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

David A. Fishman, Yueying Liu, Shawn M. Ellerbroek, and M. Sharon Stack

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 upregulated 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 β1 integrin subunit-specific antibodies, we previously demonstrated that β1 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 β1 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 LPA as an asparagine-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 GTPTases, 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 increases 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 cancer 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 acid, antirabbit IgG-peroxidase conjugates, purified mouse immunoglobulins, ConA, and 1,10-phenanthroline were all purchased from Sigma (St. Louis, MO). Synthetic lyosphospholipids (LPA 18:1 and 18:0) were products of Avanti Polar Lipid Inc. (Alabaster, AL), and the CellTiter 96 AQ kit to monitor cell proliferation and purity. Synthetic lyosphospholipids 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 increases 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.

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Leu-Dpa-Ala-Arg (25) were purchased from Peptides International (Louisville, KY). Protein concentrations were determined using the Bio-Rad D_{2} 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 10³ 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 cell viability of 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 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 replate 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, electrophoretically transferred 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 × Laemmlı 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 mM 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, gels were stained with Coomassie Blue and probed with an anti MMP-2 antibody (1:5000, 1 h). After washing, immunoreactive bands were visualized using enhanced chemiluminescence detection.

**Analysis of Migration and Invasion.** A variety of approaches were used to quantify the 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 20 μg/ml collagen in coating buffer (29) for 2 h. Cells (1 × 10⁶) 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 MMP inhibitor (10 μM). Aliquots of conditioned medium were also removed and assayed for MMP-2 activity (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. Phagokinetic tracks were performed by measuring track area by computer-assisted image analysis using NIHImage. Motility was also evaluated using a scratch wound assay as described previously (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. Semi-quantitative 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 \( \beta_1 \) integrin protein was also enhanced by LPA (Fig. 1B). In control experiments, treatment of cells with LPA (200 \( \mu M \), 72 h) did not enhance proliferation or induce toxicity (not shown). We have previously demonstrated that \( \beta_1 \) integrin clustering induces MT1-MMP-dependent proMMP-2 activation in ovarian cancer cells (12). Because the expression of \( \beta_1 \) integrins was up-regulated by LPA, cells were preincubated with LPA (80 \( \mu M \)) 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 \( \mu M \). 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 \( \mu M \) and 200 \( \mu M \) LPA, respectively. LPA-induced proMMP-2 activation was inhibited by coincubation 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).

In addition to modulating proMMP-2 activation, both \( \beta_1 \) 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 MMP activity using the quenched fluorescent substrate (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-Dpa-Ala-Arg (7.5 \( \mu M \)) 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).

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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. 3B). In untreated cells (Fig. 3B, 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 10–200 μM LPA (Fig. 4). Haptotactic migration, assessed using colloidal gold-coated coverslips overlaid with type I collagen (Fig. 5A), 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. 5B). 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. 7A) that correlated with LPA-induced MMP-2 activation (Fig. 7B). 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. 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 μM) or MMP-I (Lanes 2, 4, and 6), and extracts (20 μg) 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 μM, 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-μm pore) coated on the underside with type I collagen (10 μg/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.

Fig. 5. LPA promotes haptotactic motility. A, cells (3 × 10^5) were cultured for 24 h in the absence or presence of LPA (80 μM) or MMP-I (10 μM) as indicated before plating 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 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 μM LPA; Inh, cells treated with MMP-I (10 μM); LPA + Inh, cells treated with LPA and MMP-I. (*, 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 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 β1 integrin clustering (12). Our current results indicate that LPA up-regulates β1 integrin expression, and previous studies have shown that LPA-Edg-2 receptor signaling stimulates integrin phosphorylation and formation of β1 integrin-containing focal contacts (8), which function synergistically with β1 integrin signaling to promote cellular migration (8, 34). These data indicate that LPA-Edg binding and matrix-induced β1 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 β1 integrins on the ovarian cancer cell surface, 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.

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 relative wound closure as monitored by visual examination using a Zeiss microscope. 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 (10⁵) 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).

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