A functional comparison between the HER2^{high}/HER3 and the HER2^{low}/HER3 dimers on heregulin-β1-induced MMP-1 and MMP-9 expression in breast cancer cells

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Abstract

Overexpression of HER2 correlates with more aggressive tumors and increased resistance to cancer chemotherapy. However, a functional comparison between the HER2^{high}/HER3 and the HER2^{low}/HER3 dimers on tumor metastasis has not been conducted. Herein we examined the regulation mechanism of heregulin-β1 (HRG)-induced MMP-1 and -9 expression in breast cancer cell lines. Our results showed that the basal levels of MMP-1 and -9 mRNA and protein expression were increased by HRG treatment. In addition, HRG-induced MMP-1 and -9 expression was significantly decreased by MEK1/2 inhibitor, U0126 but not by phosphatidylinositol 3-kinase (PI-3K) inhibitor, LY294002. To confirm the role of MEK/ERK pathway on HRG-induced MMP-1 and -9 expression, MCF7 cells were transfected with constitutively active adenoviral-MEK (CA-MEK). The level of MMP-1 and -9 expressions was increased by CA-MEK. MMP-1 and -9 mRNA and protein expressions in response to HRG were higher in HER2 overexpressed cells than in vector alone. The phosphorylation of HER2, HER3, ERK, Akt, and JNK were also significantly increased in HER2 overexpressed MCF7 cells compared with vector alone. HRG-induced MMP-1 and -9 expressions were significantly decreased by lapatinib, which inhibits HER1 and HER2 activity, in both vector alone and HER2 overexpressed MCF7 cells. Finally, HRG-induced MMP-1 and MMP-9 expression was decreased by HER3 siRNA overexpression. Taken together, we suggested that HRG-induced MMP-1 and MMP-9 expression is mediated through HER3 dependent pathway and highly expressed HER2 may be associated with more aggressive metastasis than the low expressed HER2 in breast cancer cells.

Keywords: breast neoplasms; HER2 protein, human; heregulin-β1; matix metalloproteinase 1; matrix metalloproteinase 9; mitogen-activated protein kinase 1; neoplasm metastasis

Introduction

Heregulin is a family of polypeptide growth factors for the HER3/HER4 receptors, which play important roles in breast cancer cell proliferation and tumorigenesis (Falls, 2003) and are often expressed in breast cancer tissues (Dunn et al., 2004). Heregulin-β1 (HRG) enhances the formation of multicellular aggregation of the human breast cancer cells through the activation of PI3K (Tan et al., 1999). In contrast, HRG-mediated the aggregation of MCF-7 cells is inhibited by wortmannin and LY294002, a specific inhibitor of PI3K (Tan et al., 1999).

The EGFR family consists of four members and binding of ligands leads to homo- and heterodimerization (Yarden and Sliwkowski, 2001). Activation of receptors by dimerization stimulates the down-stream signaling molecules, including MAPK, Akt, and STAT (Park et al., 1996; Tan et al., 1999). HER2 is one of four members of the EGFR

(Hynes and Lane, 2005). Amplification of HER2 occurs in 15-25% of human breast cancer and is associated with an aggressive phenotype, including significant shortening of disease-free and overall survivial (Slamon *et al.*, 1987, 1989). Activation of HER2 most likely occurs through homo- or heterodimerization with HER1, HER3, and HER4 (Wallasch *et al.*, 1995; Tzahar *et al.*, 1997). The HER2/HER3 heterodimer is preferred and most tumorigenic of the possible combinations (Wallasch *et al.*, 1995; Karunagaran *et al.*, 1996).

Matrix metalloproteinases (MMPs) are a family of zinc-dependent neutral endopeptidases which collectively are capable of degrading all components of the extracellular matrix (ECM) (Duffy *et al.*, 2000). MMPs have been regarded as major critical molecules during metastasis and angiogenesis (Duffy *et al.*, 2000; Egeblad and Werb, 2002). The level of MMPs increases significantly in breast tumor cells, as well as in surrounding noncancerous breast tissue (Garbett *et al.*, 2000). MMP-1, -2, -3, -8 and -11 have been reported to have a highly expression in breast cancer (Kossakowska *et al.*, 1996; Garbett *et al.*, 2000). Overexpression of MMP-1 in epithelium is associated with increased susceptibility to chemical carcinogens (D'Armiento *et al.*, 1995) and increased growth rates of xenografts in human breast cancer (Minn *et al.*, 2005). In addition, MMP-9 expression plays a role in the digestion of collagen type IV, which is primary component of basement membranes (Nabeshima *et al.*, 2002). MMP-9 is strongly associated with an invasive phenotype in rat embryos, as well as the metastatic potential in a number of malignant tumors (Bernhard *et al.*, 1994; Jones *et al.*, 1999; Lakka *et al.*, 2003).

In this study, we verified the regulatory mechanism of HRG-induced MMP-1 and MMP-9 expression and investigated a additive role of HER2 on HER3-dependent MMP-1 and MMP-9 expression, which play a pivotal role on metastasis of various cancer cells.

