

Lysophosphatidic Acid Promotes Matrix Metalloproteinase (MMP) Activation and MMP-dependent Invasion in Ovarian Cancer Cells¹

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ABSTRACT

Ovarian cancer is an highly metastatic disease characterized by ascites formation and diffuse i.p. adhesion, invasion, and metastasis. Levels of lysophosphatidic acid (LPA) are elevated in the plasma of patients with ovarian carcinoma, including 90% of patients with stage I disease, suggesting that LPA may promote early events in ovarian carcinoma dissemination. Expression of matrix metalloproteinases (MMPs) is also up-regulated in ovarian cancer tissues and ascites, and numerous studies have provided evidence for a direct role of MMPs in i.p. invasion and metastasis. Using three-dimensional type I collagen cultures or immobilized β_1 integrin subunit-specific antibodies, we previously demonstrated that β_1 integrin clustering promotes activation of proMMP-2 and processing of membrane type 1 MMP in ovarian cancer cells (S. M. Ellerbroek *et al.*, *Cancer Res.*, 59: 1635–1641, 1999). In the current study, the effect of LPA on MMP expression and invasive activity was investigated. Treatment of ovarian cancer cells with pathophysiological levels of LPA increased cellular adhesion to type I collagen and β_1 integrin expression. A significant up-regulation of MMP-dependent proMMP-2 activation was observed in LPA-treated cells, leading to enhanced pericellular MMP activity. As a result of increased MMP activity, haptotactic and chemotactic motility, *in vitro* wound closure, and invasion of a synthetic basement membrane were enhanced. These data indicate that LPA contributes to metastatic dissemination of ovarian cancer cells via up-regulation of MMP activity and subsequent downstream changes in MMP-dependent migratory and invasive behavior.

INTRODUCTION

Ovarian cancer is an highly metastatic disease characterized by widespread i.p. metastasis and ascites (1). As the dissemination of ovarian cancer is usually confined to the surface epithelium within the peritoneal cavity, processes such as cell adhesion, migration, i.p. invasion, and proliferation likely play a predominant role in ovarian cancer pathobiology. Ascitic fluid from ovarian cancer patients potentiates the growth of ovarian cancer cells both *in vitro* and *in vivo*, and purification and characterization of this ovarian cancer-activating factor identified the lipid LPA³ as an ascites-derived mitogen (2). LPA levels are elevated in 98% of patients with ovarian cancer, including 90% of patients with stage I disease (3). These data suggest that LPA may represent a sensitive biomarker for ovarian cancer and may promote early events in ovarian cancer dissemination. Numerous studies have shown that LPA functions extracellularly, via interaction with specific G protein-coupled plasma membrane receptors (4, 5), resulting in transient increases in cytosolic free calcium, phosphorylation of specific cellular proteins including focal adhesion kinase, activation of mitogen-activated protein kinases, and formation of focal

adhesions (6, 7). Functionally, LPA triggers actin-based cytoskeletal events through Rho GTPases, influencing both random and directional migration (7, 8), and induces tumor cell invasion across host cell monolayers (9, 10).

Ovarian cancer cells secrete proteolytic enzymes including plasminogen activators and MMPs that degrade extracellular matrix proteins and promote invasion into the proteolytically modified tissue (reviewed in Ref. 11). Expression of gelatinolytic and collagenolytic MMPs including MMP-2 (gelatinase A), MMP-9 (gelatinase B), and MT1-MMP has been demonstrated in ovarian cancer ascites, cultured cells, and tissues (11–13). Furthermore, treatment of animals harboring ovarian cancer xenografts with synthetic MMP inhibitors reduces tumor burden and enhances survival (14, 15). Numerous studies have shown that MT1-MMP functions in the activation of proMMP-2 (16–18), and it has been speculated that MT1-MMP produced by ovarian epithelial carcinoma cells may initiate cell surface activation of tumor-derived or stromally derived proMMP-2, thereby facilitating motility, invasion, and metastasis (12, 19, 20). We have previously demonstrated that clustering of collagen-binding integrins on ovarian cancer cell membranes induces proMMP-2 activation (12). Furthermore, activated MMP-2 binds to the plasma membranes of ovarian cancer cells where it displays significantly enhanced catalytic efficiency relative to the solution phase enzyme (21) and promotes a 3–5-fold increase in cellular invasive activity (19). Because MT1-MMP also catalyzes direct proteolysis of extracellular matrix macromolecules, including collagens (22, 23), induction of a MT1-MMP/MMP-2 cascade is postulated to play a significant role in ovarian cancer i.p. invasion.

Previous studies have demonstrated that LPA induces secretion of urinary-type plasminogen activator (urokinase) by ovarian cancer cells (24); however, the effect of LPA on MMP production has not been evaluated. In the current study, we investigated the effect of LPA on MMP activity generated by ovarian cancer cells. Our results demonstrate that LPA stimulates proMMP-2 activation and promotes ovarian tumor cell motility and invasion in an MMP-dependent manner. Together, these data suggest that LPA-induced increases in pericellular MMP activity may function to potentiate ovarian cancer invasion and metastasis.

MATERIALS AND METHODS

Materials. BSA, gelatin (BSA), cell culture reagents, amino-phenylmercuric acetate, antirabbit IgG-peroxidase conjugates, purified mouse immunoglobulins, ConA, and 1,10-phenanthroline were all purchased from Sigma (St. Louis, MO). Synthetic lysophospholipids (LPA 18:1 and 18:0) were products of Avanti Polar Lipid Inc. (Alabaster, AL). Antihuman MT1-MMP polyclonal antibody (AB815, hinge domain) was obtained from Chemicon (Temecula, CA), anti- β_1 integrin (clone P4C10) was a product of Life Technologies, Inc. (Gaithersburg, MD), and streptavidin-FITC was purchased from Jackson ImmunoResearch. The pro-MMP-2 ELISA kit was acquired from Amersham (Arlington Heights, IL), and the CellTiter 96AQ kit to monitor cell proliferation was purchased from Promega (Madison, WI). Polyvinylidene difluoride membrane was obtained from Amersham (Hydrobond-P). SuperSignal enhanced chemiluminescence reagents were purchased from Pierce (Rockford, IL). The general hydroxamic acid MMPI INH-3850-PI and the quenched fluorescent MMP substrate (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-

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³ The abbreviations used are: LPA, lysophosphatidic acid; MMP, matrix metalloproteinase; MT-1, membrane type 1; ConA, concanavalin A; MMPI, MMP inhibitor; TIMP, tissue inhibitors of metalloproteinase; PTX, pertussis toxin.

