Lysophosphatidic Acid Induction of Vascular Endothelial Growth Factor Expression in Human Ovarian Cancer Cells

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Background: Lysophosphatidic acid (LPA) stimulates ovarian tumor growth at concentrations present in ascitic fluid. Vascular endothelial growth factor (VEGF) stimulates angiogenesis and plays a pivotal role in the formation of ovarian cancer-associated ascites. We examined whether LPA promotes ovarian tumor growth by increasing angiogenesis via VEGF.

Methods: VEGF expression was examined in a simian virus 40 T-antigen-immortalized ovarian surface epithelial cell line (IOSE-29) and in ovarian cancer cell lines (OVCAR-3, SKOV-3, and CAOV-3) treated with LPA. VEGF promoter activity was measured in OVCAR-3 cells after transfection or cotransfection with c-Fos and c-Jun, components of AP1 transcription factor, potential binding sites for which are present in the VEGF promoter. All statistical tests were two-sided.

Results: LPA treatment increased steady-state VEGF messenger RNA (mRNA) levels in OVCAR-3 cells in a time- and dose-dependent fashion and stimulated VEGF promoter activity without prolonging mRNA half-life in these cells, but LPA had little effect on IOSE-29 cells. Forced overexpression of c-Jun and c-Fos in OVCAR-3 cells stimulated VEGF promoter activity fourfold. LPA also elevated LPA protein levels by 1.5-fold in SKOV-3 cells (P = .0148), 1.9-fold in CAOV-3 cells (P < .001), and threefold in OVCAR-3 cells (P < .0001). Both Edg2 and Edg4 were detected in ovarian cancer cells; however, only Edg2 was present in normal ovarian surface epithelial cells and IOSE-29 cells.

Conclusions: LPA stimulates ovarian tumor growth, at least in part, via induction of VEGF expression through transcriptional activation. However, this LPA response is not evident in normal ovarian surface epithelial cells. Our data suggest that Edg4, but not Edg2, plays a role in LPA stimulation of ovarian tumor growth. [J Natl Cancer Inst 2001;93:762–8]

Lysophosphatidic acid (LPA), a bioactive phospholipid present in serum at concentrations of 2–20 μM (1), induces various cellular responses by interacting with specific cell surface G-protein-coupled receptors of the endothelial differentiation gene (Edg) subfamily (2). The responses to LPA include stimulation of cell proliferation by increasing cell cycle progression and inhibiting apoptosis, inhibition of cell differentiation, and stimulation of tumor cell invasion (3). Edg2 (4) and Edg4 (5) are specific LPA receptors present on many cell types. LPA stimulates ovarian cancer cell growth in vitro (6) and is found in high concentrations in ascitic fluid (7) and plasma (8) of ovarian cancer patients. LPA induces the expression of components of the AP1 transcription factor complex, including c-Jun and c-Fos (9). AP1 and hypoxia-inducible factor (HIF)-1 are involved in activation of vascular endothelial growth factor (VEGF) expression by hypoxia (10,11).

Angiogenesis is necessary for tumor growth, and a transition from limited to rapid growth accompanies neovascularization (12,13). The development of new blood vessels depends on the production of angiogenic factors released from tumor cells and/or cells in the tumor microenvironment. Among these angiogenic factors, VEGF, also known as vascular permeability factor, binds to vascular endothelial cells and is a potent inducer of angiogenesis.

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angiogenesis. Cancer patients have increased serum VEGF, and elevated VEGF messenger RNA (mRNA) levels occur in the majority of human cancers (14). Ovarian carcinoma is the most lethal gynecologic cancer in U.S. women. Ovarian carcinomas are predominantly derived from the ovarian surface epithelium and are characterized by widespread intraperitoneal carcinomatosis and formation of large volumes of ascitic fluid. We and others have reported that VEGF mRNA and protein are extensively expressed in ovarian tumors and ovarian cancer cell lines (15–19), and VEGF also plays a pivotal role in ascites formation associated with ovarian cancer by increasing vascular permeability (19).