Results

The basal levels of MMP-1 and MMP-9 mRNA and protein expression were increased by HRG in a dose-dependent manner

To verify the effect of HRG on MMP-1 and MMP-9 expression, we treated MCF7 cells with the indicated concentration for 24 h. We harvested cultured media for detecting of MMP-1 and MMP-9 protein expression and cell lysates for detecting of MMP-1 and MMP-9 mRNA expression, respectively. Our results showed that the expression of MMP-1 and MMP-9 protein was increased by HRG treatment in

a dose-dependent manner (Figure 1A). The levels of expression of MMP-1 and MMP-9 protein were increased by 474.7-fold and 78.6-fold of the control level at 50 ng/ml HRG treatment, respectively (Figure 1A). In addition, the levels of MMP-1 and MMP-9 mRNA were significantly increased by 492.3-fold and 338.0-fold of the control level at 50 ng/ml HRG treatment, respectively (Figure 1B).

To confirm these results, we treated SKBR3 and T47D cells with 50 ng/ml HRG for 24 h. The levels of MMP-9 protein expression were significantly increased by HRG in both SKBR3 and T47D cells, which are HER2- and HER3-positive cells (Supplemental Data Figure S1A).

HRG-induced MMP-1 and MMP-9 expression were mediated through MEK/ERK dependent pathway but not through PI-3K/Akt pathway

It has been reported that HRG regulates the mitogenic and tumorigenic effect of breast cancer cells through the activation of PI-3K/Akt and ERK pathway (Fiddes *et al.*, 1998; Vijapurkar *et al.*, 2003). Therefore, we next investigated the regulatory mechanism of HRG-induced MMP-1 and MMP-9 expression. As shown in Figure 2A, we treated with 50 ng/ml HRG for the indicated times. Our results also showed that the phosphorylation of ERK and Akt was increased by HRG. In addition, we examined the effect of U0126 and LY294002 on HRG-induced the phosphorylation of HER2, HER3, ERK1/2 and Akt. Our results showed that HRG-induced the phosphorylation of HER2, HER3 and Akt did not affect by U0126 and HRG-induced the phosphorylation of HER2, HER3 and ERK1/2 did not affect by LY294002 (Figure 2B).

To inhibit the activation of PI-3K/Akt and ERK pathway, we pretreated with U0126 and LY294002
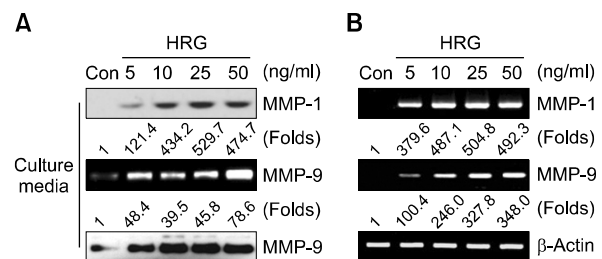

Figure 1. The levels of expression of MMP-1 and MMP-9 mRNA and protein were increased by HRG in breast cancer cells. After serum-starvation for 24 h, MCF7 cells were treated with HRG at the indicated concentrations for 24 h. MMP-1 and MMP-9 protein and mRNA expression was determined by (A) Western blotting (upper band) and Zymography (lower band) and (B) RT-PCR, respectively. The results shown are representative of three independent experiments. Con, control; HRG, heregulin-β1.
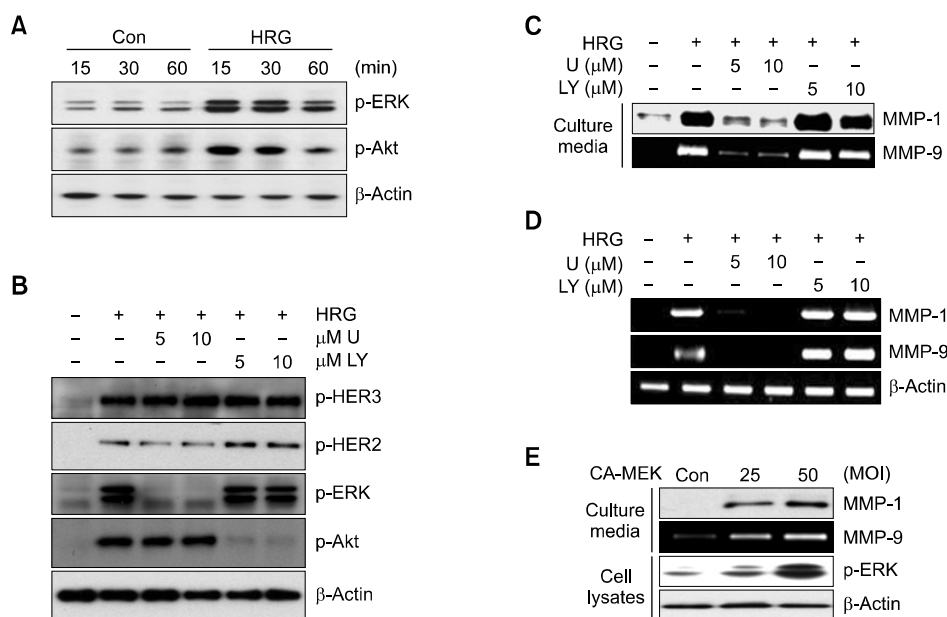
Figure 2. HRG-induced MMP-1 and MMP-9 expression was regulated by MEK/ERK dependent pathway but not by PI-3K/Akt pathway in MCF7 cells. After serum-starvation for 24 h, (A) cells were treated with or without 50 ng/ml HRG and further incubated at 37°C for the indicated times. (B-D) Cells were pretreated with U0126 or LY294002 at the indicated concentrations for 60 min, then treated with or without 50 ng/ml HRG for 30 min (B) and 24 h (C, D), respectively. MMP-1 and MMP-9 protein and mRNA expression was determined by (C) Western blotting (upper band) and Zymography (lower band) and (D) RT-PCR, respectively. (E) After transfection of CA-MEK for 24 h, cells were further incubated for 24h in serum-free culture media. p-HER3, p-HER2, p-ERK, p-Akt, and β-actin were measured in whole cell lysates by Western blotting. Con, control; HRG, heregulin-β1; U, U0126; LY, LY294002.

