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Lysophospholipids transactivate HER2/*neu* (*erb*B-2) in human gastric cancer cells

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Abstract

The ligand-less receptor HER2/*neu* (*erb*B-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.

Keywords: HER2/neu; Lysophosphatidic acid; Sphingosine 1-phosphate; Lysophospholipid; Transactivation; Receptor tyrosine kinase; Gastric cancer

The HER2/Neu (ErbB2) receptor is the protein product of the *her*2/*neu*/*erb*B-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 (S1P) is also released in large amounts [13]. Therefore, as products of the blood coagulation cascade, these two lipid mediators, LPA and S1P, 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 S1P 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). S1P 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-ERK2 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 S1P, respectively, we used 10 μM LPA and 1 μM S1P 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 S1P was added to the culture thereafter. After stimulation of starved cells with 10 μM LPA or 1 μM S1P 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