Lysophospholipids transactivate HER2/neu (erbB-2) in human gastric cancer cells

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Abstract

The ligand-less receptor HER2/neu (erbB-2) has been proposed as a prognostic marker of gastric cancer that correlates with poor clinical outcome, indicating that HER2 signals play an important role in gastric cancer progression. This study demonstrated that two major natural lysophospholipids, lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), induce rapid and transient phosphorylation of HER2 in two human gastric cancer cell lines, MKN28 and MKN74 cells. We also revealed that tyrosine phosphorylation of HER2 induced by both lysophospholipids was significantly attenuated by two inhibitors, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, AG1478, and a broad-spectrum matrix metalloproteinase inhibitor, GM6001. This suggests that the pathway of HER2 transactivation induced by these lysophospholipids is dependent on the proteolytically released EGFR ligands. Our results indicate that LPA and S1P act upstream of HER2 in gastric cancer cells, and thus may act as potent stimulators of gastric cancer.

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The HER2/Neu (ErbB2) receptor is the protein product of the her2/neu/erbB-2 proto-oncogene and belongs to the epidermal growth factor (EGFR) family of receptor tyrosine kinases (RTKs), which consists of four members, EGFR, HER2, ErbB3, and ErbB4. Each receptor of the EGFR family has in common an extracellular ligand-binding domain, a single membrane spanning region, and a cytoplasmic protein tyrosine kinase domain [1,2]. Under normal physiological conditions, activation of the EGFR family is controlled by spatial and temporal expression of their ligands, which are members of the EGF-related peptide growth factor family, such as EGF, heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor alpha (TNF-α), and neuregulin [1,2]. These growth factors are synthesized as transmembrane precursors that are released from the cell surface by proteolytic cleavage and subsequently activate RTKs of the EGFR family in an autocrine or paracrine fashion. Despite the abundance of ligands identified for EGFR, ErbB3, and ErbB4, no direct ligand for HER2 has been discovered. Instead, HER2 functions as a homo- or heterodimer with other members of the EGFR family upon interaction with agonistic ligands, such as EGF, HB-EGF, and TNF-α. HER2 is currently attracting a great deal of attention because a new adjuvant therapy using an antibody against HER2, trastuzumab (Herceptin), has proved effective in treating certain breast cancers [2,3].

As for gastric cancer, immunohistochemical studies using polyclonal HER2 antibodies have shown a 9–14% of tumor response [4–7]. In contrast, Allgayer

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et al. [8] have recently reported that, with a highly sensitive immunohistochemical method using a monoclonal antibody, HER2 was expressed in as many as 91% of gastric cancers. They also showed that staining intensity for HER2 was correlated with tumor size, serosal invasion, and lymph node metastasis, and that HER2 was an independent, functional prognostic factor for overall survival in gastric cancer, which is consistent with other previous reports [4–9]. Thus, HER2 signals appear to play an important role in the development and progression of gastric cancer as well as breast cancer.

Crosstalk between different members of receptor families has become a well-established concept in signal transduction. Tyrosine phosphorylation of various RTKs in response to activation of many G protein-coupled receptors (GPCRs), which was designated “transactivation,” has been shown to have important physiological consequences and has drawn considerable attention in recent years. One of the most intensely studied pairs of receptors has been RTK for EGF and GPCR for lysophosphatidic acid (LPA). LPA, a natural phospholipid, is also shown to transactivate HER2 as well as EGFR in Rat-1 fibroblasts, and head and neck squamous cell carcinoma (HNSCC) [10,11].

Gastric cancers are often associated with local bleeding and, thus, at the tumor site, platelets are activated and secrete lysophospholipids such as lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), and lysophosphatidylserine (LPS). These lysophospholipids are subsequently converted to LPA by lysophospholipase D [12]. From activated platelets, sphingosine 1-phosphate (SIP) is also released in large amounts [13]. Therefore, as products of the blood coagulation cascade, these two lipid mediators, LPA and SIP, are thought to be abundantly present in gastric cancer tissue, and thus to regulate various important biological responses, presumably in concert with other mediators. Both lipids have been shown to have positive roles in tumor progression such as in ovarian cancer, colorectal cancer, breast cancer, and melanoma [14–18]. In the present study, we investigated whether LPA or SIP induces phosphorylation of HER2, whose signals are crucial for the progression of gastric cancer.

