Lysophosphatidic Acid Induces Urokinase Secretion by Ovarian Cancer Cells

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ABSTRACT

Lysophosphatidic acid (LPA) is present at high concentrations in ascites from ovarian cancer patients and has potent mitogenic properties in vitro. Urokinase plasminogen activator (uPA), a critical component of the metastatic cascade, is also found at high concentrations in ovarian ascites and ovarian cancers, and the levels of uPA correlate inversely with prognosis. Because LPA stimulates the invasion of both hepatoma and lung cell lines, we investigated whether LPA could induce uPA secretion by ovarian epithelial cells and whether this process was associated with malignant transformation of ovarian epithelial cells. As indicated by zymography and Western blotting, physiologically relevant concentrations of LPA equivalent to those present in ovarian cancer ascites stimulated uPA secretion in the ovarian cancer cell lines OVCAR-3, SKOV-3, OVCA 429, OVCA 432, and OVCA 433, but not from established normal ovarian epithelial (NOE) cells as indicated by normal epithelial cell lines NOE 033 and NOE 035 or from SV40 large T antigen-immortalized normal epithelial cell lines IOSE 29 and IOSE 80. 18:1 LPA, but not 18:0 LPA, 16:0 LPA, or lysophosphatidylcholine, induced uPA secretion, concordant with previous studies of LPA receptor selectivity. Expression of the edg-2 LPA receptor was not consistently different between normal epithelial cell lines and ovarian cancer cell lines. In contrast, expression of the edg-4 LPA receptor was markedly increased in ovarian cancer cell lines as compared with NOE cell lines, raising the possibility that the edg-4 LPA receptor contributes to the ability of ovarian cancer cells but not NOE cells to produce uPA in response to LPA. LPA induced a consistent increase in uPA promoter activity and mRNA levels, suggesting that increased uPA production is, at least in part, transcriptional.

Malignant transformation may alter LPA-induced cell activation by altering the pattern of LPA receptors present and may possibly lead to more aggressive behavior by up-regulating LPA-mediated uPA secretion and stimulating extracellular stromal breakdown and invasion.

INTRODUCTION

Ovarian cancer is predominantly detected at a late stage after extensive intra-abdominal spread and is often accompanied by the formation of ascites (1). Many growth-promoting factors are known to be present in ovarian cancer ascites, frequently at low levels. LPA, in contrast, is found at significant levels (2–80 μM) in ovarian ascites (2, 3) and may play an important role in the development or progression of ovarian cancer. LPA functions as an extracellular signaling molecule, and its specific function is likely to depend on the local environment, the cell lineage, and the spectrum of LPA receptors expressed by the cell (4). LPA induces a plethora of cellular responses including smooth muscle contraction, neurite retraction, and focal adhesion assembly (4, 5). LPA also has potent mitogenic properties leading to increases in proliferation in ovarian and breast cancer cell lines (2, 6). LPA may also be involved in the metastatic process because it has recently been shown to stimulate invasion in a rat ascites hepatoma and a small cell lung cancer cell line (7).

uPA contributes to metastasis and migration because it catalyzes the conversion of plasminogen to plasmin, thus leading to degradation of the basement membrane (8). It also stimulates cellular migration and proliferation (9) potentially by binding to its specific cell surface receptor (uPA receptor). uPA has been linked to malignant transformation in ovarian (10), breast (11), and colon (12) cell lineages. In ovarian cancer, cellular uPA levels correlate inversely with prognosis (13, 14). In striking contrast to other cell lineages, uPA production can be localized to ovarian cancer cells in vivo (15). uPA is also present in significant levels in ascites (15) and is secreted by human ovarian carcinoma cell lines (10, 16, 17). Furthermore, levels of the uPA receptor are increased in ovarian cancers (18).

