Sphingosine 1-phosphate transactivates c-Met as well as epidermal growth factor receptor (EGFR) in human gastric cancer cells

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Abstract Receptor tyrosine kinases (RTKs) are transactivated by the stimulation of G protein-coupled receptors (GPCRs). Sphingosine 1-phosphate (S1P), a ligand of GPCR, is known as a tumor-promoting lipid, but its signaling pathways are not fully understood. We here demonstrated that S1P induces rapid and transient tyrosine phosphorylation of epidermal growth factor receptor (EGFR) and c-Met in gastric cancer cells, both of which have been proposed as prognostic markers of gastric cancers. The pathway of S1P-induced c-Met transactivation is Gi-independent and matrix metalloproteinase-independent, which differs from that of EGFR transactivation. Our results indicate that S1P acts upstream of various RTKs and thus may act as a potent stimulator of gastric cancer.

Keywords: Sphingosine 1-phosphate; Transactivation; Receptor tyrosine kinase; c-Met; Epidermal growth factor receptor; Gastric cancer cell

1. Introduction

Crosstalk between different members of receptor families has become a well-established concept in signal transduction. Tyrosine phosphorylation of various receptor tyrosine kinases (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. Epidermal growth factor receptor (EGFR), HER2/neu, platelet-derived growth factor β receptor (PDGFβR), insulin-like growth factor-1 receptor, and vascular endothelial growth factor (VEGF) receptors have been shown to be transactivated by various ligands of GPCRs [1–6]. One of the most intensely studied pairs of receptors has been the RTK for EGF and the GPCR for lysophosphatidic acid (LPA). LPA, a natural phospholipid, is a potent mediator of a broad range of cellular responses, including regulation of cell proliferation, protection from apoptosis, and modulation of chemotaxis [7–10]. Whereas LPA binds to three GPCRs, LPA_1/Edg-2, LPA_2/Edg-4, and LPA_3/Edg-7, it has been reported that LPA utilizes, at least in part, EGFR as a downstream signaling partner in many cell types. In various human cancer cells, LPA-induced EGFR transactivation results in cell proliferation and motility, suggesting that LPA plays an important role as a tumor promoter via EGFR transactivation [11–14].

Sphingosine 1-phosphate (S1P), a structurally related lysophospholipid, is a bioactive lipid mediator that is released in large amounts from activated platelets [15]. S1P regulates various cellular responses very similar to those induced by LPA, while acting through distinct receptors [8]. Although whether S1P acts intracellularly as a second messenger or extracellularly as a receptor ligand, or both, is unknown, exogenously added S1P binds to five GPCRs, $S1P_1$/Edg-1, $S1P_2$/Edg-5, $S1P_3$/Edg-3, $S1P_4$/Edg-6, and $S1P_5$/Edg-8. They are ubiquitously expressed and coupled to various G proteins that regulate numerous downstream signals. $S1P_1$-knockout mice exhibit intrauterine death owing to vascular abnormalities, which indicates that S1P signaling is essential during mammalian development [16]. S1P signaling is also linked to diverse biological processes in human cancer, such as cell proliferation and migration, and therefore S1P is now recognized as a tumor-promoting lipid [17–20]. Thus, the S1P signaling pathway may have important functions in both physiological and pathological conditions. Previous reports have shown that S1P can also transactivate RTKs. For example, exogenously added S1P transactivated EGFR in fibroblasts and vascular smooth muscle cells (VSMC), but not in human umbilical vein endothelial cells (HUVEC) [3,5,21]. S1P also transactivated PDGFβR in mouse embryonic fibroblasts, VSMC, and ovarian and breast cancer cells, and VEGF receptor, Flk-1/KDR and VEGFR2 in HUVEC [3,5,22,23]. These results suggest that the transactivation by S1P is dependent on cell types and possibly on S1P receptor subtypes.

In the present study, using two gastric cancer cell lines, we investigated whether S1P induces phosphorylation of two

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Abbreviations: EGF, epidermal growth factor; GPCR, G protein-coupled receptor; HB-EGF, heparin-binding EGF-like growth factor; HGF, hepatocyte growth factor; HUVEC, human umbilical vein endothelial cell; LPA lysophosphatidic acid; MMP, matrix metalloproteinase; PTX, pertussis toxin; RTK, receptor tyrosine kinase; S1P, sphingosine 1-phosphate; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cell

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RTKs, EGFR and c-Met, whose signals are crucial for progression in gastric cancer [24–26].

