Therapeutic Discovery

Trastuzumab Regulates IGFBP-2 and IGFBP-3 to Mediate Growth Inhibition: Implications for the Development of Predictive Biomarkers for Trastuzumab Resistance

Milos Dokmanovic, Yi Shen, Tabetha M. Bonacci, Dianne S. Hirsch, and Wen Jin Wu

Abstract
Activation of insulin-like growth factor-I receptor (IGF-IR) signaling is an important mechanism for trastuzumab resistance. IGF-binding proteins (IGFBP) modulate IGF-IR signaling and play important roles in the control of breast cancer progression. In this article, we report that trastuzumab treatment enhances the expression and secretion of IGFBP-3 in SKBR3 cells, a trastuzumab-sensitive breast cancer cell line, and that this upregulation of IGFBP-3 induced by trastuzumab correlates with trastuzumab-mediated growth inhibition. We describe a new role for IGFBP-3 in the regulation of IGF-I–mediated cross-talk between IGF-IR and ErbB2 signaling pathways. In particular, treatment of SKBR3 cells with recombinant IGFBP-3 blocks IGF-I–induced activation of IGF-IR and ErbB2, and stable expression of IGFBP-3 inhibits SKBR3 cell growth. We find an inverse relationship in the levels of secreted IGFBP-3 such that high levels of IGFBP-3 are associated with trastuzumab-sensitive breast cancer cells (SKBR3 and BT-474), whereas low levels of IGFBP-3 are found in trastuzumab-resistant cells (clone 3 and JIMT-1). In contrast to IGFBP-3, the secretion and expression of IGFBP-2 are upregulated in trastuzumab-resistant SKBR3 cells. Furthermore, we show that IGFBP-2 stimulates activation of ErbB2 and that trastuzumab reduces IGFBP-2–stimulated ErbB2 activation. Based on our data, we propose a novel mechanism of action whereby trastuzumab enhances the expression and secretion of IGFBP-3, which interferes with IGF-I–mediated mitogenic signaling via autocrine and paracrine mechanisms and reduces IGFBP-2–induced ErbB2 activation to mediate growth inhibition. Changes in secretion profiles of IGFBP-2 and IGFBP-3 in trastuzumab-sensitive and trastuzumab-resistant cells may promote the development of IGFBP-2 and IGFBP-3 as predictive biomarkers for trastuzumab resistance.

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Introduction
Erythroblastosis protein B2 (ErbB2)/HER2, a member of ErbB receptor tyrosine kinases, is overexpressed in approximately one third of breast cancers, and ErbB2 overexpression is associated with breast cancer progression, metastasis, poor prognosis, and poor response to therapy (1, 2). Trastuzumab, a humanized monoclonal antibody, targets the extracellular domain of ErbB2 and has been approved for the treatment of ErbB2-positive breast cancers. Multiple mechanisms of action may contribute to the efficacy of trastuzumab, including (i) trastuzumab-mediated ErbB2 internalization and degradation; (ii) interruption of ErbB2 dimerization with other ErbB family receptors; (iii) disruption of downstream signaling pathways, including phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways; (iv) induction of G1 cell-cycle arrest and apoptosis; (v) induction of antibody-dependent cellular cytoxicity; and (vi) inhibition of ErbB2 extracellular domain proteolysis (1, 2).

Treatment with trastuzumab improves outcomes for women with aggressive ErbB2-positive breast cancer. However, the majority of patients who achieve an initial response to trastuzumab acquire resistance to trastuzumab within 1 year (2–4). Ritter and colleagues reported that, in a xenograft mouse model, members of the ErbB receptor family and their ligands participated in trastuzumab resistance (5). Using the clone 3 cell line, an SKBR3 derivative and trastuzumab-resistant cell line, we identified that upregulated Rac1 activity impairs trastuzumab-induced ErbB2 internalization and degradation and contributes to trastuzumab resistance (6).
Furthermore, we found that the ErbB2 activity is enhanced in clone 3 cells as compared with SKBR3 cells. However, the mechanism leading to the upregulation of ErbB2 activity in clone 3 cells remains elusive (6).

Evidence is accumulating that the activation of insulin-like growth factor-I receptor (IGF-IR) contributes to trastuzumab resistance. Overexpression of IGF-IR followed by treatment with IGF-I in trastuzumab-resistant SKBR3 cells diminishes the growth-inhibitory effects of trastuzumab (7, 8). Inhibition of IGF-IR tyrosine kinase activity by the small molecule I-Ome-AG538 restores trastuzumab sensitivity in trastuzumab-resistant SKBR3 cells, and the IGF-IR antagonist NVP-AEW541 synergizes with trastuzumab to inhibit breast cancer cell growth (9, 10). Studies with 2 trastuzumab-sensitive cell lines, SKBR3 and BT-474, and their derived resistant sublines (pool 2 and HR20, respectively) revealed that ErbB2, ErbB3, and IGF-IR form a unique heterotrimer in trastuzumab-resistant sublines and that selective knockout of IGF-IR in these cell lines sensitized them to trastuzumab-mediated growth inhibition (11). Taken together, these studies suggest that modulation of IGF-IR-mediated signaling may be an important mechanism of action of trastuzumab, as well as of trastuzumab resistance.

