THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Lysophosphatidic Acid as a Target for Molecular Diagnosis and Therapy of Ovarian Cancer

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INTRODUCTION

Ovarian cancer has the highest rate of mortality of all gynecologic cancers. The majority of these women will eventually die from their disease. This depressing prognosis results from an inability to diagnose the tumor at an early, curable stage because there is no well-established screening method. Although there are multiple therapeutic approaches, there has been no notable increase in the cure rate and survival time in the last 25 years. Most patients rapidly develop resistance to chemotherapy and will eventually die of their disease with the overall 5-year survival rate in advanced disease remaining less than 30%. Ovarian cancer genomes are remarkably variable; an advanced cancer usually harbors many genomic changes. This genomic instability and both within and between tumor heterogeneity probably contribute to drug resistance and the lack of effective therapeutic approaches that can convert the high response rate into a high cure rate.

Lysophosphatidic acid (LPA, 1-acyl-2-lyso-sn-glycero-3-phosphate) has emerged from being considered a precursor and component of lipid remodeling to clearly playing a role as an important extracellular bioactive lipid mediating cell proliferation, migration, and survival in almost every cell type, both normal and malignant. LPA has also been shown to increase the production of factors involved in neovascularization, to induce protease production and action and invasiveness, suggesting the possibility that LPA may also play a role in metastatic competence. The concentration of LPA in plasma is normally low (100-200 nM), suggesting that the production, metabolism and clearance of LPA are strictly controlled in vivo. LPA can be produced by multiple cell lineages. The main pathway for extracellular LPA production is the activity of autotaxin/lysophospholipase D (ATX/lysoPLD) which removes a choline from lysophosphatidylcholine (LPC). ATX/lysoPLD levels are markedly increased in multiple different cancers compatible with a role in the pathophysiology of malignant disease.

LPA is efficiently metabolized maintaining LPA at low levels under physiological conditions. LPA is removed by being acylated by lipid phosphate phosphatases (LPP-s) also known as phosphatidic acid phosphohydrolases. The most likely candidates for hydrolysis of extracellular LPA, including LPP-1 (PAP2A) LPP-2 (PAP2C), and LPP-3

(PAP2B), are six transmembrane domain containing integral membrane proteins with their catalytic site and functions on the extracellular surface of the cell. The outcomes of LPA signaling are determined by a broad spectrum of LPA receptors expressed on the cell surfaces. The LPA1 (Edg 2), LPA2 (Edg 4) and LPA3 (Edg 7) members of the endothelial differentiation gene (Edg) family of G protein-coupled receptors are high affinity receptors for LPA.

Ascites from ovarian cancer patients frequently contain elevated concentrations of LPA (up to 80 μM). This suggests that ovarian cancer cells in patients are exposed to a LPA rich environment. Furthermore, LPA is a potent inducer of neovascularizing factors, vascular endothelial growth factor, IL8 and IL6 production by ovarian cancer cells, potentially contributing to neovascularization and aggressiveness of ovarian cancer. LPA is not produced in significant amounts by normal ovarian surface epithelial cells but is produced at significant levels by some ovarian cancer cell lines. The ATX/lysoPLD mRNA and protein levels are modestly elevated in malignant disease.

Both LPA2 and LPA3 are aberrantly overexpressed in ovarian cancer cells, suggesting that LPA plays an important role in the pathophysiology of this cancer. In addition to increased LPA production and LPA receptor expression, the genetic aberrations in ovarian cancer cells may contribute to increased responsiveness to LPA receptor ligation. If LPA is present at an increased concentration in ascites, it could diffuse into the systemic blood circulation where it could serve as a tumor marker for early disease detection. LPA levels have been reported to be increased in the plasma of approximately 90% of patients with ovarian cancer. We intend to evaluate the level of LPP enzyme expression in ovarian cancer and use the LPP overexpression as treatment option for ovarian cancer.

