Heregulin Regulation of Urokinase Plasminogen Activator and its Receptor: Human Breast Epithelial Cell Invasion

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ABSTRACT

Heregulin-β1, which binds human epidermal growth factor receptors 3 and 4, promotes motility and invasiveness of breast cancer cells. Considering the established role of urokinase plasminogen activator (uPA) and its receptor (uPAR) in invasion, this study was undertaken to explore the role of heregulin-β1 in regulating uPA and uPAR in breast cancer invasion. The stimulation by heregulin-β1 of noninvasive human breast cancer MCF-7 cells induced the expression of uPA mRNA, protein, and its plasminogenic activity. This uPA mRNA expression was blocked by a transcriptional inhibitor, actinomycin D, and does require de novo protein synthesis for its optimal induction in MCF-7 cells, but not in mouse mammary epithelial HC11 cells. Heregulin-β1 also induced the expression of uPAR mRNA and protein in an actinomycin D-sensitive manner and cycloheximide superinduced the uPAR mRNA. Heregulin-β1-stimulated signaling initiated the transcription from uPA- and uPAR-promoters. These results suggest that heregulin-β1 regulation of breast cancer cell invasion may be mediated in part through the up-regulation of uPA and uPAR.

INTRODUCTION

Abnormalities in the expression and action of growth factor contribute to the progression and maintenance of the malignant phenotype in breast cancer. For example, overexpression of the EGF receptor or the HER-2 family of tyrosine kinase (typical in 20–30% of breast cancers) is frequently associated with an aggressive clinical course, short disease-free survival periods, poor prognosis, and higher metastasis in human breast cancer (1). In addition to HER2 overexpression, accumulating evidence suggests that HRG that binds to HER3 and HER4 receptors also promotes the development of more aggressive/invasive phenotypes in breast cancer cells (2–7). The mechanisms and pathways by which HRG influences the biology of breast cancer cells remain elusive.

Localized breast cancer, before metastasis, can be cured with surgery. The high mortality rate associated with breast cancer, however, results from a propensity for the tumor to metastasize while the primary site is small and undetected. The process of metastasis requires, among other steps, changes in adhesion, increased migration, the production of proteases, and the invasion of the stroma. One of the important steps for tumor progression and invasion is the destruction of the ECM that separates the epithelial and stromal compartments by serine proteinases such as uPA.

In addition to growth factors, the proteolytic enzyme uPA and its receptor (uPAR; Ref. 8) also regulate cancer invasion and metastasis. The production or expression of uPA/uPAR by either the tumor cells or the neighboring stromal cells is thought to initiate the degradation of ECM components around the cancer cells and the perivascular basement membrane, thus facilitating the migration and invasation of cancer cells. Although several recent studies have established the prognostic value of uPA and uPAR in breast cancer patients (9–12), the issue of uPA/uPAR localization in tumor versus stromal cells or in both is not fully resolved. uPAR expression has been observed in breast cancer sections, predominantly in macrophages, and sometimes in tumor cells (10–13). However, using RNA in situ hybridization, the expression of uPA and uPAR has been detected in both tumor cells and stromal cells (14, 15), and Bastholm et al. (13) observed that seven of nine patients with confirmed lymph node metastasis demonstrated extensive uPAR-positive immunostaining in the primary tumor.

The uPA system consists of uPA serine protease, its cell surface-associated receptor (uPAR), plasminogen-activator inhibitors, and the proenzyme plasminogen that can be activated by uPA to form plasmin. Regulated, spatially localized degradation of the ECM is required for tissue remodeling, invasiveness, and angiogenesis. A lack of regulation in this process is the hallmark of malignancy and enables metastatic tumor cells to invade normal tissue and to obtain a blood supply. uPA is unique among serine proteases in that uPA has its own high-affinity cell-surface receptor, uPAR, which greatly enhances the action of uPA on plasminogen (16, 17). uPAR localizes on cell-cell junctions and to the leading edge of invading cells (18, 19). Thus, uPA is spatially and metabolically positioned to play a pivotal role in the directed cascade of protease activity required for tissue invasion. The binding of uPA to uPAR also initiates signaling cascades that require receptor occupancy but not uPA catalytic activity (20). Recent studies have established that uPAR is an adhesion molecule capable of interacting with components of the ECM and integrins. These activities further support the idea that the uPA system plays a role in cell motility (21–24).

