Hypoxia Enhances Lysophosphatidic Acid Responsiveness in Ovarian Cancer Cells and Lysophosphatidic Acid Induces Ovarian Tumor Metastasis In vivo

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Abstract

Lysophosphatidic acid (LPA) is elevated in ascites of ovarian cancer patients and stimulates growth and other activities of ovarian cancer cells in vitro. Tissue hypoxia is a critical factor for tumor aggressiveness and metastasis in cancers. We tested whether the asces of ovarian cancer is hypoxic and whether hypoxia influences the effects of LPA on ovarian cancer cells. We found that ovarian ascitic fluids were hypoxic in vivo. Enhanced cellular responsiveness to LPA, including migration and/or invasion of ovarian cancer cells, was observed under hypoxic conditions. This enhancement could be completely blocked by geldanamycin or a small interfering RNA targeting hypoxia-inducible factor 1α (HIF1α). LPA-induced cell migration required cytosolic phospholipase A2 (cPLA2) and LPA stimulates cPLA2 phosphorylation in a HIF1α-dependent manner under hypoxic conditions. Furthermore, we show for the first time that exogenous LPA enhances tumor metastasis in an orthotopic ovarian cancer model and HIF1α expression in tumors. 17-Dimethylaminoethylamino-17-demethoxygeldanamycin (an inhibitor of the heat shock protein 90) effectively blocked LPA-induced tumor metastasis in vivo. Together, our data indicate that hypoxic conditions are likely to be pathologically important for ovarian cancer development. HIF1α plays a critical role in enhancing and/or sensitizing the role of LPA on cell migration and invasion under hypoxic conditions, where cPLA2 is required for LPA-induced cell migration. (Cancer Res 2006; 66(16): 7983-90)

Introduction

Remaining to be one of the most deadly diseases for several decades, ovarian cancer causes ~16,000 deaths in the United States annually (1). Lack of effective early detection, the highly metastatic nature of the disease, and lack of highly effective therapeutic treatment for the late-stage cancer are main reasons for the low survival rate of patients with ovarian cancer (2–5). Thus, to further understand the mechanisms of metastasis and develop novel therapeutics for preventing and/or the treatment of the metastatic disease are pivotal. Although the mechanism of ovarian cancer spread is different from many other solid cancers, ovarian cancer is well known to be a highly metastatic disease (6). Whereas most solid tumors metastasize through intravasation and extravasation of blood vessels, ovarian cancer mainly metastasizes through direct dissemination from the primary site(s) into the peritoneal cavity (7). Our understanding of this process remains to be elusive.

Since our first publication showing that lysophosphatidic acid (LPA) is elevated in ascites from patients with ovarian cancer ~11 years ago (8, 9), numerous reports have been published showing that LPA regulates almost every aspect of ovarian cancer cell biology (reviewed in refs. 10–12). Most of these results are derived from studies using ovarian cancer cell lines in vitro. It has been proposed that LPA plays an important role in the development of ovarian cancer in vivo (10, 12). When human LPA2 (one of the receptors for LPA) is transgenically expressed in mouse ovaries, higher levels of vascular endothelial growth factor (VEGF), isomers of VEGF-A, VEGF receptors 1 and 2, and urokinase-type plasminogen activator (uPA) are produced than that from non-transgenic ovaries, suggesting a potential role of LPA in early development of ovarian cancer (13). However, direct evidence to support the role of LPA in tumorigenesis and/or tumor metastasis of ovarian cancer in vivo has yet to be presented.

Cell migration and invasion are two most important processes in tumor metastasis. We have previously shown that two phospholipase A2 (PLA2) enzymes, the calcium-independent-PLA2 (iPLA2) and cytosolic PLA2 (cPLA2), are involved in laminin-induced haptotactic cell migration (14). The PLA2 family of enzymes catalyzes the hydrolysis of the sn-2 position of phospholipids to generate free fatty acids and lysophospholipids. Whereas iPLA2 is required for laminin-induced LPA production, cPLA2 is directed required for cell migration, possibly related to its ability to produce arachidonic acid (14).

