Antihuman Epidermal Growth Factor Receptor 2 Antibody Herceptin Inhibits Autocrine Motility Factor (AMF) Expression and Potentiates Antitumor Effects of AMF Inhibitors

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ABSTRACT

Overexpression of the human epidermal growth factor receptor (HER) 2 has been linked to the development and maintenance of malignant phenotypes in breast tumors. In addition, the growth and dissemination of human cancers are regulated in part by the autocrine motility factor (AMF)/phosphoglucose isomerase shown to be up-regulated by heregulin (HRG) in breast cancer cells. This study was undertaken to explore the effect of anti-HER2 monoclonal antibody 4D5 [Herceptin (HCT)] on AMF expression and the potential of its augmentation by simple sugar AMF inhibitors. Here we show that HCT treatment of high HER2-expressing breast cancer SK-BR3, BT-474, and ZR-75R cells resulted in down-regulation of AMF mRNA and protein. HCT inhibited the ability of HRG to induce AMF expression in cells with a normal HER2 level, and HCT-mediated down-regulation could be reversed by HRG treatment in breast cancer cells with a high HER2 level. HCT also inhibited transcription from a chimeric pGL3-Luc vector-based reporter system containing the 1.8-kb promoter region of human AMF. Treatment of breast cancer cells with the combination of HCT and specific AMF inhibitors, erythrose 4-phosphate or D-mannose 6-phosphate, resulted in an additive inhibitory effect on both the growth rate and invasiveness of cells as compared with treatment with each agent alone. Results presented here suggest that HCT can 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 HCT. Accordingly, the combination of AMF inhibitor with HCT can potentiate the growth-inhibitory and anti-invasive action of HCT in breast cancer cells.

INTRODUCTION

Growth factors and their receptors play an important role in the regulation of epithelial cell growth. Abnormalities in the expression, structure, or activity of proto-oncogene products contribute to the development and the pathogenesis of cancer. For example, HER2 (1) is overexpressed and/or amplified in a number of human malignancies, including breast, ovarian, colon, lung, prostate, and cervical cancers. Recently, HER3 and HER4 have been added to the family because they share sequence homology with the tyrosine kinase domain of HER1 (2). Regulation of these receptor family members is complex, and they can be transactivated in a ligand-dependent manner. For example, binding of HRG to HER3 or HER4 can activate HER2 as a result of HER2/HER3 or HER4/HER2 heterodimeric interactions (3, 4). HER1 and HER2 have been shown to induce transformation in recipient cells, possibly due to excessive activation of signal transduction pathways. Because growth factors regulate the proliferation of cancer cells by activating cell surface receptors, one approach to controlling cell proliferation is to use anti-receptor blocking monoclonal antibodies that block growth factor receptor-mediated autocrine/paracrine growth stimulation. The humanized form of anti-HER2 monoclonal Ab, HCT, inhibits the growth of breast cancer cells overexpressing HER2 (5, 6) and is currently being used as an effective drug against some forms of breast cancer (7). Anti-receptor Abs are known to inhibit many processes, including mitogenesis, cell cycle progression, invasion and metastasis, angiogenesis, and DNA repair (8).

In addition to HRG action, it is increasingly accepted that the progression of breast cancer cells to a more invasive phenotype may also involve the AMF (9). AMF, a C-X-C cytokine, was originally distinguished by its ability to stimulate the migration of AMF-producing tumor cells via a unique seven-transmembrane receptor (gp78)-mediated pathway (10). When

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3 The abbreviations used are: HER, human epidermal growth factor receptor; HRG, heregulin b1; HCT, Herceptin; AMF, autocrine motility factor; E4P, erythrose 4-phosphate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PHI, phosphohexose isomerase; Ab, antibody; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; M5P, mannose 5-phosphate; GPI, glucose phosphate isomerase; Luc, luciferase.
AMF was originally found to be the neuroleukin/PHI, it was shown that specific PHI inhibitors (carbohydrate phosphates) inhibited both enzymatic activity and AMF-induced cell motility (11). More recently, the relationship between motile and enzymatic activities was further corroborated by us following the crystallization of the molecule with the sugars (12). Of note, in addition to the well-established neuroleukin activity of the molecule, other activities of extracellular AMF unrelated to sugar metabolism were reported in rheumatoid arthritis (13, 14), learning and memory formation (15), cavolae function (16), and angiogenesis (17).

Recently, the AMF has been identified as PHI, a molecule previously described as the extracellular cytokine neuroleukin (18). In light of their motility-regulating effects, the AMF and its receptor have been proposed to play a role in the metastasis of cancer (19). Elevated serum AMF/PHI levels were used as a tumor marker in patients with breast, colorectal, lung, kidney, and gastrointestinal carcinomas, and the relative activities are well correlated with the development of metastasis (9, 18–22). The functions of AMF have been shown to be inhibited by specific AMF sugar inhibitors including E4P and D-mannose 6-phosphate (23).