for 60 min, respectively, then treated with 50 ng/ml HRG for 24 h. We observed that HRG-induced MMP-1 and MMP-9 protein (Figure 2C) and mRNA (Figure 2D) expression was suppressed by U0126, but not by LY294002.

To directly confirm the correlation between the MEK/ERK pathway and MMP-1 and MMP-9 expression, MCF7 cells were transfected with CA-MEK. MMP-1 and MMP-9 expression was significantly increased by CA-MEK overexpression (Figure 2E). Based on these results, we demonstrated that HRG-induced MMP-1 and MMP-9 expression was regulated through a MEK/ERK-dependent pathway in MCF7 human breast cancer cells.

HRG-induced MMP-1 and MMP-9 expression were increased more in HER2-overexpressed cells than in vector alone cells

To investigate the effect of HER2 on HRG-induced MMP-1 and MMP-9 expression, we chose pBMN-HER2 cells, originally derived from MCF7 human breast cancer cells, overexpressing the wild type human HER2. As shown in Figure 3A, wild type MCF7 cells constitutively expressed endogenous HER3, while HER2 expression was very low. Using FACS analysis, we investigated modulation of the cell cycle by HRG. The data shown in Figure 3B,

revealed that HRG was not affected the regulation of cell cycle in both vector and HER2- overexpressed cells.

Next, we treated vector and HER2-overexpressed MCF7 cells with 50 ng/ml HRG for 24 h. We showed that the levels of expression of MMP-1 and MMP-9 protein in HER2-overexpressed cells were significantly increased by HRG treatment relative to in vector alone (Figure 3C). The expression of HRG-induced MMP-1 and MMP-9 protein was increased by 3.0-fold and 3.3-fold of the control level (HRG treated vector alone cells) compared with HRG-treated HER2-overexpressed cells, respectively (Figure 3C). Furthermore, HRG-induced MMP-1 and MMP-9 mRNA expression was also increased by 3.2- and 2.6-fold of the control level (HRG treated vector alone cells) compared with HRG-treated HER2-overexpressed cells, respectively (Figure 3D). Therefore, we demonstrated that HRG-induced MMP-1 and MMP-9 expression was higher in HER2-overexpressed cells than in vector alone. The formation of the HER2/HER3 heterodimer may have a greater metastatic effect compared with HER3 homodimer in MCF7 human breast cancer cells.
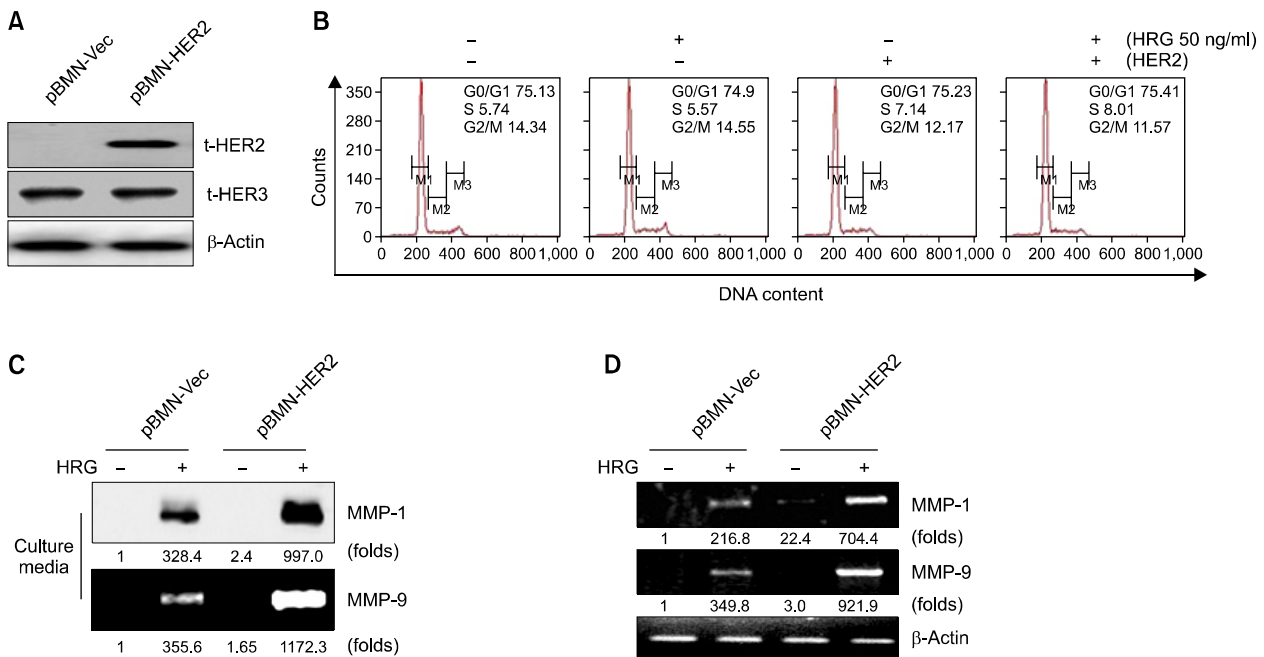
Figure 3. HRG-induced MMP-1 and MMP-9 expression was significantly increased in HER2-overexpressed cells. (A) The expression of t-HER2 and t-HER3 was measured by Western blotting in whole cell lysates. (B) The analysis of cell cycle by FACS was detected as described in *Materials and Methods*. (C, D) Cells were treated with or without 50 ng/ml HRG for 24 h. MMP-1 and MMP-9 mRNA and protein expression was determined by (C) Western blotting (upper band) and Zymography (lower band) and (D) RT-PCR, respectively. The results shown are representative of three independent experiments. Con, control; HRG, heregulin-β1.
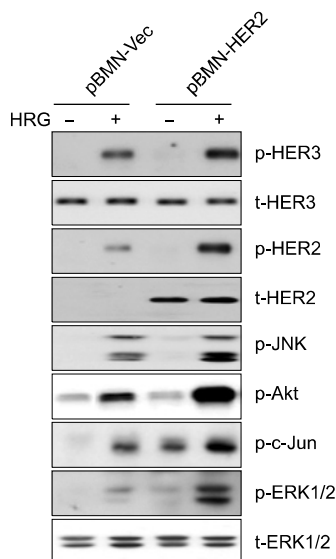