Leu-Dpa-Ala-Arg (25) were purchased from Peptides International (Louisville, KY). Protein concentrations were determined using the Bio-Rad D_c kit (Hercules, CA) with BSA as a standard.

Cell Culture and LPA Treatment. The ovarian carcinoma cell line DOV 13 was provided by Dr. Robert Bast, Jr. (M. D. Anderson Cancer Center, Houston, TX). Cell culture was maintained under standard conditions in 75-cm² cell culture flasks (26). LPA was dissolved in chloroform, evaporated, and resuspended in cold PBS containing 1% BSA (24). Cells were subcultured at a density of 10⁵ cells/well in 24-well plates under serum-free conditions in the presence of LPA (0–200 μM) for 12–48 h. The effect of LPA on cell growth was evaluated by culturing cells for 24–72 h in the presence of LPA (0–200 μM) and monitoring cellular metabolic activity using the CellTiter 96AQ kit according to the manufacturer's specifications. There was no difference in metabolic activity in cells cultured with up to 200 μM LPA for 72 h (*P* > 0.05). In control experiments, cultures included PTX (100 ng/ml), which blocks LPA signaling through its G protein-coupled receptor (27, 28). Additional control cultures included Con A (20 μg/ml) to induce proMMP-2 activation and MT1-MMP processing or MMPI (10 μM) to inhibit MMP processing (12). Conditioned media and cell lysates were collected and evaluated for MMP expression and activity as described below.

Cell Adhesion and Integrin Expression. To evaluate the effect of LPA on cell-matrix adhesion, cells were cultured for 24 h in the presence of LPA (80 μM) before evaluation of adhesion to 24-well culture plates coated with type I collagen as previously described (29, 30). Briefly, wells were coated with type I collagen or albumin (control) by passive adsorption and blocked with 3% BSA before use in adhesion assays. Control or LPA-treated cells were released by brief trypsinization (1 min, 37°C), washed in serum-containing medium to inhibit trypsin activity, and replated at a density of 1.5 × 10⁵ cells/well. Adhesion was allowed to proceed for 1 h before washing with PBS to remove unbound cells. Bound cells were fixed and stained using Diff-Quik (Fisher) and enumerated using an ocular micrometer and counting a minimum of 10 high-powered fields. To assess integrin expression levels, control or LPA-treated cells were lysed using 50 mM Tris, 150 mM NaCl, 1% NP40, and 0.1% SDS, and protein content was determined as described above. Equal concentrations of lysate (20 μg) were electrophoresed on 9% SDS-polyacrylamide gels, electroblotted to Immobilon, and probed with an anti-β₁ integrin antibody (1:1000 dilution, 3 h) followed by a peroxidase-conjugated secondary antibody (1:5000, 1 h). After washing, immunoreactive bands were visualized using enhanced chemiluminescence detection.

Analysis of MMP-2 Expression and Activity. Gelatinase activities in conditioned media were determined using SDS-PAGE gelatin zymography as previously described (12). Briefly, SDS-PAGE gels (9% acrylamide) were copolymerized with 0.1% gelatin, and samples were electrophoresed without reduction or boiling using 5× Laemmli sample buffer. SDS was removed through a 1-h incubation in 2.5% Triton X-100, and gels were incubated in 20 mM glycine, 10 mM CaCl₂, 1 μM ZnCl₂ (pH 8.3) at 37°C for 18–24 h before staining with Coomassie Blue to visualize zones of gelatinolytic activity. In some experiments, cultured cells were treated with LPA (80 μM) for 24 h to up-regulate integrin expression before seeding of control or LPA-treated cells into three-dimensional collagen gels as previously described (12). After 48 h of collagen culture, MMP-2 status was evaluated as described above. Quantitation of MMP activity was performed by monitoring hydrolysis of a quenched fluorescent peptide substrate (25) using a fluorimetric microtiter plate reader (SpectraMAX Gemini; Molecular Devices). Aliquots of conditioned media (200 μl) from control and LPA-treated cells were incubated with 7.5 μM substrate at 37°C, and kinetics of substrate hydrolysis were monitored fluorometrically using an excitation wavelength of 326 nm and monitoring emission at 396 nm. The concentration of proMMP-2 in aliquots of conditioned medium was also quantified by ELISA according to the manufacturer's specifications. Relative expression of TIMP-1 and -2 in conditioned media of LPA-treated cells (80 μM LPA, 24 h) were evaluated by reverse zymography. Reverse zymography for detection of TIMPs was performed using 15% SDS-polyacrylamide gels containing copolymerized gelatin using TIMP standards and reagents purchased from University Technologies International (Calgary, Alberta, Canada) according to the manufacturer's specifications.

Evaluation of MT1-MMP Expression, Processing, and Distribution. MT1-MMP expression and processing were evaluated by Western blotting of cell lysates as previously described (12). Briefly, control and LPA-treated cells were washed with PBS and lysed with 500 μl of lysis buffer (50 mM sodium

phosphate buffer (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% TX-100, 1 μg/ml aprotinin, 1 μM pepstatin, 10 μM leupeptin, and 10 μM E64). Lysates were collected with a cell scraper and clarified by centrifugation (10,000 × *g*, 10 min, 4°C), and protein concentration was determined using the Bio-Rad D_c protein assay. Equal concentrations of protein (20 μg) were electrophoresed on 9% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membrane, and blocked with 3% BSA in 50 mM Trizma (pH 7.5), 300 mM NaCl, 0.2% Tween 20 (TBST). Membranes were incubated for 1 h at room temperature with a 1:4000 dilution of antihuman MT1-MMP polyclonal antibody in 3% BSA/TBST. Immunoreactive bands were visualized with a peroxidase-conjugated antirabbit IgG (1:5000 in 3% BSA/TBST) and enhanced chemiluminescence.

To evaluate MT1-MMP by immunofluorescence microscopy, cells were plated onto 22-mm² glass coverslips placed into 6-well tissue culture plates and cultured in the absence or presence of LPA (80 μM) overnight. Cells were gently washed with PBS and fixed for 1 min in ice-cold methanol. Coverslips were then blocked for 45 min with PBS containing 1% BSA, followed by the addition of biotinylated anti-MT1-MMP (1:200 dilution, 1 h, room temperature). After washing three times (10 min) with PBS/1% BSA, streptavidin-FITC solution was added (1:500 dilution), and coverslips were gently agitated for 1 h at room temperature in the dark, washed as described above, and viewed using a Zeiss fluorescence microscope (Model II).

