The Human Lipid Phosphate Phosphatase-3 Decreases the Growth, Survival, and Tumorigenesis of Ovarian Cancer Cells: Validation of the Lysophosphatidic Acid Signaling Cascade as a Target for Therapy in Ovarian Cancer


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ABSTRACT

Lysophosphatic acid (LPA) is present at elevated concentrations in the ascites and plasma of ovarian cancer patients. Ovarian cancer cells produce and release LPA both constitutively and after stimulation. LPA can induce proliferation, survival, invasiveness, and resistance to chemotherapeutic drugs in a manner characteristic of human ovarian epithelial cancer cells. This suggests that LPA may be critically important for the development or progression of ovarian cancer and is thus a potential target for therapy. In this study, we demonstrate that introduction of the integral membrane protein, human lipid phosphate phosphohydrolase-3 (hLPP-3) enzyme, which hydrolyzes phosphatidic acid, LPA, sphingosine, and ceramide phosphate in vitro with selectivity for LPA, into SKOV3 and OVCAR3 ovarian cancer cells decreases colony-forming activity, increases apoptosis, and decreases tumor growth in vitro and in vivo. Strikingly, coculture of hLPP-3-expressing cells with nontransfected parental cells decreased the colony-forming activity of the parental cells, compatible with hLPP-3 decreasing levels of an extracellular mediator, likely LPA. Compatible with this contention, the expression of hLPP-3 was associated with increased rates of extracellular LPA hydrolysis. The effects of hLPP-3 on colony-forming activity were substantially reversed by the LPP-resistant LPA analogue, O-methylphosphothionate. The ability of O-methylphosphothionate to ameliorate the effects of hLPP-3, combined with the inability of an enzymatically inactive hLPP-3 to alter cellular function, suggests that the major effect of hLPP-3 was to increase the hydrolysis of extracellular LPA. Thus genetic or pharmacological manipulation of LPA metabolism, receptor activation, or downstream signaling is an attractive approach for therapy of ovarian cancer.

INTRODUCTION

LPA1 (1-acyl-2-lyso-sn-glycero-3-phosphate), the simplest phospholipid, mediates multiple functions ranging from growth promotion and increased cell cycle progression to cell survival (1–6). 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 (7–11). Levels of LPA are markedly elevated, reaching levels as high as 80 μM, in the ascites of patients with epithelial ovarian cancer (12, 13). On the basis of retrospective analyses, LPA levels are elevated in the plasma of >90% of stage I ovarian cancer patients compared with healthy individuals (14, 15). Ovarian cancer cells but not normal ovarian epithelial cells produce LPA (13), potentially contributing to the elevated LPA levels in the ascites of ovarian cancer patients. The pleiomorphic effects exerted by LPA on ovarian cancer cells suggest that LPA production or action is a potential target for molecular therapeutics or gene therapy for ovarian cancer.

The outcomes of LPA signaling are determined by the spectrum of LPA receptors expressed on the cell surfaces. The LPA1 (Edg 2), LPA2 (Edg 4), and LPA3 (Edg 7) members of the Edg family of G protein-coupled receptors are high affinity receptors for LPA and have been proposed to mediate LPA signaling in mammalian cells (16–18). Normal ovarian epithelial cells express low levels of mRNA for LPA2 and LPA3, whereas the mRNA levels for LPA2 and particularly LPA3 are markedly elevated in epithelial ovarian cancers (13, 19–21). LPA1 may exert negative effects on the growth and survival of ovarian cancer cells (19). In contrast to LPA1 and LPA2, which are activated by LPA with either saturated or unsaturated fatty acyl chains, LPA3 is preferentially activated by LPA with unsaturated fatty acyl chains (18). LPA has a modest, if any, biological activity on normal ovarian surface epithelium compatible with low-level expression of LPA2 and LPA3 by normal ovarian epithelial cells. Therefore, the high expression of LPA2 and LPA3 in ovarian cancer cells suggests that they have shifted to an LPA-dependent phenotype (13, 22).

