LPA₁ receptors mediate stimulation, whereas LPA₂ receptors mediate inhibition, of migration of pancreatic cancer cells in response to lysophosphatidic acid and malignant ascites

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Malignant ascites from pancreatic cancer patients has been reported to stimulate migration of pancreatic cancer cells through lysophosphatidic acid (LPA) and LPA₁ receptors. Indeed, ascites- and LPA-induced migration was inhibited by Ki16425, an LPA₁ and LPA₃ antagonist, in Panc-1 cells. Unexpectedly, however, in the presence of Ki16425, ascites and LPA inhibited cell migration in response to epidermal growth factor (EGF). The inhibitory migratory response to ascites and LPA was also observed in the cells treated with pertussis toxin (PTX), a G protein inhibitor, and attenuated by a small interfering RNA (siRNA) specific to the LPA₂ receptor. The inhibitory LPA action was reversed by the regulators of G-protein signaling domain of p115RhoGEF, dominant-negative RhoA or C3 toxin. Indeed, LPA activated RhoA, which was attenuated by the siRNA against the LPA₂ receptor. Moreover, LPA₁, an LPA₂ agonist, also inhibited EGF-induced migration in the PTX-treated cells. A similar inhibitory migration response through LPA₂ receptors was also observed in YAPC-PD, BxPC-3, CFPAC-1 and PK-1 pancreatic cancer cell lines. LPA also inhibited the invasion of Panc-1 cells in the PTX-treated cells in the in vitro Matrigel invasion assay. We conclude that LPA₂ receptors are coupled to the G₁₂/₁₃ protein/Rho-signaling pathway, leading to the inhibition of EGF-induced migration and invasion of pancreatic cancer cells.

Introduction

Pancreatic cancer is a highly metastatic cancer characterized by widespread intraperitoneal dissemination and ascites formation, which frequently occur even after curative resection and are the major cause of death in pancreatic cancer patients (1,2). Thus, the elucidation of the mechanisms underlying peritoneal dissemination is of great importance to develop novel tools to treat pancreatic cancer. Peritoneal dissemination is thought to be composed of several processes, including cell adhesion, migration, invasion and proliferation (3,4). Lysophosphatidic acid (LPA), one of the simplest natural phospholipids, is a lipid mediator that evokes hormone- and growth factor-like responses in almost every cell type. Five subtypes of G-protein-coupled LPA receptors (LPA₁–LPA₅) have been identified so far (5–7). LPA elicits diverse cellular responses, including proliferation, survival, morphological change and motility. LPA has been shown to be present in various biological fluids, including malignant ascites (8–10), and implicated in the cancer initiation, progression and metastasis (11–13).

We have developed an LPA receptor antagonist, Ki16425, which shows a preference for LPA₁ and LPA₂ over LPA₃ (14). Using the LPA-specific antagonist, Ki16425, and LPA₁-specific small interfering RNA (siRNA), we have recently demonstrated that LPA in the malignant ascites from pancreatic cancer patients is an active component that stimulates the migration and invasion of pancreatic cancer cells, including Panc-1 and YAPC-PD, through LPA₂ receptors (9). The stimulatory role of LPA₁ receptors for cell migration is recognized in a variety of cell types, although LPA₂ receptors seem to mediate the inhibition of migration of T lymphocytes (15). For example, DLD1 colon cancer cells (16) and GNS-3314 and CGNH-89 glioblastoma cells (17) respond to LPA, resulting in the stimulation of migration through LPA₁ receptors. Hama et al. (18) have shown that LPA₁ receptors mediate migration of SF295 glioblastoma cells, MDA-MB-231 breast cancer cells, PC-3 prostate cancer cells and A-2058 melanoma cells in response to LPA and autotaxin, an LPA-producing enzyme. Ki16425 has been shown to inhibit migration response to LPA in cancer cells that express a larger amount of LPA₁ receptors than other LPA receptor subtypes (10,17,18). Hama et al. (18) also reported that LPA did not support the migration in cancer cells that predominantly express LPA₂ receptors but only marginally express LPA₁ receptors, including MCF-7 breast cancer cells, HT-29 and KM-12 colorectal cancer cells, OVCAR-4 and OVCAR-8 ovarian cancer cells, NCI-H522 lung cancer cells, LNCaP prostate cancer cells and Hela cervical cancer cells. Ren et al. (19) reported, however, that LPA₁ and LPA₂ receptors are critical for the migration and invasion of SKOV3 and HEY ovarian cancer cells. In pancreatic cancer cells, LPA₂ receptors, in addition to LPA₁ receptors, are significantly expressed; however, the role of LPA₂ receptors remains unknown (10).

