Herulin Regulation of Autocrine Motility Factor Expression in Human Tumor Cells

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ABSTRACT

The exposure of cells to growth factors has been shown to induce cytoskeleton reorganization, leading to stimulation of cell motility and invasion. Herulin β1 (HRG), a combinatorial ligand for human epidermal growth factor receptor 3 and human epidermal growth factor receptor 4 receptors, is a regulatory secretory polypeptide with a distinctive function in promoting motility and invasiveness of breast cancer cells. In addition to HRG, motility and invasiveness of tumor cells may also involve up-regulation of expression and function of the autocrine motility factor (AMF). Here we explored the possible involvement of AMF in the motility-promoting action of HRG in the MCF-7 breast cancer cell model system. We report that HRG increases the expression of AMF mRNA by 3–8-fold in an actinomycin D-sensitive manner and does not require de novo protein synthesis. The HRG-induced stimulation of AMF expression was inhibited by specific inhibitors of p42/44MAPK and p38MAPK kinases, but not by an inhibitor of the phosphatidylinositol 3'-kinase pathway. Other HRG-responsive human cell lines demonstrated that HRG does indeed significantly up-regulate AMF expression. Furthermore, HRG-stimulated increased motility was partially suppressed by inclusion of an anti-AMF antibody to breast cancer cells, suggesting that a HRG-mediated increase in cell motility may be mediated, at least in part, via induction of AMF. The present study is the first demonstration of AMF regulation by a growth factor and suggests a potential role for AMF in HRG regulation of breast cancer cell motility and a novel function of HRG as a regulator of motility factor expression.

INTRODUCTION

Growth factors and their receptor interactions play an essential role in the regulation of epithelial cell proliferation, and abnormalities in growth factor expression and action may contribute to the progression and maintenance of the malignant phenotype. For example, HER2 overexpression is frequently (in 30% of patients with breast cancer) associated with an aggressive clinical course, shorter disease-free survival time, poor prognosis, increased sensitivity to chemotherapeutics, and increased metastasis in human breast cancer (1). Recently, additional members, HER3 and HER4, have been added to the HER2 family. All HER receptors share a sequence homology with the tyrosine kinase domain of HER1 (2–4). The regulation of HER family members is complex because HER family receptors can be transactivated by receptor-receptor interaction in a ligand-dependent manner (3) and can therefore use more than one pathway to transduce their biological functions. For example, HER3 and HER4 receptors bind to more than a dozen isoforms of HRGs or neu differentiation factors (5, 6) and can activate the HER2 receptor due to heterodimeric interactions (3, 4, 7). HRG stimulation of breast cancer cells enhances activation of PI3k, mitogen-activated protein kinase, and p38MAPK kinase (8, 9). A ligand that interacts with HER2 in the absence of other HER family members has yet to be identified. In recent years, accumulating evidence suggests that the progression of human breast cancer cells may be regulated by HRG, a combinatorial ligand for HER3 and HER4 receptors (5, 6). Recently, we and others (9–12) have demonstrated that HRG activation of breast cancer cells (in the absence of HER2 overexpression) also promotes the development of more aggressive phenotypes in breast cancer cells. The activation of HRG signaling (10, 13), activator protein 1 (14), and nuclear factor κB (15) pathways has also been linked with the progression of breast cancer cells to a more invasive phenotype. These observations suggest that ligand-driven activation of HER receptors may play an important biological role or roles in the progression of breast cancer cells to a malignant phenotype. The nature of the pathways by which HRG signals may modulate the expression of the motility factor or factors remains poorly understood.

The exposure of cells to growth factors has been shown to cause cytoskeleton reorganization, formation of lamellipodia, membrane ruffling, and altered cell morphology and has accordingly been implicated in stimulating cell migration and invasion (16, 17). Most eukaryotic cells possess the capacity to migrate over or through a substrate, and cell motility plays a key role in both normal and pathological cellular physiology, exemplified in the latter by invasion and metastasis (18). In many tissues, cells are stationary, but motility can be activated by appropriate stimuli, oncogenic transformation, or both. In fact, one of the earliest responses of cells to many extracellular growth factors is rapid reorganization of their cytoskeleton and cell shape.