The insulin-like growth factor binding protein (IGFBP) family is composed of 6 cysteine-rich secreted proteins (IGFBP-1 to IGFBP-6) that regulate IGF-mediated signaling (12). They play a key role in regulating proliferation, differentiation, and apoptosis in different organ systems including the human mammary gland. The principal mechanism of action of IGFBP5 is believed to involve binding to and influencing the actions of IGFs, although IGFBPs may also affect cellular response through an IGF-independent pathway (13). IGFBP5 may prevent interaction between IGF and IGF-IR or, when anchored to the extracellular matrix or the cell surface, act as a reservoir where they may localize and release IGFs to cell-surface receptors, thereby enhancing IGF actions (13).

Different members of the IGFBP family have important roles in the control of breast cancer cell growth and survival. IGFBP-3, the most abundant circulating IGFBP, mainly elicits antiproliferative and proapoptotic effects in breast cancer cells (14–17). Based on cDNA microarray analysis, IGFBP-3 is one of the most significantly down-regulated genes associated with ErbB2 overexpression (18). In a xenograft model, combining IGFBP-3 and trastuzumab treatments resulted in a synergistic reduction in trastuzumab-resistant tumor growth when compared with trastuzumab treatment alone (19). In addition, IGFBP-3 expression is induced by different anticancer therapeutics in breast cancer cells (20–22). In contrast to IGFBP-3, IGFBP-2 is often highly expressed in malignant tumors, but its expression decreases upon remission (23, 24). Increased IGFBP-2 levels in patient sera often correlate with tumor malignancy (25–27). In addition, IGFBP-2 expression is increased in breast cancer tissue as compared with benign tissue (28), and IGFBP-2 overexpression in breast cancer has also been proposed as a marker of antiestrogen resistance (29). In the MDA-MB-231 breast cancer cell line, overexpression of IGFBP-2 conferred a growth advantage and chemoresistance (28). At a signal transduction level, recent studies implicate relationships between IGFBP-2 expression and the PI3K/Akt signaling pathway (30, 31). However, molecular mechanisms by which IGFBP-2 contributes to cancer progression are incompletely defined.

In this article, we report a previously unappreciated relationship between IGFBP-3 and -2 and trastuzumab-mediated growth inhibition and trastuzumab resistance. Specifically, trastuzumab treatment markedly enhanced expression and secretion of IGFBP-3 in trastuzumab-sensitive SKBR3 cells, and this enhancement in IGFBP-3 expression and secretion correlated with trastuzumab-mediated growth inhibition. In addition, we show that overexpression of IGFBP-3 reduces cell proliferation of trastuzumab-sensitive SKBR3 cells as well as trastuzumab-resistant clone 3 cells. In contrast to IGFBP-3, we showed that IGFBP-2 stimulated activation of ErbB2 and that, in trastuzumab-sensitive cells, this activation was inhibited by trastuzumab treatment. We propose the ability of trastuzumab to enhance IGFBP-3 expression and secretion and to block IGFBP-2-induced activation of ErbB2 as novel mechanisms of action for trastuzumab-mediated growth inhibition. Our data suggested an inverse relationship in the levels of secreted IGFBP-3 between trastuzumab-sensitive and trastuzumab-resistant breast cancer cells, such that high levels of IGFBP-3 were associated with trastuzumab-sensitive cells (SKBR3 and BT-474), whereas low levels of IGFBP-3 were found in trastuzumab-resistant cells (clone 3 and JIMT-1). Furthermore, we found that the increased expression and secretion of IGFBP-2 positively correlated with trastuzumab resistance in SKBR3 cells. Although activating mutations in KRAS are used as a predictive biomarker for therapeutic resistance to cetuximab, a monoclonal antibody directed against ErbB1/epidermal growth factor receptor (EGFR) that is approved for the treatment of colorectal cancers, no predictive biomarkers are currently used in the clinical setting to differentiate between ErbB2-positive breast cancers that are trastuzumab-sensitive and those that would likely be resistant to trastuzumab treatment (32, 33). The findings described in this article regarding changes in secretion profiles of IGFBP-2 and IGFBP-3 in trastuzumab-sensitive and trastuzumab-resistant cells may provide important information for the development of predictive biomarkers for therapeutic resistance to trastuzumab.