2. Materials and methods

2.1. Materials

S1P was purchased from Biomol (St. Louis, MO) and dissolved in DMSO to 2×10^{-3} M, divided into aliquots and stored at −80 °C. Recombinant human hepatocyte growth factor (HGF) was purchased from R&D Systems, Inc. (Minneapolis, MN). Rabbit polyclonal anti-human c-Met antibody (C-28), rabbit polyclonal anti-human EGFR antibody and mouse monoclonal anti-human phosphotyrosine antibody (PY20) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Pertussis toxin (PTX) and the broad-spectrum matrix metalloproteinase (MMP) inhibitor GM6001 were purchased from Calbiochem (La Jolla, CA). An EGFR tyrosine kinase inhibitor AG1478 was purchased from Biomol. Sphingomyelin, sphingosine, and diphenyleneiodonium chloride were purchased from Sigma (St. Louis, MO). The pyrazolopyridine derivative JTE-013, a specific $S1P_2$ receptor antagonist, was a gift from the Central Pharmaceutical Research Institute, Japan Tobacco Inc. (Osaka, Japan) [27].

2.2. 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 Co., Grand Island, NY).

2.3. Immunoprecipitation and western blot analysis

Immunoprecipitation and Western blot analysis were performed as described previously [14,28]. 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 S1P was added to the culture thereafter. After stimulation of starved cells with 1 μM S1P for various times, cellular protein lysates (1 ml/dish/tube) were obtained and then all proteins were incubated with antibodies against two RTKs, EGFR and c-Met (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 Co., Bedford, MA) for sequential incubation with 5% reconstituted non-fat milk powder to block non-specific 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, Inc., Buckinghamshire, England). Some cells were pretreated with 100 ng/ml PTX for 24 h before stimulation and other cells were pretreated with 5–50 μM GM6001 for 30 min before stimulation. All membranes were stripped and immunoblotted with antibodies against RTKs as a control.

2.4. Preparation of total RNA and RT-PCR analysis

Total RNA was isolated from MKN28 and MKN74 cells by the acid guanidine isothiocyanate/phenol/chloroform extraction method as described by Chomczynski and Sacchi [29]. Then, 1 μg of total RNA was reverse-transcribed using a SuperScript First-Strand Synthesis System (Invitrogen Co., Carlsbad, CA, USA). The primers used for the RT-PCR analysis have been reported previously [30,31]. The sequences of the primer used were as follows: $S1P_1$ receptor (429-bp product), sense, 5′-TATCAGCGCGGACAAGGAGAACAG-3′ and antisense, 5′-ATAGGCAGGCCACCCAGGATGAG-3′; $S1P_2$ receptor (220-bp product), sense, 5′-TCGGCCTTCATCGTCATCCTCT-3′ and antisense, 5′-CCTCCCGGGCAAACCACTG-3′; $S1P_3$ receptor (394-bp product), sense, 5′-CTGCCTGCACAATCTCCCTGACTG-3′ and antisense, 5′-GGCCCGCCGCATCTCCT-3′; glyceraldehydes-3-phosphate dehydrogenase (246-bp product), sense, 5′-GATGACA TCAAGAAGGTGGTGAA-3′ and antisense, 5′-GTCTTACT CCTTGGAGGCCATGT-3′. We ran 30 PCR cycles at 94 °C (denaturation, 1 min), 62 °C (annealing, 1 min), and 72 °C (extension, 1 min). PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

3. Results

3.1. S1P induced tyrosine phosphorylation of EGFR in gastric cancer cells

We first investigated whether S1P transactivates EGFR in human gastric cancer MKN28 and MKN74 cells. These cells were incubated with 1 μM S1P or 1 ng/ml EGF for 2 min and examined for whether tyrosine phosphorylation of EGFR was induced by S1P. As shown in Fig. 1, S1P induced significant tyrosine phosphorylation of EGFR in both MKN28 and MKN74 cells.