Lipid phosphate phosphohydrolase-3 (hLPP-3 and PAP2B), a membrane-associated phosphatase, is a widely expressed member of the LPP family, which also includes LPP-1 (PAP2A) and LPP-2 (PAP2C; Ref. 23). LPP-like properties serve to terminate the receptor-directed signaling functions of LPA and related compounds (24–28). LPPs have been implicated in limiting LPA signaling in multiple systems (28–30). LPPs have been proposed to degrade extracellular LPA, particularly that associated with the cell membrane (28). Indeed, >90% of LPA degradation by ovarian cancer cells is because of the action of LPP-like enzymes (27). Alternatively, LPPs have been suggested to directly inhibit the function of G protein-coupled receptors of the LPP family (29), independent of LPA hydrolysis. Intriguingly, the effects of LPPs have under some circumstances been proposed to be independent of the known LPA receptors, implicating additional LPA receptors (30) or LPA receptor-independent effects. Although the LPPs can hydrolyze free phosphates in phospholipids, lysophospholipids, ceramide lipids, and sphingolipids, hLPP-3 shows preference for LPA (31).

LPA receptors and metabolizing enzymes are altered in expression between normal ovarian epithelium and ovarian cancer cells, suggesting that LPA and its receptors are potential targets for therapy of ovarian cancer. The purpose of this study was to determine the effects
of decreasing LPA levels by increasing LPP activity on the pathophysiology of ovarian cancer cells. These studies demonstrated that the introduction of hLPP-3 increased LPA degradation, which was associated with decreased cellular proliferation and increased death in vitro and decreased growth in vivo. The specificity of the effects of hLPP-3 for LPA was demonstrated by studies with the LPP-resistant, LPA3 receptor-specific agonist OMPT and with enzymatically inactive hLPP-3. Taken together, these studies validate LPA metabolism and function as a target for therapy in ovarian cancer.

MATERIALS AND METHODS

Cell Culture. OVCA-3, SKOV3, SKOV3 IP3, 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). IOSE 80 and IOSE 29 (SV40 T-antigen-immortalized nonmalignant ovarian surface epithelial cell lines) were grown in medium 199/MCDB 105 supplemented with 10% fetal bovine serum (32). SKOV3 IP3 is a more aggressive SKOV3 subtype developed by i.p. passage of SKOV3 (33).

G418 Selection. All of the cell lines were pretreated to evaluate G418 (Life Technologies, Inc., Grand Island, NY) resistance. OVCA-3 and all of the IOSE cell lines were cultured with 400 μg/ml G418, the A 2780 cell line with 500 μg/ml G418, and the SKOV3, SKOV3 IP3, and HEY cell lines with 800 μg/ml G418.

Construction of LPP Vectors. Four different constructs were assessed. The CMV promoter-driven pcDNA3.1-HisA vector, which adds an in-frame NH2-terminal HisA and V5 epitope tag, and pcDNA3.1-HisA-LacZ constructs were from Invitrogen (Carlsbad, CA). The human hLPP-3 complete cDNA expression vector and an inactive mutant have been described previously (31, 34, 35). To inactivate hLPP, the critical histidine in the catalytic domain was mutated to proline (from CAC to CCA at position 561) has been shown to inactivate hLPP-1 (34) and to block the activity of hLPP-3 mutated to proline (from CAC to CCA at position 561). The CMV promoter-driven pcDNA3.1-HisA vector, which adds an in-frame HisA sequence 5′-CAAAACTACAAGTA-3′ (forward) and 5′-GCTCTAGACATCATGTTGTG-3′ (reverse) using PCR. BamHI (site-921 in pcDNA3.1-HisA vector) and XbaI (−983) restriction sides 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 BglII (−13) and KpnI (−913). The validity of the expression constructs was confirmed by sequencing and restriction endonuclease analyses.

Transient and Stable Expression of hLPP-3. Cells were plated in 60-mm plastic dishes at a density of 0.5 × 106 cells/dish. At 60% confluent, cells were washed in PBS (pH 7.4); Sigma, St. Louis, MO). Cell transfection was carried out using FuGene 6 (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. Expression vector (1.5 μg) was added and incubated for 30 min at room temperature. Cotransfection with 0.5 μg of pcDNA3.1-LacZ-HisA vector, which adds an in-frame LacZ-HisA sequence 5′-CAAAACTACAAGTA-3′ (forward) and 5′-GCTCTAGACATCATGTTGTG-3′ (reverse) using PCR. BamHI (site-921 in pcDNA3.1-HisA vector) and XbaI (−983) restriction sides 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 BglII (−13) and KpnI (−913). The validity of the expression constructs was confirmed by sequencing and restriction endonuclease analyses.