Human OVCAR-3 cancer cells express VEGF mRNA and protein (16), and after receiving an intraperitoneal injection of OVCAR-3 cells, female athymic mice develop intraperitoneal carcinomatosis and massive ascites similar to clinical ovarian cancer (19,20). We hypothesized that LPA promotes ovarian tumor growth by increasing angiogenesis via VEGF. Therefore, we used OVCAR-3 cells as a model to investigate the stimulation of VEGF gene expression by LPA and mechanism(s) involved in LPA induction of VEGF expression. Because LPA stimulates ovarian tumor cell growth, we also explored Edg2/Edg4 expression in ovarian cancer cell lines and in normal ovarian surface epithelial cells.

Materials and Methods

Reagents and cell lines. The following items were purchased: 1-oleoyl-LPA and fatty acid-free bovine serum albumin (Sigma Chemical Co., St. Louis, MO) and pGL3-basic vector and all restriction enzymes (Promega Corp., Madison, WI). All cell culture reagents were obtained from the Cell Culture Facility, University of California, San Francisco. The ovarian cancer cell lines OVCAR-3, SKOV-3, and DOV-13 were obtained from American Type Culture Collection (Manassas, VA). The ovarian cancer cell line DOV-13 was provided by Dr. Robert Bast (The University of Texas M. D. Anderson Cancer Center, Houston). IOSE-29 was a simian virus 40 T-antigen-immortalized ovarian surface epithelial cell line (provided by Dr. Nelly Aueresperg), and normal ovarian surface epithelial cells were obtained by scraping the ovarian surface during surgery for nonmalignant disorders.

Cell culture and LPA stimulation. OVCAR-3 and DOV-13 cells were cultured in RPMI-1640 medium with 10% fetal calf serum (FCS), CAOV-3 and SKOV-3 cells were cultured in Dulbecco’s modified Eagle medium with 10% FCS. IOSE-29 and normal ovarian surface epithelial cells were cultured in medium 199/MCDB 105 with 10% FCS as described previously (21,22). For LPA stimulation, cells were seeded on six-well plates and cultured in complete growth medium. Upon reaching confluence, the cells were washed twice with prewarmed phosphate-buffered saline and cultured in serum-free medium overnight. LPA was added to the culture at a concentration of 20 μM, and incubation was carried out at 37°C for various times up to 24 hours. In a different experiment, various concentrations of LPA (0.02–20 μM) were added to the culture, and incubation was carried out at 37°C for 24 hours. After incubation, the conditioned medium was aspirated and saved for VEGF enzyme-linked immunosorbent assay (ELISA). The cells were harvested and used to isolate cellular total RNA for northern blotting analysis.

RNA extraction and northern blotting analysis. Total cellular RNA was isolated from the ovarian epithelial cancer cell lines and from the normal ovarian surface epithelial cells with the use of a High Pure RNA Isolation Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN) according to the manufacturer’s instructions. Equal amounts of total RNA (10 μg per lane) were separated by electrophoresis on denaturing 1.2% agarose and transferred to nylon membranes. The membranes were UV cross-linked and hybridized in the Expresshyb hybridization buffer (Clontech Laboratories, Inc., Palo Alto, CA) at 68°C for 2 hours with 32P-labeled VEGF, Edg2, Edg4, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) complementary DNA (cDNA) probes synthesized with a random primer rediPrime DNA labeling kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) according to the manufacturer’s instructions. The blots were washed at room temperature in 2x SSC (i.e., 300 mM sodium chloride and 30 mM sodium citrate)/0.05% sodium dodecyl sulfate (SDS) for 20 minutes three times and then at 50°C in 0.1x SSC/0.1% SDS for 30 minutes twice. The washed membranes were then exposed to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY) with two intensifying screens for 1–2 days at −70°C.

VEGF ELISA. The ovarian cancer cell lines were each incubated in serum-free medium overnight and then in the presence or absence of LPA. Conditioned media were centrifuged at 1100×g for 10 minutes at 4°C to remove debris and stored at −70°C until analysis. The VEGF protein level in the conditioned media was determined with the use of a Dual-Luciferase Reporter Assay Kit (Promega Corp.) according to protocols provided by the manufacturer.