Materials and Methods

Antibodies and reagents

Antibodies against ErbB2 (29D8), phospho-ErbB2 (p-ErbB2; Tyr 877, Tyr 1221/1222), Erk1/2 (L34F12), phospho-Erk1/2 (p-Erk1/2; Thr 202/Tyr 204), Akt (11E7), phospho-Akt (p-Akt; Ser 473, Thr 308), phospho-IGF-IR (p-IGF-IR; Tyr 1131), and IGF-IR were obtained from Cell Signaling Technology. Antibody against actin was
obtained from Sigma-Aldrich. Antibodies against IGFBP-2 and IGFBP-3 were purchased from Santa Cruz Biotechnology. Antiphosphotyrosine (4G10) antibody was obtained from Millipore. Trastuzumab (Genentech, Inc.) was purchased from the pharmacy at the NIH, Bethesda, MD. Recombinant human IGF-I, IGFBP-2, and IGFBP-3 were purchased from Sigma-Aldrich and were resuspended and stored according to the manufacturer’s instructions. Recombinant human epidermal growth factor (EGF) was obtained from Invitrogen. A pCMV6-AC-GFP vector encoding green fluorescent protein (GFP)-tagged IGFBP-3 and an empty vector were obtained from Origene. ELISA kits for the detection of human IGFBP-2 and IGFBP-3 in cell culture media were obtained from RayBiotech, Inc.

**Cell culture**

SKBR3 cells were obtained from the American Type Culture Collection (ATCC). Clone 3 cells, which are an SKBR3-derived clonal cell line that is trastuzumab-resistant, were a kind gift of Dr. Esteva of the M.D. Anderson Cancer Center, Houston, TX (6, 34). The standard culture media for SKBR3 cells was Dulbecco’s modified Eagle’s medium (DMEM)/F12 supplemented with 10% FBS, whereas the standard culture media for clone 3 cells was DMEM/F12 supplemented with 10% FBS and 4 mg/mL trastuzumab. The JIMT-1 cell line was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Brunswick, Germany) and has been described by Tanner and colleagues (35). The standard media for JIMT-1 cells is DMEM supplemented with 10% FBS. BT-474 cells were purchased from ATCC and were maintained in RPMI-1640 supplemented with 10% FBS. Cells were cultured in the standard culture media unless otherwise indicated.

**Generation of stable cell lines**

After electroporation of SKBR3 cells with pCMV6-AC plasmid encoding either GFP-tagged IGFBP-3 or GFP alone, the stable cell line selection was achieved using G418 as previously described (36–38).

**ELISA**

Detection of IGFBP-2 and IGFBP-3 in cell culture media was done according to the manufacturer’s instructions (RayBiotech, Inc).

**Statistical analyses**

Statistical analysis was done using GraphPad Prism 5.0. Descriptions of the specific analyses are provided in the figure legends.

**Cell-growth assays**

Cell-growth assays were conducted as previously described (39). Briefly, cells were plated in 12-well plates and cultured in the standard culture media. Trastuzumab-sensitive cells were either treated with trastuzumab (4 μg/mL) or left untreated. At the indicated times, cells were trypsinized and counted. For IGFBP-3 transient overexpression studies in clone 3 cells, the cells were transfected with plasmid encoding either GFP (control) or GFP-tagged IGFBP-3 for 48 hours using Lipofectamine and PLUS Reagent (both from Invitrogen) according to the manufacturer’s instructions. Cells were then harvested and counted at the indicated time points, and the whole-cell lysates (WCL) and cell culture media were analyzed for the levels of IGFBP-3.