Expression of uPA and uPAR has been demonstrated in every solid tumor type examined to date (25, 26). Expression is not restricted to tumor cells themselves, because several tumor-associated cell types, including macrophages, mast cells, endothelial cells, natural killer cells, and fibroblasts, are all capable of uPA and uPAR expression in various tumor types. The pattern of expression in these cells differs depending upon the type of tumor involved. For example, tumor cells that exclusively express uPAR, but not uPA, can interact with uPA that is made by surrounding stromal cells. This is seen in colon cancer (27), in which invasion and aggressiveness among cell lines correlate strongly with expression of uPAR. Expression of uPA and uPAR in breast cancer sections has been observed in tumor cells (10–13). A significant correlation was shown between advanced breast cancer and high expression levels of uPA, uPAR, or both. uPAR staining occurs only at the invasive edge (tumor-host interface) and is localized mostly in the endothelial cells in breast cancer (8, 12). Because the activation of uPA-dependent proteolysis and uPA-dependent signaling both depend on the binding of uPA to uPAR, inhibition of this interaction between uPA and uPAR has been proposed as a potential therapeutic modality to inhibit tumor progression. Preclinical tumor models using antibodies, high molecular weight inhibitors of uPA-

Received 2/7/00; accepted 10/30/00.

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1 This study was supported in part by NIH Grants CA80066 and CA65746, the Breast Cancer Research Program of the University of Texas M. D. Anderson Cancer Center (to R. K.), and NIH Grants CA58311 and CA10845 (to D. B.).

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3 The abbreviations used are: EGF, epidermal growth factor; HER, human EGF receptor; HRG, heregulin-β1; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; CAT, chloramphenicol acetyltransferase; MEK, MAP/ERK kinase; p38 MAP kinase; p38 mitogen-activated protein kinase; PI-3 kinase, phosphatiidylinositol-3 kinase; Act-D, actinomycin D; NF, nuclear factor; ECM, extracellular matrix.
uPAR interaction, and gene-expression-targeting approaches have demonstrated significant inhibitory effects on tumor growth, metastasis, and angiogenesis (28–30).

Our previous studies showed that HRG stimulates the motility and invasion of human breast cancer cells (5–7). However, it is not known whether uPA and uPAR play a role in HRG action in breast cancer cells. The purpose of this study was to investigate the regulation by HRG of the expression and function of uPA and uPAR in breast cancer cells. The present study is the first to show regulation of uPA and uPAR by HRG. Our results suggest that the uPA/uPAR system mediates, in part, the regulation by HRG of breast cancer cell invasion. Because HRG is a paracrine growth factor secreted from mesenchymal cells, our present findings raise the possibility that HRG-mediated up-regulation of uPA and uPAR in tumor cells enhances the ability of tumor cells to degrade the extracellular milieu, and thereby invade normal tissue.

MATERIALS AND METHODS

Cell Cultures and Reagents. MCF-7 human breast cancer cells were maintained in DMEM/Ham’s F-12 (1:1) supplemented with 10% FCS (5–7). Anti-uPA monoclonal antibody and recombinant HRG were purchased from Neomarkers, Inc. (Fremont, CA). Anti-vinculin antibody was purchased from Sigma, and β-catenin antibody was provided by Pierce McCrea, M. D. Anderson Cancer Center, Houston, TX (31).

