Autocrine Motility Factor Promotes HER2 Cleavage and Signaling in Breast Cancer Cells

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Abstract
Trastuzumab (Herceptin) is an effective targeted therapy in HER2-overexpressing human breast carcinoma. However, many HER2-positive patients initially or eventually become resistant to this treatment, so elucidating mechanisms of trastuzumab resistance that emerge in breast carcinoma cells is clinically important. Here, we show that autocrine motility factor (AMF) binds to HER2 and induces cleavage to the ectodomain-deleted and constitutively active form p95HER2. Mechanistic investigations indicated that interaction of AMF with HER2 triggers HER2 phosphorylation and metalloprotease-mediated ectodomain shedding, activating phosphoinositide-3-kinase (PI3K) and mitogen-activated protein kinase signaling and ablating the ability of trastuzumab to inhibit breast carcinoma cell growth. Furthermore, we found that HER2 expression and AMF secretion were inversely related in breast carcinoma cells. On the basis of this evidence that AMF may contribute to HER2-mediated breast cancer progression, our findings suggest that AMF–HER2 interaction might be a novel target for therapeutic management of patients with breast cancer, whose disease is resistant to trastuzumab. Cancer Res; 73(4); 1411–9. ©2012 AACR.

Introduction
HER2 (ERBB2/Neu), a family member of EGF receptors (HER) is overexpressed in approximately 25% of invasive breast carcinomas (1–3) and is a major approved target for breast cancer therapy. The crystal structure of HER2 suggests that its extracellular domain (ECD) exists in a constitutively active conformation resembling the ligand-bound state of the other HERs (4, 5), whereas HER2-ECD–targeting antibodies that are antagonistic or agonistic at the levels of HER2 phosphorylation and cell growth, suggest the presence of binding partner(s) necessary for complete activation of HER2 (1, 6, 7). Herceptin/trastuzumab has improved the outcome in patients with HER2-overexpressing breast carcinoma (8, 9). However, a substantial proportion of patients with HER2-positive breast cancer is intrinsically resistant to trastuzumab or acquires resistance following initial treatment (10). The mechanisms of resistance to Herceptin/trastuzumab are mainly involved in the restoration of the PI3K/AKT signaling pathways either via an epitope masking (Mucin) and escaping (truncated p95HER2), alternative compensation of receptor tyrosine kinases, or the constitutive mutations of phosphoinositide-3-kinase (PI3K) pathways (10–12). Retrospective studies suggest that the oncogenic p95HER2 variant is most likely responsible for clinical resistance to Herceptin/trastuzumab treatment (13, 14).

Phosphoglucose isomerase (EC: 5.3.1.9; PGI) is a housekeeping dimeric enzyme that catalyzes the reversible isomerization of glucose-6-phosphate and fructose-6-phosphate in glycolysis/glucoseogenesis (15). PGI belongs to the moonlighting family of proteins having multiple functions/activities within a single polypeptide chain, not resulting from multiple domains of a protein, alternative RNA splicing, gene fusions, and/or posttranslational processing (16). Secreted form of PGI in the extracellular milieu of transformed cells and several tissues was identified as neuroleukin (NLK), a neurotrophic factor that mediates the differentiation of neurons and autocrine motility factor (AMF), a tumor-secreted C-X-X-C cytokine that is involved in cell motility (17, 18). Abrupt secretion of AMF was observed in the blood and urine of patients with cancer, suggesting a prognostic value (15, 19). Functionally, AMF was shown to induce cell proliferation, differentiation, and survival of various cancer and immune cells (15). Independent reports have shown that AMF activates mitogenic mitogen-activated protein kinase (MAPK)/ERK or prosurvival PI3K/AKT pathways, similarly to the signaling mode of growth factors as emphasized in the resistance to HER2-targeted therapy (20, 21). The receptor of AMF, that is, gp78/AMFR was identified as a 7 transmembrane domain containing protein. However, gp78/AMFR-null cells still respond to AMF, suggesting the presence of yet another unidentified receptor (22, 23).

Here, we show that in human breast carcinoma cells AMF binds to HER2, induces its phosphorylation, ectodomain shedding, activates its downstream signaling pathways, and...
overcomes Heceptin/trastuzumab effect. The data suggest that AMF may be a novel therapeutic target for patients with breast cancer in conjunction with Heceptin/trastuzumab therapy.

