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Phorbol 12-Myristate 13-Acetate Stimulates Lysophosphatidic Acid Secretion from Ovarian and Cervical Cancer Cells but Not from Breast or Leukemia Cells

Zhongzhou Shen, Ph.D. Jerome Belinson, M.D. Richard E. Morton, Ph.D. Yan Xu, Ph.D. and Yan Xu, Ph.D

INTRODUCTION

Ovarian cancer ranks second in incidence among gynecological cancers and will cause an estimated 14,500 deaths in 1998 in the United States. More than 70% of women diagnosed with ovarian cancer have the disease at an advanced stage and the 5-year survival rate for these women remains very poor [1–3]. If the disease is detected at Stage I, the long-term survival rate approaches 90% [1–3]. Therefore, to improve the overall outcome of this disease, significant progress in two areas is required: (i) development of methods of early detection that are highly sensitive and highly specific and (ii) a further understanding of growth regulation in ovarian cancer through the study of growth factors and their mechanism of action, leading toward the identification of potential new targets for therapy.

The growth of ovarian tumor cells is regulated by a number of growth factors/cytokines, including epidermal growth factor (EGF), transforming growth factor β , amphiregulin, basic fibroblast growth factor, insulin-like growth factor, interleukin 1, and tumor necrosis factor α [4–7]. Ascitic fluid from ovarian cancer patients stimulates the proliferation of ovarian cancer cells both *in vitro* and *in vivo* (nude mice) [8, 9]. Ascitic fluid also stimulates intracellular calcium release from ovarian cancer cells, an activity not associated with any of the peptide factors mentioned above [8], suggesting it contains a novel factor(s).

We have purified and identified a factor in ascites from ovarian cancer patients and have termed it ovarian cancer activating factor, or OCAF. The most striking feature of this factor is that it is not a peptide factor, but a lipid molecule. OCAF is composed of several molecular species of lysophosphatidic acid (LPA)³ [10, 11]. OCAF or synthetic LPA (oleoyl) induces proliferation of ovarian cancer cells as assessed by [³H]thymidine incorporation, MTT dye reduction, and colony formation assays [10–12]. LPA is approximately four times more efficient than EGF in inducing colony formation in SKOV3 cells and 80 times more efficient in OCC1 cells [12]. Importantly, normal ovarian epithelial cells do not respond to LPA [8, 9].

³ Abbreviations used: LPA, lysophosphatidic acid; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; cPLA₂, cytosolic phospholipase A₂; iPLA₂, Ca²⁺-independent phospholipase A₂; sPLA₂, secretory phospholipase A₂; TLC, thin layer chromatography; OCAF, ovarian cancer activating factor; ER, estrogen response.

LPA is a normal constituent of serum, but it is not detectable in freshly isolated blood or plasma from healthy individuals [13–17]. Since we have found that LPA is present in ascites from ovarian cancer patients, we investigated whether LPA was also present in the plasma from patients with ovarian cancer and evaluated its potential as a marker for ovarian cancer. We found that ovarian cancer patients had significantly higher plasma LPA levels compared with controls. Higher LPA levels were also detected in the plasma from a majority of patients with other gynecologic carcinomas, but only in a minority of patients with benign gynecological diseases, and it was not detected in patients with breast cancer or leukemias [18].

While we are prospectively evaluating LPA as a potential marker for early detection, we have also begun to study the source and the mechanism underlying the elevated LPA levels in patients with ovarian and other gynecological cancers. The only LPA source that has been studied extensively is activated platelets [13, 14, 19, 20]. However, since we have observed that over 95% of gynecological cancer patients have elevated LPA levels in their plasma, whereas patients with breast cancer and leukemia do not [18], it seems unlikely that a nonspecific platelet effect could explain the unique association of gynecological cancer and elevated plasma LPA. Intriguingly, malignant effusions from ovarian cancer have been reported to contain high LPA-like activity assayed by a semiquantitative neuronal cell shape change assay, compared with effusions from other tumors, including breast and lung cancer [21]. These data suggest that there may be increased production of LPA associated with ovarian cancer. Therefore, we postulate that the malignant cancer epithelial cells might be responsible for the production and secretion of LPA into the blood.

In the present study, we tested whether ovarian, cervical, breast cancer, and leukemia cells could produce and release LPA. The enzymes involved in LPA production were also investigated.