Previously, we have shown that HRG regulates the expression and functions of AMF in breast cancer cells (24). Furthermore, HRG-induced invasiveness of human breast cancer MCF-7 cells was partially blocked by neutralizing Abs against AMF (24). Because HRG is known to promote the motility and invasiveness of breast cancer cells via the HER3/HER2 heterodimer, we questioned the significance of HER2 in the regulation of AMF expression using the therapeutic anti-HER2 blocking Ab HCT. HCT treatment of breast cancer cells resulted in down-regulation of AMF mRNA and AMF promoter activity and blocked HRG-induced stimulation of AMF expression and promoter activity. Furthermore, cotreatment of breast cancer cells with HCT and AMF sugar inhibitors was accompanied by an additive growth-inhibitory effect as compared with treatment with each agent alone. These results suggest a potential usefulness of the combination of HCT with simple sugar AMF inhibitors against human breast cancer cells.

MATERIALS AND METHODS

Cell Culture and Reagents. Human breast cancer MCF-7, SK-BR3, ZR-75R, and BT-474 cells were maintained in DMEM:Ham’s F-12 (1:1) supplemented with 10% FCS. Recombinant HRG was purchased from Neomarkers, Inc. (Free- mont, CA), and secondary Abs were purchased from the Sigma Chemical Co. (St. Louis, MO) and Molecular Probes. Monospecific polyclonal Ab directed against AMF was generated by immunization with the synthetic peptide YFQQGDMSNGKY-ITK, corresponding to amino acid sequence 351–366 of human AMF (9).

Cell Extracts, Immunoprecipitation, and Immunoblotting. To prepare cell extracts, the cells were washed three times with PBS and lysed in buffer [50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 0.5% NP40, 100 mM NaF, 200 mM NaVO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotenin] for 15 min on ice. The lysates were centrifuged in an Eppendorf centrifuge at 4°C for 15 min. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with the appropriate Abs. An equal number of cells was metabolically labeled for 12 h with 100 μCi/ml [35S]methionine in methionine-free medium or for 4 h with 1.5 mCi/ml [33P]Pi in phosphate-free medium containing 2% diazylated fetal bovine serum in the absence or presence of HCT. Cell extracts and conditioned medium (equal trichloroacetic acid-precipitable counts) were immunoprecipitated with the AMF Ab, resolved on a 10% SDS-PAGE gel, and analyzed by autoradiography.

Northern Hybridization. Total RNA was isolated using Trizol reagent (Life Technologies, Inc.), and 20 μg of each RNA were resolved on a 1.2% agarose gel after ethidium bromide staining. A 1.1-kb human AMF cDNA fragment was used as a probe (9). 28S and 18S were used to assess the integrity of the RNA, and for RNA loading and transfer control, the blots were routinely reprobed with human GAPDH cDNA.

Transfection and Promoter Assays. Cells were split in a 6-well tissue culture plate (Falcon) 24 h before transfection. Subconfluent cells were transiently transfected with pGL3-GPI-Luc (25) or with pGL3 control vector using the LipofectAMINE method (Life Technologies, Inc.). After 5 h of transfection, medium was changed to DMEM containing 10% serum. After 24 h, cultures were shifted to 0% serum for HRG (1 ng/ml) treatment or 2% serum for HCT (50 nM) or IgG (50 nM) treatment for 12 h before harvesting. Luciferase activity was measured 48 h after transfection using a luciferase assay kit (Promega). Each experiment was repeated three to five times, and transfection efficiency varied between 30% and 50%.

Cell Growth Assay. BT-474 human breast cancer cells were plated in 24-well plates in RPMI 1640 (Life Technologies, Inc.) supplemented with insulin, l-glutamine, and 10% fetal bovine serum. After an overnight incubation, cells were treated with E4P, M5P, HCT (anti-HER-2/neu Ab; a gift from Genentech, Inc., San Francisco, CA), or HCT with E4P or M5P agents. Cells were allowed to grow for 4 days at 37°C in an atmosphere of 5% CO2. Microscopic cell count showed that untreated BT-474 cells had a 1.5-fold increase in cell number over the 4-day period. The medium was removed, and 1 ml of the tetrazolium dye MTT (Sigma Chemical Co.) was added. After a 1-h incubation at 37°C in a 5% CO2 atmosphere, the MTT was removed, and isopropanol/1 N HCl (96:4) was added. Absorbance at 570 nm was determined on a Bio-tek (Bio-tek Instruments, Winooski, VT) automated microtiter plate reader. Identical concentrations and combinations were tested in 3 separate wells/assay, and the assay was performed three times, as described previously (26).

Chemoinvasion Assay. To measure invasiveness of MCF-7 cells to different treatments, 8 μm filters were coated with Matrigel (20 μg/filter). The coated filters were placed in the Boyden chambers. Cells (103) suspended in serum-free medium containing 0.1% BSA were added to the upper chamber. The filter inserts with cells were placed in wells of a 24-well culture plate containing 650 μl of serum-free medium containing 0.1% BSA as control or medium plus 50 nM HCT in the presence or absence of 100 nM E4P. Conditioned medium from mouse fibroblast NIH3T3 cells was used as a source of chemotacticant and placed in the lower compartment of the Boyden chambers. HRG (30 ng/ml) combined with fibronectin (25 μg/ml) was also used as a chemotacticant in the lower com-
partment of the chambers. After 24 h of incubation at 37°C, nonmigrating cells were scraped off, and the cells were stained with H&E. Six randomly selected fields on each filter were then counted, as described previously (27).

