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Research Article

Lysophosphatidic acid up-regulates vascular endothelial growth factor-C and lymphatic marker expressions in human endothelial cells

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Abstract. Lysophosphatidic acid (LPA) is a low-molecular-weight lipid growth factor, which binds to G-protein-coupled receptors. Previous studies have shown that LPA enhances vascular endothelial growth factor-A (VEGF-A) expression in cancer cells and promotes angiogenesis process. However, the roles of LPA in lymphatic vessel formation and lymphangiogenesis have not been investigated. Here, we demonstrated that LPA up-regulated VEGF-C mRNA and protein expressions in human umbilical vein endothelial cells (HUVECs). Furthermore, the expression levels of lymphatic markers, including Prox-1, LYVE-1 and podoplanin, were enhanced in LPA-stimulated tube forming endothelial cells *in vitro* and *in vivo*. Moreover, we showed that pretreatment with MAZ51, a VEGFR-3 kinase inhibitor, and introduction of VEGFR-3 siRNA suppressed LPA-induced HUVEC tube formation and lymphatic marker expressions. These results demonstrated that LPA enhances expression of lymphatic markers through activating VEGF-C receptors in endothelial cells. This study provides basic information that LPA might be a target for therapeutics against lymphangiogenesis and tumor metastasis.

Keywords. LPA, VEGF-C, endothelial cells, lymphatic markers, lymphangiogenesis.

Introduction

Lysophosphatidic acid (LPA) is a low-molecular-weight phospholipid present in plasma and tissues at the micromolar level [1]. By interacting with cell surface G-protein-coupled receptors (GPCRs) of the endothelial differentiation gene (Edg) subfamily [2], LPA modulates various physiological functions such as cell proliferation, differentiation, and stimulation of tumor metastasis [3, 4]. Until now, five LPA receptors, LPA_{1-5}, have been identified. In previous

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studies, LPA was shown to regulate human endothelial cell proliferation [3, 5], migration [3, 6], capillary-like tube formation *in vitro* [7, 8], activation of proteases [9], and expression of inflammation-related genes [10]. These results suggested that LPA may play an important role in regulating vessel formation in endothelial cells.

Both angiogenesis and lymphangiogenesis are events dependent on vessel formation [11, 12]. Accumulating evidence indicates that LPA plays an important role in the angiogenesis process [13, 14]. However, the importance of LPA in lymphangiogenesis has not been studied. The formation of new lymphatic vessels depends on the effects of various lymphangiogenic factors [15, 16]. Vascular endothelial growth factor (VEGF)-C has been implicated as being a potent stimulator of both angiogenesis and lymphangiogenesis [17]. By binding with VEGFR-2 and VEGFR-3, VEGF-C exerts its biological functions in various cell types [18]. Many reports indicated that VEGF-C is a ligand for VEGFR-3, while proteolytically processed VEGF-C binds to both VEGFR-2 and VEGFR-3 [19]. Since VEGFR-2 is thought to be the main mediator of angiogenesis and VEGFR-3 is crucial for lymphangiogenesis [20, 21], the promotion by VEGF-C of endothelial cell participation in the lymphangiogenesis process is predominantly mediated through binding with VEGFR-3 [22]. Previous studies indicated that VEGF-C largely acts *via* VEGFR-2 in skin [23] and is predominantly mediated through VEGFR-3 in cancer cells [24], implying that VEGF-C might regulate cellular functions through different VEGF receptors in different cell types. VEGF-C has been reported to be expressed by several cancer cells such as ovarian carcinoma cells [25], lung adenocarcinoma cells [26], breast cancer cells [27], and head-and-neck squamous carcinoma cells [28], and to promote lymphatic metastasis progression, thus promoting tumor malignancy [29].

The lack of specific lymphatic markers has in the past hampered progress with lymphangiogenesis studies. However, the recent identification of specific lymphatic markers has greatly contributed to diagnoses of lymphatic disorders and to lymphangiogenesis research [30]. Among these markers, Prox-1, LYVE-1, and podoplanin are commonly used to determine the effects of VEGF-C on lymphangiogenesis [31, 32]. These three lymphatic markers have also been used to promote lymphatic metastasis of tumors in clinical research [33].

Many reports have shown that LPA up-regulates VEGF-A, another VEGF family member recognized as a potent angiogenic factor expressed by ovarian cancer cells, and which stimulates the angiogenesis process [34]. However, the role of LPA in regulating VEGF-C and the subsequent lymphangiogenesis process has not been studied. In the present study, we first observed that LPA up-regulated VEGF-C mRNA and protein expressions in human umbilical vein endothelial cells (HUVECs) in a dose- and time-dependent fashion. Moreover, LPA also enhanced the mRNA and protein expressions of the lymphatic markers, Prox-1, LYVE-1, and podoplanin, in HUVECs. In addition, LPA induced endothelial cell tube formation and elevations of Prox-1, LYVE-1, and podoplanin expressions in these LPA-stimulated endothelial cells in which tubes formed *in vitro* and *in vivo*. LPA also stimulated endothelial cell proliferation during the lymphangiogenesis process. Moreover, LPA-induced lymphatic marker expressions could be blocked by pretreatment with the VEGFR-3 kinase inhibitor and introduction of VEGFR-3 siRNA. Our findings first demonstrate that LPA might be a potent lymphangiogenic factor, which promotes VEGF-C expression in endothelial cells, thereby stimulating lymphatic markers expression and facilitating the lymphangiogenesis process.

Materials and methods

Reagents and antibodies. LPA and MAZ51 were purchased from Sigma (St. Louis, MO). Normal mouse and goat immunoglobulin G (IgG) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human recombinant VEGF-A and VEGF-C were purchased from R&D systems (Minneapolis, MN). Human recombinant EGF was purchased from PeproTech (Rocky Hill, NJ). Fetal bovine serum (FBS) and M199 were purchased from Hyclone (Logan, UT). Trypsin-EDTA was purchased from Gibco BRL (Grand Island, NY). Endothelial cell growth medium (EGM) was purchased from Cell Applications (San Diego, CA). Penicillin, streptomycin, and L-glutamine were purchased from Invitrogen (Carlsbad, CA).

Cell culture. HUVECs were isolated from fresh umbilical cords by treatment with 1% collagenase (Sigma) in phosphate-buffered saline (PBS) at 37 °C for 10 min. After elution with M199 containing 20% FBS, HUVECs were cultured on 0.04% gelatin-coated (Sigma) 10-cm plates (Greiner Bio-One, Kremsmuenster, Austria) in M199 medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine (Invitrogen), 10% (v/v) FBS, and 25% (v/v) EGM. Cells underwent one passage weekly. Cells were subcultured after trypsinization [in a 0.5% (v/v) trypsin solution, supplemented with 0.2% (v/v) EDTA] and used throughout passages 2–4.

Reverse-transcription polymerase chain reaction (RT-PCR). Total cellular RNA was extracted from HUVECs using the TRIzol reagent (Gibco), and a Superscript kit (Gibco) was used for the RT synthesis of cDNA. PCR amplification was performed using the oligonucleotide primers of human VEGF-C (5′-CTCACTTCCTGCCGATGC-3′ and 5′-GTTCGCTGCCTGACACTG-3′), LPA_1 (5′-CGGAGACTGACTGTCAGCA-3′ and 5′-GGTCCAGAACTATGCCGAGA-3′), LPA_3 (5′-TTAGCTGCTGCCGATTTCTT-3′ and 5′-ATGATGAGGAAGGCCATGAG-3′), Prox-1 (5′-AAGACAGAGCCTCTCCTGAATC-3′ and 5′-TTGCACTTCCCGAATAAGGTGAT-3′), LYVE-1 (5′-GTGCTTCAGCCTGGTGTTG-3′ and 5′-GCTTGGACTCTTGGACTCTTC-3′), and podoplanin (5′-CCAGGAGAGCAACAACTCAA-3′ and 5′-GATGCGAATGCCTGTTACAC-3′) with 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 2 min at 72 °C. The primers used to amplify GAPDH were 5′-dACCACAGTTCATGCCATCAC and 5′-dTCCACCACCCTGTTGCTGTA with 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 2 min at 72 °C. PCR products were resolved on 2% agarose gels, stained with ethidium bromide, and photographed.