Preparation of [32P]LPA. [32P]LPA was prepared by phosphorylation of Oleoyl monoyglycerol (monooelic; Avanti Polar Lipids, Alabaster, AL) using Escherichia coli diacylglycerol kinase (Calbiochem, San Diego, CA) and [γ-32P]ATP (ICN Pharmaceuticals, Costa Mesa, CA). The reaction was terminated by addition with acidified CHCl3 and methanol, and the dried organic phase obtained was resuspended in 0.4 ml of 20:9:1 CHCl3/methanol/H2O (20:9:1) solvent A and neutralized by the addition of a small volume of 20% NH4OH in methanol. This material was applied to an Econosil NH2 5 units of high-pressure liquid chromatography column (250 × 42 mm; Alltech Associates, Deerfield, IL). The column was washed with 20 ml of solvent A and then eluted with a 40:60 linear gradient of 0–1 M ammonium acetate in solvent A. Fractions (0.5 ml) of the eluant were collected, and associated radioactivity was determined by liquid scintillation counting. 32P-labeled products were pooled and extracted from the eluant by the addition of 3 μl HCl and CHCl3 to give two phases.

hLPP Enzyme Assays. The assay procedures used were adapted from those described previously (26). In brief, assays were performed in medium containing 20 mM Tris (pH 7.5), 1 mM EGTA, and 2 mM EDTA. 32P-labeled LPA was dried under vacuum and resuspended in 6.4 mM Tris X-100. The assay volume was 100 μl, and each assay contained a final concentration of 3.2
nm Triton X-100 and 100 μM ³²P-labeled lipid substrate. Detergent-extracted membrane proteins (generally 0.1–5 μg of protein) were added directly. Assays were performed at 37°C and were terminated by addition of ice-cold 10 mg/ml BSA and 10% trichloroacetic acid. The samples were centrifuged for 5 min in a microcentrifuge, and [³²P]PO₄⁻² 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 [³²P]PO₄⁻² by quantitative extraction with ammonium molybdate.

To assess hLPP-3 activity on cell supernatants both parental and stably SKOV3-transfected cells were resuspended in OPTI-MEM medium containing 3% BSA. [³²P]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 (4 x 10⁶ cells) followed by incubation at 37°C with constant shaking. Aliquots of the suspension were removed at various times for determination of hLPP-3 activity by measurement of [³²P]PO₄⁻² release as described above.

Analysis of Bystander Effects of hLPP-3. Two days after transient transfection (see above), OVCAR-3 and SKOV3 cells were trypsinized and counted. Transfected cells (3 x 10⁴) were mixed at different ratios with their respective nontransfected parental cell line and were plated in 6-well plates. Similar approaches were performed with stably transfected SKOV3 cells. Two weeks later, colonies were stained and counted.

Immunoprecipitation and Western Blot Analysis. SDS-PAGE (12.5% SDS), Western blotting, and protein determinations were performed as previously described (35, 36) with the indicated antibodies.

Evaluation of the ERK Activation in LPP-3-transfected Cells. After 24-h starvation of 90% confluent cell cultures of LPP-3 and neo stably transfected SKOV3 cells, LPA (1 μM) was added and cell lysates prepared at the indicated time points. Western blotting was performed with antiphospho-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.

Semiquantitative RT-PCR of hLPP-3. The mRNA expression of hLPP-3 was determined using semiquantitative RT-PCR. Oligonucleotide primers were used for: HLP-3, 5’-CGGAGTATCCGCAAAACATCAAGT and 5’-CGTGTGATGATCGCGAGGATGG (306 bp); and GAPDH primers, 5’-GCCAGGGG (344 bp).

RNA samples were treated with DNase before the RT-PCR reaction following the manufacturers instructions (DNA-free, DNase Treatment and Removal Kit; Ambion, Austin, TX). The RT-PCR reaction mixtures consisted of cDNA derived from 1 μg of total RNA, 0.4 μM of sense and antisense primers, 0.2 mm of deoxynucleotide triphosphates, 0.5 units of either reverse transcriptase or Taq DNA polymerase enzymes, 5 mm DTT solution, 5 units of RNase inhibitor, and 1.5 mm MgCl₂ in a 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 reaction carried out in a Thermal Cycler (Perkin-Elmer 480, Atlanta, GA). Each cycle of PCR consisted of 30 s of denaturation at 94°C, 2 min of annealing at 54°C, and 1 min of extension at 72°C. The PCR products were visualized by electrophoresis in a 2% agarose gel with ethidium bromide.