Materials and methods

Materials. 1-Oleoyl-LPA was purchased from Sigma Chemical (St. Louis, MO). SIP and an EGFR tyrosine kinase inhibitor, AG1478, were purchased from Biomol (Plymouth Meeting, PA). Recombinant human EGF was purchased from PeproTechec (London, UK). Mouse monoclonal anti-human phosphotyrosine antibody (PY20) and mouse monoclonal anti-human HER2/neu antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse monoclonal anti-human HER2/neu antibody was purchased from Lab Vision (Fremont, CA). Pertussis toxin (PTX), the broad-spectrum matrix metalloproteinase (MMP) inhibitor, GM6001, and a HER2 tyrosine kinase inhibitor, AG825, were purchased from Calbiochem (La Jolla, CA). Rabbit polyclonal anti-phospho-ERK1/ERK2 antibody was purchased from R&D systems (Minneapolis, MN).

Cell culture. The human gastric cancer cell lines, MKN28 and MKN74, were obtained from the Riken Cell Bank (Tsukuba, Japan). MKN28 and MKN74 are cell lines established from moderately differentiated adenocarcinomas. These cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco-BRL, Grand Island, NY).

Immunoprecipitation and Western blot analysis. Since 10 μM and 1 μM are considered to be the highest physiological concentrations of LPA and SIP, respectively, we used 10 μM LPA and 1 μM SIP during these experiments [19]. Immunoprecipitation and Western blot analysis were performed as described previously [15,20]. In brief, MKN28 and MKN74 cells were grown to 80–90% confluence in 10-cm dishes. These cells were starved in serum-free medium for 24 h, and then LPA or SIP was added to the culture thereafter. After stimulation of starved cells with 10 μM LPA or 1 μM SIP for various times, cellular protein lysates (1 ml/dish/tube) were obtained and then all proteins were incubated with antibodies against HER2 (20 μl/tube). Immunoprecipitates were collected with protein A-agarose. Immunoprecipitated proteins were electrophoresed in sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gel for 35 min at 200 V. Then, the protein was transferred onto an Immobilon transfer membrane (Millipore, Bedford, MA) for sequential incubation with 5% reconstituted non-fat milk powder to block nonspecific sites, dilutions of mouse monoclonal anti-phosphotyrosine antibody, and then horseradish peroxidase-labeled sheep anti-mouse IgG, prior to development with a standard ECL kit (Amersham, Buckinghamshire, England). Some cells were pretreated with 250 nM AG1478, or 25 μM GM6001, for 30 min before stimulation, and other cells were pretreated with 100 ng/ml PTX for 24 h before stimulation. All membranes were stripped and immunoblotted with antibodies against HER2 as a control.

ERK phosphorylation. Cell lysates were electrophoresed in SDS–15% polyacrylamide gel for 45 min at 200 V. Then, the protein was transferred onto an Immobilon transfer membrane (Millipore) for sequential incubation with 5% reconstituted non-fat milk powder to block nonspecific sites, dilutions of rabbit polyclonal anti-phospho-ERK1/ERK2 antibody, and then horseradish peroxidase-labeled donkey anti-rabbit IgG, prior to development with a standard ECL kit (Amersham). Some cells were pretreated with 250 nM AG1478, or 30 μM AG825 for 30 min before stimulation. All membranes were stripped and immunoblotted with antibodies against ERK2 as a control.

Results

LPA induced tyrosine phosphorylation of HER2 in gastric cancer cells

We first investigated whether LPA transactivates HER2 in human gastric cancer cells, as previously reported in other cells [10,11,21]. MKN28 and MKN74 cells were incubated with 10 μM LPA for 2–40 min, and examined for whether tyrosine phosphorylation of HER2 was induced by LPA. As shown in Fig. 1A, LPA induced significant tyrosine phosphorylation of HER2 in both cell types. The degree of phosphorylation induced by LPA was as strong as that induced by 1 ng/ml EGF, although it was less clear than that induced by 10 ng/ml EGF (Fig. 3). Time course experiments...
revealed that phosphorylation was maximal after 2–5 min of stimulation and declined thereafter, suggesting that LPA induced rapid and transient tyrosine phosphorylation of HER2. The amount of HER2 immunoprecipitation was the same with or without LPA (Fig. 1A). In reverse experiments, phosphotyrosine immunoprecipitation and HER2 immunoblot revealed the same results (Fig. 1B).

