Research Article

Lysophosphatidic Acid Is Constitutively Produced by Human Peritoneal Mesothelial Cells and Enhances Adhesion, Migration, and Invasion of Ovarian Cancer Cells

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Abstract

Lysophosphatidic acid (LPA) is both a potential marker and a therapeutic target for ovarian cancer. It is critical to identify the sources of elevated LPA levels in ascites and blood of patients with ovarian cancer. We show here that human peritoneal mesothelial cells constitutively produce LPA, which accounts for a significant portion of the chemotactic activity of the conditioned medium from peritoneal mesothelial cells to ovarian cancer cells. Both production of LPA by peritoneal mesothelial cells and the chemotactic activity in the conditioned medium can be blocked by HELSS [an inhibitor of the calcium-independent phospholipase A_2 (iPLA$_2$)] and AACOCF$_3$ [an inhibitor of both cytosolic PLA$_2$ (cPLA$_2$) and iPLA$_2$]. Moreover, cell-based enzymatic activity assays for PLA$_2$ indicate that peritoneal mesothelial cells have strong constitutive PLA$_2$ activity. Receptors for LPA, LPA$_2$, and LPA$_3$ are involved in the conditioned medium–induced chemotactic activity. Invasion of ovarian cancer cells into peritoneal mesothelial cells has also been analyzed and shown to require PLA$_2$, LPA receptors, and the mitogen-activated protein/extracellular signal-regulated kinase kinase/extracellular signal-regulated kinase signaling pathway. Thus, we show here, for the first time, that human peritoneal mesothelial cells constitutively produce bioactive lipid signaling molecules, such as LPA, via iPLA$_2$ and/or cPLA$_2$ activities. Conditioned medium from peritoneal mesothelial cells stimulate migration, adhesion, and invasion of ovarian cancer cells, and may play similar roles *in vivo*. (Cancer Res 2006; 66(6): 3006-14)

Introduction

The "seed and soil" hypothesis of cancer spread that was proposed more than a century ago (1) has been tested and is supported by many lines of evidence generated by modern molecular and cell biology. Ovarian cancer preferentially metastasizes to the peritoneal cavity, including to organs in the cavity and to the peritoneal walls (2). Thus, the interactions between ovarian cancer cells and peritoneal mesothelial cells may play important roles in migration, attachment, and invasion of ovarian cancer cells to the peritoneal mesothelium layer. Several adhesion molecules, their receptors, matrix-degrading enzymes, growth factors, and cytokines are involved in interactions between peritoneal mesothelial cells and tumor cells (3–5).

We have previously reported that human ovarian cancer ascites contains high levels of biologically active lipid factors, such as lysophosphatidic acid (LPA; refs. 6–8). LPA levels are elevated in the blood and ascites of patients with ovarian cancer (6–11). LPA stimulates adhesion, migration, and invasion of ovarian cancer cells in culture (12–23). Two LPA receptors, LPA$_2$ and LPA$_3$, have been implicated in LPA-induced cell migration for different ovarian cancer cell lines (13, 19). LPA$_1$ is involved in cell migration in other cell types (24, 25). Malignant transformation resulted in aberrant expression of LPA$_2$ and LPA$_3$ in ovarian cancer tissues and the ratio of LPA$_2$/LPA$_1$ increased markedly during malignant transformation (26, 27). Thus, LPA is not only a potential diagnostic marker but also an important and novel therapeutic target for the treatment of ovarian cancer. The strategies to eradicate the tumor-promoting effect of LPA may include reduction of production or increase of LPA degradation, development of and testing reagents blocking the receptors for LPA, identification of the downstream signaling target(s) of LPA, and evaluation of the efficacy of blocking these targets in the treatment of ovarian cancer both *in vitro* and *in vivo* (17, 28).

LPA can be produced by activated platelets (29, 30) or by growth factor–stimulated fibroblasts and injured cells (30). LPA is also produced by ovarian cancer cells themselves under certain conditions (9, 13, 28, 31, 32). Although multiple enzymes may produce LPA (27–32), the role of lysophospholipase D in extracellular LPA production has been more extensively studied in recent years (33–38). However, despite these efforts, the source(s) of and the enzymes involved in the elevated LPA in the ascites and blood of patients with ovarian cancer remains a critical issue to be further investigated.

Materials and Methods

Reagents. Cytosolic phospholipase A_2 (cPLA$_2$) antibodies were from Upstate Cell Signaling Solutions (Lake Placid, NY) or Cell Signaling Technology (Beverly, MA). 18:1 LPA was purchased from Avanti Polar Lipids (Birmingham, AL). 1-*O*-(6-Dabcyl-Aminohexanoyl)-2-O-(6-(12-BODIPY-Dodecanoyl)Aminohexanoyl)-sn-3-Glyceryl Phosphatidyl-choline (DBPC) was prepared as described below and is from Echelon Biosciences (Salt Lake City, UT). (*E*)-6-(bromomethylene)-3-[1-Naphthalenyl-(2*H*-tetrahydro-pyran-2-one)] (HELSS) and AACOCF$_3$ were from Calbiochem (San Diego, CA). Akt inhibitor and PD98059 were from Calbiochem. Small interfering RNAs (siRNA) specific for LPA receptors LPA$_{1-3}$ were from Ambion (Austin, TX). Chemically modified siRNAs against green fluorescent protein (GFP; a negative control) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; a positive control) were from Genepharma (Shanghai, PR China). The targeting sequences for GFP and GAPDH are GCAAGCUGACCCUGAA-GUUC and CUCAUUUCCUGGUAUGACA, respectively.

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Cell culture and conditioned medium preparation. Primary human peritoneal mesothelial cells LP9 and LP3 were obtained from Coriell Cell Repository (Camden, NJ) and cultured in Medium 199 in Earle balanced salt solution/MCDB110, 1:1 mixture containing 2 mmol/L L-glutamine, 15% fetal bovine serum (FBS), 10 ng/mL epithelial cell growth factor, and 0.05 μg/mL hydrocortisone. Cells were used between the first and sixth passages. LP9 and LP3 were isolated from two patients with ovarian cancer. The species of origin were confirmed by electrophoretic analyses of nucleoside phosphorylase, glucose-6-phosphate dehydrogenase, and lactate dehydrogenase isoenzyme, and by chromosome analysis. Similar to normal human mesothelial cells, these two primary cell lines expressed insulin-like growth factor I, insulin-like growth factor–binding protein 3, and insulin-like growth factor I receptor (39). Ovarian cancer cell lines SKOV3 and HEY were cultured in RPMI 1640 containing 10% heat-inactivated FBS. To prepare conditioned medium, subconfluent cultures of peritoneal mesothelial cells were washed thrice with PBS and incubated with serum-free RPMI 1640 for different times. Conditioned medium were collected, filtered through a 0.22 μm filter, and stored at −80°C until use.