Stability of VEGF mRNA. To determine the stability of VEGF mRNA, we incubated OVCAR-3 cells in serum-free medium overnight and then in the presence or absence of LPA (20 μM) for 24 hours. The cells were harvested and washed with lysis buffer, and the luciferase activities were determined with the use of a Dual-Luciferase Reporter Assay Kit (Promega Corp.) according to protocols provided by the manufacturer. In cotransfection experiments, 0.5 μg of pCMV-Fos and 0.5 μg of pCMV-Jun expression plasmids were cotransfected with VEGF–luciferase fusion constructs and pRL-CMV plasmid at the same time; 48 hours after transfection, the cells were harvested and luciferase activities were determined.

Statistical analysis. Each experiment was performed in duplicate or triplicate. All experiments were repeated at least three times on different occasions. The results are presented as means ± 95% confidence intervals of all the values. A paired Student’s t test was used to evaluate statistically significant differences in VEGF protein levels between the LPA treatment groups and the vehicle control group. P < 0.05 was selected as the statistically significant value. All statistical tests and corresponding P values were two-sided.

Results

VEGF mRNA Induction by LPA in OVCAR-3 and IOSE-29 Cells

We first performed northern blotting to determine whether LPA affects the levels of VEGF mRNA in vitro. OVCAR-3 cells were incubated with or without LPA from 2 to 24 hours. LPA (20 μM) stimulation of VEGF mRNA in OVCAR-3 cells occurred after a 4-hour incubation, reaching a maximum level after 16 hours, then de-
clining after 24 hours, but VEGF mRNA levels were still substantially higher than control levels (0 time) (Fig. 1, A). LPA also increased VEGF mRNA levels in OVCAR-3 cells in a dose-dependent fashion (Fig. 1, B). VEGF mRNA levels were markedly enhanced by 2 μM, with a further increase induced by 20 μM, concentrations that are present physiologically.

Because ovarian cancer is thought to originate from normal ovarian surface epithelial cells, we assessed whether VEGF expression also is induced by LPA in normal ovarian surface epithelial cells. Since normal ovarian surface epithelium constitutes a small component of the ovary and survives only for five or six passages in culture, it is difficult to obtain fresh, normal ovarian surface epithelial cells in sufficient quantities for multiple experiments. OVCAR-3 cells were treated with LPA in serum-free conditioned medium after starvation for 8 hours or overnight, conditions under which normal ovarian surface epithelial cells do not survive. Therefore, we used IOSE-29 cells to investigate the effect of LPA on VEGF expression. IOSE-29 cells were treated with different concentrations of LPA for 24 hours. LPA had little effect on VEGF mRNA expression by northern blotting in IOSE-29 cells (Fig. 1, C) and did not stimulate IOSE-29 cell growth in vitro (data not shown).

VEGF Protein Induction by LPA in Ovarian Cancer Cell Lines

OVCAR-3 cells were incubated in the absence or presence of various concentrations of LPA and for various times (Fig. 2, A and B). Conditioned media were assayed for secreted VEGF by ELISA. VEGF production induced by LPA paralleled that of LPA stimulation of VEGF mRNA in a time- and dose-dependent manner. After LPA stimulation, secreted VEGF levels increased progressively up to 3.2-fold above basal levels from 4 to 16 hours of incubation, then diminished at 24 hours, but still were 2.9-fold above the control level. VEGF secretion also rose progressively when OVCAR-3 cells were treated with various concentrations of LPA.

To determine whether the effects of LPA were unique to OVCAR-3 cells, we also studied three other ovarian cancer cell lines (i.e., SKOV-3, CAOV-3, and DOV-13). Secreted VEGF levels were quantified by ELISA in the cancer cell lines treated...
with 20 μM LPA for 16 hours. Although basal VEGF levels varied among the ovarian cancer cell lines, LPA-stimulated VEGF levels were increased statistically significantly by 1.5-fold (P = .0148, SKOV-3), 1.9-fold (P < .001, CAOV-3), and threefold (P < .0001, OVCAR-3) (Fig. 2, C). VEGF levels in DOV-13 cells, however, were below the ELISA detection range in the untreated and the LPA-treated cells (data not shown).

