Role of Decreased Levels of Lipid Phosphate Phosphatase-1 in Accumulation of Lysophosphatidic Acid in Ovarian Cancer


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Abstract

The levels of lysophosphatidic acid (LPA) are consistently elevated in the ascites of ovarian cancer patients, suggesting that ovarian cancer cells are exposed to an LPA replete environment. LPA stimulates cell proliferation, cell survival, resistance to cisplatin, production and activation of proteases, invasiveness and production of the neovascularizing factors, vascular endothelial growth factor, and interleukin 8. Although ovarian cancer cells can produce LPA, this may not be the major reason for altered LPA levels in ascites. We have demonstrated that the major mechanism of degradation of LPA by ovarian cancer cells is through a lipid phosphate phosphatase (LPP)-like activity. We demonstrate herein that LPP-1 mRNA is decreased in the majority of ovarian cancers. This is recapitulated in ovarian cancer cell lines, where LPP-1 RNA levels are lower than those in normal ovarian epithelium and immortalized ovarian epithelial cells. Introduction of LPP-1 into ovarian cancer cell lines results in increased LPA hydrolysis, which is associated with a marked inhibition of cell proliferation and colony-forming activity and a marked increase in apoptosis. Thus, the LPA-rich environment of the ovarian cancer cell in vivo and the subsequent effects of cellular pathophysiology may be a consequence of both increased LPA production and decreased LPA metabolism by ovarian cancer cells.

Introduction

LPA (1-acyl-2-lyso-sn-glycero-3-phosphate) is present in elevated levels in the ascites of ovarian cancer patients. LPA, at the concentrations present in ovarian cancer ascites (up to 80 μM), is sufficient to induce growth promotion, anchorage-independent growth, increase production of growth and neovascularization factors, including interleukin 8, vascular endothelial growth factor, and LPA itself, and prevent apoptosis and anoikis (1–6). LPA also increases the production and action of proteases and increases invasiveness of ovarian cancer cells, suggesting that it may contribute to spread of ovarian cancer (6, 7). Taken together, these data suggest that an LPA-rich environment in the patient may contribute to pathophysiology of the disease and may have an adverse effect on outcome.

The source of LPA in ascites fluid from ovarian cancer patients remains to be fully elucidated. Activated platelets, adipocytes, leukocytes, fibroblasts, and endothelial cells can produce LPA (2, 4, 6, 8–10). We and others (11, 12) have demonstrated that ovarian cancer cells, but not breast or normal ovarian epithelial cells, can produce LPA in culture. Phorbol esters and LPA itself have been demonstrated to increase LPA production by ovarian cancer cells but not by breast cancer cells (11, 12). The accumulation of LPA in the supernatants of cells in culture could be attributable to increased production or decreased metabolism or a combination of both.

LPA is removed by being acylated to produce phosphatic acid, deacylated by lysophospholipases to produce glycerol phosphate, or dephosphorylated by LPPs also known as phospho-

3 The abbreviations used are: LSA, lysophosphatidic acid; LPP, lipid phosphate phosphatase; LPAAT, lysophosphatidic acid acyltransferase; LPL1, lysophospholipase-1; PLA, phospholipase A; CMV, cytomegalovirus; GFP, green fluorescent protein; PI, propidium iodide; NOE, normal epithelial cell line.
phatidic acid phosphohydrolases to produce monoacylglycerol, which is inactive on LPA receptors (2–4, 6, 10). The most likely candidates for hydrolysis of extracellular LPA, including LPP-1, LPP-2, and LPP-3, are six transmembrane domain containing integral membrane proteins with their catalytic site and functions on the extracellular surface of the cell (13–15). We have demonstrated that the major pathway for degradation of LPA by ovarian cancer cells is through a lysophosphatidic acid phosphohydrolase-like activity that is regulated by GnRH (16). Furthermore, LPPs can attenuate LPA-induced cell responses (14, 15, 17, 18). LPAAT-α and LPAAT-β catalyze the conversion of LPA to PA, however, these are intracellular proteins (19). The human lysophospholipase homologue, HU-K5, human LPL1, and lecithin cholesterol acyltransferase-like lysophospholipase can remove the acyl chain from LPA (20, 21), however, their contribution to the metabolism of extracellular LPA is unknown.

We and others (12, 22) have implicated phospholipase D and cellular and secreted PLA2 in the production of LPA by ovarian cancer cells. The recently identified phosphatidylserine- and phosphatidylcholine-specific PLA1s (23, 24) and lysophospholipase D (25) may also contribute to the production of LPA by ovarian cancer cells. In particular, PLA1 may lead to the production of polyunsaturated sn-2 LPAs that are preferential ligands for LPA3 receptor (also known as Edg 7, endothelial differentiation gene 7 receptor; Ref. 26).

In this article, we demonstrate that LPP-1 mRNA levels are decreased in ovarian cancer cells as compared with normal epithelium and cancer cells from other lineages. This translates into a decreased level of LPP-1 mRNA in ovarian cancer cell lines as compared with normal ovarian epithelial cells. Introduction of LPP-1 into ovarian cancer cell lines results in increased ectoLPA hydrolysis, which is associated with decreased colony-forming cell activity and increased apoptosis. Thus, decreased LPP-1 levels in ovarian cancer cells may contribute to elevated LPA levels in ascites and play a role in the pathophysiology of ovarian cancer.