Migration and adhesion assays. Cell adhesion and migration of ovarian cancer cells in response to conditioned medium were assayed in 96-well plates or using modified Boyden chambers with 8 μm pore size polycarbonate polyvinylpyrrolidone-free Nucleopore filters as described previously (13). The lower phase of the filter was coated with collagen I or laminin at a concentration of 10 μg/mL. Conditioned medium of peritoneal mesothelial cells (600 μL) was added to the lower chamber as the chemoattractant. Ovarian cancer cells were serum-starved overnight, and 1×10^5 cells in 100 μL serum-free RPMI 1640 was added to the upper chamber.

Cell-based enzymatic activity assays of PLA_2. The original synthesis (40) of DBPC was modified to improve yields and permit access to a variety of phospholipid head groups. The new enzyme-assisted synthesis was based on a route developed for preparing photoactive phosphatidic acid derivatives (41). Briefly, following double esterification of commercially available *sn*-glycero-3-phosphocholine with 6-dabcyl-aminohexanoic acid, the *sn*-2 acyl chain was removed by selective enzymatic hydrolysis with cobra venom PLA_2. Next, the resulting LPA analogue was reesterified with 12-(Boc-amino)dodecanoic acid. The carbamate was then removed by hydrolysis, and the resulting amino group was amidated using the activated ester C5-BODIPY, SE (Molecular Probes, Eugene, OR) to give DBPC. Full synthetic details will be published in due course.[5] The substrate DBPC was developed as a substrate for all PLA_2s [secreted PLA_2s ($sPLA_2$s), $cPLA_2$s, and calcium-independent PLA_2 ($iPLA_2$s)]. sPLA2 from different sources all use DBPC as a substrate. The substrate has also been used to measure ligand (bradykinin)-induced $cPLA_2$ activation (40).

For cell-based assays, cells were collected, washed, and resuspended in PBS and loaded on glass slides for 30 minutes to allow attachment. The cells were incubated with 1 μg DBPC (dissolved in DMSO in a minimal volume and then diluted in 30 μL PBS) for 15 minutes, and then washed twice with PBS. Cells were imaged using a Leica fluorescence microscope with excitation and emission wavelengths of 488 and 515 nm, respectively. Fluorescence intensity of each picture was measured with Image-Pro software.

Invasion assay using the Electric Cell-Substrate Impedance Sensing system. Invasion assays were conducted using the Electric Cell-Substrate Impedance Sensing (ECIS) system (Applied Biophysics, Inc., Troy, NY; refs. 42, 43). Peritoneal mesothelial cells were cultured in 8W1E ECIS arrays (Applied Biophysics), where each well for cell culture contains a small gold film circular electrode (5×10^{-4} cm^2) and a larger (0.15 cm^2) counter electrode. The electrodes were connected to the ECIS 1600R instrument that applies a small AC current (<1 μA at 4,000 Hz) between the two electrodes using culture medium as the electrolyte. The instrument monitors both the amplitude and phase of the signal and from this information reports the impedance, resistance, and capacitance of the small electrode treating the system as a simple RC series circuit. The source of the impedance has been shown to be both due to the constricted current flow in the spaces beneath the basal membrane and the electrode surface and to the resistance in the paracellular path between adjacent cells (the barrier function; ref. 44). The experiments were conducted on wells where the electrode resistance had achieved a steady-state value >5,000 mol/L of steady-state resistance.

Lipid extraction and analysis. Lipids were extracted and analyzed as described previously (8). Electrospray ionization-mass spectrometry and tandem mass spectrometry analyses were done using a Micromass Quattro Ultima Quadrupole Mass Spectrometer equipped with an electrospray ionization source (Micromass, Beverly, MA) in the Proteomics Core Facility at the Cleveland Clinic Foundation.

Transfection of siRNAs and detection of LPA receptors by reverse transcription-PCR analysis. For transient transfection, cells were cultured in six-well plates overnight and transfected with siRNAs against LPA receptors $LPA_{1\text{-}3}$ using LipofectAMINE 2000 (Invitrogen Life Technologies, Carlsbad, CA) according to the instructions of the manufacturer. The expression levels of $LPA_{1\text{-}3}$ were detected by semiquantitative reverse transcription-PCR. In brief, total RNA was extracted from cells and reverse transcribed. Derived cDNAs were used as a template in reverse transcription-PCR. Primer sequences for $LPA_{1\text{-}3}$ are as follows: LPA_1, 5′-CCCAAGCTTATGGCTGCCATCTCTACTTC and 5′-CGCGGATCCC-TAAACCACAGAGTGGTC; LPA_2, 5′-CCCAAGCTTATGGTCATCATGGGC-CAG and 5′-CGCGGATCCCTAAAGGGTGGAGTC-CATCAGT; and LPA_3, 5′-CCGGAATTCAAATGAGTGTCACTATGAC and 5′-CGCGGATCCT-TAG-GAAGTGCTTTTATTGCAG. GAPDH was amplified in a separate tube as a housekeeping gene with primers 5′-GAAGGTGAAGGTCGGAGT and 5′-GAAGATGGTGATGGGATTTC.

Results

LPA in the conditioned medium from human peritoneal mesothelial cells induces migration of ovarian cancer cells. Although protein factors have been shown to be involved in peritoneal mesothelial cell–induced migration of ovarian cancer cells, it is unknown whether bioactive lipid factors are also involved in this process. We tested the effect of the conditioned medium from two primary human peritoneal mesothelial cells, LP9 and LP3 (isolated from two patients with ovarian cancer) on migration of SKOV3 and HEY ovarian cancer cell lines.

Conditioned medium collected after incubation of LP9 cells in serum starvation medium for 24 hours (24 hr-CM) induced migration of both SKOV3 (~11-fold increase in migration to collagen I compared with treatment with control medium) and HEY (~10-fold increase in migration to laminin compared with treatment with control medium) cells (Fig. 1*A*). This effect is mainly chemotactic rather than chemokinetic, because when the conditioned medium was added to the upper chamber together with the ovarian cancer cells, only a 2-fold increase in migration of SKOV3 cells to the lower chamber was observed (data not shown). Similarly, the 24 hr-CM from LP3 cells induced migration of SKOV3 cells by ~18-fold (Fig. 1*A*). These results are consistent with previous reports (45) and confirm that peritoneal mesothelial cells are able to produce and secrete soluble chemotactic factor(s) promoting ovarian cancer cell migration.

To determine the kinetics of the chemotactic activity by peritoneal mesothelial cells, we collected conditioned medium from LP9 at different time points after they were cultured in serum starvation medium and tested their effects on migration of SKOV3 cells. The chemotactic activity in the LP9 conditioned medium was detectable from 1 hr-CM (~2.7-fold increase in cell migration) and continued to increase until 24 hours. At 48 hours, the activity was less than that of 24 hours (Fig. 1*A*, *right*). Thus, 24 hr-CM was used in most of our subsequent experiments.

[5] T. Rose and G. Prestwich, unpublished data.