Cell Extracts, Immunoblotting, and Immunoprecipitation. For preparation of cell extracts, cells were washed three times with PBS and lysed in buffer [50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 0.5% NP-40, 100 mM NaF, 200 mM NaVO₄, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotonin] 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 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with the appropriate antibodies, using an enhanced chemiluminescence (Amersham) method or alkaline phosphatase-based color reaction method (5–7).

Zymography. The proteinase activity of uPA was measured as described (5). Briefly, equal amounts of proteins from serum-free cell culture supernatants were resolved by nonreducing SDS-PAGE containing 0.1% (w/v) casein (Sigma) and 10 μg/ml plasminogen (Boehringer Mannheim). The gel was first incubated for 2.5 h at 22°C in 50 mM Tris-HCl (pH 7.5) containing 2.5% Triton X-100 (Sigma) and 0.05% sodium azide (Sigma), and then for 16 h at 37°C in 0.1 M Tris-HCI (pH 8.3) containing 0.5% sodium azide and 0.15 M NaCl. The gels were stained with Coomassie Brilliant Blue R250, and the enzymatic conversion of plasminogen to plasmin by uPA was observed as cleared zones in the blue-stained background. Supernatant from MDA-MB-231 cells were used as a positive control in zymographic gels.

Northern Hybridization and Promoter-Luciferase/CAT Assays. Total cytoplasmic RNA was analyzed by Northern hybridization using cDNA probes for human uPA and uPAR mRNA. 28S and 18S RNA were used to assess the integrity of the RNA; for RNA-loading control, the blots were routinely probed with human uPA and uPAR mRNA. 28S and 18S RNA were used to assess the presence or absence of dominant-negative mutants of MEK (35), p38MAPK (7), PI-3 kinase (5, 36), NF-κB (37), or control empty vector. The transfection efficiency was normalized by cotransfection with pSV b-Gal vector (Promega). Luciferase or CAT activity was measured at 24 h. Where indicated, cells were treated with HRG (30 ng/ml) before lysis. Each experiment was repeated with three independent transfections, and transfection efficiency varied between 40–50%.

Immunofluorescence. For indirect immunofluorescence, fixed cells were washed with PBS, blocked by incubation with 10% normal goat serum (Sigma) in PBS for 1 h at ambient temperature. Cells then were allowed to react for 1 h at ambient temperature with rabbit polyclonal antibody against β-catenin and anti-uPA monoclonal antibody. For controls, cells were treated with the secondary antibody only, omitting the primary antibody, and no signals were detected in control untreated cells. Cells were viewed with an inverted Zeiss fluorescence microscope and analyzed by confocal microscopy using the PC-based LSM-510 version 2.01 software (5, 7).

Boyden Chamber Invasion Assays. The cell migration assays were performed as described (5, 7). Briefly, the bottom of the porous 8-μm filter was coated with a thick layer of Matrigel/DMEM/Ham’s F-12 (1:2) that serves as a “chemoattractant” for the cells that are plated on the upper side of the filter. After 12 h, the cells that pass through the pores and invade the Matrigel reattach on the lower surface of the filter. The filters were fixed, stained with propidium iodide (0.1%, w/v), mounted on a slide, and analyzed by confocal microscopy. The cells that invaded the Matrigel were considered invasive, and results were expressed as the percentage of total cells/microscopic field (×40) from both sides of the filter, plus those en passage that were inside the pores. Results were expressed as the percentage of migrating cells compared with the total number of cells (cells present in all Z-sections) and represent the means ± SE of quadruplicate wells from three or more experiments.