Figure 4. Activation of downstream signaling molecules of receptor by HRG were significantly increased in HER2-overexpressed MCF7 cells. After serum-starvation for 24 h, cells were treated with or without 50 ng/ml HRG for 30 min. The phosphorylation of HER3, HER2, ERK, JNK, Akt, c-Jun, and β-actin was determined in whole cell lysates by Western blotting. The results shown are representative of three independent experiments.

Activation of down-stream signaling molecules of receptor by HRG were significantly increased in HER2-overexpressed MCF7 cells

We investigated why HRG-induced MMP-1 and MMP-9 expression was significantly increased in HER2-overexpressed cells. We treated HER2-overexpressed cells with 50 ng/ml HRG for 30 min and harvested whole cell lysates for detection the phosphorylation of various signaling molecules. Our results showed that the phosphorylation of HER3, HER2, JNK, Akt, ERK, and c-Jun was increased by HRG (Figure 4). In addition, the phosphorylation of these molecules was further increased in HER2-overexpressed cells (Figure 4). Recently, ERK1/2 cascade was shown to play an important role in EGF- or HRG-induced MMP-9 expression (Tang *et al.*, 2008; Choi *et al.*, 2009). Therefore, we demonstrated that HER2 may assist the HER3 signaling pathway to additively effect HRG-induced MMP-1 and MMP-9 expression.

Additively increased expression of MMP-1 and MMP-9 by HRG was also decreased by U0126 in HER2-overexpressed MCF7 cells

We investigated whether or not HER2 can form a direct physical complex with HER3 in HER2-
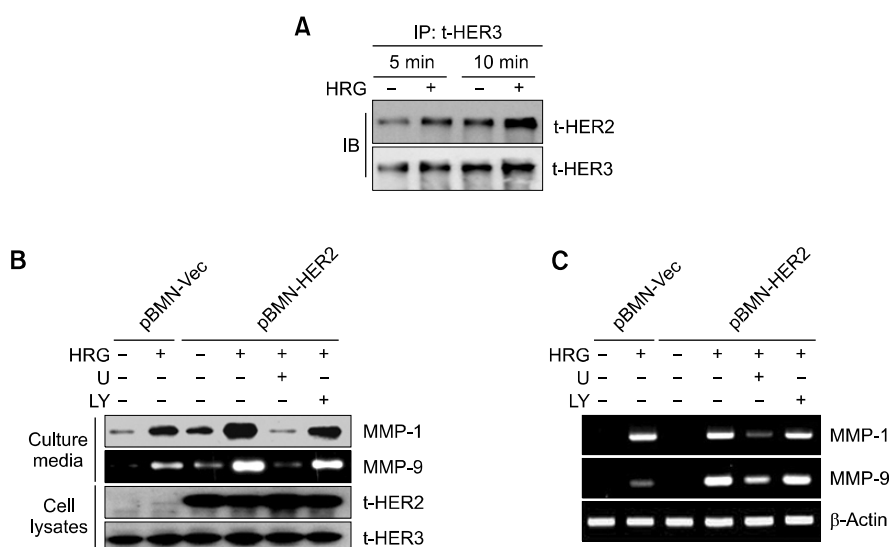
Figure 5. HRG-induced MMP-1 and MMP-9 expression of HER2-overexpressed MCF7 cells was also regulated by MEK/ERK dependent pathway. After serum-starvation for 24 h, (A) HER2-overexpressed cells were treated with or without 50 ng/ml HRG for the indicated times. Co-immnunoprecipitation was detected as described in *Materials and Methods*. (B, C) Cells were pretreated with UO126 or LY294002 at the indicated concentrations for 60 min, then treated with or without 50 ng/ml HRG for 24 h. MMP-1 and MMP-9 protein and mRNA expression was determined by (B) Western blotting (upper band) and Zymography (lower band) and (C) RT-PCR, respectively. The level of t-HER3 and t-HER2 expression was determined in whole cell lysates by Western blotting. The results shown are representative of three independent experiments. Con, control; HRG, heregulin-β1.