Materials and Methods

Analysis of Gene Expression Profiles in Ovarian Tissue Samples from Published Microarray Hybridization Data.

RNA extraction and hybridization on oligonucleotide microarrays were performed and published on 27 primary human ovarian cancer and other normal and tumors samples by previous studies (27–29). Affymetrix U95 array hybridization data are available online (27–29).4 The control samples consisted of 36 normal adult epithelial tissues (27). We combined these results with our own analysis of expression arrays from an additional 26 ovarian cancers (17 from serous and 9 from endometrioid histological subtypes). All samples were assessed and demonstrated to contain at least 80% tumor with limited stromal contamination. Control samples consisted of four separate pools of normal ovarian epithelial cells scraped directly from the surface of the ovary from 13 different patients into preservative, providing an indication of mRNA levels in normal ovarian epithelial cells in vivo.

To allow comparison across multiple arrays and the two databases, the mRNA expression data of each gene was normalized to the expression detected in normal epithelial cells. The overall expression levels in the two datasets were comparable, allowing combination the data.

Tumor Samples and RNA Preparation. Twenty-six fresh frozen primary ovarian cancers were obtained from Duke University (9 endometrioid), the Mayo Clinic (9 stage I serous), and M. D. Anderson Cancer Center (8 stage III serous). Four pools of normal ovarian surface scrapings from 13 patients were obtained from Northwestern University. The normal scrapings were collected using a cytobrush, and then the cells were resuspended and frozen in RNeasy Lysis Buffer (Qiagen, Inc., Valencia, CA). RNA was extracted using the RNeasy Kit (Qiagen, Inc.).

Gene Expression and Data Analysis. The Affymetrix GeneChip Human Genome U95 set was used to obtain gene expression data. This series tests the expression of >60,000 human genes and expressed sequence tags. The cRNA preparation, hybridization, and scanning of the microarrays were performed according to manufacturer’s protocols at the Mayo Clinic’s microarray facility. Microarray data were analyzed using the dChip software (30).

Tissue Samples, Cell Cultures, and G418 Resistance. OVCAR-3, SKOV3, SKOV3 IP1, HEY, and A 2780 (human epithelial ovarian carcinoma cell lines) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA). The IOSE 80 and IOSE 29 (SV40 T-antigen-semi-immortalized ovarian surface epithelial cell lines) were kindly provided by Nelly Auersperg (Department of Obstetrics and Gynecology, University of British Columbia, Vancouver, British Columbia, Canada; Ref. 31). The IOSE cells were grown in medium 199/MCDB 105 supplemented with 10% fetal bovine serum. All of the cell lines were tested to evaluate G418 (Invitrogen Corp., Carlsbad, CA) resistance. G418 was applied in concentration from 200 to 1000 μg/ml. Medium contained the appropriate amount of G418 and was changed every third day. The lowest concentration of G418 that killed all of the cells was applied later in the experiments. Twenty stage III, primary human epithelial ovarian tumor samples and 6 human normal ovarian surface epithelial samples were provided by the Basic Biology of Ovarian Cancer Program Project Grant (CA 64602) and the SPORE in Ovarian Cancer (CA 83639) tumor banks.

First-Strand cDNA Synthesis and Semiquantitative Reverse Transcriptase-PCR of LPP-1. The mRNA expression of LPP-1 was determined using semiquantitative reverse transcriptase-PCR. Oligonucleotide primers were used for: hLPP-1, 5′-CGCGGATCCATGTTTGACAAGCGCGG and 5′-TGCC-TATGTAGTTATCTCG (304 bp); and control glyceraldehyde-3-phosphate dehydrogenase primers, 5′-CCCCATGGCAATTC-CATGGCAGCG and 5′-GTCAATGGATGACCGTGGCCAGGG (344 bp). The RNA samples were treated with DNase before first-strand cDNA synthesis or reverse transcriptase-PCR reaction following the manufacturer’s instructions (DNA free, DNase Treat-
ment and Removal Kit; Ambion, Austin, TX). In some cases, reverse transcriptase-PCR was performed as a single step with the reverse transcriptase-PCR reaction mixture consists 1 µg of total RNA, 0.4 µM sense and antisense primers, 0.2 mM deoxynucleotide triphosphates, 0.5 units of reverse transcriptase and TaqDNA polymerase enzymes (in enzyme mix), 5 mM DTT solution, 5 units of RNase inhibitor, 1.5 mM MgCl2 in final volume of 50 µl (Titan One Tube RT-PCR System; Roche Molecular Biochemicals, Mannheim, Germany). The reverse transcriptase reaction was performed at 60°C for 30 min followed by 35 cycles of PCR in a Thermal Cycler (Perkin-Elmer 480, Atlanta, GA). Each cycle of PCR included 30 s of denaturation at 94°C, 2 min of annealing at 56°C, and 1 min of extension at 72°C.

The reverse transcriptase PCR was performed as a two step approach in the case of the primary tumor samples and the ovarian surface epithelial samples. As the first step, first-strand cDNA synthesis was done with SuperScript First-Strand Synthesis System (Invitrogen Corp.) according to the manufacturer’s instruction using 5 µg of total RNA from each primary ovarian tumor. PCR was then performed as described above using one-fifth of the reverse transcriptase product. Reverse transcriptase-PCR with glyceraldehyde-3-phosphate dehydrogenase (29 cycles) was used to demonstrate equal amounts of RNA in each sample and used to normalize results. PCR products were visualized by electrophoresis in a 2% agarose gel with ethidium bromide.

