Trastuzumab regulates IGFBP-2 and IGFBP-3 to mediate growth inhibition: implications for the development of predictive biomarkers for trastuzumab-resistance

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Abbreviation list: ErbB: erythroblastosis protein B; HER-2: human epidermal growth factor receptor 2; IGFBP(s): Insulin-like growth factor binding protein(s); IGF-I: insulin-like growth factor I; IGF-IR: insulin-like growth factor-I Receptor; EGF: epidermal growth factor; ERK: extracellular signal-regulated kinase; MAPK: mitogen-activated protein kinase; PI3K: phosphatidylinositol 3-kinase; ADCC: antibody-dependent cellular cytotoxicity; GFP: green fluorescent protein; DMEM: Dulbecco’s modified essential medium; RPMI: Roswell Park Memorial Institute; CMV: cytomegalovirus; ELISA: enzyme-linked immunosorbent assay; WCL: whole cell lysates.

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Abstract

Activation of IGF-IR signaling is an important mechanism for trastuzumab-resistance. Insulin-like growth factor binding proteins (IGFBPs) modulate IGF-IR signaling and play important roles in the control of breast cancer progression. Here, 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 up-regulation 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. Specifically, 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 JIMT1). In contrast to IGFBP-3, the secretion and expression of IGFBP-2 are upregulated in trastuzumab-resistant SKBR3 cells. We also demonstrate 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. The changes in secretion profiles of IGFBP-2 and IGFBP-3 in trastuzumab-sensitive and trastuzumab-
resistant cells may further the development of IGFBP-2 and IGFBP-3 as predictive biomarkers for trastuzumab-resistance.

**Introduction**

Erythroblastosis protein B2 (ErbB2)/human epidermal growth factor receptor 2 (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 (a) trastuzumab-mediated ErbB2 internalization and degradation; (b) interruption of ErbB2 dimerization with other ErbB family receptors; (c) disruption of downstream signaling pathways, including phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways; (d) induction of G1 cell cycle arrest and apoptosis; (e) induction of antibody-dependent cellular cytotoxicity (ADCC); (f) 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 one year (2–4). Ritter et al. 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 up-regulated Rac1
activity impairs trastuzumab-induced ErbB2 internalization and degradation and contributes to trastuzumab-resistance (6). We also found that the ErbB2 activity is enhanced in Clone 3 cells as compared to SKBR3 cells. However, the mechanism leading to the up-regulation 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-sensitive 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 two 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 knockdown of IGF-IR in these cell lines re-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 trastuzumab-resistance.

The insulin-like growth factor binding protein (IGFBP) family is comprised of 6 cysteine-rich secreted proteins (IGFBP1-6) that regulate insulin-like growth factor (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 IGFBPs 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). IGFBPs 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 anti-proliferative and pro-apoptotic effects in breast cancer cells (14-17). Based on cDNA microarray analysis, IGFBP-3 is one of the most significantly downregulated 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 as compared to trastuzumab treatment alone (19). Additionally, IGFBP-3 expression is induced by different anti-cancer therapeutics in breast cancer cells (20-22). In contrast to IGFBP-3, IGFBP-2 is often highly expressed in malignant tumors, but decreases upon remission (23, 24). Increased IGFBP-2 levels in patient sera often correlate with tumor malignancy (25-27). Additionally, IGFBP-2 expression is increased in breast cancer tissue as compared to benign tissue (28), and IGFBP-2 overexpression in breast cancer has also been proposed as a marker of anti-estrogen 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.