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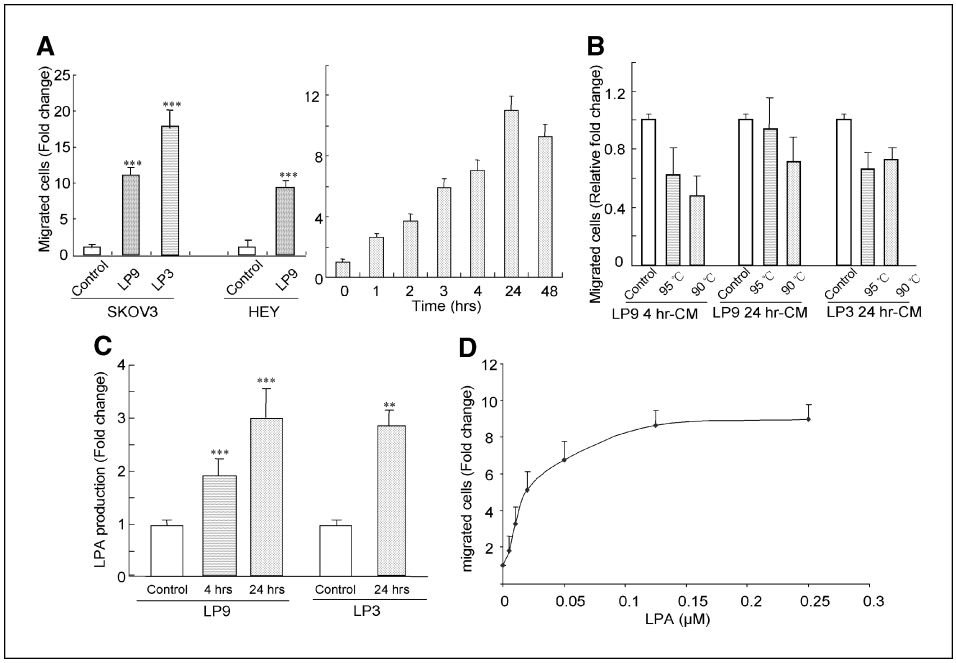

Figure 1. The conditioned medium from peritoneal mesothelial cells stimulate migration of ovarian cancer cells and contain LPA, which is a chemoattractant for ovarian cancer cells. *A, left,* effect of 24 hr-CM from LP9 or LP3 cells on SKOV3 or HEY cell migration. Conditioned medium from LP9 or LP3 cells were collected after incubation of the cells in serum-free medium for 24 hours and used as the chemoattractant (600 μL in each of the lower chambers of the Transwell plate) for the migration of SKOV3 or HEY cells. Medium without contacting cells was used as the control. ***, $P < 0.001$ versus the corresponding control (Student's *t* test). *Right,* migration of SKOV3 cells in response to conditioned medium of LP9 collected at different time points from 1 to 48 hours. *B,* heat-insensitive chemotatic activity in the conditioned medium of peritoneal mesothelial cells. Four and 24 hr-CM from LP9 or LP3 cells were heated at 95°C for 5 minutes or at 90°C for 10 minutes. The heat-insensitive chemotactic activity in the conditioned medium for SKOV3 cell migration was then evaluated using the Transwell assays as described in (*A*) and in Materials and Methods. The relative fold changes in migration were calculated by setting the cell migration in response to conditioned medium without heat treatment at 1.0. Results are from three independent experiments. *C,* mass spectrometric assays of total LPA levels in the 4 and 24 hr-CM from LP9 or LP3 cells. **, $P < 0.01$; ***, $P < 0.001$, versus the control (Student's *t* test). The LPA in 24 hr-CM from LP9 was ~80 nmol/L/10^6 cells/24 hours. *D,* dose effect of synthetic 18:1-LPA on SKOV3 cell migration. 18:1-LPA was added to the lower chamber of the Transwell plates containing ovarian cancer cells in the upper chamber. All experiments were repeated at least thrice.

To test whether bioactive lipid factor(s) is involved in the chemotactic activity of the conditioned medium, we used heat-inactivation to eliminate heat-sensitive protein factors in the conditioned medium. The LP9 conditioned medium were treated at 90°C for 10 minutes or 95°C for 5 minutes before addition to the ovarian cancer cells. Interestingly, >47% and 71% of the chemotactic activity remained after heat-treatment of 4 and 24 hr-CM, respectively (Fig. 1*B*). Similarly, >66% of the migratory activity was unaffected by heat treatment of 24 hr-CM from LP3.

To determine which factor in the heated conditioned medium was responsible for the chemotactic activity, we analyzed lysophospholipid concentrations in the conditioned medium using an electrospray mass spectrometry method that we had developed previously (8). We detected 2- to 3-fold increase in LPA levels in 4 and 24 hr-CM from LP9, when compared with control medium. LPA was also present in conditioned medium from LP3 (Fig. 1*C*). The production of LPA seemed to be constitutive because no additional stimulus was required for LPA production. It remains possible, however, that LPA production by peritoneal mesothelial cells might be inducible under certain conditions that we have not tested. To test the function of LPA in this system, we used synthetic LPA as the chemoattractant in migration assays. LPA (10-50 nmol/L) induced 3- to 7-fold increase in ovarian cancer cell migration (Fig. 1*D*), suggesting that LPA secreted by peritoneal mesothelial cells accounts for the major heat-insensitive chemotactic activity in the conditioned medium.

LPA receptors are involved in the conditioned medium–induced migration of SKOV3 cells. The LPA receptors LPA_2 and LPA_3 have been implicated in LPA-induced migration of ovarian cancer cells (13, 19). To investigate which LPA receptor is involved in mediating the migratory effect of conditioned medium from peritoneal mesothelial cells on ovarian cancer cells, we used a siRNA strategy. SKOV3 cells were transfected with siRNAs against either LPA_1, LPA_2, or LPA_3 to specifically down-regulate expression levels of each of the receptors. siRNAs against GAPDH and GFP were used as a positive control (for the action of the siRNA on its target) and negative control (to rule out nonspecific effects), respectively. The specificity and efficacy of these siRNAs was confirmed by semiquantitative reverse transcription-PCR (Fig. 2*A*). LPA receptor down-regulation did not affect the basal level of SKOV3 cell migration, because transient transfection of LPA receptors $LPA_{1\text{-}3}$ RNAi did not change the migration of SKOV3 cells toward the control medium (serum-free RPMI 1640; data not shown).