Materials and Methods

Antibodies and chemicals

Purified rabbit PGI/AMF was purchased from Sigma for AMF stimulation. Monoclonal anti-PGI (12F9A6. Pfizer) and rabbit anti-PGI (H300, Santa Cruz) antibodies were used for Western blot analysis and immunoprecipitation, p-ERK (E-4), ERK1/2(MK1), p-Tyr (PY20), anti-HER2-ICD (Neu, C-18), anti-HER2-ECD (9G6), p-HER2 antibodies, and lapatinib were purchased from Santa Cruz. Anti-p-AKT (Ser473) and AKT antibodies were from Cell Signaling. Anti-rabbit IgG-TRITC conjugated, tetramethylrhodamine isothiocyanate [TRITC]-conjugated, fluorescein isothiocyanate [FITC]-conjugated, tetravinylrhodamine isoioxyanate [TRITC]-conjugated) were also incubated separately. Immunofluorescence images were obtained using a Zeiss Confocal Laser Microscope LSM 510. Four micrometer sections obtained from formalin fixed, paraffin-embedded tissue blocks were deparaffinized, rehydrated, and microwaved on high for 2 minutes. Fixation, paraffinization, rehydration, and microwaving were repeated twice. Statistical analysis was done using Student t test. P < 0.005 was regarded as significant.

Immunofluorescence and immunohistochemistry

For immunofluorescence studies, SkBr3 cells grown on 12-mm round coverslips (Fisher) were processed after AMF-V5 treatment for 15 minutes. The cells were washed and fixed with 4% paraformaldehyde for 10 minutes and permeabilized with 0.2% Triton X-100 for 5 minutes. For double immunofluorescence labeling, the cells were incubated with primary antibodies (anti-HER2 and anti-V5) for 1 hour separately to minimize the cross-reactivity. After 3 washes, appropriate secondary antibodies (fluorescein isothiocyanate [FITC]-conjugated, tetramethylrhodamine isoioxyanate [TRITC]-conjugated) were also incubated separately. Immunofluorescence images were obtained using a Zeiss Confocal Laser Microscope LSM 510. Four micrometer sections obtained from formalin fixed, paraffin-embedded tissue blocks were deparaffinized, rehydrated, and microwaved on high for 5 minutes in 1 mmol/L sodium citrate buffer, pH 6.0. The sections were washed 3× in PBS and blocked with Super Block (Skytek Laboratories) for 10 minutes. Sequential sections were then incubated in PBS and linked with biotinylated mouse anti-AMF antibodies at 4°C overnight. The sections were then washed 3× for 10 minutes each in PBS and linked with biotinylated anti-mouse secondary antibodies (Vector Laboratories) followed by Texas Red conjugated, avidin. After washing, the sections were incubated at 4°C overnight with rabbit anti-HER2 antibodies (Invitrogen) and with fluorescein-conjugated anti-rabbit secondary antibodies. Images were documented with an OLYMPUS BX40 microscope supported by a DP72 CCD Camera, and CellSens Dimension Imaging Software (Olympus).

Cell growth assay

BT474 cells were seeded at a density of 5 × 10^4 cells per well in 24-well plates. After 24 hours, cells were treated with either 40 μg/mL trastuzumab or a combination of AMF (90 mmol/L) with trastuzumab. Cell growth was monitored at 550 nm after MTT assay. All experiments were carried out in quadruplicates and repeated twice. Statistical analysis was done using Student t test. P < 0.005 was regarded as significant.

Cell migration assay

Transwell (Corning Costar) migration assays were conducted on SkBr3 cells following pretreatment with trastuzumab for 16 hours. A total of 5 × 10^4 SkBr3 cells in serum-free medium were introduced into the upper compartment of Transwell chambers (8 μmol/L pore) and were allowed to migrate in lower chambers with 10% FBS or AMF (0.1 μg/mL) for 16 hours. Migrated cells were fixed and stained with Hema-3 stain kit (Fisher). Each condition was assayed in triplicate and each experiment was repeated 3 times.
Results