overexpressed MCF7 and endogenous HER2 and HER3 expressed SKBR3 breast cancer cells. After treatment of HER2-overexpressed MCF7 breast cancer cells with 50 ng/ml HRG for the indicated times, cell lysates were immunoprecipitated with anti-HER3 antibody (Figure 5A) and the immunopreciptates were analyzed by immunoblotting with anti-HER2 and anti-HER3 antibodies. As shown in Figure 5A, the immunoblot analyses revealed an association with HER2 and HER3. In addition, we confirmed the interaction of HER2 and HER3 in endogenous HER2 and HER3 expressed SKBR3 breast cancer cells (Supplemental Data Figure S1B).

To elucidate the mechanism of the additive effect of HER2 on MMP-1 and MMP-9 expression, we pretreated with U0126 and LY294002 for 60 min prior to HRG treatment, then harvested culture media (protein) and cell lysates (mRNA), respectively, for detecting the expression of MMP-1 and MMP-9. As expected, the expression of MMP-1 and MMP-9 protein (Figure 5B) and mRNA (Figure 5C) was more increased in HER2-overexpressed cells. In contrast, these effects were significantly decreased by U0126, but not by LY294002 (Figures 5B and 5C).

HRG-induced MMP-1 and MMP-9 expression was completely decreased by lapatinib in both vector alone and HER2-overexpressed cells

We investigated the effect of lapatinib on HRG-induced

MMP-1 and MMP-9 expression. Lapatinb is known as a small molecule inhibitor of the HER1 and HER2 tyrosine kinase activity (Xia *et al.*, 2002). Thus, we pretreated cells with 2 μM lapatinib for 60 min to block the kinase activity of HER2, then treated with 50 ng/ml HRG for 24 h. Our results showed that HRG-induced MMP-1 and MMP-9 expression was completely blocked by lapatinib in both vector alone and HER-2-overexpressed cells (Figure 6A).

In accordance with previous study (Xia *et al.*, 2002), we showed that HRG-induced phosphorylation of HER2, ERK, and Akt was inhibited by lapatinib (Figure 6B). Interestingly, we observed that HRG-induced phosphorylation of HER3 was suppressed by lapatinib (Figure 6B). Although HER3 does not have kinase activity, these results suggest that lapatinib can suppress the conformational change of HER3 by ligand stimulation.

Finally, to verify the role of HER3 and HER2 on HRG-induced MMP-1 and MMP-9 expression, MCF7 cells were transfected with HER3 and HER2 siRNA, respectively. Our results showed that HRG-induced MMP-1 and MMP-9 expression was significantly decreased by HER3 (Figure 6C) as well as HER2 (Supplemental Data Figure S1C) siRNA overexpression in MCF7 breast cancer cells Therefore, we demonstrated that HRG-induced MMP-1 and MMP-9 expression was mediated through HER3/HER2-dependent pathway.
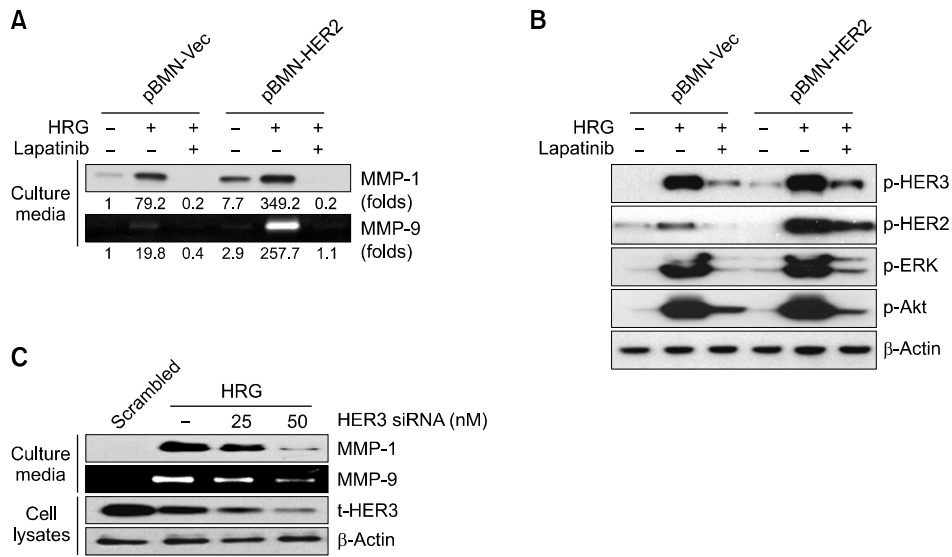
Figure 6. HRG-induced MMP-1 and MMP-9 expression was completely eliminated by lapatinib in both vector alone and HER2-overexpressed cells. After serum-starvation for 24 h, (A) cells were pretreated with 2 μM lapatinib for 60 min, then treated with or without 50 ng/ml HRG for 24 h. MMP-1 and MMP-9 protein expression was determined in culture media by Western blotting (upper band) and Zymography (lower band), respectively. (B) Cells were pretreated with 2 μM lapatinib for 60 min and then treated with or without 50 ng/ml HRG for 30 min. The phosphorylation of HER3, HER2, ERK, Akt, and β-actin was determined in whole cell lysates by Western blotting. (C) After transfection of scrambled and 25 and 50 nM HER3 siRNA for 48 h, respectively, cells were further incubated for 24 h in serum-free culture media, then treated with or without 50 ng/ml HRG at 37°C for 24 h. MMP-1 and MMP-9 protein expression was determined by Western blotting (upper band) and Zymography (lower band) in culture media. The results shown are representative of three independent experiments. Con, control; HRG, heregulin-β1.