In the present study, we extended the previous study and examined the effects of Ki16425 on the change in pancreatic cancer cell motility by LPA and ascites. Consistently with the previous study (10), Ki16425 potently inhibited the migration response to LPA and ascites in the pancreatic cancer cells. Unexpectedly, however, we observed that both LPA and ascites inhibited epithelial growth factor (EGF)-induced migration in the presence of Ki16425. This result suggests that LPA-rich ascites has the potential not only to stimulate but also to inhibit cell migration. We characterized the mechanisms underlying LPA- and ascites-induced inhibitory migration and found that the LPA₂ receptor/RhoA-signaling pathways mediate the inhibitory response to LPA and ascites when the functions of LPA₁ receptors and/or G proteins are blocked.

Materials and methods

Materials

I-Oleyl-sn-glycero-3-phosphate (LPA) was purchased from Cayman Chemical Co. (Ann Arbor, MI); fatty acid-free bovine serum albumin (BSA) was from Calbiochem-Novabiochem Co. (San Diego, CA); pertussis toxin (PTX) was from List Biological Laboratories (Campbell, CA); EGF was from Sigma-Aldrich (St Louis, MO); EZ-Detect Activation Kit for RhoA was from PIERCE (Rockford, IL) and monoglyceride (MG) lipase was from Asahi Kasei Corp.

Abbreviations: BSA, bovine serum albumin; CHO, Chinese hamster ovary; EGF, epidermal growth factor; LPA, lysophosphatidic acid; MG lipase, mono-glyceride lipase; mRNA, messenger RNA; MT, 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide; PTX, pertussis toxin; RGS, regulators of G-protein signaling; siRNA, small interfering RNA; S1P, sphingosine 1-phosphate.
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As the ascites were prepared from pancreatic cancer patients at the Division of Thoracic and Viscerai Organ Surgery, Gunnma Graduate University School of Medicine, from 2001 to 2003. Informed consent was obtained from each patient for the use of samples. Ascites was collected in the presence of ethylenediaminetetraacetic acid (at a final concentration of 2-3 mM) and centrifuged at 1000 g for 20 min to remove cells as described previously (10). The cell-free fluid was stored at –20°C until use. In the present study, we used the same ascites as those used in the previous study (10). The ascites A1, A2 and A3 used in the present study corresponds to the ascites from case 2, case 3 and case 4, in the previous study, respectively. Their LPA levels evaluated by a bioassay based on the ability to inhibit cyclic adenosine 3',5'-monophosphate accumulation in LPA-expressing RH7777 cells were 534 nM for case 2, 2318 nM for case 3 and 6637 nM for case 4, respectively (10).

Cell culture

Human pancreatic cancer cell lines, Panc-1 and PK-1, were kindly provided by the Cancer Cell Repository, Tohoku University (Sendai, Japan), and BxPC-3 and CFPAC-1 were purchased from the American Type Culture Collection (Rockville, MD). APC-YD pancreatic cancer cell line was established from the pancreatic cancer patient (10). All pancreatic cancer cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum. Twenty-four hours before the experiments, the medium was changed to a fresh medium (without serum) containing 0.1% (wt/vol) BSA (fraction V) unless otherwise specified. Where indicated, PTX (100 ng/ml) was added to the culture medium 2 h before the experiments.

Construction of adenoviral vector and infection of recombinant adenovirus

The recombinant adenovirus containing the regulators of G-protein signaling (RGS) domain of p115RhoGEF (p115RGS), C3 toxin and the dominant-negative RhoA (T199RhoA) was constructed as described previously (24). The adenovirus expressing green fluorescent protein was used for controls. For the infection with recombinant adenoviruses, pancreatic cancer cells were cultured on 6 or 10 cm dishes. When the cells had become 90% confluent, the cells were harvested by trypsin, seeded in RPMI 1640 containing 0.1% BSA and test agents and then further incubated at 37°C. All experiments were performed in duplicate or triplicate. The results of multiple experiments were expressed as mean ± SE.
from more than three different batches of cells, unless otherwise stated. Statistical significance was assessed by analysis of variance or the Student's *t*-test; values were considered significant at *P* < 0.05 (*).