Tumor Growth in Nude Mice. SKOV3 parental and SKOV3 hLPP-3 or SKOV3 IP₁ parental and SKOV3 IP₁-hLPP-3 stably transfected ovarian cancer cells (4 x 10⁶ cells) were injected s.c. into the left and right thigh of 6–8 week-old female nude mice (Harlan Laboratories, Ltd., Indianapolis, IN). Tumor sizes (in width and length) were measured, and tumor volumes (mm³) were estimated according to the formula: tumor volume = (long dimension) x (short dimension) x 0.52 (37). On day 50 (SKOV3) or day 26 (SKOV3 IP₁), the mice were euthanized, and autopsy was carried out.

I.p. growth was assessed by injection of 1 x 10⁷ cells of SKOV3 parental and SKOV3 hLPP-3 or SKOV3 IP₁ parental and SKOV3 IP₁-hLPP-3 cells. Abdominal circumference and body weight were assessed every second day after day 9 (SKOV3 IP₁) or day 21 (SKOV3). The mice were euthanized on day 41 (SKOV3) and day 30 (SKOV3 IP₁) after injection. At the time of euthanasia, autopsy was performed. Animals with any detectable tumor were counted. Animals free of detectable tumor were excluded from the analysis.

Statistical Analyses. Unpaired continuous outcomes were compared using Wilcoxon rank sum tests. Paired continuous outcomes were compared using Wilcoxon sign-rank tests. Proportions were compared using χ² analyses. Longitudinal data were compared using repeated measures of ANOVA. Significance was set at P < 0.05.

RESULTS

HLP-3 Decreases Colony-forming Activity of Ovarian Cancer Cells. To assess the role of LPA, either produced by ovarian cancer cells (13) or present in serum, in the growth of ovarian cancer cells, we determined the effects of introduction of hLPP-3, which degrades LPA, on the ability of ovarian cancer cells to form colonies (>500 cells and >1 mm in diameter). After transient transfection of hLPP-3 driven by either the CMV or human telomerase promoter [both of which are highly active in ovarian cancer cells (38)], hLPP-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; Fig. 1A). As shown in Fig. 1B, the ability...
of hLPP-3 to decrease colony-forming activity of ovarian cancer cells was dependent on an intact catalytic activity, compatible with the degradation of LPA because transfection of mutant hLPP-3 lacking catalytic activity (34, 35) had no effect on colony-forming ability. In contrast to SKOV3 and OVCAR-3 ovarian cancer cell lines, transient expression of hLPP-3 under the control of the CMV promoter only modestly (2-fold, 56% decrease) decreased colony-forming activity of the MCF-7 breast cancer cell line. On the basis of observations from our (13) and other laboratories (39) that breast cancer cell lines both constitutively and inducibly produce much lower levels of LPA than ovarian cancer cell lines, the MCF-7 breast cancer cell line may not be dependent on LPA for proliferation.

To additionally characterize the effect of hLPP-3 on the growth of ovarian cancer cells, we attempted to establish stable cell lines. We were able to establish stable hLPP-3-expressing SKOV3 and SKOV3 IP1 cell lines. The cell lines contained modestly increased amounts of hLPP-3 as assessed by RT-PCR and Western blotting (Fig. 2, A and B). There is selective overexpression of the higher molecular weight species of hLPP-3 in the stable cell line, which corresponds to the mature plasma membrane localized form of the enzyme (35).

**hLPP-3 Expression Increases LPA Hydrolysis.** The hLPP-3 expressed in the SKOV3 cell lines was functional as assessed by hydrolysis of phosphate from labeled LPA. As compared with parental SKOV3 cells where 32 pmol LPA were hydrolyzed/30-min assay/mg protein, 44 pmol LPA were hydrolyzed/30-min assay/mg protein in the hLPP-3-expressing SKOV3 ovarian cancer cells.
Thus expression of hLPP-3 resulted in a 4.4-fold increase in LPA hydrolysis. The expression of LPP-3 on the cell surface was confirmed by analysis of the ability of transfected cells to hydrolyze radiolabeled LPA added to the media. As indicated in Fig. 2C, transfected cells demonstrate increased ability to hydrolyze radiolabeled LPA in the media compared with the parental line (P = 0.03). This was reflected in an increased rate and magnitude of LPA hydrolysis.