Here, 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. Additionally, we demonstrate 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 demonstrated 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 JIMT1). Furthermore, we found that the increased expression and secretion of IGFBP-2 positively correlated with trastuzumab-resistance in SKBR3 cells. While activating mutations in KRAS are used as a predictive biomarker for therapeutic-resistance to cetuximab, a monoclonal antibody directed against ErbB1/EGFR that is approved for the treatment of colorectal cancers, no predictive biomarkers are currently used in the clinic 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 here regarding the changes in secretion
profiles of IGFBP-2 and 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 (Tyr 877, Tyr 1221/1222), Erk1/2
(L34F12), phospho-Erk1/2 (Thr 202/Tyr 204), Akt (11E7), phospho-Akt (Ser 473, Thr
308), phospho-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. Anti-
phosphotyrosine (4G10) antibody was obtained from Millipore. Trastuzumab
(Genentech, Inc) was purchased from the pharmacy at the National Institutes of Health
(NIH), Bethesda, MD, USA. Recombinant human IGF-I, IGFBP-2, and IGFBP-3 were
purchased from Sigma-Aldrich and were re-suspended and stored according to the
manufacturer’s protocol. Recombinant human EGF was obtained from Invitrogen.
pCMV6-AC-GFP vector encoding GFP-tagged IGFBP-3 and 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 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 M.D. Anderson Cancer Center, Houston, Texas (6, 34). The standard culture media for SKBR3 cells was DMEM/F12 supplemented with 10% fetal bovine serum, whereas the standard culture media for Clone 3 cells was DMEM/F12 supplemented with 10% fetal bovine serum and 4 μg/ml trastuzumab. The JIMT-1 cell line was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Brunswick, Germany) and described by Tanner M. et al. (35). The standard media for JIMT-1 cells is DMEM supplemented with 10% fetal bovine serum. BT-474 cells were purchased from ATCC and were maintained in RPMI-1640 supplemented with 10% fetal bovine serum. 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).

**Enzyme-linked immunosorbent assay (ELISA)**

Detection of IGFBP-2 and IGFBP-3 in cell culture media was done according to the manufacturer’s protocol (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 performed 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 (4μg/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 according to the manufacturer’s protocol. The 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 cells and Clone 3 cells (an SKBR3- derivative that is trastuzumab-resistant). SKBR3 cells were treated with trastuzumab for three days
or six days or left untreated. Clone 3 cells were maintained in their standard culture media which contains 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 Figure 1A, secreted IGFBP-3 was slightly increased from day three to day six 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 (Figure 1B). However, an increase in the secretion of IGFBP-3 (~ seven-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 three-fold decrease in cell numbers at day six as compared to the cells not treated with trastuzumab (Figure 1B). Additionally, analysis of the secreted levels of IGFBP-3 in Clone 3 cells at days three and six indicated that very low concentrations of IGFBP-3 were detected in the media despite the increase in cell numbers from day 3 to day 6 (Figures 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 (Figure 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 tyrosine 1131 (Figure 2A). Consistent with another report (19), pretreatment with recombinant human IGFBP-3 effectively blocked IGF-I-induced
tyrosine phosphorylation of IGF-IR (Figure 2A). After the pretreatment of cells with IGFBP-3, we found that IGF-I-induced phosphorylation of Erk1/2 was abrogated (Figure 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 IGFBP3 (Figure 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 Figure 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 (Figure 2C, right panels).

Additionally, the effects of IGFBP-3 were specific to the IGF-I-mediated activation of ErbB2, as addition of IGFBP-3 was unable to block EGF-induced ErbB2, Erk, and Akt phosphorylation (Figure 2D). These data, when taken together with Figure 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**
We next asked if 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 whole cell lysates was detected by Western blot analysis. As shown in Figure 3A, the expression of endogenous IGFBP-3 was increased after SKBR3 cells were treated with trastuzumab for 3 or 6 days. The increase in IGFBP-3 protein expression induced by trastuzumab parallels the changes in the trastuzumab-induced IGFBP-3 secretion in SKBR3 cells (Figures 1A and B). When IGFBP-3 expression in WCL of Clone 3 cells was examined, we found that IGFBP-3 protein levels were reduced compared to that in SKBR3 cells, consistent with the data shown in Figure 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 three-fold by day seven as compared to SKBR3 cells stably expressing empty vector (Figure 3B). Based on data shown in Figures 1, 2, and 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 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 post-transfection, whereas Figure 3C (graph) showed that the amount of IGFBP-3 in cell culture media collected at day 1 and day 3 was increased in Clone 3 cells transiently expressing IGFBP-3-GFP as compared to 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 demonstrated that expression of exogenous IGFBP-3 reduced Clone 3 cell growth, especially at earlier time points (day1 and day3), consistent with the inhibitory effect of IGFBP-3 on SKBR3 cells (Figure 3B).