MATERIAL AND METHODS

Materials. Precoated silica gel 60 TLC plates were obtained from EM Science (Gibbstown, NJ). Lysophosphatidic acids (palmitoyl, oleoyl, and stearoyl LPAs) were purchased from Avanti Polar-lipids, Inc. (Birmingham, AL). Lysophosphatidylcholine, lysophosphatidylinositol, lysophosphatidylserine, lysophosphatidylethanolamine, 8-anilino-1-naphthalenesulfonic acid, ammonium hydroxide, and PMA were obtained from Sigma, Inc. (St. Louis, MO). All other organic solvents were chromatographic grade and from Fisher (Pittsburgh, PA). HEY cells were a gift from G. Mills, M.D. Anderson, Texas [9]. OCC1, Hela, Jurkat, K562, MCF7, and MB-MDA-231 were purchased from the American Type Culture Collection (Rockville, MD). Fetal bovine serum (FBS), trypsin-EDTA, glutamine, RPMI 1640, and DMEM/F12 were obtained from Fisher Scientific (Pittsburgh, PA). Gentamycin was obtained from GIBCO-BRL (Grand Island, NY).

Mammalian cell culture. HEY, OCC1, Hela, Jurkat, K562, MCF7, and MB-MDA-231 were cultured in complete RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, and 50 $\mu\text{g/ml}$ gentamycin in T75 flasks till cells reached 80–90% confluence. Cells were starved in 10 ml DMEM/F12 (50/50) with 2 mM glutamine and 50 $\mu\text{g/ml}$ gentamycin for 48 h before PMA stimulation. The supernatants from different cells were collected and processed for LPA analysis.

LPA analysis. LPA extraction and purification were performed with a similar method as described by Tokumura *et al.* [15]. Briefly, the supernatants from the various cell populations were acidified and extracted by methanol/chloroform (2:1, v/v). After mixing with chloroform and H_2O , LPA was extracted into the chloroform phase and the extract was concentrated at 40°C under nitrogen. The residue was dissolved in methanol/chloroform (2:1, v/v) and loaded on a silica gel plate. The developing solvent was chloroform/methanol/ammonium hydroxide (65:35:5.5, v/v/v). LPA and other phospholipids were detected by spraying the plates with 0.1% 8-anilino-1-naphthalenesulfonic acid in water and visualized under ultraviolet light. LPA spots resolved by TLC were scraped from the silica gel plates and hydrolyzed in 1 M ethanolic KOH. The resulting fatty acids were transmethylated in the presence of behenic acid (Nu Check Prep. Inc., Elysian, MN) as an internal standard with BCl_3 -methanol reagent (Supelco, Inc., Pittsburgh, PA). After transmethylation, the fatty acid esters were extracted with petroleum ether and quantified by Hewlett-Packard Model 5890 gas chromatograph equipped with a column (1.83 m \times 2 mm) coated with 3% SP-2310, 2% SP-2300, on 100/120 Chromosorb W AW (Supelco, Inc. Bellefonte, PA). Two fatty methyl esters standard mixtures were obtained from Nu Check Prep. Inc. Concentrations of LPA and each individual species were derived from the fatty acid ester content and calibrated with internal standard.

Statistics. Determination of statistically significant differences between experimental groups was performed using the nonparametric Mann-Whitney test [22]. Differences between each group were considered to be significant when *P* values of <0.05 were obtained.

RESULTS

PMA-stimulated LPA secretion from HEY cells in a dose- and time-dependent manner. LPA was not detected in the supernatant from HEY, OCC1, Hela, Jurkat, K562, MCF7, and MB-MDA-231 cultured in serum-free medium, suggesting that tumor cells do not produce LPA when growth factors are depleted. To produce LPA, PLA_2 activity is likely to be involved [23]. The activation of PLA_2 can be conveniently and quickly achieved *in vitro* by PMA stimulation, since it activates PKC, which in turn, activates some isoforms of PLA_2 and PLD [19, 23–27].

PMA stimulated significant LPA production which could be detected in the supernatant of ovarian cancer HEY cells. This

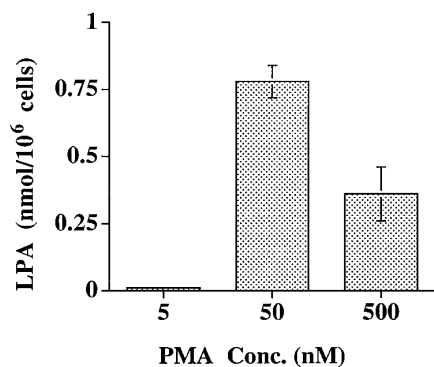


FIG. 1. Dose dependence of PMA-stimulated LPA release into the culture supernatant of HEY cells. HEY cells were cultured as described under Material and Methods. PMA was added to the medium at the indicated concentrations and incubated for 5 min at 37 C. The culture supernatant of HEY cells was collected for LPA analysis. The cell numbers were counted for normalization. LPA analysis was carried as described under Material and Methods. Values are calculated as means \pm S.D. from three experiments.

stimulation was dose and time dependent. As shown in Fig. 1, LPA release was the highest with 50 nM PMA stimulated cells and lower for both 5 and 500 nM PMA. LPA formation and release was rapid (5 min) and very little LPA was detected in the supernatant from HEY cells after 24 h treatment of 50 nM PMA (Fig. 2).