Analysis of Migration and Invasion. A variety of approaches were used to evaluate the functional effect of LPA on motility and invasion. Chemotactic directional migration was evaluated using a modified Boyden chamber (31). Porous filters (8-μm pores) were coated on the underside by passive adsorption with type I collagen (Sigma) by incubating with 10 μg/ml collagen in coating buffer (29) for 2 h. Cells (1 × 10⁵) pretreated with LPA as described above were plated in the upper chamber in the presence or absence of LPA as indicated and allowed to migrate for 4 h. Nonmigrating cells were removed from the upper chamber with a cotton swab, filters were stained with Diff-Quik Stain, and migrating cells adherent to the underside of the filter were enumerated using an ocular micrometer and counting a minimum of 10 high-powered fields. Data are expressed as relative migration (number of cells/field) and represent the mean and SD of quadruplicate experiments. Invasive activity was quantified by plating cells (5 × 10⁴) onto a Boyden chamber coated with Matrigel (11 μg/filter) followed by incubation for 48–72 h. Staining of filters and quantitation of invading cells was performed as described above. In some experiments, invasion was quantified in the presence of the MMPI (10 μM). Aliquots of conditioned medium were also removed and assayed for proMMP-2 activation as described above. (It should be noted that the zymogram incubation buffer did not contain MMPI, thus allowing visualization of MMP-2 species.)

Haptotactic motility was assessed as previously described (30, 32) by plating cells (3000/coverslip) on coverslips coated with colloidal gold overlaid with type I collagen (50 μg/ml). Cells were allowed to migrate for 26 h, and phagokinetic tracks were monitored by visual examination using a Zeiss microscope with darkfield illumination. Semiquantitative analysis of phagokinetic tracks was performed by measuring track area by computer-assisted image analysis using NIHImage. Motility was also evaluated using a scratch wound assay as previously described (33). Confluent monolayers of cells were wounded with a uniform scratch, rinsed with PBS, and incubated in the presence or absence of LPA or MMP-I for 0–24 h. Wound closure was monitored by visual examination using a Zeiss microscope. Semiquantitative analysis of wound closure was obtained by measuring the wound width on photographic images using Fowler digital needlepoint calipers accurate to 0.01 mm.

RESULTS

LPA Promotes Ovarian Carcinoma Cell Adhesion and MMP Processing. LPA levels are elevated in 90% of stage I ovarian cancer patients and 98% of patients with advanced disease (3). Previous studies have demonstrated that LPA promotes β₁ integrin-dependent migration and induces tumor cell invasion of host monolayers (8–10, 34). Further, preferential adhesion of ovarian cancer cells to interstitial type I collagen is mediated via β₁ integrins (12, 29, 30). To examine the effect of LPA on β₁ integrin expression and function,

cells were cultured with LPA for 24 h and adhesion to type I collagen-coated surfaces was evaluated. A significant increase in adhesion to collagen was apparent in LPA-treated cells relative to controls (Fig. 1A; $P < 0.005$). Concomitant with increased adhesion, expression of β_1 integrin protein was also enhanced by LPA (Fig. 1B). In control experiments, treatment of cells with LPA (200 μM , 72 h) did not enhance proliferation or induce toxicity (not shown). We have previously demonstrated that β_1 integrin clustering induces MT1- MMP-dependent proMMP-2 activation in ovarian cancer cells (12). Because the expression of β_1 integrins was up-regulated by LPA, cells were preincubated with LPA (80 μM) for 24 h before seeding into three-dimensional collagen cultures to induce integrin clustering. Preincubation with LPA enhanced the response of DOV13 cells to three-dimensional collagen culture, resulting in increased proMMP-2 processing (Fig. 1C).

To further evaluate the effect of LPA on MMP activity, cells were cultured in uncoated dishes in serum-free medium in the presence of increasing concentrations of LPA 18:1, and MMP expression was evaluated by zymography. Although MMP-2 expression levels were not significantly altered, a concentration-dependent increase in proMMP-2 processing was observed relative to untreated cells (Fig. 2A), with changes observed using LPA concentrations as low as 5–10 μM . Control cultures treated with LPA 18:0 showed no changes in proMMP-2 processing (not shown). These results were confirmed by an ELISA specific for proMMP-2, demonstrating a decrease in proMMP-2 levels from 36.2 ± 0.85 ng/ml in untreated controls to 21.5 ± 1.7 and 14.3 ± 0.5 ng/ml in cells treated with 80 μM and 200 μM LPA, respectively. LPA-induced proMMP-2 activation was inhibited by coinubation with PTX (Fig. 2B), which was shown to block LPA-dependent signaling via G_i protein-coupled receptors in ovarian cancer cells (2). Relative expression levels of the MMP inhibitors TIMP-1 and TIMP-2 were unaltered by LPA treatment, as shown by reverse zymography (Fig. 2C). Quantitative evaluation of