AIM OF THE STUDY AND HYPOTHESIS

1. 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. Although ovarian cancer cells can produce LPA, this may not be the major reason for altered LPA levels in ascites. We try to demonstrate that the major pathway of degradation of LPA by ovarian cancer cells is through a lipid phosphate phosphatase (LPP)-like activity.
2. At first step we evaluate the LPPs' mRNA expression to see any difference between the normal epithelial cells and ovarian cancer cells.
3. We intend to introduce LPP-1 or LPP-3 into ovarian cancer cell lines in order to increase LPA hydrolysis with transient and stable transfection.
4. Our purpose is to determine the effects of decreasing LPA levels by increasing LPP activity on the pathophysiology of ovarian cancer cells. We try to demonstrate that the introduction of LPP-1 and LPP-3 will increase LPA degradation, which is associated with decreased cellular proliferation and increased death in vitro and decreased growth *in vivo*.
5. The specificity of the effects of LPP-3 on LPA would be demonstrated by studies involving the LPP-resistant, LPA3 receptor-specific agonist, 1-Oleoyl-Sn-2-O-Methyl-Rac-Glycero-3-Phosphothionate (OMPT) and with enzymatically inactive LPP-3. Taken together, we expect that these studies validate LPA metabolism and function as a target for therapy in ovarian cancer.
6. If it is successful, we plan to apply this method not only *in vitro* but also *in vivo* in animal models.
7. We would further manipulate the LPA production and metabolism to evaluate the relationship between the metabolizing and producing enzyme.

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 tumor samples. Affymetrix U95 array hybridization data are available (http://www.gnf.org/cancer/epican.) The control samples consisted of 36 normal adult epithelial tissues. 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. 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 of the data.

Gene Expression and data analysis

Twenty-six fresh frozen primary ovarian cancer samples were obtained from Duke University (9 endometrioid), the Mayo Clinic (9 stage I serous), and MD Anderson Cancer Center (8 stage III serous). 4 pools of normal ovarian surface scrapings from 13 patients were obtained from Northwestern University (Chicago). RNA was extracted using the RNeasy Kit (Qiagen Inc, Valencia, CA). The Affymetrix GeneChip® Human Genome U95 set was used to obtain gene expression data. This series tests the expression of more than 60,000 human genes and ESTs. The cRNA preparation, hybridization, and scanning of the microarrays was performed according to manufacturer's protocols at the Mayo Clinic's microarray facility. Microarray data was analyzed using the dChip software (http://www.biostat.harvard.edu/complab/dchip/).

Cell cultures and G418 selection and first-strand cDNA synthesis and semi-quantitative RT-PCR of LPP-1

OVCAR-3, SKOV3, SKOV3 IP_1, HEY and A 2780 (human epithelial ovarian carcinoma cell lines) were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA). The IOSE 80 and IOSE 29 (simian virus 40 T-antigen-semi-immortalized ovarian surface epithelial cell lines) were were grown in medium 199/MCDB 105 supplemented with 10 % FBS. All the cell lines were pre-tested to evaluate G418 (Gibco BRL, Grand Island, NY) resistance. G418 was applied in concentration from 200 μg/ml to 1000 μg/ml. The medium contained the appropriate amount of G418 and was changed every third day. The lowest concentration of G418 that killed all the cells was applied later in the experiments. The mRNA expression of LPP-1 was determined using semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Oligonucleotide primers were used for: LPP-1 primers: 5`-CGCGGATCCATGTTTGACAAGACGCG and 5`-TGGCTATGTAGTTATTCCTG, (304 bp) and control GAPDH primers: 5`-CCCATGGCAAATTCCATGGCACCG and 5`-GTCATGGATGACCTTGGCCAGGGG (344 bp). Oligonucleotide primers were used for LPP-3 primers: 5`-CGCGGATCCATGCAAAACTACAAGTA and 5`-CGTGATGATCGCGAGGATGG (306 bp) GAPDH primers: 5`-CCCATGGCAAATTCCATGGCACCG and 5`-GTCATGGATGACCTTGGCCAGGGG (344 bp). The RNA samples were treated with DNAse before first strand cDNA synthesis or RT-PCR reaction following the manufacturer's instructions (DNA-free, DNAse Treatment and Removal Kit) (Ambion, Austin, Texas, USA). In some cases, RT-PCR was performed as a single step with the RT-PCR reaction mixture consists 1μg of total RNA, 0.4 μM of sense and antisense primers, 0.2 mM of dNTPs, 0.5 U of reverse transcriptase and Taq-DNA polymerase enzymes (in enzyme mix), 5 mM DTT-solution, 5 U RNAse inhibitor, 1.5 mM $MgCl_2$ in final volume of 50 μl (Titan One Tube RT-PCR System, Roche Molecular Biochemicals, Mannheim, Germany).