In addition to HRG action, it is increasing accepted that the progression of breast cancer cells to a more invasive phenotype may also involve the AMF (19). AMF was originally distinguished by its ability to stimulate the migration of AMF-producing tumor cells via a receptor (gp78)-mediated pathway (20–22). Recently, the AMF has been identified as phosphohexose isomerase, a molecule previously described as the extracellular cytokine neuroleukin (23). In light of their motility-regulating effects, the AMF and its receptor have been proposed to play a role in the metastasis of cancer (24). Expression of the AMF pathway correlates well with disease progression in numerous cancers including colorectal, bladder, esophageal, and gastric cancer (25–28).

HRG was shown to stimulate the motility and invasiveness of human breast cancer cells (9, 13), but it is still unknown whether there is any role for AMF in the noticed action of HRG in breast cancer cells. Here we investigate HRG’s potential regulation of AMF expression and function in breast cancer cells and demonstrate that HRG stimulation of noninvasive MCF-7 human breast cancer cells leads to the induction of expression of AMF mRNA and protein. Extension of these observations to two other HRG-responsive human cell lines also demonstrates a significant capacity of HRG to up-regulate AMF expression. The HRG-induced stimulation of AMF expression was suppressed by specific inhibitors of p42/44MAPK and p38MAPK kinases, but not by an inhibitor of the PI3k pathway. Furthermore, HRG-stimulated enhancement of cell motility-associated changes are not due to a direct effect of HRG on the expression of other motility factors.

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3 The abbreviations used are: HER, human epidermal growth factor receptor; HRG, herulin β1; AMF, autocrine motility factor; PI3k, phosphatidylinositol 3'-kinase; Ab, antibody; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FACS, fluorescence-activated cell sorting.

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such as cell scattering and actin reorganization in breast cancer cells was partially suppressed by monospecific anti-AMF polyclonal Ab. The present study is the first to show regulation of AMF expression by a polypeptide growth factor and suggests that AMF may have an augmenting role in HRG’s regulation of breast cancer cells.

MATERIALS AND METHODS

Cell Cultures and Reagents. Human breast cancer MCF-7 cells (9) and colorectal carcinoma cell lines LS174T, CaCo-2, and FET (29) were maintained in DMEM:Ham’s F-12 (1:1) supplemented with 10% FCS. Recombinant HRG was purchased from Neomarkers, Inc. (Freemont, CA), and secondary Abs were purchased from Sigma Chemical Co. (St. Louis, MO) and Molecular Probes. Monospecific polyclonal Ab directed against AMF was generated by immunization with a synthetic peptide YFQQGDMESNGKY-ITK, corresponding to amino acids 351–366 of human AMF, as described previously (19).

Cell Extracts and Immunoprecipitation. 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 Na2VO3, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin] for 15 min on ice. The lysates were centrifuged in an Eppendorf centrifuge at 4°C for 15 min. Cell lysates containing equal amounts of protein were resolved on a SDS-PAGE, transferred to nitrocellulose, and probed with the appropriate Abs. An equal number of cells were metabolically labeled for 12 h with 100 μCi/ml [35S]methionine in methionine-free medium containing 2% dialyzed fetal bovine serum in the presence or absence of HRG. Cell extracts (equal permeable trichloroacetic acid charge) were immunoprecipitated with the desired Ab or control Ab, and resolved on a SDS-PAGE gel, and analyzed by autoradiography (30).

Northern Hybridization. Total RNA was isolated using Trizol reagent, and 20 μg of each RNA were resolved on a 1% agarose gel after ethidium bromide staining. A 1.1-kb human cDNA fragment was used as a probe (19). GAPDH levels were used to assess the integrity of the RNA and of the RNA-loading control.

Analysis of F-Actin Distribution and Cell Scattering. For F-actin staining, cells cultured on glass coverslips or in chamber slides were fixed with 3.7% paraformaldehyde, followed by a short incubation with acetone at −20°C as indicated by the manufacturer. In some cases, coverslips were washed twice with PBS and lysed in buffer [50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 0.5% NP40, 100 mM NaF, 200 mM Na2VO3, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin] for 15 min on ice. The lysates were centrifuged in an Eppendorf centrifuge at 4°C for 15 min. Cell lysates containing equal amounts of protein were resolved on a SDS-PAGE, transferred to nitrocellulose, and probed with the appropriate Abs. An equal number of cells were metabolically labeled for 12 h with 100 μCi/ml [35S]methionine in methionine-free medium containing 2% dialyzed fetal bovine serum in the presence or absence of HRG. Cell extracts (equal permeable trichloroacetic acid charge) were immunoprecipitated with the desired Ab or control Ab, and resolved on a SDS-PAGE gel, and analyzed by autoradiography (30).