Tumor hypoxia is a common feature of solid tumors and associated with tumor growth, angiogenesis, resistance to apoptosis, and compromise in radiotherapy and chemotherapy, as well as tumor metastasis (15). It becomes a central issue in tumor pathology and cancer treatment. Rapid growth of solid i.p. tumors and large volumes of ascitic fluid characterize ovarian cancer. In particular, large numbers of ovarian tumor cells are present in ascitic fluids. Whereas the hypoxic conditions have been characterized in solid ovarian tumors, the potential hypoxia in human ovarian ascites and its effect on tumor cells have not previously been studied.
Hypoxia-inducible factor 1α (HIF1α) is an essential component in changing the transcriptional response of tumors under hypoxia by regulating transcription of more than 60 genes involved in many aspects of cancer biology, including cell survival, glucose metabolism, cell invasion, and angiogenesis (16). Among them, VEGF, interleukin 8 (IL-8), and uPA are present in human ovarian cancer ascites and have been implicated in cancer angiogenesis and metastasis (17, 18). Interestingly, LPA induces secretion of IL-8, VEGF, and uPA from ovarian cancer cells (19–22).

Based on these previous publications, we tested whether ovarian cancer ascites is hypoxic and whether hypoxic conditions have any effect on actions of LPA in ovarian cancer cells. We have examined the responsiveness of ovarian cells to LPA under hypoxic versus normoxic conditions. Moreover, we used an orthotopic ovarian cancer mouse model to determine the effect on LPA in tumor metastasis in vivo. The potential involvement of HIF1α in LPA-induced cell migration, invasion, and tumor metastasis has been tested both in vitro and in vivo.

Materials and Methods

Reagents. Epidermal growth factor (EGF) and fatty acid–free bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). 1-Oleoyl-2-hydroxyl-sn-glycerol-3-phosphate (18:1-LPA) and 1-myristoyl-2-hydroxy-sn-3-phosphate were purchased from Avanti Polar Lipids (Birmingham, AL). Bio-safe NA was from Research Products International Corp. (Mt. Prospect, IL). 1-(S)-Stearoyl-2-hydroxy-sn-glycerol-3-phosphocholine was purchased from Amersham Pharmacia Biotech (Upssala, Sweden). Geldanamycin was purchased from Alexis Biochemicals (San Diego, CA). Antibodies against phosphorylated cPLA2 (Ser505) and total cPLA2 were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Human collagen I and other extracellular matrix proteins were from Chemicon International (Temecula, CA). 17-Dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) was from Invitrogen (San Diego, CA). HIF1α small interfering RNA (siRNA; sense, 5′-GGCCUCUGUGAUGAGGCUUt-3′; antisense, 5′-AAGCCUCAUCA-CAGAGGCTT-3′) and green fluorescent protein siRNA were obtained from Ambion, Inc. (Austin, TX).

Patients and gas analysis of ascites. Twelve ovarian cancer patients were recruited in this study for gas analysis of ascites at the Chonbuk National University Medical School (Chonju, Chonbuk, Korea). The Institutional Review Board of Chonbuk National University Hospital and the Ethics Committee of Institute for Medical Science, Chonbuk National University Medical School approved the study. The patients were informed about the gas analysis test and the surgical procedure (all patients were over 18 years). The pumps were replaced every 15 days. To test the effect of 17-DMAG, 500 µL of Sigmacote (Sigma-Aldrich Co., St. Louis, MO) was added to the peritoneal cavity. The pumps were first siliconized by putting in 100 mL of Sigmacote (Sigma-Aldrich Co., St. Louis, MO), followed by removal of the liquid and letting it dry. The pumps were replaced every 15 days. To test the effect of 17-DMAG, 500 µL of PBS or 0.8 mg of 17-DMAG in 500 µL of PBS were given i.p., thrice a week. Primary tumors were allowed to develop for 21 days, at which point the mice were sacrificed and secondary metastatic disease was scored on the individual organs depending on the number of secondary foci, the size, and the spread of the disease. The disease score was compared with the number of metastatic foci on each organ and was categorized into three classes: small (<1 mm), medium (<2 mm), and large (>2 mm). The organs scored were bowels, mesentery, pancreas, liver, diaphragm, body wall, lungs, and the thoracic cavity. The primary tumor size was measured using calipers.