**Analysis of Total LPP and LPP-1 Activity in Ovarian Cancer Cell Lines.** Cells were grown in 35-mm diameter dishes to ~80% confluence, washed once in PBS, and lysed by scraping in buffer containing 20 mM Tris (pH 7.4), 2 mM EDTA, and protease inhibitors (benzamidine and phenylmethylsulfonyl fluoride). The cells were disrupted by brief probe sonication. LPP activity in the lysates was determined using [32P]LPA as described previously (32, 33). For the data shown in Fig. 3 assays contained 1–5 µg of protein. Immunoprecipitations were performed as described previously (32–34). LPP activity in washed immune complexes was determined as above using [32P]LPA.

**The LPP-1 cDNA Containing Vectors Driven by CMV and hTERT Promoters.** The complete cDNA of LPP-1 (33) was amplified using primers with the sequences 5’-CGCGATCCATGGTTGACAAGACCGG-3’ (forward) and 5’-GCTCTAGAAGGCCTGTTGATTGCTG-3’ (reverse) using PCR, cut with BamHI and XhoI, and inserted into BamHI and XhoI restriction sites of the pcDNA3.1-HisA epitope-tagged eukaryotic expression vector (Invitrogen Corp.). The CMV promoter was replaced in the pcDNA3.1-HisA vector with the hTERT promoter (35) using BglII and KpnI. The activity of all PCR products was confirmed by sequencing and of the vectors by restriction endonuclease analyses.

**Transient and Stable Expression of LPP-1.** SKOV3 or OVCAR-3 cells were plated in 60-mm plastic dishes at a density of 0.5 × 10^6 cells/dish. After 48 h, when they were ~60–80% confluent, cells were washed twice in PBS (pH 7.4; Sigma, St. Louis, MO). Cell transfection was carried out using FuGene 6 carrier (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s instructions with minor modifications. Briefly, 10 µl of FuGene 6 were added into 100 µl of serum-free medium and incubated 10 min at room temperature. A total of 1.5 µg of expression vector was mixed and incubated for an additional 30 min at room temperature. Cotransfection with 0.5 µg of GFP-expressing vector was performed as a control for transfection efficiency. This mixture was applied into the 60-mm plate containing 3 ml of medium.

Quantitation of transfection efficiency was done by flow cytometry using a FACScam with Cellquest 3.3 software package (Becton Dickinson, San Jose, CA). GFP was identified using a single parameter histogram display of log green fluorescence. Cells transfected with an empty vector were used as a negative control to develop a gate to determine the percentage of GFP-positive transfected cells. The transfection efficiency varied from 4–21%. To create stable lines, 48 h after transient transfection, medium containing the appropriate amount of G418 (selection medium) was changed every third day for 6–8 weeks.

**GFP-targeted Cell Cycle Progression Assay with Transient Transfection.** Cells were transiently transfected using FuGene 6 when they were 60–80% confluent. The parental lines were transfected with either pcDNA3.1-LacZ-HisA, pc-hTERT-DNA3.1-HisA, pcDNA3.1-LPP-1-HisA, or pcTERT-DNA3.1-LPP-1-HisA constructs separately and cotransfected with pgFP. Forty-eight h later, the medium was removed, and the cells were washed twice in PBS and trypsinized. Both floating and adherent cells were harvested and subjected to flow cytometry. Cells were fixed with 0.25% parafomaldehyde (Poly-Scientific, Bay Shore, NY) in PBS solution followed by DNA staining with PI staining buffer (10 µg/ml PI, 0.1% Tween 20, and 100 µg/ml RNase A in PBS). To assess cell cycle progression, a two-color flow cytometric analysis was performed on a FACScam flow cytometer (Becton Dickinson) using Cellquest 3.3 software for acquisition and analysis. GFP-positive cells were gated and analyzed for cellular DNA content.

**Colony Formation Assay.** The colony formation assay was performed as previously described with slight modifications with both transiently and stably transfected cell lines (36, 37). Two days after transient transfection, cells were trypsinized, washed in PBS twice, and counted and 3 × 10^6 cells were reseeded. For stable-transfected cells, 2 × 10^6 stably transfected cells were plated into 30-mm 6-well plates. Two weeks later, colonies were stained with 0.1% Coomassie blue (Bio-Rad, Hercules, CA) in 30% methanol and 10% acetic acid. The number of the colonies (>300 cells) was counted by two independent investigators, and the variation was <10%.

**Analysis of the Bystander Effect of hLPP-1 Using Transient and Stable Transfection.** Two days after transient transfection, cells were trypsinized and counted. Cells transfected with hLPP-1 protein were mixed with equal numbers (3 × 10^6) of neo-transfected control cells and cultured in 30-mm dishes the presence of G418. Stably transfected SKOV3 cells (2 × 10^6) were mixed with an equal number of nontransfected SKOV3 cells and plated into 30-mm dishes in the absence of G418. Two weeks later, colonies were stained and counted as described above.

**Migration Assays of LPP-1 Overexpressing SKOV3 Cells.** Migration assays were performed using a transwell chamber membrane (8-µm pore size; Biocoat, Becton Dickinson Labware, Franklin Lakes, NJ). LPA (10 µM) was added to the lower chambers. Cells were initially starved of serum. Cells
(5 × 10⁶) were added to the upper chamber and allowed to migrate for 24 h at 37°C. Cells that had not migrated were removed from the upper chamber with a cotton swab. The remaining cells were fixed and stained with crystal violet.