Human Tissue Samples. Human breast tissue samples were obtained from patients who had surgery for breast cancer, frozen in liquid nitrogen, and stored at −80°C (13). Three samples each of grade 2 and grade 3 disease were analyzed (13). The tissue samples were homogenized in TritonX-100 lysis buffer [20 mM HEPES, 150 mM NaCl, 1% Triton X-100, 0.1% deoxycholate (v/v), 2 mM EDTA, 2 mM DTA, 2 mM sodium orthovanadate, and protease inhibitor mixture (Boehringer Mannheim)] and equal amounts of proteins were analyzed by Western blotting.

RESULTS AND DISCUSSION

Regulation of AMF mRNA Expression by HRG. To examine the potential involvement of AMF in the action of HRG, we explored the regulation of AMF mRNA expression in MCF-7 cells, which are known to respond to HRG by increased cell motility (9, 13). Total RNA was isolated from control cells and HRG-treated cells, and expression of AMF mRNA was analyzed by Northern hybridization using a cDNA to human AMF (19). Data in Fig. 1A demonstrate that HRG increases the steady-state levels of the 1.6-kb transcript of AMF by 2–6-fold in MCF-7 cells, with the maximal induction taking place between 3 and 12 h after HRG treatment, followed by a decline to near basal levels by 24 h. The observed up-regulation of AMF mRNA in MCF-7 cells was a specific effect of HRG because there was no effect of HRG on the expression of the AMF receptor gp78 (Fig. 1B).

HRG may enhance the expression of AMF mRNA due to increased synthesis of newly transcribed mRNA, increased stability, or both. To determine which of these was the case, we examined the effect of actinomycin D, an inhibitor of transcription, on specific mRNA expression. Treatment of cells with actinomycin D completely abol-

Fig. 1. HRG regulation of AMF mRNA expression. A, MCF-7 cells were treated with HRG for the indicated times. Total RNA (20 μg) was analyzed by Northern blotting using an AMF cDNA probe. The blot was reprobed with GAPDH cDNA probe. Quantitation of AMF mRNA is shown in the bottom panel. B, total RNA levels from MCF-7 cells were analyzed by Northern blotting using AMF cDNA and reprobed with gp78 cDNA.
ished the HRG-mediated induction of AMF mRNA, suggesting that HRG regulates AMF at the transcriptional level (Fig. 2). To study translational regulation, we used cycloheximide, a translational inhibitor. Cycloheximide inhibited the expression of AMF mRNA (Fig. 2, compare Lane 4 with Lane 1), while HRG treatment further stimulated the expression of AMF mRNA compared to the level in cycloheximide treated culture (Fig. 2, compare Lane 3 with Lane 4), suggesting no requirement for a de novo protein synthesis for HRG-mediated stimulation of AMF mRNA expression.

HRG is known to activate a number of signaling pathways, including PI3k, p42/44 MAPK, and p38 MAPK pathways (9). As expected, HRG treatment of MCF-7 cells stimulated the activation of PI3k, p42/44 MAPK, and p38 MAPK via a kinetic mechanism similar to HER2 phosphorylation (date not shown). To understand the nature of the HRG signaling pathway leading to AMF mRNA expression, we used three inhibitors (Ly294002, PD980599, and SB203580) that specifically inhibit PI3k, p42/44 MAPK, and p38 MAPK, respectively. Pretreatment of cells with Ly294002 had no inhibitory effect on the HRG-mediated up-regulation of AMF mRNA (Fig. 3, bottom panel). In brief, these results suggest that p42/44MAPK and p38MAPK pathways may be involved in HRG-mediated stimulation of AMF.