Cell culture and exposure to hypoxia. All ovarian cells were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS) at 37°C with 5% CO2. HEY ovarian cancer cells were from Dr. G. Mills (the Cleveland Clinic Foundation, Cleveland, OH). For the hypoxia-exposure experiments, cells were cultured in RPMI 1640 (without FBS) in a hypoxia chamber (1% O2) at 37°C for 12 to 17 hours, and then for different cellular functional assays under normoxic conditions.

Cell migration and invasion assays. Cell migration assays were done under the aerobic condition as previously described (14). Briefly, the bottom of the insert of 24-well modified Boyden’s chamber (Corning Life Sciences, Corning, NY) was coated with 10 µg/mL collagen I and 5.0 × 104 cells in RPMI 1640 were added into the insert. RPMI 1640 containing LPA (5 µmol/L) or EGF (20 ng/mL) was added to the lower chambers. The migration assays were conducted for 4 hours at 37°C. After 4 hours, the insert was washed with 1× PBS and unimmigrated cells were removed by a cotton swab. The lower side of the insert was fixed in 100% methanol for 30 minutes and then stained with hematoxylin for 30 minutes. The excess stain was washed off with H2O. The cells were identified under a microscope and different fields were counted.

Invasion assay was done under aerobic condition as previously described (23). Briefly, matrigel-coated 24-well Boyden’s invasion chambers (Becton Dickinson Labware, Bedford, MA) were used and 5.0 × 104 cells (SKOV3, HEY, or other ovarian cancer cells) in RPMI 1640 were added into the insert. RPMI 1640 containing LPA (5 µmol/L) and EGF (20 ng/mL) was added to the lower chambers. The invasion assays were conducted for 18 hours at 37°C. The same method for fixing and counting cells as described above (for cell migration) was used. For siRNA experiments, before doing invasion assays, cells were transfected with green fluorescent protein (100 nmol/mL) siRNA or HIF1α (100 nmol/mL) siRNA for 48 hours using LipofectAMINE 2000 according to the protocol of the manufacturer.

Western blot analysis. Protein samples were loaded on a 7.5% to 12% SDS-PAGE gel. After electrophoresis at 120 V for 90 minutes, separated proteins were transferred to polyvinylidene difluoride membranes (Amerham Pharmacia Biotech, Piscataway, NY) by the wet transfer method (300 mA for 180 minutes). Non-specific sites were blocked with 5% nonfat milk in TBST buffer [25 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, and 0.1% Tween 20] for 1 hour and the blots were then incubated overnight at 4°C with specific antibodies. Binding of the specific antibodies was visualized by chemiluminescence.

The orthotopic mouse ovarian cancer model. All the animal experiments were done according to the Cleveland Clinic Foundation, Biological Resources Unit Protocol #ARC 6661. Nu/Nu mice were obtained from Charles River Labs (Wilmington, MA) and irradiated with 250 to 500 rads. The following day, 5×103 SKOV3 cells were injected s.c. into both the rear flanks of the irradiated mice. Tumors were allowed to develop for nearly 2 to 3 weeks until they were about 15 to 20 mm² in size. These mice were subsequently sacrificed and 3 × 3 × 2 mm portions of the tumor were excised and orthotopically transplanted onto the ovaries of additional Nu/Nu female mice using 7-0 Prolene suture. At this point, Alzet microosmotic pumps (model 1002) containing 100 µL PBS (for control) or 4 mmol/L LPA (in 100 µL PBS) were placed into the peritoneal cavity. The pumps were first siliconized by putting in 100 µL of SigmaD repeat (Sigma-Aldrich Co., St. Louis, MO), followed by removal of the liquid and setting it dry. The pumps were replaced every 15 days. To test the effect of 17-DMAG, 500 µL of PBS or 0.8 mg of 17-DMAG in 500 µL of PBS were given i.p., thrice a week. Primary tumors were allowed to develop for 21 days, at which point the mice were sacrificed and secondary metastatic disease was scored on the individual organs depending on the number of secondary foci, the size, and the spread of the disease. The disease score was compared with the number of metastatic foci on each organ and was categorized into three classes: small (<1 mm), medium (<2 mm), and large (>2 mm). The organs scored were bowels, mesentery, pancreas, liver, diaphragm, body wall, lungs, and the thoracic cavity. The primary tumor size was measured using calipers.