RESULTS

HRG Enhances the Expression of uPA in Breast Cancer Cells. The motility and invasiveness of human breast cancer cells are regulated by both HRG (5–7) and uPA (reviewed in Ref. 8). Because both HRG and uPA regulate breast cancer cell invasion, we explored the possible role of HRG regulation of uPA expression and activity using a noninvasive MCF-7 breast cancer cell model system. MCF-7 cells were treated with HRG, and the level of secreted uPA in the conditioned medium was assayed by zymography. HRG induced the secretion of uPA in a time-dependent manner over absent or undetectable levels of uPA in untreated control cells (Fig. 1A). This uPA secretion was caused by up-regulation of uPA mRNA expression in HRG-treated cells, demonstrated by Northern blot analysis hybridization using a human uPA cDNA. HRG increased 3- to 4-fold the steady-state levels of the 2.5-kb transcript of uPA compared with untreated MCF-7 cells. The maximal induction occurred 1–4 h post-HRG treatment, and the uPA levels of uPA transcript were maintained up to 24 h (Fig. 1B); this was dose-dependent (Fig. 1C). The enhanced expression of uPA mRNA observed in MCF-7 cells was a specific effect of HRG, because the related transforming growth factor, TGF-α, did not influence the expression of uPA mRNA (Fig. 1D).

Since Grimaldi et al. (38) have shown a close correlation between the proliferative state of cells and uPA transcription, we next examined the expected mitogenic effects of HRG and TGF-α in MCF-7 cells. Results suggested that both HRG and TGF-α stimulated the growth of MCF-7 cells under serum-free culture conditions (Fig. 1E), but only HRG was able to induce the expression of uPA (Fig. 1D). These observations suggested that the observed up-regulation of uPA in HRG-treated cells may not be a consequence of growth-promoting action of HRG. In addition, there was no change in the rate of [3H]thymidine incorporation into DNA between 4–12 h after HRG, a time point of maximum uPA expression (data not shown).

The observed HRG-mediated increase in uPA mRNA could have been caused by increased synthesis of newly transcribed mRNA, by enhanced stability of uPA mRNA, or by both. To evaluate these possibilities, we examined the effect of the transcription inhibitor Act-D. Pretreatment of cells with Act-D abolished HRG-mediated induction of uPA mRNA, suggesting that continuous RNA synthesis was necessary for the increased expression of uPA mRNA in HRG-
treated cells (Fig. 2). To evaluate translational regulation, we used cycloheximide, a translational inhibitor. Treatment of cells with cycloheximide partially blocked the level of uPA expression compared with levels in cells treated with HRG alone, and there was a stabilizing effect of cycloheximide on the level of uPA mRNA. The results also suggest that HRG regulates uPA expression at a pretranslational level.

To understand the role of uPA in HRG’s effect on normal mammary epithelial cells, we investigated the effects of HRG on both the invasive potential of and the uPA expression in a noninvasive mouse mammary epithelial HC11 cell line. Results of the Boyden chamber invasion assay indicated that HRG promotes the ability of HC11 cells to invade through the ECM (Fig. 3A). The HRG up-regulated the expression of uPA in HC11 cells, and this was in an Act-D-sensitive and cycloheximide-insensitive manner (Fig. 3B). In brief, these results suggest that HRG regulation of uPA may regulate motility and invasion in both normal and pathological conditions in breast epithelial cells.

HRG Enhances uPAR mRNA Expression in Breast Cancer Cells. uPA binds to its high-affinity, heavily glycosylated cell-surface receptor, uPAR (39). Expression of uPAR is believed to be involved in a wide range of functional activities that are not necessarily dependent on uPA binding to uPAR. For example, uPAR interaction with the extracellular domain of integrins regulates cell adhesion and migration (21–24).

Because MCF-7 cells express low levels of uPAR (40, 41), we next evaluated the potential regulation of uPAR mRNA expression in MCF-7 cells, which are known to respond to HRG with increased cell motility and invasion (5–7). Total RNA was isolated both from control and from HRG-treated cells, and expression of uPAR mRNA was analyzed by Northern blot analysis. Results in Fig. 4A demonstrate the kinetics of uPAR mRNA expression in HRG-treated MCF-7 cells. HRG rapidly increased the steady-state levels of uPAR mRNA several-fold over the undetectable uPAR mRNA level in control cells. Cotreatment of cultures with cycloheximide superinduced the uPAR mRNA, and cycloheximide stabilized the uPAR mRNA (Fig. 4B). HRG induction of uPAR mRNA was sensitive to Act-D, a transcription inhibitor. Pretreatment of cells with Act-D abolished the HRG-mediated induction of uPAR mRNA, suggesting that HRG may regulate uPAR expression at a transcriptional level (Fig. 4B).