The increased ability to hydrolyze radiolabeled LPA translated into a decrease in extracellular LPA. When 1 μM 18:1 LPA was added to media alone, there was no detectable change in LPA concentrations over time (time: 0 min, 780 nM; 10 min, 740 nM; 1 h, 845 nM; and 8 h, 819 nM). In the presence of SKOV3 cells, LPA was hydrolyzed [time: 0 min, 727 nM; 10 min, 580 nM; 1 h, 555 nM; and 8 h, <100 nM (levels > 100 nM were readily detectable in calibration curves)]. Expression of hLPP-3 resulted in a marked decrease in LPA levels (time: 0 min, 808 nM; 10 min, 474 nM; 1 h, 229 nM; and 8 h, <100 nM), particularly at early time points, compatible with increased LPP activity.

As indicated above, expression of hLPP-3 resulted in increased rates
of LPA hydrolysis and LPA concentrations in media. To determine whether this resulted in functional consequences, we assessed the effect of expression of hLPP-3 on LPA-induced phosphorylation of ERKs, a sensitive indicator of LPA signaling. As indicated in Fig. 2, D and E, expression of hLPP-3 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 h), 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.

hLPP-3 Decreases Growth of Ovarian Cancer Cells through LPA Hydrolysis. As indicated above, the catalytic activity of hLPP-3 is required for the ability to decrease cell growth and further the expression of hLPP-3 results in a decrease in extracellular LPA levels. To determine whether the effects of hLPP-3 on the growth of ovarian cancer cells was attributable to hydrolysis of extracellular LPA, we assessed the ability of addition of exogenous LPA to reverse the effects of hLPP-3 expression. Strikingly, addition of exogenous LPA up to 50 μM failed to reverse the effects of hLPP-3 expression (data not presented). As indicated above, LPA phosphatase activity was increased 4.4-fold by stable expression of hLPP-3, resulting in considerable LPA hydrolytic activity. However, as indicated in Fig. 3, A and B, OMPT was able to substantially reverse the effects of hLPP-3 on both colony-forming activity and on apoptosis with an OMPT concentration of 100 nM proving optimal in both assays. We assessed whether OMPT could be a competitive inhibitor of hLPP-3 enzyme activity. Although at high concentrations (10 μM), OMPT modestly inhibited hLPP-3 activity, at the concentrations used in this study (10 and 100 nM), OMPT did not alter the ability of hLPP-3 to hydrolyze LPA (data not presented). The ability of exogenous OMPT to reverse the effects of hLPP-3 suggests that the major effect of hLPP-3 on the growth of ovarian cancer cells was because of hydrolysis of extracellular LPA.

Over time in culture, the stably transfected SKOV3 cell lines demonstrated decreased hLPP-3 levels as assessed by RT-PCR, Western blotting, and ability to hydrolyze LPA in cell supernatants. This may reflect selection against cells expressing high levels of hLPP-3. Thus the stably transfected cells were freshly thawed for most experiments. Strikingly, despite the ability to establish neo-resistant cell lines, we were unable to establish hLPP-3 overexpressing lines in OVCAR-3, HEY, or A2780 ovarian cancer cell lines or in TAg expressing normal ovarian epithelium cells. This failure to establish stable cell lines is compatible with the growth inhibition after transient transfection (Fig. 1A). Intriguingly, SKOV3 constitutively produces very high levels of LPA as compared with other ovarian cancer cell lines (13), potentially contributing to the ability to tolerate LPP-3.

As indicated in Fig. 1C, the ability of the stable hLPP-3-expressing SKOV3 cell lines to form colonies was markedly decreased (5.1-fold, 80% decrease) as compared with neo-resistant cells (P = 0.0002). Thus either transient or stable expression of hLPP-3 markedly decreases the ability of ovarian cancer cell lines to form colonies.