LPP-1 and LPP-3 enzyme assays and immunoprecipitation

The assay volume was 100 μl, and each assay contained a final concentration of 3.2 mM Triton X-100 and 100 μM ^{32}P-labeled lipid substrate. Detergent-extracted membrane proteins (generally 0.1-5 μg of protein) were added directly. The (^{32}P)PO_4^{2-} released into the supernatant was quantitated by liquid scintillation counting. This assay was validated by demonstrating that the water-soluble radioactivity released from the substrate was (^{32}P)PO_4^{2-} by quantitative extraction with ammonium molybdate. The 12.5 % SDS-polyacrylamid gel was used for SDS-polyacrylamide gel electrophoresis. To assess LPP-1 or LPP-3 activity on cell supernatants both parental and stably SKOV3 transfected cells were resuspended in OPTI-MEM medium containing 3 % bovine serum albumin. ^{32}P LPA was added to a final concentration of 20 μM by bath sonication. Assays were initiated by adding 1 ml of this substrate preparation to 1 ml of cells ($4x10^5$ cells) followed by incubation at 37 C° with constant shaking. Aliquots of the suspension were removed at various times for determination of LPP-3 activity by measurement of [^{32}P]PO^{-2}_4 release as described above.

Cytoplasmic $[Ca^{2+}]_i$ assay

After starvation for 12-24 hours, the cells were harvested and loaded with 1 μM Indo-1 AM in phosphate-buffered saline (PBS) for 30 min at 37 °C. Cytoplasmic $[Ca^{2+}]_i$ was determined at an excitation wavelength of 331 nm and an emission wavelength of 410 nm using a fluorescence spectrophotometer (F-4000, Hitachi). Approximately 3×10^6 cells were used for $[Ca^{2+}]_i$ determination in a stirred quartz cuvette kept at 37 °C. PC-3M cells were plated onto opaque, 96-well clear bottom plates at about 80% of confluence. Calcium signals were recorded using a fast kinetic fluorometer (FLEXStation, Molecular Devices, Inc.).

Evaluation of the ERK activation in LPP-3 transfected cells and Western Blot

After 24 hours of starvation of 90 % confluent cell cultures of LPP-3 and stably transfected SKOV3 cells, LPA (1μM) was added and cell lysates prepared at the indicated time points. Western blotting was performed with anti-phospho-ERK1/2 (Cell Signaling Technology, Inc. Beverly, MA) and anti-ERK2 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Relative density of specific bands was determined using NIH Image 1.62.

The LPP-1 and LPP-3 cDNA containing vectors driven by CMV and hTERT promoters

The complete cDNA of LPP-1 was amplified using primers with the sequences: 5-CGCGGATCCATGTTTGACAAGACGCG -3 (forward) and 5-GCTCTAGAAGGCTGGTGATTGCTCG -3 (reverse) using PCR, cut with Bam HI and Xba I and inserted into Bam HI and Xba I restriction sites of the pcDNA3.1-HisA epitope tagged eukaryotic expression vector (Invitrogen, Carlsbad Ca). The CMV promoter was replaced in the pcDNA3.1-HisA vector with the hTERT promoter using Bgl II and Kpn I. The validity of all PCR products was confirmed by sequencing the product and by restriction endonuclease analyses of the vectors. The complete cDNA of LPP-3 was amplified using primers with the sequences: 5-CGCGGATCCATGCAAAACTACAAGTA -3 (forward) and 5-GCTCTAGACATCATGTTGTGGTGAT-3 (reverse) using polymerase chain reaction (PCR). Bam HI (site-921 in pcDNA3.1-HisA vector) and Xba I (-983) restriction sites were built into the primer pairs allowing the cDNA to be ligated into the pcDNA3.1-HisA epitope tagged eukaryotic expression vectors. The CMV promoter was deleted, and the hTERT378 promoter was inserted into the pcDNA3.1-HisA expression vector with Bgl II (-13) and Kpn I (-913). The validity of the expression constructs was confirmed by sequencing and restriction endonuclease analyses.

Transient and stable expression of LPP-1 and LPP-3 and Colony Formation Assay

Cell transfection was carried out using FuGene 6 carrier (Roche Molecular Biochemicals, Indianapolis, USA). Quantitation of transfection efficiency was done by flow cytometry using a FACScan with CELLQUEST 3.3 software package (Becton-Dickinson, San Jose, California). Green fluorescent protein (GFP) was identified using a single parameter histogram display of log Green Fluorescent. Cells transfected with an empty vector were used as a negative control to develop a gate to determine the percent of GFP positive transfected cells. The transfection efficiency varied from 4-21 %. To create stable lines, 48 hours after transient transfection, medium containing the appropriate amount of G418 (selection medium) was changed every third day for 6-8 weeks. Transiently transfected ovarian carcinoma cells ($3x10^4$ cells) or $2x10^3$ stably transfected SKOV3 cells were seeded into 30-mm 6-well plates. Two weeks later, colonies were stained with 0.1 % Coomassie brilliant blue R-250 (Bio-Rad, Hercules, CA) in 30 % methanol and 10 % acetic acid. Colonies (greater than 500 cells or 1mm in diameter) were counted by two investigators and the variation was under 10 %.

