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The NF-κB pathway mediates lysophosphatidic acid (LPA)-induced VEGF signaling and cell invasion in epithelial ovarian cancer (EOC)☆

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ABSTRACT

Objectives. Our previous report has implicated the involvement of VEGF-VEGFR-2 h signaling in LPA-induced EOC invasion. However, the mechanism by which LPA regulates VEGF and VEGFR-2 expression remains to be elucidated. In the present study, we systematically examined the signal transduction pathways activated by LPA and further evaluated whether LPA's effect on VEGF-VEGFR-2 signaling and EOC invasion was mediated by the activation of NF-κB pathway.

Methods. Using a signal transduction PathwayFinder PCR array, we examined the expression change of 86 key genes representing 18 signal transduction pathways in DOV13 and SKOV3 cells upon LPA (20 μM) treatment. We also used quantitative PCR, Western blotting and ELISA to evaluate the effect of NF-κB pathway inhibition on $VEGF_{121}$, $VEGF_{165}$ and VEGFR-2 mRNA and protein expression/secretion with or without the presence of LPA (20 μM) in SKOV3. Cell invasion under various treatment conditions was assessed by Matrigel invasion assay and MMP-2 secretion was detected by gelatin zymography.

Results. Our results showed that in both DOV13 and SKOV3, several of the NF-κB pathway components, such as TNF, are consistently activated by LPA stimulation. In addition, treatment with an NF-κB pathway activation inhibitor, at 10 μM, significantly decreased LPA-induced $VEGF_{121}$, $VEGF_{165}$ and VEGFR-2 mRNA expression and VEGF secretion, as well as LPA-induced SKOV3 invasion ($p<0.05$). When combined with an EGFR inhibitor, NF-κB pathway inhibition exhibited a significantly stronger effect than used alone ($p<0.05$) on reducing LPA-induced VEGF secretion and cell invasion. Additionally, NF-κB inhibition also decreased LPA-induced MMP-2 secretion and MMP-1 expression ($p<0.05$).

Conclusions. These results suggest that the NF-κB pathway plays an important role in LPA-induced VEGF signaling and EOC invasion and targeting this pathway may reveal potential therapeutic options for metastatic EOC.

Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy in the United States, with more deaths from this disease alone than all the other gynecologic malignancies combined [1]. This dismal statistic is attributed to the fact that most women (75%) are diagnosed at an advanced stage, when the disease already presents with widespread metastatic dissemination, characterized by rampant intraperitoneal carcinomatosis and large volumes of ascites [2]. Additionally, although approximately 80% of women respond to first-line chemotherapy regimens, most of them will relapse after 1 year with metastases and the development of chemo-resistance [3]. Therefore, effectively controlling ovarian metastasis represents a very important strategy to eradicate this deadly disease.

Ovarian cancer metastasis is a complex process that requires a coordinated effort between tumor cells and the surrounding tumor microenvironment where proteases, growth factors, cytokines, and chemokines are released from both tumor cells and stromal cells and interact with each other to promote tumor growth, invasion and metastasis. Among those bio-molecules, lysophosphatidic acid (LPA) stands out as a unique lysophospholipid growth factor regulating multiple events within the ovarian tumor microenvironment to promote EOC invasion and metastasis [4,5]. LPA is released by malignant ovarian epithelial cells or mesothelial cells [6] and mediates many cellular processes, such as DNA synthesis, cell apoptosis, proliferation, cell survival, adhesion, invasion and migration. LPA acts through the activation of G protein coupled receptors (GPCRs), LPA1-6 [7,8]. Previous studies have shown that LPA induces the expression of VEGF and IL-8 to promote EOC invasion and migration [9,10].

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LPA's effects on VEGF have attracted much attention given VEGF's role in angiogenesis as well as cell migration, proliferation and survival. VEGF is a dimeric glycoprotein, commonly released during episodes of hypoxia, as well as following LPA stimulation, via its transcriptional [10] and translational regulation [11]. Upon activation of its receptors, mostly VEGFR-2, VEGF induces endothelial cell invasion, migration, proliferation and angiogenesis. Many studies have also shown the association of increased VEGF and VEGFR-2 levels with tumor progression and poor prognosis in malignancies such as breast, colon, lung and ovarian cancer [12–16]. We have recently reported that LPA-induced EOC invasion is partially mediated by the VEGF-VEGFR-2 signaling axis [15]. Additionally, when VEGFR-2 is blocked with a small molecular inhibitor (SU1498) or siRNA, the secretion and activity of MMP-2 and uPA are significantly reduced, which thereby decreases LPA's invasion and migration promoting effects [15]. However, the exact molecular mechanism by which LPA affects the VEGF-VEGFR-2 signaling axis in EOC remains unclear. To explore the signaling pathways involved in LPA-induced VEGF-VEGFR-2 signaling, in the present study, we first systematically examined gene expression changes of common signal transduction pathways affected by LPA stimulation, then we used small molecule inhibitor against the most dysregulated pathway to test its efficacy on reducing LPA-induced VEGF-VEGFR-2 expression, as well as EOC invasion.

Materials and methods

Materials

LPA (18:1) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL); VEGFR-2 antibody, anti-GAPDH antibody and anti-phospho-paxillin antibody were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-actin antibody was a product of Sigma Inc. (St. Louis, MO). NF-κB activation inhibitor II and EGFR inhibitors were purchased from VWR Scientific (Bridgeport, NJ) and diluted in DMSO to 10 mM stock concentration.