Because LPA and uPA are both present at elevated levels in ovarian cancer, and LPA can induce invasiveness in other cell lineages, we investigated whether LPA could induce uPA secretion in normal and ovarian cancer cell lines. We demonstrate that LPA induces an increase in uPA promoter activity, mRNA levels, protein levels, and enzyme activity in ovarian cancer cell
**MATERIALS AND METHODS**

**Cell Culture.** OVCAR-3 and SKOV-3 ovarian cancer cells and SKBR 3 breast cancer cells were obtained from ATCC (Manassas, VA). They were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated FCS (Sigma), 10% L-glutamine, 1% penicillin, and 1% streptomycin (Life Technologies, Inc.). NOE cells were cultured from fresh surgical specimens according to an institutional review board-approved protocol at The University of Texas M. D. Anderson Cancer Center. Cells were scraped from the surface and cultured in a 1:1 mixture of MCDB 105 medium (Sigma) and Medium 199 (Life Technologies, Inc.) supplemented with 10 ng/ml epidermal growth factor (Sigma), 10% FCS, 10% L-glutamine, 1% penicillin, and 1% streptomycin (NE media; Ref. 19). Immortalized NOE cells (IOSE 029 and IOSE 80; Ref. 19) were obtained from Dr. N. Auersperg (University of British Columbia, Vancouver, Canada) and were also maintained in the media used to culture NOE cells, but without the addition of epidermal growth factor and the reduction of FCS from 10% to 5%. NIH3T3 cells (ATCC) were cultured in DMEM (Life Technologies, Inc.) containing 10% FCS, 10% L-glutamine, 1% penicillin, and 1% streptomycin. OVCA 429, OVCA 432, and OVCA 433 (a generous gift from Dr. R. Bast, University of Texas M. D. Anderson Cancer Center) were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 1% penicillin, and 1% streptomycin.

**LPA Stimulation.** A 20 nM LPA stock solution was prepared in chloroform (Sigma) and stored at −20°C. Immediately before use, the required aliquot was dried down under sterile conditions, and the LPA pellet was gently resuspended in 1% delipidated BSA (Sigma) for 30 min on ice. The cell lines were cultured to near confluence in complete medium, washed with SFM, and then starved in SFM for 16–24 h. Fresh SFM was replaced before LPA addition.

**Zymography.** Zymography was performed by the method outlined by Heussen and Dowdle (20). Serum-free supernatants from each cell culture, after normalization for viable cell number by the MTT assay (21), were analyzed under nonreducing conditions on 7.5% SDS-PAGE (Sigma) containing 0.1% casein and 4 mg of plasminogen. The gel was then incubated at room temperature in a 2.5% Triton X-100 solution containing 0.05% sodium azide and 0.05 M Tris (pH 7.5; Sigma) followed by incubation at 37°C in 0.1 M Tris-HCl (pH 8.3)/0.5 M sodium chloride solution for 16 h. The gel was then stained with a 0.25% Coomassie Blue/11% acetic acid/45.5% methanol solution for 40 min, followed by destaining in a 25% methanol/10% acetic acid mixture. Enzyme activity was visualized as clear zones on a blue background after conversion of plasminogen to plasmin by uPA. UMSSC (a squamous carcinoma cell line kindly provided by Dr. T. Carey, University of Michigan, Ann Arbor, MI) was used as a positive control.

**Western Blot Analysis.** Serum-free cell culture supernatant was concentrated 10–30-fold by Centricron 10 columns (Amicon, Beverly, MA). Sample volumes were normalized for cell number by the MTT dye conversion assay (19) and/or BCA protein quantitation of cell supernatants (Pierce, Rockford, IL) as indicated. Supernatant proteins were separated by 12.5% SDS-PAGE, transferred to Immobilon-P membrane (Millipore, Bedford, MA), blocked with 3% BSA or 4% skim milk powder, and probed using a 1:1000 dilution of rabbit polyclonal antibody to uPA. The arrow on the right represents the migration of recombinant uPA in a parallel lane.