Discussion

All of HER family members are highly expressed in human breast cancer and overexpression of HER2 has been associated with a poor prognosis (Slamon *et al.*, 1989). Although a specific ligand of HER2 has not been identified, HER2 activates through heterodimerization with HER1, HER3, or HER4 (Wallasch *et al.*, 1995; Tzahar *et al.*, 1997; Vijapurkar *et al.*, 2003). To date, the critical role of HER2 on HER3-dependent MMP-1 and MMP-9 expression in breast cancer cells has not been fully elucidated. Therefore, we investigated the regulatory mechanism of HRG-induced MMP-1 and MMP-9 expression and compared the metastatic effect between HER2^{high}/HER3 and HER2^{low}/HER3 in a HER2-overexpressed *in vitro* model.

Heregulins are subdivided into two major isotypes (α and β) and have been identified as the ligands for HER3 and HER4 (Chang *et al.*, 1997). Heregulins are expressed in approximately 25% of breast cancer carcinomas (Dunn *et al.*, 2004), and increases breast cancer cell proliferation and tumorigenesis as well as promotes the aggressive and invasive characteristics of cancer cells (Atlas *et al.*, 2003; Falls, 2003). The elevated expression of many MMPs is associated with tumor progression, such as poor prognosis, invasion, and metastasis (Deryugina and Quigley, 2006). Recently, the

activity of MMP-7 promoter was significantly increased by STAT-3 activation in MCF7 cells overexpressing HER2 in compare with parental MCF7 cells (Yuan *et al.*, 2008). Our results showed that HRG increases the basal level of MMP-1 and MMP-9 expression in a dose-dependent fashion which is involved in the breakdown of the ECM during tissue remodeling.

Furthermore, heregulins bind to HER3 and HER4 with low and high affinity, respectively, and activates multiple signaling pathways, including the PI-3K/Akt and ERK pathway in breast cancer cells (Fiddes *et al.*, 1998; Yen *et al.*, 2000; Neve *et al.*, 2002). The activation of these signaling pathways by heregulins plays a critical role in the mitogenic and tumorigenic effects (Fiddes *et al.*, 1998; Neve *et al.*, 2002). In agreement with these reports, our data showed that HRG-induced MMP-1 and MMP-9 expression is suppressed by U0126, but not by LY294002. In addition, HRG-induced MMP-1 and MMP-9 expression was significantly decreased by HER3 siRNA overexpression. Therefore, we have demonstrated that the metastatic effect of HRG through the induction of MMP-1 and MMP-9 depends on the HER3 → MEK/ERK dependent pathway in breast cancer cells.