HRG Induces the Expression of AMF Protein. Recently, AMF has been shown to exist in five variant forms, Mr 65,000, Mr 57,000, Mr 46,000, Mr 38,000, and Mr 31,000 in HT1080 fibrosarcoma cells (19). It was proposed that these AMF forms may be derived from a single gene by alternative splicing, posttranslational modifications, or both (19). To determine whether the observed increase in the level of AMF mRNA in HRG-treated MCF-7 breast cancer cells was associated with an increase in the expression of AMF protein, Western blot analysis was performed. The results in Fig. 4A demonstrate that MCF-7 cells responded to HRG by a significant specific increase in AMF protein.

Fig. 2. HRG induces AMF transcription. MCF-7 cells were treated with cycloheximide (50 μg/ml) or actinomycin D (10 μg/ml) in the presence or absence of HRG (10 ng/ml) for 3 h. Total RNA was isolated, and the levels of AMF mRNA were as detected by Northern blotting. The blot was reprobed with a GAPDH cDNA probe. Quantitation of AMF mRNA is shown in the bottom panel.

Fig. 3. Effects of inhibitors PD98059, SB203580, and LY294002 on AMF expression in HRG-treated cells. MCF-7 cells pretreated for 30 min with or without inhibitors were cultured with or without HRG for 4 h. Total RNA was isolated, and expression of AMF mRNA was detected by Northern blotting. The blot was reprobed with a GAPDH cDNA probe. Quantitation of AMF mRNA is shown in the bottom panel.

Fig. 4. HRG up-regulates the level of AMF protein. A, MCF-7 cells were treated with HRG for the indicated times. Total lysates were subjected to SDS-PAGE, blotted with anti-AMF Ab, and subsequently reprobed with preimmune serum. B, conditioned medium from control and HRG-treated MCF-7 cells was concentrated, analyzed by SDS-PAGE, immunoblotted with anti-AMF Ab (Lanes 1–3), and reprobed with preimmune serum (Lanes 1’–3’). C, MCF-7 cells were stimulated with HRG for 8 h and metabolically labeled with [35S]methionine during the last 4 h before harvesting. Cell lysates were immunoprecipitated with an anti-AMF Ab and analyzed by SDS-PAGE, followed by fluorography. *, AMF protein.
the level of the Mr 38,000 form of AMF protein, which was not detected by preimmune serum. Because earlier studies have shown that the Mr 57,000 form of AMF may be secreted in HT1080 fibrosarcoma cells (19), we also examined the expression of AMF in the conditioned medium of MCF-7 cells treated with or without HRG (Fig. 4B). In contrast to the fibrosarcoma cells, HRG treatment appears to increase the accumulation of the Mr 38,000 form of AMF in epithelial cells. To further validate the induction of AMF in HRG-treated cells, we examined the synthesis of AMF protein in MCF-7 cells metabolically labeled with [35S]methionine. HRG treatment resulted in the increased expression of newly synthesized AMF within 8 h (Fig. 4C).

To further characterize the regulation of AMF expression in HRG-treated cells, we next examined the redistribution of the AMF protein by indirect immunofluorescence and by confocal analysis. To identify the nuclei, we used the DNA intercalating agent 4',6-diamidino-2-phenylindole. The AMF protein was detected using a FITC-coupled secondary goat Ab directed against the polyclonal rabbit anti-AMF
Ab. The AMF protein was localized primarily in the cytoplasm in MCF-7 cells (Fig. 5A). The observed AMF staining was specific because there was no such immunoreactivity with the preimmune serum (data not shown). After the HRG treatment, AMF accumulated in the cytoplasm, as shown by an increased intense staining (Fig. 5). However, in HRG-treated cells, a significant amount of AMF was also localized at intercellular boundaries as well as in the plasma membrane. Taken together, the results demonstrated that HRG-induced AMF expression was accompanied by an alteration in the subcellular distribution of AMF.

An enhancement of AMF immunoreactivity at the plasma membrane suggested that HRG stimulation may be related to an increase in membrane-bound AMF in MCF-7 cells. To explore this possibility, we analyzed the cell surface expression of AMF by flow cytometry using anti-AMF Ab and fluorescence (FITC)-tagged antirabbit IgG on unfixed cells (Fig. 6). The mean fluorescence of control cells was 15–20%, and this was increased to 45% by HRG treatment. These results indicated that HRG-mediated up-regulation of AMF was accompanied by an increase of cell surface expression of AMF in MCF-7 cells. The increase in the level of cell surface AMF was specific because there was no such increase when preimmune serum was used.