**Results**

**Trastuzumab-induced IGFBP-3 secretion correlates with trastuzumab-mediated growth inhibition**

In a mouse model, addition of recombinant IGFBP-3 has been shown to inhibit growth of ErbB2-positive breast tumors as well as to sensitize the tumor’s response to trastuzumab (19). To obtain a better understanding of molecular mechanisms by which IGFBP-3 coordinates with trastuzumab to mediate growth inhibition of breast cancer cells, we analyzed IGFBP-3 secretion in SKBR3 and clone 3 cells (an SKBR3 derivative that is trastuzumab-resistant). SKBR3 cells were treated with trastuzumab for 3 or 6 days or left untreated. Clone 3 cells were maintained in their standard culture media containing 4 μg/mL trastuzumab. The cell culture media were harvested at the indicated times and then IGFBP-3 levels were determined by ELISA. As shown in Fig. 1A, secreted IGFBP-3 was slightly increased from day 3 to day 6 in SKBR3 cells that were not treated with trastuzumab. This increase in secreted IGFBP-3 in the media was most likely due to the increase in cell numbers (Fig. 1B). However, an increase in the secretion of IGFBP-3 (∼7-fold) in media was observed from day 3 to day 6 when SKBR3 cells were treated with trastuzumab. Furthermore, the increase in secretion of IGFBP-3 correlated with trastuzumab-mediated growth inhibition, such that SKBR3 cells treated with trastuzumab exhibited an approximately 3-fold decrease in cell numbers at day 6 as compared with untreated cells (Fig. 1B). In addition, analysis of the secreted levels of IGFBP-3 in clone 3 cells at days 3 and 6 indicated that very low concentrations of IGFBP-3 were detected in the media in spite of the increase in cell numbers from day 3 to day 6 (Fig. 1A and B). The relative IGFBP-3 was derived by dividing the total IGFBP-3 in cell culture media at day 3 and day 6 by the fold increase in cell number at the indicated time points (Fig. 1C).

**IGFBP-3 specifically inhibits IGF-I-induced tyrosine phosphorylation of IGF-IR and ErbB2**

We next addressed the question of whether secreted IGFBP-3 interfered with IGF-I-mediated signaling. As expected, addition of recombinant human IGF-I led to tyrosine phosphorylation of IGF-IR at Tyr 1131 (Fig. 2A). Consistent with another report (19), pretreatment with recombinant human IGFBP-3 effectively blocked IGF-I-induced tyrosine phosphorylation of IGF-IR (Fig. 2A). After the pretreatment of cells with IGFBP-3, we found...
that IGF-I–induced phosphorylation of Erk1/2 was abrogated (Fig. 2B). Moreover, phosphorylation of Akt at both T308 and S473 residues was enhanced when cells were treated with IGF-I. However, this IGF-I–induced phosphorylation of Akt at both T308 and S473 residues was not observed when cells were pretreated with IGFBP-3 (Fig. 2B). Cross-talk between IGF-IR and ErbB2 occurs in SKBR3 cells (9, 40). We examined whether IGF-I was able to activate ErbB2 in SKBR3 cells. As shown in Fig. 2C (left panels), we found that IGF-I induced tyrosine phosphorylation of ErbB2 at both Y877 and Y1221 residues in a time-dependent manner. More importantly, IGF-I–induced increases in ErbB2 phosphorylation at Y877 and Y1221 were not observed when cells were pretreated with IGFBP-3 (Fig. 2C; right panels). In addition, the effects of IGFBP-3 were specific to the IGF-I–mediated activation of ErbB2, as addition of IGFBP-3 could not block EGF-induced ErbB2, Erk, and Akt phosphorylation (Fig. 2D). These data, together with Fig. 1, support the idea that the increase in IGFBP-3 secretion at the onset of trastuzumab-induced growth arrest in SKBR3 cells may act, via paracrine and/or autocrine mechanisms, as a tumor-suppressive signal, which may potentiate the growth-inhibitory effects of trastuzumab by counteracting the IGF-I–mediated activation of both IGF-IR and ErbB2 signaling pathways in SKBR3 cells.

Trastuzumab enhances expression of endogenous IGFBP-3 in SKBR3 cells, and overexpression of IGFBP-3 inhibits SKBR3 and clone 3 cell growth