**Northern Blot Analysis.** Northern blot analysis for the quantitation of uPA-specific mRNA was performed as described previously (22). Briefly, total RNA isolated from OVCAR-3...
cells by the method of Chomczynski and Sacchi (23) was electrophoresed (10 μg/lane) through 1% agarose gels containing 0.22 M formaldehyde (Sigma). uPA (ATCC), edg-2, edg-4, and GAPDH probes were radiolabeled by random prime labeling using the Redi-Prime labeling kit (Amersham). Membranes were incubated with radiolabeled probes in 50% formamide, 5 × Denhardt’s solution, 0.5% SDS, 50 mM HEPES (pH 7.0), 4.8 × SSC, and 100 μg/ml salmon sperm single-strand DNA (Sigma) at 42°C for 18 h. Blots were washed three times in 0.1 × SSC containing 0.1% SDS at room temperature and then washed three times (for 20 min each) at 50°C in 0.1 × SSC/0.1% SDS before autoradiography at −80°C for 3 days with one screen or with a phosphorimager. Stripped blots were probed for GAPDH expression to standardize RNA loading.

**Transient Transfection and the Luciferase Reporter Assay.** Trypsinized and washed ovarian cancer cells (OVCAR-3 and SKOV-3 cells) were resuspended in complete medium at 5 × 10⁶ cells/ml, and 700 μl were reserved for each electroporation. The cells were gently mixed with 10 μg of the luciferase construct (pRSV-luc or puPA-luc; provided by Dr. T. Silverman, NIH, Bethesda, MD) and pulsed at 250 V/940 μF using a Gene Pulser II system (Bio-Rad). The cells were then recovered in 10 ml of complete medium and cultured in dishes for 24 h. The cells were starved in SFM for 12 h before LPA addition in fresh SFM. LPA stimulation was performed over an additional 12-h incubation. After a 48-h total incubation, the cell monolayer was washed three times with ice-cold PBS and lysed using Promega (Madison, WI) reporter lysis buffer for 10 min on ice. The cells were scraped and collected in a microfuge tube and vortexed vigorously, and the lysate was recovered after full-speed centrifugation at 4°C for 10 min. For the luciferase assay, 15 μg of total lysate protein, as determined by the BCA assay, were resuspended in 50 μl of lysis buffer and autosampled using the Analytical Luminescence Monolight 2010 (San Diego, CA).

**RESULTS**

LPA induces a striking increase in the level of uPA in the supernatants of OVCAR-3 and SKOV-3 cells, as shown by Western blot analysis (Fig. 1) and by zymography. Low but significant levels of uPA protein are present in resting supernatants of SKOV-3 cells, but not OVCAR-3 cells. This may reflect the constitutive production of LPA by SKOV-3 cells but not OVCAR-3 cells (data not shown). LPA-induced uPA secretion could be detected by 12 h, increased until 24 h (Fig. 2), and persisted for at least 48 h. LPA also induced an increase in uPA production by other ovarian cancer cell lines including OVCA.
LPA-induced increases in uPA secretion by OVCAR-3 cells were detectable upon exposure to 10 μM LPA, with optimal concentrations ranging from 20–80 μM (Fig. 3). Similar concentrations of LPA have previously been shown to induce significant proliferative effects on ovarian cancer cells and to parallel the LPA concentrations found in the serum and ascites of ovarian cancer patients (2, 3, 6).

We have demonstrated previously that 18:1 LPA efficiently induces cellular changes in cytosolic calcium and cellular proliferation in ovarian cancer cells, whereas 16:0 LPA and 18:0 LPA are relatively inefficient in mediating such effects (2, 6). In the ovarian cancer cell line OVCAR-3, LPA containing an unsaturated fatty acyl chain (18:1 LPA) effectively stimulated uPA secretion, whereas LPA with a saturated fatty acyl chain...
(18:0 LPA and 16:0 LPA) had no effect (Fig. 4). Alteration of the phosphate group (LPC) rendered the lysosphospholipid ineffective in increasing uPA secretion (Fig. 4). This suggests that the structure of LPA is important in regulating function and potential interaction with the LPA receptors, mediating uPA production in ovarian cancer cells.

