Phosphatidylinositol Inhibits Vascular Endothelial Growth Factor-A–Induced Migration of Human Umbilical Vein Endothelial Cells

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Abstract. Phosphatidylinositol (PI), a phospholipid in component of cell membranes, is widely distributed in animals, plants, and microorganisms. Here, we examined in vitro whether PI inhibits the angiogenesis induced by vascular endothelial growth factor-A (VEGF-A). PI concentration-relatedly and significantly (at 10 and 30 µg/ml) inhibited VEGF-A–induced tube formation in a co-culture of human umbilical vein endothelial cells (HUVECs) and fibroblasts. PI also inhibited the migration, but not proliferation, induced in HUVECs by VEGF-A. Furthermore, PI at 30 µg/ml inhibited the VEGF-A–induced phosphorylation of serine/threonine protein kinase family protein kinase B (Akt) and p38 mitogen activate kinase (p38MAPK), key molecules in cell migration, but not phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), a key molecule in cell proliferation. These findings indicate that PI inhibits VEGF-induced angiogenesis by inhibiting HUVECs migration and that inhibition of phosphorylated-Akt and -p38MAPK may be involved in the mechanism. Therefore, PI may be expected to prevent some diseases caused by angiogenesis.

Keywords: angiogenesis, phosphatidylinositol, vascular endothelial growth factor-A, human umbilical vein endothelial cell, serine/threonine protein kinase family protein kinase B (Akt)

Introduction

Angiogenesis is defined as the generation of new vascular networks from existing blood vessels, with physiological angiogenesis being observed in endometrial, ovarian, and wound healing. However, abnormal angiogenesis has been reported to lead to retinal diseases [for example, age-related macular degeneration (AMD), diabetic retinopathy (DR), and neovascular glaucoma] and to aid the progress of invading cancerous cells (1–3). Angiogenesis involves multiple steps: 1) detachment of pre-existing mural pericytes (vascular destabilization), 2) degeneration of the extracellular matrix, 3) migration of endothelial cells, 4) proliferation of endothelial cells, 5) tube formation, and 6) reattachment of pericytes (vascular stabilization). Previous studies have revealed that angiogenesis is stimulated and/or regulated by various factors. Vascular endothelial growth factor-A (VEGF-A), a representative angiogenesis-promoting factor, is activated via VEGF receptor-2 (VEGFR-2) and causes endothelial cells to proliferate and migrate via elevations of phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) (proliferation), phosphorylated-serine/threonine protein kinase family protein kinase B (Akt) (migration), and p38 mitogen activated protein kinase (p38MAPK) (migration) (4–7).

Phosphatidylinositol (PI), a phospholipid component of cell membranes, is widely distributed in every organism. In particular, PI is abundant (at 20%–30% of total phospholipids) in seeds and yeast, and it consists of one mole of glycerol, two moles of fatty acid, one mole of a hexahydric alcohol (namely, inositol), and one mole of phosphoric acid. Yanagida (8) has reported that...
feeding mice with PI reduces the serum concentration of triacylglycerol (TAG) by 50%, serum cholesterol and phospholipid by 30%–35%, and the liver TAG concentration by 25% (versus TAG-fed mice). The proposed mechanism involves PI inhibiting phosphohydrolase (PAP) and diacylglycerol acyltransferase (DGAT) (8).

In the last few years, some metabolites of PI (for example, oleic acid, conjugated linoleic acid, and myoinositol trisphosphate) have been reported to have antiangiogenic effects in vitro and/or in vivo (9–12). Furthermore, phosphatidylinositol-3-phosphate (PI3P), a metabolite of PI, plays an important role in the intracellular signaling system that involves phosphorylation of Akt and induces an elevation of activated Rac (13). Therefore, PI might be expected to exert some influence over angiogenesis. At present, however, little is known about its effects on angiogenesis in vitro. The purpose of the present study was to clarify the effects of PI on angiogenesis in vitro (tube formation and cell proliferation and migration), and the phosphorylation of Akt, ERK1/2, and p38MAPK induced by VEGF-A.