**Results**

*Ki16425 modulates the migratory activity of Panc-1 cells in response to malignant ascites from pancreatic cancer patients and LPA*

Consistent with the previous study (10), LPA and malignant ascites from pancreatic cancer patients stimulated the migration of Panc-1, a pancreatic cancer cell line (Figure 1A, left panel), and the stimulatory migration response to LPA and ascites was markedly inhibited by Ki16425, an LPA\(_1\) and LPA\(_3\) antagonist (Figure 1B, left panel). EGF also stimulated the cell migration maximally, and the EGF-induced activity was not further enhanced by LPA or ascites (Figure 1A, right panel). Unexpectedly, however, LPA and ascites significantly inhibited the EGF-induced migration in the presence of Ki16425 (Figure 1B, right panel). Thus, LPA and ascites have the potential not only to stimulate but also to inhibit the migration response of Panc-1 cells. We have shown previously that MG lipase, an LPA-degrading enzyme, inhibited the stimulatory migration response to LPA and ascites (10). As shown in Figure 1C, prior treatment of LPA and ascites with MG lipase reversed their inhibitory migration activity in the presence of EGF, suggesting that not only stimulatory but also inhibitory actions of ascites on the migration response are mediated by LPA existing in the ascites. Both stimulatory and inhibitory actions were observed at a similar range of LPA concentrations (Figure 1D). In the present study, we used 1–10% ascites, in which LPA exists at the concentrations of ~0.005–0.66 μM (see Materials and Methods), which is enough to significantly exert both a stimulatory and an inhibitory migration responses.

**Inhibition of cancer cell motility by LPA and ascites**

LPA\(_1\) receptors stimulate, whereas LPA\(_2\) receptors inhibit, the migration of Panc-1 cells

In Panc-1 cells, LPA\(_1\) and LPA\(_2\) are major LPA receptor subtypes (Figure 2A). In order to examine the roles of the LPA receptor subtypes in the migration response, an siRNA strategy was employed: the respective siRNAs against the LPA\(_1\) and LPA\(_2\) receptors specifically inhibited their mRNA expression (Figure 2B). Under the conditions, siRNA specific to LPA\(_1\) receptors (siLPA\(_1\)) markedly inhibited the stimulatory migration response to LPA and ascites (Figure 2C, middle panel), whereas the siRNA specific to LPA\(_2\) (siLPA\(_2\)) was ineffective for inhibiting the stimulatory action (Figure 2C, right panel). These results support the notion that the stimulatory migration response is mediated by LPA\(_1\) receptors but not by LPA\(_2\) receptors. Similarly to the Ki16425-treated cells shown in Figure 1B, LPA and ascites inhibited EGF-induced migration in the siLPA\(_1\)-treated cells, whereas siLPA\(_2\) had no appreciable effect on the migration response to any of the agents employed (Figure 2C).

The lack of the significant effect by siLPA\(_2\), however, does not mean that the LPA\(_2\) receptor has no ability to regulate the migration activity. The inhibitory migration response was also observed when the cells were treated with PTX, which inhibits the function of G\(_i\) proteins (Figure 2D, left panel). These results suggest that the allowance of LPA and ascites to inhibit the migration response to EGF requires the blockage of the LPA\(_1\) receptor/G\(_i\) protein system. On the other hand, treatment of the cells with siLPA\(_2\), but not with siLPA\(_1\), almost completely reversed the inhibitory migration response induced by LPA and ascites in the PTX-treated cells (Figure 2D). Similarly, siLPA\(_2\) also reversed the inhibitory activity of LPA and ascites in the presence of Ki16425 (Figure 2E). These results suggest that LPA\(_2\) receptors mediate the LPA- and