Mechanism of LPA Stimulation of VEGF Expression

We first investigated VEGF mRNA stability by examining its half-life. OVCAR-3 cells were incubated with or without LPA for 24 hours. Dactinomycin was then added at various times. Total RNA was isolated for northern blotting. The half-life of VEGF mRNA in both untreated and treated OVCAR-3 cells was similar (Fig. 3, A), indicating that LPA-evoked increases were not attributable to increased mRNA stability by prolonging its half-life. We speculated that LPA might increase the VEGF transcription rate by stimulating VEGF promoter activity. Therefore, two VEGF promoter–reporter constructs were transfected transiently into OVCAR-3 cells. The luciferase activity of the pGL2–2274VEGF construct, which contains the entire VEGF promoter, was considerably increased by twofold after LPA treatment. However, LPA had no effect on the luciferase activity of the shorter construct (pGL2–25VEGF, which contains a minimal promoter fragment [-25 to +50 bp relative to the transcription start site]) or pGL2–basic vector (Fig. 3, B).

LPA induces expression of c-Jun and c-Fos (9); Hu YL, Jaffe RB: unpublished data. Three putative AP1-binding sites are present in the VEGF promoter region (24). To determine if LPA stimulation of VEGF expression is mediated through c-Jun and c-Fos, we performed forced expression of c-jun and c-fos cDNAs with either pGL2–2274VEGF or pGL2–
25VEGF in OVCAR-3 cells. Cotransfection with pCMV-Jun and pCMV-Fos expression plasmids led to a dramatic fourfold increase in luciferase activity of pGL2-2724VEGF but not of pGL2-25VEGF (Fig. 3, C).

**LPA Receptors in Normal Ovarian Surface Epithelial Cells, IOSE-29 Cells, and Ovarian Cancer Cell Lines**

We further analyzed the expression of LPA receptors in normal ovarian surface epithelial cells (passages 3 and 4), IOSE-29 cells, and ovarian cancer cell lines. Edg4 (1.8-kb transcript) was readily detectable by northern blotting in all cancer cell lines studied but not in normal ovarian surface epithelial or in IOSE-29 cells. In contrast, Edg2 (4.6-kb transcript) was expressed at similar levels by all cell lines (although at a low level in OVCAR-3) (Fig. 4). Similar results were obtained by reverse transcription–polymerase chain reaction and by western blotting with antibodies to Edg2 and Edg4 (data not shown).

**DISCUSSION**

LPA can be derived from activated platelets (1) and from ovarian tumors ([25]; Mills GB: unpublished data). In the present study, LPA stimulation of ovarian cancer growth could be attributed, at least in part, to specific overexpression of Edg4 and induction of VEGF. It is likely that two distinct mechanisms are involved in LPA stimulation of ovarian tumor growth in vivo, since both Edg4 and VEGF are overexpressed, but only VEGF is likely to induce angiogenesis and vascular permeability leading to ascites formation.

We demonstrated that LPA enhances VEGF mRNA and protein levels in ovarian carcinoma cell lines. LPA increases the VEGF transcription rate rather than the mRNA stability. However, VEGF levels in untreated and LPA-treated DOV-13 cells were undetectable. This result is not unexpected, since this is the only cell line that does not have gene amplification of phosphoinositol-3 kinase (PI3-K) among about 80% of ovarian cancer cell lines and primary ovarian tumors, and both LPA and VEGF signal via a PI3-K pathway (26). Although the increase in VEGF protein is modest (1.5-fold to threefold), a twofold to fourfold increase in VEGF leads to considerable tumorigenic competence in colorectal cancer (27). LPA stimulation of ovarian cancer but not of IOSE-29 cell proliferation in vitro can be associated with VEGF induction by LPA. Because we examined only one IOSE cell line, it would be of interest to confirm these observations with the use of other IOSE cell lines. Thus, our present data suggest that LPA stimulation of VEGF expression is a common response of ovarian cancer cell lines and that LPA stimulates tumor growth indirectly through increasing expression of VEGF and perhaps other angiogenic factors (e.g., interleukin 8 and basic fibroblast growth factor [bFGF]) to provide the blood supply essential both for progressive growth of primary malignancies and for development of metastatic disease.