Enzyme-linked immunosorbent assay (ELISA). The VEGF-C concentrations in the culture supernatant were measured by ELISA kits purchased from R&D Systems. An antibody specific for the cytokine to be studied was coated onto the wells of a 96-well ELISA plate. Samples, including standards with known cytokine contents, control specimens, and unknowns, were pipetted into these wells, followed by the addition of a biotinylated second antibody. During the first incubation, the cytokine antigen simultaneously bound to the captured antibody on one site and to the solution phase biotinylated antibody on a second site. After removal of any excess of the secondary antibody, conjugated streptavidin peroxidase (Chemicon International, Temecula, CA) was added. This enzyme bound to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove all unbound enzyme, a substrate solute was added, which was acted upon by the bound enzyme to produce color. The intensity of this colored product was directly proportional to the concentration of cytokines present in the original specimen.

Cyflow analysis. Sub-confluent HUVECs were starved for 16 h and treated as indicated. Suspensions of 10^6 cells were permeabilized with methanol (–20 °C). Permeabilized cells in 200 µl PBS with 0.1% fatty acid-free bovine serum albumin (BSA) were mixed with 2 µl of the antibodies against human Prox-1, LYVE-1, or podoplanin and were incubated for 1 h at 4 °C. Antibody-conjugated cells were washed with PBS three times and incubated with FITC-conjugated secondary antibodies (Pierce Chemical, Rockford, IL) for 2 h at 4 °C. Fluorescence signals were determined by CyFlow® SL (Partec, Münster, Germany) and analyzed by WinMDI version 2.8 software.

***In vitro* Matrigel tube formation assay.** Matrigel (BD Pharmingen, San Diego, CA) at 0.4 ml/well was plated evenly in a 24-well plate, and incubated at 37 °C for 30 min before seeding the HUVECs (0.5×10^5 cells/well). Tube formation was studied over 6 h and photographed by phase-contrast microscopy. The original magnification used was ×100. The Matrigel was fixed with methanol (–20 °C), blocked, permeabilized, and stained with a mouse anti-human antibody (clone: WM59) against PECAM-1 (BD Pharmingen) and a goat anti-human Prox-1 antibody (clone: AF2727, R&D systems) followed by incubation with an FITC-conjugated goat anti-mouse secondary antibody (DAKO, Carpinteria, CA) or an AlexaFluor-555-conjugated donkey anti-goat secondary antibody (Molecular Probes, Eugene, OR). The Matrigel was also stained with goat anti-human LYVE-1 (clone: AF2089, R&D systems) or mouse anti-human podoplanin (clone: 4D5a55E6, Santa Cruz Biotechnology) followed by incubation with an AlexaFluor-555-conjugated donkey anti-goat secondary antibody (Molecular Probes) or Cy5-conjugated goat anti-mouse secondary antibody (DAKO). After a series of further washes with PBS, samples were mounted on glass slides and viewed using a Zeiss fluorescence microscope (Oberkochen, Germany). The original magnification used was ×100.

***In vivo* Matrigel plug assay.** Eight-week-old BALB/c mice were given a subcutaneous injection at the abdominal midline with 0.4 ml Matrigel supplemented with media, and 5 µM LPA. After 7 days, the mice were killed, and the plugs were removed and then processed for hematoxylin and eosin (H&E) staining. Images were photographed by phase-contrast microscopy. The vessels penetrating the Matrigel were subjected to an immunohistochemical assay and visualized by Zeiss fluorescence microscopy. The original magnification used was ×40. BALB/c mice were obtained from laboratory animal center of National Taiwan University medical school (Institutional Animal Care and Use Committee approval no: 096046).

Immunohistochemical assay. Subcutaneously injected Matrigel plugs were dissected away from BALB/c mice and fixed in 4% paraformaldehyde before being immunostained for Prox-1 and PECAM-1. Briefly, Matrigel plug sections were blocked and permeabilized

in PBS containing 20% fetal calf serum and 0.5% Triton X-100. After incubation, the mouse anti-human antibody (clone: Mec13.3) against PECAM-1 (BD Pharmingen) and rabbit anti-mouse Prox-1 antibody (clone: AB5475, Chemicon International) were followed by incubation with the FITC-conjugated goat anti-rat secondary antibody (DAKO) and AlexaFluor-555-conjugated goat anti-rabbit secondary antibody (Molecular Probes). Sections were also stained with goat anti-mouse LYVE-1 (Clone: AF2125, R&D Systems) or goat anti-mouse podoplanin (Clone: AF3244, R&D Systems) followed by incubation with an AlexaFluor-555-conjugated donkey anti-goat secondary antibody (Molecular Probes). After three washes with PBS, samples were mounted on glass slides and viewed using a Zeiss fluorescence microscope.

Cell proliferation detection by 5-bromo-2′-deoxyuridine (BrdU). To detect proliferating cells during the course of tube formation, media or 5 μM of LPA-treated HUVECs seeded on Matrigel-coated plates were incubated with 10 μM of the thymidine analog BrdU (Sigma), which is selectively incorporated into cellular DNA during the S-phase. After 24 h of incubation, samples were stained with the FITC-conjugated anti-BrdU antibody (BD Pharmingen) and then visualized by Zeiss fluorescence microscope.

siRNA transfections. siRNAs targeting VEGFR-3 were obtained from Santa Cruz Biotechnology. Sequences of 21-nucleotide scrambled siRNA were UUCUCCGAACGUGUUCACGUdTdT, and ACGUGACACGUUCGGAGAAdTdT. HUVECs were transfected using an optimized protocol for electroporation of HUVECs with the Nucleofector apparatus (Amaxa Biosystems, Köln, Germany). Cells at 80% confluence were trypsinized and centrifuged. Cells (1×10^6) were resuspended in 100 μl of supplemented HUVECs Nucleofector™ solution (Amaxa Biosystems) and electroporated in the presence of 2 μg of various siRNA oligonucleotides or constructs. Transfected cells were seeded onto gelatin-coated plastic dishes and used after 24 h.

Statistical analysis. Significant differences between treatment groups were tested using analysis of variance (ANOVA) followed by Duncan's new multiple-range tests (StatView; Abacus Concept, Berkeley, CA). Each experiment was repeated at least three times. A value of $p<0.05$ was considered statistically significant.

Results

LPA enhances VEGF-C mRNA expression in HUVECs. LPA promotes angiogenesis through enhancing the expressions of various genes, including VEGF-A [13, 34]. We further investigated if LPA up-regulates mRNA expression of the lymphangiogenic factor VEGF-C. LPA up-regulated VEGF-C mRNA expression in HUVECs in a concentration-dependent manner (Fig. 1A). The expression of VEGF-C mRNA appeared at 1 μM and peaked at 5 μM LPA treatment, and the effect was sustained at 10 μM of LPA treatment. Thus, we used 5 μM LPA for the rest of the experiments. Because the enhancement of VEGF-C mRNA expressions by LPA treatment in HUVECs was concentration dependent, we next investigated whether the effects of LPA on VEGF-C mRNA levels in HUVECs were time dependent (Fig. 1C). HUVECs were incubated with 5 μM LPA for different time intervals as indicated. The enhanced VEGF-C mRNA expressions in HUVECs were first observed as early as 1 h after treatment was initiated, peaked at 2 h, were sustained at a high level until 4 h, and then declined thereafter. These results indicated that LPA enhances VEGF-C mRNA expressions in HUVECs.

LPA enhances VEGF-C protein expression in HUVECs. Since mRNA levels were increased by LPA, we further investigated if the elevated mRNA level of VEGF-C was also correlated with protein expression levels. The total VEGF-C protein levels in LPA-treated cells were detected using ELISA. Our results showed that LPA enhanced VEGF-C protein expressions in concentration- (Fig. 1B) and time-dependent (Fig. 1D) manners. Consistent with the RT-PCR results, the enhancement effects of LPA on VEGF-C protein expression in HUVECs peaked at a concentration of 5 μM, and the effect was sustained at 10 μM of LPA treatment. In the time-course experiments, the enhancement effects of LPA on VEGF-C protein expressions peaked at 8 h, and the effect was sustained at 24 h after treatment (Fig. 1D). These results indicated that LPA enhances mRNA and protein expressions of VEGF-C in HUVECs.