**Cell Preparation for Analysis of LPP-1 Activity.** For studies using SKOV3 cell membranes or detergent-extracted membrane proteins, the monolayer of infected cells was washed gently with PBS and lysed by the addition of 4 ml of ice-cold lysis buffer [20 mM Tris (pH 7.5), 5 mM EGTA, 0.1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride] and scraping (34). The cell suspension was transferred to a 15-ml conical tube, and the cells were disrupted by sonication (Vertis Systems Sonifier), 10-s pulses on ice. The disrupted cells were centrifuged at 20,000 × g at 4°C for 20 min. The cytosolic fraction was removed, and the membrane fraction was resuspended in ice-cold lysis buffer. Detergent extracts were prepared from the membranes by the addition of Triton X-100 to final concentration of 1% followed by incubation at 4°C with constant rocking for 1 h. The solubilized material was centrifuged at 26,000 × g at 4°C for 30 min, and the supernatant was removed.

**Preparation of LPA as Substrate.** [³²P]LPA was prepared by phosphorylation of oleoyl monoacylglycerol (mole) (Avanti Polar Lipids, Alabaster, AL) using *Escherichia coli* diacylglycerol kinase (Calbiochem, San Diego, CA) and [γ³²P]ATP (ICN Pharmaceuticals, Costa Mesa, CA). The reaction was terminated by extraction with acidified CHCl₃ and methanol, and the dried organic phases obtained were resuspended in 0.4 ml of 20:9:1 CHCl₃/methanol/H₂O (solvent A). A total of 0.5-ml fractions of the eluant was removed from the upper chamber with a cotton swab. The °C. Cells that had not migrated were migrated for 24 h at 37

**LPA Determination in Cell Supernatants.** LPA 18:1 (1 μM) was added to SKOV3 cells, and LPP-1-transfected SKOV3 and cell supernatants were collected at the indicated times. An unnatural LPA, 17:0, was added to the supernatants after collection to monitor efficiency of isolation and detection of LPA. LPA was extracted from 1 ml of cell supernatant using Waters Oasis HLB (1 ml) and 30 mg of solid phase extraction cartridges (Millford, CT) preconditioned with 1 ml of methanol and 1 ml of water. Cartridges were washed twice with 1 ml of water and dried under vacuum for 5 min. LPA was eluted from the cartridges using 1 ml of 95:5.5 methanol/chloroform:1 mM NH₄OH. Twenty-five μl of eluant were injected into the liquid chromatograph/mass spectrometer/mass spectrometer using a Waters X Terra 3.5-μm C18 2 × 100-mm micropore column in an Agilent 1100 binary HPLC. The column was run in the isocratic mode using a mobile phase of 90:5.5 methanol/chloroform:1 mM ammonium hydroxide. LPA isoforms were detected using a MicroMass QuattroUltima triple quadrupole mass spectrometer (Beverly, MA) using electrospray-negative ionization with the instrument operating in a multiple reaction-monitoring mode. Specific transitions for each LPA are as follows: LPA 18:1 is 435.24 > 152.8 and LPA 17:0 423.1 C2 × 100-mm micropore column in an Agilent 1100 binary HPLC. The column was run in the isocratic mode using a mobile phase of 90:5.5 methanol/chloroform:1 mM ammonium hydroxide. LPA isoforms were detected using a MicroMass QuattroUltima triple quadrupole mass spectrometer (Beverly, MA) using electrospray-negative ionization with the instrument operating in a multiple reaction-monitoring mode. Specific transitions for each LPA are as follows: LPA 18:1 is 435.24 > 152.8 and LPA 17:0 423.1.

**Statistical Analyses.** Unpaired continuous outcomes were compared using Wilcoxon rank-sum tests and exact permutation tests. Paired continuous outcomes were compared using Wilcoxon sign-rank tests. Proportions were compared using χ² analyses. Significance was set at P < 0.05.

**Results**

**LPP-1 and Human Lysophospholipase Homologue mRNA Expression Is Decreased in Ovarian Carcinomas.** Using Affymetrix array data, we were able to analyze levels of expression of a number of enzymes involved in hydrolysis of LPA in multiple tumor types (Table 1). LPP-1 mRNA levels in ovarian cancer samples were decreased ~5-fold as compared with normal epithelium. Furthermore, LPP-1 RNA levels were lower in ovarian cancers as compared with other tumor lineages. For example, the levels of LPP-1 RNA in prostate and kidney samples were at least 10-fold higher than the levels in ovarian cancer cells. In contrast, LPP-2 and LPP-3 RNA levels were similar between ovarian cancer samples and normal epithelium. The overall total of LPP-1, LPP-2, and LPP-3 RNA levels was lower in ovarian cancer than in any other tumor type analyzed, compatible with decreased LPP and particularly LPP-1 levels contributing to the elevated LPA levels in ovarian cancer patients.