Next, we asked whether trastuzumab enhanced the expression of IGFBP-3 in SKBR3 cells. The cells were treated with trastuzumab or left untreated for the indicated times, and IGFBP-3 in WCLs was detected by Western blot analysis. As shown in Fig. 3A, the expression of endogenous IGFBP-3 was increased after SKBR3 cells were treated with trastuzumab (4 μg/mL) or left untreated and of clone 3 cells grown in their standard media, which contains 4 μg/mL trastuzumab. A total of 1 x 10^6 cells were plated in 10-cm dishes and cultured in the standard cell culture media (see Materials and Methods). Media without cells were used for blank readings. At the indicated time points, cell culture media were collected. Following centrifugation to remove cell debris, the media were concentrated 10-fold using centrifugal filter units (Millipore). IGFBP-3 levels were determined by ELISA. For ELISA, each sample was run in duplicates, and the standard curve was established according to the manufacturer’s instructions. Columns, mean of 3 independent experiments; bars, SD. *, P < 0.01; **, P < 0.001 as determined by 2-way ANOVA followed by Bonferroni posttests. B, growth profiles for clone 3 cells and SKBR3 cells grown as described in A. Cells were plated at a density of 5 x 10^4 cells per well of a 12-well plate in triplicate. At the indicated time points, cells were trypsinized and counted by trypan blue exclusion using a hemocytometer. Data represent 3 independent experiments. Points, mean of triplicates; bars, SD. C, the relative IGFBP-3 was derived from A and B. The total IGFBP-3 in cell culture media at day 3 and day 6 was obtained by multiplying the concentration of IGFBP-3 in media (pg/mL) shown in A by the total volume of the cell culture media (10 mL). The fold increase in cell number was the ratio of the number of cells at the indicated time points (days 3 and 6) to the number of cells at day 0. The relative IGFBP-3 was normalized by dividing the total IGFBP-3 in cell culture media by the fold of increase in cell number at the indicated time points.
treated with trastuzumab for 3 or 6 days. The increase in IGFBP-3 protein expression induced by trastuzumab parallels changes in the trastuzumab-induced IGFBP-3 secretion in SKBR3 cells (Fig. 1A and B). When IGFBP-3 expression in WCLs of clone 3 cells was examined, we found that IGFBP-3 protein levels were reduced compared with that in SKBR3 cells, consistent with the data shown in Fig. 1A. We then examined the effects of IGFBP-3 on SKBR3 and clone 3 cell proliferation. Stable expression of GFP-tagged IGFBP-3 in SKBR3 cells reduced their growth 3-fold by day 7 as compared with SKBR3 cells stably expressing empty vector (Fig. 3B). Based on data shown in Figs. 1–3, we propose a model whereby trastuzumab-induced IGFBP-3 expression and secretion is an additional mechanism of action for trastuzumab-induced growth inhibition. In addition, the escape from trastuzumab-induced IGFBP-3 expression and secretion, as is the case for clone 3 cells, may contribute to trastuzumab resistance. To address the question of whether the expression of exogenous IGFBP-3 affects clone 3 cell growth, cells were transiently transfected with a plasmid encoding either GFP vector control or GFP-tagged IGFBP-3. Figure 3C (Western blot) showed IGFBP-3-GFP expression in Clone 3 cells 24 hours posttransfection, whereas Fig. 3C (graph) showed that the amounts of IGFBP-3 in cell culture media collected at day 1 and day 3 were increased in clone 3 cells transiently expressing IGFBP-3-GFP as compared with that in GFP vector control cells. These data provided additional line of evidence to show the transient expression of IGFBP-3-GFP in Clone 3 cells. Figure 3D shows that expression of exogenous IGFBP-3 reduced clone 3 cell growth, particularly at earlier time points (days 1 and 3), consistent with the inhibitory effect of IGFBP-3 on SKBR3 cells (Fig. 3B).

Levels of secreted IGFBP-3 in BT-474 and JIMT-1 breast cancer cells are associated with their sensitivity to trastuzumab

BT-474 and JIMT-1 are 2 breast cancer lines often used to study the mechanisms of trastuzumab resistance. Although both cell lines overexpress ErbB2, BT-474...
cells are sensitive to trastuzumab whereas JIMT-1 cells are resistant to trastuzumab (11, 35). We, therefore, questioned whether there was a difference in the IGFBP-3 secretion in these 2 cell lines. To determine the levels of secreted IGFBP-3, BT-474 and JIMT-1 cells were seeded at equal numbers, and the cell culture media were harvested at the indicated time points. As shown in Fig. 4, the levels of secreted IGFBP-3 were lower in JIMT-1 cells than in BT-474 cells (Fig. 4A), although JIMT-1 cells replicated faster than BT-474 cells from day 2 to day 4 (Fig. 4B). Taken together, our data revealed an inverse relationship in the levels of secreted IGFBP-3 between