Immunostaining of primary tumors. Primary tumors were removed after sacrificing the mice from the different groups. Frozen sections were made from the primary tumors, stained with rabbit anti-HIF1α (Abcam, Inc., Cambridge, MA) at a dilution of 1:40, and color detected with rabbit goat-horseradish peroxidase.

Statistical analysis. Student’s t test was used for statistical analyses and P < 0.05 was considered to be significant.

Results

Ovarian cancer ascites is hypoxic. We hypothesized that when compared with organ surfaces and peritoneal walls, which are enriched with blood vessels, ascitic fluids in the peritoneal cavity...
are hypoxic. To test this, we determined the O$_2$ pressure in ascitic fluids from patients with ovarian cancer. As shown in Table 1, we found that ascitic fluids were hypoxic, with ~50% reduction of the O$_2$ pressure and ~87% reduction of oxygen content (O$_2$CT) measured in ascites when compared with normal blood gas values. On the other hand, the CO$_2$ pressure was increased by ~10% whereas pH values of cancer ascitic fluids were not significantly changed (Table 1).

**Hypoxic conditions enhanced LPA-induced cell migration.**

Cell migration is one of the important steps of tumor metastasis and we have shown that LPA is a potent chemotactic factor for ovarian cancer cells (14, 24, 25). Here, we determined the effect of hypoxia on the LPA-induced cell migration. Ovarian cancer cells usually migrate and invade into the peritoneal wall, which is a normal, well-vascularized tissue as compared with abnormal tumor tissue and ascites. Thus, we hypothesize that ovarian cancer cells are first situated in a hypoxic condition (in ascites, which is hypoxic), but then migrate to and invade organs under rather normoxic conditions. We have found that hypoxia is not cytotoxic for ovarian cancer cells for at least 12 to 24 hours (data not shown). Therefore, we pretreated ovarian cancer cells in hypoxia and then conducted the cell migration and invasion (see below) assays under normoxic conditions. LPA (100 nmol/L to 10 μmol/L) potently induced migration to collagen I in SKOV3 ovarian cancer cells (Fig. 1A). Interestingly, hypoxia pretreatment resulted in an increased responsiveness to LPA without significantly changing the basal-level haptotactic cell migration toward collagen I (in the absence of LPA; Fig. 1A and B).

HIF1α is one of the most important factors involved in hypoxia-induced cellular activities. It activates the transcription of genes that are involved in crucial aspects of cancer biology, including angiogenesis, cell survival, glucose metabolism, and invasion (16). Geldanamycin, an inhibitor of heat shock protein 90 (Hsp90), has been shown to be an effective inhibitor of HIF1α by inducing degradation of HIF1α (26–28). Interestingly, whereas geldanamycin (10 μmol/L) did not significantly affect cell migration under normoxic condition, it reduced the basal level of cell migration and completely blocked the enhanced LPA responsiveness in hypoxia-pretreated cells (Fig. 1B). These data strongly suggest that Hsp90 and HIF1α are likely to be involved in the enhanced cellular responsiveness to LPA in ovarian cancer cells under hypoxic conditions. EGFR, a growth factor involved in ovarian cancer (29), displayed less chemotactic activities than LPA, and the EGFR-induced cell migration was insensitive to geldanamycin in either normoxia- or hypoxia-treated cells as assessed by the fold stimulation when compared with controls under the same condition (Fig. 1B), suggesting that hypoxia conditions more specifically affect cellular responsiveness to LPA in SKOV3 cells.

The median concentration of LPA is 19.4 μmol/L in ascites with 1 to 5 μmol/L of 18:1 LPA (30). We observed that 5 μmol/L of 18:1-LPA induced a maximal differential cell migratory response (1.9-2.7-fold difference) in SKOV3 cells when the hypoxic versus

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**Table 1. Gas analysis of ovarian cancer ascites (n = 12)**