GFP targeted cell cycle progression assay.

The parental lines were transfected with either pcDNA3.1-LacZ-HisA, pc-hTERT-DNA3.1-HisA, pcDNA3.1-LPP-1/3-His A or pchTERT-DNA3.1-LPP-1/3-HisA, constructs separately, and co-transfected with pGFP. To assess cell cycle progression, a two-color cytometric analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) using CELLQUEST 3.3 software for acquisition and analysis. GFP positive cells were gated and analyzed for cellular DNA content.

Analysis of bystander effects of LPP-1 and LPP-3

Two days after transient transfection, OVCAR-3 and SKOV3 cells were trypsinized and counted. Transfected cells ($3x10^4$) were mixed at different ratios with their respective non-transfected parental cell line and were plated in 6 well plates. Stably

transfected SKOV3 cells ($2x10^3$) were mixed with an equal number of respective non-transfected parental SKOV3 cells and plated and colony forming assay was done.

Migration assay and tumor growth in nude mice

Migration assays were performed using a transwell chamber membrane (8μm pore size; Biocoat, Becton Dickinson Labware, Franklin Lakes, NJ). LPA, 10μM, was added to the lower chambers. Cells were initially starved of serum. Cells ($5x10^4$) were added to the upper chamber and allowed to migrate for 24 h at 37 ºC. The migrated cells were fixed and stained with crystal violet.

SKOV3 parental and SKOV3 LPP-3 or SKOV3 IP_1 parental and SKOV3 IP_1-hLPP-3 stably transfected ovarian cancer cells were injected subcutaneously ($4x10^6$ cells) or intraperitoneally ($1x10^7$ cells) into of 6-8 week old female nude mice (Harlan Laboratories, LTD, Indianapolis, Indiana). Tumor sizes (in width and length) were measured and tumor volumes (mm^3) were estimated according to the formula: Tumor volume = (long dimension) x (short dimension)^2/2.

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 chi-square analyses. Longitudinal data were compared using repeated measures of analysis of variance. Significance was set at $P<0.05$.

RESULTS

The LPP-1 mRNA expression is decreased in ovarian cancer samples by Affymetrix microRNA database.

LPP-1 and human lysophospholipase homolog 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. LPP-1 mRNA levels in ovarian cancer samples were decreased approximately 5 fold as compared to normal epithelium. Further, LPP-1 RNA levels were lower in ovarian cancers as compared to 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. The overall total of LPP-1, -2 and –3 RNA levels were 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.

Semi-quantitative RT-PCR analysis of human ovarian carcinoma cell lines and primary ovarian tumor samples

Based on the microarray data, we decided to further evaluate LPP-1 mRNA expression levels in ovarian cancer cells as compared to normal epithelium. We characterized the expression of LPP-1 in 20 primary ovarian tumor samples, all with greater than 80% tumor cell content, 6 normal surface epithelium samples, one normal surface epithelial short term culture (NOE), and 5 ovarian carcinoma cell lines, including OVCAR-3, SKOV3, SKOV3-IP_1, HEY, and A 2780. Using the lowest normal ovarian epithelial sample as a cut-off, 16/20 ovarian cancer samples and 4/5 ovarian cancer cell lines demonstrated decreased LPP-1 RNA levels, with some of the levels being 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

HEY, OVCAR-3 and SKOV3 demonstrated lower total LPP activity (representing the activity of all three LPP isoforms), than the non-tumorigenic ovarian surface epithelial cell line (IOSE). When LPP-1 was immunoprecipitated with LPP-1 specific antibodies, approximately 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 of 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.

Functionally competent expression of LPP-3 in SKOV-3 cancer cell line

To characterize the effect of LPP-s on the growth of ovarian cancer cells, we attempted to establish stable cell lines. We were able to establish stable LPP-3 expressing SKOV3 and SKOV3 IP_1 cell lines. Ovarian cancer cell lines and normal control epithelial cells were transiently and stably transfected with LPP-1- and LPP-3-expressing vectors, and enzyme overexpression was confirmed by semiquantitative RT-PCR, immunoprecipitation, Western blotting and immunofluorescence . There is selective over expression of the higher molecular weight species of LPP-3 in the stable cell line, which corresponds to the mature plasma membrane localized form of the enzyme. These cells demonstrate increased ability to hydrolyze radiolabeled LPA in the media compared to the parental line ($P=0.03$). This was reflected in an increased rate and magnitude of LPA hydrolysis.

Evaluation of the ERK activation and intracellular calcium mobilization in LPP-3-transfected cells compared to non-transfected cells.