Western blot analysis was performed to determine whether or not the observed increase in the level of uPAR mRNA in HRG-treated MCF-7 breast cancer cells was associated with an increase in the expression of uPAR protein. Western blot analysis showed that the
level of the M subunit of uPAR protein in the MCF-7 cells was significantly increased after HRG treatment (Fig. 4C). These results indicated that HRG-mediated up-regulation of uPAR was accompanied by an increase in the level of uPAR expression, and may play a role in HRG regulation of the invasion of breast cancer cells.

**HRG Regulation of uPA and uPAR Promoter Activities.** To further confirm the role of HRG in the transcriptional regulation of the uPA and uPAR, we transiently transfected MCF-7 cells with a chimeric CAT gene fused with the 5′ region of the uPA- or uPAR-promoter (32–34). In the transfected MCF-7 cells treated with HRG, there was a significant stimulation of both the uPA- and uPAR-promoter activities (Fig. 5A).

Different functions of HRG are known to be regulated by distinct signaling pathways. For example, HRG uses p38MAPK and PI-3 kinase pathways to regulate the cell-spreading and formation of lamellipodia, respectively (5–7). To understand the nature of the HRG signaling pathways that stimulate transcription from the uPA promoter, we used cotransfection of dominant-negative mutants that specifically inhibit MEK (35), p38MAPK (7), PI-3 kinase (5, 36), and NF-κB (37) activation in HRG-treated MCF-7 cells. Inclusion of dominant-negative mutants of MEK and p38MAPK suppressed both the constitutive and HRG-inducible stimulation of uPA promoter activity (Fig. 5B). Inclusion of dominant-negative NF-κB also inhibited the constitutive uPA promoter activity; that inhibition, however, was partially overcome by HRG treatment. Next, we evaluated whether HRG-inducible signaling pathways were necessary in the activation of uPA promoter. As shown in Fig. 5C, uPA promoter activity was below the detection limits in cells cotransfected with dominant-negative mutants of MEK and NF-κB 66. Inclusion of the dominant-negative p38MAPK mutant inhibited the constitutive uPA promoter activity; this inhibition, in contrast to that of uPAR promoter, was modestly overcome by HRG treatment (Fig. 5C). Blocking the PI-3 kinase pathway by dominant-negative p85 [which effectively blocks the activation of PI-3 kinase pathway (36)] did not significantly affect the HRG-induced stimulation of uPAR or uPA promoters. In brief, these results suggested the possible involvement of MEK, p38MAPK, and NF-κB 66, but not PI-3 kinase, in the baseline regulation of uPAR- and uPA-promoter activities.

**Regulation of HRG-mediated Cell Invasion by the uPA-uPAR System.** In addition to proteolysis, the uPA/uPAR system plays an important role in cell motility (reviewed in Ref. 8). Because HRG is a potent mitogen (5–7) and induces the expression of uPA and uPAR expression (this study), and because uPA is known to stimulate cell motility in MCF-7 cells (40, 41), we explored whether uPA/uPAR system acts as a mediator during HRG-induced cell motility and invasion for cells grown in Matrigel. The modulation in cell shape and membrane-bound uPA was subsequently measured. The formation of motile structures and an increase in the level of membrane-bound uPA immunostaining accompanied HRG stimulation of MCF-7 cells (Fig. 6A). Parallel quantitation of the ability of cells to invade was confirmed by the Boyden chamber invasion assay (Fig. 6B). Together, these results suggested a significant increase in the level of membrane-bound uPA upon HRG treatment.