Materials and Methods

Reagents

VEGF-A was purchased from Kurabo (Osaka). Antibodies against phosphorylated Akt (Ser 473) and total Akt and phosphorylated ERK1/2 (Thr 202/Tyr 204) and total ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA, USA).

Anti–phosphorylated-p38MAPK antibody and anti–total-p38MAPK antibody were purchased from Promega (Madison, WI, USA) and Cell Signaling Technology, respectively. PI was a gift from Asahi Kasei Pharma Corporation (Tokyo). PI was extracted using soy bean lecithin. Y294002 was purchased from Kasei Pharma Corporation (Tokyo). PI was a gift from Asahi Promega (Madison, WI, USA) and Cell Signaling Technology (Beverly, MA, USA).

Cell culture

Human umbilical vein endothelial cells (HUVECs, Kurabo) were cultured in growth medium (HuMedia-EB2, Kurabo) at 37°C in a humidified atmosphere of 5% CO2 in air. The HuMedia-EB2 medium consists of a base medium (HuMedia-EB2, Kurabo) supplemented with 2% fetal bovine serum (FBS), 10 ng/ml recombinant human epidermal growth factor (hEGF), 1 µg/ml hydrocortisone, 50 µg/ml gentamicin, 50 ng/ml amphotericin B, 5 ng/ml recombinant human basic fibroblast growth factor-B (hFGF-B), and 10 µg/ml heparin.

Tube-formation assay

An angiogenesis assay kit (Kurabo) was used according to the manufacturer’s instructions. Briefly, HUVECs co-cultured with fibroblasts were cultivated in the presence or absence of various concentrations of test drugs plus VEGF-A (10 ng/ml). After 11 days, cells were fixed in 70% ethanol. The cells were incubated with diluted primary antibody (mouse anti-human CD31, 1:4000) for 1 h at 37°C and then incubated with the secondary antibody (goat anti-mouse IgG alkaline phosphatase-conjugated antibody, 1:500) for 1 h at 37°C; visualization was achieved by using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT). Images were obtained from five different fields (5.5 mm2 per field) for each well; and then tube area, length, joints, and paths were quantified using Angiogenesis Image Analyzer Ver. 2 (Kurabo). Tube area means the total area measured 2-dimensionally. Tube length means the total length of the various tubes. A joint is where two different tubes intersect (i.e., a “branch point”). Path means the total number of pieces of tube branching from all joints.

Cell-proliferation assay

HUVECs were seeded into 96-well plates at a density 2,000 cells/well at 37°C for 12 h and preincubated in HuMedia-EB2 with 2% FBS at 37°C for 6 h. They were then incubated for 72 h in fresh medium containing VEGF-A (10 ng/ml) with or without various concentrations of PI. After incubation, the viable cell numbers were measured by means of a WST-8 assay. Briefly, 10 µl of CCK-8 (Dojindo, Kumamoto) was added to each well, incubated at 37°C for 3 h, and the absorbance measured at 492 nm (reference wave length, 660 nm).