*S1P induced tyrosine phosphorylation of HER2 in gastric cancer cells*

S1P has been shown to transactivate EGFR in rat fibroblasts and vascular smooth muscle cells, but not HER2 in any cell type [22–24]. MKN28 and MKN74 cells were incubated with 1 μM S1P for 2–40 min, and examined for whether tyrosine phosphorylation of HER2 was induced by S1P. With the same time course as LPA, S1P significantly induced rapid and transient tyrosine phosphorylation of HER2 in both MKN28 and MKN74 cells (Fig. 2A). The degree of phosphorylation induced by S1P was almost the same as that induced by LPA, and the amount of HER2 immunoprecipitation was the same with or without S1P (Fig. 2A). In reverse experiments, phosphotyrosine immunoprecipitation and HER2 immunoblot revealed the same results (Fig. 2B).

**Transactivation of HER2 was dependent on EGFR tyrosine kinase activity and metalloproteinase function in gastric cancer cells**

To elucidate the mechanisms of the transactivation, we performed several experiments using specific inhibitors. The EGFR inhibitor AG1478 (250 nM) markedly inhibited LPA-induced tyrosine phosphorylation of HER2 in both MKN28 and MKN74 cells, which suggests that transactivation of HER2 requires EGFR tyrosine kinase activity (Fig. 3). These results seem reasonable, since HER2 is activated through forming homo- and heterodimers with EGFR.

LPA-induced EGFR transactivation is also known to require MMP activation, which results in cleavage of the membrane-anchored growth factor precursor pro HB-EGF in some cells [11,25]. To address the question of whether such a ligand-dependent mechanism is also involved in HER2 transactivation in gastric cancer cells, we next examined the effect of the MMP inhibitor, GM6001. As shown in Fig. 3, LPA-induced tyrosine phosphorylation of HER2 in response to S1P in human gastric cancer cells. (A) Human gastric cancer MKN28 and MKN74 cells were serum-starved for 24 h and then incubated with 1 μM S1P for 2–40 min. After cell lysis, HER2 was IP using monoclonal anti-HER2 antibody, and immunoprecipitates were immunoblotted with monoclonal anti-phosphotyrosine antibody. Then the membrane was stripped and immunoblotted with anti-HER2 to detect HER2 at Mr 185 kDa, as a control. (B) Human gastric cancer MKN28 and MKN74 cells were serum-starved for 24 h and then incubated with 10 μM LPA for 2–10 min. These cell lysates were IP with anti-phosphotyrosine antibody, and immunoprecipitates were immunoblotted with anti-HER2 antibody.
phosphorylation of HER2 was almost abolished by 30-min preincubation with GM6001 (25 μM) in both cell types. These results in gastric cancer cells are compatible with the previous report on LPA-induced transactivation of HER2 in HNSCC [11].

Similar experiments were performed on S1P-induced transactivation. As shown in Fig. 4, both AG1478 (250 nM) and GM6001 (25 μM) partly abolished tyrosine phosphorylation of HER2 after S1P treatment, although the inhibitory effects on S1P-induced transactivation were less prominent than those on LPA-induced transactivation. Taken together, these results implicate that the regulation of MMPs and intrinsic EGFR tyrosine kinase activity have major roles in LPA- or S1P-induced transactivation of HER2, although S1P and LPA might be involved in different mechanisms.

**Tyrosine phosphorylation of HER2 induced by LPA or S1P was not inhibited by pertussis toxin**

It has been reported that LPA-induced EGFR transactivation is PTX sensitive, suggesting involvement of the Gi protein in this signaling pathway [26,27]. We also confirmed this phenomenon in both MKN28 and MKN74 cells (data not shown). Then, to see whether Gi is also involved in HER2 transactivation in gastric cancer cells, we examined the effect of PTX. As shown in Fig. 5, 24-h pretreatment with 100 ng/ml PTX did not significantly inhibit tyrosine phosphorylation of HER2 in response to either LPA or S1P. Even with 200 ng/ml PTX pretreatment, or even with 48-h pretreatment, PTX did not significantly inhibit HER2 tyrosine phosphorylation (data not shown). This suggests that both LPA- and S1P-induced HER2 transactivation are Gi-independent.

**HER2 was implicated in the lysophospholipid-induced ERK/MAPK pathway in gastric cancer cells**

Activation of the ERK/MAPK pathway is a key step in the regulation of important cellular responses such as cell proliferation. Both LPA and S1P were known to be able to induce ERK activation [10,11,21,23]. We therefore investigated the effect of both lysophospholipids on ERK activity in gastric cancer cells, by immunoblotting cell lysates with an anti-phospho-ERK1/ERK2 antibody. In time course experiments, LPA (10 μM)-induced ERK activation was detectable as early as 5 min after stimulation and peaked within 10 min in MKN28 cells (Fig. 6A). S1P (1 μM)-induced ERK activation was also detectable as early as 5 min after stimulation and peaked within 10 min in MKN28 cells, which is almost the same time course as that induced by LPA (Fig. 6A). MKN74 cells showed mostly the same responses (data not shown).