**DISCUSSION**

The results presented here indicate that HRG treatment of noninvasive MCF-7 breast cancer cells significantly enhances cell invasion and motility. Our conclusion that HRG is an active inducer of the uPA/uPAR pathway is supported by several findings: (a) the HRG-
mediated increase in the levels of expression of uPA mRNA is accompanied by an enhanced secretion of uPA in the conditioned medium; (b) HRG stimulated expression of uPAR mRNA and protein; (c) HRG promoted the appearance of motile structures and membrane-bound uPA immunoreactivity at the free edge of motile cells; and (d) HRG stimulates the transmigration of cells across a porous membrane. These data provide evidence that up-regulation of the uPA/uPAR system contributes to the invasive function of HRG in breast cancer cells.

Our finding that incubation of breast cancer cells with a uPAR-inhibitor significantly suppresses HRG-induced invasion is important because it suggests that autocrine/paracrine stimulation of the uPA/uPAR pathway by cellulyarly derived factors such as HRG is involved in the regulation of uPAR expression in breast cancer cells. In breast cancer cells such as MCF-7, HRG may also up-regulate the expression and secretion of uPA. This raises the possibility that functional autocrine interaction exists between uPA and uPAR.

We also demonstrated that HRG stimulation of breast cancer cells was accompanied by the simultaneous stimulation of expression of uPA mRNA and uPA protein and of uPA secretion. Regulation of uPA by HRG appears to be pretranslational; it was completely inhibited by Act-D. This view is further supported by the observation that HRG can stimulate uPA-promoter activity in breast cancer cells. This was in contrast to other growth factors where the effect could be at the level of uPAR mRNA stability. Lund et al. (42) reported that phorbol ester 12-myristate 13-acetate and TGF-β1 increased the stability of the uPAR mRNA in the human lung cancer cell line A549. Further, Shetty et al. (43) demonstrated that the increase in uPA mRNA in mesothelioma cells by the phorbol ester was attributable to a 4-fold increase in mRNA half-life. In brief, our observations raise the possibility that HRG uses the uPA/uPAR autocrine pathway to transduce its signal(s), leading to invasion.

Additional support for the idea that HRG regulates the uPAR pathway is provided by earlier published studies showing that highly invasive breast cancer MDA-MB-231 cells, which secrete HRG (44), express very high levels of the uPA/uPAR system. These observations also raise the possibility that reported variations in the levels of basal uPA/uPAR expression among different breast cancer cells are caused in part by variable degrees of interaction between endogenous HRG and its receptors, HER3 and HER4. It is possible that the endogenous HRG, the overexpressed HER2 family members, or both may act as one potential cellular factor that controls the action of the uPA/uPAR system in breast cancer, and may have functional implications in the deregulation of the uPA pathway in breast cancer. In addition, it is also possible that the reported variability in the localization of uPA or uPAR on tumor or stroma cells may be influenced by the presence or absence of soluble factors such as heregulin.

The observed induction of the uPA/uPAR pathway in breast cancer epithelial cells by HRG, a paracrine growth factor that is secreted by the mesenchymal cells, may have functional implications in the invasion of breast cancer cells. Any potential up-regulation of uPAR by HRG in breast cancer epithelial cells is likely to promote further the ability of tumor cells to invade their surrounding environment because uPA ligation to uPAR may trigger proteolytic pathways and, thus, the ability of tumor cells to degrade the extracellular milieu. Our findings that HRG regulates the levels of expression of uPA and its receptor and the invasion of breast cancer epithelial cells open a new avenue for investigation to closely link HRG-signalizing, the urokinase pathway, and breast cancer invasion.

ACKNOWLEDGMENTS

We thank Ratna Vadlamudi, Mahiots Mandal, and Amjad Talukder for their informative suggestions in this project, Shao-Cong Sun for I primaries, and Dan Medina for providing the HC11 cells.

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