We demonstrated that LPA specifically stimulates the VEGF promoter activity, and we are continuing to localize LPA response element(s) in the promoter region. Analysis of the VEGF promoter region reveals potential binding sites for transcription factors AP1, AP2, and SP1, which are involved in the activation of VEGF expression by platelet-derived growth factor, tumor necrosis factor-α, bFGF, and transforming growth factor-α (24,28–30). Our data also demonstrate that forced expression of c-jun and c-fos cDNAs can stimulate VEGF promoter activity fourfold in OVCAR-3 cells. Interaction of c-Jun with SP1 transcription factor stimulates expression of cardiac-specific atrial natriuretic factor (31). Such an interaction might also occur in the VEGF promoter and accounts, in part, for the fourfold increase in promoter activity. To examine this possibility, we created two 5′ deletional promoter–luciferase constructs at −268 and −131 that have potential AP2 and SP1 sites but lack three AP1 sites. After either LPA treatment or forced expression with c-jun and c-fos cDNAs, the luciferase activities of these constructs were intermediate between constructs pGL2-2724VEGF and pGL2-25VEGF (data not shown). This observation suggests that LPA stimulation of ovarian tumor growth via VEGF can occur through activation of both AP1 and SP1. AP1 promotes tumor progression and metastasis (32–34). However, the specific mechanisms by which LPA induces VEGF via Fos-Jun and Jun-Sp1 interaction with the VEGF promoter are still unclear and await further exploration with the use of site-directed mutagenesis and gel mobility shift assays. LPA may also activate other transcription factors (e.g., HIF-1) to further induce VEGF expression, since LPA can activate a PI3-K (2), which is involved in hypoxia induction of VEGF expression through HIF-1 (35).

The four ovarian cancer cell lines that we studied, as well as OV202 primary ovarian cancer cells (36), express Edg2 and Edg4, while normal and immortalized ovarian surface epithelial cells express only Edg2. LPA stimulates DNA synthesis and activates serum response element–luciferase reporter activity in ovarian cancer cells but not in IOSE-29 cells. Furthermore, pertussis toxin and mitogen-activated protein kinase kinase inhibitor (PD98059), which block LPA receptor-signaling pathways, suppress ovarian cancer cell responses to LPA (36). Thus, our data suggest that Edg4, but not Edg2, may be a key receptor involved in LPA stimulation of ovarian tumor growth, together with the activation of VEGF expression. Other Edg receptors may also be involved, and the factor(s) that induce Edg4 expression in ovarian cancer cells remain to be explored.

Results of treatment of ovarian epithelial cancer have changed minimally (37), likely because of the failure to detect the disease at an early stage. Our data show that Edg4 was detected at relatively high levels in ovarian cancer cell lines but not in normal ovarian surface epithelial cells. Thus, Edg4 may be a novel biomarker for...
ovarian cancer, as LPA may be. However, the levels of LPA receptors, especially of Edg4, do not appear to correlate precisely with the VEGF response of ovarian cancer cells to LPA. We speculate that Edg4 is necessary but not sufficient (e.g., a PI3-K-signaling pathway also is needed) for LPA stimulation of VEGF in ovarian cancer cells. Since LPA can stimulate ovarian tumor growth by inducing angiogenesis through increasing VEGF expression, inhibition of cell growth could be achieved by creating soluble dominant negative Edg4 mutant(s) that can block the effects of LPA. Thus, an Edg4 dominant negative mutant(s) and/or antisense Edg4 sequence may be useful in inhibiting ovarian tumor growth in vivo and in vitro.

REFERENCES


NOTES

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