AMF induces PI3K/MAPK signaling pathways in a HER family dependent manner

To study whether AMF acts independently of HER2 signaling, we initially established whether AMF induces PI3K/MAPK signaling pathways through gp78/AMFR in low HER2-expressing breast cancer cell line T47D. Treatment with purified AMF showed that it activates both PI3K/AKT and MAPK/ERK (Fig. 1A–C). Furthermore, gp78/AMFR knockdown study revealed that gp78/AMFR mediates AMF-induced p-ERK but not p-AKT, suggesting involvement of another AMF receptor for AKT activation (Supplementary Fig. S1). Because participation of a pertussis-toxin sensitive G protein in the signal transduction of AMF-stimulated cell motility was previously reported (24), we ventured that presence of yet another AMF receptor might be a G protein coupled receptor (GPCR), which transactivates the HER family through ectodomain shedding of membrane-bound HER ligand precursors by matrix metalloproteinases (MMP), resulting in the activation of PI3K/MAPK pathways (25). Therefore, we tested whether AMF is involved in the trans-activation of the HER family for AKT activation. Interestingly, the results show that AMF-induced p-AKT/p-ERK was partially suppressed by lapatinib [dual kinase inhibitor of EGF receptor (EGFR) and HER2] compared with control and EGF, indicating that AMF-induced p-AKT/p-ERK is mediated by HER receptors and implying the responsibility of gp78/AMFR for partial suppression by lapatinib (Fig. 1D). To study whether a GPCR mediates AMF-induced p-AKT/p-ERK, we conducted suppression studies using pertussis-toxin (G protein inhibitor) or Marimastat (MMPs inhibitor) for blocking GPCR signaling. We found that these inhibitors did not suppress AMF-induced p-AKT/p-ERK (Fig. 1E), signifying that AMF signaling is not linked to GPCR but independently activates EGFR and/or HER2.

AMF induces phosphorylation of HER2 and enhances its cleavage

As HERs phosphorylation is induced by homo- and heterodimerization following the engagement of HER ligand and a cognate receptor (2), we addressed whether AMF induces the phosphorylation of EGFR and/or HER2. As shown in Fig. 2A, phosphorylation of both EGFR and HER2 were induced by exogenous EGF, which does not bind to HER2 (2, 3), implying EGFR/HER2 heterodimerization. While AMF did not induce phosphorylation of EGFR and kinetic studies indicated that AMF induces the phosphorylation of HER2 within a very short time frame of 2 minutes (Fig. 2B). Moreover, we found that both background p-HER2 and unphosphorylated p185HER2 vanished concomitantly with the emergence of cleaved p95HER2. As ectodomain shedding of p185HER2 was previously reported to generate membrane-anchored p95HER2 (95 to 100 kDa) by...
sheddases such as MMPs at the juxtamembrane region of HER2 (26), we addressed whether HER2 cleavage is directly affected by AMF or is mediated by MMPs. MMPs inhibitor suppressed AMF-induced cleavage of p185HER2 and also stabilized phosphorylated HER2 by AMF (Fig. 2C), indicating that AMF is sufficient for the phosphorylation of HER2 and AKT activation and also cooperates with MMPs for HER2 cleavage.

Parental EBNA293 cells did not show AKT/ERK activation after AMF treatment (Supplementary Fig. S2). To verify that AMF indeed triggers phosphorylation and participates in the cleavage of HER2, we tested the interaction of exogenously added AMF and HER2, using a membrane impermeable cross-linker (DTSSP), which establishes extracellular association of proteins. Exogenous AMF was coimmunoprecipitated with HER2 (Fig. 3A, top). To further verify this interaction V5-tagged AMF (AMF-V5) was expressed and purified to rule out any possible background experimental noise from the endogenous AMF. The interaction of exogenous AMF-V5 with HER2 was clearly observed by reciprocal coimmunoprecipitation studies (Fig. 3A, middle and bottom). In addition, immunofluorescence studies revealed the colocalization of HER2 and exogenous AMF-V5 (Fig. 3B).

**Figure 2.** AMF induces phosphorylation of HER2 and its cleavage. A, T47D cells starved and stimulated by AMF or EGF were immunoprecipitated with anti-phosphotyrosine antibodies (p-Y). Input lysate indicates lysate used for immunoprecipitation. B, HER2 phosphorylation and cleavage were monitored following AMF treatment in T47D cells at indicated time points, using immunoprecipitation (IP) with anti-HER2-ICD (epitope: intracellular domain). C, Marimastat-pretreated T47D cells were stimulated by AMF for 15 minutes and immunoprecipitation was conducted with anti-HER2-ECD (epitope: ectodomain, 9G8 clone). D, HER2 status in EBNA 293 cells transiently transfected with CMV-HER2 (wild-type) or vector (−) for 24 hours and then starved and stimulated with AMF for 15 minutes. E, HER2-transfected and starved EBNA 293 cells were pretreated with Marimastat and stimulated with AMF for 15 minutes. NS, nonspecific. F, T47D cells were pretreated with siRNA-HER2 (20 nmol/L) for 48 hours and then stimulated by AMF for 5 minutes after cell starvation. −, control siRNA. Control indicates bovine serum albumin.