Both MMP-1 and MMP-9 contain a TATA box and an activator protein-1 (AP-1) site on their promoters, which binds members of the Fos and Jun family
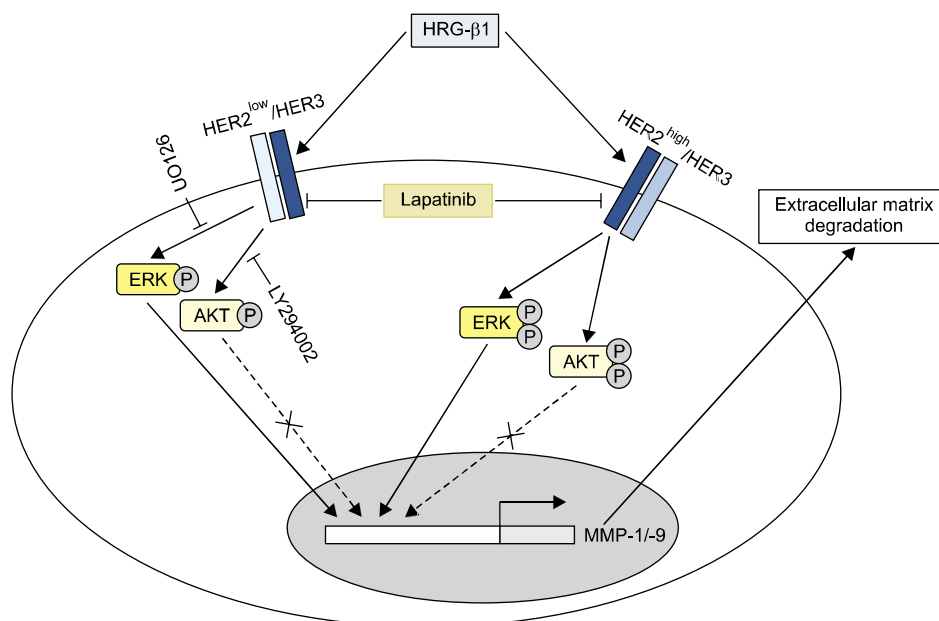
Figure 7. Schematic models. Heregulin-β1 triggers the phosphorylation of HER3 and HER4 and then activates several signaling molecules such as ERK and Akt. These activations are additively augmented by HER2 overexpression. In particular, the phosphorylation of ERK and Akt is higher in HER2^{high}/HER3 MCF7 cells than in HER2^{low}/HER3 MCF7 cells. MMP-1 and -9 expressions also significantly increase in HER2^{high}/HER3 MCF7 cells, compared with in HER2^{low}/HER3 MCF7 cells.

(Fingleton, 2006). The sequential activation of Raf, MEK, and ERK can trigger the expression of multiple MMPs, including MMP-1 and MMP-9 (Kajanne *et al*., 2007; Kim *et al*., 2009). Constitutive ERK activation significantly increases the activity of MMP-1 and MMP-9 promoter through activation of AP-1 (Kajanne *et al*., 2007). Our results showed that the phosphorylation of ERK, JNK, and c-Jun by HRG is additively increased in HER2-overexpressed MCF7 cells. Therefore, we have demonstrated that elevated phosphorylation of c-Jun may directly increase the transcriptional activity of MMP-1 and MMP-9 expression.

Lapatinib is a dual-specificity tyrosine kinase inhibitor (TKI) with a reversible high affinity for the TK domains of HER1 and HER2 (Rusnak *et al*., 2007). Lapatinib has been approved for treatment in patients with HER2-amplified breast cancer (Geyer *et al*., 2006). In this study, we treated with lapatinib for inhibition of HRG-induced HER2 phosphorylation. Our finding also showed that lapatinib suppresses HRG-induced the phosphorylation of Akt and ERK, which are signaling molecules of HER2 downstream, as well as inhibition of HER2 phosphorylation. Because HER3 lacks intrinsic kinase activity, the formation of the HER2/HER3 heterodimer activates the most mitogenic signaling pathway (Pinkas-Kramarski *et al*., 1996). Interestingly, HRG-stimulated phosphorylation of HER3 is completely eliminated by lapatinib. Based on these results, we suggest that lapatinib suppresses the comformational change of ligand-dependent HER3 for recruiting HER2.

As shown in Figure 7, HRG-induced MMP-1 and MMP-9 expression is regulated by a MEK/ERK-

dependent pathway in MCF7 cells. In addition, the phosphorylation of ERK, JNK, and Akt is additively amplified in HER2-overexpressed MCF7 cells. The high activation of ERK up-regulates MMP-1 and MMP-9 expression through the induction of AP-1 activity in HER2-overexpressed MCF7 cells. Furthermore, lapatinib suppresses the phosphorylation of HER3 in both vector alone and HER2-overexpressed MCF7 cells. Therefore, we also speculate that lapatinib may have a strong therapeutic potential in HER1 and/or HER2 expressing tumors, as well as in HER3 expressing tumors. HER3 plays an important role on HRG-induced MMP-1 and MMP-9 expression in breast cancer cells.

Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), RPMI1640, antibiotics, and 10% zymogram gel were purchased from Life Technologies (Rockville, MD). FBS was purchased from Hyclone (Logan, Utah). Rabbit monoclonal anti-p-HER2, t-HER2, p-ERK, p-SAPK/JNK, and p-Akt antibodies were purchased from Epitomics (Burlingame, CA). Lapatinib was a generous gift from Dr. Young-Hyuck Im (Sungkyunkwan University, Seoul, Korea). Mouse monoclonal anti-β-actin, t-HER3, and secondary peroxidase-conjugated antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). U0126 and LY294002 were purchased from Tocris (Ellisville, MO). HRG was purchased from Peprotech (Rocky Hill, NJ).

Cell cultures

Vector alone and HER2 overexpressed MCF7 human breast cancer cells were a generous gift from Dr. Incheol Shin (HanYang University, Seoul, Korea). The wild type, vector alone, and HER2 overexpressed MCF7 human breast cancer cell lines were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. SKBR3 and T47D human breast cancer cells were cultured in RPMI1640 supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. For experiments, cells were maintained in culture medium supplemented without FBS for 24 h and were further incubated with HRG (1, 2, and 5 ng/ml) for 24 h in fresh serum-free media. Lapatinib (HER1 and HER2 tyrosine kinase inhibitor), U0126 (MEK1/2 inhibitor), and LY294002 (PI-3K inhibitor) were added 60 min prior to HRG treatment, as indicated.