LPA enhances Prox-1, LYVE-1, and podoplanin mRNA and protein expressions in HUVECs. We next determined whether LPA-induced VEGF-C mRNA expression contributes to lymphangiogenesis. The mRNA expression level of the three lymphatic markers, Prox-1, LYVE-1, and podoplanin, were up-regulated by 5-μM LPA treatment at 8 and 12 h (Fig. 1C). By Cyflow analysis, we observed that 5 μM LPA, 10 ng/ml EGF, and 100 ng/ml VEGF-C enhanced Prox-1, LYVE-1, and podoplanin protein

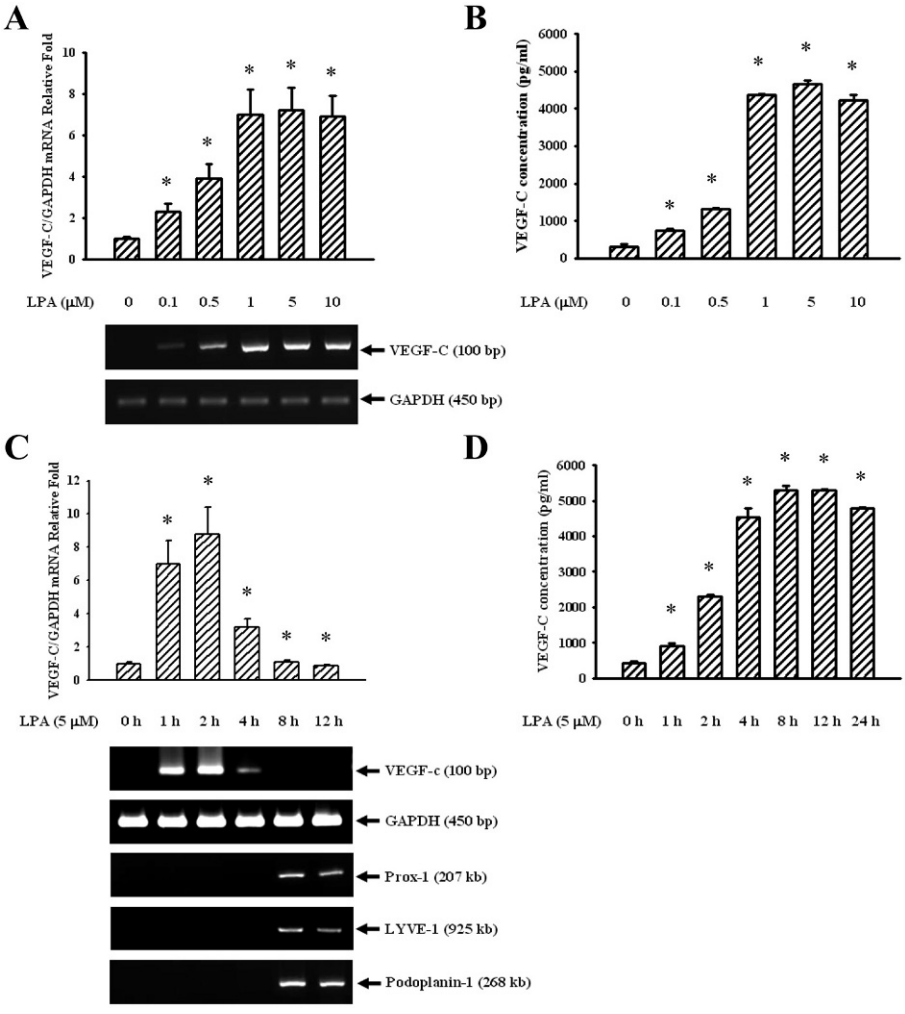

Figure 1. Lysophosphatidic acid (LPA) up-regulates vascular endothelial growth factor-C (VEGF-C) mRNA expression in human umbilical vein endothelial cells (HUVECs) in a dose- and time-dependent manner. (*A*) HUVECs were incubated with LPA for 4 h at various concentrations as indicated. RNAs from treated cells were harvested and subjected to RT-PCRs using specific primer sets for human VEGF-C or GAPDH. The reaction products were separated on 2 % agarose gels and photographed. (*B*) Levels of VEGF-C in supernatant of cultured HUVECs were measured by ELISA analysis after incubation with LPA for 24 h at various concentrations as indicated. (*C*) HUVECs were also incubated with LPA (5 μM) for various times as indicated and then subjected to RT-PCRs using specific primer sets for human VEGF-C, Prox-1, LYVE-1, Podoplanin or GAPDH as described above. Histograms represent quantification of RT-PCR corrected with GAPDH and analyzed by PhosphorImager® using ImageQuaNT® software. All data are relative multiples of expression compared to untreated cells. (*D*) Patterns of VEGF-C protein expressions were determined after HUVECs were treated with 5 μM LPA for various times as indicated and were measured by ELISA analysis as described above. All ELISA analysis data are expressed as the mean ± SE. * Statistically different as compared to the control ($p<0.05$). Similar experiments were repeated three times, and a representative result is shown.

expressions in HUVECs. However, treatment with VEGF-A (100 ng/ml) showed no enhancement effects on these lymphatic marker expressions in HUVECs (Fig. 2). In the presence of control antibodies, media- and LPA-treated cells were negative for these lymphatic marker expressions (Fig. 2). These results confirmed the specificity of antibodies against human Prox-1, LYVE-1, and podoplanin. Consistent with the RT-PCR findings (Fig. 1C), these results further confirmed that LPA might be a specific lymphangiogenic factor, which stimulates lymphatic marker expression in human endothelial cells.

LPA induces HUVEC tube formation and specific lymphatic marker expressions *in vitro*. To determine whether LPA-induced VEGF-C mRNA expression has any physiological significance, we next investigated LPA-induced HUVEC tube formation *in vitro*. HUVECs were treated with media or LPA (5 μM) and then seeded onto Matrigel-coated plates. We observed that LPA profoundly enhanced HUVEC tube formation *in vitro* (Fig. 3A). Using an *in vitro* Matrigel tube formation assay followed by immunostaining with a specific lymphatic marker, Prox-1, we found a significant increase in Prox-1 staining in tube-forming