PMA-stimulated LPA secretion from ovarian cancer and cervical cancer cells, but much lower amounts from breast cancer and leukemia cells. We tested LPA levels in the supernatant from other cells after the treatment of 50 nM PMA for 5 min. As shown in Fig. 3, the two ovarian cancer cell lines HEY and OCC1 produced significant amounts of LPA (0.78 cells and 0.56 nmol/10⁶ cells). Hela cells, a cervical cancer cell line, also released LPA, although at lower amounts (0.20 nmol/10⁶ cells), into the supernatant after PMA treatment. On the contrary, MCF7, an estrogen-response (ER) positive breast cancer cell line and MB-MDA-231, an ER negative breast cancer cell line, only released a very small amount of LPA

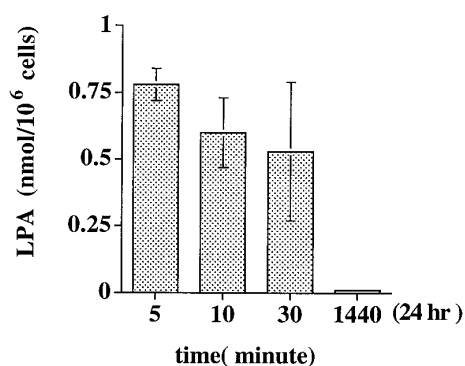


FIG. 2. Time course of PMA (50 nM)-stimulated LPA release into the culture supernatant of HEY cells. HEY cells were cultured and stimulated with 50 nM PMA for different times as indicated.

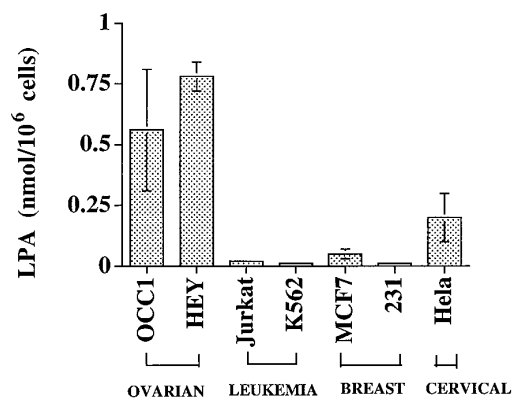


FIG. 3. PMA-stimulated LPA release into the culture supernatant of multiple cell types HEY, OCC1, Hela, Jurkat, K562, MCF7, and MB-MDA-231 cells were stimulated with 50 nM PMA for 5 min.

(0.05 cells and 0.01 nmol/10⁶ cells, respectively). In addition, two leukemia cell lines (Jurkat and K562) secreted negligible LPA after PMA stimulation.

Statistically, the PMA-stimulated LPA levels in ovarian cancer cells were significantly higher than the PMA-stimulated LPA levels in breast cancer cells ($P = 0.002$) or leukemia cells ($P = 0.002$). The PMA-stimulated LPA levels in cervical cancer cells were also significantly higher than that in breast cancer cells ($P = 0.01$) and leukemia cells ($P = 0.02$).

LPA secretion from ovarian cancer cells by PMA stimulation was AACOCF₃- and tamoxifen-sensitive. To investigate the role of phospholipase A₂ in LPA production in ovarian cancer cells, HEY cells were pretreated with 100 μ M AACOCF₃, an inhibitor for both cPLA₂ and iPLA₂, for 12 min prior to treatment with 50 nM PMA for 5 min. Figure 4 shows that 75.6% of PMA-stimulated LPA release was inhibited by AACOCF₃ pretreatment. Tamoxifen citrate (50 μ M, 20 min pretreatment) also inhibited PMA-stimulated LPA release (72.2% inhibition) (data not shown).

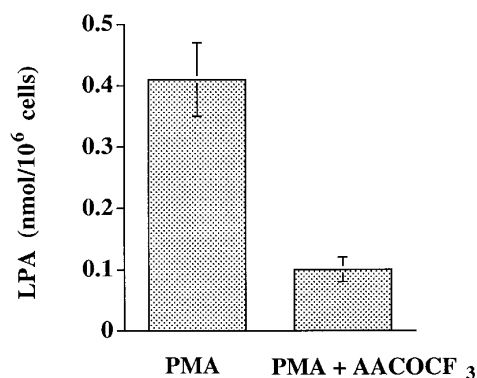


FIG. 4. AACOCF₃ inhibited PMA-stimulated LPA release into the supernatant of HEY cells. HEY cells were pretreated with 100 μ M AACOCF₃ for 12 min, prior to the stimulation of 50 nM PMA for 5 min.