Figure 3. Trastuzumab treatment increases endogenous IGFBP-3 levels in SKBR3 cells and overexpression of IGFBP-3 inhibits SKBR3 and clone 3 cell growth. A, SKBR3 and clone 3 cells were cultured in the standard cell culture media. SKBR3 cells were then treated with trastuzumab (4 μg/mL) or left untreated for the indicated time. The standard cell culture media for clone 3 cells always contains trastuzumab at a concentration of 4 μg/mL (see Material and Methods). Cells were harvested at the indicated time points and the levels of IGFBP-3 in WCLs were detected by Western blot analysis. B, SKBR3 cells stably expressing empty vector (control) or GFP-tagged IGFBP-3 (IGFBP-3-Clone 1 and IGFBP-3-Clone 2) were seeded in triplicate at a density of 15,000 cells per well on a 12-well plate. At the indicated time points, cells were trypsinized and counted. Points, mean of 2 independent experiments in triplicate; bars, SD. *, P < 0.001 as determined by 2-way ANOVA followed by Bonferroni posttests. Western blot, levels of stable expression of GFP-IGFBP-3 in each clone were determined using antibody directed against IGFBP-3. C, clone 3 cells were seeded at a density of 5 × 10^4 cells per well on a 12-well plate in triplicate and cultured in the standard culture media. Cells were then transfected with the plasmid encoding either GFP empty vector (control) or GFP-tagged IGFBP-3. WCLs were harvested 24 hours posttransfection (day 1) and analyzed with Western blot for the overexpression of GFP-tagged IGFBP-3. Cell culture media were harvested at days 1 and 3 and analyzed by ELISA for the secreted IGFBP-3 in the cell culture media. D, growth profiles of clone 3 GFP control cells and clone 3 cells expressing IGFBP-3-GFP. Points, mean of 3 independent experiments run in triplicate. Bar, SD. *, P < 0.05; **, P < 0.001 as determined by 2-way ANOVA followed by Bonferroni posttests.
trastuzumab-sensitive and -resistant breast cancer cells, such that the levels of secreted IGFBP-3 were decreased in trastuzumab-resistant cells (clone 3 and JIMT-1 cells) and increased in trastuzumab-sensitive cells (SKBR3 and BT-474 cells). It should be noted that IGFBP-3 secretion was not enhanced in BT-474 cells treated with trastuzumab (data not shown). We hypothesize that this may be due to the high levels of IGFBP-3 in BT-474 cells creating a negative feedback loop that prevents trastuzumab from inducing IGFBP-3 secretion. Nonetheless, data shown in Fig. 4 are consistent with the results obtained in SKBR3 and clone 3 cells that IGFBP-3 may potentiate trastuzumab-mediated growth inhibition. Downregulation of IGFBP-3 may render cells resistant to trastuzumab treatment.

Increases in IGFBP-2 expression and secretion are associated with trastuzumab resistance in SKBR3 cells

Overexpression of IGFBP-2 has been reported to contribute to resistance to chemotherapy and antiestrogen treatment in breast cancers (28, 29). We set out to investigate whether IGFBP-2 was also involved in trastuzumab resistance. The cell culture media used for determining the levels of secreted IGFBP-3 described in Fig. 1 were also assayed for secreted IGFBP-2. As shown in Fig. 5A, IGFBP-2 levels detected in the cell culture media of clone 3 cells were more than 4-fold greater than IGFBP-2 levels detected in the culture media of SKBR3 cells. Moreover, the difference in levels of secreted IGFBP-2 between SKBR3 and clone 3 cells was due to differences in cell-growth rate as the SKBR3 and clone 3 cell numbers were comparable (Fig. 1B). A modest decrease in IGFBP-2 secretion by SKBR3 cells treated with trastuzumab was found at both day 3 and day 6 as compared with untreated SKBR3 cells (Fig. 5A). This may be attributable to the decrease in SKBR3 cell numbers after trastuzumab treatment (Fig. 1B). The relative IGFBP-2 was normalized by dividing the total IGFBP-2 in the cell culture media at day 3 and day 6 by the fold increase in cell number at the indicated time points (Fig. 5B). As shown in Fig. 5C, protein levels of endogenous IGFBP-2 were elevated in clone 3 cells as compared with SKBR3 cells. Interestingly, we also found an increase in IGFBP-2 expression after the SKBR3 cells were treated with trastuzumab for 3 or 6 days (Fig. 5C). This suggests that expression of IGFBP-2 may be a survival mechanism used by SKBR3 cells to counteract the growth inhibition mediated by trastuzumab. Chronic exposure of SKBR3 cells to trastuzumab, as is the case for clone 3 cells, increases IGFBP-2 expression and secretion, and this may override trastuzumab-induced growth arrest, and contribute to trastuzumab resistance.

IGFBP-2 stimulates the activation of ErbB2-coupled signaling in SKBR3 cells

IGFBP-2 functions as an oncoprotein to stimulate breast cancer cell growth (13, 15, 28). High levels of IGFBP-2 in clone 3 cells prompted the question as to whether the increased IGFBP-2 secretion contributes to the upregulated ErbB2 phosphorylation in clone 3 cells, which we reported previously (6). As shown in Fig. 6A, tyrosine phosphorylation of ErbB2 was increased in a time-dependent manner following IGFBP-2 treatment of serum-starved SKBR3 cells. IGFBP-2-induced ErbB2 tyrosine phosphorylation was also dose-dependent (Fig. 6A). We next examined whether downstream effectors of ErbB2 were affected by IGFBP-2 stimulation. As shown in Fig. 6B, IGFBP-2 treatment stimulated activation of Erk and Akt, both of which, when aberrantly upregulated, have been reported to contribute to trastuzumab resistance. Based on these findings, we propose that increased IGFBP-2 expression and secretion contributes to trastuzumab resistance by counteracting trastuzumab-induced
growth inhibition through attenuation of ErbB2-coupled signaling.