Migration of HUVECs in wound-healing assay

An in vitro wound-healing assay was performed to measure unidirectional migration by HUVECs. HUVECs were seeded at 4 × 104 cells per well into a 12-well plate, incubated for 48 h at 37°C in a humidified atmosphere of 5% CO2, washed with PBS twice, and then incubated in HuMedia-EB2 with 1% FBS. After 24-h incubation, the monolayers of HUVECs were scratch-wounded to approximately 1-mm depth in a straight line using TR-222-C (1 to 200 µl) pipet tips (Axygen Scientific, Central Avenue, CA, USA). For stimulation, VEGF (10 ng/ml) was added, with or without PI at 3 to 30 µg/ml and LY294002 at 20 µM, and incubation continued for 24 h. Images were taken at the time of the wounding and at 24-h intervals thereafter using a phase-contrast microscope (Olympus, Tokyo). Migrated cells were estimated by counting the cell numbers in 4 randomly chosen fields (0.036 mm2 per field) within the wounded region.
Immunoblotting
Subconfluent HUVECs were incubated in HuMedia-EG2 at 37°C in a 5% CO₂ atmosphere. Then, the medium was replaced with Dulbecco’s modified Eagle medium (D-MEM) containing 25 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) (Invitrogen, Grand Island, NY, USA) and 0.5% FBS (for Akt detection) or 2% FBS (for ERK1/2 and p38MAPK detection), and then incubation was allowed to proceed for a further 18 or 1 h, respectively, at 37°C. Next, the medium was changed to fresh medium (constituents as above) containing VEGF-A (10 ng/ml) with or without PI (30 µg/ml), and then incubation was continued for 10 (for Akt detection) or 5 (for ERK1/2 and p38MAPK detection) min. The HUVECs were washed 2 times with 10 mM NaF in PBS, lysed in RIPA buffer (Sigma) supplemented with protease inhibitor cocktail (Sigma), phosphatase inhibitor cocktail 1 (Sigma), and phosphatase inhibitor cocktail 2 (Sigma), and stocked at −80°C. Equal amounts of the various samples were electrophoresed on an SDS-PAGE gel and then transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with Blocking One-P (Nacarai Tesque, Kyoto) for 30 min, the membranes were incubated with one of the following, as the primary antibody: anti-phosphorylated Akt, anti-total Akt, anti-phosphorylated ERK1/2, anti-total ERK1/2, anti-phosphorylated p38MAPK, or anti-total p38MAPK. After this incubation, the membrane was incubated with the secondary antibody: HRP-conjugated goat anti-rabbit IgG (Pierce Biotechnology, Rockford, IL, USA). The immunoreactive bands were visualized using Super Signal® West Femto Maximum Sensitivity Substrate (Pierce Biotechnology) and measured using GelPro (Media Cybernetics, Silver Spring, MD, USA).

Statistical analyses
Data are presented as means ± S.E.M. Statistical comparisons were made using a one-way ANOVA followed by Dunnett’s multiple-comparison test or a Student’s t-test. A value of P<0.05 was considered to indicate statistical significance.

Results
Effects of PI on VEGF-A–induced tube formation in HUVECs co-cultured with fibroblasts
Representative images of the tube formation induced by VEGF-A (with or without 3 to 30 µg/ml PI) are shown in Fig. 1A. Quantitative analysis showed that PI inhibited the tube formation (tube area, length, joints, and paths) induced by VEGF-A in a concentration-related manner (Fig. 1: B – E), with 10 µg/ml or more PI significantly decreasing all four parameters (versus the VEGF-A–treated control level) (Fig. 1: B – E).

Effects of PI on HUVEC proliferation
VEGF-A at 10 ng/ml increased cell proliferation in HUVECs to approximately 170% of the control (Fig. 2). Addition of PI at 3 – 30 µg/ml did not inhibit this VEGF-A–induced proliferation. PI alone (without VEGF-A) increased the HUVEC proliferation in a concentration-related manner, its effect being significant at 30 µg/ml (Fig. 2).

Effects of PI on HUVEC migration
To investigate whether PI would inhibit the migration of HUVECs, we performed a wound-healing assay under 1% FBS conditions. Briefly, HUVECs were scratch-wounded and treated with VEGF at 10 ng/ml with or without PI at 3 – 30 µg/ml. VEGF-A at 10 ng/ml increased cell migration in HUVECs to approximately 190% of the control (Fig.3B). PI inhibited this migration, its effect being significant at both 10 and 30 µg/ml (Fig. 3). Activation of PI3K-Akt signaling is known to be an important step in the VEGF-induced migration of HUVECs. Therefore, we ascertained that inhibition of PI3K inhibit VEGF-A–induced migration of HUVECs. LY294002 (20 µM), a PI3K inhibitor, significantly inhibited VEGF-A–induced migration (Fig. 3C). Moreover, LY294002 at 20 µM alone significantly inhibited basal cell migration (Fig. 3C).