We next tested the ability of peritoneal mesothelial cell conditioned medium to induce migration of SKOV3 cells that were transiently transfected with the siRNAs. As shown in Fig. 2*B*, we found that SKOV3 chemotaxis induced by the heat-treated conditioned medium from LP9 was reduced when LPA receptor expression was down-regulated in the SKOV3 cells. The effect of knocking down the different receptors varied with LPA_2 showing the strongest effect (~60% inhibition of conditioned medium–induced SKOV3 migration), followed by LPA_3 (30-40% inhibition) and LPA_1 (20-30% inhibition). The heat-treated conditioned

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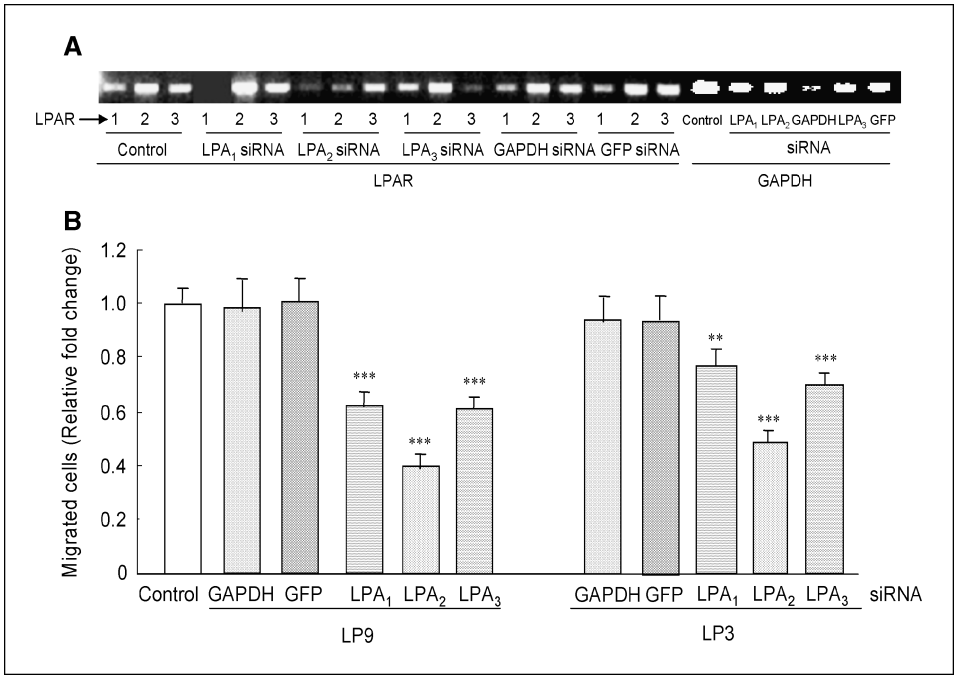

Figure 2. Receptors for LPA (*LPAR*), $LPA_{1\text{-}3}$, are involved in heated-treated peritoneal mesothelial cell conditioned medium–induced cell migration. *A,* effect of siRNAs against $LPA_{1\text{-}3}$ on the mRNA levels of $LPA_{1\text{-}3}$ in SKOV3 cells. SKOV3 cells were transiently transfected with siRNAs against $LPA_{1\text{-}3}$, and the RNA expression levels of $LPA_{1\text{-}3}$ were detected by reverse transcription-PCR. siRNAs against GAPDH and GFP were used as a positive and a negative control. *1,* LPA_1; *2,* LPA_2; *3,* LPA_3. *B,* siRNAs against $LPA_{1\text{-}3}$ reduced the chemotactic effect of heat-treated 24 hr-CM from LP9 and LP3 on the migration of SKOV3 cells. **, $P < 0.01$, ***, $P < 0.001$ versus each corresponding control (Student's *t* test). Results are from three independent experiments.

medium from LP3 showed similar results when used in migration assays containing siRNA-transfected SKOV3 cells (Fig. 2*B*). In contrast to the $LPA_{1\text{-}3}$ siRNAs, siRNAs against GAPDH or GFP did not have any significant effect on conditioned medium–induced cell migration, although the siRNA against GAPDH did effectively reduce expression of its target gene (Fig. 2*A* and *B*). These results are consistent with previous reports showing that LPA_2 or LPA_3 is important for migration of SKOV3 or HEY cells (13, 19). They also provide additional support that LPA is an important active component of the chemotatic activity in the conditioned medium from peritoneal mesothelial cells.

$iPLA_2$ and $cPLA_2$ are required for LPA production by peritoneal mesothelial cells. We have recently shown that $iPLA_2$ and $cPLA_2$ are required for LPA production and/or LPA-induced cell migration in HEY cells (13). To determine whether these enzymes are involved in LPA production by peritoneal mesothelial cells, we included $AACOCF_3$ (an $iPLA_2$ and $cPLA_2$ inhibitor; 25 μmol/L) or HELSS (an $iPLA_2$ inhibitor; 1 μmol/L) during the time when the conditioned medium from peritoneal mesothelial cells were generated. Both inhibitors blocked LPA production and the inhibitory effect of $AACOAF_3$ was stronger than that of HELSS, suggesting that both enzymes are involved in LPA production (Fig. 3*A*). At the concentrations of the PLA_2 inhibitors used here, we did not observe any toxicity effect (assessed by cell viability test in the presence of trypan blue). These inhibitors also greatly reduced the migratory activity of the heat-treated conditioned medium (Fig. 3*B*). To account for the possibility that the inhibitors might have a direct effect on cell migration independent of lipid production, we also added the inhibitors directly to the conditioned medium after the conditioned medium were produced. Indeed, under these conditions, we observed an inhibitory effect (30-40% inhibition) on cell migration. However, when the inhibitors were included during conditioned medium production, stronger inhibition (52-73%) was observed (Fig. 3*B*), suggesting that $iPLA_2$ and/or $cPLA_2$ are involved in lipid factor production by peritoneal mesothelial cells. Although the molecular mechanisms of the action of $cPLA_2$ remains to be further investigated, we have shown previously that PLA_2 inhibitors inhibit cell migration (13). In contrast, a specific inhibitor for $sPLA_2$, thioether amide-PC (10 μmol/L; Biomol, Plymouth Meeting, PA), did not have any significant effect on cell migration when added either during or after conditioned medium production (Fig. 3*B*). Another enzyme, lysophospholipase D, has been shown to play a major role in LPA production via conversion of lysophosphatidylcholine in serum (34, 38, 46). Using [^{3}H]lysophosphatidylcholine as the substrate, we did not detect any conversion of lysophosphatidylcholine to LPA in the conditioned medium from peritoneal mesothelial cells (data not shown). These results suggest that $sPLA_2$ and lysophospholipase D do not play a significant role in the production/secretion of LPA in the conditioned medium of peritoneal mesothelial cells.

Cell-based enzymatic assays were conducted to further assess the PLA_2 activity in peritoneal mesothelial cells using the fluorogenic substrate DBPC (40). In this assay, fluorescence intensity is generated when PLA_2 cleaves the fluorescent fatty acid analogue from the *sn*-2 position, releasing it from the intramolecular quenching effect of the Dabcyl. Using this assay, we found that both LP9 and LP3 had strong endogenous and constitutively active PLA_2 activity because FBS or other stimuli were not required for the activity (Fig. 3*C, a-e*). In contrast, very low levels of endogenous and constitutively active PLA_2 activity were observed in ovarian cancer cells compared to peritoneal mesothelial cells, and increase in PLA_2 activity using the DBPC assay is dependent on transfection and expression of PLA_2 genes (data not show). Phosphorylation of $cPLA_2$ is an indication of its activation (47). We found that $cPLA_2$ was extensively phosphorylated in LP3 and LP9 cells regardless of the presence of FBS (Fig. 3*D*), suggesting that $cPLA_2$ is constitutively active in peritoneal mesothelial cells.