**The levels of secreted IGFBP-3 in BT-474 and JIMT1 breast cancer cells are associated with their sensitivity to trastuzumab**

BT-474 and JIMT1 are two 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 JIMT1 cells are resistant to trastuzumab (11, 35). We therefore questioned if there was a difference in the IGFBP-3 secretion in these two cell lines. To determine the levels of secreted IGFBP-3, BT-474 and JIMT1 cells were seeded at equal numbers, and the cell culture media were harvested at the indicated time points. As shown in Figure 4, the levels of secreted IGFBP-3 were lower in JIMT1 cells than in BT-474 cells (Figure 4A), although JIMT1 cells replicated faster than BT-474 cells from day 2 to day 4 (Figure 4B). Taken together, our data revealed an inverse relationship in the levels of secreted IGFBP-3 between trastuzumab-sensitive and -resistant breast cancer cells, such that the levels of secreted IGFBP-3 were decreased in trastuzumab-resistant cells (Clone 3 and JIMT1 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.
Nevertheless, data shown in Figure 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 anti-estrogen 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 Figure 1 were also assayed for secreted IGFBP-2. As shown in Figures 5A IGFBP-2 levels detected in the cell culture media of Clone 3 cells were more than four-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 not due to differences in cell growth rate as the SKBR3 and Clone 3 cell numbers were comparable (Figure 1B). A modest decrease in IGFBP-2 secretion by SKBR3 cells treated with trastuzumab was found at both days 3 and 6 as compared to untreated SKBR3 cells (Figure 5A). This may be attributable to the decrease in SKBR3 cell numbers after trastuzumab treatment (Figure 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 (Figure 5B). As shown in Figure 5C, the protein levels of endogenous IGFBP-2 were elevated in Clone 3 cells as compared to 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 (Figure 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 with 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). The high levels of IGFBP-2 in Clone 3 cells prompted the question as to whether the increased IGFBP-2 secretion contributes to the up-regulated ErbB2 phosphorylation in Clone 3 cells, which we reported previously (6). As shown in Figure 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 (Figure 6A). We next examined whether downstream effectors of ErbB2 were affected by IGFBP-2 stimulation. As shown in Figures 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 (Figure 5C). We then questioned if 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 one hour. As shown in Figure 6C, IGFBP-2 treatment caused a dose-dependent increase in ErbB2 phosphorylation in serum-starved SKBR3 cells (Figure 6C, upper panel, lanes 1-4), consistent with the results shown in Figure 6A. However, trastuzumab pretreatment of SKBR3 cells gave rise to an increase in ErbB2 tyrosine phosphorylation independent of IGFBP-2 treatment (Figure 6C, compare lane 2 with lane 5) and treatment with IGFBP-2 did not result in further increase in ErbB2 phosphorylation (Figure 6C, upper panel, lanes 5-7). Trastuzumab-induced ErbB2 phosphorylation in SKBR-3 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 (6) (Figure 6C, lower panel, compare lanes 2-4 with lanes 5-7). However, data shown in Figure 6C provided important information with regards to the difference in tyrosine phosphorylation of ErbB2 induced by IGFBP-2 or trastuzumab, such that trastuzumab-induced tyrosine phosphorylation of ErbB2 was accompanied by the receptor degradation, whereas IGFBP-2 was unable to induce ErbB2 degradation (Figure 6A and C). Taken together, data shown in Figure 6C indicate that trastuzumab interferes with IGFBP-2-mediated tyrosine phosphorylation.
phosphorylation of ErbB2 in SKBR3 cells. To further address whether trastuzumab affects IGFBP-2-mediated activation of ErbB2 signaling, we performed another experiment where we assayed the effect of trastuzumab on the kinetics of IGFBP-2-mediated increase in ErbB2 phosphorylation in SKBR3 cells. SKBR3 cells were serum-starved and treated with trastuzumab overnight, and cells then were stimulated with IGFBP-2 for the indicated time. As shown in Figure 6D, IGFBP-2 treatment transiently increased ErbB2 phosphorylation at 30 minute time point. However, ErbB2 tyrosine phosphorylation was blocked at the rest of the time points (Figure 6D). Figure 6D (bottom two panels) also showed that no increase in IGFBP-2-induced Erk1/2 phosphorylation was detected after SKBR3 cells were serum-starved and pretreated with trastuzumab (Figure 6D, bottom panels). Although a slight increase in Erk phosphorylation was observed at the 360 minute time point (Figure 6D), this activation was not typical of IGFBP-2-induced activation of Erk, which usually occurs within 15 minutes post IGFBP-2 stimulation (Figure 6B). 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.

