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Original Research Article

Lysophosphatidic acid (LPA) effects on endometrial carcinoma *in vitro* proliferation, invasion, and matrix metalloproteinase activity

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ABSTRACT

Objectives. Lysophosphatidic acid (LPA) has potent growth-regulatory effect in many cell types and has been linked to the *in vivo* tumor growth and metastasis in several malignancies. The goal of this study was to assess the regulation of (EC) microenvironment by LPA through the examination of its effect on cell proliferation, migration, invasion, uPA activity, and matrix metalloproteinase (MMP) secretion/activation.

Methods. All experiments were performed *in vitro* using an EC cell line, HEC-1A. Cell proliferation was determined using the Promega MTS proliferation assay following 48 h of exposures to different concentrations of LPA (0.1, 1.0 and 10.0 μM). Cell invasion was assessed using a modified Boyden chamber assay with collagen I coated on the membrane. HEC-1A motility was examined by Boyden chamber migration assay as well as the scratch wound closure assay on type I collagen. MMP secretion/activation in HEC-1A conditioned medium was detected by gelatin zymography. MMP-7 mRNA expression was determined using real-time PCR. uPA activity was measured using a coupled colorimetric assay.

Results. LPA, at the concentrations of 0.1 and 1.0 μM, significantly induced the proliferation of HEC-1A cells ($p<0.01$). At 10 μM, LPA- induced HEC-1A proliferation to a less extent and showed no significant effect on HEC-1A invasion and migration ($p>0.05$). Gelatin zymogram showed that HEC-1A cells secreted high levels of MMP-7, while MMP-2 and MMP-9 are barely detectable. In addition, LPA significantly enhanced uPA activity in HEC-1A conditioned medium in a concentration-dependent manner.

Conclusions. LPA is a potent modulator of cellular proliferation and invasion for EC cells. It also has the capacity to stimulate the secretion/activity of uPA and MMP-7. Those results suggest that LPA is a bioactive modulator of EC microenvironment and may have a distinct regulation mechanism as observed in epithelial ovarian cancer.

Introduction

Endometrial cancer (EC) is the most common gynecologic malignancy in the United States. In 2009, there are 42,160 estimated new cases of EC, and 7780 deaths are expected from this disease in US [1]. EC often causes abnormal postmenopausal bleeding as an initial symptom and is commonly detected as an early stage malignancy, with more than 75% of women with this disease diagnosed at stage I/II and the majority of them surgically curable [2,3]. Thus, the overall prognosis for EC is excellent, with a 5-year survival rate approximating 80% [4,5]. However, the good prognosis associated with the diagnosis of endometrial carcinoma is limited to endometrioid tumors and does not characterize clear cell or serous carcinoma; those women diagnosed with such disease carry a dismal prognosis.

Tumor growth and metastasis requires a coordinated succession of events within the tumor microenvironment that involves proliferation, loss of cellular adhesion, degradation of the extracelluar matrix, invasion into host stroma, cell migration, and angiogenesis [6,7]. Growth factors and cytokines, serine proteases, matrix metalloproteinases (MMPs), cellular adhesion molecules, tumor cell–stromal interactions, and phospholipids are actively involved in the regulation of these processes [8–10]. Thus, understanding the tumor microenvironment and its regulatory mechanism is critical for the development of effective therapies to treat metastatic endometrial carcinoma.

Lysophosphatidic acid (LPA) is a bioactive phospholipid that has potent growth-regulatory effect in many cell types, influencing proliferation, survival, migration, invasion, wound-healing, as well as changes in cell morphology and differentiation [11]. LPA mainly acts through the endothelial differentiation gene (Edg) family of G protein-coupled receptors currently referred to as LPA1-3 (Edg-2, Edg-4, and Edg-7) to mediate its effect. Those LPA receptors are aberrantly expressed in various types of cancer cells, and may be

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involved in the mechanism of tumor growth and metastasis [12–18]. Other LPA receptors identified to date include LPA4 (p2y9/GPR23) [19], LPA5 (GPR92) [20] and LPA6 (p2y5) [21], which are more closely related orphan G protein receptors and are less well studied than LPA1-3. Due to the importance of LPA-LPA receptor signaling in tumor growth and metastasis, LPA receptors have become potential targets for the development of antitumor agents particularly in ovarian cancer.

LPA levels were found to be elevated in the plasma and ascites of more than 90% of ovarian cancer patients [22]. Its levels were also found elevated in the plasma of patients with cervical and endometrial cancer, as well as multiple myeloma [22–24]. While there is a significant amount of published and ongoing research on the roles of LPA in ovarian cancer, currently few studies have examined the regulation effect of LPA on EC growth and metastasis. We have recently reported that LPA2 mediates LPA-induced EC invasion using HEC-1A cells [25]. In this study, we will continue our effort to extensively examine the effect of LPA on HEC-1A cell proliferation, migration, invasion, uPA activity, and MMPs secretion/activation to provide a better understanding of its roles in EC growth, invasion, and migration.