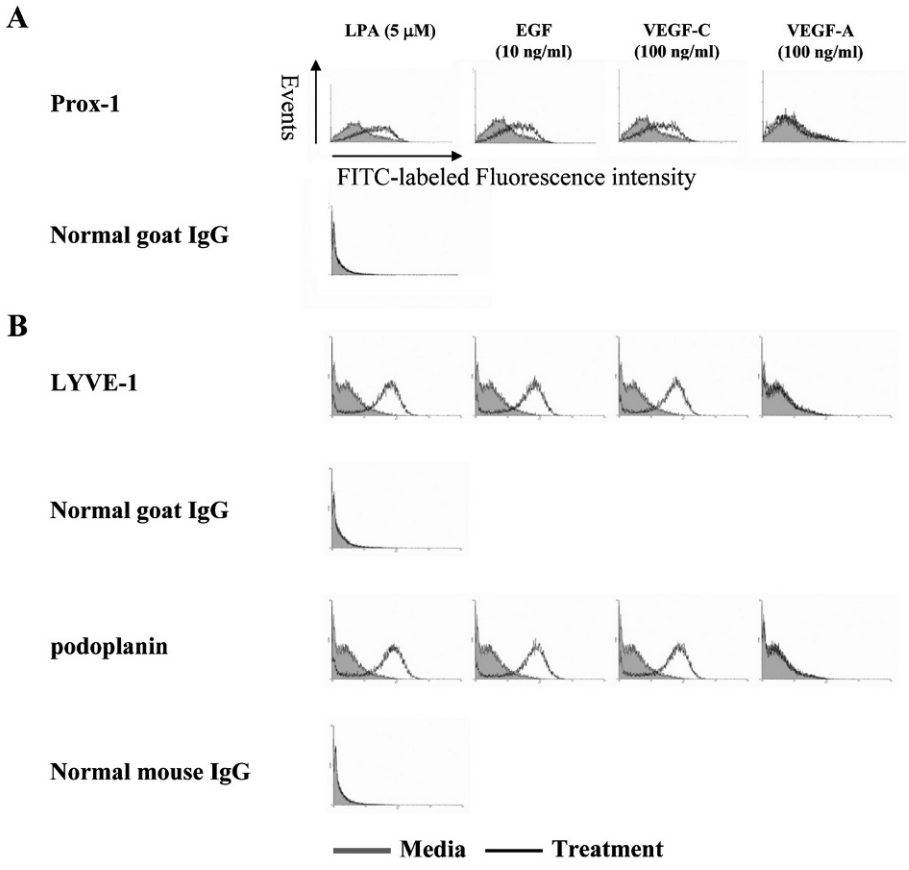

Figure 2. LPA up-regulates Prox-1, LYVE-1, and podoplanin protein expressions in HUVECs. (*A*) HUVECs were treated with media, LPA (5 μM), endothelial growth factor (EGF; 10 ng/ml), VEGF-C (100 ng/ml), or VEGF-A (100 ng/ml) for 8 h. Cells were dissociated by trypsinization and fixed with a 4% paraformaldehyde solution. Fixed cells were incubated with a goat anti-human Prox-1 antibody for 1 h at 4 °C, then treated with an FITC-conjugated anti-goat secondary antibody for 30 min at 4 °C and analyzed by Cyflow. Media- and LPA-treated cells were also stained with a control antibody, normal goat IgG followed by an FITC-conjugated anti-goat secondary antibody, which was used for a negative control. (*B*) Trypsinized cells were incubated with the goat anti-human LYVE-1 or mouse anti-human podoplanin antibody or control IgG for 1 h at 4 °C, then treated with an FITC-conjugated secondary antibody for 30 min at 4 °C and analyzed by Cyflow. Media- and LPA-treated cells were also stained with control antibodies, normal goat IgG, or mouse goat IgG followed by FITC-conjugated anti-goat or anti-mouse secondary antibody, which was used as a negative control.

HUVECs in response to LPA (Fig. 3B). Moreover, we also observed profound increases in LYVE-1 and podoplanin staining in LPA-induced tube-forming HUVECs (Fig. 3C). In addition, we found that Prox-1 signals were detected in cell nucleus, whereas LYVE-1 and podoplanin were expressed in cell membrane as well as PECAM-1 (Fig. 3B, C), which is consist with previous findings [35]. By staining with PECAM-1, a well-known endothelial cell marker, we observed that only LPA-treated HUVECs expressed PECAM-1 (Fig. 3B). In contrast, media-treated samples displayed less green signals due to treatment of media stimulating no HUVEC branch formation. These results demonstrated that LPA might be a potent regulator, which enhances human endothelial cells tube formation and specific lymphatic marker expressions *in vitro*.

LPA induces HUVEC tube formation and specific lymphatic marker expressions *in vivo*. Since LPA enhances human endothelial cell tube formation and specific lymphatic marker expressions *in vitro* (Fig. 3), we further investigated the effects of LPA on endothelial cell tube formation and specific lymphatic marker expressions *in vivo*. Matrigel-containing media or LPA (5 μM) was injected subcutaneously into the abdominal area of BALB/c mice. The vascularized plugs were removed and processed by H&E staining to identify if the area was covered by vessels. As shown in Figure 4A, new vessel formation was observed in LPA-containing Matrigel plugs. These results confirmed that LPA can induce endothelial cell tube formation *in vivo*. Moreover, LPA also enhanced Prox-1, LYVE-1, and podoplanin expressions by endothelial cells in Matrigel plugs removed from the mouse abdominal subcutaneous midline

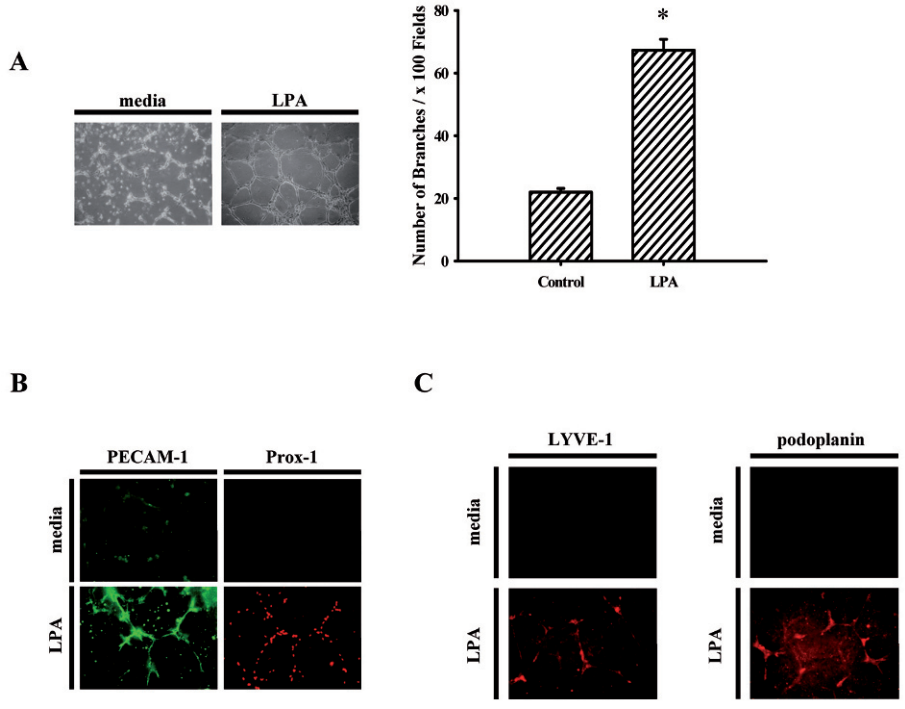

Figure 3. LPA stimulates endothelial cell tube formation and specific lymphatic markers expression *in vitro*. (*A*) HUVECs were starved and treated with media or 5 μM LPA for 8 h and then seeded onto Matrigel-coated plates. Images were taken at 6 h after plating and visualized by phase-contrast microscopy. The histogram represents branches from each cell, which were counted from three representative ×100 fields/well. * Statistically different as compared to the control ($p<0.05$). (*B*) Serum-starved HUVECs were treated with media or LPA (5 μM) for 8 h and then seeded on Matrigel-coated plates. At 6 h after plating, the Matrigel of each experiment was fixed and subjected to an immunocytochemical assay. Staining of a mouse anti-human PECAM-1 or a goat anti-human Prox-1 primary antibody followed by an FITC-conjugated anti-mouse or an Alexa-555-conjugated donkey anti-goat secondary antibody is shown. (*C*) Media or LPA-treated cells seeded on Matrigel-coated plates were also stained with a goat anti-human LYVE-1 or mouse anti-human podoplanin antibody followed by an AlexaFluor-555-conjugated donkey anti-goat or Cy5-conjugated goat anti-mouse secondary antibody. All images were visualized by fluorescence microscopy (original magnification, ×100).

(Fig. 4B, C). Similar to *in vitro* Matrigel tube formation results, we observed that less PECAM-1 signals could be detected in media-treated samples. Moreover, Prox-1 was expressed in cell nucleus, whereas LYVE-1 and podoplanin were expressed on cell membrane (Fig. 4B, C). These results suggest that LPA might regulate the formation of lymphatic vessel endothelial cells *in vivo*.

LPA stimulates endothelial cell lymphatic marker expression and cell proliferation *in vitro*. Here we further verify whether LPA stimulates tube-formed endothelial cell proliferation. As shown in Figure 5, significant BrdU incorporation was observed in Prox-1-positive cells in response to 5 μM LPA treatment. These results indicated that LPA might modulate specific lymphatic marker expressions and proliferation in human endothelial cells.