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>$P_{CO2}$ (mm Hg)</th>
<th>$P_{O2}$ (mm Hg)</th>
<th>O$_2$CT (%)</th>
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<tbody>
<tr>
<td>Average</td>
<td>7.40</td>
<td>42.77</td>
<td>45.65</td>
<td>2.52</td>
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<tr>
<td>SD</td>
<td>0.08</td>
<td>8.02</td>
<td>8.68</td>
<td>0.55</td>
</tr>
<tr>
<td>Max</td>
<td>7.50</td>
<td>54.00</td>
<td>57.00</td>
<td>3.10</td>
</tr>
<tr>
<td>Min</td>
<td>7.31</td>
<td>35.10</td>
<td>37.40</td>
<td>1.50</td>
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**NOTE:** Normal blood gas values: pH, 7.35 to 7.45; partial pressure of carbon dioxide ($P_{CO2}$), 35 to 45 mm Hg; partial pressure of oxygen ($P_{O2}$), 75 to 100 mm Hg; oxygen content (O$_2$CT), 15% to 23%. The age range of these patients was 44 to 55 years (50.17 ± 4.02 years).
normoxic conditions were compared. Thus, in most experiments conducted in this work, we have used 5 μmol/L of 18:1-LPA, unless specified.

Hypoxic conditions enhanced LPA-induced cell invasion. To further assess the effects of hypoxia on the metastatic potential of ovarian cancer cells, we conducted in vitro invasion assays. The enhanced LPA-induced cell invasion was observed in both SKOV3 and HEY ovarian cancer cells (Fig. 2A and B). A similar augmentation of LPA effect was observed in hypoxia-pretreated cells. In addition, similar to the migratory activity, LPA-induced cell invasion was sensitive to geldanamycin in hypoxia-treated but not in normoxia-treated cells as assessed by the fold stimulation (Fig. 2A and B). The EGF-stimulated cell invasion, on the other hand, was significantly reduced in hypoxia-treated SKOV3 cells (Fig. 2A). Other ovarian cancer cells, such as ovca420, also responded to LPA in cell invasion (data not shown). To more specifically address the potential involvement of HIF1α, we employed a siRNA strategy (Fig. 2C). siRNA against HIF1α, but not the control siRNA against GFP, reduced the expression of HIF1α and LPA-induced cell invasion under hypoxia conditions (Fig. 2C, a and b).

cPLA2, but not iPLA2, activity is required for cell migration to collagen I. We have previously shown that both cPLA2 and iPLA2 activities are required for cell migration to laminin in HEY ovarian cancer cells (14). Exogenously added LPA can replace the requirement of iPLA2, but not cPLA2, indicating iPLA2 is mainly involved in LPA production and cPLA2 is directly involved in cell migration (14). Moreover, we found that laminin, but not collagen I, induces LPA production. To determine the role of cPLA2 and iPLA2 in migration of SKOV3 cells to collagen I, cells were pretreated

Figure 2. Effect of LPA on invasion of SKOV3 and HEY cells. A, effect of geldanamycin (10 μmol/L) on LPA-induced invasion of SKOV3 cells under normoxic and hypoxic conditions. B, effect of geldanamycin (10 μmol/L) on LPA-induced invasion of HEY cells under normoxic and hypoxic conditions. C, effect of siRNA targeting HIF1α on LPA-induced invasion of SKOV3 cells (a) and HIF1α expression (b) detected by Western blot analyses. Cell invasion assays and Western blotting were conducted as described in Materials and Methods. Numbers on top of columns indicate the fold increase of cell migration over control under the same conditions. Columns, mean for three independent replicates; bars, SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001, Student’s t test (two tailed).
with inhibitors of cPLA2 and iPLA2. As shown in Fig. 3A, cell migration to collagen I was almost completely inhibited in the presence of AACOCF3 (100 μmol/L), an inhibitor for both cPLA2 and iPLA2. In contrast, HELSS (1 μmol/L), an inhibitor specific for iPLA2, did not have significant effect on migration of SKOV3 cells to collagen I. Moreover, we found that LPA (5 μmol/L, 24 hours) enhanced cPLA2 expression in hypoxia-treated, but not in normoxia-treated, SKOV3 cells (Fig. 3B, a). This increase in cPLA2 expression is independent of HIF1α, as inhibition by geldanamycin did not result in significant decrease in expression of cPLA2 in hypoxia (Fig. 3B, a). On the other hand, LPA-induced (5 μmol/L for 30 minutes) cPLA2 activation (as measured by cPLA2 phosphorylation) in hypoxia-treated cells was geldanamycin sensitive, suggesting that HIF1α is involved in this activation (Fig. 3B, b).