**Trastuzumab interferes with IGFBP-2–mediated activation of ErbB2 signaling**

Elevation of IGFBP-2 expression was observed within a week when SKBR3 cells were incubated with trastuzumab (Fig. 5C). We then questioned whether trastuzumab can reduce IGFBP-2–mediated activation of ErbB2. SKBR3 cells were serum-starved and incubated in media with or without trastuzumab overnight. Cells were then treated with IGFBP-2 at the indicated concentrations for 1 hour. As shown in Fig. 6C, IGFBP-2 treatment caused a dose-dependent increase in ErbB2 phosphorylation in serum-starved SKBR3 cells (Fig. 6C, top panel, lanes 1–4), consistent with the results shown in Fig. 6A. However, trastuzumab pretreatment of SKBR3 cells gave rise to an increase in ErbB2 tyrosine phosphorylation independent of IGFBP-2 treatment (Fig. 6C; compare lane 2 with lane 5), and treatment with IGFBP-2 did not result in further increase in ErbB2 phosphorylation (Fig. 6C, top panel, lanes 5–7). Trastuzumab-induced ErbB2 phosphorylation in SKBR3 cells has been reported by others previously (41). This might be the reason why the dose-dependent increase in ErbB2 tyrosine phosphorylation induced by IGFBP-2 was not observed in cells pretreated with trastuzumab. Trastuzumab-mediated endocytic degradation of ErbB2 was observed in cells pretreated with trastuzumab, consistent with our previous report (ref. 6; Fig. 6C, bottom panel, compare lanes 2–4 with lanes 5–7). However, data shown in Fig. 6C provided important information with regard to the difference in tyrosine phosphorylation of ErbB2 induced by IGFBP-2 or trastuzumab, such that trastuzumab-induced tyrosine phosphorylation of ErbB2 was companied by the receptor degradation whereas IGFBP-2 could not induce ErbB2 degradation (Fig. 6A and C). Taken together, these data suggest that trastuzumab may, at least partially, inhibit IGFBP-2–mediated activation of ErbB2 signaling, which may be an additional mechanism of action for trastuzumab-induced growth inhibition.
Figure 6. IGFBP-2 mediates the activation of ErbB2-coupled signaling in SKBR3 cells and trastuzumab interferes with IGFBP-2–mediated activation of ErbB2 signaling. A, SKBR3 cells were serum-starved overnight and then treated with recombinant human IGFBP-2 for the indicated times. Cells were then harvested, and WCLs were subjected to immunoprecipitation using antibody directed against ErbB2 (29D8). Immunoprecipitates were probed with anti-phospho-tyrosine antibody (4G10) and then stripped and reprobed for ErbB2 (top 2 panels). SKBR3 cells were serum-starved overnight and then treated with recombinant human IGFBP-2 with the indicated doses for 60 minutes. Immunoprecipitates were probed with anti-phospho-tyrosine antibody (4G10) and then stripped and reprobed for ErbB2 (bottom 2 panels). B, SKBR3 cells were serum-starved overnight and then treated with recombinant human IGFBP-2 for the indicated times. The levels of p-Erk1/2 in WCLs were determined by Western blot analysis. SKBR3 cells were serum-starved overnight and then treated with recombinant human IGFBP-2 at the indicated doses for 60 minutes. Levels of p-Erk1/2, total Erk1/2, p-Akt (T308), and total Akt in WCLs were determined by Western blot analysis. C, SKBR3 cells were serum-starved and either left untreated or treated with 10 µg/mL trastuzumab overnight. Cells were then incubated with recombinant human IGFBP-2 at the indicated concentrations (250 and 500 ng/mL) for 1 hour. Cells were then harvested, and WCLs were subjected to immunoprecipitation using anti-ErbB2 antibody. Immunoprecipitates were probed with anti-phospho-tyrosine antibody (4G10) and then stripped and reprobed for immunoprecipitated ErbB2. D, SKBR3 cells were serum-starved and either left untreated or treated with 10 µg/mL trastuzumab overnight. Cells were then incubated with the recombinant human IGFBP-2 (250 ng/mL). Cells were then harvested at the indicated time points and subjected to immunoprecipitation as described in C. Immunoprecipitates were probed with anti-phospho-tyrosine antibody (4G10) and then stripped and reprobed for immunoprecipitated ErbB2. The levels of p-Erk1/2 and total Erk1/2 in WCLs were determined by Western blot analysis.
Discussion