Enhancement by LPA of Prox-1, LYVE-1, and podoplanin expressions in HUVECs is mediated through a VEGF-C-dependent mechanism. Since LPA induces VEGF-C mRNA and protein expression in HUVECs (Fig. 1A, B) and LPA also enhances Prox-1, LYVE-1, and podoplanin mRNA and protein expressions in HUVECs (Figs. 1, 2), we further investigated if the effect of LPA on these lymphatic markers expression is mediated through the induction of VEGF-C expression in endothelial cells. We addressed this question using MAZ51, a VEGFR-3 kinase inhibitor [36]. By Cyflow analysis, pretreatment with 10 μM MAZ51 for 12 h significantly suppressed the enhancement effects of LPA on Prox-1, LYVE-1, and podoplanin protein expressions in HUVECs (Fig. 6). These results indicated that enhancement of Prox-1, LYVE-1, and podoplanin expressions in endothelial cells by LPA is VEGF-C dependent. To further clarify the role of VEGF-C/VEGFR-3 axis on LPA-induced HUVEC tube formation and lymphatic marker expression, HUVECs were transfected with scrambled or VEGFR-3 siRNA followed by media or 5-μM LPA treatment, and then subjected to *in vitro* Matrigel tube formation assay. Our results showed that introduction of VEGFR-3

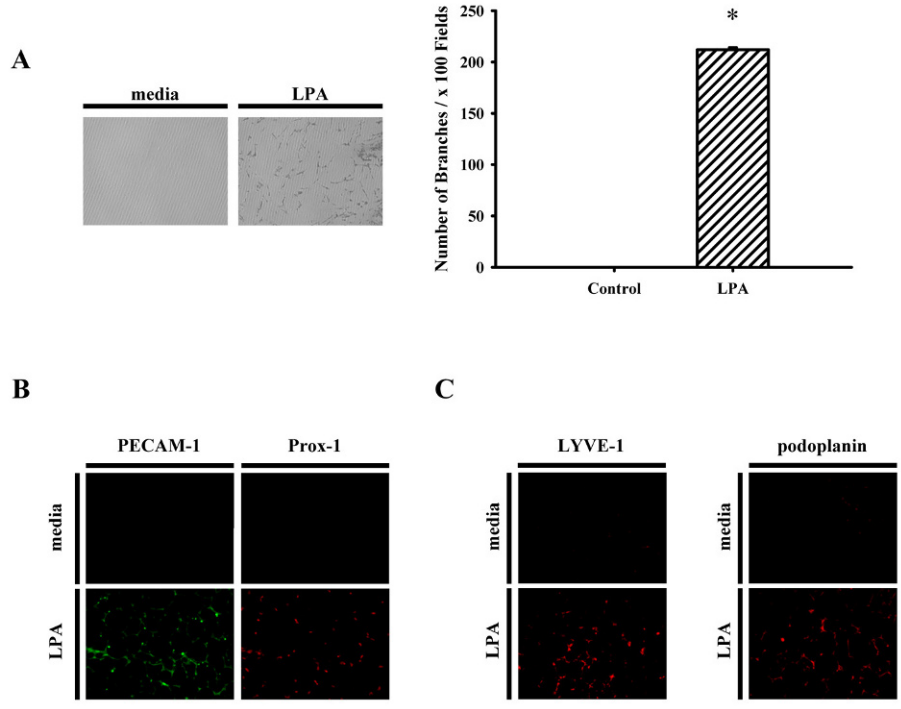

Figure 4. LPA stimulates endothelial cell tube formation and specific lymphatic markers expression *in vivo*. (*A*) Matrigel plugs were generated by a subcutaneous injection of Matrigel containing media or LPA (5 μM) into the abdominal region of BALB/c mice. The plugs were removed 7 days later and processed for hematoxylin and eosin (H&E) staining. The histogram represents the number of vessels penetrating the Matrigel, which were counted from three representative fields. * Statistically different as compared to the control ($p<0.05$). (*B*) Sections of the Matrigel plugs were incubated with a rat anti-mouse PECAM-1 or a rabbit anti-mouse Prox-1 primary antibody followed by an FITC-conjugated goat anti-rat or an Alexa-555-conjugated goat anti-rabbit secondary antibody (*C*) Sections were also stained with a goat anti-mouse LYVE-1 or podoplanin antibody followed by AlexaFluor-555-conjugated donkey anti-goat secondary antibodies. All images were visualized by fluorescence microscopy (original magnification, ×100).

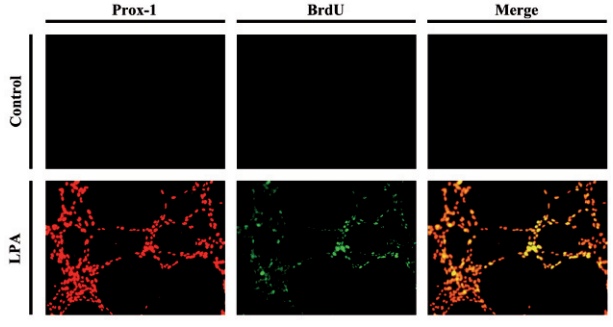

Figure 5. Effects of LPA on HUVEC lymphatic marker expression and cell proliferation *in vitro*. BrdU were added to starved HUVECs for 16 h, and cultures were treated with media or LPA (5 μM) for 8 h. Treated cells were seeded on Matrigel-coated plates and stained with a goat anti-human Prox-1 primary antibody followed by an Alexa-555-conjugated donkey anti-goat secondary antibody or FITC-conjugated BrdU antibody. All images were visualized by fluorescence microscopy (original magnification, ×100).

siRNA profoundly suppressed PECAM-1 signals in both LPA- and media-treated samples. Moreover, we found that Prox-1 signals detected in LPA-treated samples were significantly inhibited by VEGFR-3 siRNA (Fig. 6B). These results further confirmed that LPA-stimulated endothelial tube formation and lymphatic marker expression are mediated through a VEGF-C/VEGFR-3-dependent mechanism.

Pretreatment of MAZ51 suppressed LPA-induced HUVEC tube formation and Prox-1 expression *in vitro*. To further investigate if VEGF-C/VEGFR-3 axis mediates LPA-enhanced endothelial tube formation, HUVECs were pretreated with vehicle or MAZ51 (10 μM) for 12 h followed by treatments with media, LPA (5 μM), or VEGF-A (100 ng/ml) for 8 h. Treated cells were subjected to *in vitro* Matrigel tube formation assay. Our data showed that pretreatment of MAZ51 significantly suppressed LPA- but not VEGF-A-enhanced HUVEC tube formation *in vitro* (Fig. 7A). Furthermore, our results demonstrated that MAZ51 profoundly suppressed PECAM-1 signals in LPA- but not VEGF-A-treated samples. In addition, we found that Prox-1 signals were significantly enhanced in LPA- but not VEGF-A-treated samples and these enhancement effects were remarkably abrogated by MAZ-51 (Fig. 7B). These findings suggested that VEGR-3 is required for LPA but not

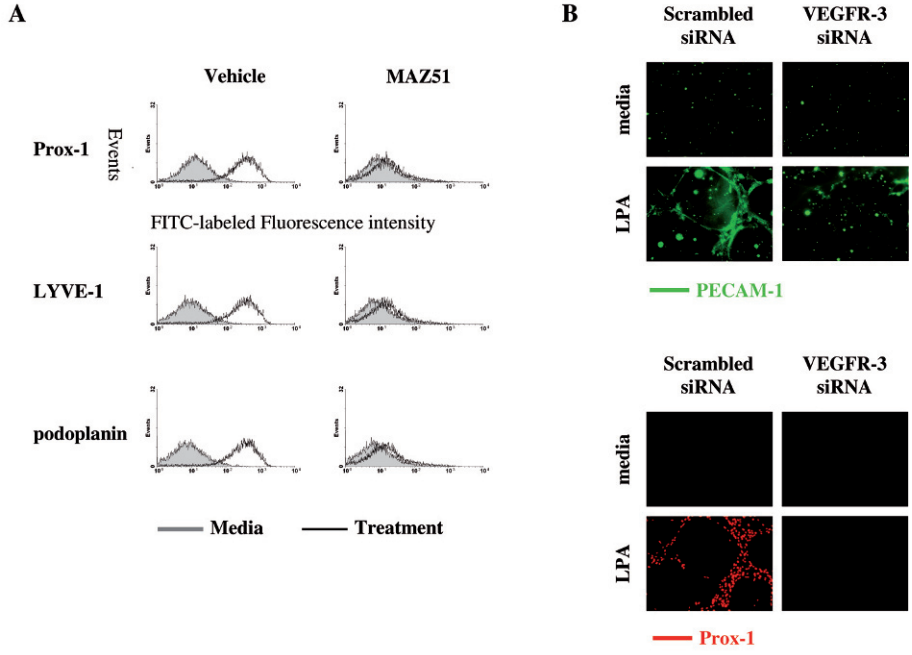

Figure 6. LPA-induced Prox-1, LYVE-1, and podoplanin protein expressions in HUVECs were inhibited by VEGFR-3 kinase inhibitor and VEGFR-3 siRNA. (*A*) HUVECs were pretreated with vehicle control or 10 μM of MAZ51 for 12 h. Treated cells were then treated with control media or 5 μM LPA for 8 h. Cells were dissociated by trypsinization and fixed by a 4% paraformaldehyde solution. Fixed cells were incubated with a goat anti-human Prox-1 antibody for 1 h at 4 °C, then treated with an FITC-conjugated anti-goat secondary antibody for 30 min at 4 °C and analyzed by Cyflow. Trypsinized cells were also incubated with the goat anti-human LYVE-1 or mouse anti-human podoplanin antibody at 4 °C, then treated with an FITC-conjugated secondary antibody for 30 min at 4 °C and analyzed by Cyflow. (*B*) HUVECs were transfected with scrambled or VEGFR-3 siRNA. At 24 h after transfection, HUVECs were treated with media or LPA (5 μM) for 8 h and then seeded on Matrigel-coated plates. At 6 h after plating, Matrigel of each experiment was permeabilized with methanol (–20 °C) and subjected to an immunocytochemical assay. Staining of a mouse anti-human PECAM-1 or a goat anti-human Prox-1 primary antibody followed by an FITC-conjugated anti-mouse or an Alexa-555-conjugated donkey anti-goat secondary antibody is shown.