LPA stimulates ovarian cancer metastasis in vivo in an orthotopic mouse model. Despite the fact that numerous articles describing the potential role of LPA in ovarian cancer cells have been published since our first report on ovarian cancer ascites LPA in 1995 (8, 9) and the detection of elevated levels of LPA in ascites of ovarian cancer patients (8, 9, 30, 31), the effect of LPA on ovarian tumor growth and/or metastasis in vivo has not been directly shown. To address the role of LPA in tumor development in vivo, we used an orthotopic model of ovarian cancer metastasis in mice with SKOV3 cells based on a mouse model using RMG1 clear cell ovarian carcinoma cells (ref. 32; see Materials and Methods). LPA was delivered i.p. in release-controlled osmotic pumps (Alzet Osmotic Pumps, Cupertino, CA). LPA exerted a significant effect on tumor metastasis (Fig. 4A and B). To quantify metastatic loci, we arbitrarily divided the metastatic colonies into three groups by their size, small (<1 mm), medium (<2 mm), and large (>2 mm), and counted each of the colonies. LPA-treated mice had metastases to the mesentery, the bowel, the liver, the body wall, and the diaphragm, which highly resembles the human ovarian cancer disease. Orthotopically implanted tumors derived from SKOV3 cells in the PBS control mice developed tumors in ovaries, but metastases were minimally observed in the PBS control group (Fig. 4B). This is the first direct evidence of the role of LPA in ovarian tumor metastasis. The effect of LPA on tumor growth at the primary sites (the tumor-transplanted ovaries) was minimal and no statistical significant difference in tumor sizes was observed between the control and the LPA-treated groups. Although it has been shown that SKOV3 cells constitutively actively produce LPA in vitro under tissue culture conditions (33), we did not detect any significant amount of LPA in either blood or peritoneal washings in the PBS control mice implanted with SKOV3 tumors (data not shown), suggesting that SKOV3 cells may not be able to produce LPA in vivo in mice.

The established mouse model described above provides a very useful system to test potential therapeutic reagents for the treatment of ovarian cancer. To confirm the importance of HIF1α in LPA-induced tumor metastasis, we tested the effect of 17-DMAG (a water-soluble geldanamycin derivative, which is more suitable for in vivo studies) on blocking metastasis in vivo. At a concentration of 40 mg/kg body weight, 17-DMAG (administered thrice per week) significantly inhibited metastasis induced by LPA (Fig. 4A and B). These results suggest that
HIF1α represents a useful target for the treatment of ovarian cancer.

**LPA stimulates HIFα expression in tumor tissues.** To further confirm the role of HIFα in LPA-induced tumor metastasis, we examined the expression of HIFα in tumor tissues collected from LPA- and PBS-treated mice. Immunocytochemistry staining clearly showed that LPA greatly enhanced the HIF1α expression in tumor sections (Fig. 5). These results provide the first line of evidence that LPA is a regulator of HIFα expression in vivo and suggest that the enhanced responsiveness of cancer cells to LPA under hypoxic conditions may be directly related to the ability of LPA to upregulate and/or stabilize the expression levels of HIF1α.

**Discussion**

**LPA stimulates metastasis of ovarian cancer in vivo.** Eleven years after we reported that LPA was a growth factor in ovarian cancer ascites (8, 9), we provide the first line of direct evidence here that LPA stimulates ovarian cancer metastasis in vivo using an orthotopic mouse model (Fig. 4), which further supports LPA as a critical and novel therapeutic target for ovarian cancer (10, 12, 34). In most previous ovarian cancer mouse work, either s.c. or i.p. injection was used (35, 36). The i.p. injection models mimic human ovarian cancer at late stages but cannot accurately assess tumor metastasis from ovaries. The model that we used here clearly distinguishes tumors at the primary site (the ovary) and at the secondary sites, and the tumor spread closely resembles the human disease. Our experiments using 17-DMAG provides proof-of-principle evidence that this mouse model can be very useful in testing therapeutic reagents targeting metastatic ovarian cancer. Our results are also consistent with a previous report showing the antitumor activity of 17-DMAG in mouse model studies in metastatic pancreatic carcinoma, s.c. xenograft melanoma, and small-cell lung carcinomas (37).