**AMF interacts with the extracellular domain of HER2**

Because the data indicate that AMF induces phosphorylation of HER2, we tested the interaction of exogenously added AMF and HER2, using a membrane impermeable cross-linker (DTSSP), which establishes extracellular association of proteins. Exogenous AMF was communoprecipitated with HER2 (Fig. 3A, top). To further verify this interaction V5-tagged AMF (AMF-V5) was expressed and purified to rule out any possible background experimental noise from the endogenous AMF. The interaction of exogenous AMF-V5 with HER2 was clearly observed by reciprocal communoprecipitation studies (Fig. 3A, middle and bottom). In addition, immunofluorescence studies revealed the colocalization of HER2 and exogenous AMF-V5 (Fig. 3B).
To elucidate whether AMF interacts with the ectodomain of HER2, HER2-transfected cells were extracellularly cross-linked with either exogenous AMF or AMF-V5. We observed that AMF or AMF-V5 cross-linked HER2 molecules were not immunoprecipitated with anti-HER2-ECD antibodies and immunoprecipitated HER2 levels were reduced compared with trastuzumab (juxtamembrane) or anti-HER2-ICD antibodies (Fig. 3C), suggesting that AMF–HER2–binding motif is either overlapping or close to the epitope of anti-HER2-ECD antibody. Next, taking advantage of the data obtained from the crystal structures of AMF and the ECD of HER2 (4, 27), we used the ZDOCK software (http://zdock.bu.edu) to predict their physical interactions. AMF binding to HER2 ectodomain was mapped to the molecular region of HER2 between domain I and III (Fig. 3D).

**AMF overcomes the Hereceptin/trastuzumab suppression of PI3K/MAPK signaling pathways**

As HER2 overexpressing cells have a high expression level of endogenous p-HER2 via a ligand-independent mechanism (1–3), we examined how AMF is able to affect endogenous p-HER2 and HER2 in HER2-overexpressing cells. Also, we questioned whether AMF-induced downstream events might be blocked by anti-HER2-ECD antibodies, which can compete with AMF for binding to HER2. As shown in Fig. 4A, the treatment with anti-HER2-ECD antibodies alone showed that it induced p-HER2 levels in concert with HER2 cleavage and AKT/ERK activation, mimicking AMF actions. Combination of AMF and anti-HER2-ECD antibodies shows synergistic effect. Interestingly, HER2 shedding was induced by exogenous AMF regardless of the presence of Herceptin/trastuzumab in the conditioned media. AMF treatment overcame Herceptin/trastuzumab–mediated suppression of HER2 cleavage and p-AKT/p-ERK levels.

Next, as AMF-induced p95HER2 is an indicator of trastuzumab resistance through epitope escaping (10), we proceeded to validate the effect of AMF on cell growth and motility with trastuzumab treatment. We observed AMF-induced HER2 cleavage in cells treated with trastuzumab in complete medium (Fig. 4B). As a result, exogenous AMF partially overcame the inhibitory effect of trastuzumab on cell growth and motility (Fig. 4C and D).

**AMF secretion is controlled by HER2 expression**

Next, we examined the relationship between AMF and HER2 expression. AMF secretion was observed to be positively correlated with the expression of both p95HER2 and p185HER2 proteins (Supplementary Fig. S3). Sequentially, we found that HER2 knockdown inhibits AMF secretion, suggesting that AMF
secretion is regulated, at least in part by HER2 signaling in breast carcinoma cells (Fig. 5A). AMF knockdown did not affect HER2 expression, whereas reduced p-HER2 levels along with suppression of AKT/ERK activation in T47D cells (Fig. 5B). In addition, the blocking of AMF function partially inhibited the phosphorylation of HER2, resulting in the reduction of p-AKT/p-ERK levels (Fig. 5C) and endogenously secreted AMF does not significantly affect the cleavage of HER2 in HER2-overexpressing cells (Fig. 5C).

Finally, to further convince that AMF–HER2 relationship is not only an in vitro phenomena, we conducted 2 independent immunohistochemistry studies using human breast cancer tumors and MCF10A DCIS.com xenograft, which is a mouse model of human comedo ductal carcinoma in situ (28). Compared with the surrounding stroma, both HER2 and AMF were found to be strongly expressed in the human breast carcinoma epithelium, and especially colocalized on the cell membranes of MCF10A DCIS.com xenograft (Fig. 5D).