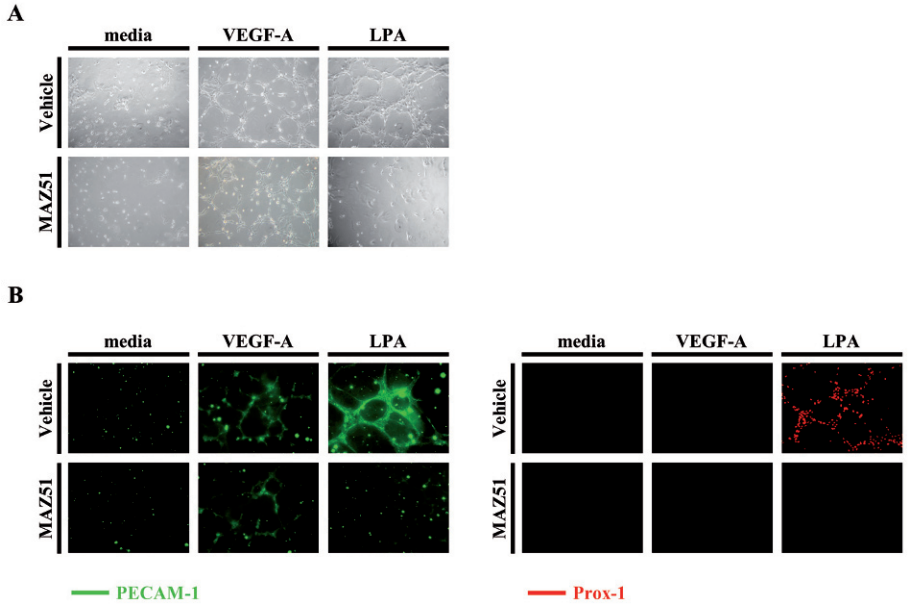

Figure 7. Pretreatment with the VEGFR-3 kinase inhibitor significantly suppressed LPA-induced HUVEC tube formation and Prox-1 expression *in vitro*. (*A*) HUVECs were pretreated with vehicle control or 10 μM of MAZ51 for 12 h. Treated cells were treated with media, LPA (5 μM), or VEGF-A (100 ng/ml) for 8 h and then seeded on Matrigel-coated plates. At 6 h after plating, images were taken and visualized by phase-contrast microscopy. (*B*) Matrigel of each experiment was permeabilized with methanol (–20 °C) and subjected to immunocytochemical assay. Staining of a mouse anti-human PECAM-1 or a goat anti-human Prox-1 primary antibody followed by an FITC-conjugated anti-mouse or an Alexa-555-conjugated donkey anti-goat secondary antibody is shown.

VEGF-A to stimulate endothelial cell tube formation and subsequent lymphatic marker expressions.

Discussion

Many growth factors have been reported to stimulate the differentiation of various endothelial cell types into lymphatic endothelial cells. Interleukin-3 (IL-3) is a potential stimulator for promoting non-lymphatic endothelial cell differentiation into lymphatic endothelial cells that positively express Prox-1. IL-3 therefore regulates non-lymphatic endothelial cell differentiation into lymphatic endothelial cells, thus promoting progression of the lymphangiogenesis process [35]. These studies demonstrated that not only lymphatic endothelial cells but also other types of endothelial cells can be used to study lymphangiogenesis. Groger et al. [35] also reported that the basal mRNA expression levels of podoplanin and LYVE-1 are relative high in lymphatic endothelial cells, while mRNA expression levels of these two lymphatic markers are low in HUVECs. Our results also confirmed that basal mRNA expression levels of Prox-1, LYVE-1 and podoplanin are low in HUVECs (Fig. 1). These findings suggested that HUVECs, an easily obtained endothelial cell type, is suitable for studying lymphatic marker expressions in response to agonists. In the present study, we found that LPA induced VEGF-C mRNA expression in endothelial cells, and subsequently Prox-1, LYVE-1, and podoplanin expressions in endothelial cells *in vitro* and *in vivo*. Our previous study showed that LPA modulates endothelial cell proliferation [18]. In this study, we further demonstrated that LPA stimulates lymphatic marker expressions in these proliferating endothelial cells (Fig. 5).

Pro-inflammatory factors such as IL-1β and tumor necrosis factor-α (TNF-α) have been shown to enhance VEGF-C expression in HUVECs [37]. In this study, we also demonstrated that LPA stimulated VEGF-C mRNA and protein expressions in HUVECs (Fig. 1). Since LPA has been reported to be a proinflammatory factor [38], our data suggest that these proinflammatory factors might be potent regulators of VEGF-C expression in human endothelial cells. In our previous study, we demonstrated that LPA stimulates IL-1β expression in HUVECs [39], implying that LPA might also regulate VEGF-C expression through up-regulating IL-1β expression in human endothelial cells. In addition, proinflammatory factors including TNF-α and interferon-γ (IFN-γ) enhance Prox-1 and podoplanin expressions in endothelial cells, and these enhancement effects are dependent on IL-3 [40, 41]. These findings further illustrate that proinflammatory factors may play critical roles in normal endothelial cells differentiating into lymphatic endothelial cells, which is consistent with our current results. One recent study reported that elevation of circulating interleukin-8 (IL-8) is highly correlated to VEGF-C but not VEGF-A level elevation in metastatic esophageal squamous carcinoma patient [42]. Since our previous study found that LPA enhances IL-8 expression in HUVECs in an IL-1-dependent manner [39], we suggested that LPA induces IL-1-mediated IL-8 expression in human endothelial cells might contribute to VEGF-C rather than VEGF-A production and subsequent lymphatic markers expressions in human endothelial cells.

The role of VEGF-C in association with VEGFR-3 on promoting lymphangiogenesis process has been well established [19, 22]. Our results showed that LPA-induced Prox-1, LYVE-1, and podoplanin protein expressions in HUVECs were significantly suppressed by pretreatment with MAZ51, a VEGFR-3 kinase inhibitor (Fig. 6). These results suggested that LPA might up-regulate these lymphatic marker expressions through up-regulating VEGF-C expression in endothelial cells.

VEGF-A is a well-known key regulator of angiogenesis process [43, 44]. Using an *in vitro* Matrigel tube formation assay and *in vivo* Matrigel plug assay, VEGF-A was shown to significantly enhance HUVECs tube formation *in vitro* [45, 46] and vessels penetrating the Matrigel *in vivo* [47, 48]. Moreover, pre-incubation of VEGFR-2 kinase inhibitor, ZD6474 largely suppressed VEGF-A-induced endothelial cell tube formation *in vitro* and *in vivo* [48], implying that VEGF-A/VEGFR-2 axis might play critical role on modulating endothelial cell tube formation and subsequent angiogenesis process. Our results demonstrated that VEGF-C but not VEGF-A showed enhancement effects on protein expressions of the lymphatic markers, Prox-1, LYVE-1, and podoplanin in HUVECs (Fig. 2). Consistent with previous study [41], our observation suggest that involvement of VEGF-C *via* VEGFR-3 might be the major signaling pathway mediating LPA-stimulated endothelial cell tube formation and the subsequent lymphangiogenesis process, while the VEGF-A/VEGFR-2 axis might not regulate lymphatic marker expressions in endothelial cells.