The heat-insensitive factor(s) in conditioned medium from peritoneal mesothelial cells activates extracellular signal-regulated kinase and Akt in ovarian cancer cells. We have previously shown that LPA activates extracellular signal-regulated kinase (ERK) and Akt in ovarian cancer cells (16). To test whether

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the heat-insensitive factor(s) in conditioned medium could also regulate these signaling molecules, we conducted Western blot analyses on ERK and Akt. We found that incubation of SKOV3 cells with heat-treated conditioned medium from either LP9 or LP3 cells for 5 to 10 minutes resulted in ERK phosphorylation indicative of its activation (Fig. 4*A* and *B*). Akt activation involves two phosphorylation events, T308, which is required for Akt activity, and A473, which further enhances the activity. We observed that heat-treated conditioned medium induced Akt A473 phosphorylation after 30 to 60 minutes of incubation. Phosphorylation of Akt T308 induced by the conditioned medium showed two phases, which peaked at 5 minutes and 1 hour (Fig. 4*A* and *B*). These results are consistent with activities of LPA in ovarian cancer cells that have been previously described (6, 7, 16).

Conditioned medium from peritoneal mesothelial cells enhances adhesion of ovarian cancer cells to collagen I. Ovarian cancer cells shed from the primary tumor sites and migrate to secondary sites, such as the peritoneal wall. To form a metastasis, they need to attach or adhere to the peritoneum and invade through it. Thus, we tested whether conditioned medium from peritoneal mesothelial cells also had an effect on adhesion of ovarian cancer cells. It was found conditioned medium of LP9 made from different time points (1-48 hours) increased SKOV3 adhesion on collagen I up to 1.8-fold, with the 24 hr-CM having the strongest effect (Fig. 5*A*). Similar to the migratory effect of the peritoneal mesothelial cells conditioned medium, >85% of the adhesive activity of the conditioned medium was retained after heat treatment (Fig. 5*B*). Furthermore, synthetic 18:1-LPA induced a dose-dependent enhancement of SKOV3 adhesion to collagen I (Fig. 5*C*), which is consistent with our previous observations in HEY ovarian cancer cells (13). These results suggest that LPA present in the conditioned medium of peritoneal mesothelial cells is likely to be a major factor in its adhesive activity.

To confirm the involvement of LPA signaling in conditioned medium–induced SKOV3 adhesion, we tested the effect of transfection of siRNAs against $LPA_{1\text{-}3}$ into the SKOV3 cells. As shown in Fig. 4*D*, transfection with siRNA against LPA_2 had the strongest inhibitory effect on adhesion, followed by siRNA against LPA_3. siRNA against LPA_1 had no significant effect on the adhesive activity of the conditioned medium (Fig. 5*D*). Control siRNAs against GAPDH or GFP also had no significant effect on cell adhesion (Fig. 5*D*).

Conditioned medium from peritoneal mesothelial cells stimulates invasion of ovarian cancer cells into peritoneal mesothelial cell monolayers. Cell invasion is one of the characteristics of malignant tumor cells compared with normal

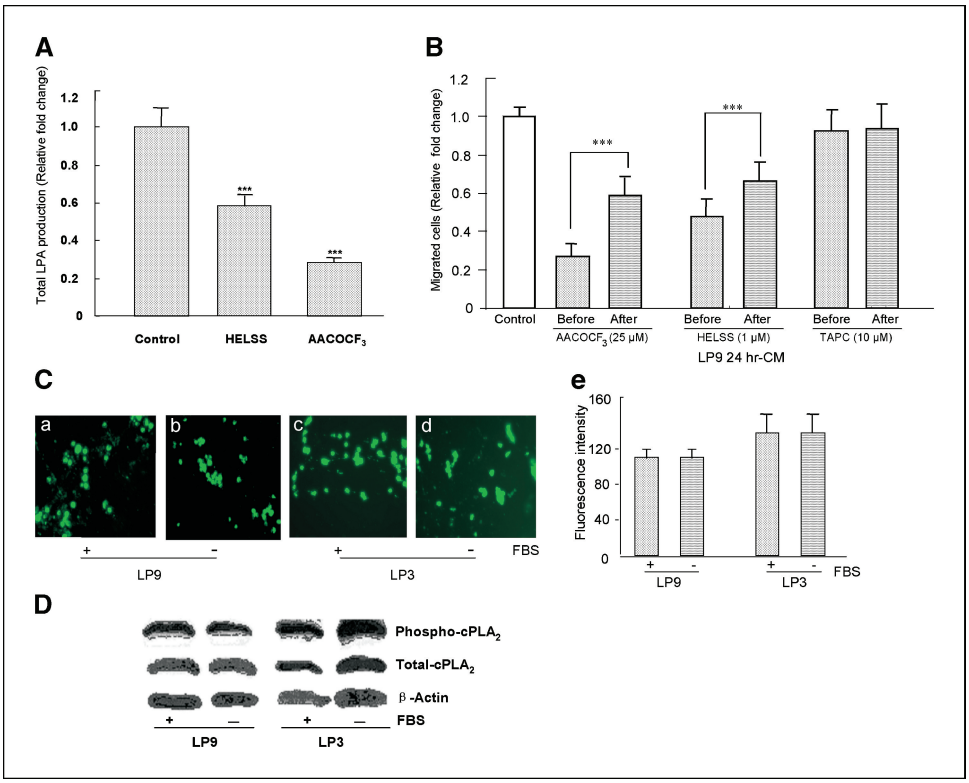

Figure 3. $iPLA_2$ and $cPLA_2$ activities are involved in generating the chemotactic activity of peritoneal mesothelial cell conditioned medium. *A,* total LPA production in the conditioned medium from LP9 was inhibited by HELSS (1 μmol/L) and $AACOCF_3$ (25 μmol/L). ***, $P < 0.001$ versus the corresponding control (Student's *t* test). The LPA in 24 hr-CM from LP9 is ~80 nmol/L/10^6 cells/24 hours. *B,* PLA_2 inhibitors reduced the chemotactic activity of 24 hr-CM from LP9 on SKOV3 cell migration. When LP9 cells were incubated for conditioned medium production, $AACOCF_3$ (25 μmol/L), HELSS (1 μmol/L), or thioether amide-PC (*TAPC*; 10 μmol/L) was added to the medium during the incubation for 24 hours before collecting conditioned medium (*Before*). We also collected conditioned medium produced in the absence of inhibitors, but added inhibitors into the lower chamber after conditioned medium were collected to assess the direct effects of these inhibitors during the migration assays (*After*). The effects of these conditioned medium on migration of SKOV3 cells were analyzed. Relative fold changes in migration were determined by setting cell migration in response to conditioned medium without treatment at 1.0. ***, $P < 0.001$ versus the control (Student's *t* test). *C, a* to *d*, cell-based enzymatic activities of PLA_2 in peritoneal mesothelial cells with or without overnight serum (FBS) starvation. Cells (10^4) were incubated with DBPC for 30 minutes. See Materials and Methods for the assay conditions. *e,* summary of three independent cell-based enzymatic assays. Intensities were measured as described in Materials and Methods. *D,* $cPLA_2$ is constitutively phosphorylated in peritoneal mesothelial cells. Phosphorylated $cPLA_2$ and total $cPLA_2$ expression in peritoneal mesothelial cells were detected by corresponding antibodies in Western blot analyses. β-actin was used as a loading control. Cell lysates from LP9 and LP3 grown with or without overnight starvation from FBS were used for Western blot analyses.