Effects of PI on phosphorylation of Akt
Phosphorylation of Akt is known to be an important step in the VEGF-induced migration of HUVECs. Therefore, we examined the effects of PI at 30 µg/ml on the VEGF-A–induced phosphorylation of Akt. Treatment with VEGF-A at 10 ng/ml for 10 min increased the phosphorylation of Akt approximately 2.4-fold, and PI at 30 µg/ml significantly inhibited this increase (Fig. 4A).

Effects of PI on phosphorylation of ERK1/2 and p38MAPK
We examined that effects of PI on phosphorylated-ERK1/2 and p38MAPK induced by VEGF-A. Treatment with VEGF-A at 10 ng/ml for 5 min increased the phosphorylation of ERK1/2 and p38 MAPK to approximately 3.2- and 5.0-fold, respectively. PI did not affect the phosphorylation of ERK1/2 induced by VEGF-A (Fig. 4B). However, PI at 30 µg/ml significantly inhibited VEGF-A–induced phosphorylation of p38MAPK (Fig. 4C).
Discussion

In the present study, we found that PI inhibited angiogenesis in vitro, and our results suggest that its effect may be due in part to a reduction in cell migration through an inhibition of Akt phosphorylation.

VEGF promotes many of the events necessary for angiogenesis (such as the proliferation and migration of endothelial cells, the remodeling of the extracellular matrix, and the formation of capillary tubules) both in vitro and in vivo (14). It has been reported that antibodies against VEGF, supplementation with soluble VEGF receptor-1 (sVEGFR-1), and expression of antisense VEGF sequences all lead to inhibition of angiogenesis (15–17). These previous findings suggest that VEGF is one of the most important factors in angiogenesis. In the present study, we examined the effects of PI on VEGF-A-induced angiogenesis. In our in vitro assay, PI was used at 3 to 30 µg/ml, and it concentration-relatedly inhibited VEGF-A–induced tube formation in HUVECs co-cultured with human fibroblast cells.
Angiogenesis occurs through tube formation and adhesion of pericytes, with the tube formation involving degradation of the extracellular matrix, and migration and proliferation of endothelial cells (ECs). We therefore considered that the observed inhibitory effect of PI on VEGF-A–induced tube formation in this assay model might be due to an inhibition of the proliferation and/or migration of HUVECs. Hence, we examined whether PI inhibits the VEGF-A–induced proliferation and/or migration of HUVECs. PI at 3 to 30 µg/ml had no effects on VEGF-A–induced proliferation, but it concentration-relatedly inhibited the VEGF-A–induced-migration of HUVECs, its effects being significant at both 10 and 30 µg/ml. These results indicate that the inhibitory effect of PI on VEGF-A–induced tube formation may be attributed to an inhibition of the migration, rather than proliferation of ECs.

Jensen et al. (18) reported that some lysophosphatidyl lipids (LPL) [lysophosphatidylinositol (LPI), lysophosphatidylcholine (LPC), and lysophosphatidylserine (LPS)] and certain amphiphilic substances [Triton X-100, octyl-β-glucoside, arachidonic acid (AA), DHA, 5,8,11,14-eicosatetraynoic acid (ETYA), and capsaicin] inhibit the migration of ECs. They suggested that physical changes in the cell membrane may contribute to the effects of amphiphilic substances on EC migration. However, it seems likely that the inhibitory action of PI on migration involves another mechanism, in addition to or instead of the above mechanisms (see below). This is the first report to demonstrate that PI can inhibit the VEGF-A–induced migration of HUVECs.

Akt is recognized as a key regulator of cell-viability systems, and it is involved in the survival of cultured HUVECs (19, 20). Furthermore, the VEGF-A–induced migration of ECs is known to be Akt-phosphatidylinositol-3-kinase (PI3K)–dependent (21 – 23). In the present study, PI at 30 µg/ml inhibited the Akt phosphorylation induced by VEGF-A, indicating that PI induces a direct and/or upstream inhibition of Akt in the signaling cascade induced by VEGF-A. Ali et al. (24) indicated that, the PI3K inhibitor LY294002 at 10 µM inhibits the VEGF-induced migration of HUVECs. Therefore, these
results suggest that inhibition of phosphorylated-Akt inhibits the VEGF-A–induced migration. On the other hand, PI alone at 30 µg/ml did not have any effect on basal cell migration or on the basal phosphorylated-Akt level. We therefore consider that a physical change in the cell membrane upon supplementation with PI does not affect the signaling pathways mediating HUVEC migration, at least. On the other hand, LY294002 alone significantly inhibited basal cell migration. Therefore, it was considered that PI moderately inhibited VEGF-A–induced phosphorylation of Akt without affecting basal level of phosphorylated-Akt in contrast to LY294002.