Fig. 4. Transient transfection of hLPP-3 increases apoptosis. A, Forty-eight h after transient transfection with LPP and a GFP-containing vector, cells were harvested and fixed by 0.25% paraformaldehyde in PBS solution followed by propidium iodide (10 μg/ml) DNA staining. Two-color cytometric analysis was performed, and the percentages of hypodiploid cells as an indication of apoptosis was determined using CellQuest software. Primary flow cytometric graphs from SKOV3 cell line transfected with CMV promoter 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 are the mean ± SE of three separate experiments. B, the experiment was repeated with a mutated hLPP-3 (LPP-3M, catalytically inactive) expression vector.
hLPP-3 Markedly Increases Apoptosis in Ovarian Cancer Cells. The decreased ability of ovarian cancer cells to form colonies could either be attributable to decreased cell cycle progression or attributable to increased rates of apoptosis. To assess these possibilities, SKOV3 and OVCAR-3 cells were cotransfected with GFP (to mark transfected cells) and hLPP-3 under either the CMV or hTERT promoters and assessed for cell cycle progression and apoptosis (hypodiploid peak) by staining with propidium iodide. There were no obvious differences in cell cycle progression as indicated by number of cells in G1, S, or G2-M (Fig. 4B) in control or hLPP-3-expressing SKOV3 or OVCAR-3 cells. However, there was a significant and consistent increase in the hypodiploid peak in hLPP-3-expressing cells (P < 0.0001) compatible with an increased rate of apoptosis (Fig. 4A). Thus the decreased ability of hLPP-3-expressing ovarian cancer cells to form colonies is associated with an increased rate of cellular apoptosis. As indicated in Fig. 4B, a mutant hLPP-3, which is unable to hydrolyze LPA (34, 35), did not alter the apoptosis rate, confirming a need for intact enzyme activity in the effect of hLPP-3 on cell death.

hLPP-3 Decreases the Growth of Nontransfected Bystander Cells. As indicated above, overexpression of LPP-3 results in decreased LPA levels in cellular supernatants. Thus by decreasing extracellular LPA, expression of hLPP-3 in one population of ovarian cancer cells could decrease the colony-forming cell activity of bystander nontransfected cells. This would be particularly important if hLPP-3 were used in a gene therapy type of approach where it is difficult to transflect all cells. To assess this possibility, we performed a series of cell mixing assays with hLPP-3-transfected cells and control parental cells.

We initially determined 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). As previously described (Fig. 1, A and B), expression of hLPP-3 either transiently or stably resulted in a marked decrease in colony-forming cell activity. To assess the effects of hLPP-3-transfected cells on the growth of nontransfected cells, we combined equal amounts of hLPP-3-expressing cells and parental cells. Thus the expected number of colonies in the combination experiment would be the number of colonies produced by parental cells plus the number of colonies produced by the transfected cells. As indicated in Fig. 5, A and B, with both transient transfection and with stable cell lines, there was a marked decrease in the number of colonies observed compared with the expected number of the colonies (P = 0.028, P = 0.012). Therefore, hLPP-3-transfected cells were able to decrease the proliferation of parental cells compatible with the effect of hLPP-3 being related to the effects on an extracellular mediator, likely LPA.

hLPP-3 Decreases Tumor Growth of Ovarian Cancer Cells in Vivo. After s.c. injection of SKOV3 cells, tumors developed in 9 of 10 mice as compared with 4 of 10 mice that received injections of hLPP-3-expressing SKOV3 cells. After s.c. injection of SKOV3-IP1 cells, a more aggressive SKOV3 subline developed by i.p. passage of SKOV3 (35), tumors developed in 10 of 10 mice as compared with 5 of 10 mice that received injections of hLPP-3-expressing SKOV3 IP1 cells. Thus the take rate of the tumors expressing hLPP-3 was markedly less than that of the parental lines. In addition to a decreased take rate, as indicated in Fig. 6, the growth rate of both the SKOV3 and SKOV3-IP1, hLPP-3-expressing tumors was markedly decreased as compared with the parental cell lines (note: only mice that developed tumors are used to derive the growth curves; P for both SKOV3 and SKOV3-IP1 < 0.0001, Fig. 6, A and B). At the termination of the study (mandated by tumor size and American Association of Laboratory Animal Care guidelines), the average SKOV3 parental tumor was 0.48 ± 0.15 g in weight, whereas the hLPP-3-expressing SKOV3 tumors averaged 0.13 ± 0.04 g in weight. Similarly, with the SKOV3 IP1 parental tumor, the average weight was 0.44 ± 0.11 g, whereas hLPP-3-expressing tumors had an average weight of 0.12 ± 0.02 g. Thus with both tumor lines, for the animals that developed tumors, the average tumor weight was four times greater in the parental than in the hLPP-3-expressing tumors.