3.2. S1P induced tyrosine phosphorylation of c-Met in gastric cancer cells

The HGF receptor, c-Met, is also a member of RTKs, and binding of HGF induces autophosphorylation of tyrosine residues in c-Met. Recently, c-Met was reported to be transactivated by prostaglandin E2 (PGE2) in colon cancer cells, so we hypothesized that S1P can transactivate c-Met [32]. Human gastric cancer cells MKN28 and MKN74 were incubated with 1 μM S1P for 2–60 min, and examined for whether tyrosine phosphorylation of c-Met was induced by S1P. As shown in Fig. 2A, S1P induced significant tyrosine phosphorylation of c-Met in both MKN28 and MKN74 cells. Time course experiments revealed that phosphorylation was maximal after 2–5 min of stimulation and declined thereafter, suggesting that S1P induced rapid and transient tyrosine phosphorylation of c-Met. A polyclonal antibody against c-Met was used to detect $p140^{c\text{-}met}$ β-chain expression, as well as the $170^{c\text{-}met}$ precursor. The amount of c-Met immunoprecipitation was the same with or without S1P (Fig. 2A). In reverse experiments, phosphotyrosine immunoprecipitation and anti-c-Met immunoblot revealed the same results (Fig. 2B). Tyrosine phosphorylation of c-Met induced by 1 μM S1P was as strong as that induced by 5 ng/ml HGF (Fig. 3).

3.3. Tyrosine phosphorylation of c-Met induced by S1P was not inhibited by pertussis toxin

To elucidate the mechanism by which S1P transactivates c-Met, we used several inhibitors. To address the question of

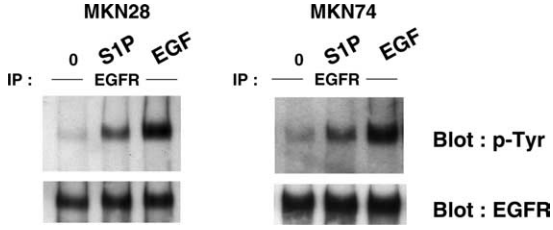

Fig. 1. Rapid tyrosine phosphorylation of EGFR in response to S1P in human gastric cancer cells. Human gastric cancer cells MKN28 and MKN74 were serum-starved for 24 h and then stimulated for 2 min with 1 μM S1P. After cell lysis, EGFR was immunoprecipitated (IP) using polyclonal anti-EGFR antibody and immunoprecipitates were immunoblotted with monoclonal anti-phosphotyrosine antibody. Cells treated with 1 ng/ml EGF for 2 min were used as a positive control for tyrosine phosphorylation. Then, the membrane was stripped and immunoblotted with anti-EGFR to detect EGFR at M_r 170 kDa, as a control.

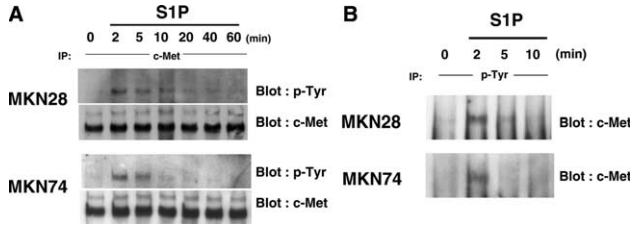

Fig. 2. Rapid tyrosine phosphorylation of c-Met in response to S1P in human gastric cancer cells. (A) Human gastric cancer cells MKN28 and MKN74 were serum-starved for 24 h and then incubated with 1 μM S1P for 2–60 min. After cell lysis, c-Met was IP using polyclonal anti-c-Met antibody and immunoprecipitates were immunoblotted with monoclonal anti-phosphotyrosine antibody. Then, the membrane was stripped and immunoblotted with anti-c-Met as a control. Anti-c-Met antibody was used to detect the β-chain of the c-Met receptor at M_r 140 kDa as well as its precursor form at M_r 170 kDa. (B) Human gastric cancer cells MKN28 and MKN74 were serum-starved for 24 h and then stimulated with 1 μM S1P for 2–10 min. These cell lysates were immunoprecipitated with anti-phosphotyrosine antibody and immunoprecipitates were immunoblotted with anti-c-Met antibody.