To determine whether this resulted in functional consequences, we assessed the effect of expression of LPP-3 on LPA-induced phosphorylation of ERK kinases, a sensitive indicator of LPA signaling. Expression of LPP3 resulted in a decrease in maximal levels of ERK phosphorylation, which was associated with a rapid decrease in erk phosphorylation levels. At later time points (2-3 hrs), ERK phosphorylation returned to baseline in both parental and transfected SKOV3 cells, compatible with the decrease in LPA levels to undetectable levels at late times as described above in both cell lines. ERK activation is based on LPA receptor activation, but we wanted to know whether LPP-3 overexpression had any effect on LPA receptor binding. Once LPA binds the receptor, it may be inactivated and internalized within a few minutes. The intracellular calcium mobilization of the parental line was compared with that of the LPP-3-overexpressing ovarian tumor cell line, and no difference was observed. This indicates that the ability of LPA to bind to the receptor is independent from the presence of the LPP-3 enzyme on the surface, and the activity is due to LPA hydrolysis rather than an effect on receptor function.

LPP-1 and LPP-3 decreases colony-forming ability of carcinoma cells

LPA is a potent regulator of multiple activities in ovarian cancer cells. We evaluated the effect of increasing LPP-1 and LPP-3 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. As indicated by the ability to form colonies, stable expression of LPP-1 decreased the growth of the SKOV3 ovarian cancer cells approximately 1.9 fold (49 ± 8.4 vs. 26 ± 4.3 $p=0.0002$). Due to 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. Transient expression of LPP-1 caused a marked growth inhibition in

both OVCAR-3 and SKOV3. The inhibitory effect ranged from 2.3 fold 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 *in vitro*. Following transient transfection of LPP-3 driven by either the CMV or human telomerase promoter, LPP-3 markedly decreased the ability of both SKOV3 (7.3 fold (86%decrease) with hTERT-hLPP-3 and 7.1 fold (86% decrease) with CMV-hLPP-3) and OVCAR-3 (6.8 fold with (85% decrease) hTERT-hLPP-3 and 6.7 fold (85% decrease) with CMV-hLPP-3) ovarian cancer cells to form colonies (P=0.002).

LPP-1 and LPP-3 markedly increases the apoptosis rate in ovarian cancer cell lines

The decreased ability of ovarian cancer cells to form colonies could either be due to decreased cell cycle progression or due to increased rates of apoptosis. There were no obvious differences in cell cycle progression as indicated by number of cells in G1, S or G2M in control or LPP-1 expressing SKOV3 or OVCAR-3 cells. 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. Thus the decreased ability of LPP-1 expressing ovarian cancer cells to form colonies is associated with an increased rate of cellular apoptosis. There was also a significant and consistent increase in the hypodiploid peak in LPP-3 expressing cells (P<0.0001) compatible with an increased rate of apoptosis. Thus the decreased ability of LPP-3 expressing ovarian cancer cells to form colonies is associated with an increased rate of cellular apoptosis. This experiment was repeated with the mutant, inactive LPP-3 using the same condition, and a mutant LPP-3, which is unable to hydrolyze LPA did not alter the apoptosis rate, confirming a need for intact enzyme activity in the effect of LPP-3 on cell death.

LPP-3 decreases growth of ovarian cancer cells through LPA hydrolysis

As indicated above, the catalytic activity of LPP3 is required for the ability to decrease cell growth and further the expression of LPP3 results in a decrease in extracellular LPA levels. To determine whether the effects of LPP-3 on the growth of ovarian cancer cells was due to hydrolysis of extracellular LPA, we assessed the ability of addition of exogenous LPA or a non-hydrolysable LPA analog, OMPT (1-Oleoyl-Sn-2-O-Methyl-Rac-Glycero-3-Phosphothionate), to reverse the effects of LPP-3 expression. As indicated earlier, LPA phosphatase activity was increased 4.4-fold by stable expression of LPP-3 resulting in considerable LPA hydrolytic activity. However, OMPT was able to substantially reverse the effects of LPP-3 on both colony forming activity and on apoptosis with an OMPT concentration of 100nM proving optimal in both assays. The ability of exogenous OMPT to reverse the effects of LPP-3 suggests that the major effect of LPP-3 on the growth of ovarian cancer cells was due to hydrolysis of extracellular LPA.