As shown in Figure 7A, we demonstrated that morphology of LPA- and VEGF-A-enhanced endothelial cell tubes are different, which is consistent with the results in previous study that morphology of VEGF-C- and VEGF-A-enhanced endothelial cell tubes differed [49]. Moreover, our data revealed that MAZ51 significantly suppressed LPA-enhanced Prox-1 expression in endothelial cells (Fig. 7B), further suggesting that LPA might be a lymphangiogenic factor.

Many agonists have been reported to up-regulate both VEGF-A and VEGF-C. Mancino et al. [50] reported that estrogen can stimulate both VEGF-A and VEGF-C in the human cholangiocarcinoma cell line, HuH-28 cells. In addition, IL-1α and IL-6 have been reported as agonists for both VEGF-A and VEGF-C expression in CAPAN-1 cells, a pancreatic cancer cell line [51]. Ristimaki et al. [37] further indicated that IL-1β up-regulates both VEGF-A and VEGF-C expression in HUVECs. These studies suggested that various pro-inflammatory cytokines stimulate VEGF-A and VEGF-C expression in various types of cells. Previous studies reported that LPA could stimulate VEGF-A expression in cancer cells [13, 34]. In the present study, we further observed that LPA stimulated VEGF-C expression in HUVECs, suggesting that LPA might affect angiogenesis and lymphangiogenesis process.

In summary, this study first demonstrates that LPA might enhance VEGF-C mRNA and protein expressions and subsequent mRNA and protein expressions of the lymphatic markers, Prox-1, LYVE-1, and podoplanin, in human endothelial cells. In addition, LPA also enhanced endothelial cell tube formation and lymphatic marker expressions *in vitro* and *in vivo*, implying that LPA might up-regulate VEGF-C expression, which contributes to lymphatic marker expression in endothelial cells, thus promoting progression of the lymphangiogenesis process. LPA also stimulated endothelial cell proliferation as the lymphangiogenesis process proceeds. Furthermore, LPA might enhance lymphatic marker expressions through up-regulating VEGF-C expression in endothelial cells, therefore facilitating progression of the lymphangiogenesis process. Our study clarifies the role of LPA in lymphangiogenesis, and these findings also suggest some potential targets for anti-lymphangiogenesis therapies.

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1 Eichholtz, T., Jalink, K., Fahrenfort, I. and Moolenaar, W. H. (1993) The bioactive phospholipid lysophosphatidic acid is released from activated platelets. Biochem. J. 291, 677–680.
2 Moolenaar, W. H., Kranenburg, O., Postma, F. and Zondag, G. C. (1997) Lysophosphatidic acid: G-protein signalling and cellular responses. Curr. Opin. Cell. Biol. 9, 168–173.
3 Lee, H., Goetzl, E. J. and An, S. (2000) Lysophosphatidic acid and sphingosine 1-phosphate stimulate endothelial cell wound healing. Am. J. Physiol. Cell. Physiol. 278, C612-C618.
4 Panetti, T. S. (2002) Differential effects of sphingosine 1-phosphate and lysophosphatidic acid on endothelial cells. Biochim. Biophys. Acta 1582, 190–196.
5 Panetti, T. S., Chen, H., Misenheimer, T. M., Getzler, S. B. and Mosher, D. F. (1997) Endothelial cell mitogenesis induced by LPA: Inhibition by thrombospondin-1 and thrombospondin-2. J. Lab. Clin. Med. 129, 208–216.
6 Panetti, T. S., Nowlen, J. and Mosher, D. F. (2000) Sphingosine-1-phosphate and lysophosphatidic acid stimulate endothelial cell migration. Arterioscler. Thromb. Vasc. Biol. 20, 1013–1019.
7 Chen, S. U., Chou, C. H., Lee, H., Ho, C. H., Lin, C. W. and Yang, Y. S. (2008) Lysophosphatidic acid up-regulates expression of interleukin-8 and -6 in granulosa-lutein cells through its receptors and NF-kappaB-dependent pathways: Implications for angiogenesis of corpus luteum and ovarian hyperstimulation syndrome. J. Clin. Endocrinol. Metab. 93, 935–943.
8 Langlois, S., Gingras, D. and Beliveau, R. (2004) Membrane type 1-matrix metalloproteinase (MT1-MMP) cooperates with sphingosine 1-phosphate to induce endothelial cell migration and morphogenic differentiation. Blood 103, 3020–3028.
9 Wu, W. T., Chen, C. N., Lin, C. I., Chen, J. H. and Lee, H. (2005) Lysophospholipids enhance matrix metalloproteinase-2 expression in human endothelial cells. Endocrinology 146, 3387–3400.
10 Lin, C. I., Chen, J. N., Lin, P. W., Chang, K. J., Hsieh, F. J. and Lee, H. (2007) Lysophosphatidic acid regulates inflammation-related genes in human endothelial cells through LPA_1 and LPA_3. Biochem. Biophys. Res. Commun. 363: 1001–1008.
11 Risau, W. (1997) Mechanisms of angiogenesis. Nature 386, 671–674.
12 Ryan, T. J., Mortimer, P. S. and Jones, R.L. (1986) Lymphatics of the skin. Int. J. Dermatol. 25, 411–419.
13 English, D., Kovala, A. T., Welch, Z., Harvey, K. A., Siddiqui, R. A. Brindley, D. N. and Garcia, J. G. (1999) Induction of endothelial cell chemotaxis by sphingosine 1-phosphate and stabilization of endothelial monolayer barrier function by lysophosphatidic acid, potential mediators of hematopoietic angiogenesis. J. Hematother. Stem. Cell. Res. 8, 627–634.
14 van Meeteren, L. A., Ruurs, P., Stortelers, C., Bouwman, P., van Rooijen, M. A., Pradere, J. P., Pettit, T. R., Wakelam, M. J., Saulnier-Blache, J. S., Mummery, C. L., Moolenaar, W. H. and Jonkers, J. (2006) Autotaxin, a secreted lysophospholipase D, is essential for blood vessel formation during development. Mol. Cell. Biol. 26, 5015–5022.
15 Makinen, T., Veikkola, T., Mustjoki, S., Karpanen, T., Catimel, B. and Nice, E. C. (2001) Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. EMBO J. 20, 4762–4773.
16 Skobe, M., Hawighorst, T., Jackson, D. G., Prevo, R., Janes, L., Velasco, P., Riccardi, L., Alitalo, K., Claffey, K. and Detmar, M. (2001) Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. Nat. Med. 7, 192–198.
17 Skobe, M., Hamberg, L.M., Hawighorst, T., Schirner, M., Wolf, G.L., Alitalo, K. and Detmar, M. (2001) Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. Am. J. Pathol. 159, 893–903.
18 Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lahtinen, I., Kukk, E., Saksela, O., Kalkkinen, N. and Alitalo, K. (1996) A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J. 15, 290–298.
19 Joukov, V., Sorsa, T., Kumar, V., Jeltsch, M., Claesson-Welsh, L., Cao, Y., Saksela, O., Kalkkinen, N. and Alitalo, K. (1997) Proteolytic processing regulates receptor specificity and activity of VEGF-C. EMBO J. 16, 3898–3911.
20 Lohela, M., Saaristo, A., Veikkola, T. and Alitalo, K. (2003) Lymphangiogenic growth factors, receptors and therapies. Thromb. Haemost. 90, 167–184.
21 Millauer, B., Wizigmann-Voos, S., Schnurch, H., Martinez, R., Moller, N. P., Risau, W. and Ullrich, A. (1993) High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. Cell 72, 835–846.

22 Kukk, E., Lymboussaki, A., Taira, S., Kaipainen, A., Jeltsch, M., Joukov, V. and Alitalo, K. (1996) VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. Development 122, 3829–3837.

23 Goldman, J., Rutkowski, J. M., Shields, J. D., Pasquier, M. C., Cui, Y., Schmokel, H. G., Willey, S., Hicklin, D. J., Pytowski, B. and Swartz, M. A. (2007) Cooperative and redundant roles of VEGFR-2 and VEGFR-3 signaling in adult lymphangiogenesis. FASEB J. 21, 1003–1012.

24 Roberts, N., Kloos, B., Cassella, M., Podgrabinska, S., Persaud, K., Wu, Y., Pytowski, B. and Skobe, M. (2006) Inhibition of VEGFR-3 activation with the antagonistic antibody more potently suppresses lymph node and distant metastases than inactivation of VEGFR-2. Cancer Res. 66, 2650–2657.