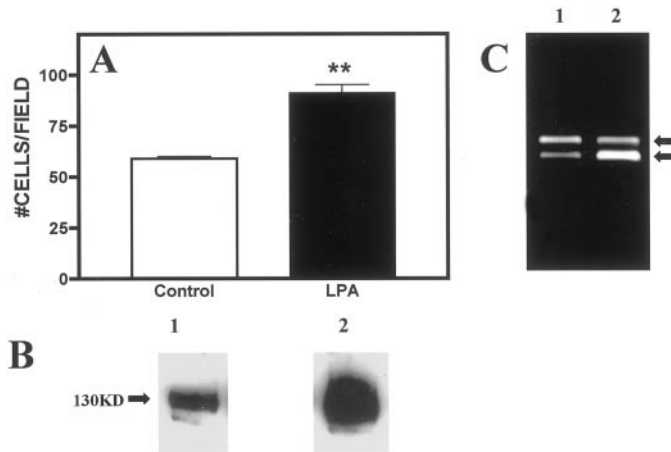


Fig. 1. Effect of LPA on ovarian cancer cell adhesion. A, LPA increases cellular adhesion to type I collagen. Cells were cultured for 18 h in the presence (■, 80 μM) or absence (□) of LPA and analyzed for adhesion to wells coated by passive adsorption with type I collagen. Cells (1.5×10^5) were added to wells for 1 h at 37°C. After washing to remove nonadherent cells, bound cells were fixed and enumerated using an ocular micrometer by counting 10 high-power fields. Data represent the mean and SD from triplicate experiments (**, $P < 0.005$ relative to control). B, LPA induces expression of β_1 integrin. Cells were cultured in the absence (Lane 1) or presence (Lane 2) of LPA as described above. Cells were lysed and lysates (20 μg) were electrophoresed on 9% SDS-polyacrylamide gels and immunoblotted with anti- β_1 integrin, followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection. C, LPA enhances collagen-induced proMMP-2 processing. Cells were cultured for 24 h in the absence or presence of LPA (80 μM) before subculturing onto three-dimensional collagen gels (12). Conditioned media were analyzed for processing of proMMP-2 by gelatin zymography. Lane 1, control cells; Lane 2, LPA-pretreated cells. Arrows, the migration positions of pro- (top) and active-MMP-2 (bottom).

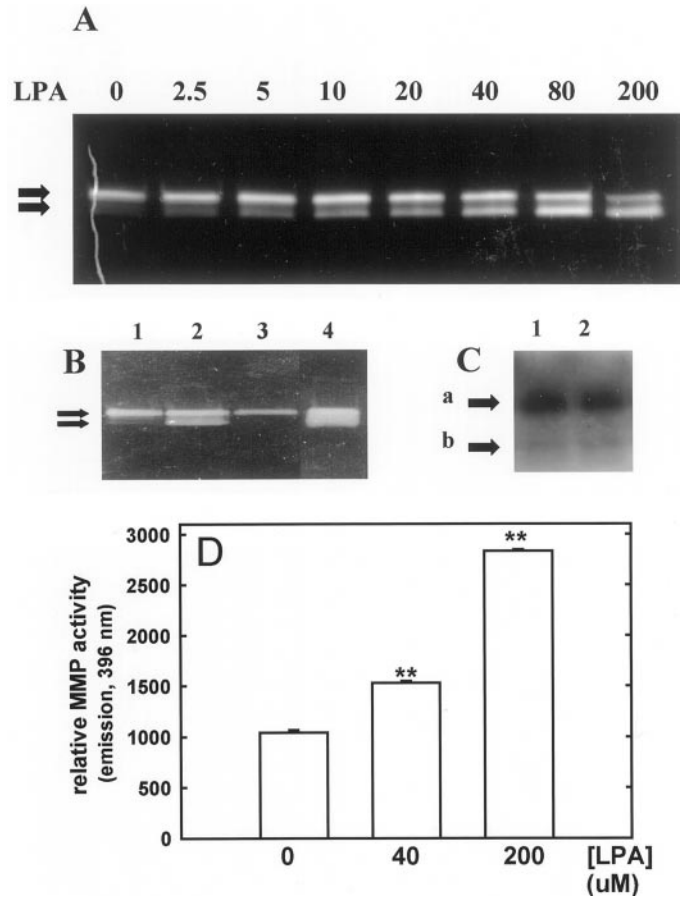


Fig. 2. LPA promotes proMMP-2 processing and enhances net MMP activity. A, concentration dependence of LPA-induced proMMP-2 processing. Cells (10^5) were cultured for 24 h with increasing concentrations of LPA as indicated (μM), and conditioned media were analyzed for activation of proMMP-2 by gelatin zymography. Arrows, the migration positions of pro- (top) and active MMP-2 (bottom). B, LPA-induced proMMP-2 processing is PTX sensitive. Cells were cultured in the absence of LPA (Lanes 1 and 4) or in the presence of LPA (80 μM , Lanes 2 and 3). Addition of PTX (100 ng/ml) abrogated LPA-induced proMMP-2 processing (Lane 3). Control cells (Lane 4) were treated with Con A (20 $\mu\text{g}/\text{ml}$) to induce proMMP-2 activation. Arrows, the migration positions of pro- (top) and active MMP-2 (bottom). C, reverse zymogram of TIMP activity. Cells were cultured in the absence (Lane 1) or presence (Lane 2) of LPA (80 μM) and analyzed for TIMP-1 and -2 expression by reverse zymography on 15% SDS-polyacrylamide gels. Arrows a and b, the migration positions of TIMP-1 and -2 standards, respectively. D, LPA enhances net MMP activity. Cells were treated for 24 h with LPA at the concentrations indicated, and conditioned media (200 μl) were evaluated for MMP activity using the quenched fluorescent substrate (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-Dpa-Ala-Arg (7.5 μM) and by monitoring fluorescence emission at 396 nm using an excitation wavelength of 326 nm (**, $P < 0.005$ relative to untreated control).

MMP activity in conditioned medium using a synthetic quenched fluorescent peptide substrate confirmed the net increase in MMP-2 activity, showing a threefold increase in substrate cleavage in LPA-treated cultures (Fig. 2D).

In addition to modulating proMMP-2 activation, both β_1 integrin clustering and ConA treatment of ovarian cancer cells induce processing of MT1-MMP to M_r 55,000 and 43,000 species (12). Cleavage of the M_r 55,000 specie to the M_r 43,000 form is MMP dependent and can be blocked using a broad-spectrum hydroxamic acid-based MMP inhibitor such as MMP-I (12). To determine whether LPA regulates proMT1-MMP processing, lysates from control and LPA-treated cells were analyzed for MT1-MMP by Western blotting. As previously reported, ConA promoted efficient MT1-MMP processing (Fig. 3, Lane 3), and accumulation of the M_r 55,000 active specie was captured in the presence of MMP-I (Fig. 3, Lane 4). Although LPA-induced up-regulation of MMP-2 activation was readily apparent (Fig. 2), only a slight increase in the M_r 55,000 active MT1-MMP specie was detectable, as captured in LPA-treated cells in the presence of