In some cells, EGFR have been implicated in ERK activation induced by LPA or S1P [20,23]. Therefore, we next analyzed the role of HER2 as well as EGFR in ERK activation of gastric cancer cells, by using a HER2 tyrosine kinase inhibitor, AG825, as well as an EGFR tyrosine kinase inhibitor, AG1478. MKN28 cells were deprived of serum overnight, pretreated with AG1478 (250 nM) or AG825 (30 μM) for 30 min, and then stimulated with 10 μM LPA, or 1 μM S1P, for 10 min. As shown in Fig. 6B, both AG1478 and AG825 significantly reduced the lysophospholipid-mediated ERK phosphorylation signal. Inhibitory effects of AG825 were less prominent than those of AG1478. Whereas AG1478 is known as an EGFR specific inhibitor, it also blocked HER2 signals as shown in Figs. 3
Taken these results together, HER2 as well as EGFR were implicated in the lysophospholipid-induced ERK pathway in gastric cancer cells.

Discussion

LPA and S1P are serum-borne lysophospholipids that signal through their cognate GPCRs. LPA, the simplest glycerophospholipid, mediates a broad range of cellular responses, including smooth muscle cell contraction, platelet aggregation, regulation of cell proliferation, protection from apoptosis, modulation of chemotaxis, and transcellular migration [28–30]. S1P also regulates various cellular responses very similar to those induced by LPA, while acting through distinct receptors [28–30]. Some of these cellular responses implicate these lysophospholipids as mediators of tumor progression [17]. In this study, we demonstrated that both LPA and S1P can induce tyrosine phosphorylation of HER2 in gastric cancer cells. This fact indicates that these lipid mediators may be potent stimulators of gastric cancer that act upstream of HER2 signaling pathways (Fig. 7).

It has been known that treatment of cells with ligands for GPCRs results in the subsequent activation of RTKs, a phenomenon termed “transactivation.” Stimulation of LPA has been found to lead to increased tyrosine phosphorylation, and hence activation, of EGFR in several different cell types [10]. LPA has also been reported to induce tyrosine phosphorylation of HER2 in Rat-1 fibroblasts and HNSCC, but not in HeLa cells [10,11,21]. Thus, RTK transactivation induced by GPCR ligands seems to be cell type specific [15,22–24]. In this study, we used two gastric cancer cell lines and revealed that LPA induced HER2 transactivation in both. We newly found that HER2 transactivation by LPA reached a maximum in a few minutes, which is mostly consistent with previous reports on EGFR transactivation by LPA [10,26]. We also revealed that regulation of MMPs and intrinsic EGFR tyrosine kinase activity is crucial for LPA-induced transactivation of HER2 in gastric cancer cells, which is also compatible with previous reports on HNSCC [11]. Taken together, these results indicate that LPA rapidly induces proteolytic cleavage of transmembrane precursors such as pro-HB-EGF via MMP activation, which results in activation of HER2 as well as EGFR in gastric cancer cells.

SIP, similar to LPA, can activate various RTKs such as EGFR, platelet-derived growth factor β receptor (PDGFβR), and vascular endothelial growth factor (VEGF) receptors, Flk-1/KDR and VEGFR2 [22–24,31,32]. In addition to these RTKs, we newly found that HER2 was also transactivated by S1P in our study of gastric cancer cells. The time course of S1P-induced HER2 transactivation was almost the same as that of

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Fig. 6. HER2 as well as EGFR mediates lysophospholipid-induced ERK activation in human gastric cancer cells. (A) Human gastric cancer MKN28 cells were serum-starved for 24 h and then incubated with 10 μM LPA or 1 μM S1P for 5–60 min. After cell lysis, cell lysates were immunoblotted with polyclonal anti-phospho-ERK1/ERK2 antibody. Then the membrane was stripped and immunoblotted with monoclonal anti-ERK2 antibody, as a control. (B) Human gastric cancer MKN28 cells were serum-starved for 24 h, pretreated with 250 nM AG1478, 30 μM AG825, or an equal volume of vehicle (DMSO) for 30 min, and stimulated with 10 μM LPA or 1 μM S1P for 10 min. After cell lysis, cell lysates were immunoblotted with polyclonal anti-phospho-ERK1/ERK2 antibody. Then the membrane was stripped and immunoblotted with monoclonal anti-ERK2 antibody, as a control.