The human lysophospholipase homologue (HU-K5) mRNA expression was decreased in ovarian cancers ~2.5-fold when compared with normal epithelium, however, it was not decreased as compared with several other tumor lineages. LPAAT-β mRNA expression was decreased ~30% in ovarian cancer cells, whereas no significant changes were detectable in LPAAT-α or LPL1.
We evaluated changes in LPP and HU-K5 mRNA levels in individual patients by combining data from the published database with our own data using the same U95 Affymetrix arrays (Fig. 1). This combined analysis of 53 ovarian cancer samples compared with four separate pools of normal epithelial cells scraped directly from the ovaries of 13 patients demonstrated that LPP-1 mRNA levels were markedly decreased in all samples, and in many cases, LPP-1 levels were decreased 10-fold. LPP-1 expression was decreased at least 2-fold in 89% of the tumors as compared with normal epithelium. When LPP-2 and LPP-3 mRNA levels were considered, 2 of 3 of patients showed some decrease in levels (30%). HU-K5 mRNA levels were decreased at least 2-fold in 77% of ovarian cancers.

Table 1: Analysis of the DNA array data

<table>
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<tr>
<th>Enzyme</th>
<th>Genbank accession no.</th>
<th>Normal ovarian epithelium (n=27)</th>
<th>Breast (n=25)</th>
<th>Lung (n=27)</th>
<th>Colon (n=23)</th>
<th>Prostate (n=26)</th>
<th>Kidney (n=11)</th>
<th>Bladder (n=6)</th>
<th>Ovary/normal (n=27)</th>
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<td>663</td>
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<td>815</td>
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</table>

* Values at the medians (range) of expressed DNA array results in each tumor type in Ref. 27–29.

Fig 1 Human LPP-1, LPP-2, LPP-3, and HU-K5 gene expression in ovarian cancer. The normalized values of hybridization signal of LPP-1, LPP-2, LPP-3, and HU-K5 transcripts from 53 ovarian carcinoma samples (see “Materials and Methods”). The average of the expression of the transcripts in the normal epithelial tissues normalized to 1 is shown for comparison as a horizontal dashed line.

We evaluated changes in LPP and HU-K5 mRNA levels in individual patients by combining data from the published database with our own data using the same U95 Affymetrix arrays (Fig. 1). This combined analysis of 53 ovarian cancer samples compared with four separate pools of normal epithelial cells scraped directly from the ovaries of 13 patients demonstrated that LPP-1 mRNA levels were markedly decreased in all samples, and in many cases, LPP-1 levels were decreased 10-fold. LPP-1 expression was decreased at least 2-fold in 89% of the tumors as compared with normal epithelium. When LPP-2 and LPP-3 mRNA levels were considered, 2 of 3 of patients showed some decrease in levels (>30%). HU-K5 mRNA levels were decreased at least 2-fold in 77% of ovarian cancers.

Semiquantitative Reverse Transcriptase-PCR Analysis of Human Ovarian Carcinoma Cell Lines and Primary Ovarian Tumor Samples. On the basis of the microarray data, we decided to further evaluate LPP-1 mRNA expression levels in ovarian cancer cells as compared with normal epithelium. We characterized the expression of LPP-1 in 20 primary ovarian tumor samples, all with >80% tumor cell content, 6 normal surface epithelium samples, 1 normal surface epithelial short-term culture (NOE), and 5 ovarian carcinoma cell lines, including OVCAR-3, SKOV3, SKOV3 IP1, HEY, and A 2780. Expression levels were normalized to that of a SKOV3 LPP-1 transfectant, which was run on all gels and designated as 1. As indicated in Fig. 2, LPP-1 levels in normal ovarian epithelium from multiple individuals were tightly clustered. All ovarian tumors samples and ovarian cancer cell lines were lower than the average of the NOE. Using the lowest normal ovarian
epithelial sample as a cutoff, 16 of 20 ovarian cancer samples and 4 of 5 ovarian cancer cell lines demonstrated decreased LPP-1 RNA levels, with some of the levels markedly decreased. These data suggested the possibility that decreased LPP-1 levels contribute to the increased LPA levels in ascites of ovarian cancer patients and to the increased levels of LPA in ovarian cancer cell supernatants.

LPP-1 Activity Is Decreased in Ovarian Cancer Cell Lines. As indicated in Fig. 3, HEY, OVCAR-3, and SKOV3 demonstrated lower total LPP activity, representing the activity of all of the LPP isoforms than the nonneoplastic ovarian surface epithelial cell line (IOSE). When LPP-1 was immunoprecipitated with LPP-1-specific antibodies (the antibodies effectively and specifically immunoprecipitate LPP-1 but do not function as efficiently in Western blotting), ~10% of the LPP-1 activity present in IOSE was accounted for by LPP-1. Strikingly, HEY, OVCAR-3, and SKOV3 demonstrated markedly lower levels of LPP-1 activity than did IOSE. Indeed, the difference in LPP-1 activity between IOSE and the tumor cells was much greater than that for total LPP activity, compatible with the contention that LPP-1 activity is selectively decreased in ovarian cancer cells. The LPP-1 activity correlated with that of the mRNA levels with SKOV3 having the lowest levels in both assays.