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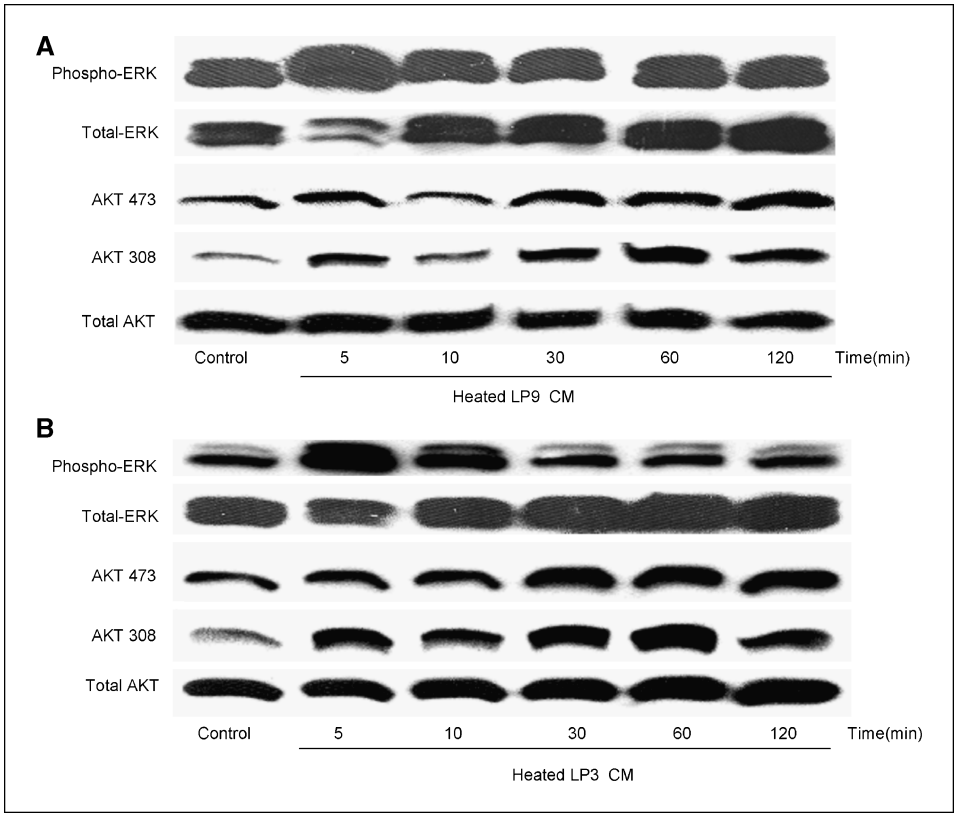

Figure 4. Heat-treated conditioned medium from peritoneal mesothelial cells activate ERK and Akt in SKOV3 cells. *A,* heat-treated conditioned medium (*CM*) from LP9 activate ERK and Akt. SKOV3 cells were serum-starved overnight, then treated with heat-treated 24 hr-CM from LP9 for different times (from 5 minutes to 120 minutes). Western blot analyses for total and phosphorylated forms of ERK and Akt were conducted as described in Materials and Methods. *B,* heat-treated conditioned medium from LP3 activate ERK and Akt in SKOV3 cells.

cells or benign tumor cells. To assess the ability of ovarian cancer cells to invade peritoneal mesothelial cell monolayers, an ECIS system was used, which monitors the invasive process in real-time based on impedance changes due to disruption of the monolayer of cells (42). We have adopted this technology to assess the ability of SKOV3 cells to invade into the mesothelial cell monolayer (the natural target of ovarian cancer cell invasion). SKOV3 cells were cultured with peritoneal mesothelial cell (after peritoneal

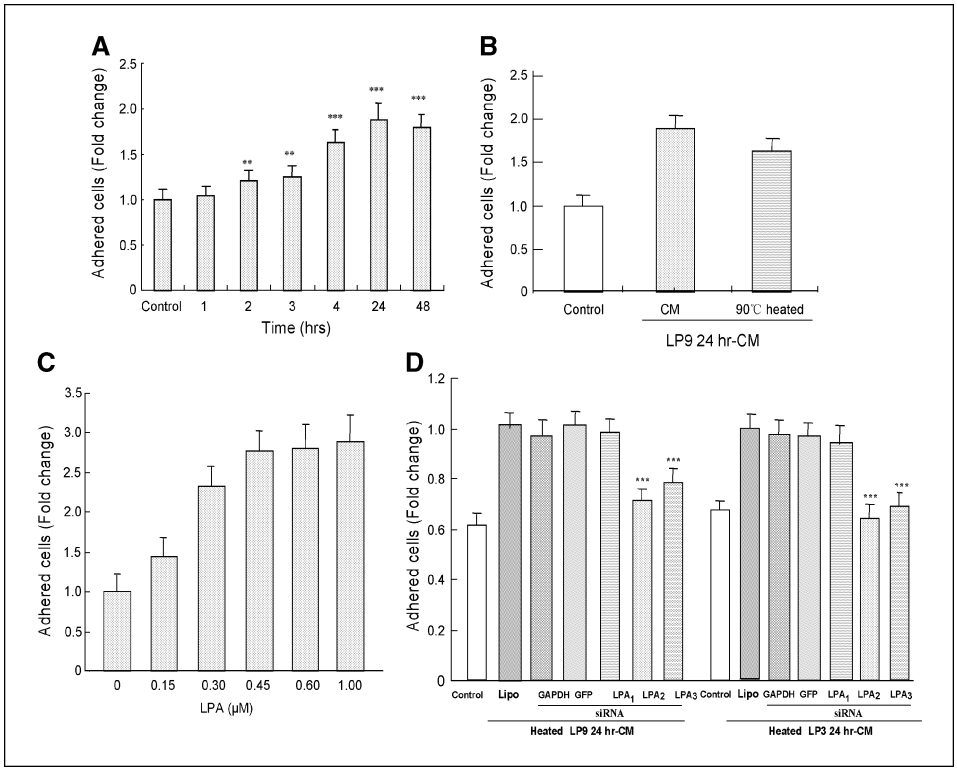

Figure 5. Conditioned medium produced by peritoneal mesothelial cells enhance adhesion of SKOV3 cells to collagen I. *A,* conditioned medium from LP9 were collected at different time points (1-48 hours) and tested for their effect on SKOV3 cell adhesion to collagen I. SKOV3 cells were collected and resuspended in control medium or in conditioned medium from LP9 and adhesion assays were conducted as described in Materials and Methods. **, $P < 0.01$; ***, $P < 0.001$ versus the control (Student's *t* test). *B,* heat-insensitive adhesive activity in the conditioned medium. LP9 24 hr-CM was heated at 90°C for 10 minutes. SKOV3 cells were collected and resuspended in heat-treated or nonheated conditioned medium and adhesion assays were conducted. *C,* 18:1-LPA enhances SKOV3 adhesion to collagen I. *D,* siRNAs against LPA_2 and LPA_3 transiently transfected into SKOV3 cells reduced cell adhesion induced by heat-treated 24 hr-CM from LP9 or LP3. siRNAs against GAPDH and GFP were used as controls. *Lipo,* LipofectAMINE was used as a negative control. Data were from three independent experiments.