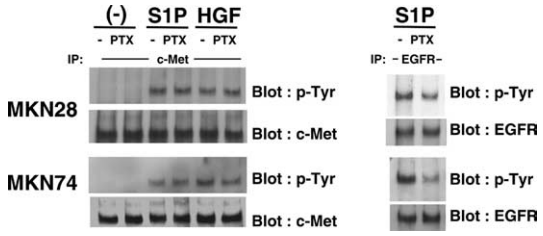

Fig. 3. Effect of pertussis toxin, a Gi inhibitor, on S1P-stimulated c-Met transactivation in gastric cancer cells. Human gastric cancer MKN28 and MKN74 cells were serum-starved for 24 h with or without 100 ng/ml PTX and then stimulated with 1 μM S1P or 5 ng/ml HGF for 2 min. After cell lysis, c-Met was IP using anti-c-Met antibody. Immunoprecipitates were immunoblotted with anti-phosphotyrosine antibody. Then, the membrane was stripped and immunoblotted with anti-c-Met as a control. The same experiments were performed using anti-EGFR antibody as a control for the inhibitory effect of PTX.

whether a Gi-dependent mechanism is involved in S1P-induced c-Met transactivation in gastric cancer cells, we examined the effect of PTX, a Gi inhibitor. As shown in Fig. 3, pretreatment with PTX partly decreased the phosphorylation of EGFR induced by S1P, which was compatible with previous reports on VSMC [3]. In contrast, the phosphorylation of c-Met induced by S1P was not altered by PTX pretreatment. Thus, S1P-induced c-Met transactivation in gastric cancer cells seems to be Gi-independent.

3.4. *Tyrosine phosphorylation of c-Met induced by S1P was not inhibited by GM6001*

Previous reports have shown that GPCR-induced EGFR transactivation requires MMP activation and cleavage of the membrane-anchored growth factor precursor pro heparin-binding EGF-like growth factor (HB-EGF) in COS-7, HEK-293 cells and HNSCC [2,11]. As shown in Fig. 4, pretreatment with the MMP inhibitor GM6001 decreased the phosphorylation of EGFR induced by S1P in a dose-dependent manner, which was compatible with previous reports. To address the question of whether such a ligand-dependent mechanism is also involved in c-Met transactivation in gastric cancer cells, we examined the effect of GM6001. As shown in Fig. 4, in contrast to EGFR transactivation, GM6001 did not significantly inhibit S1P-induced tyrosine phosphorylation of c-Met at any concentration. Thus, S1P-induced c-Met transactivation in gastric cancer cells does not require MMP activation.

3.5. *S1P Induced tyrosine phosphorylation of both c-Met and EGFR via $S1P_2$ receptor in gastric cancer cells*

We examined S1P-related molecules such as sphingomyelin and sphingosine, in order to investigate whether this transactivation is mediated by S1P receptor or by a direct effect of S1P on the plasma membrane. As shown in Fig. 5A, only S1P, but not sphingomyelin or sphingosine, induced transactivation of c-Met and EGFR, which suggests that transactivation of both is mediated by S1P receptor. Therefore, we next analyzed the mRNA expression of different S1P receptors by RT-PCR, to determine which isoforms of S1P receptor are expressed on gastric cancer cells. As shown in Fig. 5B, both MKN28 and MKN74 cells exclusively expressed the $S1P_2$ receptor among three major S1P receptors. We confirmed the reliability of our RT-PCR by using HUVEC as positive control for $S1P_1$ and $S1P_3$, and also confirmed that the RT-PCR product obtained without the addition of reverse transcriptase did not contain a DNA band in the gel (data not shown). We then examined the effect of JTE-013, a specific antagonist of the $S1P_2$ receptor, on transactivation induced by S1P. As shown in Fig. 5C, JTE-013 completely inhibited S1P-induced tyrosine phosphorylation of c-Met as well as that of EGFR in both cells. This compound did not inhibit HGF-induced tyrosine phosphorylation of c-Met or EGF-induced tyrosine phosphorylation of EGFR in gastric cancer cells (Fig. 5C). Taken together, these results indicate that S1P induced both c-Met phosphorylation and EGFR phosphorylation via $S1P_2$ receptor in gastric cancer cells.

3.6. *S1P-induced c-Met transactivation is independent from EGFR kinase activity in gastric cancer cells*

Transactivation of c-Met induced by PGE2 was shown to be dependent on EGFR kinase activity in colon cancer cells. However, very recent report revealed that c-Met transactivation induced by LPA and thrombin was independent on EGFR kinase activity in hepatocellular and pancreatic cancer cells [32,33]. Therefore, we examined the effect of an EGFR