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 radiation, pro-apoptotic, and chemotherapeutic agents (42). The results presented here indicate that trastuzumab...
treatment enhances IGFBP-3 expression and secretion and that this up-regulation 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 demonstrated to contribute to trastuzumab-resistance (9). Here, 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, 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 and trastuzumab no longer enhances IGFBP-3 production, and that restoration of IGFBP-3 by transiently expressing exogenous IGFBP-3 may potentiante trastuzumab activity in trastuzumab-resistant Clone 3 cells. Given that ErbB2 activity is markedly elevated in Clone 3 cells as compared to 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 here, 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 (Figure 5C). Given that IGFBP-2 is part of a mechanism to compensate for the mitogenic and anti-apoptotic 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 month trastuzumab treatment), IGFBP-2 levels and secretion are elevated in cells, as is the case for Clone 3 cells (Figure 5A, 5B and 5C). 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 (Figure 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 (JIMT1). Approximately, 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 one
year (1, 4). No predictive biomarkers are currently used in the clinic 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. Based on our data we also 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 here warrant further clinical investigation as potential predictive biomarkers for therapeutic resistance to trastuzumab.

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Disclaimer: The information presented in this article reflects the views of the authors and does not represent the policy of the U.S. Food and Drug Administration.

References:


26. Elminger MW, Bell M, Schütt BS, Langkamp M, Kutoh E, Ranke MB.


Figure legends

Figure 1

Trastuzumab-induced IGFBP-3 secretion is correlated with its inhibitory effects on SKBR3 cell proliferation

A. IGFBP-3 secretion profiles of SKBR3 cells 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. 1 x 10^6 cells were plated in 10 cm dishes and cultured in the standard cell culture media (see Materials and Reagents). 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 three independent experiments; bars, standard deviation (SD). *, p < 0.01; **, p< 0.001 as determined by two-way ANOVA followed by Bonferroni post tests. B. Growth profiles for Clone 3 cells and SKBR3 cells grown as described in A. Cells were plated at 5 x 10^4 cells per well of 12 well plate in triplicate. At the indicated time points cells were trypsinized and counted by trypan blue exclusion using a hemocytometer. Data represent three independent experiments. Points, mean of triplicates; bars, SD. C. The relative IGFBP-3 was derived from Figure 1A and B. The total IGFBP-3 in cell culture media at the Day 3 and Day 6 was obtained by multiplying the concentration of IGFBP-3 in media (pg/ml)
shown in Figure 1A 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 the 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.

Figure 2

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

A. SKBR3 cells were serum-starved and incubated with recombinant human IGFBP-3 (500 ng/ml) overnight and then treated with IGF-I (100 ng/ml). At the indicated time points, cells were harvested, and the levels of phosphorylated and total IGF-IR in whole cell lysates (WCL) were determined by Western blot analysis. B. The experimental procedures were essentially the same as in A, except levels of phospho-Erk1/2, phospho-Akt, total Erk1/2, and total Akt in WCL were determined by Western blot analysis. Western blot of actin was done to confirm equal protein loading (From here on, all of the actin Western blots were performed to confirm equal protein loading.) C. The experimental procedures were essentially the same as in A, except levels of phospho-ErbB2 (Y877 and Y1221) and total ErbB2 in WCL were determined by Western blot analysis. D. SKBR3 cells were incubated with recombinant human IGFBP-3 (500 ng/ml) overnight and then treated with EGF (50 ng/ml). At the indicated time points, cells were harvested, and the levels of phospho-ErbB2 (Y877), phospho-Erk1/2, total Erk1/2,
phospho-Akt (T308 and S473), and total Akt in WCL were determined by Western blot analysis.

**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 (4μg/ml) or left untreated for the indicated time. The standard cell culture media for Clone 3 cells always contains trastuzumab at 4μg/ml (see Material and Methods). Cells were harvested at the indicated time points and the levels of IGFBP-3 in WCL were detected by Western blot analysis. B. SKBR3 cells stably expressing empty vector (control) or GFP-tagged IGFBP-3 (IGFBP-3-clone 1 and 2) were seeded in triplicate at 15,000 cells per well of a 12-well plate. At the indicated times, cells were trypsinized and counted. Points: mean of two independent experiments in triplicate; bars: SD. *, p < 0.001 as determined by two-way ANOVA followed by Bonferroni post tests. 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 5 x 10⁴ cells per well of 12 well plate in triplicate and cultured in the standard culture media. Cells were then transfected the plasmid encoding either GFP empty vector (control) or GFP-tagged IGFBP-3. WCL were harvested 24h post-transfection (Day1) and analyzed by 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. **Growth profiles of Clone 3 GFP control cells and Clone 3 cells expressing IGFBP-3-GFP. Points: mean of three independent experiments run in triplicate. Bar: SD. *, p < 0.05; **, p < 0.001 as determined by two-way ANOVA followed by Bonferroni post tests.**