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mesothelial cell formed a monolayer) when ECIS assays were conducted. Presumably, the factors (including LPA) secreted by peritoneal mesothelial cell will directly exert their effect on SKOV3. After peritoneal mesothelial cell reached a relatively stable impedance (the data are represented as normalized resistance R_{norm}; ref. 42) by forming a confluent monolayer on the gold electrode at ~5 hours, SKOV3 cells (1×10^5) were added to these cells (arrows indicated "challenge" in Fig. 6) and the electric resistance changes were recorded. Forty to 50 hours after SKOV3 cells were added, a dramatic drop in resistance was observed (Fig. 6*A*), indicating cell invasion through the mesothelial cell layer. To confirm if this resistance drop really reflects cell invasion, we tested whether other cell types also cause a nonspecific drop in resistance. The control cell lines chosen included NIH3T3 cells, which are noninvasive and noncancerous, and MCF7 cells, which are breast cancer cells with low invasive potential. In addition, being a breast cancer cell line, we predicted that MCF7 would not invade mesothelial cells from the peritoneal cavity, which is not a natural metastatic site for breast cancer. The same conditions used for SKOV3 cells were used for these two cell lines in the ESIC system. Neither NIH3T3 nor MCF7 cells induced a significant drop in resistance (Fig. 6*A*). These data indicate that the resistance drop observed when SKOV3 cells were used reflects a true ability of SKOV3 cells to invade into peritoneal mesothelial cell monolayers.

To determine whether the receptors for LPA are involved in cell invasion, we transfected cells with siRNAs against $LPA_{1\text{-}3}$, as well as control siRNAs against GAPDH or GFP. We found that siRNAs against the LPA_2 and LPA_3 receptors, but not that of LPA_1, had significant inhibitory effect on cell invasion (Fig. 6*B*). siRNAs against LPA_2 and LPA_3 greatly delayed the invasion process without significantly affecting the extent of invasion (Fig. 6*B*). In contrast, siRNA against LPA_1, as well as control siRNAs against GAPDH and GFP, had no significant effect on cell invasion (Fig. 6*B*). These data are consistent with the results of the cell migration and adhesion assays shown above and suggest that LPA_2 and LPA_3 play important roles in cellular functions of SKOV3 cells.

To test whether $iPLA_2$ and $cPLA_2$ activities were required for SKOV3 cell invasion, we pretreated peritoneal mesothelial cell LP9