LPP-1 and LPP-3 decreases the growth of non-transfected bystander cells

The ability of LPP-1 and LPP-3 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 or LPP-3 in one population of ovarian cancer cells could decrease the colony forming cell activity of bystander non-transfected cells. To assess this possibility, we performed a series of cell mixing assays with LPP-1 transfected cells and control parental cells. We combined equal numbers of stably or transiently LPP-1/LPP-3 transfected cells and parental cells. There was a marked decrease in the number of colonies observed as compared to the expected number of colonies (P=0.01, P=0.01). Therefore, LPP-1 or LPP-3 transfected cells were able to decrease the proliferation of parental cells compatible with the effect of LPP-1 being related to the effects of an extracellular mediator, likely LPA.

LPP-1 overexpression decreases the ability of the tumor cells to migrate

LPA has been demonstrated to markedly increase cellular migration. We assessed whether overexpression of LPP-1 in ovarian cancer cells would also decrease LPA induced cellular migration. LPA (10μM) stimulated migration of empty vector pcDNA3.1 transfected SKOV3 cells. In contrast, LPA failed to induce migration in LPP-1 over-expressing SKOV3 cells. Thus the increased hydrolysis of LPA by LPP-1 is translated into alterations in cellular motility. This is compatible with the decrease in LPP-1 in ovarian cancer cells contributing to these important components of the transformation cascade.

LPP-3 decreases tumor growth of ovarian cancer cells *in vivo*

Following subcutaneous injection of SKOV3 cells into athymic female nude mice, tumors developed in 9/10 mice as compared to 4/10 mice injected with LPP-3 expressing SKOV3 cells. Following subcutaneous injection of SKOV3-IP_1 cells, a more aggressive SKOV3 subline developed by intraperitoneal passage of SKOV3, tumors developed in 10/10 mice as compared to 5/10 mice injected with LPP-3 expressing SKOV3 IP_1 cells. Thus the take rate of the tumors expressing LPP-3 was markedly less than that of the parental lines. In addition to a decreased take rate, the growth rate of both the SKOV3 and SKOV3-IP_1 LPP-3 expressing tumors was significantly decreased as compared to the parental cell lines (P value for both SKOV3 and SKOV3-IP_1<0.0001,). At the termination of the study (mandated by tumor size and AALAC guidelines), the average SKOV3 parental tumor was 0.48$\pm$0.15 g in weight while the hLPP-3 expressing SKOV3 tumors averaged 0.13$\pm$0.04 g in weight. Similarly, with the SKOV3 IP_1 parental tumor the average weight was 0.44$\pm$0.11 g while LPP-3 expressing tumors had an average weight of 0.12$\pm$0.02 g. With both tumor lines, for the animals that developed tumors the average tumor weight was 4 times greater in the parental than in the LPP-3 expressing tumors.

When SKOV3 or SKOV3-IP_1 cells were injected into the orthotopic site in the peritoneal cavity, LPP-3 expression also decreased the take rate (1 of 5 from SKOV3

LPP-3 injected and 3 of 5 from SKOV3 IP_1-LPP-3 injected mice with all parental cell injections (10/10) resulting in tumor growth). With both SKOV3 and SKOV3 IP_1 cells, LPP-3 expression decreased intraperitoneal growth as assessed by both animal weight ($P=0.005$, $P<0.0001$) and abdominal circumference ($P=0.042$, $P=0.001$).

As indicated above, LPP-3 resulted in a marked decrease in take rates and in those cases where tumors formed, a decrease in growth rates. However, after a delay in growth, the LPP-3 expressing tumors appeared to enter a more rapid growth phase. As the LPP-3 construct was not under selective pressure *in vivo*, it was possible that the eventual increase in growth rate was due to loss of LPP-3. The RT-PCR showed that this was indeed the case. Following *in vivo* growth, LPP-3 levels in the transfected lines were markedly decreased. Even more striking, however, following *in vivo* growth, LPP-3 levels were markedly decreased in the parental cell lines. It thus appears that *in vivo* growth of SKOV3 cells is associated with a down regulation of expression of both endogenous LPP-3 and transfected LPP-3. This suggests that a very strong negative selection exists against LPP3 expression in ovarian cancer cells *in vivo*.