Fig. 7. Summary of the effects of LPA and S1P on human gastric cancer cells.
LPA-induced HER2 transactivation. We also found that S1P-induced transactivation of HER2 was significantly inhibited by GM6001 and AG1478, suggesting an overlapping mechanism between LPA- and S1P-induced HER2 transactivation. As for transactivation induced by S1P, S1P-stimulated transactivation of EGFR requires MMP activation, whereas S1P-stimulated transactivation of PDGFR and VEGFR2 does not require MMP activation [24,32]. This suggests a different pathway of S1P-induced transactivation between members of the EGFR family and other RTKs. Since MMP activation induced proteolytic cleavage of transmembrane precursors such as pro-HB-EGF, which subsequently activates RTKs of the EGFR family, it seems to be reasonable that S1P-induced transactivation of HER2 also requires MMP activation [25,26]. The inhibitory effects of GM6001 and AG1478 on HER2 transactivation were not as clear with S1P as with LPA. The reason is not clear, but might depend on their receptor systems. A mechanistic study is currently underway.

Previous reports showed that Gi-, Gq-, and G13-coupled receptors can transactivate EGFR after agonist stimulation in diverse cell systems [26,33]. Thus, EGFR transactivation seems to occur via both PTX-insensitive and PTX-sensitive pathways. We confirmed that EGFR transactivation by both LPA and S1P was PTX sensitive in gastric cancer cells (data not shown). In contrast, nothing is known about the involvement of G proteins in HER2 transactivation. In this study, we found that HER2 transactivation either by LPA or S1P in gastric cancer was not significantly inhibited by PTX in any experimental conditions. This suggests that other G proteins play a major role in their signaling pathways, since each LPA receptor and each S1P receptor couples with several heterotrimeric G proteins such as Gq and G13.

Whereas many studies have repeatedly demonstrated the involvement of EGFR in LPA-induced, or S1P-induced, ERK activation, the involvement of HER2 was not shown yet [10,11,21,23]. Thus, in order to establish the role of the HER2 pathway in mitogenic signaling by LPA and S1P, we measured ERK1/2 activation under the various experimental conditions. Using an EGFR specific inhibitor, AG1478, and a HER2 specific inhibitor, AG825, we revealed that not only EGFR but also HER2 was certainly implicated in the lysophospholipid-induced ERK pathway in gastric cancer cells. Since AG1478 is known as an EGFR specific inhibitor, many previous studies, by using AG1478, concluded that the EGFR was involved in LPA-induced ERK activation [10,23]. However, AG1478 also blocked the phosphorylation of HER2 molecules as we showed in Figs. 3 and 4. Therefore, the inhibitory effect of AG1478 on ERK activation induced by both lysophospholipids may be partially dependent on HER2 related signals.

Whereas the involvement of EGFR in LPA-induced ERK activation was thus reported, the EGFR-mediated signaling pathway may not be the only signaling pathway that LPA employs to activate ERK. For examples, Andreev et al. [34] reported that LPA could stimulate ERK activation in mouse embryonic fibroblasts derived from EGFR knockout mice, suggesting the negative contribution of EGFR signal on LPA-induced ERK activation. Although the exact reason for this discrepancy is not clear, some explanations are possible. First, EGFR transactivation by GPCRs is proposed to be mediated by both intracellular and extracellular processes [25,35]. Andreev et al., as they described in their article, investigated only an intracellular process of transactivation. In contrast, in our present study, since an MMP inhibitor mostly inhibited tyrosine phosphorylation of HER2 induced by LPA and S1P, transactivation in our experiments seems to be mediated mainly via an extracellular process. Second, expression profiles of LPA receptors or S1P receptors are different between gastric cancer cells and embryonic fibroblasts. Both MKN28 and MKN74 cells express LPA2 receptor and S1P2 receptor, both of which are ubiquitously expressed in various cancer cells as well as normal tissues [20,36]. In contrast, embryonic fibroblasts were shown to exceptionally express LPA4 receptor [37]. Although the role of LPA4 receptor is still unclear, it may play specific roles in EGFR-independent ERK activation.

In summary, this study demonstrated that both LPA and S1P transactivate HER2 at physiological concentrations. Our results indicate that these lipid mediators utilize HER2 as a downstream signaling partner and play roles as tumor promoters via HER2 transactivation. Since gastric cancer tissues frequently express the HER2 antigen, and may be exposed to sufficient concentrations of S1P and LPA, the modulation of HER2 signals by these two lipid mediators may critically determine the fate of cancer cells. The regulation of HER2 as well as other RTK signals by specific antagonists of these lipid mediators or their receptors may be a new and effective treatment strategy for gastric cancer.

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References

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