LPP-1 Decreases Colony-forming Ability of Ovarian Carcinoma Cells. LPA is a potent regulator of multiple activities in ovarian cancer cells. To evaluate the potential role of decreased LPP-1 levels in the pathophysiology of ovarian cancer, we assessed the effect of increasing LPP-1 levels on the ability of ovarian cancer cells to proliferate as indicated by colony-forming cell activity, a sensitive method to determine effects of mediators on cellular function (36, 37). We attempted to establish stable cell lines expressing LPP-1 in IOSE 29, IOSE 80, OVCAR-3, HEY, and A2780. Despite multiple attempts and a ready ability to develop neomycin-resistant lines, we were unable to develop stable LPP-1-expressing cell lines based on these lines. This suggests that LPP-1 decreases the growth or survival of ovarian epithelial and ovarian cancer cell lines. We have demonstrated that the SKOV3 cell line releases high levels of LPA into cell supernatants (12). Consistent with this observation, we were able to develop stable LPP-1-expressing cell lines in SKOV3 and in SKOV3 IP1, a variant of SKOV3 selected for the ability to grow i.p. These cells demonstrated a ~4.9-fold increase in LPP-1 RNA levels as assessed by reverse transcriptase-PCR analysis (data not presented). However, even on the SKOV3 background, LPP-1 mRNA levels and activity decreased during cell passage. Thus, for analysis of LPP-1 activity, early passage-transfected cells were used.

We examined the ability of stably transfected and parental SKOV3 cell lines to hydrolyze LPA presented in the medium. This assay reflects the activity of all of the LPP isoforms on the cell surface. The LPA hydrolysis activity of LPP-1 stably transfected SKOV3 was 7.7 times higher (32 ± 2.3 versus 246 ± 4.3 pmol LPA hydrolyzed/30 min/mg protein) than in the nontransfected parental line. As indicated by immunoprecipitation of cell lysates with LPP-1-specific antibodies (34), SKOV3 cell lysates demonstrated decreased LPP-1 enzyme activity (10 ± 1.1 versus 23.1 ± 1.9 pmol/30 min/mg protein immunoprecipitated) as compared with NOE. Stably transfected SKOV3 (late passage) demonstrated 27.0 ± 0.9 pmol/30 min/mg protein immunoprecipitated LPP-1. This activity data were compatible with the 4.9-fold increase in LPP-1 mRNA expression in the stable lines. Thus, ectopic expression of LPP-1 in ovarian cancer cells results in a marked increase in the ability of cells to hydrolyze extracellular LPA.

The increased ability of LPP-1-transfected SKOV3 to hydrolyze radiolabeled LPA suggested that LPP-1 transfection would decrease the concentration of LPA in the culture media. When 1 µM 18:1 LPA was added to media alone, there was no detectable change in LPA concentration over time (time 0, 780 nM; 10 min, 740 nM; 1 h, 845 nM; and 8 h, 819 nM). SKOV3 induced hydrolysis of extracellular LPA cells [time 0; 727 nM; 10 min, 580 nM; 1 h, 555 nM; and 8 h, <100 nM] levels above 100 nM were readily detectable in calibration curves]. The expression of LPP-1 in SKOV3 cells resulted in a marked increase in the hydrolysis of LPA (time 0, 701 nM; 10 min, 633 nM; 1 h, 364 nM; and 8 h <100 nM), compatible with increased LPP-1 activity. Thus, the extracellular concentration of LPA is markedly decreased by transfection of LPP-1.

As indicated by the ability to form colonies, stable expression of LPP-1 decreased the growth of the SKOV3 ovarian cancer cells ~1.9-fold (49 ± 8.4 versus 26 ± 4.3, P = 0.0002; Fig. 4B). Because of the difficulty in obtaining high-level LPP-1 expression in stable cell lines, we assessed the effect of transient transfection of LPP-1 on colony-forming cell activity of SKOV3 and OVCAR-3 ovarian cancer cell lines. As indicated in Fig. 4A, transient expression of LPP-1
caused a marked growth inhibition in both OVCAR-3 and SKOV3. The inhibitory effect ranged from 2.3- to 5.8-fold when LPP-1 was driven by the CMV and hTERT promoters in the OVCAR-3 and SKOV3 cell lines \( (P = 0.0022, P = 0.0022, P = 0.0416, P = 0.0022) \), respectively. Thus, ectopic expression of LPP-1 strongly inhibits the ability of ovarian cancer cell lines to proliferate as indicated by colony-forming cell activity \textit{in vitro}.

Fig. 3 Determination of LPP activity in ovarian cancer cell lysates and LPP-1 immune precipitates. Data normalized to 1 µg of protein in lysates. The data shown are means ± SD of triplicate determinations from a single representative experiment.

Fig. 4 Growth inhibitory effect of LPP-1 overexpression assessed by colony-forming cell activity. A, 2 days after transient transfection, cells were trypsinized, washed in PBS twice, and counted. A total of \( 3 \times 10^5 \) cells was seeded into 30-mm 6-well plates. The selection medium supplemented with G418 was changed every third day. Two weeks later, colonies were stained by 0.1% Coomassie blue in 30% methanol and 10% acetic acid. Average number of colonies/dish is presented. B, growth inhibition effect of LPP-1 in stably transfected SKOV3 cell line. A total of \( 2 \times 10^3 \) of cells from the stable SKOV3 line was seeded into 30-mm 6-well plates. Complete culture medium supplemented with G418 was changed every third day. Two weeks later, colonies were stained by 0.1% Coomassie blue in 30% methanol and 10% acetic acid. The data are the mean of six separate experiments ± SD.
LPP-1 Increases the Apoptosis Rate in Ovarian Carcinoma Cell Lines. The decreased ability to form colonies could reflect decreased cell cycle progression or increased rates of apoptosis. There were no obvious differences in cell cycle progression as indicated by number of cells in G1, S, or G2-M in control or LPP-1-expressing SKOV3 or OVCAR-3 cells (Fig. 5). However, as indicated by an accumulation of a hypodiploid peak on cell cycle analysis, LPP-1 expression increased the rate of apoptosis in both SKOV3 and OVCAR-3 (Fig. 5). Thus, the decreased ability of LPP-1-expressing ovarian cancer cells to form colonies is associated with an increased rate of cellular apoptosis.