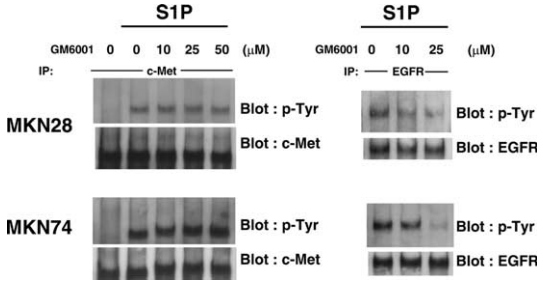

Fig. 4. Effect of GM6001, an MMP inhibitor, on S1P-stimulated c-Met transactivation in gastric cancer cells. Human gastric cancer cells MKN28 and MKN74 were serum-starved for 24 h and pre-incubated with the indicated concentrations of GM6001 for 30 min. After incubation with 1 μM S1P for 2 min, cell lysates were obtained and IP with anti-c-Met. Immunoprecipitates were immunoblotted with anti-phosphotyrosine antibody. Then the membrane was stripped and immunoblotted with anti-c-Met as a control. The same experiments were performed using anti-EGFR antibody as a control for the inhibitory effect of GM6001.

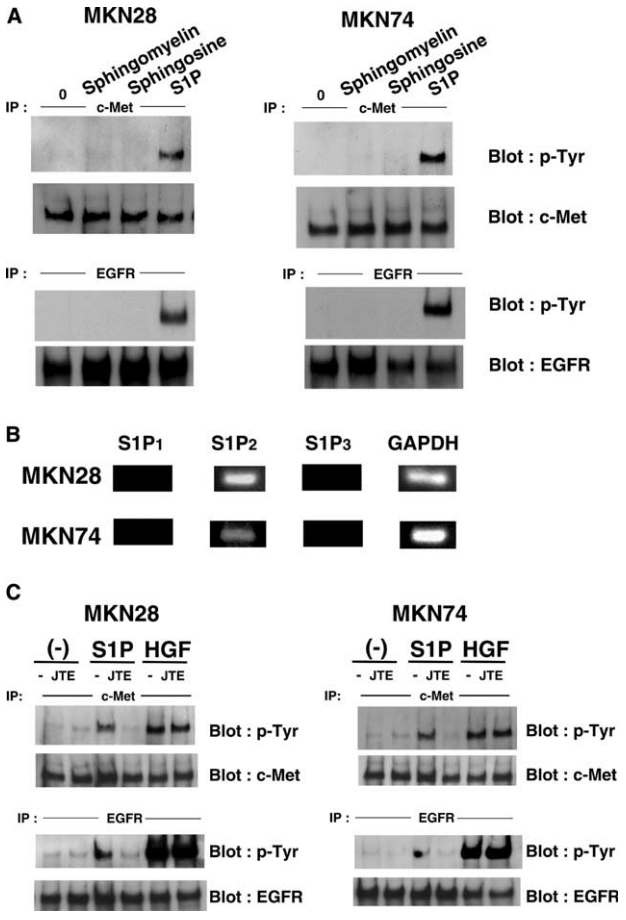

Fig. 5. Involvement of $S1P_2$ receptor with S1P-induced transactivation in gastric cancer cells. (A) Human gastric cancer cells MKN28 and MKN74 were serum-starved for 24 h and then stimulated for 2 min with 1 μM sphingomyelin, 1 μM sphingosine, and 1 μM S1P. After cell lysis, c-Met or EGFR was IP using polyclonal anti-c-Met or anti-EGFR antibody, respectively, and immunoprecipitates were immunoblotted with monoclonal anti-phosphotyrosine antibody. Then, the membrane was stripped and immunoblotted with anti-c-Met or anti-EGFR as a control. (B) RT-PCR analysis was performed on mRNA isolated from both MKN28 and MKN74 cells. (C) Human gastric cancer cells MKN28 and MKN74 were serum-starved for 24 h and then pre-incubated with 10 μM JTE-013 for 10 min. After incubation with 1 μM S1P for 2 min, cell lysates were obtained and IP with anti-c-Met or anti-EGF antibody. Immunoprecipitates were immunoblotted with anti-phosphotyrosine antibody. Then, the membrane was stripped and immunoblotted with anti-c-Met or anti-EGFR as a control.

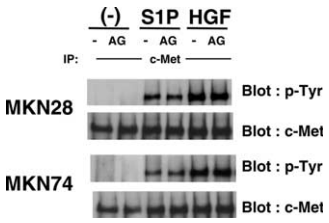

Fig. 6. Effect of AG1478, an EGFR tyrosine kinase inhibitor, on S1P-stimulated c-Met transactivation in gastric cancer cells. Human gastric cancer MKN28 and MKN74 cells were serum-starved for 24 h and pre-incubated with 250 ng/ml AG1478 for 30 min. After incubation with 1 μM S1P for 2 min, cell lysates were obtained and IP with anti-c-Met. Immunoprecipitates were immunoblotted with anti-phosphotyrosine antibody. Then, the membrane was stripped and immunoblotted with anti-c-Met as a control.