Cell culture

The EOC cell line SKOV3 was obtained from American Tissue Culture Collection (ATCC) and cultured in Modified McCoy's 5A Medium (Sigma Inc, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), 100 IU penicillin, 100 μM streptomycin and 2.5 μg/mL amphotericin. This cell line was maintained under standard conditions (37 °C and 5% CO_2) in 75 cm^2 tissue culture flasks. The experiments were performed using cells in the logarithmic phase of growth.

cDNA microarray

The Human Signal Transduction PathwayFinder™ RT²*Profiler*™ PCR Array (SA Biosciences, Frederick, MD, PAHS-014A), which includes the cDNAs of 86 key genes representative of 18 different signal transduction pathways was used to examine the major signal transduction pathways, activated by LPA stimulation in EOC DOV13 and SKOV3 cells. Briefly, total RNA from non-treated DOV13 and SKOV3 cells as well as LPA (20 μM)-treated cells was extracted by using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma, St. Louis, MO), and 1 μg of total RNA was then reverse transcribed (RT) to first-strand complementary DNA (cDNA) according to the protocol provided by the RT^2 First Strand Kit (SABiosciences, Frederick, MD). The resultant cDNA was then diluted and mixed with 2× SABiosciences RT^2 qPCR Master Mix and ddH_2O to a total volume of 2700 μL, with 25 μL of reaction mixture loaded into each well of the 96 well plate and the real time amplification was performed on a iCycler IQ5™ (Bio-Rad Laboratoris, Hercules, CA) PCR detection system following the instructions provided by the PCR array kit. The mRNA levels were normalized against housekeeping genes included in the PCR array (beta glucuronidase, *B2M*; hypoxanthine guanine phosphoribosyl transferase 1, *HPRT1*; heat shock protein 90 kDa alpha, *RPL13A*; glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*; and beta actin, *ACTB*). Gene expression fold changes were calculated according to the ΔΔCt method using the RT² Profiler PCR Array Data Analysis Template v3.3 software. Unsupervised clustergram were generated by using the RT^2 PCR array data analysis web portal (http://www.sabiosciences.com/pcrarraydataanalysis.php).

Quantitative real time-PCR

DOV13 and SKOV3 cells in the logarithmic growing phase were trypsinized, centrifuged and resuspended at a concentration of 1×10^5/mL and then 2.5 mL cells were plated into each well of 6-well plate to grow for 24 h. For the validation of NF-κB, TNF, IL-8, ICAM-1 and IL1-α, cells were treated with 20 μM LPA or vehicle control (0.1% BSA/PBS) after overnight starvation in serum free medium. In order to test the effect of the NF-κB activation inhibitor on VEGF, VEGFR-2 and MMPs expression, SKOV3 cells were treated with vehicle control (DMSO + 0.1% BSA/PBS), various concentrations of inhibitor, 20 μM LPA and 20 μM LPA plus inhibitor for 6 h. After treatment, total RNA was extracted from cells using the GenElute™ Mammalian Total RNA kit (Sigma). Following DNase I treatment with the Turbo™ DNA-free kit (Ambion Inc, Austin, TX), 0.5 μg of total RNA was reverse transcribed (RT) and subsequently amplified using the iQ™ SYBR GREEN Supermix (Bio-Rad) for real time-PCR as previously described [15]. The primer pairs used for VEGF121, VEGF165, VEGFR-2 are as described previously [15], the primer pairs sequences for NF-κB, TNF, IL-8, ICAM-1, and IL1-α are listed in supplemental Table 1. Real-time PCR reactions were carried out on the iCycler IQ5™ (Bio-Rad Laboratoris, Hercules, CA) with the following cycling conditions: denaturing at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 55 s for 40 cycles. The gene expression change upon treatment was presented as relative expression (fold over non-treated control or percentage of control) after normalizing to GAPDH, and was calculated as previously described using the $2^{-\Delta\Delta Ct}$ method [17,18].

Western blotting

SKOV3 cells were coated on 6-well plates at a concentration of 1×10^5/mL, with 2.5 mL cells plated into each well. When reaching 80–90% confluence, cells were serum starved overnight and then treated in SFM with vehicle control (DMSO + 0.1% BSA/PBS) or various concentrations of inhibitor with or without LPA (20 μM) for 6 h. Then, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% triton X-100, and 5 mmol/L EDTA] to lyse protein. Collected lysate was subsequently centrifuged for 15 min at 14,000 rpm and supernatant containing protein was saved for subsequent assays. Equal amounts of protein (50 μg) were loaded and separated by SDS-PAGE and then transferred to nitrocellulose membranes via the iBlot apparatus (Invitrogen, Carsbad, CA). Primary antibodies were used with the following dilution: actin (1:10,000), GAPDH (1:5000), VEGFR-2 (1:200) and phospho-paxillin (1:200). Blotting was performed using procedures as previously described [17]. Each experiment was repeated at least three times.

VEGF ELISA

Conditioned medium was collected from cells treated with LPA and NF-κB inhibitor at various concentration for 6 h, and centrifuged for 15 min at 14,000 rpm. Supernatants were then collected and stored at −20 °C until further analysis. ELISA was performed following the protocol provided by Chemicon of Millipore Inc