DISCUSSION

Under physiologic circumstances, LPA levels in bodily fluids, cell membranes, or cells are low (submicromolar), likely reflecting rapid clearance or degradation of LPA. Elevated LPA concentrations in ascites of ovarian cancer patients could be a consequence of altered levels or altered activity of enzymes involved in LPA production, an increase in the number of LPA producing cells (i.e. tumor cells), or altered clearance. These changes in LPA levels and its possible effects on the tumor cells seemed to be an attractive field for evaluation. Using public transcriptional profiling databases as well as the CGAP SAGE database and the transcriptional profiling databases of M.D. Anderson Cancer Center, we found that there were obvious decreases in the levels of LPPs, which degrade LPA in ovarian cancer. There was also an increase of the ATX/LysoPLD mRNA levels up to 200 fold in a proportion of ovarian cancer cell preparations obtained directly from the patient. The combined expected effects of these enzymes would be to increase LPA levels in the local tumor environment. In contrast to ATX/lysoPLD action and compatible with the increased LPA levels in ascites, the overall total levels of all three LPP mRNA was lower in ovarian cancer tissue samples and cell lines than it was in normal epithelial tissues and cell lines derived from normal ovarian epithelium. The biggest part of the difference in total LPP mRNA levels could be attributed to decreased LPP-1, which was, on average, five times lower in ovarian cancers than in normal epithelial tissues. Data suggest that ovarian cancer cells have an increased ability to produce LPA compared to normal ovarian epithelium. In addition to alterations in the production or action of enzymes involved in LPA production, even a modest ability of ovarian cancer cells to produce LPA could result in marked aberrations in levels of LPA in ovarian cancer patients. Taken together, these studies suggest that the ovarian cancer environment is LPA rich due to aberrations in both LPA production and metabolism.

The evidence that LPA levels and metabolizing enzymes are aberrant in ovarian cancer suggests that LPA production or action could provide novel effective targets for therapy of ovarian cancer. We have validated LPA production, metabolism and action as potential therapeutic targets in ovarian cancer both *in vitro* and *in vivo* by evaluating the effects of decreasing LPA production by ovarian cancer cells by transfecting LPP into

ovarian cancer cells. Ovarian cancer cell lines and control normal epithelial cells were transiently and stably transfected with LPP-1- and LPP-3-expressing vectors, with enzyme overexpression being confirmed by semiquantitative RT-PCR and immunoprecipitation. Overexpression of either the LPP-1 or LPP-3 isoenzymes decreased cell proliferation and survival as indicated by colony-forming activity of ovarian cancer cells. The ability of LPP-3 and LPP-1 to decrease the colony-forming activity of ovarian cancer cells was dependent on LPA degradation as mutant, biologically inactive LPP-3 had no effect on colony-forming activity. To further determine whether the effects of LPP-3 on the growth of ovarian cancer cells were due to hydrolysis of extracellular LPA, we assessed the ability of a nonhydrolyzable LPA3 receptor-selective agonist (OMPT), to reverse the effects of LPP-3 expression. OMPT was able to substantially reverse the inhibition of both colony-forming activity and apoptosis by LPP-3. The ability of exogenous OMPT to reverse the effects of LPP-3 suggests that the major effect of hLPP-3 on the growth of ovarian cancer cells is due to hydrolysis of extracellular LPA. LPP-1 and LPP-3 overexpression markedly increased the apoptosis rate in ovarian carcinoma cell lines with lesser effects on cell cycle progression suggesting that the decreased growth rate was a consequence of both decreased proliferation and increased cell death.

LPA has been shown to markedly increase cellular migration which could contribute to tumor aggressiveness or to metastases. We thus assessed whether overexpression of LPP-1 in ovarian cancer cells would alter LPA-induced migration. LPA (10 μM) stimulated migration of control transfected SKOV3 cells in a transwell assay. Strikingly, LPA failed to induce migration in LPP-1-overexpressing SKOV3 cells. These results indicate that the increased extracellular hydrolysis of LPA by LPP-1 is translated into alterations of cellular motility as well as proliferation, colony formation, and survival. These findings are compatible with the hypothesis that decreases in LPP-1 levels and activity in ovarian cancer cells contribute to the pathophysiology of ovarian cancer through both increased proliferation and survival as well as the metastatic cascade. This is supported by our and other previous studies indicating that LPA increased the activity and amount of a number of proteases involved in invasion and metastases.

If expression of LPP-1 and LPP-3 decreases extracellular LPA, then the overexpression of it in one population of ovarian cancer cells should also influence the colony-forming ability of nontransfected tumor cells. This could prove very important in gene therapy approaches as LPP-1 or LPP-3 could then induce death of non-transfected bystander cancer cells. To evaluate this theory, LPP-1- and LPP-3-transfected cells were mixed with an equal number of parental cells. Both LPP-1- andLPP-3-transfected cells decreased the proliferation of non-transfected tumor, which is compatible with the hypothesis that the effect of LPP-1 or LPP-3 is related to degradation of an extracellular mediator, likely LPA.