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*Figure 1.* Effect of Ki16425, an LPA\(_3\) and LPA\(_3\) antagonist, on migration response to ascites and LPA. (A and B) The effect of Ki16425 on Panc-1 cell migration response to LPA, ascites, and EGF was examined. Panc-1 cells were treated for 10 min with (B) or without (A) 3 μM Ki16425 and then assayed for migration response to 1 μM LPA and 10% ascites prepared from three pancreatic cancer patients (A1, A2 and A3) in the presence or absence of 10 ng/ml EGF. The LPA concentration in 10% ascites was estimated to be ~0.005–0.66 μM as shown in Materials and Methods. The effect of LPA or ascites in the presence of EGF was significant. (C) LPA and ascites were treated with MG lipase as described in Materials and Methods and then migration assay was performed similarly as described in (B) except that final concentration of ascites was 1%. The inhibitory actions of LPA and ascites were reversed by MG lipase treatment. (D) Panc-1 cells were treated with or without 3 μM Ki16425 (Ki) and then incubated for migration response to the indicated concentrations of LPA in the presence or absence of 10 ng/ml EGF. The results are expressed as migrated cell numbers in four places under microscopy at ×400 magnification. Data are the means ±SEs of three separate experiments in (A–C) and the means ±SDs of a representative result in (D). Other experiment gave a similar result.
ascites-induced inhibitory migration response. The specificity of the siLPA2 was confirmed in Figure 2F. Sphingosine 1-phosphate (S1P), although small when compared with LPA, similarly inhibited the migratory response to EGF in the PTX-treated cells (Figure 2F, left panel). Unlike LPA, however, the S1P-induced inhibition of the migration response was hardly affected by the siLPA2 treatment.

The role of the inhibitory migration activity of LPA2 receptors was confirmed by the LPA2 agonist, LP-105, which has been reported to also have a weak antagonistic activity for LPA1 and LPA3 receptors (25). The LPA derivative inhibited the EGF-induced migration response in PTX-treated cells (supplementary Figure 1A is available at Carcinogenesis Online). The inhibitory activity of LP-105 was, as expected, attenuated by siLPA2 treatment (supplementary Figure 1B is available at Carcinogenesis Online).

Involvement of G_{12/13} proteins and RhoA in the inhibitory action via LPA2 receptors

Recent studies have suggested that G_{12/13} proteins mediate the inhibitory migration response to S1P through S1P2 receptors (26). Since LPA2 receptors are known to be coupled to G_{12/13} proteins (5), we examined the role of G_{12/13} proteins on the inhibitory migration response. The RGS domain of p115RhoGEF has been shown to interact with G_{12} or G_{13}, resulting in the inhibition of G_{12} or G_{13} signaling (27). As shown in Figure 3A, p115RGS did not affect the stimulatory action of LPA. However, the inhibitory LPA action in the presence of EGF was significant in (C–F).
downstream signaling pathways of G_{12/13} proteins, we employed a dominant-negative form of the RhoA gene (DN-RhoA), T19N-RhoA and C3 toxin genes. Neither DN-RhoA nor C3 toxin affected the LPA-induced stimulation of migration (Figure 3D), whereas both DN-RhoA and C3 toxin attenuated the LPA-induced inhibition of the migration response to EGF (Figure 3E). The expression of DN-RhoA protein (~21 kDa) was confirmed by the increase in the band by western blotting with RhoA antibody (F). The cells were then assayed for cell migration with or without 1 μM LPA and 10 ng/ml EGF. The results are expressed as migrated cell numbers. Data are the means ± SEs of three separate experiments.

LPA activates RhoA through the LPA_2 receptor
As shown in Figure 4A, LPA but not EGF stimulated formation of GTP-RhoA, an active form of RhoA, as measured by a pull-down assay. The LPA-induced RhoA activation was not appreciably affected by the treatment of the cells with either Ki16425 or PTX regardless of the presence of EGF (Figure 4A). On the other hand, siLPA_2 treatment inhibited the LPA-induced RhoA activation (Figure 4B). These results suggest that LPA activates RhoA through the LPA_2 receptor regardless of the activation state of the LPA_1 receptor/G_i protein.