In striking contrast to the consistent response of ovarian cancer cell lines to LPA, LPA did not induce detectable increases in uPA secretion by NOE cell lines NOE 033 and NOE 035 or viral SV40 large T antigen-immortalized NOE cell lines IOSE 29 and IOSE 80 (Ref. 19; Fig. 1). LPA also did not induce uPA secretion by the SKBR 3 breast cancer cell line or by NIH3T3 murine fibroblasts, despite the ability of LPA to induce the proliferation of these cells (data not shown). Thus, LPA specifically up-regulates uPA secretion in ovarian cancer cell lines, but not in NOE cell lines. Because LPA increases the level of secreted and active uPA protein, we investigated whether LPA altered the expression of uPA transcripts. LPA induced a 5.5-fold increase in the level of uPA mRNA in OVCAR-3 cells within 24 h of treatment, as shown by densitometry (Fig. 5). After 48 h of LPA stimulation, a marked 35-fold increase in uPA mRNA levels was seen (Fig. 5). This is similar to the time course of LPA-induced uPA secretion (Fig. 1). Taken together, these findings strongly suggest that LPA stimulation induces increased steady-state uPA-specific mRNA levels and promotes the concomitant elevation of uPA protein synthesis and secretion.

The effect of LPA on the nuclear transcription of uPA was then evaluated using a promoter-linked luciferase transfection assay. A 2–3-fold increase in luciferase activity was consistently seen in the presence of LPA (Fig. 6). The modest but consistent response of the uPA promoter to LPA treatment indicates that LPA-induced increases in uPA transcription likely contribute to the ability of LPA to increase uPA levels, mRNA levels, and protein levels in cellular supernatants.

The ability of LPA to consistently induce uPA secretion by ovarian cancer cells but not by normal ovarian epithelium led us to explore the expression of the edg-1, edg-2, and edg-4 LPA receptors (4) by ovarian epithelial cell lines. mRNA levels of edg-1, which mediates responses to LPA and sphingosine-1-phosphate (4), are low in the ovarian cancer cell lines studied (data not shown). As indicated in Fig. 7, edg-2 mRNA levels differed markedly between normal and malignant ovarian epithelial cell lines. Although edg-2 mRNA levels were low in NOE 033 and NOE 035, similar edg-2 mRNA levels were present in SKOV-3 and OVCAR-3, which secreted uPA in response to LPA, and IOSE 80, which did not produce uPA in response to LPA. Thus edg-2 expression was not sufficient to explain the ability of LPA to induce uPA secretion. In contrast, edg-4 mRNA levels were consistently increased in ovarian cancer cell lines as compared with NOE cell lines. Thus, the ability of LPA to induce the secretion of uPA from ovarian cell lines correlates with edg-4 mRNA levels but not with edg-1 or edg-2 mRNA levels.

**DISCUSSION**

As in other cancers, the presence of the uPA correlates with the aggressiveness of ovarian cancer (10, 13–15, 24, 25). For example, a correlation was noted between higher uPA levels and increased metastatic lymph node involvement, increased ascites, and higher-grade ovarian tumors (10, 13–15, 24, 25). Additionally, higher tissue and ascites levels of uPA were associated with malignant transformation of ovarian epithelium (10). uPA is important in the metastatic process because it disrupts the basement membrane and facilitates spread (26). Once secreted, uPA binds to its specific receptors, and the ligand-receptor complex induces the conversion of plasminogen to plasmin (27). Plasmin then degrades the surrounding stroma, which contains fibrin, fibronectin, proteoglycans, laminin, and collagen type IV (28). However, the mechanisms regulating uPA production by ovarian cancer cells are not well characterized.

LPA is markedly elevated in ascites and plasma of patients with ovarian cancer when compared with normal healthy women (2, 29). It has recently been suggested that LPA may provide an early diagnostic marker for ovarian cancer (29). LPA has multiple biological roles but has only recently been shown to stimulate invasion in certain cancer cell lines. Our data suggest that LPA is involved in invasion and metastases, because LPA consistently induces the secretion of uPA by ovarian cancer cells.