DISCUSSION

We present data here to show that ovarian and cervical cancer cells can produce LPA under certain conditions. To our knowledge, this is the first demonstration that cancer cells can produce LPA.

Our data show that ovarian cancer cells, but not breast cancer (MCF7 and MB-MDA-231) or leukemia (K562 and Jurkat) cells, secrete LPA. The lack of LPA production in breast and leukemia cells, we believe, is not due to the lack of responsiveness of these cells to PMA. We have previously shown that pretreatment with PMA blocks LPA-induced calcium release in MCF7 and MB-MDA-231 cells (unpublished observation). PMA is known to cause Jurkat cells to proliferate and produce IL-2 [28]. It is well documented that K562 cells differentiate in response to PMA [29, 30]. In addition, PMA has been shown to stimulate aromatase activity in MCF7 cells [31]. PMA also induces a dose-dependent increase in prostaglandin E₂ production, inhibits cell growth, and stimulates aromatase activity in 231 cells [32, 33]. The mechanisms that underlie these differences remain to be investigated. It is possible that under different conditions these breast and leukemia cells may secrete LPA into the medium.

Interestingly, the LPA produced by different cancer cells *in vitro* showed the same tumor type specificity as we observed when measuring LPA in plasma [18]. These results suggest that malignant ovarian epithelial cells may represent an important source of the elevated LPA detected in the plasma of patients with ovarian cancers [18].

We observed that LPA was not secreted by ovarian cancer cells *in vitro* when growth factors were depleted. Under physiological and/or pathological conditions, however, ovarian cancer cells are under the influence of many growth factors and cytokines, including EGF, transforming growth factor β , amphiregulin, basic fibroblast growth factor, insulin-like growth factor, interleukin 1, and tumor necrosis factor α [4–6]. Some of these factors have been shown to activate PLA₂ in other cell systems *in vitro*. For example, EGF activates cPLA₂ in human squamous carcinoma A431 cells and fibroblasts [34–37]. TGF- β regulates the expression of phospholipase A₂(s) and induces an increase in cytosolic 85 kDa PLA₂ (activity and protein) in macrophages after a prolonged exposure (>12 h) [38]. IL-1 and TNF- α , but not IL-6, regulate the expression of group II phospholipase A₂ in glomerular mesangial cells [39, 40]. Identification of the physiological stimulus for the production of LPA in ovarian cancer cells is under investigation. Studies of LPA production under physiological and pathological conditions, using an animal model, tumor tissue, or primary tumor culture will be necessary to confirm the source of elevated plasma LPA.

Our inhibitor studies strongly suggest the involvement of a cPLA₂ or an iPLA₂, but do not rule out the potential involvement of a secretory phospholipase A₂ (sPLA₂) in LPA production. cPLA₂ has been shown to be required for activation of

sPLA₂, and both enzymes are involved in cell activation and signal transduction in P388D₁ macrophage [41]. Recently, a nonpancreatic sPLA₂ was reported to be involved in LPA production in platelets [42]. The role of sPLA₂ and other enzymes in LPA production and secretion in ovarian cancer cells remains to be determined. If the source and enzymes associated with the elevated LPA levels in patients with ovarian cancer are defined, they may serve as important targets for early intervention of ovarian cancer.

LPA in the medium was diminished after prolonged treatment with PMA, presumably due to the downregulation of PKC and/or rapid conversion of LPA to either phosphatidic acid through reacylation or dephosphorylation to monoacylglycerol through a LPA phosphohydrolase action [23]. *In vivo*, LPA binds strongly to serum albumin. Albumin not only carries LPA in the serum, but also prolongs its physiological half-life [23]. Therefore, LPA might have a longer half-life *in vivo* than we have observed in cell culture.

Our previous data on plasma LPA levels [18] do not rule out a potential contribution from platelets to the elevated LPA levels found in gynecological cancer patients [18]. Although we used plasma samples to avoid platelet aggregation during sample collection and analysis, and we did not observe significant difference in platelet counts between patients and normal controls, it is still possible that a platelet abnormality in gynecological patients contributed, in part, to the elevated LPA levels. However, the data we present here indicate that tumor epithelial cells are capable of producing LPA under certain conditions and suggest that they are potential sources for elevated plasma LPA *in vivo*.

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