Involvement of the LPA_2 receptor in the inhibition of migration of pancreatic cancer cell lines
In Figure 5, we examined the migration activity in pancreatic cancer cell lines, including YAPC-PD, BxPC-3, CFPAC-1 and PK-1, other than Panc-1. Although the expression profile of LPA receptor subtypes varies from cell to cell in all pancreatic cell lines (10), the mRNA expression of both LPA_1 and LPA_2 receptors is commonly observed (Figure 5A–D). Moreover, both LPA and EGF stimulated cell migration, PTX treatment abolished the LPA-induced migration activity without having any significant effect on the EGF-induced action and finally LPA significantly inhibited EGF-induced migration in the PTX-treated cells. The role of LPA_2 receptors in the inhibitory LPA action was confirmed by the siLPA_2 treatment: LPA-induced...
inhibition of the migration response to EGF was almost completely reversed (Figure 5E–H) under the conditions in which siLPA2 specifically inhibited LPA2 mRNA expression (Figure 5A–D).

LPA inhibits Matrigel invasion in the cells treated with PTX
Proteolysis of extracellular matrix proteins is an important step for the invasion and metastasis of cancer cells. The possibility that LPA inhibits the invasion of pancreatic cancer cells was studied using a Matrigel invasion assay. Panc-1 cells were loaded on Matrigel-coated pore filters for 24 h in the absence or presence of EGF or LPA in the lower chamber, and the cells that had migrated to the lower surface of the filters were counted. Consistently with the migration activity, LPA stimulated invasion and its activity was blocked by PTX treatment (Figure 6A and B); LPA inhibited EGF-induced invasion in the PTX-treated cells (Figure 6B) and moreover, the LPA-induced inhibition of invasion was attenuated by treatment of the cells with siLPA2 but not with siLPA1 (Figure 6C and D), suggesting that LPA, through LPA2 receptors, also has the potential to inhibit invasion in PTX-treated cells.

Viability and adhesion activity of the cells are not regulated by LPA, ascites and other agents, which modulate the motility activity
We finally examined if agents used in the present study changed the viability and adhesion activity of the cells rather than their invasive property and thereby affected the migration and invasive activity of the cells. For this purpose, the cells were treated with PTX and/or siLPA2. The treatment of the cells with PTX and/or siRNA hardly affected their viability, as assessed by MTT reduction assay (supplementary Figure 2A is available at Carcinogenesis Online). A part of the cells were harvested and seeded on the collagen-coated plates in the presence or absence of LPA, ascites, EGF and Ki16425. Four hours later, attached cell numbers were measured by the MTT assay. As shown in supplementary Figure 2B (available at Carcinogenesis Online), the adhesion activity was hardly affected by the agents employed. Thus, it is unlikely that the agents employed in the present study affected motility of the cells by changing cell viability and adhesion activity.

Discussion
Ki16425 is an LPA1 and LPA3 antagonist (14). Consistently with the previous study (10), the LPA antagonist inhibited the migration of pancreatic cancer cells by malignant ascites from pancreatic cancer patients. Unexpectedly, however, the ascites inhibited EGF-induced migration in the presence of the LPA antagonist. The present report, to our knowledge, is the first indication that potent migration-stimulating ascites also has the potential to inhibit the migration activity of invasive pancreatic cancer cells. The elucidation of the inhibitory mechanism of cancer cell migration by ascites may provide a novel insight into the control of migration and, hence, invasion and metastasis of cancer cells.

The component of ascites that contributes to the inhibition of cell migration may be LPA. Indeed, LPA mimicked the ascites-induced inhibition of migration in the presence of Ki16425. The ascites- and LPA-induced inhibition of migration response to EGF was suppressed by MG lipase, an LPA-degrading enzyme. Moreover, the inhibitory response to ascites and LPA was attenuated by siRNA specific to LPA2 receptors, suggesting that LPA2 receptors mediate the inhibitory action. The inhibition of migration by LPA is also uncommon, but this is not the first report. Recently, Takuwa et al. reported that LPA inhibited the insulin-like growth factor-I-induced migration of Chinese hamster ovary (CHO) cells when the function of G1 proteins was inhibited by PTX, whereas LPA stimulated cell migration in the absence of the
toxin treatment (28). Jaganathan et al. (29) recently reported that LPA induces divergent migration responses depending on the activation status of Rho in human mesenchymal stromal cells. LPA inhibited the migration response in association with the activation of RhoA, whereas this was reversed to an induction of migration by LPA when RhoA was inactivated by the C3 toxin. In human mesenchymal stromal cells, LPA1 and LPA2 receptors are expressed; however, the role of the LPA receptor subtypes in the migration responses has not been examined in experiments (29).