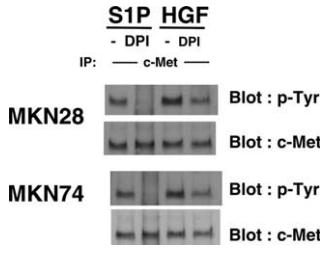

Fig. 7. Effect of DPI, an NADPH oxidase-specific inhibitor, on S1P-stimulated c-Met transactivation in gastric cancer cells. Human gastric cancer cells MKN28 and MKN74 were serum-starved for 24 h and then pre-incubated with 10 μM DPI for 30 min. After incubation with 1 μM S1P for 2 min, cell lysates were obtained and IP with anti-c-Met. Immunoprecipitates were immunoblotted with anti-phosphotyrosine antibody. Then, the membrane was stripped and immunoblotted with anti-c-Met as a control.

tyrosine kinase inhibitor, AG1478, on S1P-induced c-Met transactivation. Whereas AG1478 (250 nM) markedly inhibited EGF-induced tyrosine phosphorylation of EGFR (data not shown), it did not significantly inhibit c-Met phosphorylation induced by S1P as well as HGF (Fig. 6). These results suggest that S1P-induced c-Met transactivation was independent from EGFR kinase activity in gastric cancer cells.

3.7. S1P-induced c-Met transactivation is mediated with reactive oxygen species in gastric cancer cells

In a recent paper by Fischer et al. [33], the transactivation of c-Met by both LPA and thrombin has been shown to be mediated by reactive oxygen species (ROS). Thus, we examined the effect of an NADPH oxidase-specific inhibitor, diphenyleneiodonium chloride (DPI). Because recent lines of research implicated membrane-bound NADPH oxidases in the growth factor stimulated production of ROS, DPI interferes with NAPDH oxidase-dependent ROS production [33,34]. As shown in Fig. 7, blockade of NADPH oxidase function abolished c-Met phosphorylation induced by S1P in both cells, whereas only marginally affected c-Met phosphorylation induced by HGF.

4. Discussion

RTKs regulate many key processes, such as cell proliferation, differentiation, motility and survival. Dysregulation of RTKs by mutation, gene rearrangement, gene amplification, and overexpression of both receptors and ligands has been implicated as causative factors in the development and progression of numerous human cancers. Two RTKs, EGFR and c-Met, serve as prognostic markers in human gastric cancer, which indicate that both RTKs play an important role in gastric cancer development and progression [24–26]. In this study, we demonstrated that S1P transactivates EGFR and c-Met in gastric cancer cells. This intercellular receptor crosstalk between S1P receptors and these two RTKs suggests that S1P may be a potent stimulator of gastric cancer progression by acting upstream of various RTK signaling pathways (Fig. 8).

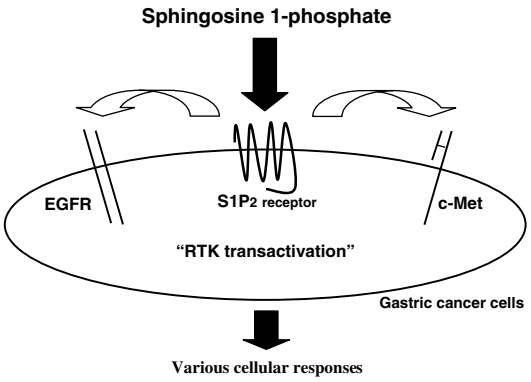

Fig. 8. Summary of effects of S1P on human gastric cancer cells.