Materials and methods

Cell lines and culture conditions

The human endometrioid adenocarcinoma HEC-1A cell line and fibrosarcoma HT-1080 cell line were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in our laboratory in a humidified atmosphere incubator at 37 °C with 5% CO_2. Briefly, HEC-1A cells were grown in modified McCoy's 5A medium, and HT-1080 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma, St. Louis, MO), all containing 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA). All experiments were carried out with the cells in the logarithmic phase of growth. Synthetic LPA (18:1) was purchased from Avanti Polar Lipids (Alabaster, AL).

Preparation of HT-1080 conditioned media

HT-1080 cells were cultured to 80% confluence, washed three times with PBS, and then incubated in serum-free medium (SFM) for 24 h. The culture media was then collected and centrifuged at 500×g for 15 min, and then at 800×g for 10 min. The supernatants were stored at 4 °C for a maximum of 24 h before their use. Aliquots of conditioned media that were not used within 24 h were stored at −80 °C.

Proliferation assay

HEC-1A cells were plated in 96-well plate at a concentration of 5×10^3 cells/well and starved overnight in SFM before treatment. Cells were then treated with vehicle control (0.1% BSA/PBS) or LPA at different concentrations (0.1, 1.0 and 10.0 μM) in triplicate for 48 h. Then, the MTS solution was mixed with phenazine methosulfate (PMS) at a ratio of 20:1 and 20 μl of the mixture was added to the wells and incubated at 37 °C for 4 h according to the protocol provided by the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (also known as the MTS assay) kit (Promega, Madison, WI). Absorbance at 490 nm was then measured using a 96-well microplate reader from Molecular Devices (Sunnyvale, CA). Hepatocyte growth factor (HGF, Sigma, St. Louis, MO) at 50 ng/ml was used as a positive control as previously described [26]. Results are presented as the means ± SE absorbance at 490 nm (A490) of triplicate assays.

The HEC-1A proliferation upon LPA treatment at the concentrations described above was also assessed with the addition of HT-1080 serum-free conditioned media in a 50 μl:50 μl mixture with McCoy's 5A SFM to evaluate the effects of LPA on HEC-1A proliferation with the introduction of an exogenous source of pro-MMP-2.

In vitro invasion assay using collagen I

The ability of cells to invade through collagen I was tested in a modified Boyden chamber assay. Boyden chamber inserts in 24-well plate with an 8 μm porous membrane (BD Biosciences, Bedford, MA) were coated with 25 μg/50 μl of rat tail collagen type I (Upstate Cell Signaling Solutions, Lake Placid, NY) in phosphate buffered saline (PBS) and incubated for 1 h at 37 °C. The redundant collagen I was then removed by pipeting and HEC-1A cells (1.5×10^5 cells/well) were added to the top of the collagen coated chamber and incubated in SFM for 48 h with the addition of 0.1, 1 and 10 μM LPA in triplicate wells. The solution to dissolve LPA (0.1% BSA/PBS) was used as vehicle control and HGF (50 ng/ml) was used as a positive control. At the end of incubation, noninvading cells on the top chamber of the inserts were removed with cotton swabs and the inserts were then fixed and stained with the Diff-Quik® staining kit (Dade Behring Inc, Newark, DE). The membrane on the air dried inserts were then cut and mounted on glass slides with the bottom side facing up. The number of cells invading through the membrane was then counted using the Verta VU 7000 series optical microscope (Wesco, Pittsburg, PA) at 100× magnification and the average number of cells per field was calculated.

Cell migration assay

Chemotactic migration of HEC-1A cells was examined using a modified Boyden chamber. The bottom chambers were filled with 5% FBS (control) or 5% FBS containing LPA (0.1, 1.0 and 10 μM) or 50 ng/ml HGF (positive control). HEC-1A cells (1.5×10^5/well) in SFM were plated in the upper chamber of the noncoated porous polycarbonate membrane inserts (8 μm diameters), and incubated at 37 °C with 5% CO_2 for 24 h. Nonmigrated cells were removed from the upper chamber with a cotton swab and the filters were stained using the Diff-Quick® staining kit. Migrated cells adherent to the bottom side of the filters were then counted as described previously in the invasion assay. Data were expressed as the average number of migrated cells/field representing triplicate experiments.

Wound healing assay

HEC-1A cells were plated onto six-well plates and grown until confluence in culture medium containing 10% FBS. Then, the cultures were serum deprived for 12 h. A 1000 μl pipette tip was used to make a single uniform scratch wound that crossed each well. The wells were washed twice with PBS to remove floating cell debris, and then incubated in the absence or presence of LPA (0.1, 1.0 and 10 μM) or 50 ng/ml HGF in SFM for 48 h. Wound closure was monitored by visual examination using a Nikon TE 2000 microscope. A total of five photographs were taken for each well, one at each of five mark points made on the underside of each well. Photographs were taken at 0, 24 and 48 h. Five measurements of the wound width on each photographic image were made using the MetaMorph Imaging System software (Universal Imaging Corporation, Downington, PA). The five measurements for each point were averaged and normalized to their corresponding value at time 0. The data for each experimental condition come from 25 measurements and the experiments were performed three times.

RNA isolation and RT-PCR analysis

Total cellular RNA was prepared from HEC-1A cells using the GenElute Mammalian Total RNA kit (Sigma, St. Louis, MO). Following