When SKOV3 or SKOV3-IP1 cells were injected into the orthotopic site in the peritoneal cavity, hLPP-3 expression also decreased the take rate [1 of 5 from SKOV3 hLPP-3-injected and 3 of 5 from SKOV3 IP1, hLPP-3-injected mice with all parental cell injections (10 of 10), resulting in tumor growth]. With both SKOV3 and SKOV3 IP1 cells, LPP-3 expression decreased i.p. growth as assessed by both animal weight (P = 0.005, P < 0.0001) and abdominal circumference (P = 0.042, P = 0.001; Fig. 6, C–F).

As indicated above, the hLPP-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 hLPP-3-expressing tumors appeared to enter a more rapid growth phase. As the hLPP-3 construct was not under selective pressure in vivo, it was possible that the eventual increase in growth rate was attributable to loss of hLPP-3. As indicated in Fig. 7, this was indeed the case. After in vivo growth, hLPP-3 levels in the transfected lines were markedly decreased. Even more striking, however, after in vivo growth, hLPP-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 hLPP-3 and transfected hLPP-3. This suggests that a very strong negative selection exists against hLPP-3 expression in ovarian cancer cells in vivo.

**DISCUSSION**

In 2001, there were an estimated 23,400 new cases of ovarian cancer in the United States (41). The majority of these women will eventually die from their disease, at least, in part, because 75–80% of all patients present with late stage disease for which current treatment is inadequate. Late-stage epithelial ovarian cancers grow in an environment composed of ascitic fluid, which we have demonstrated to contain activities that support growth of both primary cultures of epithelial ovarian cancer cells and of epithelial ovarian cancer cell lines in vitro and in vivo (12, 13). One of these factors was originally called ovarian cancer-activating factor (OCAF). It was later established that the major growth factor comprising ovarian cancer-activating factor in ascites is LPA (15). Because LPA increases epithelial...
We were readily able to establish hLPP-3-expressing stable lines on the SKOV3 and SKOV3 IP1 background but not with other ovarian carcinoma cell lines. SKOV3 constitutively produces much higher levels of LPA than the other ovarian carcinoma cell lines (13), potentially contributing to the ability to form stable hLPP-3-expressing lines on this background. An increase in hLPP-3 in the SKOV3 lines was manifest on semiquantitative RT-PCR, Western blotting, and by an increased ability to hydrolyze LPA. The inability to stably express hLPP-3 in other ovarian cell lines is compatible with a strong negative selection for high-level expression of hLPP-3. This selection appears to be particularly powerful in vivo where the hLPP-3-transfected cells as well as the parental cell lines expressed markedly decreased levels of hLPP-3. This suggests that normal tissue culture in the presence of FCS that contains high levels of LPA as well as precursors for LPA production (13) is permissive for expression of hLPP-3.

The data are most compatible with the hypothesis that hLPP-3 exerts its effects on ovarian cancer cells through decreasing extracellular LPA. Both the ability to decrease colony-forming activity and to induce apoptosis was dependent on an intact catalytic activity suggestive of hydrolysis of a free phosphate in glycerol, sphingosine, or ceramide lipids. The likelihood that the target was LPA was supported by the observation that addition of a nonhydrolysable LPA analogue, OMPT (40), to the medium substantially reversed the effects of hLPP-3 on both colony-forming activity and cellular apoptosis. Compatible with this contention, LPA levels were decreased in the supernatants of LPP-3-expressing cell lines. Furthermore, signaling downstream of LPA as indicated by phosphorylation of ERKs was curtailed in hLPP-3-expressing cells. HLP-3 demonstrated a clear bystander effect also consistent with the effects of hLPP-3 being attributable to hydrolysis of an extracellular mediator, likely LPA. This also suggests the potential that hLPP-3 gene therapy could not only alter the growth and survival of transfected or infected cells but also of neighboring cells, resulting in a marked effect on tumor growth.

In summary, our results demonstrate that expression of exogenous hLPP-3 causes apoptosis and growth inhibition in ovarian cancer cell lines in vitro and in vivo. The ability of exogenous OMPT to reverse the effect of hLPP-3 in vitro and a requirement for an intact enzyme activity suggest that the major effect of hLPP-3 on the growth of ovarian cancer cells is because of hydrolysis of extracellular LPA. Taken together, the data suggests that LPA production, metabolism, receptor binding, and downstream signaling pathways warrant additional investigation as targets for therapy in ovarian cancer.


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