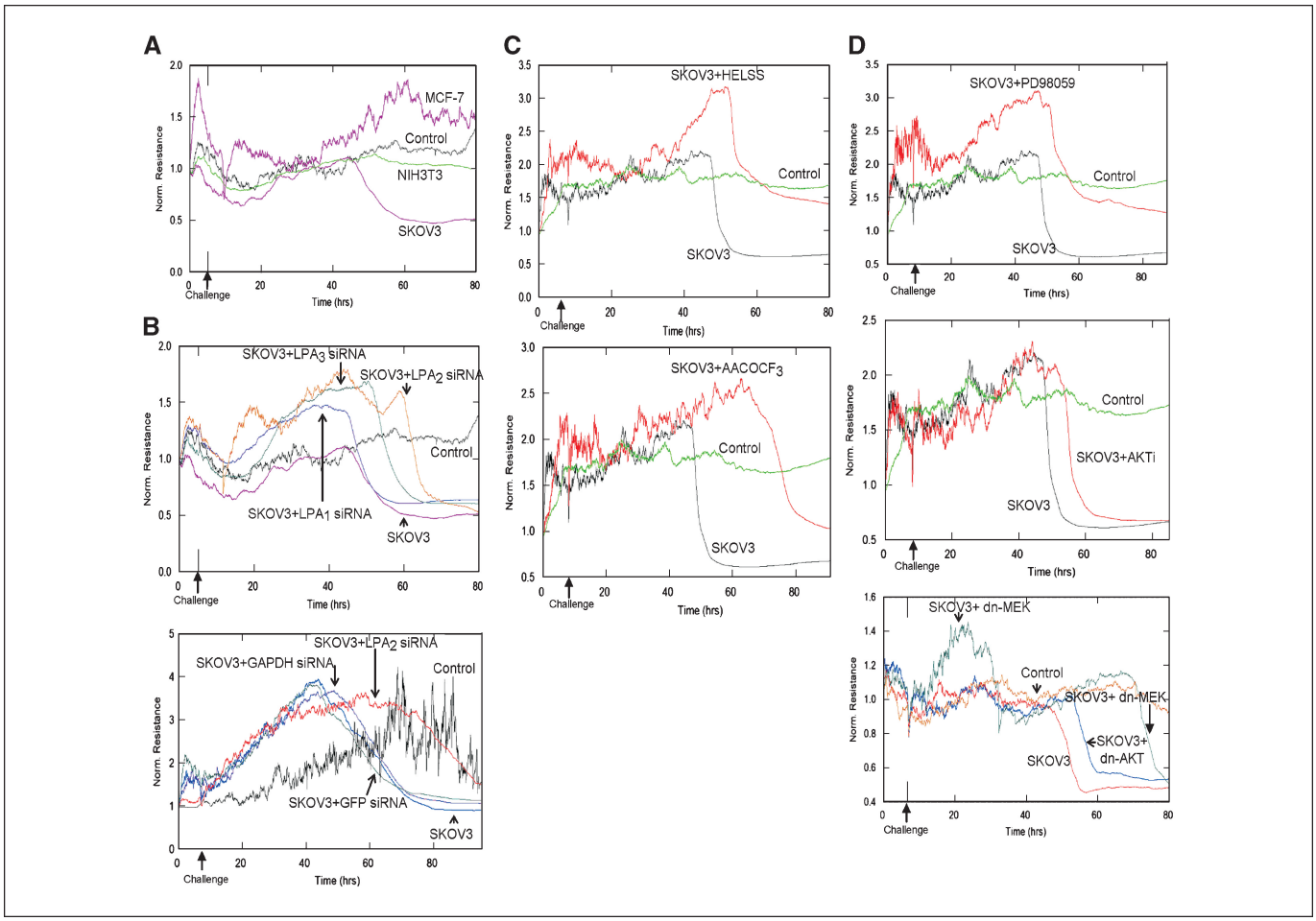

Figure 6. Invasion of SKOV3 cells into peritoneal mesothelial cells requires LPA_2, LPA_3, $iPLA_2$, $cPLA_2$, and MEK-ERK activity. *A,* invasion assays were conducted in the ECIS system (see Materials and Methods). LP9 (1×10^5 cells in 100 μL medium) were added to the gelatin-coated wells of the ECIS chambers and allowed to form a monolayer covering the gold electrode in each well. On the next day, SKOV3, NIH3T3, or MCF7 cells (1×10^5 cells in 300 μL medium) were added on top of the LP9 cell layer. In the control, 300 μL medium without cells was added on top of the LP9 monolayer. *B,* SKOV3 cells were transiently transfected with siRNAs against $LPA_{1\text{-}3}$, GAPDH, or GFP, and tested for invasion into LP9 in the ECSI system. *C,* for inhibitor treatments, LP9 cell monolayers were pretreated with HELSS (1 μmol/L, 30 minutes) or $AACOCF_3$ (100 μmol/L, 30 minutes) before addition of SKOV3 cells. *D,* SKOV3 cells were pretreated with the MEK inhibitor PD098059 (10 μmol/L, 30 minutes; *top*) or an Akt inhibitor (*Akti*, 10 μmol/L, 30 minutes; *middle*) before their application to the LP9 monolayers in the ECIS wells. *Bottom,* SKOV3 cells were transient transfected with dominant negative (*dn*) MEK or Akt and tested for invasion into LP9.

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with HELSS (1 μmol/L, 30 minutes) and $AACOCF_3$ (100 μmol/L, 30 minutes) and the invasive behavior of the SKOV3 cells into peritoneal mesothelial cell monolayer was recorded (Fig. 6*C*). Both HELSS and $AACOCF_3$ inhibited invasion of SKOV3 cells into the peritoneal mesothelial cells, as judged by the delayed invasion time and reduced invasion extent. These inhibitory effects were more prominent with $AACOCF_3$ (Fig. 6*C*), suggesting that both of the PLA_2 enzymes are likely to be involved in the invasion process.

Because we found that LP9 and LP3 conditioned medium activated both ERK and Akt in SKOV3 cells, we determined whether they were functionally involved in cell invasion. We pretreated SKOV3 cells with a mitogen-activated protein/ERK kinase (MEK) inhibitor (PD98059) or an Akt inhibitor before addition of these cells to the top of the mesothelial cell monolayer in our invasion assays. Our results show that the MEK inhibitor (presumably through inhibiting ERK) inhibited cell invasion by both increasing the time required for invasion and reducing the overall extent of invasion compared with control cells (Fig. 6*D, top*). On the other hand, although Akt inhibitor delayed the invasion process by 5 to 6 hours, it did not affect the final extent of invasion (Fig. 6*D, middle*). To confirm these results, we transiently transfected dominant negative forms of MEK and Akt into SKOV3 cells and tested their ability to invade peritoneal mesothelial cells. Results similar to those from the experiments using inhibitors were obtained (Fig. 6*D, bottom*), supporting a role for MEK-ERK in the invasion process, whereas AKT is involved to a much lesser extent.

Discussion

One of the important ways to block the tumor-promoting effect of LPA is to reduce LPA production. Thus, identifying the source of the elevated LPA in the ascites and blood of patients with ovarian cancer is pivotal. The current work provides the first line of evidence that peritoneal mesothelial cells from ovarian cancer patients possess constitutive PLA_2 activity, which is involved in LPA production.

Our data suggest that both ovarian cancer cells and peritoneal mesothelial cells contribute to the elevated LPA levels found in ovarian cancer ascites (refs. 7, 8 and the current work). Thus, development of effective and novel strategies to treat ovarian cancer should take both ovarian cancer cells and peritoneal mesothelial cells into consideration.

In addition, our data are consistent with the importance of LPA_2 and LPA_3 in cellular functions of LPA (13, 19) and the fact that the expression of LPA_2 and LPA_3 is elevated in malignant ovarian cancer tissues (26).

The discovery of secreted lysophospholipase D has brought much attention and it has been considered the main enzyme responsible for extracellular LPA production. However, we have not found direct evidence for lysophospholipase D or $sPLA_2$ activity from either ovarian cancer cells or peritoneal mesothelial cells. Instead, our data suggest that both $iPLA_2$ and $cPLA_2$ are likely to be involved in LPA production by peritoneal mesothelial cells. PLA_1, PLA_2, and glycerophosphate acyltransfease (33, 36, 48, 49) are possibly involved in LPA intracellular production. The critical issues are whether they can be directly involved in extracellular LPA production (e.g., through microvesicles shed from cells; ref. 50) or whether it is possible that intracellularly produced LPA secretes extracellularly. These issues remain to be further studied.

ECIS-based assays have been established to measure the cell morphologic, adhesive, and invasive properties in a real-time, automated, and quantitative fashion (42–44). Monolayer disruption via the retraction of endothelial cell or peritoneal mesothelial cell junctions induced by tumor cells may be the major or at least part of the causes for the observed electrical resistance changes. The ability of tumor cells to induce these changes reflects their invasive potential. As a similar scenario, when endothelial cell monolayers were challenged with highly metastatic cell lines, observations were recorded by using scanning and transmission electron microscopy as well as phase-contrast time-lapse microscopy that showed the binding of the cells to endothelial cell layer, the retraction of the endothelial cell junctions, and finally the penetration of the cells through the endothelial monolayer. This *in vitro* sequence of events has been suggested to represent similar invasive activities that take place during the metastatic process *in vivo*. These activities have also been monitored using the ESIC system. As the metastatic cells invade the endothelial monolayer, they break down the barrier function of the endothelial cell layer, resulting in large drops in impedance. These impedance changes are automatically followed over time and used to quantify the *in vitro* invasive activities of the cells (42). In our work, we have used three different cell lines (two with low or no metastatic potential versus metastaic ovarian cancer SKOV3 cells) and our results support the idea that the measured resistance changes reflect the ability of cells to affect cell junctions and penetrate monolayer peritoneal mesothelial cells, which reflects the metastatic potential of the cells. Invasion assays conducted using the ECIS system represented several advantages and/or unique features when compared with other invasion measurement methods used. First, invasion of ovarian cancer cells into the peritoneal mesothelial cell monolayer is measured, which better mimics *in vivo* events compared with Matrigel-coated Boyden chamber assays. Second, the ECIS system has high sensitivity. Finally, ECIS follows real-time changes during cell invasion, illustrating the kinetics of this process, whereas the Boyden chamber method only measures one end-point result at a time. This becomes very important when assessing the effect of different reagents on the invasion process. As shown in our results, different reagents have different modes of effects, involving the timing of invasion, the overall extent of invasion, or both. If a reagent does not affect the final invasion outcome (as in the case of Akt inhibitor), its effect may not be detected in traditional Boyden chamber assays.

In summary, our data reveal that peritoneal mesothelial cells may be an important source of the elevated LPA levels found in ovarian cancer ascites, which stimulate ovarian cancer development. This indicates that, although treatment of the cancer is an important goal, the host environment should also be taken into serious consideration because it may play a pivotal role in the pathophysiology of the disease.

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