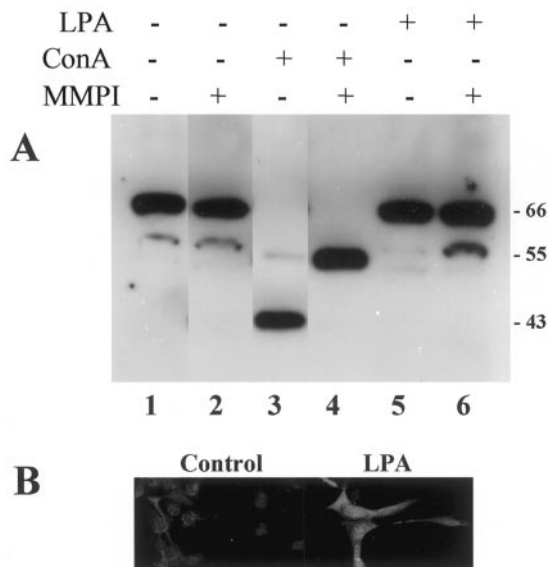


Fig. 3. Effect of LPA on MT1-MMP expression, processing, and distribution. *A*, Western blot of MT1-MMP. Cells (10^5) were cultured in the absence (Lanes 1–4) or presence (Lanes 5 and 6) of LPA ($80 \mu\text{M}$) or MMP-I (Lanes 2, 4, and 6), and extracts ($20 \mu\text{g}$) were analyzed for MT1-MMP processing by electrophoresis on 9% SDS-polyacrylamide gels under reducing conditions and immunoblotting for MT1-MMP followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection. The migration position of molecular weight standards is indicated; numbers on right, M_r in thousands. *B*, immunofluorescence of MT1-MMP. Cells were cultured in the absence (left) or presence (right) of LPA ($80 \mu\text{M}$, 24 h) and processed for immunofluorescence microscopy using biotinylated anti-MT1-MMP (1:200) and streptavidin-conjugated FITC as described in “Materials and Methods.”

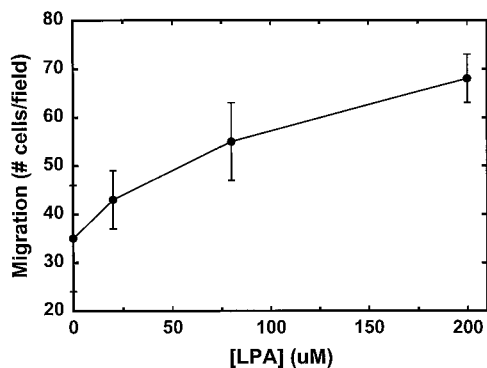


Fig. 4. LPA promotes chemotactic migration. Cells (10^5) were cultured for 24 h in the presence of LPA at the concentration indicated before seeding onto a porous polycarbonate filter ($8\text{-}\mu\text{m}$ pore) coated on the underside with type I collagen ($10 \mu\text{g}/\text{ml}$). After incubation for 4 h to permit migration, nonmigrating cells were removed from the upper chamber, filters were stained, and migrating cells adherent to the underside of the filter were enumerated using an ocular micrometer and counting a minimum of 10 high-powered fields. Data are expressed as relative migration (number of cells/field) and represent the mean and SD of quadruplicate experiments.

MMP-I (Fig. 3, Lanes 5 and 6), suggesting that LPA may function via a mechanism distinct from that of integrin clustering to up-regulate cellular MMP activation. Because it has been reported that physical clustering of MT1-MMP may also potentiate proMMP-2 activation (35), the distribution of MT1-MMP in control and LPA-treated cells was evaluated by immunofluorescence microscopy (Fig. 3*B*). In untreated cells (Fig. 3*B*, left panel) MT1-MMP expression is predominantly peri-nuclear, whereas MT1-MMP is more widely distributed at the cell surface in LPA-treated cells (right panel), suggesting an LPA-induced change in MT1-MMP trafficking or localization.

LPA Enhances Ovarian Cancer Cell Migration and Invasion. To assess the functional consequences of enhanced integrin expression and MMP activity on cellular events associated with i.p. metas-

tasis, the effect of LPA on cellular motility and invasive activity was evaluated. Using the Boyden chamber assay to assess chemotactic motility, a concentration-dependent increase in migration toward collagen was observed in the presence of $10\text{--}200 \mu\text{M}$ LPA (Fig. 4). Haptotactic migration, assessed using colloidal gold-coated coverslips overlaid with type I collagen (Fig. 5*A*), demonstrated that addition of LPA strongly enhanced collagen-driven motility. Quantitation of phagokinetic tracks using computer-assisted image analysis indicated an approximately threefold increase in the relative migration areas of control and LPA-treated cells, respectively (Fig. 5*B*). Additionally, LPA-stimulated motility was reduced to control levels in the presence of MMP-I (Fig. 5, *A* and *B*), implicating MMP activity in LPA-induced migration. Similar results were obtained in an *in vitro* scratch wound assay, wherein LPA-induced wound closure was significantly increased relative to control wounded cultures, and the LPA-induced increase in motility was blocked by the addition of MMP-I (Fig. 6, *A* and *B*). To examine the effect of LPA on *in vitro* invasion, cells were seeded into Boyden chambers overlaid with Matrigel to provide a three-dimensional, protein-rich barrier to migration. LPA treatment resulted in a significant increase in invasive activity (Fig. 7*A*) that correlated with LPA-induced MMP-2 activation (Fig. 7*B*). Both MMP-2 processing and invasive activity were blocked by the MMP-I, providing additional evidence for the role of LPA-induced MMP activity in ovarian cancer invasion.

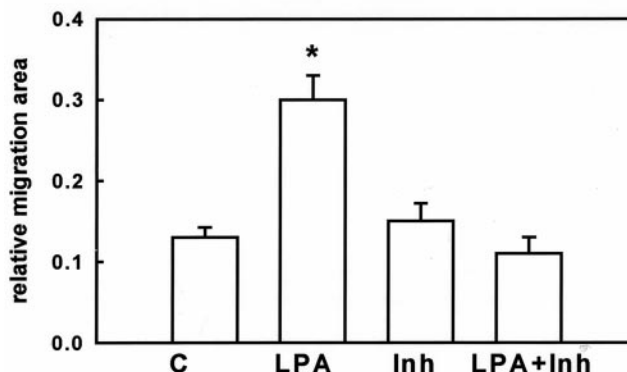
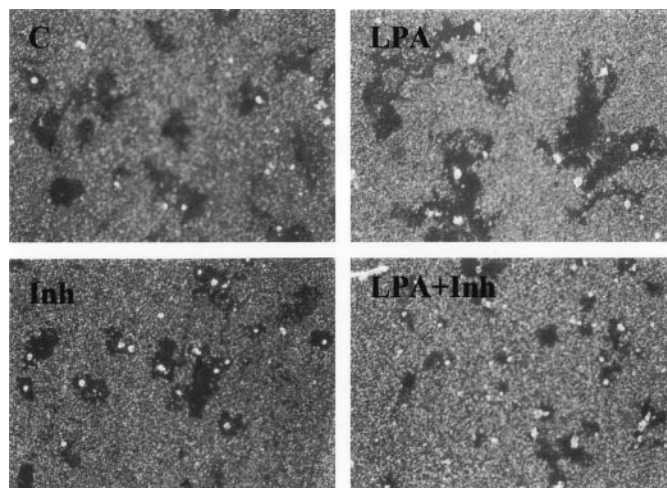