In the CHO cell system, LPA1 receptors seem to mediate both the stimulatory and the inhibitory action (28). In our pancreatic cancer cell system, however, LPA2 receptors, rather than LPA1 receptors, mediate the inhibitory action, whereas LPA1 receptors mediate the stimulatory action. First, in pancreatic cancer cells, LPA1, LPA2 and LPA3 receptors are significantly expressed. As reported above, LPA2-siRNA, but not LPA1-siRNA, attenuated the LPA-induced inhibition of the migration response to EGF. Second, an LPA2 receptor agonist, LP-105, inhibited the EGF-induced migration response. Third, the

Fig. 5. LPA2 receptor-mediated inhibition of migration response also occurs in other pancreatic cancer cell lines, including YAPC-PD, BxPC-3, CFPAC-1 and PK-1. (A–D) The expression of LPA1 and LPA2 receptor mRNA in the cells treated with siLPA2, YAPC-PD cells (A), BxPC-3 cells (B), CFPAC-1 cells (C) and PK-1 cells (D) were treated with non-silencing RNA (NS) or siLPA2 and then the expression of LPA1 and LPA2 receptor mRNA was measured. The receptor mRNA content was first evaluated as the relative ratio to glyceraldehydes 3-phosphate dehydrogenase (GAPDH) mRNA content (×10³), which was 15.87 and 2.37 for YAPC-PD, 13.71 and 1.66 for BxPC-3, 15.34 and 4.21 for CFPAC-1 and 1.85 and 1.54 for PK-1 in LPA1 and LPA2 mRNA, respectively, in the NS-treated cells. In the figures, the respective receptor mRNA expression in the siLPA2-treated cells is shown as percentages of the respective mRNA expression obtained in the NS-treated cells. (E–H) LPA inhibits EGF-induced migration in the PTX-treated pancreatic cell lines through LPA2 receptors. The NS- or siLPA2-treated YAPC-PD cells (E), BxPC-3 cells (F), CFPAC-1 cells (G) or PK-1 cells (H) were further treated with PTX or without the toxin (Control) and then assayed for cell migration with or without 1 μM LPA and 10 ng/ml EGF. The results are expressed as migrated cell numbers. Data are the means ± SEs of three or four separate experiments. *The effect of LPA in the presence of EGF was significant.

Fig. 6. LPA inhibits EGF-induced invasion of Panc-1 cells through LPA2 receptors. (A–D) Panc-1 cells were first treated with non-silencing RNA (NS) (A and B), siLPA1 (C) or siLPA2 (D) and then treated with or without PTX. The cells were incubated for Matrigel invasion assay with or without 1 μM LPA and 10 ng/ml EGF. Data are the means ± SEs of three or four separate experiments. *The effect of LPA in the presence of EGF was significant.
inhibitory LPA action was observed in the presence of an LPA1 and LPA3 antagonist, Ki16425, suggesting that the inhibitory action is mediated by neither LPA1 nor LPA3 receptors. Finally, LPA1-siRNA allowed LPA to inhibit EGF-induced migration, whereas the siRNA inhibited the LPA-induced stimulatory migration response.

Although the LPA receptor subtype involved in the inhibitory migration response was different, the mechanisms leading to the inhibition of migration by LPA were very similar in CHO cells and Panc-1 cells. The inhibitory LPA action in the pancreatic cancer cells was attenuated by p115RGS, an RGS domain of p115RhoGEF that specifically blocks Gαq or Gα13 signaling; C3 toxin, a toxin that specifically deactivates Rho and T19NRhoA, the dominant-negative RhoA. This suggests that LPA inhibits the EGF-induced migration response through the G12/13 protein/Rho-signaling pathways, which is essentially the same signaling cascade for the LPA1-mediated inhibition of migration in PTX-treated or G1 protein-inactivated CHO cells (28). Both LPA1 and LPA2 receptors have been shown to be coupled to G12/13 proteins, which may cause Rho activation (5,12). Although previous studies showed that Rho is required for a migration response to a variety of growth factors (30–32), strong activation of Rho, via S1P2 receptor-mediated G12/13 protein activation, induces the inhibition of migration of CHO cells (33), B16 melanoma cells (34), glioblastoma cells (22,35), mouse embryo fibroblasts (36) and vascular smooth muscle cells (37). The activation of Rho induced by melatonin (38) and oligodendrocyte lineage transcription factor 2 (OLIG2) (39) has also been shown to inhibit the migration of MCF-7 breast cancer cells and U12-1 glioma cells, respectively. Consistently with the inhibitory role of Rho GTase in cell migration, cyclin D1 has been shown to stimulate the migration of mouse embryo fibroblasts in association with the inhibition of Rho/Rho kinase (40,41). Moreover, a Rho inhibitor, C3 toxin and a Rho kinase inhibitor, Y27632, have been shown to stimulate the migration of astrocytoma cells (42) and mouse hematopoietic progenitor cells (43).