EGFR is well-known to not only transduce EGF signals, but also integrate diverse stimuli such as LPA, bombesin, thrombin, and S1P [1,2,5,6,21]. This transactivation of EGFR represents the paradigm for cross-talk between GPCRs and RTK signaling pathways. From this knowledge, a novel picture of EGFR as a central cellular network element has been established in recent years, which broadens both its physiological as well as pathological significance [35–37]. In gastric cancer, *Helicobacter pylori* (*H. pylori*), a pathogenic Gram-negative bacterium that colonizes human gastric mucosa, is known to induce EGFR activation [38,39]. Since *H. pylori* infection has a strong association with the development of gastric cancer, S1P as well as other GPCR ligands may also be involved in the carcinogenesis of gastric cancer via EGFR transactivation [40,41].

c-Met is another RTK that is overexpressed in many solid tumors. HGF, the natural ligand for c-Met, induces autophosphorylation of tyrosine residues in c-Met and can lead to proliferation, increased survival, altered motility, and enhanced invasion into the extracellular matrix. Recently, c-Met was shown to be transactivated by PGE2 in colon cancer cells, which resulted in the promotion of colon cancer cell invasion [32]. In this study, we revealed that S1P induced definite tyrosine phosphorylation of c-Met in gastric cancer cells MKN28 and MKN74. We also revealed that tyrosine phosphorylation of c-Met induced by S1P was rapid and transient, which is the same as that of EGFR.

Previous studies indicate that S1P-stimulated transactivation of EGFR requires MMP activation, whereas S1P-stimulated transactivation of both PDGFβR and VEGFR2 does not, suggesting a different pathway of RTK transactivation by S1P [3,23]. In our study of gastric cancer cells, S1P-induced transactivation of c-Met was not significantly inhibited by GM6001. These data suggest that c-Met transactivation by S1P does not require MMP activation and that the pathway of S1P-induced c-Met transactivation in gastric cancer cells seems to differ from that of EGFR transactivation. Since MMP activation induced proteolytic cleavage of transmembrane pro-HB-EGF, which subsequently activates EGFR, it seems acceptable that c-Met as well as PDGFβR and VEGFR2 do not have such transmembrane precursors [2].

It has been shown that both S1P-induced EGFR transactivation and S1P-induced PDGFβR transactivation in VSMC are Gi-dependent [3]. In contrast, transactivation of c-Met induced by S1P in this study did not show inhibition by a Gi inhibitor. This is a novel finding in this field. Previous studies of other GPCR ligands have shown that not only Gi, but also Gq plays a role in EGFR transactivation [1,6,42]. In order to investigate the possible role of Gq in c-Met transactivation by GPCR ligands, we examined the effect of bombesin, whose receptor is known to couple with Gq. However, bombesin did not induce the significant phosphorylation of c-Met in gastric cancer cells under any experimental conditions (data not shown). It does not directly mean the negative contribution of Gq to c-Met transactivation, since the expression level of bombesin receptor is not characterized in these cells. Because of the lack of a well-known inhibitor against Gq, we could not obtain evidence for a role of Gq in S1P-induced c-Met transactivation.

We demonstrated that gastric cancer cells expressed $S1P_2$ receptor, and that its antagonist JTE-013 totally abrogated both c-Met and EGFR transactivation induced by S1P, suggesting that $S1P_2$ receptor mediates this transactivation. Previous reports have shown that $S1P_1$ receptor or $S1P_3$ receptor mediated cross-talk between S1P receptor and PDGFR [22,43–45]. Our results suggest the receptor specific cross-talk between RTKs and GPCR.

Pai et al. [32] have reported that PGE_2 transactivates c-Met in a manner dependent on functional EGFR in colon cancer cells. Others also reported that activated EGFR can phosphorylate and activate c-Met in thyroid cancer cells and hepatocytes [46,47]. In clear contrast, Fischer et al. [33] have recently reported that EGFR activity is not essential for c-Met transactivation induced by LPA or thrombin. In this study, we used AG1478 and found that S1P-induced c-Met transactivation was independent of EGFR kinase activity, which is compatible with the report of Fischer et al. [33].

Finally, we revealed that S1P-induced c-Met transactivation requires NAPDH oxidase activity. Plasma membrane-bound NAPDH oxidases have previously been implicated in the acute and rapid production of ROS in response to growth factor treatment [33,34]. Taken together, our results indicate that c-Met transactivation induced by S1P is mediated by ROS, which is compatible with previous report on that induced by LPA or thrombin [33].

In summary, this study demonstrates that S1P, at physiological concentration, activates two RTKs, whose signals are crucial for gastric cancer progression, although the mechanisms are somewhat different. Gastric cancers are often associated with local bleeding and thus, at the tumor site, platelets are activated and release various growth factors including S1P. Therefore, a significant amount of extracellular S1P is thought to be present in gastric cancer tissue and thus able to induce various important biological responses. The development of an antagonist for S1P or the appropriate spectrum of S1P receptors may also be new and effective therapy for gastric cancer.

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