Fig. 5. LPA promotes haptotactic motility. *A*, cells (3×10^3) were cultured for 24 h in the absence or presence of LPA ($80 \mu\text{M}$) or MMPI ($10 \mu\text{M}$) as indicated before plating on coverslips coated with colloidal gold overlaid with type I collagen ($50 \mu\text{g}/\text{ml}$). Cells were allowed to migrate for 26 h, and phagokinetic tracks were visualized using darkfield illumination. *B*, semiquantitative analysis of migration area. The area of phagokinetic tracks was evaluated using computer-assisted image analysis and NIHImage. *C*, untreated controls; *LPA*, cells treated with $80 \mu\text{M}$ LPA; *Inh*, cells treated with $10 \mu\text{M}$ MMPI; *LPA+Inh*, cells treated with LPA and MMPI. (*, $P < 0.001$ relative to control untreated cells).

DISCUSSION

Women with ovarian cancer exhibit significantly elevated LPA levels in both plasma and ascites (2–80 μM ; Refs. 2 and 3), suggesting that this bioactive lipid may contribute to early events that promote tumor cell dissemination. This is supported by a recent report demonstrating that LPA induces expression of urokinase (24), a serine proteinase correlated with ovarian cancer aggressiveness (11). The current study demonstrates that LPA also up-regulates MMP activation in ovarian cancer cells. As a functional consequence of LPA-induced MMP activation, cellular motility and invasive activity are enhanced. Immunohistochemical and *in situ* hybridization studies have demonstrated that MT1-MMP mRNA and protein are prevalent in ovarian carcinomas, particularly at the invasive front, whereas MMP-2 is produced by both tumor cells and associated stromal elements (20, 36). Also, we have previously reported that ovarian cancer cells with the capacity to bind and activate either endogenous or exogenous MMP-2 have significantly enhanced invasive activity (21, 30). This likely results from direct hydrolysis of matrix collagens and adhesive glycoproteins that impede cellular migration and invasion by both MMP-2 and MT1-MMP (22, 23, 37). Together, these data

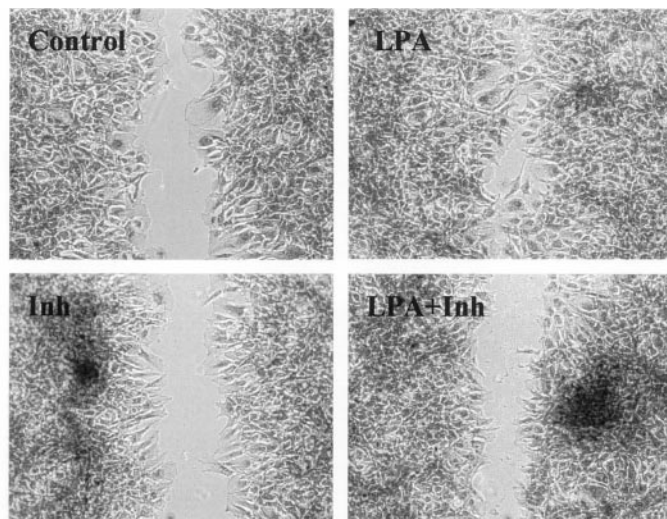


Fig. 6. LPA enhances *in vitro* wound closure. *A*, confluent monolayers of cells were wounded with a uniform scratch, rinsed to remove debris, and incubated in the absence or presence of LPA (80 μM) or MMPI (10 μM) as indicated for 24 h. Photographs indicate relative wound closure as monitored by visual examination using a Zeiss microscope. Fields shown are representative of the width of quadruplicate wounds made in triplicate cultures. *B*, semiquantitative analysis of wound closure. Relative wound closure was determined by measuring the width of the wounds on photographic images using digital needlepoint calipers. Data are expressed as percentage wound closure, relative to the width of control wounds photographed at time zero (not shown). *C*, untreated controls; *LPA*, cells treated with 80 μM LPA; *Inh*, cells treated with MMPI (10 μM); *LPA+Inh*, cells treated with LPA and MMPI. (*, $P < 0.001$ relative to control untreated cells).

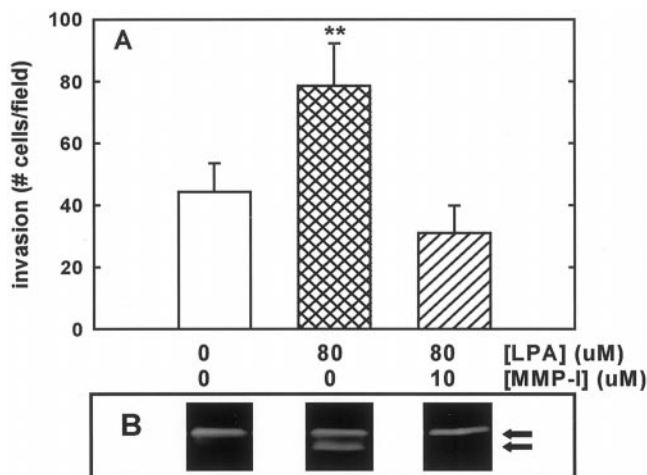


Fig. 7. Effect of LPA on invasive activity. Cells (10^5) were added to porous polycarbonate filters (8- μm pore) coated with Matrigel (11 μg) in the absence or presence of LPA (80 μM) or MMPI (10 μM) as indicated. After incubation for 48 h, conditioned media and filters were collected. *A*, filters were stained, and invading cells were enumerated using an ocular micrometer to count a minimum of 10 high-powered fields. Data are expressed as relative invasion (number of cells/field) and represent the mean and SD of quadruplicate experiments. (**, $P < 0.005$ relative to controls in the absence of LPA and MMPI). *B*, conditioned media were analyzed for MMP activity by gelatin zymography. Arrows, the migration positions of pro- (top) and active MMP-2 (bottom).

suggest that LPA-induced MMP processing may function to promote ovarian cancer *i.p.* dissemination.