The mechanisms by which overexpression of LPP isoenzymes alter cellular function appears to be through degradation of LPA and limiting LPA-induced signaling. LPA induces changes within seconds of addition to the media. In contrast, other signaling events such as phosphorylation of Erk kinases are delayed and prolonged. If the effects of LPPs are primarily due to degradation of LPA, then LPP overexpression should not alter changes in cytosolic calcium which occur prior to LPA degradation but in contrast LPP overexpression would be expected to decrease Erk phosphorylation. As already showed in this study, there was no difference in the ability of LPA to induce increases in intracellular calcium mobilization in parental or LPP-transfected cells. In contrast, transfection of LPP-3 resulted in a decrease in maximal levels of Erk phosphorylation, and further in a more rapid decline in Erk phosphorylation levels to baseline. The differential rate of decline in erk phosphorylation was compatible with the rate of degradation of LPA by LPP transfected and parental cells.

The ability of LPPs to decrease cellular proliferation and apoptosis *in vitro* was reflected by a marked decrease in tumor take rates and growth rates *in vivo*. After subcutaneous injection of SKOV3 cells, tumors developed in 90% of the mice, compared with only 40% of the mice injected with LPP-3-expressing SKOV3 cells. In addition, the growth rate of the LPP-3-expressing SKOV3 tumor cells was markedly decreased compared with that of the parental cell lines. At the end of the study, the average weight of SKOV3 parental tumors was 4 fold higher than that of LPP-3-expressing SKOV3 tumors. When SKOV3 cells were injected into the orthotopic site for ovarian cancer, the peritoneal cavity, LPP-3 expression decreased the take rate to 20% in the injected mice,

whereas all parental cell injections resulted in tumor growth. LPP-3 expression decreased intraperitoneal growth as assessed by both animal weight and abdominal circumference. However, after a delay in growth, the LPP-3-expressing tumors appeared to enter a more rapid growth phase. It was a possibility that the eventual increase in the growth rate was due to loss of LPP-3. Strikingly, when the rapidly growing tumors were excised and assessed for the presence of the LPP-3 transgene, LPP-3 levels in the transfected lines were markedly decreased. Furthermore, LPP-3 levels were also decreased in the parent lines *in vivo*. It therefore appears that *in vivo* growth of SKOV3 cells is associated with downregulation of expression of transfected LPP-3. This suggests that a very strong negative selection exists against LPP-3 expression in ovarian cancer cells *in vivo*.

The changes of the LPA signaling cascade in ovarian cancer offers multiple further field for investigations. ATX/LysoPLD is a particularly attractive target for therapy. Indeed, application of RNAi to ATX/LysoPLD potently downregulates cellular proliferation and survival. A series of adenoviral vectors have been developed with broadly active promoters and also with ovarian cancer specific promoters - such as telomerase. Although a wide variety of LPA receptor-selective agonists and antagonists have been investigated but a highly effective therapeutic agent was nsot found yet.

Taken together, these studies suggest that an autocrine LPA loop is critical for the proliferation, survival, motility and *in vivo* growth of ovarian cancer cells. Further, the results suggest that this autocrine loop is a potential target for therapy of ovarian and likely other cancers.

CONCLUSIONS

1. LPP-1 expression is decreased in ovarian cancer cells. Our data suggest that decreased LPP-1 expression could result in increased extracellular LPA levels.
2. Introduction of LPP-1 and LPP-3 into ovarian cancer cells significantly decreased the ability of ovarian cancer cells to form colonies and to grow both *in vitro* and *in vivo*.
3. The decreased colony forming cell activity caused by increased expression of LPP-1 and LPP-3 was associated with a marked increase in apoptosis with no obvious differences in cell cycle progression.
4. The inability to stably express LPP-1 or LPP-3 in other ovarian cell lines is compatible with a strong negative selection for high-level expression of LPP-1, LPP-3 both in *in vivo* and *in vitro.*
5. The LPP-1 and LPP-3 exerts its effects on ovarian cancer cells through decreasing extracellular LPA. This was supported by the observation that addition of a non-hydrolysable LPA analog, OMPT, to the medium substantially reversed the effects of LPP-3 on both colony forming activity and cellular apoptosis.
6. Both the ability to decrease colony forming activity and to induce apoptosis was dependent on an intact catalytic activity of LPP-3
7. The downstream signaling of LPA as indicated by phosphorylation of ERK kinases was curtailed in LPP-3 expressing cells.
8. Both LPP-1 and LPP-3 demonstrated a clear bystander effect, also consistent with the theory that LPP-s hydrolyse an extracellular mediator, likely LPA.
9. Overexpression of LPP-1 in SKOV3 cells markedly decreased cellular motility.
10. Taken together these data suggests that LPA production, metabolism, receptor binding and downstream signaling pathways are thus attractive targets for therapy in ovarian cancer.

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