For the studies reported in this article, pumps were filled with 100 µL of a 4 mmol/L LPA solution and implanted for 14 days. This effectively delivers 1 nmol LPA/h per mouse. Considering that the mouse peritoneum has an average volume of 3 mL, this will translate to a concentration of LPA in the peritoneum of ~0.4 µmol/L/hr. Taking LPA degradation in vivo into consideration, the
actual concentrations per mice would be in the low nanomolar range. We measured LPA concentrations in mice using the mass spectrometry-based method and indeed found that the LPA concentrations were in the low nanomolar range. However, due to the sensitivity limitations of the instrumentation used, LPA measurements in this concentration range were not highly accurate or reproducible. Therefore, these data were not presented. Nevertheless, our estimate that LPA concentrations are in the 10 to 100 nmol/L range is consistent with our experimental results. We have found that LPA, at as low as 10 nmol/L, induces migration and/or invasion of ovarian cancer cells in vitro (25). We have also found that LPA at these low concentrations minimally affects primary ovarian tumor growth as reported in the current manuscript. This is consistent with our published results (8, 9) and with results reported by other laboratories showing that the mitogenic activity of LPA usually requires higher concentrations (1-20 μmol/L).

**Human ovarian cancer ascites is hypoxic and hypoxia enhances LPA effects in ovarian cancer cells.** Hypoxia has been known as a key regulatory factor in tumor development. We have reported here, for the first time, that cancer ascites is hypoxic, which is an important factor to be considered in understanding the mechanism of tumorigenesis and metastasis of cancers, as well as in therapeutic treatment, because hypoxia has been shown to increase resistance to chemotherapy and radiation therapy (15, 38).

The rapidly growing and disseminated ovarian cancer cells in the peritoneal cavity encounter hypoxic conditions. In addition to surviving under these conditions, they acquire the ability to migrate and invade through extracellular matrix proteins. We provide evidence in this work to show that ovarian malignant tumor cells gain an enhanced responsiveness to LPA (one of the important ovarian cancer stimulating factors) under hypoxic conditions. The hypoxia-enhanced effect may be LPA specific in tumor cells because the cellular responsiveness to EGF, another important growth factor for ovarian cancer, is not increased under hypoxic condition. We and others have shown that ovarian cancer ascites contains elevated levels of LPA (8–10, 30, 31, 39); thus, our findings may have important pathologic significance.

**The molecular mechanisms of hypoxia enhancement of LPA effects.** We provide some insightful information on the molecular mechanisms of the enhancement effect of hypoxia, although extensive mechanistic studies are warranted for our further understanding of this process. In vivo, hypoxia may stimulate tumor metastasis via increase of both LPA production and cellular responsiveness to LPA. One of our important findings is that LPA increases HIF1α expression in vivo, which may represent the major mechanism of enhanced LPA effects. Our data suggest that HIF1α plays a central role in increased cellular responsiveness to LPA under hypoxic conditions. Targeting the Hsp90-HIF1α axis for cancer therapy has been very attractive (16, 37, 40). A recent work by Kamal et al. (41) shows that tumor Hsp90 is present in multichaperone complexes whereas Hsp90 from normal tissues is in a latent, uncomplex form. An analogue of geldanamycin, 17-AAG (or 17-DMAG) has much higher affinity to the Hsp90 in a complex form than in an uncomplex form, which may confer an inhibition with tumor selectivity. To our knowledge, our data have made the first connection between the LPA signaling pathways and the HIF1α signaling system in cancer.

We show here that LPA stimulates geldanamycin-sensitive cPLA2 activation, which is required for cell migration under hypoxic conditions, although LPA-induced up-regulation of cPLA2 expression is not geldanamycin sensitive. cPLA2 activation and its involvement in cell migration have previously been reported almost exclusively in the immune system and noncancerous cell types involved in inflammation, including hematopoietical cells (42), endothelial cells (43, 44), fibroblasts (45), and smooth muscle cells (46, 47). We have first reported that cPLA2 activity is required for LPA-induced cell migration in ovarian cancer cells, which is an essential component of cell invasion and tumor metastasis (14, 25). Although substantial work has been conducted in the PLA2 field,
ovarian cancer cellular responses to LPA and LPA stimulates LPA actions and its role in ovarian cancer warrants further studies. In summary, we have shown that hypoxia conditions enhance ovarian cancer cellular responses to LPA and LPA stimulates LPA actions and its role in ovarian cancer warrants further studies. Our work indicates that cPLA2 is a signaling molecule of therapy.


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