RESULTS AND DISCUSSION

AMF, an Anti-HER2 Ab-regulated Gene Product. To examine the potential involvement of AMF in the action of HER2, we examined the effect of the anti-HER2 blocking Ab HCT on the levels of AMF mRNA expression in SK-BR3, BT-474, and ZR-75R breast cancer cells, which are known to express high levels of HER2 (28). Data in Fig. 1A demonstrate that HCT decreases the steady-state levels of the 1.6-kb transcript of AMF by 2–6-fold in SK-BR3 cells in a time-dependent manner. The down-regulation of AMF mRNA was also observed in other high HER-2-expressing cells such as BT-474 (Fig. 1B) and ZR-75R (Fig. 1C).

Because HRG is known to up-regulate AMF expression (24), we questioned whether HRG could reverse the HCT-induced down-regulation of AMF mRNA. The SK-BR3 cells were treated with HCT in the presence or absence of HRG, and the effect on the expression of AMF mRNA was examined. As shown in Fig. 2A, HRG treatment of SK-BR3 cells (high HER2-expressing cells) was accompanied by a modest up-regulation of AMF mRNA expression level as compared with the relatively high AMF mRNA level in untreated control cells. However, HCT treatment resulted in a significant down-regulation of AMF mRNA level compared with the level in control cells, and this was prevented by cotreatment with HCT and HRG. Because HCT interferes with HER2 function (27), and because HRG enhances AMF mRNA expression via the HER2/HER3 complex, these results imply that HER2 regulates AMF expression in breast cancer cells.

Regulation of AMF Promoter Activity by HCT. To determine whether the observed down-regulation of AMF mRNA expression by HCT resulted from inhibition of AMF promoter activity, we examined the effect of HCT on transcription of a chimeric pGL3-Luc vector-based reporter system containing the 1.8-kb promoter region of human GPI/AMF (19). MCF-7 cells and BT-474 cells were transiently transfected with pGL3-GPI, and activity was tested in the presence or absence of HRG, HCT, or both. As illustrated in Fig. 2B, HCT down-regulated AMF promoter activity by 2.5–3-fold as compared with the level in untreated control cells. In contrast, HRG significantly stimulated AMF promoter activity in a HCT-sensitive manner (Fig. 2C).

HCT Down-Regulates AMF Protein. Recently, AMF has been shown to undergo posttranslation modification and to exist in five variant forms (Mr 65,000, Mr 57,000, Mr 46,000, Mr 38,000, and Mr 31,000); the Mr 46,000 form exists in HT1080 fibrosarcoma cells (9). The AMF forms are derived from a single gene by alternative splicing, posttranslational modifications, or both (9). To determine, whether the observed decrease in the level of AMF mRNA by HCT in human breast cancer
cells was associated with a decrease in the expression of AMF protein and its phosphorylation. We examined the synthesis of AMF protein in BT-474 and SK-BR3 cells metabolically labeled with [35S]methionine or [32P]Pi. HCT treatment led to a decreased expression of a newly synthesized M, 38,000 AMF protein species (Fig. 3, A and B) and its phosphorylation (Fig. 3C).

**HCT Potentiates the Action of AMF Inhibitors.** Because HCT reduced the expression of AMF, we explored whether it could potentiate the activity of specific AMF sugar inhibitors (17). Breast cancer BT-474 cells were treated with E4P, D-mannose 6-phosphate, and HCT, alone or in combination, to determine the effect of these sugar inhibitors on cell proliferation in the presence of HCT. As shown in Fig. 4A, treatment with E4P and HCT alone inhibited the growth of breast cancer cells by 15% and 42%, respectively, compared with the growth rate of untreated cells, whereas the combination of the two agents resulted in an additive growth inhibition of 65%. Similarly, treatment of MCF-7 cells with HCT and E4P resulted in a reduction of cell motility by 21 ± 3% and 24 ± 3%, respectively (Fig. 4B), whereas the combination of HCT and E4P reduced the motility of the cells by 53 ± 4%. These results suggest the potential usefulness of combining HCT with AMF sugar inhibitors against human breast cancer cells.

Abnormality in the action of HER2 has been shown to be frequently associated with increased motility and progression of breast cancer cells to a more invasive and aggressive phenotype. Because motility and invasiveness of human tumors have been shown to be associated with AMF expression, we investigated the effect of HER2 blockade by HCT on AMF expression and whether AMF inhibitors could augment the effect of HCT on the growth and invasion of breast cancer cells. Results presented here have shown that HCT down-regulates the expression of AMF in breast cancer cells with HER2 overexpression. Furthermore, HCT, in combination with specific AMF inhibitors, led to an additive inhibitory effect on both the growth rate and invasiveness of cells as compared with treatment with each agent alone. These findings imply a role of the AMF pathway in the action of HCT and that AMF inhibitor can potentiate the action of HCT in breast cancer cells.

**REFERENCES**