Western blotting

Proteins of cell lysates were boiled for 5 min in Laemmli sample buffer and electrophoresed in 10% SDS-PAGE gels. Proteins were transferred to PVDF membrane, and the membranes were then blocked in 10% skim milk in TBS with 0.01% Tween-20 (TBS/T) for 15 min. The blots were incubated with anti-p-HER2, p-HER3, p-ERK, or p-JNK (1/1,000), in TBS/T buffer at 4°C overnight. Blots were washed four times for 10 min in TBS/T buffer, and subsequently incubated in anti-rabbit peroxidase-conjugated antibody (1/2,000 dilution) in TBS/T buffer. After 1 h incubation at room temperature (RT), the blots were washed 4 times, and ECL plus reagents were used for development. Signal densities were quantified using a desitometric program (Bio 1D; Vilber Lourmat, Marne La Vallec, France).

Zymography

Zymography was performed in 10% SDS-PAGE gels that had been cast in the presence of gelatin as described previously (Kim *et al.*, 2009). Briefly, samples (100 μl) were resuspended in loading buffer and run on without prior denaturation. After electrophoresis, gels were washed to remove SDS and incubated for 30 min at RT in a renaturing buffer (50 mM Tris, 5 mM CaCl₂, 0.02% NaN₃, 1% Triton X-100). In next steps, gels incubated for 48 h at 37°C in a developing buffer [50 mM Tris-HCl (pH 7.8) 5 mM CaCl₂, 0.15 M NaCl, and 1% Triton X-100]. Gels were subsequently stained with Coomassie Brilliant Blue G-250 and destained in 30% methanol, 10% acetic acid to detect gelatinase secretion. Signal densities were quantified using a desitometric program (Bio 1D).

Immunoprecipitation

For immunoprecipitation experiments, HER2-overexpressed cells were grown to 70-80% confluence and performed, as described previously (Kim *et al.*, 2010). Briefly, cells were washed with ice cold PBS, and lysed in a buffer containing 20 mM Tris-HCl (pH 7.4), 300 mM NaCl, 2 mM EDTA, 2 mM EGTA (pH 8.0), 0.4 mM sodium vanadate, 0.4 mM PMSF, and 2% Triton X-100. The cell lysates were centrifuged at 12,000 rpm for 15 min at 4°C and the supernatants were subjected to immunoprecipitation with anti-HER3 antibody (1-5 μg/ml each). The immunocomplexes were absorbed to ProteinA/G PLUS-Agarose (Santa Cruz Biotechnology, Inc.) and washed 3 times with lysis buffer. After extensive washing, samples were resolved by the addition of 2 × sample buffer, separated by 10% SDS-PAGE gel, and transferred to PVDF membranes. Blots were then probed by immunoblot analysis with anti-HER2 and anti-HER3 antibodies (1/1,000 dilution).

RT-PCR

Total RNA was extracted from MCF7 human breast cancer cells using TRIzol (Invitrogen, Carlsbad, CA), according to manufacturer's protocol. Extracted RNA was electrophoresed in 1% agarose gels to confirm its quality and quantity. Equal amounts of RNA (1 μg) were reverse transcribed using a first-strand cDNA synthesis kit (MBI Fermentas, Vilnius, Lithuania). Semiquantitative PCR was performed using specific primers for human genes listed in Supplemental Data Table S1. Reaction products were electrophoresed in 2% agarose gels and visualized with ethidium bromide (EtBr).

Flow cytometry analysis (FACS)

Cells were trypsinized and harvested by centrifugation at 1,500 rpm for 5 min. The cell pellets were then resuspended in 1 ml PBS and fixed in 70% ethanol for 20 min at RT. Fixed cells were centrifuged and washed twice in PBS to wash out any apoptotic cells. The cells were resuspended in 1 ml of PBS with 100 μg/ml of DNase-free RNase A (Biopure, Canada) then incubated for 30 min in a 37°C water bath. The cells were collected by centrifugation at 1,500 rpm, the cell pellets were washed twice with PBS, resuspended in PBS containing 50 μg/ml of propidium iodide (Sigma) then analyzed using the FACS-vantage (Becton-Dickinson, San Diego, CA).

HER3 and HER2 siRNA transfection

HER3 and HER2 siRNA were purchased from Dharmacon Research (Lafayette, CO). We found that the optimal siRNA knock-down conditions involved transfecting MCF7 breast cancer cells at 80% confluence maintained in DMEM with 10% FBS; Effectene (Qiagen, Valencia, CA) was used for transfections with HER3 or HER2 siRNA (25, 50 nM or as noted) following protocols provided by the manufacturer. Fresh serum-free media with or without 50 ng/ml HRG were added 24 h after the 48 h transfection.

Adenovirus transfer

The empty (*Lac Z*) and adenoviral human CA-MEK cDNA were the gifts of Dr. Hyunil Ha (Seoul National University, Korea). Recombinant adenovirus-expressing human CA-MEK was reproduced into 293A cells. The expression of this construct was confirmed by Western blotting.

Supplemental data

Supplemental data include a figure and a table and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-44-8-02.pdf.

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