The activation of Rho may be induced by several GTP-binding regulatory protein-coupled receptors; however, only S1P2 receptors (22,33–37) and LPA1 receptors (28) have been known to be able to inhibit cell migration. Based on the results of the present study, the LPA2 receptor may be listed as a third GTP-binding regulatory protein-coupled receptor to inhibit cell migration through the G12/13 protein/Rho-signaling pathways. The LPA-induced inhibition of the migration response to EGF in the G1 protein-inactivated Panc-1 cells was almost completely reversed by siLPA2 but not by siLPA1 (Figure 2D). Thus, in contrast to CHO cells, the LPA1 receptor is unable to inhibit the migration response in pancreatic cancer cells even under G1 protein-inactivated conditions. The difference in the LPA1 receptor expression level and its coupling efficiency to the effectors may explain the difference in the LPA1-mediated responses between the two cell lines. In any event, in order to detect the inhibitory LPA action, some specific modifications of the cells, i.e. inactivation of either LPA1 receptors or G1 proteins, are required. This may be the reason why the inhibitory role of LPA2 receptors was missing in the previous study.

However, it remains uncharacterized why the inhibitory action through the LPA2 receptor/G12/13 protein/Rho signaling on the Rac-mediated migration response requires inactivation of the LPA1 receptor/G1 protein signaling. LPA-induced Rho activation was inhibited by siLPA1 but was not affected by Ki16425 and PTX, suggesting that the LPA1 receptor/G1 protein signaling may modulate the downstream signaling of Rho rather than the LPA2 receptor-mediated Rho activation step. Although we have not measured the Rac activity in the present study, in the CHO cell system characterized by Sugimoto et al. (28), Rac activity is positively regulated by G1 proteins and negatively regulated by the G12/13 protein/Rho, although the downstream signaling leading to Rac inhibition by Rho remains unknown (26). Thus, they showed that, in the presence of functional G1 proteins, the negative signal is masked by the positive signal at the Rac activation step.

Although the further investigation is required to determine the mechanism underlying the LPA2 receptor-mediated inhibition of migration response, the result of the present study may justify the usage of LPA1 antagonists against the invasion and metastasis of cancer cells. In the cells expressing both LPA1 and LPA2 receptors, LPA1 antagonists and/or G1 protein inhibitors are expected to not only inhibit LPA1-mediated stimulation of migratory activity but also allow LPA, through LPA2 receptors, to inhibit the migration in response to growth factors. In other words, LPA becomes the inhibitory modulator for cancer cell motility in the presence of LPA1 antagonists or G1 protein inhibitors. LPA1 and LPA2 receptors are recognized to be widely expressed in a variety of cancer cells (11–13), including pancreatic cancer cells (10) and glioma cells (17,18). In the present study, even though we investigated only the pancreatic cancer cell line, all five strains of the cells examined showed the inhibitory migration response to LPA through LPA2 receptors under G1 protein-inactivated conditions. Although it remains uncharacterized whether or not the inhibition of motility of pancreatic cancer cells through LPA2 receptors occurs in vivo, Ki16425 has been shown to effectively attenuate the in vivo bone metastases (44). The in vivo effects of Ki16425 on the intraperitoneal dissemination and metastasis of pancreatic cancer cells are our current project.

In conclusion, blockage of LPA1 receptors and/or G1 proteins allows malignant ascites and LPA to actively inhibit the motility of pancreatic cancer cells through the LPA2 receptor/G12/13 protein/Rho-signaling pathways.

**Supplementary material**

Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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