Increased expression of endogenous IGFBP-3 or treatment with recombinant human IGFBP-3 was shown not only to inhibit cancer cell growth in a variety of experimental systems but also to enhance the efficacy of radioactive, proapoptotic, and chemotherapeutic agents (42). The results presented in this article indicate that trastuzumab treatment enhances IGFBP-3 expression and secretion and that this upregulation of IGFBP-3 induced by trastuzumab is correlated with trastuzumab-mediated growth inhibition of SKBR3 cells. Cross-talk between IGF-IR and ErbB2 signaling pathways has been shown to contribute to trastuzumab resistance (9). In this article, we describe a novel role for IGFBP-3 in the regulation of IGF-I–mediated cross-talk between IGF-IR and ErbB2 signaling pathways. In particular, our results show that addition of exogenous human IGFBP-3 blocks not only IGF-I–induced activation of IGF-IR but also inhibits IGF-I–induced activation of ErbB2 and that stable expression of IGFBP-3 inhibits SKBR3 cell growth. Taken together, we propose a novel mechanism of action whereby trastuzumab enhances the production of IGFBP-3 in trastuzumab-sensitive SKBR3 cells, leading to the enhanced expression and secretion of IGFBP-3. Subsequently, the enhanced levels of IGFBP-3 interfere with IGF-I–mediated mitogenic signaling, via autocrine and/or paracrine mechanisms, and mediate the growth inhibition. The role played by IGFBP-3 in contributing to the mechanism of action of trastuzumab is underscored by the fact that, in clone 3 cells, IGFBP-3 expression and secretion are decreased, trastuzumab no longer enhances IGFBP-3 production, and that restoration of IGFBP-3 by transiently expressing exogenous IGFBP-3 may potentiate trastuzumab activity in trastuzumab-resistant clone 3 cells. Given that ErbB2 activity is markedly elevated in clone 3 cells as compared with trastuzumab-sensitive SKBR3 cells (6), this inverse relationship between trastuzumab-sensitive and -resistant SKBR3 cells in IGFBP-3 expression and secretion is further supported by the finding that IGFBP-3 is one of the most significantly downregulated gene associated with ErbB2 overexpression (18). Consistent with data we described in this article, resistance to gefitinib, which is an EGFR tyrosine kinase inhibitor, was also reported to be associated with the marked decrease in IGFBP-3 expression and secretion in A431 squamous cancer cells (43).

Interestingly, we have also found that IGFBP-2 expression can be enhanced after SKBR3 cells are treated with trastuzumab (Fig. 5C). Given that IGFBP-2 is part of a mechanism to compensate for the mitogenic and antiapoptotic effects of IGF (15), this enhancement of IGFBP-2 production may be a survival mechanism that is used by the cells to counteract the inhibitory effects mediated by trastuzumab. After chronic exposure of SKBR3 cells to trastuzumab (i.e., 3–5 months of trastuzumab treatment), IGFBP-2 levels and secretion are elevated in cells, as is the case for clone 3 cells (Fig. 5A–C). This may eventually override the inhibitory effects mediated by trastuzumab, resulting in trastuzumab resistance. However, at the early stage of trastuzumab treatment, our data support the idea that blocking IGFBP-2–stimulated ErbB2 activation and downstream ErbB2 signaling is another important mechanism of action for trastuzumab-mediated growth inhibition (Fig. 6).

The finding that changes in secreted levels of IGFBP-3 reflect the sensitivity of SKBR3 breast cancer cells to trastuzumab may provide important information for the development of predictive biomarkers for trastuzumab resistance. Furthermore, the secreted levels of IGFBP-3 are markedly reduced in breast cancer cells that show primary trastuzumab resistance (JIMT-1). Approxi- mately two thirds of ErbB2-positive breast cancers show primary resistance to trastuzumab treatment, and a majority of patients who achieve an initial response to trastuzumab acquire resistance to trastuzumab within 1 year (1, 4). No predictive biomarkers are currently used in the clinical setting to differentiate these populations of breast cancers from trastuzumab-sensitive disease. There is an urgent need for the identification of predictive biomarkers for trastuzumab resistance. Based on our data, we propose that the dramatic decrease in the circulating levels of IGFBP-3 in breast cancer patients who are receiving trastuzumab may serve as a predictive biomarker for trastuzumab resistance. On the other hand, high circulating levels of IGFBP-3 or circulating levels of IGFBP-3 that can be enhanced by the treatment with trastuzumab may be a therapeutic indicator of tumor responsiveness to trastuzumab treatment. Furthermore, based on our data, we propose that high circulating levels of IGFBP-2 may be another predictive biomarker for trastuzumab resistance. The changes in secretion profiles of IGFBP-3 and IGFBP-2 described in this article warrant further clinical investigation as potential predictive biomarkers for therapeutic resistance to trastuzumab.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Milos Dokmanovic, Yi Shen, Tabetha M. Bonacci, et al.

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