**Effect of Anti-AMF Ab on HRG-mediated Increased Cell Motility.** Because HRG has previously been shown to be a potent motogen (9, 13) and induces AMF expression, and because AMF stimulates cell motility in other cell types (this study) (31, 32), we explored the possibility of a meditative role of AMF during HRG-induced cell motility. As seen in Fig. 7, anti-AMF Ab, but not preimmune serum, suppressed the cell scattering by 40% in HRG-treated MCF-7 cells, suggesting that the HRG-mediated increase in cell motility may involve, at least in part, AMF in MCF-7 cells.

**HRG Regulates AMF Expression in Diversified Cell Types.** To determine whether the observed induction of AMF expression is an effect restricted to MCF-7 cells or more general to HRG-responsive cells, we examined the HRG-AMF relationship in HRG-responsive human colorectal FET and LS174T cells (28). As illustrated in Fig. 8, HRG treatment led to a significant up-regulation of AMF mRNA expression in tumor cells (Fig. 8A). The observed induction of AMF mRNA expression may not be a universal property of stimulatory growth factors, because transforming growth factor α and HB-epider-
eral growth factor only modestly induced AMF mRNA expression in FET cells (Fig. 8B). These results suggest that AMF expression could be up-regulated more effectively by a polypeptide factor with motility-promoting action, such as HRG.

AMF Expression and Breast Cancer. Because HER family members are known to be overexpressed in a number of tumors and cancer cell lines, we explored the potential relationship between the levels of HER family members and AMF expression. There was no correlation between the status of HER family members and expression of AMF variants, at least in the seven breast cancer cell lines analyzed in this study (Fig. 9A). However, the M, 38,000 form of AMF was readily detectable in cells with tumorigenic potential (such as BT-474 and MDA-453 cells) or metastatic potential (such as MDA-435 cells). These results suggested that breast cancer cells may express certain variants of AMF, the regulation of which may be complex. We also examined AMF expression in a small number of tumor biopsy samples. Three samples each from grade 2 and grade 3 breast tumors were analyzed. In general, grade 3 specimens had a higher level of AMF variants than grade 2 specimens (Fig. 9B). Additional studies using a large number of clinical samples are needed to confirm these findings.

Abnormality in the action of the HER2 receptor and HRG, which activates the HER2 receptor via its binding to HER3 or HER4 receptors, has been shown to be frequently associated with increased motility and progression of breast cancer cells to a more invasive and aggressive phenotype. Because both motility and invasiveness of human tumors have been shown to be influenced by AMF, we questioned the regulation of AMF expression by HRG and showed that HRG enhances the AMF expression of AMF mRNA without de novo protein synthesis. The observed HRG-induced stimulation of cell motility was accompanied by increased expression of cellular AMF protein and localization of AMF at the cell surface, and cotreatment with anti-AMF Ab (but not preimmune serum) partially blocked HRG-induced cell motility. Another notable finding was the possible involvement of p44/42MAPK and p38MAPK in mediating the stimulation of AMF mRNA by HRG. In this context, we recently demonstrated that inhibition of p38MAPK leads to suppression of HRG-induced stimulation of cell spreading and scattering of breast cancer cells (13, 33). Because AMF has been shown to promote cell spreading (33), and because HRG may use the p38MAPK-dependent signal transduction pathway to induce both cell spreading (33) and AMF expression (this study), the role of p38MAPK in the action of HRG may be important. However, inducible expression of AMF may also involve distinctive HRG-initiated signals such as p44/42MAPK, which may not be required for HRG-induced cell spreading and scattering effects (33). These results suggest that different functions of HRG may be regulated by a combination of specific and overlapping signaling pathways. This hypothesis is further supported by our recent finding that HRG stimulation of PI3k is required for actin reorganization (9) but does not influence cell spreading (13) or AMF expression (this study). In summary, we show here that HRG may also induce the expression of other motogen molecules such as AMF that in concept may contribute to the motility and invasive phenotypes.

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