The proliferation rate of SKBR3/IGF-IR cells was 125% that of SKBR3/neo cells, and trastuzumab reduced their proliferation by only 20% (Fig. 2, A). In soft-agar assays (Fig. 2, B), trastuzumab reduced SKBR3 colony formation by 62%, but it reduced SKBR3/IGF-IR colony formation by only 12% (Fig. 2, B). Trastuzumab was a statistically significantly better inhibitor of SKBR3/neo cells than of SKBR3/IGF-IR cells under both anchored (P = .02) and anchorage-independent (P = .004) conditions.

In the presence of IGF-I, SKBR3/neo cells but not SKBR3/IGF-IR cells were growth inhibited by trastuzumab. Trastuzumab statistically significantly reduced (P = .004) the proliferation of SKBR3/neo cells but did not affect (P = .92) the proliferation of SKBR3/IGF-IR cells (Fig. 2, C). Addition of IGFBP-3 restored or even enhanced the trastuzumab-induced growth inhibition of SKBR3/IGF-IR cells in the presence of IGF-I (Fig. 2, C). In SKBR3/IGF-IR cells cultured with IGF-I and trastuzumab, where trastuzumab had no growth-inhibitory effect on cell proliferation, immunoprecipitation experiments detected the highest level of IGF-IR phosphorylation (i.e., activation); addition of IGFBP-3 to these cultures reduced IGF-IR phosphorylation by 75% (data not shown).
Overexpression of IGF-IR and Trastuzumab-Induced G₁-Phase Arrest

An increased proportion of cells in G₁ phase is associated with the reduced rate of cell proliferation induced by trastuzumab or antibody 4D5 (19,36). Flow cytometry revealed that trastuzumab increased the percentage of SKBR3/IGF-IR cells and SKBR3/neo cells in G₁ phase, but the increase was less in SKBR3/IGF-IR cells (Fig. 2, D). In SKBR3/IGF-IR cells, this increase was blocked completely by IGF-I and restored by IGFBP-3.
Modification of Downstream Effects of Trastuzumab by IGF-I

Anti-HER2/neu antibodies induce the Cdk inhibitor p27^Kip1, which associates with Cdk2 and contributes to growth inhibition (36). By using western blots, we investigated the effect of IGF-IR signaling on Cdk inhibitors. Baseline levels of p27^Kip1 and p21^Cip1 were substantially lower in SKBR3/IGF-IR cells than in SKBR3/neo cells. Trastuzumab induced p27^Kip1 expression in SKBR3/neo cells but not in SKBR3/IGF-IR cells. An increased amount of Cdk2-associated p27^Kip1 was observed in trastuzumab-treated SKBR3/neo cells but not in trastuzumab-treated SKBR3/IGF-IR cells (data not shown).
Early- and mid-G1 cyclins were reported previously to be inhibited by anti-HER2 antibodies (36). We observed that cyclin E levels were higher in SKBR3/IGF-IR cells than in SKBR3/neu control cells. Although trastuzumab reduced cyclin E levels in both lines, the reduction was greater in SKBR3/neu cells. IGF-I reversed this reduction in SKBR3/IGF-IR cells (data not shown).

**DISCUSSION**

The common occurrence of clinical resistance to trastuzumab suggests either that many molecular alterations can confer such resistance or that a few mechanisms conferring such resistance arise frequently. In the latter case, there would be a particularly strong rationale for determining whether targeting both HER2/neu and a pathway commonly implicated in resistance would be clinically beneficial. Our results suggest that molecular alterations resulting in enhanced activation of IGF-I and/or pathways distal to IGF-IR represent one family of mechanisms of resistance to trastuzumab.

Specific molecular lesions that might confer such resistance to trastuzumab include overexpression of IGF-I or IGF-II, underexpression of growth-inhibitory IGFBPs, overexpression of IGFBP proteases, or reduced activity of intracellular phosphatases, such as PTEN, that normally limit IGF-I signaling. Although these alterations have been described in breast cancer, it has not been possible to observe sequential changes in gene expression as trastuzumab-sensitive cancers become resistant to trastuzumab, because women are not generally subjected to repeat biopsy after trastuzumab resistance has been observed clinically.

IGF-IR signaling has not been reported previously to confer resistance to trastuzumab-induced growth inhibition. This mechanism is plausible, however, because IGF-I and trastuzumab affect downstream events, such as cyclin E expression, in opposite directions (9,36–38). Given recent insights into signaling networks (39–45), it is likely that IGF-I and trastuzumab perturb these networks in opposite directions, rather than acting independently on two linear signaling pathways that intersect at one node.

Our results suggest that targeting several signal transduction pathways simultaneously may lead to more effective control of neoplastic growth than targeting a single pathway and add to the evidence (25–29) that the IGF-IR pathway is an attractive target. However, we urge the establishment of banks of sequential tumor biopsy specimens to determine if changes in signal transduction networks (particularly IGF-IR signaling) associated with the development of resistance to trastuzumab or to other drugs in patients correlate with findings in laboratory models.

**REFERENCES**


NOTES

Editor’s note: Representatives of Protigen Incorporated reviewed and approved this report. D. Mascarenhas is the chief executive officer of Protigen Incorporated, which supplied the insulin-like growth factor-binding protein-3 for this research.

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