25 Hsieh, C. Y., Chen, C. A., Chou, C. H., Lai, K. P., Jeng, Y. M., Kuo, M. L., and Wei L. H. (2004) Overexpression of Her-2/NEU in epithelial ovarian carcinoma induces vascular endothelial growth factor C by activating NF-kappa B: Implications for malignant ascites formation and tumor lymphangiogenesis. J. Biomed. Sci.11, 249–259.

26 Su, J. L., Shih, J. Y., Yen, M. L., Jeng, Y. M., Chang, C. C., Hsieh, C. Y., Wei, L. H., Yang, P. C., and Kuo M. L. (2004) Cyclooxygenase-2 induces EP1- and HER-2/Neu-dependent vascular endothelial growth factor-C up-regulation: A novel mechanism of lymphangiogenesis in lung adenocarcinoma. Cancer Res. 64, 554–564.

27 Timoshenko, A. V., Chakraborty, C., Wagner, G. F. and Lala P. K. (2006) COX-2-mediated stimulation of the lymphangiogenic factor VEGF-C in human breast cancer. Br. J. Cancer 94, 1154–1163.

28 O-charoenrat, P., Rhys-Evans, P., Modjtahedi, H. and Eccles, S. A. (2000) Vascular endothelial growth factor family members are differentially regulated by c-erbB signaling in head and neck squamous carcinoma cells. Clin. Exp. Metastasis 18, 155–161.

29 Mandriota, S. J., Jussila, L., Jeltsch, M., Compagni, A., Baetens, D. and Prevo, R. (2001) Vascular endothelial growth factor-C mediated lymphangiogenesis promotes tumour metastasis. EMBO J. 20, 672–682.

30 Oliver, G. (2004) Lymphatic vasculature development. Nat. Rev. Immunol. 4, 35–45.

31 Weninger, W., Partanen, T. A., Breiteneder-Geleff, S., Mayer, C., Kowalski, H., Mildner, M., Pammer, J., Stürzl, M., Kerjaschki, D., Alitalo, K. and Tschachler, E. (1999) Expression of vascular endothelial growth factor receptor-3 and podoplanin suggests a lymphatic endothelial cell origin of Kaposi's sarcoma tumor cells. Lab. Invest. 79, 243–251.

32 Wigle, J. T., Harvey, N., Detmar, M., Lagutina, I., Grosveld, G. and Gunn, M. D. *(2002)* An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. EMBO J. 21, 1505–1513.

33 Ji, R. C. (2006) Lymphatic endothelial cells, tumor lymphangiogenesis and metastasis: New insights into intratumoral and peritumoral lymphatics. Cancer Metastasis Rev. 25, 677–694.

34 Hu, Y. L., Albanese, C., Pestell, R. G. and Jaffe, R. B. (2003) Dual mechanisms for lysophosphatidic acid stimulation of human ovarian carcinoma cells. J. Natl. Cancer Inst. 95, 733–740.

35 Groger, M., Loewe, R., Holnthoner, W., Embacher, R., Pillinger, M., Herron, G. S., Wolff, K. and Petzelbauer, P. (2004) IL-3 induces expression of lymphatic markers Prox-1 and podoplanin in human endothelial cells. J. Immunol. 173, 7161–7169.

36 Kirkin, V., Thiele, W., Baumann, P., Mazitschek, R., Rohde, K., Fellbrich, G., Weich, H., Waltenberger, J., Giannis, A. and Sleeman, J. P. (2004) MAZ51, an indolinone that inhibits endothelial cell and tumor cell growth *in vitro*, suppresses tumor growth *in vivo.* Int. J. Cancer 112, 986–993.

37 Ristimaki, A., Narko, K., Enholm, B., Joukov, V. and Alitalo, K. (1998) Proinflammatory cytokines regulate expression of the lymphatic endothelial mitogen vascular endothelial growth factor-C. J. Biol. Chem. 273, 8413–8418.

38 Zhang, Z., Liu, Z. and Meier, K. E. (2006) Lysophosphatidic acid as a mediator for pro-inflammatory agonists in a human corneal epithelial cell line. Am. J. Physiol. Cell. Physiol. 291, C1089–1098.

39 Lin, C. I., Chen, C. N., Chen, J. H. and Lee H. (2006) Lysophospholipids increase IL-8 and MCP-1 expressions in human umbilical cord vein endothelial cells through an IL-1-dependent mechanism. J. Cell. Biochem. 99, 1216–1232.

40 Geleff, S., Schoppmann, S. F. and Oberhuber, G.. (2003) Increase in podoplanin-expressing intestinal lymphatic vessels in inflammatory bowel disease. Virchows Arch. 442, 231–237.

41 Kerjaschki, D., Regele, H. M., Moosberger, I., Nagy-Bojarski, K., Watschinger, B., Soleiman, A., Birner, P., Krieger, S., Hovorka, A., Silberhumer, G., Laakkonen, P., Petrova, T., Langer, B. and Raab, I. (2004) Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates. J. Am. Soc. Nephrol. 15, 603–612.

42 Krzystek-Korpacka, M., Matusiewicz, M., Diakowska, D., Grabowski, K., Blachut, K., Konieczny, D., Kustrzeba-Wojcicka, I., Terlecki, G. and Banas, T. (2008) Elevation of circulating interleukin-8 is related to lymph node and distant metastases in esophageal squamous cell carcinomas – Implication for clinical evaluation of cancer patient. Cytokine 41, 232–239.

43 Distler, J. H., Hirth, A., Kurowska-Stolarska, M., Gay, R. E., Gay, S. and Distler, O. (2003) Angiogenic and angiostatic factors in the molecular control of angiogenesis. Q. J. Nucl. Med. 47, 149–161.

44 Hoeben, A., Landuyt, B., Highley, M. S., Wildiers, H., Van Oosterom, A.T. and De Bruijn, E. A. (2004) Vascular endothelial growth factor and angiogenesis. Pharmacol. Rev. 56, 549–580.

45 Kim, H. S., Shin, H. S., Kwak, H. J., Cho, C. H., Lee, C. O. and Koh, G. Y. (2003) Betacellulin induces angiogenesis through activation of mitogen-activated protein kinase and phosphatidylinositol 3'-kinase in endothelial cell. FASEB J. 17, 318–320.

46 Sini, P., Wyder, L., Schnell, C., O'Reilly, T., Littlewood, A., Brandt, R. Hynes, N. E and Wood, J. (2005) The antitumor and antiangiogenic activity of vascular endothelial growth factor receptor inhibition is potentiated by ErbB1 blockade. Clin. Cancer Res. 11, 4521–4532.

47 Michaud-Levesque, J., Demeule, M. and Béliveau R. (2007) *In vivo* inhibition of angiogenesis by a soluble form of melanotransferrin. Carcinogenesis 28, 280–288.

48 Wu, W., Onn, A., Isobe, T., Itasaka, S., Langley, R. R., Shitani, T., Shibuya, K., Komaki. R., Ryan. A, J, Fidler, I. J., Herbst, R. S. and O'Reilly, M. S. (2007) Targeted therapy of orthotopic human lung cancer by combined vascular endothelial growth factor and epidermal growth factor receptor signaling blockade. Mol. Cancer Ther. 6, 471–483.

49 Podgrabinska, S., Braun, P., Velasco, P., Kloos, B., Pepper, M. S. and Skobe, M. (2002) Molecular characterization of lymphatic endothelial cells. Proc. Natl. Acad. Sci. USA 99, 16069–16074.

50 Mancino, A., Mancino, M. G., Glaser, S. S., Alpini, G., Bolognese, A., Izzo, L., Francis, H., Onori, P., Franchitto, A., Ginanni-Corradini, S., Gaudio, E. and Alvaro, D. (2008) Estrogens stimulate the proliferation of human cholangiocarcinoma by inducing the expression and secretion of vascular endothelial growth factor. Dig. Liver Dis. [Epub ahead of print].

51 Tang, R. F., Wang, S. X., Zhang, F. R., Peng, L., Wang, S. X., Xiao, Y. and Zhang, M. (2005) Interleukin-1alpha, 6 regulate the secretion of vascular endothelial growth factor A, C in pancreatic cancer. Hepatobiliary Pancreat. Dis. Int. 4, 460–463.