Discussion

The studies presented earlier show that exogenous AMF contributes to Herceptin/trastuzumab resistance of human breast carcinoma cells via generation of p95HER2 fragment. Because AMF interacts with HER2 and AMF–interacting HER2 is sensitive to HER2 shedding by MMPs, we hypothesize that

the interaction between AMF and HER2 could structurally expose the MMPs enzymatic clip site at HER2 juxtamembrane region. The expression levels and activation of MMPs might be considered a critical limiting step of HER2 shedding due to partial recovery by AMF in trastuzumab-inhibited cell growth and motility (Fig. 4C and D). Despite the inhibition of MMPs, presence of AMF was sufficient for phosphorylation of HER2 and activation of PI3K/MAPK (Fig. 2C and D), suggesting that AMF can act as a HER2-binding partner enhancing its activation. Weather AMF-interacting HER2 underdoes heterodimerization with another member of HER2 family for PI3K/MAPK activation is yet to be determined.

Because both AKT and ERK activation occurs mostly in the cellular signaling of receptor tyrosine kinase (RTK), the mechanism in which gp78/AMFR knockdown showed the suppression of AMF-activated ERK but not AMF-induced AKT is poorly understood (Supplementary Fig. S1). Of note, gp78 signaling-suppress ERK activation and also acts as an E3 ligase in the cytoplasm (29).

Transgenic mouse models harboring rat full-length p185HER2 (Neu) recapitulated the initial events of HER2-induced mammary tumorigenesis. A long latency period for tumor initiation is required for the acquisition of activating mutations such as in-frame deletion or insertion at the juxtamembrane region of Neu transgene (30–32). As these
mutations have not been found in human breast cancer, several studies have suggested epigenetic mechanisms for the expression of truncated p95HER2, for example, alternative translation initiation or an alternative spliced form of HER2 resembling Neu-derived somatic mutations (33–35). In this respect, p95HER has been found in subgroups of human patients with breast carcinoma, resulting in constitutively hyperactive dimers with intermolecular disulfide bonds (26, 36). Transgenic recapitulation studies showed that p95HER2 transgene-induced tumors lead to more aggressive and metastatic phenotype with relatively short latency period compared with full-length HER2-induced tumors (37). p95HER2 fragments are suggested to arise through mechanisms of proteolytic shedding by MMPs or translation of HER2 internal initiation codons (26, 38). In the present study, AMF-enhanced HER2 shedding suggests that AMF participates in HER2 oncogenic and aggressive progression through the epigenetic event of AMF secretion. As HER2 overexpression positively controls AMF secretion (Fig. 5A), the positive feedback loop of HER2 overexpression, AMF secretion and p95HER2 generation could be speculated in HER2-induced cancer progression. AMF secretion may also be controlled by other mechanisms, as the secretion level of AMF is higher in high motility cells (MDA-MB-231) compared with low motility cells (T47D), regardless of the similar expression levels of HER2 by both the cell lines (Supplementary Fig. S4).

Although the mechanisms of resistance to Herceptin/trastuzumab by breast tumors have been delineated at a preclinical level, it is still to be established in patients and remain a challenge for the clinicians. In vitro studies suggested Herceptin/trastuzumab could inhibit the shedding of p185HER2 (36, 39) and retrospective studies in patients with breast cancer revealed a high association between the predisposition of p95HER2 and clinical resistance to Herceptin/trastuzumab (8, 9). Previously, AMF was identified as a serum tumor marker in patients with breast cancer (18). It was also reported that Herceptin could effectively block both ligand-induced and constitutive expression of AMF associated with high HER2 overexpression, implying a role of the AMF pathway in the action of Herceptin (40). Here, we have documented that AMF treatment of breast carcinoma cells suppresses the effect of Herceptin/trastuzumab by overcoming both p-AKT/p-ERK levels and protecting cell growth through p95HER2 generation (Fig. 4). We suggest that high AMF-secreting HER2-positive cancer cells could initially and intrinsically have the advantage of resistance to Herceptin/trastuzumab. Therefore, we propose that AMF might be a novel therapeutic target in patients with HER2-overexpressing breast cancer and this target should
be considered in combination with Heceptin/trastuzumab therapy. In summary, AMF might play a role in HER2-driven breast cancer progression and initial resistance of trastuzumab as depicted in Fig. 6.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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