**Figure 4**

**The levels of secreted IGFBP-3 in BT-474 and JIMT1 breast cancer cells.**

A. The indicated cells (BT-474 and JIMT1) were seeded at 1 x 10^6 per 10 cm dish in triplicate and were cultured in standard cell culture media (see Material and Methods). IGFBP-3 levels were determined by ELISA as described in Figure 1A. *, p < 0.001; **, p < 0.0001 as determined by an unpaired two-tailed student’s T-test. B. After harvesting the cell culture media, cells were then trypsinized and counted. Data represent two independent experiments. Columns: mean of triplicate; bars: SD.

**Figure 5**

**Trastuzumab-resistant Clone 3 cells are characterized by an increase in IGFBP-2 expression and secretion.**

A. The analysis of IGFBP-2 secretion was done using the same cell culture supernatants described in Figure 1A. The experimental procedures were the same as used in Figure 1A, except cell culture media were not concentrated prior to analysis by ELISA. Columns, mean of three independent experiments; bars, SD. *, **, p < 0.01 as determined by two-way ANOVA followed by Bonferroni post tests. B. The calculation of the relative IGFBP-2 was the same as that of the relative IGFBP-3 described in Figure
Following the collection of cell culture media for ELISA, cells were harvested and the levels of IGFBP-2 in WCL were determined by Western blot analysis.

**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 WCL were subjected to immunoprecipitation using antibody directed against ErbB2 (29D8). Immunoprecipitates were probed with anti-phospho-tyrosine antibody (4G10) and then stripped and re-probed for ErbB2 (upper two panels). SKBR3 cells were serum-starved overnight and then treated with recombinant human IGFBP-2 with the indicated doses for 60 min. Immunoprecipitates were probed with anti-phospho-tyrosine antibody (4G10) and then stripped and re-probed for ErbB2 (lower two panels). **B.** SKBR3 cells were serum-starved overnight and then treated with recombinant human IGFBP-2 for the indicated times. The levels of phospho-Erk1/2 in WCL 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 min. Levels of phospho-Erk1/2, total Erk1/2, phospho-Akt (T308), and total Akt in WCL 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 harvested, and
WCL were subject 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 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 phospho-Erk1/2 and total Erk1/2 in WCL were determined by Western blot analysis.
Figure 1: Dokmanovic et al.
Figure 2: Dokmanovic et al.
Figure 3: Dokmanovic et al.

A. 

Day3 Day6

<table>
<thead>
<tr>
<th>SKBR3</th>
<th>Clone 3</th>
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IGFBP-3
Actin
trastuzumab

B. 

- Control
- IGFBP-3-clone 1
- IGFBP-3-clone 2

Cell number (x10^5)

Day 0 1 4 7

0 1 2 3

Day 1 Day 3

0 20 40 60

IGFBP-3 in media (pg/ml)

C. 

IGFBP-3-GFP
Actin

GFP-IGFBP-3

D. 

GFP Control
IGFBP-3-GFP

Cell number (x10^5)

Day 0 1 3 5

0 1 2 3

** Figure 3: Dokmanovic et al. **

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A.

![Graph showing IGFBP-3 in media (pg/ml) over days 2 and 4 for BT474 and JIMT1 cell lines.](image)

B.

![Bar graph showing cell number (x10^5) over days 2 and 4 for BT474 and JIMT1 cell lines.](image)

Figure 4: Dokmanovic et al.
A. IGFBP-2 in media (x 10^-3 pg/ml)

B. Relative IGFBP-2

C. IGFBP-2 and Actin

Figure 5: Dokmanovic et al.
Figure 6: Dokmanovic et al.
Molecular Cancer Therapeutics

Trastuzumab regulates IGFBP-2 and IGFBP-3 to mediate growth inhibition: implications for the development of predictive biomarkers for trastuzumab-resistance

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