Although the detailed mechanism by which LPA functions to promote proMMP-2 activation has not been elucidated, previous studies have demonstrated that LPA binds to ovarian cancer cells via the *Edg* (endothelial cell differentiation gene) receptor family (27, 39). Many *Edg* family members including *Edg-2* and *Edg-4* are widely expressed in ovarian cancer cells (27) and function as PTX-sensitive G protein-coupled receptors to activate multiple signal transduction pathways, including Rho GTPases (24, 38, 39). We have previously described a MMP-2/MT1-MMP activation cascade in ovarian cancer cells induced by three-dimensional collagen culture via signal transduction activated by β_1 integrin clustering (12). Our current results indicate that LPA up-regulates β_1 integrin expression, and previous studies have shown that LPA-*Edg-2* receptor signaling stimulates integrin phosphorylation and formation of β_1 integrin-containing focal contacts (8), which function synergistically with β_1 integrin signaling to promote cellular migration (8, 34). These data indicate that LPA-*Edg* binding and matrix-induced β_1 integrin clustering may function via convergent signal transduction pathways to promote MMP expression and/or processing, with subsequent downstream changes in migratory and invasive behavior. As MT1-MMP immunoreactivity is colocalized with β_1 integrins on the ovarian cancer cell surface,⁴ it is interesting to speculate that LPA- or matrix-induced integrin clustering may function in part to “focalize” MT1-MMP proteolytic activity to sites of cell-matrix contact, thereby promoting pericellular proteolysis and subsequent *i.p.* invasion.

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⁴ S. M. Ellerbroek and M. S. Stack. Functional interplay between type I collagen and cell surface matrix metalloproteinase activity, manuscript submitted.