VEGF-induced activation of PI3K catalyzes the phosphorylation of inositol phospholipids to generate 3'-phosphorylated phosphoinositides (25). The downstream target of PI3K is Akt, and the PI3K/Akt pathway has been focused upon in various studies on the role of PI3K in angiogenesis (25). Therefore, it might be expected that Akt would be activated by supplementation with PI, but we found that the VEGF-A–induced activation of Akt was inhibited by PI. Among the possible mechanisms, PI, or one of its metabolites, might operate as a negative feedback factor.

Several lines of studies have reported that ERK1/2 is mainly related to cell proliferation (4, 26). In the present study, PI did not inhibit the VEGF-A–induced proliferation of HUVECs or phosphorylation of ERK1/2. These results suggest that PI inhibits VEGF-A–induced phosphorylation of Akt, but does not affect the ERK pathway.

Previously studies have reported that p38MAPK induces phosphorylation of the heat shock protein-27 (HSP27), a molecular chaperone, and it positively regulates VEGF-induced actin reorganization and migration (7, 27). Therefore, we examined the effect of PI on VEGF-A–induced phosphorylation of p38MAPK. PI significantly inhibited VEGF-A–induced phosphorylation of p38MAPK, but PI alone did not affect basal phosphorylation. These findings suggest that the inhibitory effects of PI on VEGF-induced migration of HUVECs may be due to attenuation of Akt and p38MAPK phosphorylation. Furthermore, Rousseau et al. (7) showed that a p38MAPK inhibitor, SB203580, did not inhibit VEGF–A–induced proliferation of HUVECs or phosphorylation of ERK1/2. Therefore, these findings suggest that the inhibitory effects of PI on phosphorylated-p38MAPK do not affect cell proliferation or phosphorylation of ERK1/2.

Matrix metalloproteases (MMPs), specific proteases that degrade matrix components, are produced by ECs, fibroblasts, and vascular smooth muscle cells. Previous studies have suggested that there is a direct relationship between VEGF and the expressions of MMPs, their
expressions being enhanced by VEGF (28, 29). MMP-2 and membrane type-1-MMP (MT-1-MMP) are enzymes with roles in angiogenesis. Sroka et al. (30) suggested that in human prostate cancer cells (PC-3), MT-1-MMP expression is regulated by specificity protein-1 (Sp1), a zinc finger transcription factor of the Sp/Kruppel-like factor family, through activation of the Akt pathway. Hence, PI could also inhibit the VEGF-induced upregulation of MMPs through an inhibition of Akt activation in HUVECs. However, our results demonstrated that when applied alone, PI increased the proliferation of HUVECs. Similarly, when GM6001 (an MMP inhibitor) was applied alone, it increased the proliferation of HUVECs in our preliminary experiments (N. Matsunaga et al., unpublished data). GM6001 increased the proliferation of breast cancer cells (MDA-MB-435) (31). These findings indicate that MMP inhibitors may inhibit the MMP-induced degradation of growth factors and that PI may possibly act as an MMP inhibitor. However, further studies will be needed to clarify whether PI does indeed inhibit the VEGF-induced activation of MMPs.

In conclusion, our findings indicate that PI inhibits VEGF-induced angiogenesis and that this effect is mediated by an inhibition of cell migration, not of proliferation, through inhibitions of the Akt and p38MAPK pathways. On the basis of these findings, it might be expected that dietary PI would help to prevent angiogenesis-related diseases such as cancer, AMD, DR, and neovascular glaucoma, although this idea will need to be tested in further studies.

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