LPP-1 Decreases the Growth of Nontransfected By-stander Cells. The ability of hLPP-1 expression to increase the hydrolysis of extracellular LPA suggests that LPP-1 may function to decrease extracellular LPA concentrations. If this were the case, expression of LPP-1 in one population of ovarian cancer cells could decrease the colony-forming cell activity of bystander-nontransfected cells. To assess this possibility, we performed a series of cell mixing assays with LPP-1-transfected cells and control parental cells.

Our preliminary data indicated that the number of colonies formed demonstrated essentially a linear relationship related to the number of cells plated, i.e., there were no effects related to cell crowding at higher concentrations (data not presented). To assess the effects of LPP-1-transfected cells on the growth of nontransfected cells, we combined equal numbers of stably or transiently LPP-1-transfected cells and parental cells. The predicted number of colonies in the combination experiment would be the sum of the number of colonies produced by parental cells and the number of colonies produced by the transfected cells cultured separately. As indicated in Fig. 6, A and B, with either transient transfection or stable cell lines, there was a marked decrease in the number of colonies observed compared with the expected number of colonies (\(P = 0.01, \ P = 0.01\)). Therefore, LPP-1-transfected cells were able to decrease the proliferation of parental cells compatible with the effect of LPP-1 related to the effects of an extracellular mediator, likely LPA.

LPP-1 Overexpression Decreases the Ability of the Tu-mor Cells to Migrate. LPA has been demonstrated to markedly increase cellular migration. We thus assessed whether overexpression of LPP-1 in ovarian cancer cells would also decrease LPA-induced cellular migration. As indicated by the ability of cells to migrate toward LPA in a transwell assay (Fig. 7), LPA (10 \(\mu M\)) stimulated migration of empty vector pcDNA3.1-transfected SKOV3 cells. In contrast, LPA failed to induce migration in LPP-1-overexpressing SKOV3 cells. Thus, the increased hydrolysis of LPA of LPP-1 is translated into alterations in cellular motility as well as in proliferation, colony formation, and survival, compatible with the decreases in LPP-1 in ovarian cancer cells contribution to these important components of the transformation cascade.

Discussion

LPA levels are consistently elevated in the ascites of ovarian cancer patients (41, 42) and likely contributing to the patho-

![Fig. 5 LPP-1 increases apoptosis in ovarian cancer cell lines. Forty-eight h after transient transfection, cells were harvested and fixed by 0.25% paraformaldehyde in PBS solution followed by PI (10 \(\mu g/ml\)) for DNA staining. Two-color cytometric analysis was performed, and the percentages of hypodiploid cells as an indication of apoptosis determined using CellQuest software. Primary flow cytometric graphs from SKOV3 cell line transfected with hTERT-driven constructs are presented. M1 represents hypodiploid cells. Percentage of cells in G1, S, and G2-M is presented with (right) and without (left) correction for hypodiploid cells. The data shown in the bar graph are the mean of three separate experiments \(\pm SD\).](image-url)
physiology of ovarian cancer (6). The mechanisms leading to the elevated levels of LPA in ovarian cancer ascites are not currently clear. Although ovarian cancer cells can release LPA into cell supernatants (11, 12), it is not known whether ovarian cancer cells or other cellular components in ascites are the primary source of LPA. Furthermore, it is not known whether the increased levels are because of increased rates of production or decreased rates of degradation. We have demonstrated that the major mechanism of LPA degradation by ovarian cancer cells is through an LPP-like activity (16).

The LPP family includes at least three members, LPP-1, LPP-2, and LPP-3 isoforms (13, 38, 39). LPP-like properties serve to terminate the receptor-directed signaling functions of LPA and hydrolyze LPA, PA, ceramide 1-phosphate, and sphingosine 1-phosphate (14, 15, 38, 43). Little is known about the biological function or reason for existence of multiple LPP isoforms (13).

Based upon the decrease in LPP-1 mRNA expression in ovarian cancers as compared with normal epithelium and other tumor lineages indicated by transcriptional profiling and semi-quantitative reverse transcriptase-PCR analysis combined with the decreased LPP-1 enzyme activity in ovarian cancer cell lines, we focused on the LPP-1 isoform as a potential contributor to the increased levels of LPA in ascites and supernatants of ovarian cancer cells. LPP-1 demonstrates modest selectivity for LPA with a rank order preference of LPA > PA > sphingosine 1-phosphate > ceramide 1-phosphate (17, 33, 34). LPP-1 is an integral membrane protein that likely degrades extracellular or membrane associated LPA, playing a major role in limiting responses to LPA (11, 17, 44–46). LPA levels are low intracellularly, in the cell membrane, and in plasma from normal individuals, suggesting that LPA-degrading enzymes are highly active (2, 3, 47).