REFERENCES

- Hoskins, W. J. Prospective on ovarian cancer: Why prevent? *J. Cell. Biochem. Suppl.*, 23:189–199, 1995.
- Xu, Y., Gaudette, D. C., Boynton, J. D., Franker, A., Fang, X. J., Sharma, A., Hurteau, J., Casey, G., Goodboey, A., Mellors, A., Holub, B. J., and Mills, G. B. Characterization of an ovarian cancer activating factor in ascites from ovarian cancer patients. *Clin. Cancer Res.*, 1: 1223–1232, 1995.
- Xu, Y., Shen, Z., Wiper, D. W., Wu, M., Morton, R. E., Elson, P., Kennedy, A. W., Belinson, J., Markman, M., and Casey, G. Lysophosphatidic acid as a potential biomarker for ovarian and other gynecologic cancers. *J. Am. Med. Assoc.*, 280: 719–723, 1998.
- An, S., Bleu, T., Hallmark, O. G., and Goetzl, E. J. Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. *J. Biol. Chem.*, 273: 7906–7910, 1998.
- Moolenaar, W. H., Kranenburg, O., Postma, F. R., and Zondag, G. Lysophosphatidic acid: G protein signaling and cellular responses. *Curr. Opin. Cell. Biol.*, 9: 168–176, 1997.
- Xu, Y., Fang, X., Casey, G., and Mills, G. B. Lysophospholipids activate ovarian and breast cancer cells. *Biochem. J.*, 309: 933–940, 1995.
- Chranowaka-Wodnicka, M., and Burridge, K. Tyrosine phosphorylation is involved in reorganization of the actin cytoskeleton in response to serum or LPA stimulation. *J. Cell Sci.*, 107: 3643–3654, 1994.
- Sakai, T., de la Pena, J. M., and Mosher, D. F. Synergism among lysophosphatidic acid, β_1 A integrins, and epidermal growth factor or platelet-derived growth factor in mediation of cell migration. *J. Biol. Chem.*, 274: 15480–15486, 1999.
- Imamura, F., Horai, T., Mukai, M., Shinkai, K., Sawada, M., and Akedo, H. Induction of *in vitro* tumor cell invasion of cellular monolayers by lysophosphatidic acid or phospholipase D. *Biochem. Biophys. Res. Commun.*, 193: 497–503, 1993.
- Mukai, M., Imamura, F., Ayaki, M., Shinkai, K., Iwasaki, T., Murakami-Murofushi, K., Murofushi, H., Kobayashi, S., Yamamoto, T., Nakamura, H., and Akedo, H. Inhibition of tumor invasion and metastasis by a novel lysophosphatidic acid (cyclic LPA) *Int. J. Cancer*, 81: 918–922, 1999.
- Stack, M. S., Ellerbroek, S. M., and Fishman, D. A. The role of proteolytic enzymes in the pathology of ovarian carcinoma (review). *Int. J. Oncol.*, 12: 569–576, 1998.
- Ellerbroek, S. M., Fishman, D. A., Kearns, A. S., Bafetti, L. M., and Stack, M. S. Ovarian carcinoma regulation of matrix metalloproteinase-2 and membrane type 1 matrix metalloproteinase through β_1 integrin. *Cancer Res.*, 59: 1635–1641, 1999.
- de Nictolis, M., Garbisa, S., Lucarini, G., Goteri, G., Masiero, L., Ciavattini, A., Garzetti, G. G., Stetler-Stevenson, W. G., Fabris, G., Biagini, G., and Prat, J. 72 kilodalton type IV collagenase, type IV collagen, and K_1 67 antigen in serous tumors of the ovary: a clinicopathologic, immunohistochemical, and serological study. *Int. J. Gynecol. Pathol.*, 15: 102–109, 1996.
- Davies, B., Brown, P. D., East, N., Crimmin, M. J., and Balkwill, F. R. A synthetic matrix metalloproteinase inhibitor decreases tumor burden and prolongs survival of mice bearing human ovarian carcinoma xenografts. *Cancer Res.*, 53: 2087–2091, 1993.
- Brown, P. D. Matrix metalloproteinase inhibitors: a novel class of anti-cancer agents. *Adv. Enzyme Regul.*, 35: 293–301, 1995.
- Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. A matrix metalloproteinase expressed on the surface of invasive tumor cells. *Nature (Lond.)*, 370: 61–65, 1994.
- Butler, G. S., Butler, M. J., Atkinson, S. J., Will, H., Tamura, T., van Westrum, S. S., Crabbe, T., Clements, J., d'Ortho, M. P., and Murphy, G. The TIMP-2 membrane type 1 metalloproteinase receptor regulates the concentration and efficient activation of progelatinase A. *J. Biol. Chem.*, 273: 871–880, 1998.
- Lehti, K., Lohi, J., Valtanen, H., and Keski-Oja, J. Proteolytic processing of membrane-type 1 matrix metalloproteinase is associated with gelatinase A activation at the cell surface. *Biochem. J.*, 334: 345–353, 1998.
- Fishman, D. A., Bafetti, L. M., and Stack, M. S. Membrane-type matrix metalloproteinase expression and matrix metalloproteinase-2 activation in primary human ovarian epithelial carcinoma cells. *Invasion Metastasis*, 16: 150–159, 1996.
- Afzal, S., Lalani, E., Poulos, R., Stubbs, A., Rowlinson, G., Sato, H., Seiki, M., and Stamp, G. W. H. MT1-MMP and MMP-2 mRNA expression in human ovarian tumors: possible implications for the role of desmoplastic fibroblasts. *Hum. Pathol.*, 29: 155–165, 1998.
- Young, T. N., Pizzo, S. V., and Stack, M. S. A plasma membrane-associated component of ovarian adenocarcinoma cells enhances the catalytic efficiency of matrix metalloproteinase-2. *J. Biol. Chem.*, 270: 999–1002, 1995.
- d'Ortho, M. P., Stanton, H., Butler, M., Atkinson, S. J., Murphy, G., Hembry, R. M. MT1-MMP on the cell surface causes focal degradation of gelatin films. *FEBS Lett.*, 421: 159–164, 1998.
- Ohuchi, E., Imai, K., Fuji, Y., Sato, H., Seiki, M., and Okada, Y. Membrane type 1 matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. *J. Biol. Chem.*, 272: 2446–2451, 1997.
- Pustilnik, T. B., Estrella, V., Wiener, J. R., Mao, M., Eder, A., Watt, M. V., Bast, R. C., Jr., and Mills, G. B. Lysophosphatidic acid induces urokinase secretion by ovarian cancer cells. *Clin. Cancer Res.*, 5: 3704–3710, 1999.
- Knight, C. G., Willenbrock, F., and Murphy, G. A novel coumarin-labelled peptide for sensitive continuous assay of matrix metalloproteinases. *FEBS Lett.*, 296: 263–266, 1992.
- Rodriguez, G. C., Berchuch, A., Whitaker, R. S., Schlossman, D., Clarke-Pearson, D., Bast, R. C., Jr. Epidermal growth factor receptor expression in normal ovarian epithelium and ovarian cancer. *Am. J. Obstet. Gynecol.*, 164: 745–750, 1991.
- Fang, X., Gaudette, D., Furui, T., Mao, M., Estrella, V., Eder, A., Pustilnik, T., Sasagawa, T., Lapushin, R., Yu, S., Jaffe, R. B., Wiener, J. R., Erickson, J. R., and Mills, G. B. Lysophospholipid growth factors in the initiation, progression, metastases, and management of ovarian cancer. *Ann. NY Acad. Sci.*, 188–208: 1999.
- Nietgen, G. W., and Durieux, M. E. Intercellular signaling by lysophosphatidate. *Cell Adhes. Commun.*, 5: 221–235, 1998.
- Moser, T. L., Pizzo, S. V., Bafetti, L. M., Fishman, D. A., and Stack, M. S. Evidence for preferential adhesion of ovarian epithelial carcinoma cells to type I collagen mediated by the $\alpha_2\beta_1$ integrin. *Int. J. Cancer*, 67: 695–701, 1996.
- Fishman, D. A., Kearns, A., Chilikuri, K., Bafetti, L. M., O'Toole, E. A., Georgacopoulos, J., Ravosa, M. J., and Stack, M. S. Metastatic dissemination of human ovarian epithelial carcinoma is promoted by $\alpha_2\beta_1$ integrin-mediated interaction with type I collagen. *Invasion Metastasis*, 18: 15–26, 1998.
- Pilcher, B. K., Dumin, J. A., Sudbeck, B. D., Krane, S. M., Welgus, H. G., and Parks, W. C. The activity of collagenase-1 is required for keratinocyte migration on a type I collagen matrix. *J. Cell Biol.*, 137: 1445–1457, 1997.
- Albrecht-Buehler, G. The phagokinetic tracks of 3T3 cells. *Cell*, 11: 395–404, 1977.
- Albuquerque, M. L., Waters, C. M., Salva, U., Schnaper, H. W., and Flozak, A. S. Shear stress enhances human endothelial cell wound closure *in vitro*. *Am. J. Physiol. Heart Circ. Physiol.*, 279: 293–302, 2000.
- Sakai, T., Peyruchaud, O., Fassler, R., and Mosher, D. F. Restoration of β_1 A integrins is required for lysophosphatidic acid-induced migration of β_1 -null mouse fibroblastic cells. *J. Biol. Chem.*, 273: 19378–19382, 1998.
- Kinoshita, T., Sato, H., Okada, A., Ohuchi, E., Imai, K., Okada, Y., and Seiki, M. TIMP-2 promotes activation of progelatinase A by membrane type 1 matrix metalloproteinase immobilized on agarose beads. *J. Biol. Chem.*, 273: 16098–16103, 1998.
- Afzal, S., Lalani, E. N., Foulkes, W. D., Boyce, B., Tickle, S., Cardillo, M. R., Baker, T., Pignatelli, M., and Stamp, G. W. H. Matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 expression and synthetic matrix metalloproteinase-2 inhibitor binding in ovarian carcinomas and tumor cell lines. *Lab. Invest.*, 74: 406–421, 1996.
- MacDougall, J. R., and Matrisian, L. M. Contribution of tumor and stromal matrix metalloproteinases to tumor progression, invasion and metastasis. *Cancer Metastasis Rev.*, 14: 351–362, 1995.
- Goetzl, E. J., and An, S. Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine 1-phosphate. *FASEB J.*, 12: 1589–1598, 1998.
- Furui, T., LaPushin, R., Mao, M., Khan, H., Watt, S. R., Watt, M. V., Lu, Y., Fang, X., Tsutsui, S., Siddik, Z. H., Bast, R. C., Jr., and Mills, G. B. Overexpression of Edg-2/vzq-1 induces apoptosis and anoikis in ovarian cancer cells in a lysophosphatidic acid-independent manner. *Clin. Cancer Res.* 5: 4308–4318, 1999.