**Fig. 6** Induction of uPA promoter activity by LPA. Cells were mixed with a luciferase construct (pRSV-luc or puPA) and electroporated at 250 V. After allowing 24 h for recovery, cells were starved in SFM. LPA was then added for 12 and 24 h. Cells were washed and lysed with the Promega reporter lysis buffer, and the lysate was recovered after centrifugation. Total lysate protein (15 μg) was analyzed for luciferase activity. The data are presented as relative luciferase activity, normalizing the RLU (relative light units) to that of puPA in the absence of LPA. A 100% increase indicates a doubling from control levels. LPA did not alter the activity of a pRSV-luc construct, indicating specificity for puPA-luc.
cancer cell lines including OVCAR-3, SKOV-3, OVCA 429, OVCA 432, and OVCA 433. As shown by Western blotting in Fig. 1, SKOV-3, but not OVCAR-3, secretes significant levels of uPA protein in the absence of exogenous LPA. Strikingly, SKOV-3, but not OVCAR-3 or normal ovarian epithelium, constitutively produces low levels of LPA (data not shown). NOE cells have been reported to secrete uPA, but at levels 17–34-fold lower than those of ovarian cancer cell lines (30). LPA does not, however, increase the secretion of uPA by NOE cells. Malignant transformation may render ovarian cancer cells susceptible to LPA-induced increases in uPA secretion, contributing to invasion and metastases. mRNA expression levels of the edg-1 and edg-2 LPA receptors did not correlate with the ability of LPA to induce uPA expression. In contrast, higher levels of edg-4 were present in ovarian cancer cells than in normal ovarian epithelium, correlating with the ability of LPA to induce uPA secretion. Thus, signaling through edg-4 may account for the ability of LPA to induce uPA secretion by ovarian cancer cells. Alternatively, LPA receptors may link to different signaling and physiological responses in ovarian cancer cells than in normal ovarian epithelium.

LPA also demonstrates structural specificity because only 18:1 LPA induced uPA secretion, whereas forms of LPA with saturated fatty acyl chains did not. This implies that lipid structure is tightly linked to function and is likely regulated through a highly specific cellular receptor. LPA induced a significant increase in uPA mRNA steady-state levels and a modest increase in activity of the uPA promoter, suggesting that sustained elevations in uPA protein levels in cell supernatants are a result of increased uPA promoter activation. However, the modest increase in promoter activity is much less than the increase in uPA mRNA levels induced by LPA, suggesting that LPA may also alter mRNA stability.

In conclusion, LPA likely plays a role in the invasiveness and metastasis of ovarian cancer cells, and further studies are needed to understand the molecular mechanisms involved.

Fig. 7  RNA levels of edg-2 and edg-4. RNA from normal ovarian epithelium and ovarian cancer cell lines was probed for the presence of edg-2 and edg-4. A, representative Northern blots. B and C, densitometry from several Northern blots normalized for 18S RNA. To compare across Northern blots, OVCAR-3 was run on each blot, and samples were normalized to the intensity of the signal for OVCAR-3.
outcome of ovarian cancer through the production of uPA. LPA may thus be involved in the metastatic process because it induces proliferation and stimulates secretion of uPA in ovarian cancer cells. Mechanisms targeting LPA production or action might thus be useful in treating this aggressive cancer. Indeed, we and others (2) have identified potential LPA receptor antagonists.

Note Added in Proof

A novel LPA receptor, edg-7, with selectivity for LPA with unsaturated fatty acyl chains has been recently identified (31). The requirement of LPA-induced uPA secretion for LPA with an unsaturated fatty acyl chain (Fig. 4), combined with a markedly increased edg-7 mRNA expression in ovarian cancer cells as compared with normal ovarian epithelium, suggests that edg-7 may contribute to LPA-induced uPA secretion.

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