LPP-1 mRNA levels are decreased in the majority of ovarian cancers. In contrast, LPP-1 and total LPP levels are markedly elevated in kidney and prostate cancers. The RNA expression data would support the contention that the LPPs are not acting redundantly, and LPP-1 has a function distinct from LPP-2 and LPP-3, although the genes are expressed in overlapping patterns, and the proteins appear to be able to catalyze the same reactions. Thus, the effects of decreased LPP-1 levels may be relatively selective to ovarian cancers and may also argue that the initiation or progression of ovarian cancer may be particularly dependent on the action of LPA. The decrease in LPP-1 levels may contribute to the ability of ovarian cancer cell lines to produce high amounts of LPA as compared with normal ovarian epithelium or breast tumor samples (11, 12). We and others (47) have demonstrated that ovarian cancer cells exhibit increased expression of LPA2 (edg4) and LPA3 (edg7) LPA receptors. The increased LPA levels in ascites, decreased ability to hydrolyze membrane-associated LPA because of decreased LPP-1 levels, and altered LPA receptor expression may act together to contribute to the transformation of ovarian cancer cells. In particular, the altered LPA degradation and receptor
expression could contribute to the ability of ovarian cancer cells to survive in suspension in ascites (anoikis conditions) to proliferate and to implant and invade in the peritoneal cavity. Taken together, this suggests that LPA production, degradation, or action are potential targets for therapy for this devastating disease.

Intriguingly, differentiation of preadipocytes into mature adipocytes is associated with a decrease in the ability to hydrolyze extracellular LPA and a decrease in LPP-1, LPP-2, and LPP-3 mRNA levels (44). This appears to be similar to the case in ovarian cancer wherein LPP and in particular LPP-1 levels are markedly decreased. However, in contrast to ovarian cancer cells where LPA2 and LPA3 receptors (2) are increased on the cell surface rendering the cells more sensitive to LPA (7), mature adipocytes exhibit a marked down-regulation of LPA receptor expression (48). Thus, the net outcome during differentiation of adipocytes as compared with ovarian cancer cells may be decreased LPA signaling.

Compatible with the decreased LPP-1 levels in ovarian cancer cells playing a part in the pathophysiology of ovarian cancer, ectopic expression of LPP-1 decreased the ability of ovarian cancer cells to form colonies, a sensitive assay for cellular proliferation (36, 37). This effect was associated with an increased apoptotic rate rather than a decrease in cell cycle progression, suggesting that a major role for LPA in this system is maintenance of cell viability. The effects of LPP-1 were striking in terms of long-term cell growth because we were unable to develop stable overexpressing LPP-1 ovarian epithelial cell lines in any but the SKOV3 background despite multiple attempts. SKOV3, as compared with other ovarian cancer cells, produces high levels of LPA (12), potentially contributing to the ability to establish stable cell lines expressing LPP-1. Even with the SKOV3 cell line, we were able to only modestly overexpress LPP-1 (4.9-fold increase in RNA levels, 7.7-fold increase in ectoLPP activity). Furthermore, the overexpression proved unstable, with LPP levels declining with cell passage. We established multiple SKOV3-overexpressing cell lines on several occasions in all cases, overexpression of LPP-1 was associated with a decrease in cell proliferation and colony-forming cell activity. Transient transfection studies demonstrated, however, that the effects observed with stably overexpressing SKOV3 cell lines could be generalized to other ovarian cancer cell lines. Thus, the decrease in LPP-1 in ovarian cancer may contribute to increased survival, proliferation, and colony-forming cell activity.

In addition to changes in cellular proliferation, LPA can also induce increases in cellular motility (25). This became readily apparent with the cloning of the lysophospholipase D responsible for the production and LPA and the demonstration that lysophospholipase D is identical to the previously cloned autocrine motility factor, autotaxin (25). Compatible with the effects of LPP-1 on cellular proliferation and extracellular LPA
levels, overexpression of LPP-1 in SKOV3 cells markedly decreased cellular motility. Taken together, the results suggest that decreased LPP-1 levels in ovarian cancer cells have the potential to alter multiple facets of cellular function.

LPP-1 demonstrates ectoLPA hydrolysis activity. This suggests that loss of LPP-1 expression could result in increased extracellular LPA levels. As a corollary, ectopic expression of LPP-1 should decrease extracellular LPA levels as demonstrated herein. The ability of LPP-1-expressing ovarian cancer cells to decrease the proliferation of bystander cells (Fig. 6) is compatible with this hypothesis. This also suggests that delivery of LPP-1 to ovarian cancer cells through a gene therapy approach may not only decrease the growth, survival, motility, and metastases of transfected or infected cells but could also decrease the growth, survival, and metastases of nearby nontransfected or infected cells.

GnRH agonists have been demonstrated to inhibit the proliferation of ovarian carcinoma cells (16). Strikingly, occupation of GnRH receptors increases the LPP activity of ovarian cancer cell membranes and ovarian cancer cell lines. This suggests that pharmacological manipulation of LPP-1 expression or activity could alter the outcome in ovarian cancer.

Taken together, the data are compatible with a hypothesis that decreased LPP-1 expression by ovarian cancer cells contributes to the accumulation of LPA in the ascites of ovarian cancer patients. It additionally suggests that LPA production, degradation, and action are targets for therapy in ovarian cancer. One mechanism to achieve this goal would be to increase the levels or activity of LPP-1 by pharmacological or gene therapy approaches.

References


Role of Decreased Levels of Lipid Phosphate Phosphatase-1 in Accumulation of Lysophosphatidic Acid in Ovarian Cancer

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